

UNIVERZITA PALACKÉHO v OLOMOUCI

PŘÍRODOVĚDECKÁ FAKULTA

LABORATOŘ RŮSTOVÝCH REGULÁTORŮ

**Brassinosteroidy: chemie jako nástroj studia jejich
biologických účinků**

Habilitační práce

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„The ultimate aim of the modern movement in biology is to explain all biology in terms of physics and chemistry.“

Francis Crick (1966)

Prohlašuji, že jsem tuto habilitační práci vypracoval samostatně a že jsem uvedl všechny použité literární zdroje včetně mých autorských a spoluautorských prací. Při zpracování textu této habilitační práce byly využity nástroje umělé inteligence k dílčím jazykovým a stylistickým korekcím a v jednom případě také jako pomocný nástroj při návrhu grafického schématu. Umělá inteligence nebyla použita k tvorbě odborného obsahu, interpretaci výsledků ani formulaci vědeckých závěrů.

V Olomouci dne 20. 8. 2026

RNDr. Miroslav Kvasnica, PhD.

Poděkování

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Zkratky

ABC	ATP-binding cassette
ABCB1	ATP-BINDING CASSETTE SUBFAMILY B MEMBER 1
ABCB19	ATP-BINDING CASSETTE SUBFAMILY B MEMBER 19
ACC	1-aminocyklopropan-1-karboxylová kyselina
Ac ₂ O	anhydrid kyseliny octové
AFCs	Alexa Fluor 647–castasteron
ATP	Adenosin trifosfát
BAK1	BRI1-ASSOCIATED RECEPTOR KINASE 1
BES1	BRI1-EMS-SUPPRESSOR 1
BIN2	BRASSINOSTEROID INSENSITIVE 2
BKI1	BRI1 KINASE INHIBITOR 1
BKK1	BAK1-LIKE KINASE 1
BL	brassinolid
BR	brassinosteroid
BRI1	BRASSINOSTEROID INSENSITIVE 1
BRL1	BRI1-LIKE 1
BRL2	BRI1-LIKE 2
BRL3	BRI1-LIKE 3
BSK1	BR-SIGNALING KINASE 1
BSU1	BRI1 SUPPRESSOR 1
BZR1	BRASSINAZOLE RESISTANT 1
CDG1	CONSTITUTIVE DIFFERENTIAL GROWTH 1
CSA	alkynylovaný derivát castasteronu (castasterone-alkyne)
CS	castasteron
DHQD-CLB	dihydrochinidin p-chlorbenzoát
DMF	<i>N,N</i> -dimethylformamid
DMSO	dimethylsulfoxid
EDC	1-ethyl-3-(3-dimethylaminopropyl)karbodiimid
ETD-300	Ethylene Detector 300
EtOH	ethanol

GC-FID	plynová chromatografie s plamenoionizační detekcí
GCI	Grating-Coupled Interferometry
GFP	Green Fluorescent Protein
HG2	Hoveydův–Grubbsův katalyzátor 2. generace
HOBt	1-hydroxybenzotriazol
HWE	Hornerova–Wadsworthova–Emmonsova (reakce)
KIB1	KINK SUPPRESSED IN BZR1-1D
LRR	Leucine-Rich Repeat
LRR-RLK	Leucine-Rich Repeat Receptor-Like Kinase
MCPBA	kyselina meta-chlorperbenzoová
MeOH	methanol
NMO	<i>N</i> -methylmorfolin- <i>N</i> -oxid
<i>n</i> -BuLi	<i>n</i> -butyllithium
PP2A	PROTEIN PHOSPHATASE 2A
SERK	SOMATIC EMBRYOGENESIS RECEPTOR KINASE
SERK1	SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1
SERK3	SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3
SERK4	SOMATIC EMBRYOGENESIS RECEPTOR KINASE 4
TBAF	tetra- <i>n</i> -butylamonium fluorid
<i>t</i> -BuOH	2-methylpropan-2-ol (<i>t</i> -butanol)
TFPAA	kyselina trifluorperoxyoctová
THF	tetrahydrofuran
TsOH	kyselina <i>p</i> -toluensulfonová
VH1	VASCULAR HIGHWAY 1

1 Úvod

Rostlinná říše zahrnuje široké spektrum strukturně rozmanitých sloučenin, které působí jako hormony a významně ovlivňují téměř všechny životní funkce rostlin. Mezi tyto látky patří brassinosteroidy, polyhydroxylované steroidní sloučeniny. Přestože první práce o brassinech, látkách obsažených v pylu řepky, byla publikována již v roce 1970 [1], jejich intenzivní výzkum započal až koncem 70. let 20. století, kdy se podařilo z téhož zdroje izolovat a následně charakterizovat základní brassinosteroidní hormon – brassinolid [2]. Jak bývá u objevů nových sloučenin obvyklé, této problematice se ujali jak analyticky orientovaní chemici, kteří začali vyhledávat příbuzné sloučeniny, tak i organičtí chemici usilující o nalezení syntetických přístupů k brassinosteroidům. v počáteční fázi výzkumu tak bylo publikováno značné množství prací popisujících nové přírodní brassinosteroidy, syntetické postupy jejich přípravy i syntézy nepřirodních analogů.

Postupem času začala ve výzkumu převládat biologicky orientovaná část. Pozornost se soustředila na studium fyziologických účinků brassinosteroidů u rostlin, identifikaci a charakterizaci brassinosteroidních receptorů a s nimi souvisejících signálních, transportních a biosyntetických drah, stejně jako na vliv brassinosteroidů na genovou expresi [3,4,5]. Významnou oblastí výzkumu se rovněž stal tzv. cross-talk brassinosteroidů s dalšími rostlinnými hormony [6]. Veškeré tyto chemicky i biologicky orientované studie přitom směřovaly k zodpovězení klíčové otázky, zda je možné brassinosteroidy prakticky využít v zemědělství. Zatímco chemici se tuto problematiku snažili řešit prostřednictvím syntézy levnějších a biologicky účinnějších analogů, biologicky orientovaný výzkum se zaměřoval na možnosti úpravy signálních a biosyntetických drah vedoucí ke zvýšení odolnosti rostlin a ke zlepšení výnosu [7,8].

Přestože je naprostá většina výzkumu brassinosteroidů tradičně zaměřena na rostlinnou říši, od počátku 21. století se začíná rozvíjet také studium jejich účinků na živočišné, resp. lidské buňky [9]. Tento směr výzkumu vychází mimo jiné z určité strukturní podobnosti brassinosteroidů s lidskými steroidními hormony. Předpokládalo se, že by tato podobnost mohla vést k interakcím s vybranými buněčnými procesy u živočichů, zejména v oblasti regulace buněčného cyklu, proliferace a buněčné smrti. v počátečních fázích výzkumu byla proto pozornost zaměřena především na hodnocení cytotoxicity a antiproliferačních účinků [10,11]. Výzkum byl následně dále rozšířen například o studium antiangiogenních [12], antivirových [13] či neuroprotektivních účinků [14]. v řadě případů brassinosteroidy sloužily jako strukturní předloha pro syntézu biologicky aktivnějších analogů.

Souhrn dosavadních poznatků dokládá, že brassinosteroidy představují významnou skupinu biologicky aktivních sloučenin s potenciálem jak pro základní výzkum, tak pro praktické aplikace [15]. Zájem o jejich studium přitom dlouhodobě nepolevuje; jen v letech 2024–2025 bylo podle databáze Web of Science publikováno více než 700 vědeckých prací.

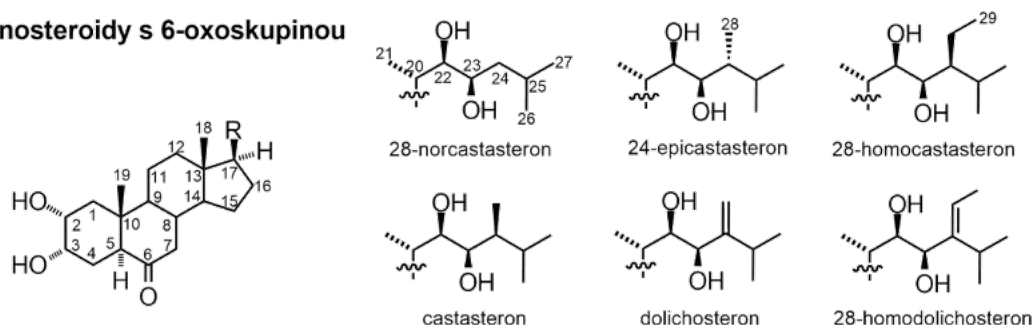
Tato habilitační práce si klade za cíl ukázat, že chemie zůstává i přes výrazný rozvoj biologického výzkumu brassinosteroidů důležitým nástrojem jejich studia.

2 Struktura přírodních brassinosteroidů

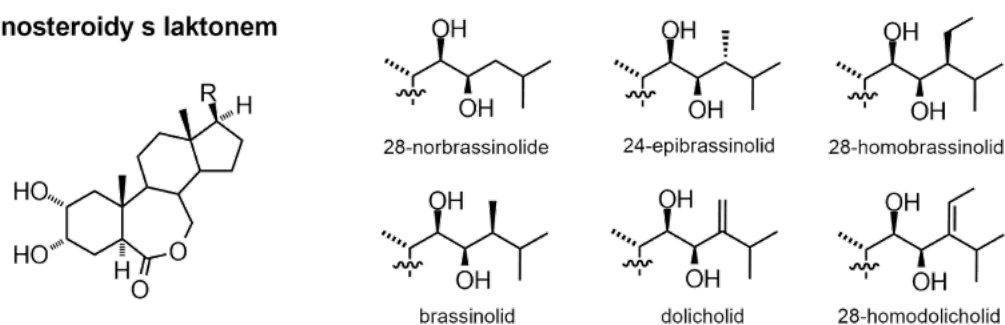
Jak již bylo zmíněno, brassinosteroidy jsou polyhydroxylované steroidy. Jejich základem je tetracyklický 5 α -cholestanový skelet substituovaný zejména na kruzích A a B a dále na postranním řetězci. Strukturní variabilita přírodních brassinosteroidů je dána především počtem, polohou a konfigurací kyslíkatých funkčních skupin, stejně jako typem alkylového substituentu v pozici C-24, výjimečně také v pozici C-25. Biologická aktivita brassinosteroidů je přitom významně ovlivněna jak typem substituce steroidního skeletu, tak konfigurací jednotlivých funkčních skupin [16].

V závislosti na přítomnosti a typu alkylového substituentu lze brassinosteroidy rozdělit do tří skupin podle celkového počtu uhlíků na C27, C28 a C29 brassinosteroidy. První skupina zahrnuje 28-norbrassinosteroidy, které nemají v poloze C-24 žádný alkylový substituent. Druhá, nejpočetnější skupina, obsahující methylový substituent v poloze C-24, zahrnuje biologicky nejaktivnější brassinosteroidy – castasteron a brassinolid (základní řada), dále jejich izomery, 24-epibrassinosteroidy a také brassinosteroidy obsahující methylidenovou skupinu v poloze C-24, tedy dolichosteron a dolicholid. Poslední skupinu tvoří brassinosteroidy s ethylovým substituentem v poloze C-24, označované jako 28-homobrassinosteroidy. Základní rozdělení přírodních brassinosteroidů je uvedeno na obrázku 1.

brassinosteroidy s 6-oxoskupinou

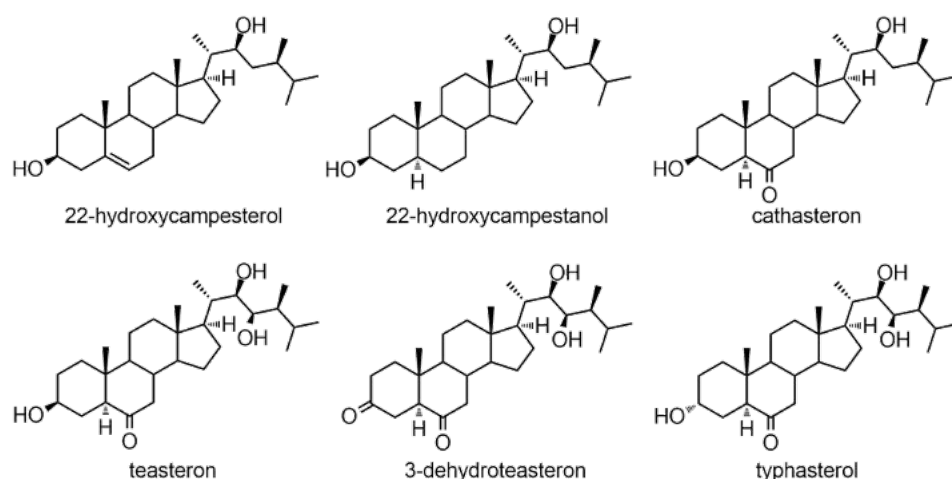


brassinosteroidy s laktonem



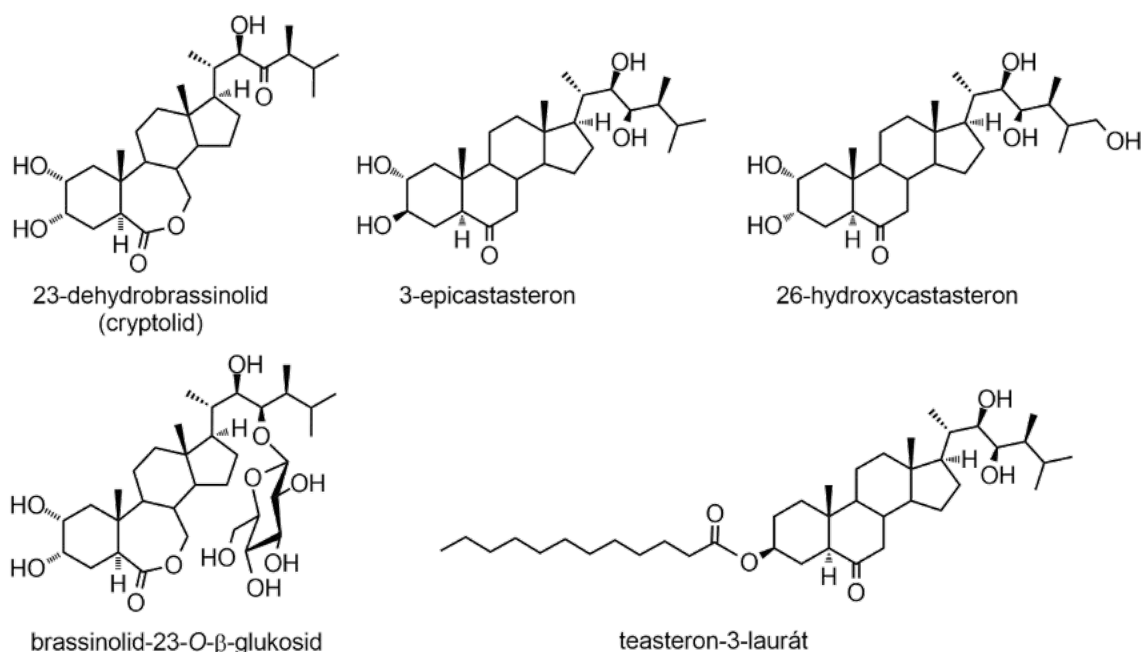
Obrázek 1: Struktury základních přírodních brassinosteroidů s číslováním skeletu.

Kromě skupiny dvanácti aktivních derivátů však existuje rozsáhlá skupina jejich biosyntetických prekurzorů [17,18]. Za brassinosteroidy jsou v tomto případě považovány až deriváty obsahující minimálně dvě hydroxylové skupiny v pozicích C-3 a C-22. Některé prekurzory castasteronu a brassinolidu jsou zobrazeny na obrázku 2. v neposlední řadě byla izolována a charakterizována také celá řada biologicky neaktivních metabolitů brassinosteroidů, včetně jejich konjugátů, jejichž význam dosud není zcela objasněn (obrázek 3) [18-21].



Obrázek 2: Příklady brassinosteroidních prekurzorů.

V případě metabolitů dochází nejčastěji k hydroxylacím a epimerizacím hydroxylových skupin, v menší míře pak také ke štěpení C–C vazeb vedoucímu ke zkracování postranního řetězce. Mezi izolovanými konjugáty byly identifikovány jak polární glykosidy, tak nepolární estery mastných kyselin. Je však nutné zdůraznit, že tyto metabolity byly převážně izolovány v rámci metabolických studií po aplikaci aktivních brassinosteroidů v koncentracích převyšujících jejich přirozený obsah v rostlinách. Přítomnost příslušného enzymatického aparátu a experimentální důkazy jejich metabolismu nicméně umožňují předpokládat existenci také endogenních metabolitů. Jejich detekce však bude pravděpodobně vyžadovat vyšší citlivost analytických metod.



Obrázek 3: Příklady struktur brassinosteroidních metabolitů.

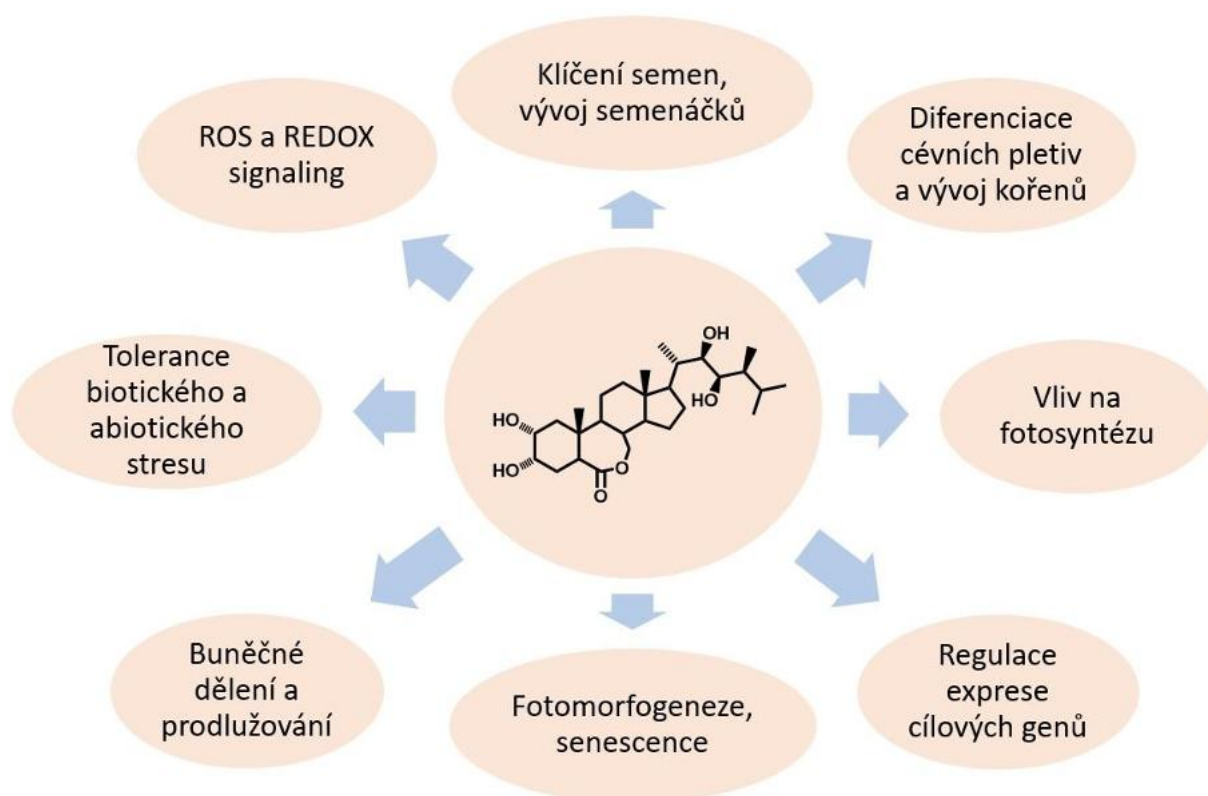
3 Hodnocení biologické aktivity brassinosteroidů

Brassinosteroidy podporují růst a vývojové procesy ve všech rostlinných pletivech a orgánech, ovlivňují také klíčení semen a reprodukci rostlin. Na buněčné úrovni regulují prodlužování buněk, buněčné dělení a diferenciaci. [22-24]. Brassinosteroidy koordinují a integrují různé procesy nezbytné pro růst částečně prostřednictvím interakcí s dalšími hormony [6]. Kromě své klíčové role v růstu a vývoji rostlin se také podílejí na mechanismech zajišťujících toleranci vůči různým environmentálním stresům, včetně vysokých a nízkých teplot, sucha, salinity, poškození herbicidy a napadení patogeny [25-27]. Některé důležité aktivity brassinosteroidů jsou uvedeny na obrázku 4.

Pro studium biologické aktivity brassinosteroidů se využívá zejména jejich vliv na buněčné dělení a prodlužování, vliv na růst kořene a také vliv na produkci rostlinného hormonu ethylen. v následujících podkapitolách jsou popsány čtyři základní biotesty pro studium brassinosteroidů a jejich syntetických derivátů, které v naší laboratoři využíváme.

3.1 Rýžový inkлинаční biotest

Rýžový inkлинаční test (rice lamina inclination assay) představuje jeden z nejčastěji využívaných biotestů pro hodnocení biologické aktivity brassinosteroidů [28]. Princip testu vychází ze schopnosti brassinosteroidů stimulovat prodlužovací růst buněk v lamina joint oblasti mezi čepelí a pochvou listu mladých rostlin rýže (*Oryza sativa*). v důsledku tohoto růstu

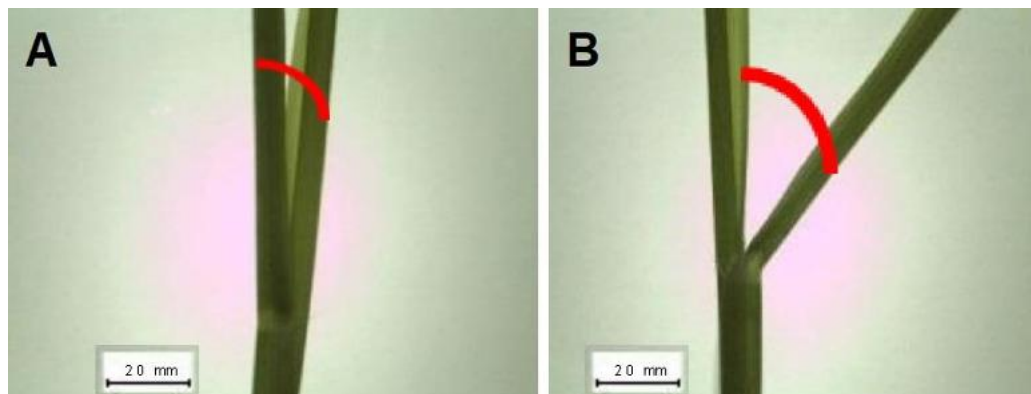


Obrázek 4: Schéma základních fytohormonálních účinků brassinosteroidů.

dochází ke zvětšování úhlu mezi listovou čepelí a stonkem, přičemž velikost vzniklého úhlu koreluje s biologickou aktivitou testované látky. Tento test, původně vyvinutý pro hodnocení přirozených brassinosteroidů, se stal standardní metodou pro studium biologické aktivity syntetických analogů a vztahu mezi strukturou a aktivitou [28,29]. Výhodou rýžového inkлинаčního testu je zejména jeho relativní jednoduchost, citlivost a dobrá reprodukovatelnost výsledků. Současně umožňuje porovnávat biologickou aktivitu různých derivátů vůči standardům, nejčastěji 24-epibrassinolidu, 24-epicastasteron nebo brassinosteroidy 28-homo řady. Test nicméně neposkytuje informace o mechanismu účinku sledovaných derivátů a hodnotí především výsledný fyziologický účinek spojený s buněčnou elongací. Určité omezení představuje také skutečnost, že biologická aktivita stanovená pomocí rýžového inkлинаčního testu nemusí vždy přímo korelovat s účinky pozorovanými u jiných biotestů. I přes tato negativa zůstává rýžový inkлинаční test důležitým nástrojem pro primární hodnocení fytohormonální aktivity brassinosteroidů.

Standardní protokol dříve používaný v naší laboratoři pro měření rýžového inkлинаčního testu může vypadat následovně [30]. Semena kultivaru rýže Bahia jsou namočena do vody a inkubována v růstové komoře při fotoperiodě 8 hodin světla : 16 hodin tmy při 30 °C po dobu 2 dnů. Naklíčená semena jsou poté vysazena na povrch 0,5% vodného agarového média a inkubována za výše uvedených podmínek po dobu 4 dnů. Vybraným semenáčkům je pomocí mikrostříkačky aplikován roztok brassinosteroidu (v 95% ethanolu) na lamina joint druhého listu. Ošetřené i neošetřené (kontrolní) semenáčky jsou poté vráceny do růstové

komory při 30 °C ve tmě na další 2 dny. Následně je měřen vnitřní úhel mezi čepelí druhého listu a jeho listovou pochvou za účelem stanovení aktivity testovaných brassinosteroidů (obrázek 5). Příslušný laboratorní postup je možné v literatuře nalézt v různých modifikacích a s různými kultivary rýže.



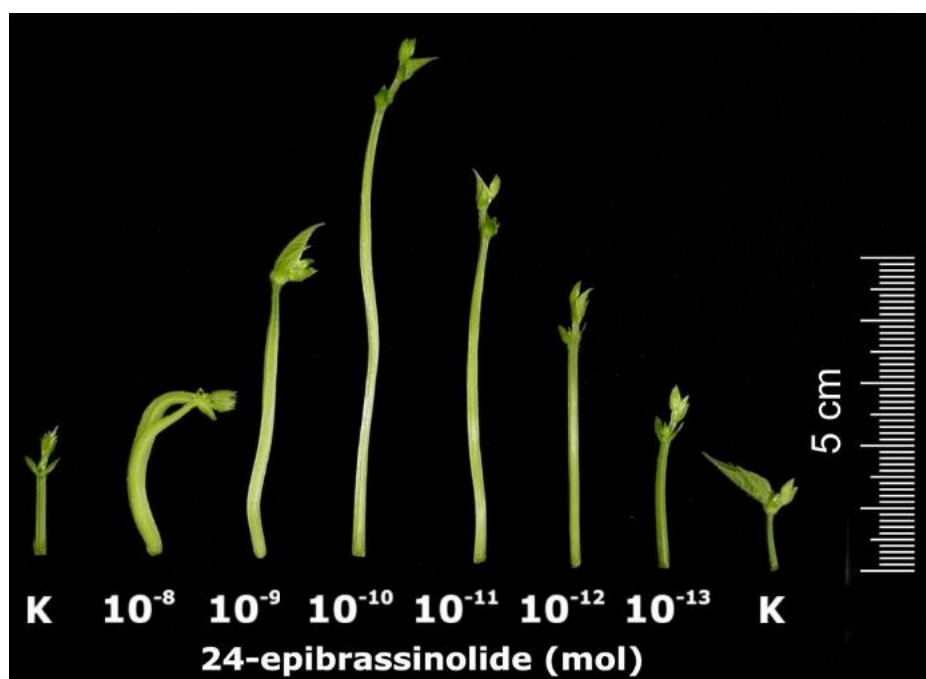
Obrázek 5: Ukázka výsledků rýžového inkлинаčního testu, A - negativní kontrola B - brassinolid (10 nM). Převzato z Ferrer K., 2021 [30].

3.2 Biotest růstu druhého internodia fazolu

Fazolový biotest založený na prodlužování druhého internodia semenáčků (bean second internode assay) představuje další často využívanou metodu pro hodnocení biologické aktivity brassinosteroidů [31]. Mechanismus tohoto testu je obdobný rýžovému inkлинаčnímu testu, protože biologická odpověď je rovněž založena na schopnosti brassinosteroidů ovlivňovat růst rostlinných buněk. v případě fazolového testu je však výsledná reakce výrazně závislá na koncentraci aplikovaného brassinosteroidu. Při nízkých koncentracích (cca 10^{-9} - 10^{-13} M) převládá stimulace prodlužovacího růstu buněk ve druhém internodiu rostlin fazolu obecného (*Phaseolus vulgaris*) [32], přičemž míra elongace slouží jako ukazatel biologické aktivity testovaných látek. Se zvyšující se koncentrací brassinosteroidů se však charakter odpovědi mění. Dochází ke stimulaci biosyntézy ethylenu, jehož účinek postupně převládne nad přímým růstovým působením brassinosteroidů. Výsledkem je komplexní morfologická odpověď připomínající klasickou triple response vyvolanou ethylenem, charakterizovaná inhibicí prodlužovacího růstu, radiálním ztloustnutím internodia, jeho zakřivením a v některých případech i podélným roztržením pletiva (obrázek 6).

Jeden z protokolů modifikovaných a využívaných v naší laboratoři je následující [33]. Semena fazolu (např. kultivar Pinto) jsou nechána klíčit po dobu 2 dnů. Vybraná naklíčená semena se zasadí do květináčů obsahujících perlit a modifikovaný Hoaglandův roztok. Květináče jsou následně umístěny do kultivačního prostoru s kontrolovanými podmínkami. Skupiny 7denních semenáčků s druhými internodii o délce 1-2 mm jsou ošetřeny testovanými brassinosteroidy aplikovanými v lanolinu. Kontrolní skupina rostlin se ošetří pouze lanolinem.

Délka druhého internodia je stanovena po 5 dnech kultivace a rozdíl mezi ošetřenými a kontrolními rostlinami představuje míru biologické aktivity testovaných látek.



Obrázek 6: Výsledek testu na druhém internodu fazolu po aplikaci různých koncentrací 24-epibrassinolidu.

3.3 Inhibiční test růstu kořene *Arabidopsis*

Tento test, někdy nazývaný jako test citlivosti růstu kořenů, vychází z fyziologického účinku brassinosteroidů na růst kořene *Arabidopsis thaliana* [34,35]. Podobně jako u předchozích testů se i v tomto případě biologická aktivita projevuje ovlivněním procesů buněčného dělení a prodlužovacího růstu buněk v kořenových pletivech. Zatímco nižší koncentrace brassinosteroidů mohou růst kořene stimulovat, při vyšších koncentracích dochází k opačnému efektu, tedy k inhibici kořenového růstu. Obdobný koncentračně závislý účinek lze pozorovat také u auxinů. Tento jev představuje charakteristickou fyziologickou odpověď a tvoří základ využití kořenového testu při hodnocení biologické aktivity těchto rostlinných hormonů. Významnou výhodou tohoto testu je jeho vysoká citlivost, která umožňuje sledovat účinky brassinosteroidů již při velmi nízkých koncentracích. Test je proto často využíván nejen pro hodnocení biologické aktivity nových analogů, ale také při studiu mutantů se změněnou citlivostí k brassinosteroidům a při objasňování mechanismů jejich signalizace [36]. Na modelu *Arabidopsis* lze také stanovovat délku hypokotylu či epikotylu. v obou případech dochází při vyšší koncentraci brassinosteroidů k inhibici jejich růstu. Analogicky je možno provádět tyto experimenty i na jiných rostlinách, např. hrachu (*Pisum*).



Obrázek 7: Výsledek kořenového biotestu s 24-epibrassinolidem, kořeny nenataženy.

Stručně lze experimentální uspořádání popsat následovně [37]. Semena *Arabidopsis thaliana* L. (ekotyp Columbia, Col-0) jsou po dobu 2 dnů stratifikována při 4 °C a následně klíčena na vertikálně orientovaném agarovém médiu obsahujícím poloviční koncentraci Murashigeho–Skoogova média doplněného o 1 % (w/v) sacharózy. Rostliny jsou kultivovány při konstantní teplotě a za jasně daných podmínek střídání světla a tmy. Kultivační média obsahují 24-epibrassinolid jako pozitivní kontrolu (obrázek 7) a testované deriváty brassinosteroidů. Po 7 dnech kultivace jsou kořeny narovnány na povrchu pevného média, naskenovány pomocí vysokorozlišovacího skeneru a následně je stanovena jejich celková délka.

3.4 Stanovení produkce ethylenu

Je již známo, že brassinosteroidy ovlivňují produkci ethylenu v různých rostlinných pletivech. Jedním z hlavních mechanismů je stabilizace ACC syntázy, klíčového enzymu biosyntézy ethylenu [38]. Účinek brassinosteroidů na tvorbu ethylenu však závisí na jejich koncentraci – nízké koncentrace mohou produkci ethylenu potlačovat, zatímco vyšší koncentrace ji výrazně zvyšují. Tento proces souvisí s působením transkripčních faktorů BES1 a BZR1, které regulují expresi genů pro ACC syntázu [39]. Za fyziologických podmínek BR biosyntézu ethylenu tlumí, zatímco při vyšších koncentracích společně s auxiny podporují jeho tvorbu. z tohoto důvodu představuje měření produkce ethylenu vhodnou metodu pro hodnocení biologické aktivity nových brassinosteroidů a jejich analogů. Ve srovnání s klasickými BR bioesey, jako je test druhého internodia fazole, rýžový inklinální test nebo inhibiční test na kořenech *Arabidopsis*, poskytuje tato metoda nejen informaci o fenotypové odpovědi rostliny, ale také umožňuje propojit pozorovaný účinek s molekulárními mechanismy BR signalizace [40].

Běžný protokol pro stanovení produkce ethylenu v naší laboratoři [40] spočívá v tom, že semenáčky hrachu ošetřené testovanými brassinosteroidy (BR) jsou umístěny na 24 hodin ve tmě do uzavřené skleněné nádoby. Následně je odebrán vzorek plynu z prostoru nad rostlinami a analyzován pomocí plynové chromatografie s plamenoionizační detekcí (GC-FID). Alternativně lze obsah ethylenu stanovit také pomocí fotoakustického detektoru ethylenu, například přístrojem ETD-300.

4 Chemické přístupy ke studiu brassinosteroidů

Zatímco struktura přírodních brassinosteroidů podléhá poměrně přesně definovaným pravidlům vycházejícím z dosud izolovaných sloučenin [15], u syntetických derivátů je zpravidla nutné zachovat pouze určitou strukturní podobnost s přírodními brassinosteroidy. Vždy však záleží na účelu, pro který jsou dané deriváty připravovány. v případě vývoje nových analogů a testování jejich biologické aktivity může být strukturní podobnost s přírodními brassinosteroidy výrazně menší než v případech, kdy jsou syntetické deriváty využívány k získávání nových poznatků o přírodních brassinosteroidech. Mezi syntetické deriváty lze zařadit také izotopicky značené sloučeniny, např. deuterované brassinosteroidy pro analytické experimenty [41].

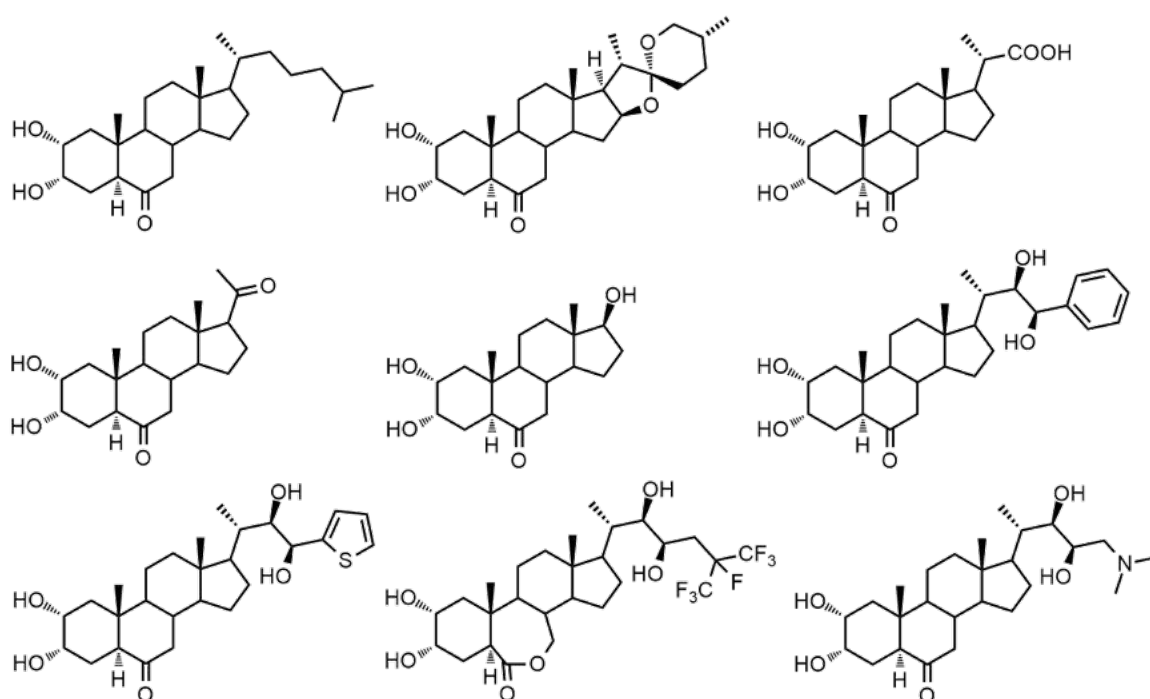
4.1 Syntetické analogy brassinosteroidů a jejich fytohormonální aktivita

Hledání nových analogů s cílem získat vyšší fytohormonální aktivitu provází výzkum brassinosteroidů již od určení jejich struktury na konci 70. let minulého století. Vzhledem k jejich chemické struktuře byly modifikace směřovány především na postranní řetězec a v menší míře také na kruhy A a B. Takto připravené analogy byly následně testovány pomocí rostlinných biotestů, zejména z hlediska jejich prorůstových účinků a uplatnění v zemědělství. v menší míře byly připravovány také deriváty určené k inhibici brassinosteroidních receptorů. s ohledem na rozsah těchto strukturních modifikací budou v této kapitole představeny pouze vybrané sloučeniny připravené v Laboratoři růstových regulátorů, na jejichž syntéze jsem se podílel já nebo moji studenti.

4.1.1 Modifikace na postranním řetězci

Modifikace postranního řetězce představují nejrozsáhlejší oblast strukturních úprav brassinosteroidů, jelikož právě tato část molekuly významně ovlivňuje jejich biologickou aktivitu a současně poskytuje široké možnosti chemických modifikací. Pozornost byla věnována zejména konfiguraci chirálních center postranního řetězce, přítomnosti různých kyslíkatých funkčních skupin v pozicích C-22 a C-23 a také inkorporaci dalších funkčních skupin či heteroatomů [42-49]. Vedle jednodušších modifikací byly připravovány také deriváty se zkráceným, prodlouženým, zcela odstraněným či nově vystavěným postranním řetězcem s cílem objasnit vztah mezi strukturou a fytohormonální aktivitou [50-54]. Bylo prokázáno, že i relativně malé změny v uspořádání postranního řetězce mohou vést k výrazným změnám biologické aktivity.

Z chemického hlediska lze při přípravě derivátů s modifikovaným postranním řetězcem uplatnit dva přístupy. První, relativně jednodušší, přístup spočívá ve využití již existujícího postranního řetězce výchozího steroidu a chemických úpravách omezených pouze na kruhy A a B. Výhodou tohoto postupu je především snadná dostupnost řady přírodních steroidních prekurzorů a menší syntetická náročnost. Tímto způsobem byla připravena brassinosteroidní analoga odvozená od různých steroidních skeletů, například androstanového [50,52,53], pregnanového [51], cholestanového [55] či spirostanového [56]. Druhý přístup zahrnuje vybudování nového postranního řetězce, což je vhodné zejména pro přípravu steroidů s atypickými postranními řetězci, například obsahujícími aromatický kruh, heterocyklus nebo inkorporovaný heteroatom (obrázek 8) [47,57-59]. Tento přístup umožňuje cíleně měnit strukturní vlastnosti molekuly a současně poskytuje širší možnosti studia vztahu mezi strukturou a biologickou aktivitou.



Obrázek 8: Příklady syntetických brassinosteroidů s různými postranními řetězci.

Na uvedený koncept navazovaly i naše práce zaměřené na přípravu nových brassinosteroidních analogů s aromaticky modifikovaným postranním řetězcem [58,59]. Hlavním cílem bylo ověřit, jak zavedení fenylové skupiny a jejích substituentů ovlivní prostorové uspořádání molekuly, její interakci s receptorem BRI1 a následně biologickou aktivitu připravených látek. Návrh těchto sloučenin vycházel ze znalostí krystalových struktur receptoru BRI1 a předpokladu, že hydrofobní aromatická skupina může vhodně interagovat s nepolární oblastí vazebného místa receptoru. Tyto práce tak představují racionální přístup k návrhu nových brassinosteroidních analogů založený na propojení strukturních poznatků, molekulárního modelování a experimentálního ověření biologické aktivity.

V první sérii našich prací byla připravena nová skupina analogů obsahujících para-substituovanou fenylovou skupinu v postranním řetězci. Pro syntézu těchto derivátů byla

využita Hornerova–Wadsworthova–Emmonsova (HWE) reakce, která umožnila cílené vybudování nového postranního řetězce a zavedení různých aromatických substituentů [58]. Následné transformační kroky zahrnovaly Sharplessovu asymetrickou dihydroxylaci a Baeyerovu–Villigerovu oxidaci vedoucí k přípravě dvou typů derivátů – 6-keto sloučenin a odpovídajících B-laktonů (schéma 1). Tento přístup umožnil sledovat vliv i obou modifikací B-kruhu.

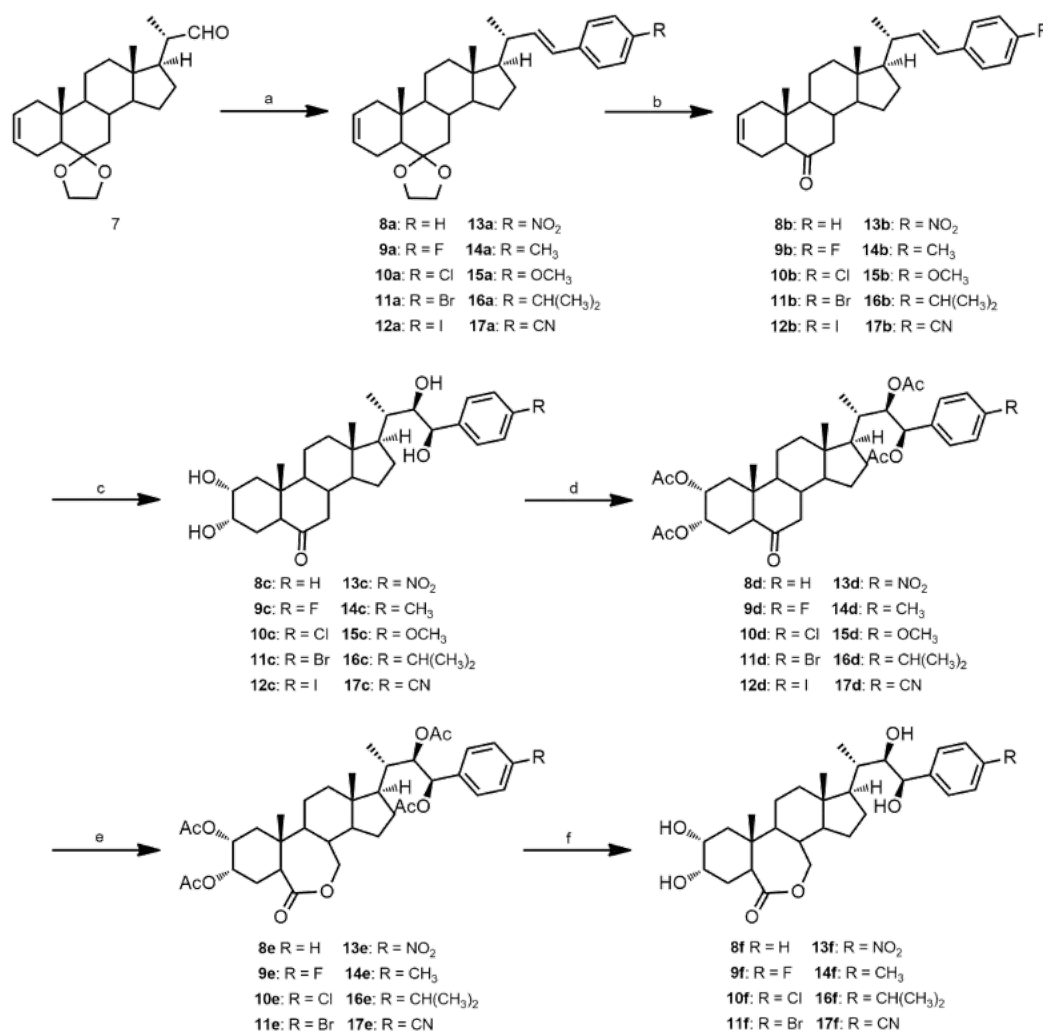


Schéma 1: Syntéza fenyl analogů pomocí HWE reakce: (a) benzyltrifenylfosfonium bromid nebo diethyl arylphosphonát, n-BuLi/THF; (b) 5% HCl/THF; (c) OsO₄, CH₃SO₂NH₂, K₃[Fe(CN)₆], K₃CO₂, DHQD CLB/t-BuOH, H₂O; (d) Ac₂O/pyridin; (e) trifluorperoxyoctová kyselina/CH₂Cl₂; (f) NaOH/THF, H₂O. Převzato a upraveno dle Kvasnica M., 2016 [58].

Na tuto studii následně navázala další práce, která rozšířila rozsah připravovaných aromatických derivátů a současně využila odlišnou syntetickou strategii založenou na alkenové zkřížené metatezi neboli cross-metatezi (schéma 2) [59]. Tato metoda představovala efektivní alternativu k předchozímu přístupu a umožnila konstrukci nových postranních řetězců reakcí steroidního dienového prekursoru s komerčně dostupnými substituovanými styreny (tabulka 1). Výhodou této metodiky byla zejména syntetická flexibilita a možnost připravit širší spektrum derivátů lišících se nejen povahou substituentu, ale také jeho polohou

na aromatickém jádře. Tím se podařilo významně rozšířit soubor studovaných sloučenin a podrobněji analyzovat vztahy mezi strukturou a biologickou aktivitou. v rámci této práce byly připraveny jen 6-keto deriváty.

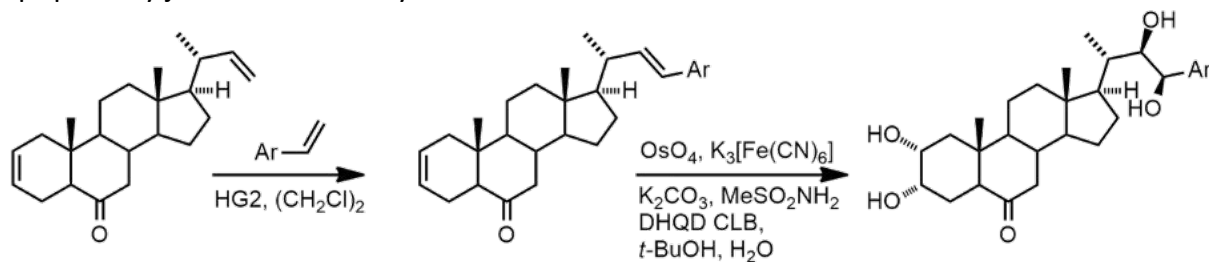
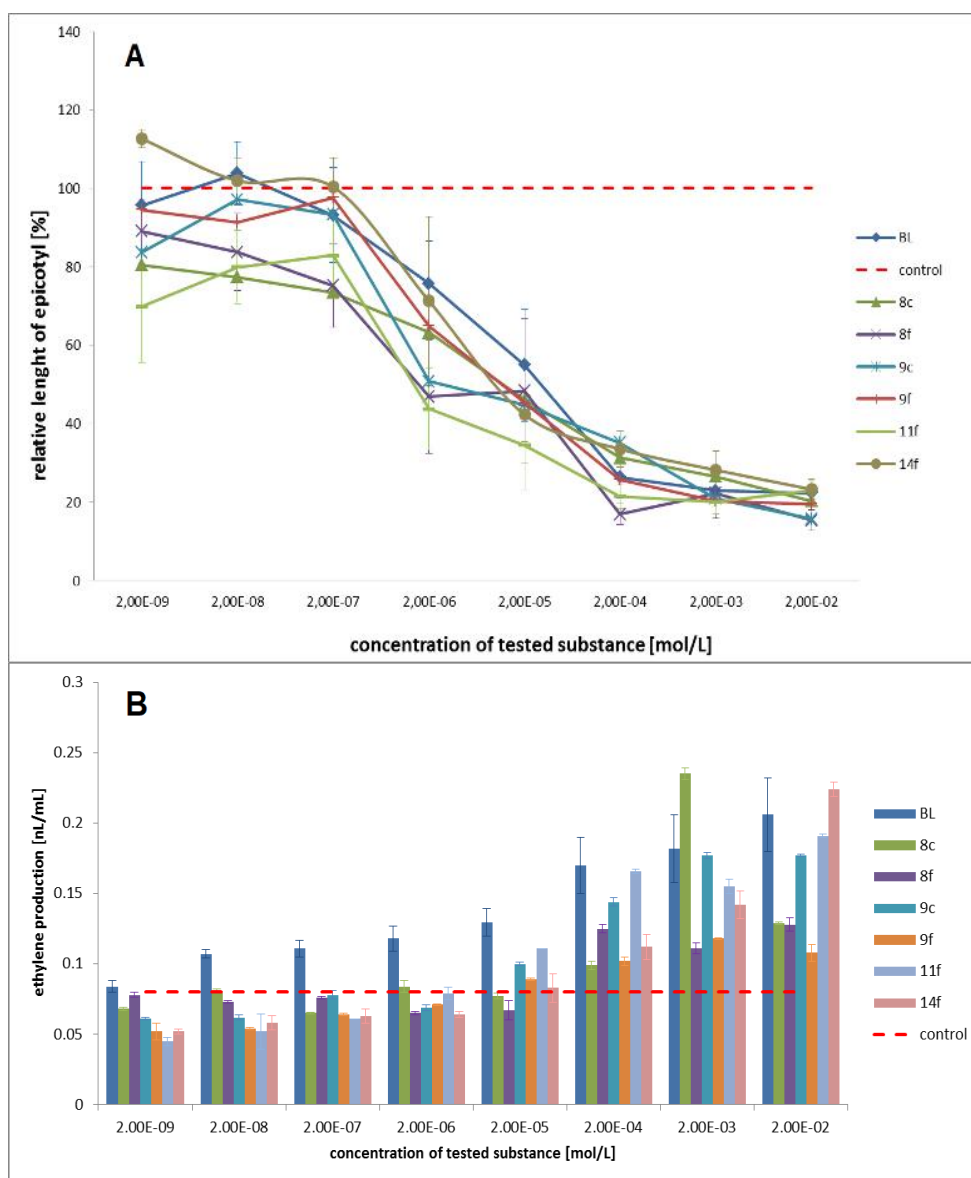


Schéma 2: Syntéza fenylových analogů pomocí cross-metateze.

Entry	Ar	Yield (%)	Entry	Ar	Yield (%)
a		81	l		69
b		65	m		71
c		73	n		57
d		75	o		66
e		64	p		71
f		70	q		61
g		75	r		58
h		59	s		60
i		66	t		32
j		73	u		na
k		65	v		na

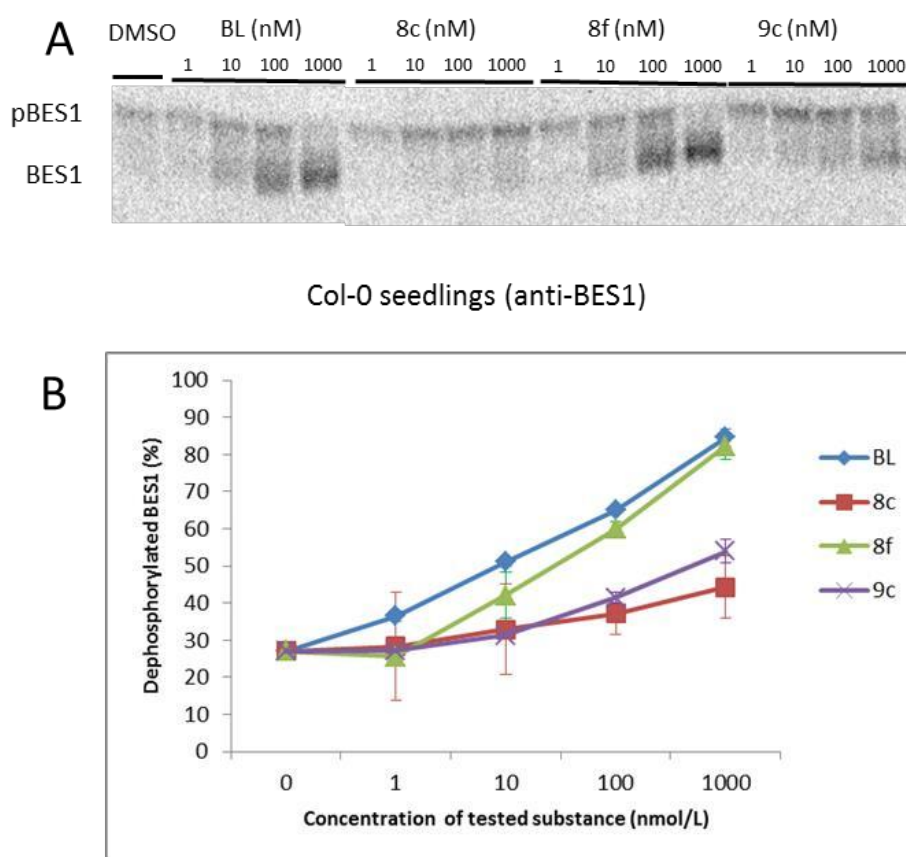
Tabulka 1: Seznam fenylových derivátů připravených metatezí podle schématu 2 s uvedením výtěžků. (na – sloučenina neizolována)

Biologické hodnocení připravených látek společně s molekulárním modelováním ukázalo, že charakter a prostorové uspořádání substituentů v aromatickém postranním řetězci významně ovlivňují interakce s receptorem BRI1 a následnou biologickou odpověď. Primární biologická aktivita byla provedena pomocí standardních rostlinných bioesejí, zejména inhibičního testu na etiolovaných hrachových rostlinách a testů citlivosti kořenů a hypokotylů modelové rostliny *Arabidopsis thaliana*. Měřena byla také produkce ethyleny (obrázek 9). Tyto experimenty umožnily porovnat účinky jednotlivých derivátů s přirozenými brassinosteroidy a identifikovat neaktivnější sloučeniny. Některé z připravených analogů vykazovaly biologickou aktivitu srovnatelnou nebo dokonce vyšší než přirozený brassinolid.



Obrázek 9: Grafy znázorňující účinek fenylových analogů připravených pomocí HWE reakce na délku epikotylu *Arabidopsis thaliana* (A) a produkci ethyleny (B). Převzato z Kvasnica M., 2016 [58].

U nejaktivnějších derivátů však hodnocení nebylo omezeno pouze na fenotypové projevy pozorované v základních bioesajích. Za účelem ověření, zda připravené sloučeniny skutečně ovlivňují brassinosteroidní signální dráhu na molekulární úrovni, byly provedeny také experimenty sledující aktivaci intracelulární signalizace. k tomu byla využita Western blot analýza transkripčního faktoru BES1, který představuje jeden z klíčových regulačních prvků brassinosteroidní signalizační dráhy [60]. Bylo sledováno relativní zastoupení fosforylované a defosforylované formy proteinu BES1, přičemž aktivace signalizační dráhy vede k akumulaci jeho aktivní defosforylované formy (obrázek 10). U nejaktivnějších látek, tj. čistý fenyl analog brassinolidu a castasteronu a *p*-fluorfenyl analog castasteronu, bylo prokázáno zvýšení podílu defosforylovaného BES1, což poskytlo přímý důkaz, že pozorované fenotypové změny nejsou pouze sekundárním účinkem, ale souvisejí s aktivací vlastní brassinosteroidní signalizační kaskády. Toto zjištění významně rozšířilo interpretaci biologických výsledků a umožnilo hlubší mechanistické pochopení účinku připravených analogů.



Obrázek 10: (A) Imunoblotová analýza proteinu BES1 u semenáčků *Arabidopsis thaliana* znázorňující defosforylaci BES1 po ošetření brassinosteroidy. (B) Graf podílu defosforylovaného BES1 z celkového množství BES1 v *Arabidopsis* (2 biologické replikáty, S.E.M.). Převzato z Kvasnica M., 2016 [58].

V další části výzkumu byla pozornost zaměřena na analogy brassinosteroidů obsahující dusík v postranním řetězci [37]. Na rozdíl od dříve studovaných fenylových derivátů byly připraveny série látek nesoucí amidové, aminové a amoniové funkční skupiny, a to jak v krátkém, tak dlouhém postranním řetězci. Syntéza vycházela z běžných steroidních

prekursorů a zahrnovala vícestupňové transformace vedoucí k dusíkatým derivátům (schéma 3). Součástí studie bylo také molekulární modelování pomocí dockingu, které umožnilo odhadnout možné uspořádání připravených derivátů ve vazebné kapse receptoru BRI1 a jejich předpokládanou vazebnou energii.

Biologická aktivita byla následně hodnocena pomocí testu citlivosti růstu kořenů u *Arabidopsis thaliana*. Přestože některé deriváty vykazovaly v *in silico* srovnatelné vazebné energie jako brassinolid, experimentální výsledky tuto predikci nepotvrdily a dusíkaté analogy vykazovaly pouze slabou nebo žádnou inhibiční aktivitu. U několika látek byl při nízkých koncentracích pozorován mírný stimulační účinek na prodlužování kořenů, avšak celkově byla jejich aktivita výrazně nižší než u přírodních brassinosteroidů. Výsledky tak ukázaly, že samotný docking není pro odhad biologické aktivity brassinosteroidních analogů dostačující, protože výsledný účinek nezávisí pouze na předpokládané afinitě k receptoru, ale také na schopnosti látky pronikat a transportovat se v rostlinných pletivech až k místu působení. Současně se ukázalo, že zatímco přítomnost nepolárních aromatických substituentů může biologickou aktivitu podporovat, zavedení polárnějších funkčních skupin, jako jsou aminy, amidy nebo amoniové soli, je z hlediska biologického účinku méně příznivé.

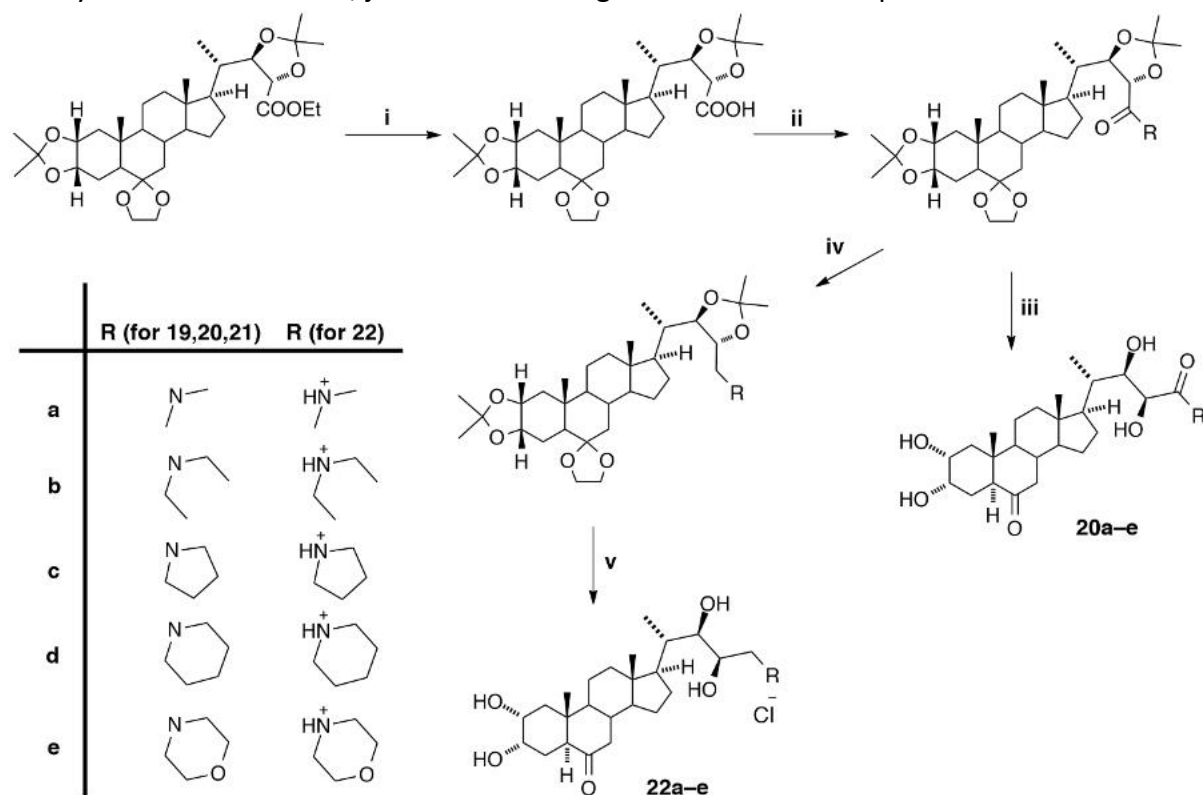


Schéma 3: Syntéza dusíkatých analogů s delším postranním řetězcem: (i) KOH, MeOH, H₂O, reflux, pak HCl; (ii) amin, HOBt, EDC, DMF, 40 °C; (iii) HCl, THF, H₂O, 45 °C, pak NaHCO₃; (iv) LiAlH₄, dioxan, reflux; (v) HCl, THF, H₂O, 45 °C.

Jako významný příspěvek k pochopení vztahů mezi strukturou a biologickou aktivitou postranního řetězce lze uvést také práci Wendeborna a kol. [47], kteří provedli systematické srovnání široké série známých i nových analogů brassinosteroidů s různě modifikovaným C-17 postranním řetězcem. Na základě krystalografických dat receptorového komplexu BRI1–

SERK1 autoři předpokládali, že tato část molekuly poskytuje větší prostor pro strukturní variace než samotné steroidní jádro. Experimentálně bylo potvrzeno, že malé substituenty, jako například cyklopropylová skupina, vedou ke ztrátě biologické aktivity, pravděpodobně v důsledku nedostatečných hydrofobních interakcí s vazebnou kapsou receptoru. Naproti tomu některé heteroaromatické deriváty, zejména thiofenové analogy, vykazovaly významnou biologickou aktivitu, zatímco polárnější oxazolové deriváty byly prakticky neaktivní.

Nejzajímavější výsledky byly získány opět u aromaticky substituovaných derivátů. Fenylové analogy a jejich para-halogenované deriváty si zachovaly vysokou biologickou aktivitu, která byla v některých případech srovnatelná s přirozenými brassinosteroidy. Naopak zavedení polárních karboxylových skupin do aromatického jádra vedlo k výraznému poklesu nebo úplné ztrátě aktivity. Obdobně se negativně projevil také příliš velký objem substituentu, jak ukázaly naftylové deriváty. Tyto výsledky potvrdily, že biologická aktivita je v této části molekuly úzce spojena se zachováním vhodných hydrofobních interakcí a optimálních sterických parametrů.

Výsledky publikované Wendebornem a kol. [47] dále podporují závěry vyplývající z našich studií aromaticky modifikovaných brassinosteroidů, zejména pokud jde o schopnost aromatických substituentů zachovat vysokou biologickou aktivitu při modifikaci postranního řetězce. v obou případech se ukázalo, že receptor BRI1 toleruje nahrazení přirozeného alkylového zakončení postranního řetězce aromatickými substituenty, což je v souladu s existencí volného prostoru v hydrofobní části vazebné kapsy receptoru. Současně však obě studie naznačily existenci sterických limitů, protože příliš objemné substituenty vedly ke snížení biologické aktivity. Přestože ve Wendebornově práci vykazoval p-chlorfenylový derivát mírně vyšší aktivitu než odpovídající p-fluorfenylový analog, zatímco v našem souboru látek byl trend opačný, nelze tento rozdíl považovat za obecný strukturně-aktivitní vztah. v obou studiích totiž mezi nejaktivnější deriváty patřil nesubstituovaný fenylový analog, což naznačuje, že rozhodující je především přítomnost aromatického systému schopného vhodně vyplnit hydrofobní oblast vazebné kapsy receptoru. Pozorované rozdíly mohou navíc souviset s odlišnou strukturou hodnocených analogů i použitými biologickými testy, které sledovaly různé aspekty brassinosteroidní odpovědi – od elongačního účinku až po aktivaci vlastní signalizační dráhy prostřednictvím BES1. Současně je však třeba zdůraznit, že aromatická modifikace postranního řetězce nevedla k jednoznačnému překonání biologické aktivity přirozených brassinosteroidů, zejména brassinolidu a castasteronu.

4.1.2 Modifikace na steroidních kruzích

Vedle rozsáhlých studií zaměřených na modifikace postranního řetězce byla pozornost věnována také změnám ve steroidním jádře [61-68]. Modifikace A a B kruhu představují další významný nástroj pro studium vztahu mezi chemickou strukturou brassinosteroidů a jejich biologickou aktivitou. v již zmiňované práci Wendeborna a kol. [47] byly kromě změn v postranním řetězci systematicky hodnoceny také změny v oblasti A- a B-kruhu steroidního

skeletu. Získané výsledky poskytly podrobnější pohled na význam jednotlivých funkčních skupin steroidního jádra pro biologickou aktivitu brassinosteroidů a jejich interakce s receptorovým komplexem BRI1–SERK1. Největší pozornost byla věnována hydroxylovým skupinám v polohách C-2 a C-3, které patří mezi charakteristické strukturní prvky biologicky aktivních brassinosteroidů. Systematické srovnání připravených analogů ukázalo, že odstranění obou hydroxylových skupin vedlo prakticky k úplné ztrátě biologické aktivity, což potvrdilo jejich zásadní význam pro rozpoznání ligandu receptorem. Zajímavé výsledky však přineslo hodnocení derivátů obsahujících pouze jednu z těchto hydroxylových skupin. Zatímco analog nesoucí pouze hydroxyl v poloze C-3 vykazoval pouze střední biologickou aktivitu, derivát obsahující výhradně hydroxylovou skupinu v poloze C-2 si zachoval překvapivě vysokou účinnost [47]. Tento výsledek byl v souladu s krystalovou strukturou receptorového komplexu, která ukázala, že hydroxylová skupina v poloze C-2 vytváří přímé interakce s koreceptorem SERK1 a významně přispívá ke stabilizaci aktivního receptorového komplexu. Naopak methylace hydroxylových skupin nebo jejich náhrada objemnějšími substituenty vedly k výraznému snížení biologické aktivity. Významnou část studie představovaly také modifikace B-kruhu. Autoři zkoumali zejména vliv fluorace v poloze C-5 a methylace v poloze C-7, tedy změn, které zachovávají základní uspořádání steroidního skeletu, avšak mění jeho elektronové a sterické vlastnosti. Fluorované deriváty vykazovaly ve srovnání s odpovídajícími nefluorovanými analogy nižší biologickou aktivitu. Podobně byla aktivita snížena také u derivátů nesoucích methylovou skupinu v poloze C-7, přičemž rozsah tohoto poklesu závisel na struktuře konkrétního analogu.

Výsledky tak ukázaly, že i relativně malé změny v oblasti B-kruhu mohou významně ovlivnit biologické vlastnosti brassinosteroidů. Soubor získaných dat dále rozšířil poznatky o vztazích mezi strukturou a biologickou aktivitou brassinosteroidů. Zatímco výsledky diskutované v předchozí kapitole ukázaly, že vysokou biologickou aktivitu lze v některých případech zachovat i po rozsáhlejších modifikacích postranního řetězce, studie změn v oblasti A- a B-kruhu potvrdila mimořádný význam správného uspořádání funkčních skupin steroidního jádra pro biologické působení těchto rostlinných hormonů.

Na poznatky získané studiem modifikací hydroxylových skupin na A-kruhu navázala naše práce zaměřená na přípravu 2,3-seco analogů brassinosteroidů [69]. Základní myšlenkou bylo ověřit, zda je pro biologickou aktivitu rozhodující samotná přítomnost hydroxylových skupin v polohách C-2 a C-3, nebo zda je nezbytné také jejich přesné prostorové uspořádání dané rigidní strukturou A-kruhu. Rozštěpením vazby mezi atomy C-2 a C-3 měl vzniknout flexibilnější systém, ve kterém by se obě hydroxylové skupiny mohly orientovat nezávisleji než u přirozených brassinosteroidů. v průběhu syntetických studií se však ukázalo, že otevření A-kruhu významně ovlivňuje chemické vlastnosti připravovaných sloučenin. Pokusy o přípravu odpovídajících 2,3-seco-2,3-dihydroxy-6-etonů opakovaně vedly k intramolekulárním cyklizačním reakcím a vzniku termodynamicky stabilnějších produktů. U cholestanové řady byly pozorovány přeměny vedoucí nejprve ke vzniku cyklického enoletheru **5a** a následně intramolekulárního ketalu obsahujícího nově vytvořené pěti- a šestičlenné kruhy **5b** (schéma 4). Obdobné chování bylo zaznamenáno také u stigmastanových analogů, přičemž pouze

v případě 2,3-seco-28-homocasteronu se podařilo izolovat odpovídající dihydroxyketon, a to pouze v omezeném výtěžku a čistotě. Ani příprava B-laktonových analogů nevedla k úplnému odstranění těchto komplikací. U některých derivátů byly pozorovány další intramolekulární přeměny vedoucí ke vzniku strukturálně odlišných laktonů, které byly zřejmě termodynamicky stabilnější než původně očekávané produkty, viz derivát **6** (schéma 4). Tyto výsledky ukázaly, že rozštěpení A-kruhu vytváří předpoklady pro vznik nových intramolekulárních interakcí a výrazně ovlivňuje stabilitu vznikajících struktur.

Biologické hodnocení připravených seco derivátů neprokázalo očekávané zachování biologické aktivity vyplývající z větší konformační volnosti hydroxylových skupin. Interpretace těchto výsledků však není jednoznačná, protože syntetické studie současně odhalily výraznou tendenci otevřených 2,3-seco struktur k intramolekulární cyklizaci. Nelze proto vyloučit, že k obdobným přeměnám docházelo alespoň částečně i během biologických experimentů a že pozorovaná aktivita nebo její absence byla ovlivněna přítomností cyklických produktů vznikajících z původně zamýšlených seco derivátů. Získané výsledky tak neumožňují jednoznačně posoudit vliv samotné flexibility hydroxylových skupin na biologickou aktivitu brassinosteroidů. Současně však ukázaly, že zachování integrity A-kruhu je důležité nejen z hlediska biologické funkce, ale také z pohledu chemické stability molekuly.

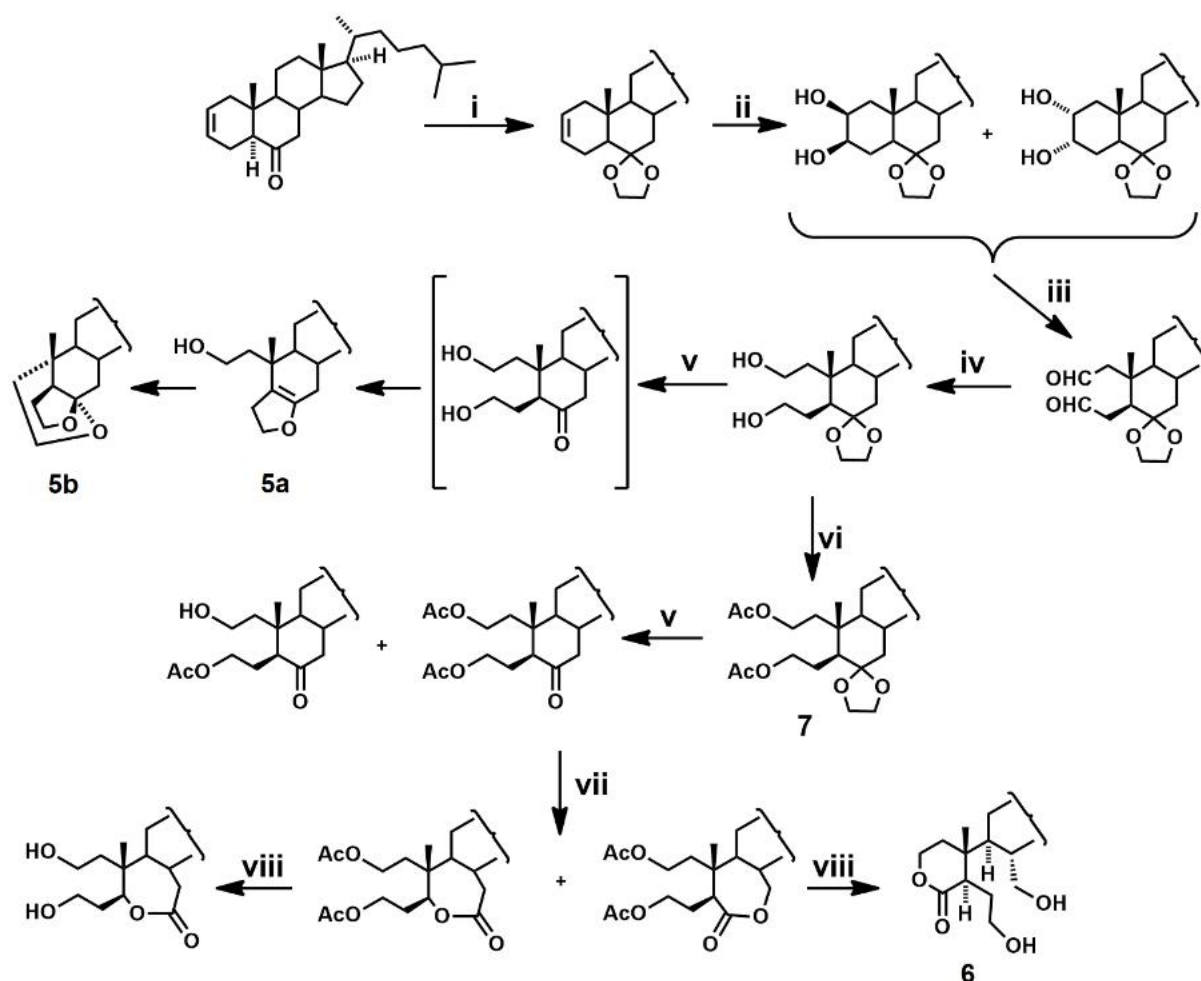


Schéma 4: Syntéza 2,3-secocholestanových derivátů: (i) $(\text{CH}_2\text{OH})_2$, TsOH, PhMe, reflux; (ii) OsO_4 , NMO, THF, aceton; (iii) NaIO_4 , silikagel, CH_2Cl_2 ; (iv) LiAlH_4 , THF, reflux; (v) HCl, THF, H_2O , 40 °C; (vi) Ac_2O , pyridin; (vii) $\text{CF}_3\text{CO}_3\text{H}$, CH_2Cl_2 ; (viii) MeONa, MeOH, THF.

Studium biologické aktivity syntetických analogů významně rozšířilo poznatky o vztazích mezi strukturou a účinkem brassinosteroidů. Získané výsledky současně ukázaly, že samotné hodnocení fytohormonální aktivity neumožňuje vždy jednoznačně vysvětlit molekulární podstatu pozorovaných rozdílů mezi jednotlivými deriváty. Pro hlubší pochopení těchto vztahů je proto nezbytné zohlednit také jejich interakce s brassinosteroidními receptory.

4.2 Chemická biologie brassinosteroidních receptorů

4.2.1 Brassinosteroidní receptorové kinázy

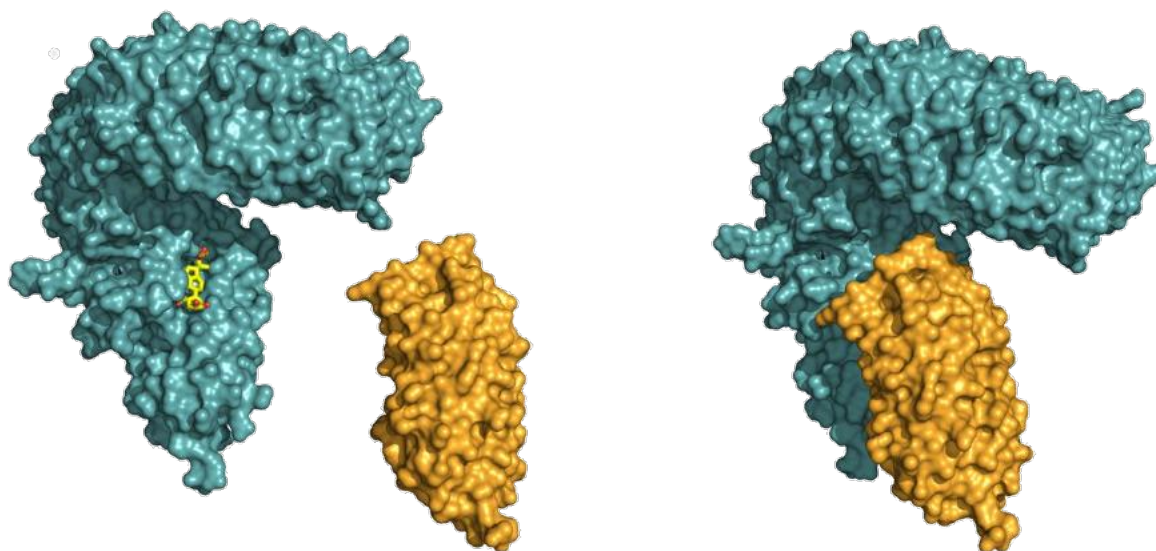
Brassinosteroidní receptor BRI1 (BRASSINOSTEROID INSENSITIVE 1) představuje důležitý prvek brassinosteroidní signalizace a jeden z nejlépe charakterizovaných receptorů rostlinných hormonů [70,71]. Jeho identifikace poskytla první důkaz, že percepce steroidních hormonů u rostlin probíhá prostřednictvím membránově lokalizovaných receptorových kináz [72-74]. BRI1 náleží do rozsáhlé rodiny leucinem bohatých receptor-like kináz (LRR-RLK) a je tvořen extracelulární doménou obsahující na leucin bohaté repetice, jednou transmembránovou oblastí a intracelulární serin/threoninovou kinázovou doménou. Charakteristickým strukturním prvkem receptoru je přítomnost tzv. ostrovní (island) domény vložené mezi repetice extracelulární části, která společně s LRR doménou vytváří vazebnou kapsu pro brassinosteroidní ligandy.

Pozdější studie odhalily existenci dalších členů receptorové rodiny BRI1, označovaných jako BRL1, BRL2 a BRL3 (*BRI1-LIKE 1–3*) [75]. Receptory BRL1 a BRL3 vykazují vysokou sekvenční i strukturní podobnost s BRI1 a jsou exprimovány převážně ve vaskulárních pletivech. Strukturální analýzy ukázaly, že jejich vazebné kapsy jsou téměř identické s receptorem BRI1, což se odráží také v podobném spektru vázaných brassinosteroidních ligandů [75,76]. Funkční studie navíc prokázaly, že oba receptory jsou za určitých podmínek schopny částečně komplementovat defekty vznikající při narušení funkce BRI1, což svědčí o značné míře funkční redundance v rámci této receptorové rodiny [75,76].

Odlišné postavení zaujímá receptor BRL2, známý rovněž pod označením VASCULAR HIGHWAY 1 (VH1) [75]. Přestože byl dlouhou dobu považován za receptor bez přímé účasti na percepci brassinosteroidů, recentní strukturní a biochemické studie prokázaly přítomnost zachovalé steroid-vazebné kapsy schopné interagovat s vybranými brassinosteroidy [77]. Ve srovnání s BRI1, BRL1 a BRL3 však vykazuje nižší afinitu k testovaným ligandům a jeho fyziologická funkce zůstává předmětem dalšího výzkumu. Současné poznatky naznačují, že evoluční diverzifikace receptorové rodiny není spojena pouze se změnami ligandové specifity, ale také s odlišnou tkáňovou expresí a regulací následných signalizačních procesů.

Klíčovým krokem aktivace brassinosteroidní signalizace je vytvoření receptorového komplexu zahrnujícího kromě receptoru také koreceptor ze skupiny SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK), zejména SERK1 a SERK3/BAK1 [78-80]. Vazba brassinosteroidu do extracelulární kapsy receptoru umožňuje asociaci obou proteinů a vznik

aktivního komplexu receptor–ligand–koreceptor. Brassinosteroid zde funguje jako tzv. „molekulární lepidlo“ stabilizující interakci mezi oběma receptory (obrázek 11). Následná trans-fosforylace intracelulárních kinázových domén vede k aktivaci signalizační kaskády zakončené transkripčními faktory BES1 a BZR1, které představují hlavní prvky brassinosteroidní odpovědi.



Obrázek 11: Receptor BRI1 (modrozelená) s navázaným brassinolidem a následná tvorba receptorového komplexu BRI1–SERK1 (oranžová). Vytvořeno dle Santiago J. et al., 2013 [78].

4.2.2 Brassinosteroidní signální dráha

Vazba brassinosteroidu na receptorový komplex představuje pouze počáteční krok rozsáhlé signální kaskády, která převádí extracelulární signál na změny genové exprese v buněčném jádře. Bez přítomnosti brassinosteroidů je receptor BRI1 udržován v neaktivním stavu prostřednictvím proteinu BKI1 (BRI1 KINASE INHIBITOR 1), který se váže na jeho cytoplazmatickou doménu a omezuje aktivaci receptorového komplexu [81]. Po navázání brassinosteroidu dochází k fosforylaci BKI1 receptorem BRI1 a jeho disociaci od plazmatické membrány a samotného receptoru. Fosforylovaný BKI1 následně interaguje s proteiny rodiny 14-3-3, čímž přispívá k efektivní aktivaci brassinosteroidní signalizační dráhy. Aktivovaný receptorový komplex současně fosforyluje časně cytoplazmatické komponenty signalizace, mezi které patří především receptor-like cytoplazmatické kinázy BSK1 (BR-SIGNALING KINASE 1) [82] a CDG1 (CONSTITUTIVE DIFFERENTIAL GROWTH 1) [83]. Zatímco BSK1 interaguje s fosfatázou BSU1 (BRI1 SUPPRESSOR 1), CDG1 ji přímo fosforyluje a podporuje její aktivaci [84].

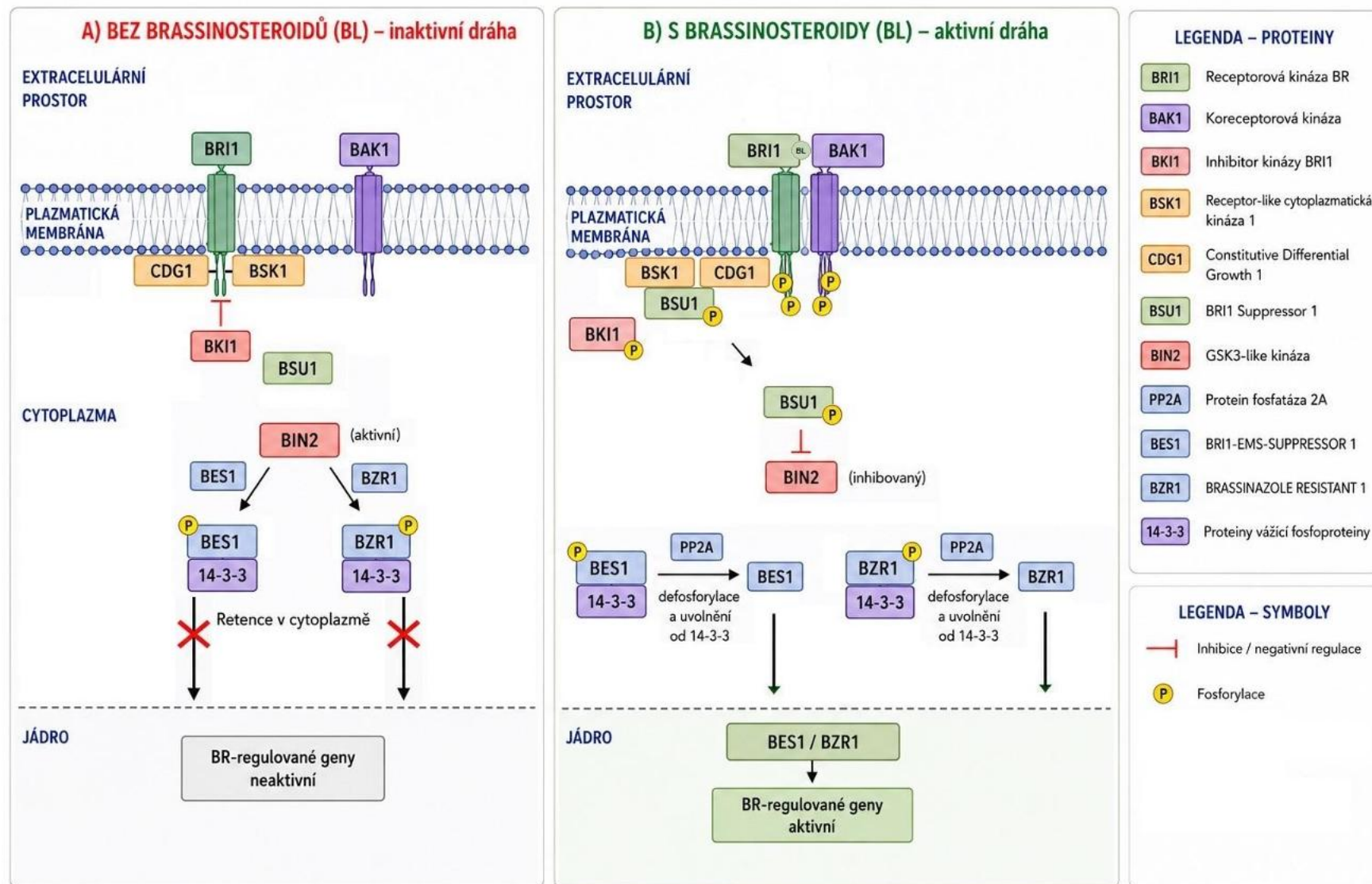
Klíčovým regulačním uzlem celé signalizační dráhy je serin/threoninová kináza BIN2 (BRASSINOSTEROID INSENSITIVE 2), která v nepřítomnosti brassinosteroidů funguje jako hlavní negativní regulátor odpovědi na tyto fytohormony [85]. Aktivní BIN2 fosforyluje transkripční regulátory BES1 (BRI1-EMS-SUPPRESSOR 1) [60,86] a BZR1 (BRASSINAZOLE

RESISTANT 1) [87,88], čímž omezuje jejich schopnost regulovat expresi cílových genů. Fosforylované formy BES1 a BZR1 navíc interagují s proteiny rodiny 14-3-3, které podporují jejich retenci v cytoplazmě nebo export z jádra [89]. Tím je zabráněno aktivaci brassinosteroidy regulovaných genů a signalizační dráha zůstává utlumená.

Aktivace receptorového komplexu postupně vede k odstranění represivního vlivu BIN2. Podle klasického modelu brassinosteroidní signalizace se na tomto procesu podílí fosfatáza BSU1, která defosforyluje tyrosinový zbytek Tyr200 v molekule BIN2 [90]. Tento krok je spojován se snížením aktivity kinázy a současně umožňuje interakci BIN2 s E3 ubikvitin ligázou KIB1 (KINK SUPPRESSED IN BZR1-1D). KIB1 následně zprostředkovává polyubikvitinaci BIN2 a její degradaci prostřednictvím 26S proteazomu, což vede k dalšímu omezení represivního působení této kinázy [91]. Přestože je role KIB1 v degradaci BIN2 dobře experimentálně podložena, přesný význam defosforylace Tyr200 pro regulaci aktivity BIN2 zůstává předmětem výzkumu.

Potlačení aktivity BIN2 posouvá rovnováhu ve prospěch defosforylovaných forem transkripčních regulátorů BES1 a BZR1. Na jejich aktivaci se podílí protein fosfatáza PP2A (PROTEIN PHOSPHATASE 2A), která oba proteiny přímo defosforyluje [92]. Defosforylované formy BES1 a BZR1 ztrácejí schopnost vázat proteiny 14-3-3, akumulují se v buněčném jádře a regulují expresi velkého počtu brassinosteroidy responzivních genů. Výsledkem je aktivace fyziologických procesů spojených s elongací buněk, diferenciací pletiv, vývojem cévních svazků, reprodukčním vývojem a dalšími růstovými odpověďmi rostlin na brassinosteroidy.

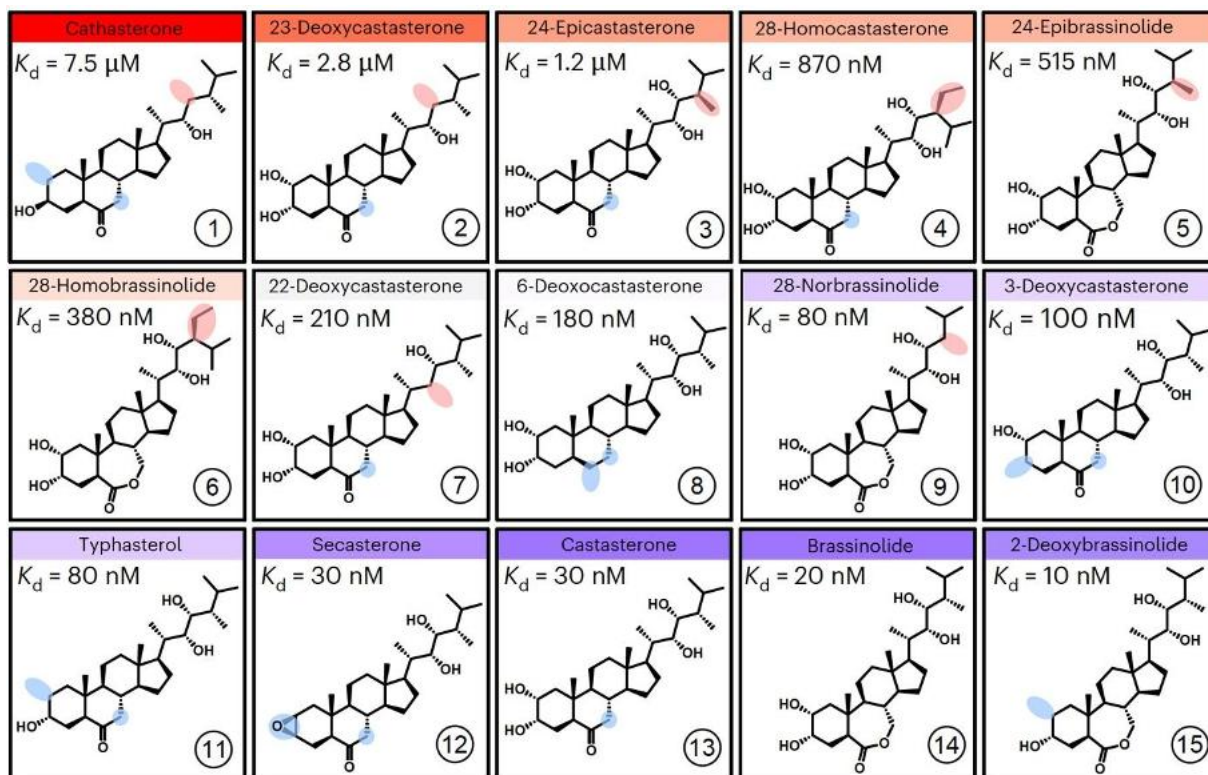
Přestože základní uspořádání brassinosteroidní signalizační dráhy je dnes dobře popsáno, některé její části zůstávají předmětem intenzivního výzkumu. Zejména není dosud zcela objasněn přesný mechanismus, kterým fosfatáza BSU1 reguluje aktivitu BIN2, ani význam jednotlivých kroků navrženého modelu zahrnujícího defosforylaci Tyr200 a následnou degradaci BIN2 zprostředkovanou ligázou KIB1. Určité nejasnosti přetrvávají rovněž v případě proteinu BSK1, jehož přesná molekulární funkce v přenosu signálu od receptorového komplexu k downstream komponentám nebyla dosud plně objasněna. Naproti tomu role CDG1 jako kinázy fosforylující BSU1, stejně jako funkce BIN2, PP2A a transkripčních regulátorů BES1/BZR1 patří mezi nejlépe experimentálně podložené části celé brassinosteroidní signalizační dráhy. Zjednodušené schéma celé dráhy je na obrázku 12.



Obrázek 12: Zjednodušené schéma brassinosteroidní signální dráhy (popis viz text). Schéma navrženo ChatGPT a upraveno autorem.

4.2.3 Vliv struktury brassinosteroidů na afinitu k receptoru BRI1

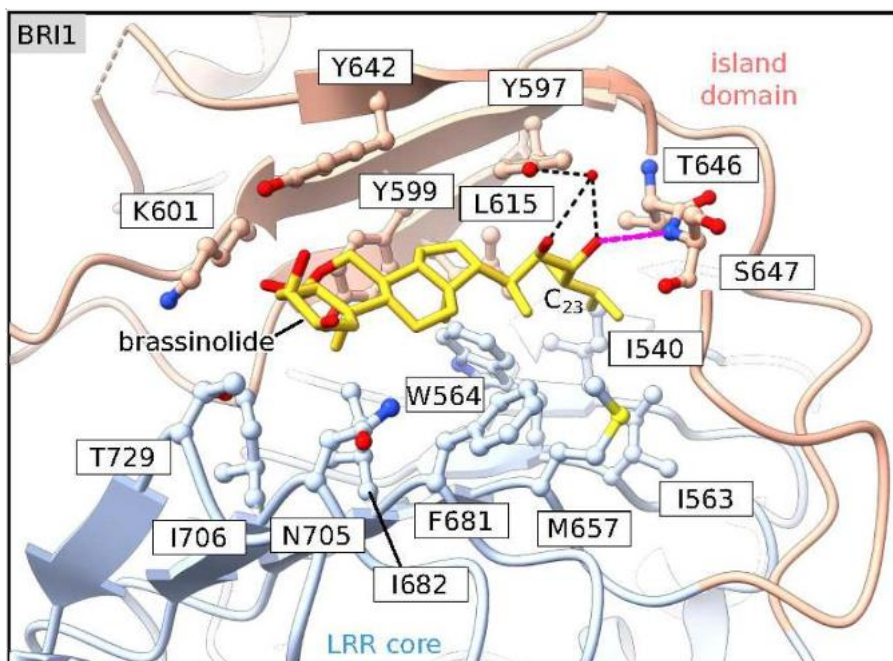
Přestože je brassinolid tradičně považován za hlavní ligand receptoru BRI1, rostliny obsahují celou řadu dalších brassinosteroidů, které se od sebe liší strukturou steroidního jádra i postranního řetězce. Teprve systematická studie zaměřená na vazbu jednotlivých brassinosteroidů k receptoru BRI1 umožnila podrobněji určit, které strukturální prvky molekuly jsou pro vysokou afinitu k receptoru skutečně důležité [77]. v rámci této studie jsme ve spolupráci s týmem profesora Michaela Hothorna z Univerzity v Ženevě zjistili, že receptor BRI1 je schopen vázat poměrně široké spektrum přirozených brassinosteroidů a že jeho ligandová specifita je méně striktní, než se původně předpokládalo. Pomocí metody GCI (Grating-Coupled Interferometry) [93-94] jsme stanovili rovnovážné disociační konstanty (K_d) komplexů receptoru BRI1 s jednotlivými ligandy (obrázek 13). Hodnota této konstanty vyjadřuje afinitu ligandu k receptoru, přičemž nižší hodnota K_d odpovídá vyšší afinitě. z naměřených hodnot vyplynulo, že ze všech testovaných brassinosteroidů vykazoval nejvyšší afinitu k BRI1 receptoru 2-deoxybrassinolid, jehož rovnovážná disociační konstanta byla přibližně dvakrát nižší než v případě přirozeného ligandu brassinolidu. Příprava mnou připravených testovaných analogů je znázorněna ve schématech 5 a 6.



Obrázek 13: Struktura brassinosteroidů a jejich rovnovážná disociační konstanta s receptorem BRI1. Seřazeno od nejnižší afinity (červená) k nejvyšší (modrá). Ve struktuře znázorněny rozdíly proti struktuře brassinolidu, červená – změna snižuje afinitu, modrá – změna má malý vliv. Kinetika vazby ligandů byla analyzována metodou GCI. Převzato z Caregato A. et al., 2026 [77].

Nejvýznamnější vliv na afinitu k receptoru má struktura postranního řetězce. Klíčovou roli hraje především hydroxylová skupina na uhlíku C23, jejíž odstranění vede k výraznému

poklesu afinity. Strukturní analýzy ukázaly, že právě tato hydroxylová skupina vytváří silnou vodíkovou vazbu s island doménou receptoru a podílí se na stabilizaci receptor–ligandového komplexu. Naproti tomu hydroxylová skupina na uhlíku C22 se jeví jako méně důležitá, protože její absence snižuje afinitu pouze částečně (obrázek 14). Významná je rovněž stereochemie na uhlíku C24. Epimery brassinolidu a castasteronu nesoucí opačnou konfiguraci v této pozici se vážou podstatně hůře než přirozené ligandy. Podobný účinek byl pozorován také u C29 brassinosteroidů s prodlouženým postranním řetězcem, což naznačuje, že receptor citlivě reaguje nejen na konfiguraci, ale i na velikost této části molekuly.



Obrázek 14: Interakce brassinolidu s receptorem BRI1. Převzato z Caregato A. et al., 2026 [77].

Naopak změny ve steroidním jádře ovlivňují vazbu k receptoru výrazně méně. Bylo například zjištěno, že přítomnost sedmičlenného laktonu v B-kruhu není pro vazbu nezbytná, protože brassinolid a castasteron vykazují srovnatelnou afinitu. Podobně je receptorem dobře tolerována absence ketoskupiny na uhlíku C6 i některých hydroxylových skupin na A-kruhu. Vysokou afinitu si tak zachovávají i některé biosyntetické intermediáty, například typhasterol nebo 6-deoxycasteron. Tyto výsledky naznačují, že z hlediska samotného rozpoznání ligandu hraje steroidní skelet spíše podpůrnou úlohu.

Souhrnně lze říci, že afinitu brassinosteroidů k receptoru BRI1 určují především strukturní vlastnosti postranního řetězce, zejména přítomnost hydroxylové skupiny na C23 a správná konfigurace substituentu na uhlíku C24. Naproti tomu receptor vykazuje poměrně vysokou toleranci vůči modifikacím steroidního jádra. Tato kombinace selektivity a současně určité flexibility pravděpodobně umožňuje BRI1 rozpoznávat více přirozeně se vyskytujících brassinosteroidů, které se mohou podílet na regulaci signalizace v různých pletivech a vývojových stádiích rostlin.

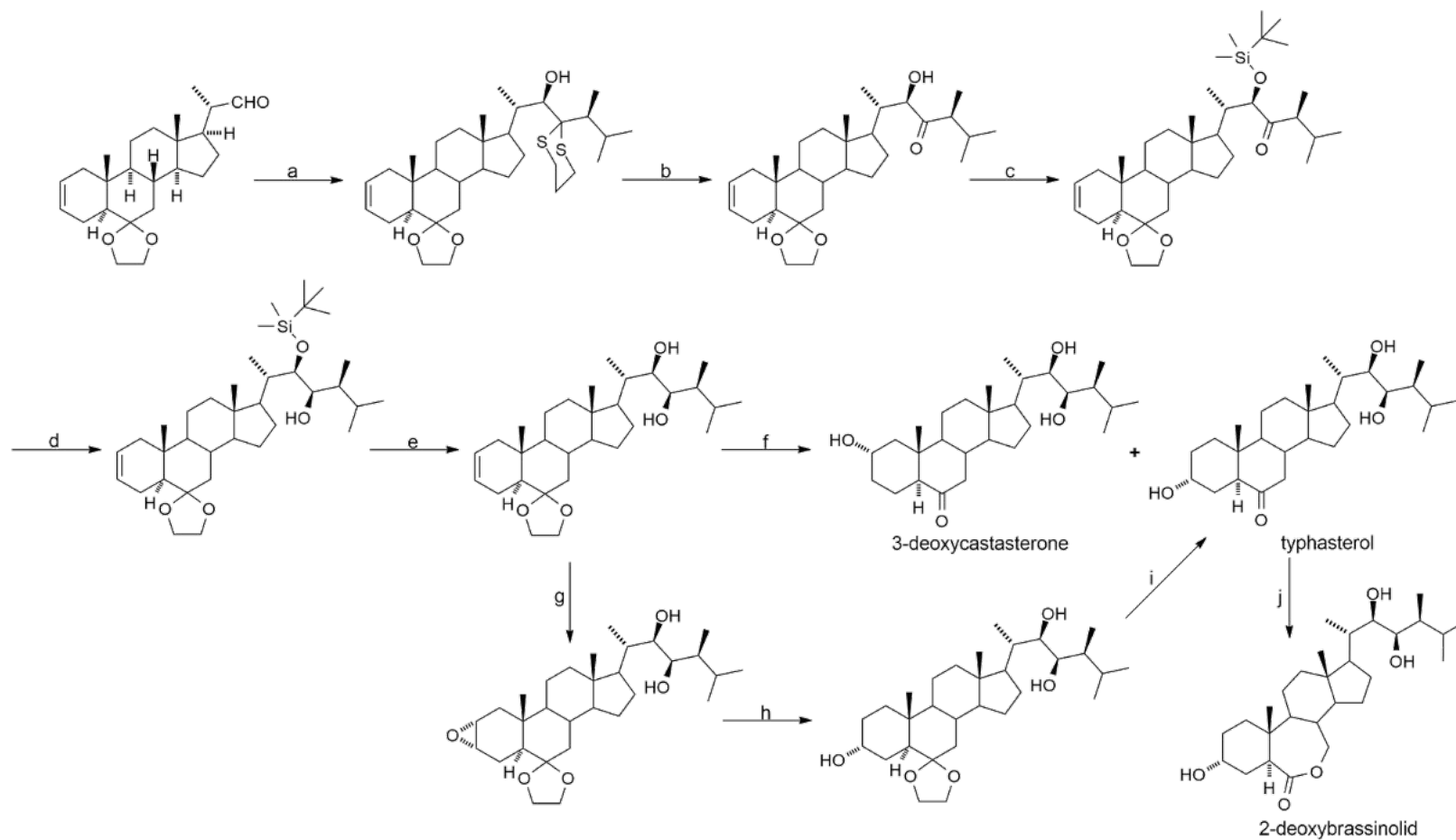


Schéma 5: Syntéza 3-deoxycastasteronu (7), typhasterolu (8) a 2-deoxybrassinolidu (11). a: (S)-2-(3-methylbutan-2-yl)-1,3-dithian/n-BuLi/THF, r.t. pak -78 °C, 1h; b: NaH₂PO₄/2-methyl-2-buten/ NaClO₂/H₂O/ethanol, 0 °C, 10 min; c: TBSOTf/2,6-lutidin/CH₂Cl₂, 0 °C, 1 h; d: DIBAL-H/toluen, -78 °C, 1h; e: TBAF/THF, r.t., 1h; f: 1, BH₃/THF, r.t., 6h; 2, NaOH/H₂O₂/H₂O/THF, r.t., 12h; g: MCPBA/EtOAc, r.t., 4h; h: LiAlH₄/THF/Et₂O, 40 °C, 3h; i: HCl/H₂O/THF, 40 °C, 4h; j: TFPA/CH₂Cl₂, r.t., 4h.

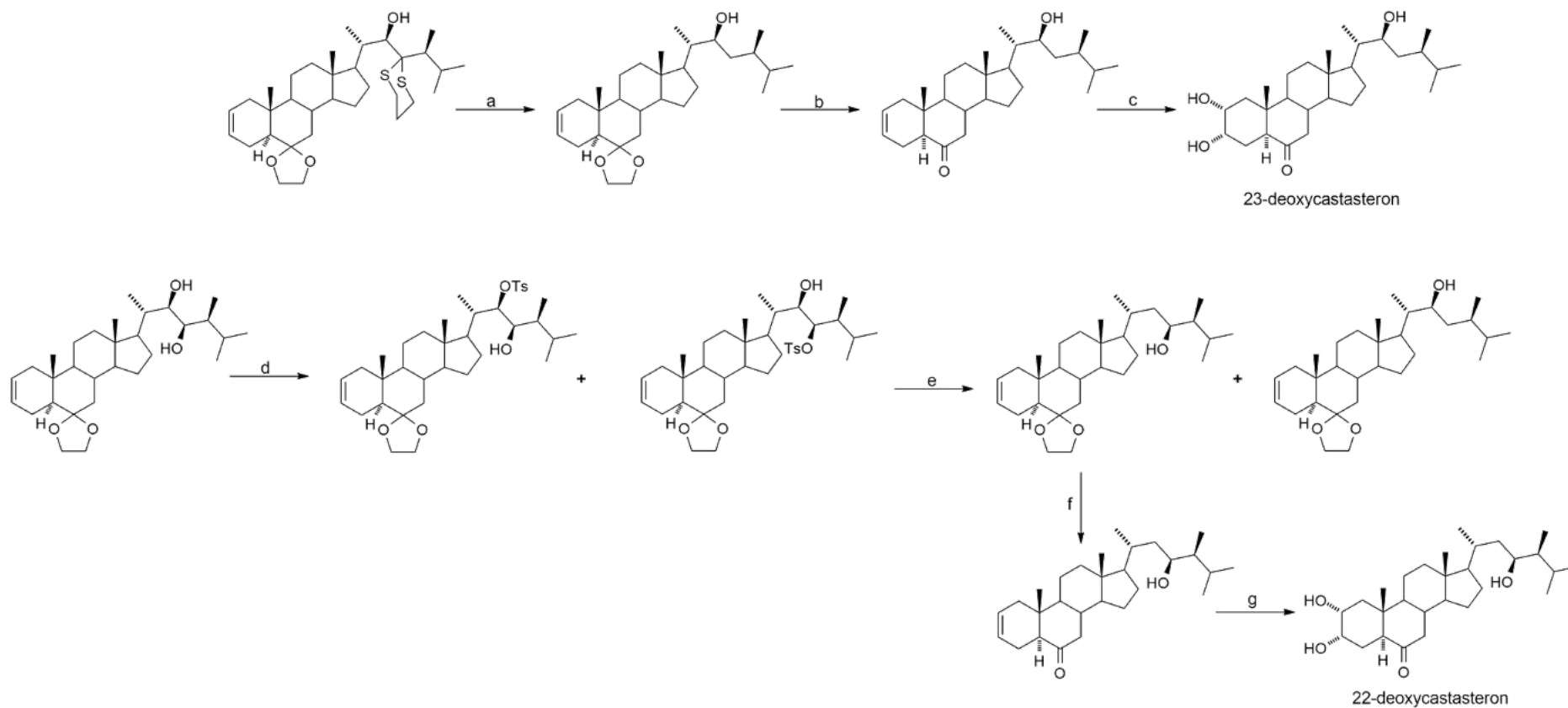
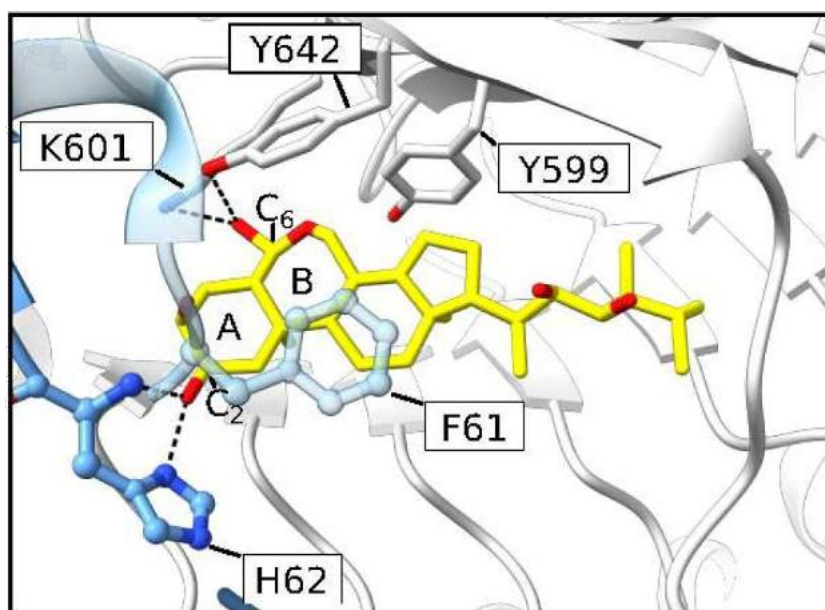


Schéma 6: Syntéza 23-deoxycastasteronu (15) a 22-deoxycastasteronu (20). a: Ra-Ni/EtOH, r.t., 6h; b: HCl/H₂O/THF, 40 °C, 4h; c: OsO₄/K₂CO₃/K₂[Fe(CN)₆]/CH₃SO₂NH₂/HQ-4-ClBz/t-BuOH/H₂O, r.t., 10 h; d: *p*-TsCl/pyridin, r.t., 3 dny; e: LiAlH₄/THF/Et₂O, r.t., 3h; f: HCl/H₂O/THF, 40 °C, 2h; g: OsO₄/K₂CO₃/K₂[Fe(CN)₆]/CH₃SO₂NH₂/HQ-4-ClBz/t-BuOH/H₂O, r.t., 10 h.

4.2.4 Vliv struktury brassinosteroidů na afinitu ke koreceptorům SERK

Schopnost brassinosteroidu navázat se na receptor BRI1 však sama o sobě nezaručuje aktivaci signalizace. Po navázání ligandu dochází ke konformačním změnám extracelulární části receptoru, které umožňují vytvoření aktivního receptorového komplexu s členy rodiny SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) [78,79]. U modelové rostliny *Arabidopsis thaliana* bylo pro signalizaci brassinosteroidů popsáno několik členů této rodiny, především SERK1, SERK3/BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) a SERK4/BKK1 (BAK1-LIKE 1), které fungují jako koreceptory a po vytvoření komplexu s BRI1 iniciují fosforylační kaskádu vedoucí k aktivaci BR signalizace. z tohoto důvodu je vedle samotné afinity k BRI1 nezbytné zohlednit také schopnost ligandu podporovat vznik receptor–koreceptorového komplexu.



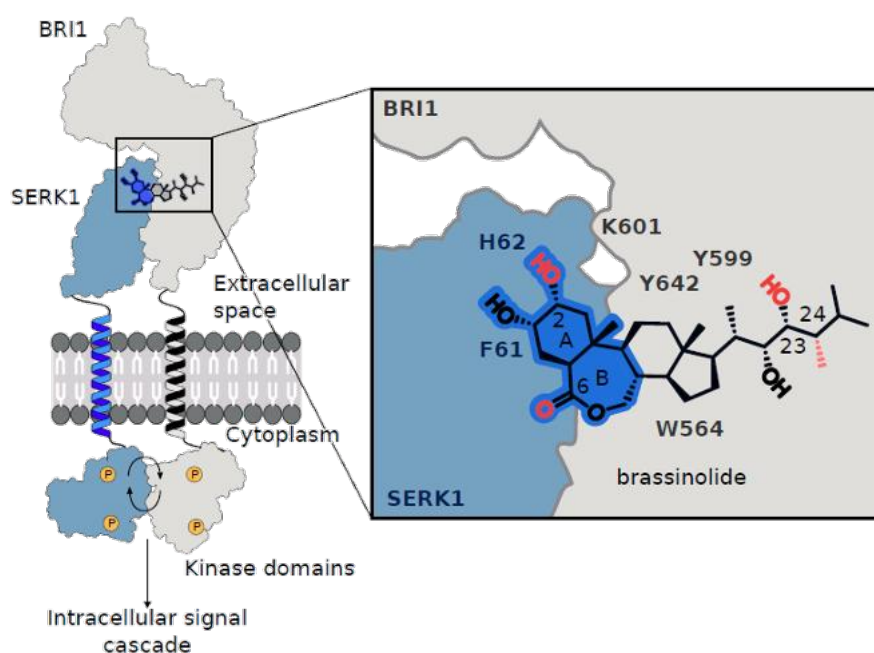
Obrázek 15: Interakce brassinolidu s koreceptorem SERK1. Převzato z Caregato A. et al., 2026 [77].

Ve spolupráci se skupinou profesora Michaela Hothorna z Univerzity v Ženevě jsme se z tohoto důvodu zabývali také otázkou, které strukturní prvky brassinosteroidů jsou nezbytné pro vytvoření aktivního receptorového komplexu [77]. Zatímco naše předchozí výsledky ukázaly, že receptor BRI1 je schopen vázat poměrně široké spektrum přirozených brassinosteroidů, ukázalo se, že požadavky na následnou interakci s koreceptorem jsou podstatně specifitější.

Podrobnější strukturní a biochemické studie ukázaly, že požadavky na vazbu koreceptoru jsou v některých ohledech odlišné od požadavků na samotnou vazbu k BRI1. Zatímco vysokou afinitu k receptoru určují především vlastnosti postranního řetězce, pro efektivní interakci se SERK proteiny jsou důležité některé strukturní prvky steroidního jádra. Klíčovou roli zde hraje hydroxylová skupina na uhlíku C2, která vytváří přímý kontakt s histidinem His62 v extracelulární doméně SERK proteinů. Ligandy postrádající tuto hydroxylovou skupinu si mohou zachovávat vysokou afinitu k BRI1, avšak jejich schopnost podporovat tvorbu aktivního receptorového komplexu je výrazně snížena (obrázek 15).

Typickým příkladem je typhasterol nebo 2-deoxybrassinolid. Obě sloučeniny se vážou na BRI1 s relativně vysokou afinitou, avšak ve srovnání s brassinolidem nebo castasteronem podstatně hůře podporují vazbu SERK koreceptorů. Tyto výsledky ukázaly, že vazbu ligandu k receptoru a následnou aktivaci signalizace nelze považovat za totožné procesy. Některé brassinosteroidy jsou schopny obsadit vazebnou kapsu BRI1, aniž by současně zajistily správné prostorové uspořádání nezbytné pro asociaci se SERK proteiny.

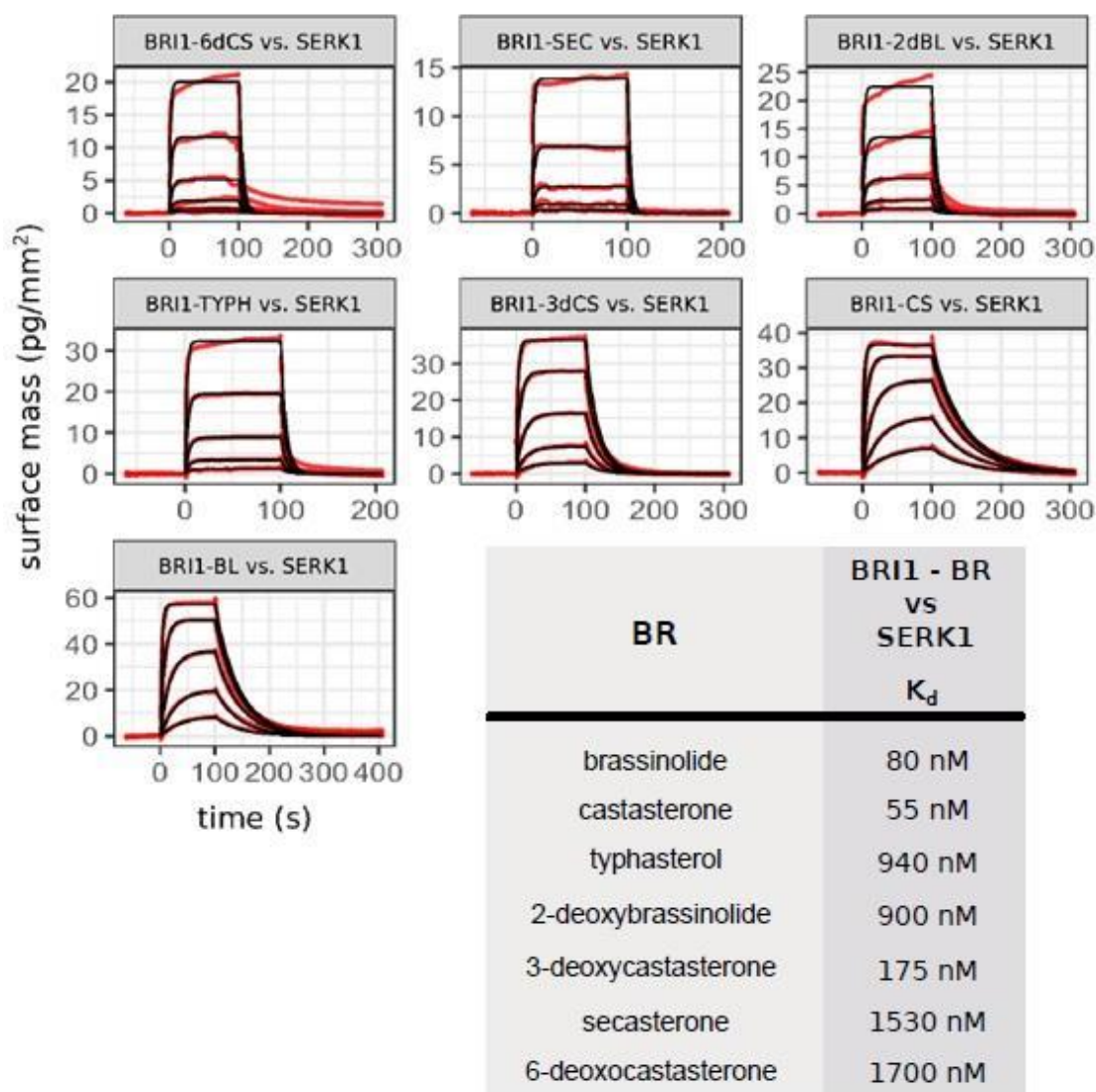
Význam správné orientace ligandu v receptorové kapse dále potvrzují naše výsledky získané pro secasteron a 6-deoxycasteron. Přestože se oba ligandy vážou k BRI1 poměrně účinně, změny ve struktuře steroidního jádra vedou ke zhoršené tvorbě receptor–koreceptorového komplexu. Ukazuje se tedy, že některé funkční skupiny, které nejsou nezbytné pro samotnou vazbu k receptoru, mohou být důležité pro přesné uspořádání ligandu na rozhraní mezi BRI1 a SERK proteiny. Právě toto rozhraní vytváří aktivní signální jednotku schopnou přenášet informaci do cytoplazmy. Funkční skupiny důležité pro receptor či koreceptor jsou znázorněny na obrázku 16.



Obrázek 16: Schéma receptorového komplexu BRI1 (šedá) – SERK1 (modrá) a klíčových interakcí brassinolidu ve vazebné kapse. Klíčové funkční skupiny brassinolidu znázorněny červeně. Převzato z Caregato A. et al., 2026 [77].

Tyto poznatky významně rozšířily současné chápání mechanismu percepce brassinosteroidů. Ukázalo se totiž, že biologická aktivita jednotlivých brassinosteroidů není dána pouze jejich afinitou k receptoru BRI1, ale také schopností stabilizovat komplex s koreceptory rodiny SERK (obrázek 17). Některé přirozeně se vyskytující biosyntetické intermediáty tak mohou působit jako slabí agonisté nebo dokonce antagonisté signalizace, protože obsazují vazebné místo receptoru, avšak neumožňují efektivní nábor koreceptorů. Tvorba aktivního komplexu BRI1–SERK se tak jeví jako druhý stupeň molekulární selekce, který

společně s afinitou ligandu k receptoru určuje výslednou intenzitu brassinosteroidní odpovědi.



Obrázek 17: GCI senzogramy znázorňující stabilitu komplexu BRI1-SERK1 a několika brassinosteroidů jako ligandů. v tabulce rovnovážné disociační konstanty komplexů. Převzato z Caregato A. et al., 2026 [77].

4.2.5 Vliv struktury brassinosteroidů na afinitu k receptorům BRL1-3

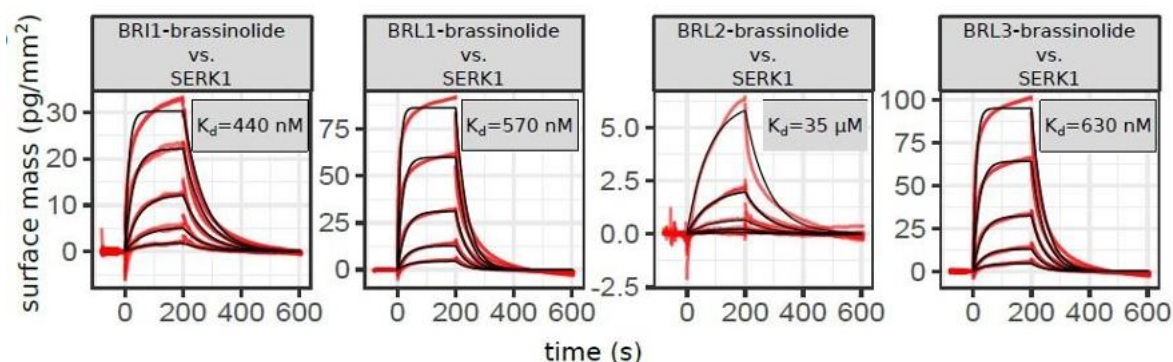
Přestože jsou receptory BRL1 a BRL3 exprimovány v odlišných pletivech než BRI1, jejich extracelulární domény vykazují vysoký stupeň sekvenční i strukturní podobnosti. Tomu odpovídají také výsledky našich vazebných studií [77], které ukázaly, že oba receptory rozpoznávají prakticky stejné spektrum brassinosteroidů jako BRI1. BRL1 i BRL3 vážou brassinolid s vysokou afinitou a obdobné hodnoty byly pozorovány také pro castasteron, typhasterol, secasteron, 6-deoxycastasteron nebo 28-norbrassinolid. Naopak brassinosteroidy s modifikovanou konfigurací postranního řetězce, například 24-epibrassinolid, 24-epicastasteron nebo C29 deriváty nesoucí dodatečnou ethylovou skupinu

v poloze C24, vykazují výrazně nižší afinitu. Stejně jako u BRI1 se tedy ukazuje, že rozhodující roli při rozpoznání ligandu hrají především vlastnosti postranního řetězce, zatímco změny ve steroidním jádře jsou receptorem tolerovány podstatně lépe.

Tato podobnost byla následně potvrzena i krystalografickými studiemi receptoru BRL3. Struktury komplexů s brassinolidem, castasteronem a typhasterolem ukázaly prakticky totožné uspořádání vazebné kapsy jako v případě BRI1. Zachovány zůstávají i klíčové interakce mezi ligandem a island doménou receptoru, zejména konzervovaná vazba zahrnující hydroxylovou skupinu na uhlíku C23. z dostupných dat proto vyplývá, že BRL1 a BRL3 představují funkčně velmi blízké homologické receptory BRI1, které se od něj liší především svým expresním profilem, nikoliv mechanismem rozpoznání brassinosteroidů. Tento závěr je navíc v souladu s dřívějšími genetickými studiemi, které ukázaly, že při vhodné expresi jsou BRL1 a BRL3 schopny alespoň částečně nahradit funkci BRI1.

Odlišnou pozici v rámci této receptorové rodiny zaujímá receptor BRL2. Na rozdíl od BRL1 a BRL3 nebyl dlouhou dobu považován za receptor brassinosteroidů a byl spojován především s regulací vývoje cévních svazků. Přestože jeho extracelulární doména vykazuje značnou podobnost s ostatními členy rodiny BRI1, naše vazebné experimenty odhalily výrazně odlišný profil ligandové specifity. BRL2 je schopen vázat některé brassinosteroidy, avšak s podstatně nižší afinitou. Brassinolid a castasteron se vážou přibližně o jeden řád slaběji než k receptoru BRI1 a ještě výraznější rozdíly byly pozorovány u dalších ligandů. Například typhasterol nebo 28-norbrassinolid, které patří mezi silné ligandy BRI1, vykazují vůči BRL2 pouze slabou vazbu. U některých sloučenin, například 24-epibrassinolidu, 24-epicastasteronu nebo 28-homocastasteronu, nebyla vazba za použitých experimentálních podmínek detekována vůbec.

Získané výsledky tak naznačují, že BRL2 nelze považovat za klasický brassinosteroidní receptor ve stejném smyslu jako BRI1, BRL1 nebo BRL3. Přestože si zachovává strukturálně podobnou vazebnou kapsu a je schopen interagovat s vybranými brassinosteroidy, jeho ligandová specifita i afinita jsou výrazně odlišné. Navíc vykazuje podstatně slabší schopnost vytvářet komplex s koreceptory rodiny SERK (obrázek 18). Fyziologický význam těchto interakcí proto zatím zůstává nejasný a nelze vyloučit, že BRL2 v rostlině rozpoznává dosud neidentifikovaný ligand nebo plní specifickou regulační funkci odlišnou od klasické brassinosteroidní signalizace.



Obrázek 18: GCI sensorgram stability komplexů brassinosteroidních receptorů s brassinolidem a SERK1 s uvedením výrazného rozdílu K_d u BRL2 receptoru. Převzato z Caregato A. et al., 2026 [77].

Zajímavým výsledkem studie bylo zjištění, že vysokou afinitu k receptorům BRI1, BRL1 a BRL3 nevykazuje pouze brassinolide, ale také několik dalších přirozeně se vyskytujících brassinosteroidů. Mezi nimi vyniká zejména castasteron, který se váže s afinitou srovnatelnou s brassinolidem a zároveň účinně podporuje tvorbu receptorového komplexu s koreceptory SERK. v kombinaci s výrazně vyššími endogenními koncentracemi castasteronu tato pozorování podporují hypotézu, že castasteron představuje vedle brassinolidu další fyziologicky významný ligand brassinosteroidních receptorů.

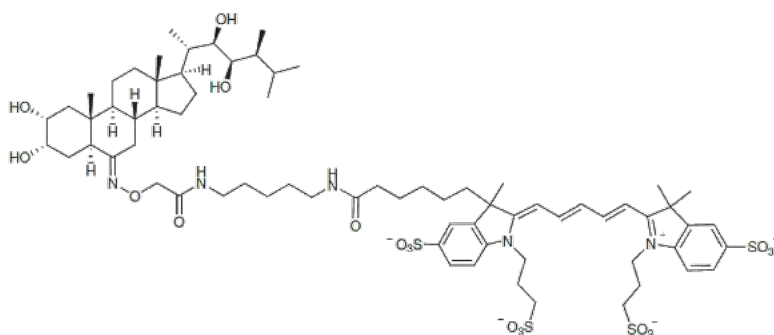
Přestože studium receptorů významně přispělo k objasnění mechanismů percepce a signalizace brassinosteroidů, samo o sobě neposkytuje úplný obraz o jejich působení v rostlině. Pro výslednou fyziologickou odpověď je totiž důležitá nejen schopnost ligandu navázat se na receptor, ale také jeho prostorová a časová distribuce v rostlinných pletivech. k objasnění těchto procesů začaly být v posledních letech využívány speciálně navržené chemické sondy umožňující sledovat lokalizaci a transport brassinosteroidů přímo v živých rostlinách.

4.3 Chemické sondy pro studium transportu brassinosteroidů

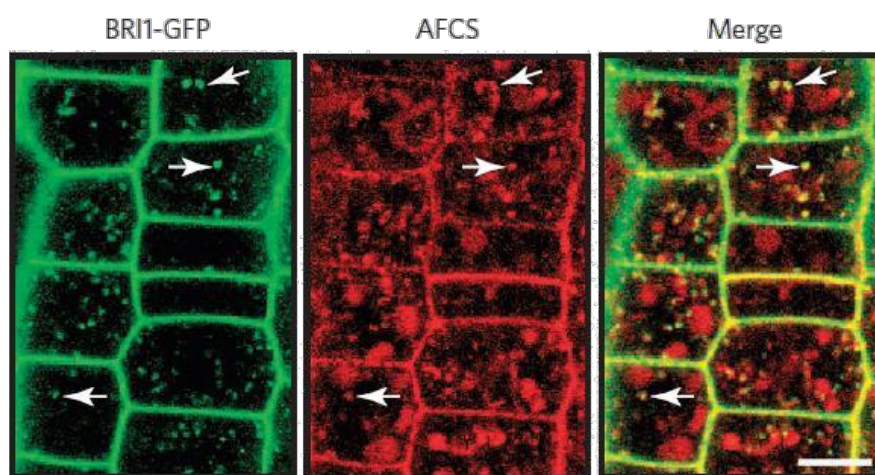
Důležitým aspektem regulace hormonální signalizace je také způsob distribuce hormonů v rostlinném organismu. U řady fytohormonů, například auxinů, cytokininů nebo kyseliny abscisové, hraje transport mezi jednotlivými orgány a pletivy zásadní úlohu při koordinaci růstu a vývoje rostlin [95,96]. v případě brassinosteroidů je však situace odlišná. Již dřívější studie [97] ukázala, že brassinosteroidy nepodléhají významnému dálkovému transportu mezi orgány rostliny a jejich účinek je proto považován za převážně lokální. Přesto však dlouhou dobu chyběly experimentální nástroje, které by umožnily sledovat pohyb brassinosteroidů přímo v živých rostlinných buňkách a objasnit mechanismy odpovědné za jejich distribuci.

Významný metodický pokrok přinesla práce Irani a kol. [98], ve které byl připraven fluorescenčně značený derivát castasteronu označený jako AFCS (Alexa Fluor 647–castasterone, obrázek 19). Tato molekula si zachovala biologickou aktivitu a umožnila poprvé přímo sledovat osud brassinosteroidu, v tomto případě castasteronu, po navázání na receptor

BRI1 v živých buňkách. Pomocí konfokální mikroskopie bylo prokázáno, že komplex receptor–ligand podléhá endocytóze a následně putuje až do vakuoly. Současně bylo ukázáno, že hlavní část brassinosteroidní signalizace probíhá na plazmatické membráně a že endocytóza receptorového komplexu slouží především k utlumení signálu (obrázek 20). Tato studie představovala jeden z prvních příkladů využití chemické biologie v oblasti brassinosteroidního výzkumu a ukázala, že vhodně navržené chemické sondy mohou poskytnout přímý pohled na pohyb hormonů a receptorových komplexů v rostlinných buňkách.



Obrázek 19: Struktura AFCS.



Obrázek 20: Mikroskopické snímky epidermálních buněk kořenové špičky exprimujících BRI1-GFP po 15minutové expozici 20 μ M AFCS. AFCS (červeně) kolokalizuje s BRI1-GFP (zeleně) na plazmatické membráně a v endosomech (označených šipkami), přičemž se současně akumuluje ve vakuolách. Měřítka: 5 μ m. Převzato z Irani N. G., 2012 [98].

Použití fluorescenčně značených hormonů však přináší i určité komplikace. Fluorescenční značky představují ve srovnání s molekulou brassinosteroidu objemné substituenty, které mohou ovlivňovat průnik buněčnými membránami, vazbu k receptorům i následný intracelulární transport. Nelze proto vyloučit, že sledované chování takto označených derivátů nemusí vždy plně odpovídat vlastnostem přirozených hormonů. Snahou obejít tato omezení byl rozvoj bioortogonálních přístupů, při nichž je do molekuly hormonu zabudována pouze malá reaktivní skupina a samotná fluorescenční značka je připojena až

následně přímo v biologickém systému (schéma 7). Díky tomu je možné sledovat distribuci hormonu s podstatně menším rizikem, že jeho transport nebo biologická aktivita budou ovlivněny přítomností objemného fluoroforu.

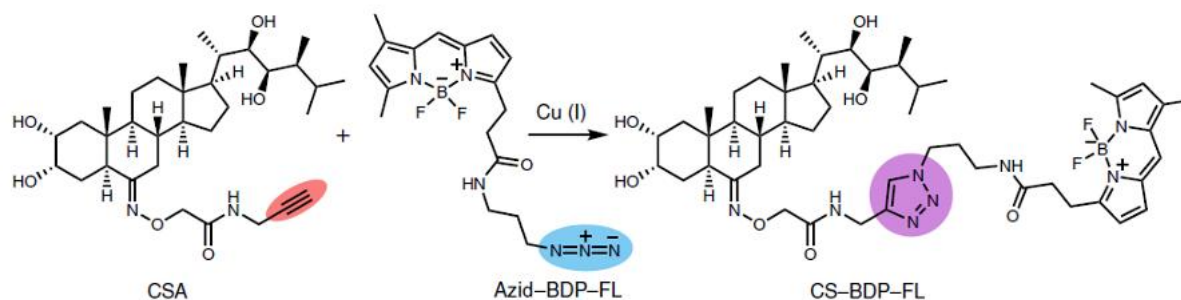
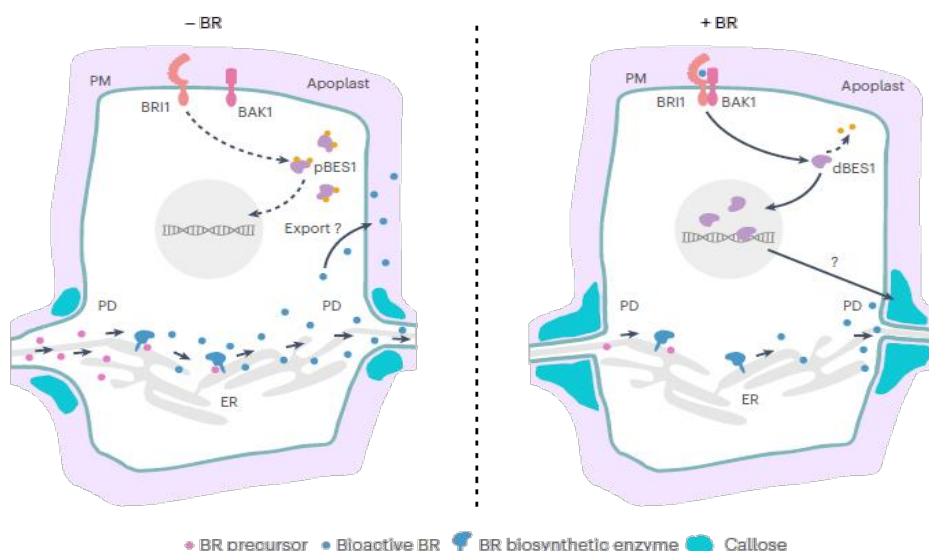


Schéma 7: Bioorthogonální reakce pro vizualizaci brassinosteroidů v buňkách.

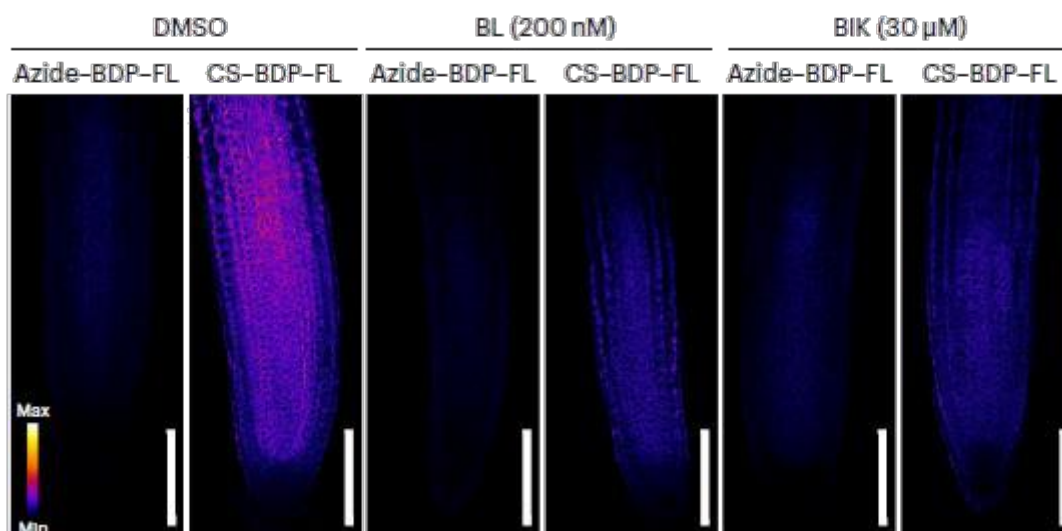
Další významný posun přinesla naše společná studie s týmem dr. Jenny Russinové z VIB Ghent [99] zaměřená na prostorovou organizaci biosyntézy brassinosteroidů v kořeni modelové rostliny *Arabidopsis thaliana*. Pomocí transgenních linií exprimujících fluorescenčně značené biosyntetické enzymy bylo prokázáno, že většina buněk neobsahuje kompletní soubor enzymů potřebných k syntéze biologicky aktivního hormonu. Jednotlivé kroky biosyntetické dráhy jsou rozděleny mezi různé buněčné vrstvy a dokončení biosyntézy proto vyžaduje pohyb meziproductů mezi sousedními buňkami. Současně bylo zjištěno, že exprese biosyntetických enzymů dosahuje maxima v elongační zóně kořene, kde se překrývá s maximem brassinosteroidní signalizace. Tyto výsledky vedly k formulaci modelu, podle kterého je distribuce brassinosteroidů určována především lokální biosyntézou a omezeným pohybem biosyntetických prekurzorů mezi sousedními buňkami.



Obrázek 21: Schéma úlohy plasmodesmat v transportu prekurzorů brassinosteroidů a regulaci jejich signalizace. Zvýšená hladina brassinosteroidů snižuje propustnost plasmodesmat ukládáním kalózy a přispívá k udržování hormonální homeostázy. Převzato z Wang Y., 2023 [100].

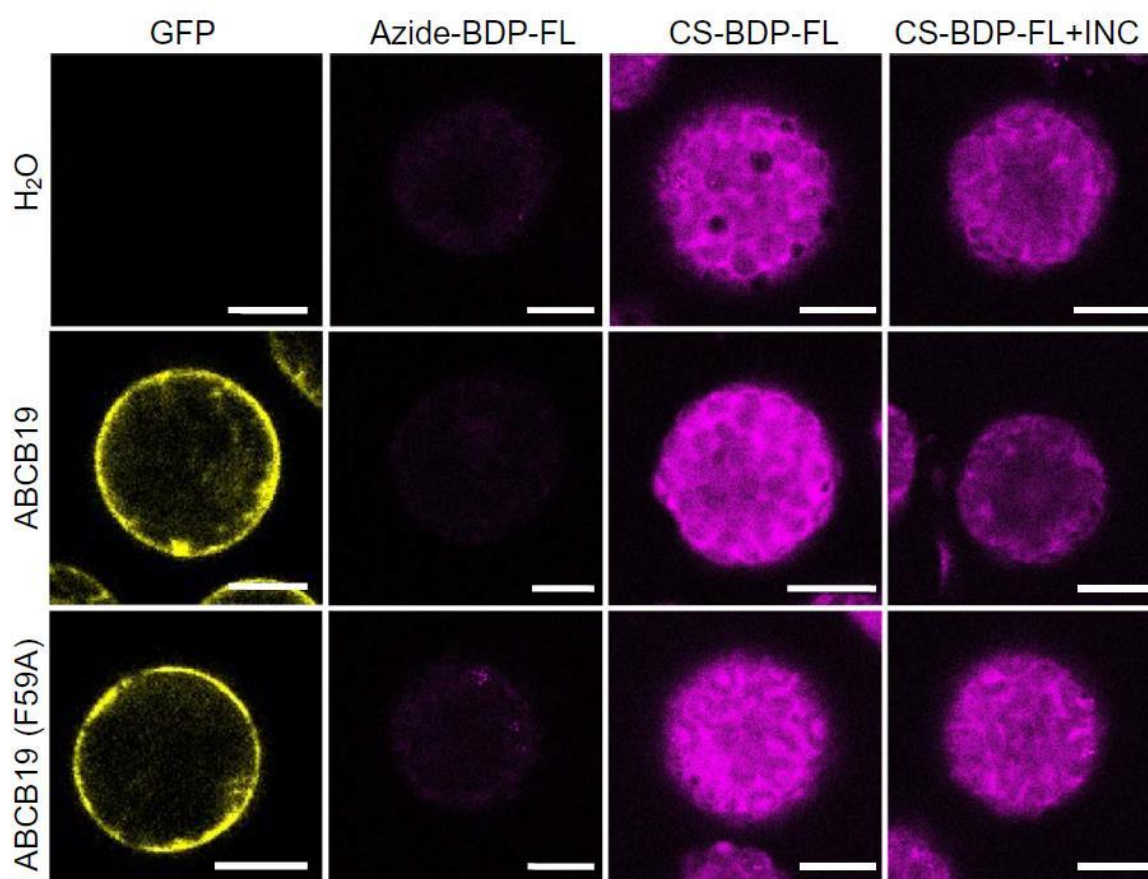
Přesto však zůstávala nezodpovězena otázka, jakým způsobem k tomuto mezibuněčnému transportu dochází. Odpověď přinesla až naše další společná práce [100] s dr. Russinovou využívající kombinaci genetických přístupů, chemické biologie a již zmiňované bioortogonální chemie. Pro studii jsme připravili bioaktivní alkynylovaný derivát castasteronu (CSA, schéma 7), který umožňoval následnou fluorescenční detekci pomocí click chemie. Díky této sondě bylo možné přímo sledovat pohyb brassinosteroidních molekul mezi buňkami. Experimenty ukázaly, že brassinosteroidy a jejich prekurzory (např. v práci použitý 21-hydroxycampesterol) mohou přecházet mezi sousedními buňkami prostřednictvím plasmodesmat (obrázek 21). Současně bylo prokázáno, že omezení propustnosti plasmodesmat vede ke snížení brassinosteroidní signalizace a k narušení růstu kořene. Zajímavé bylo také zjištění, že samotná hladina brassinosteroidů ovlivňuje propustnost plasmodesmat, čímž vzniká regulační mechanismus propojující transport hormonu s jeho biosyntézou a signalizací (obrázek 22). Podobně účinkuje i chemický inhibitor kinázy BIN2, bikinin, což vede ke zvýšené aktivitě brassinosteroidních transkripčních faktorů [101]. Tato práce tak poprvé poskytla přímý důkaz, že plasmodesmata představují hlavní cestu krátkodobého mezibuněčného transportu brassinosteroidů.

Další dlouhodobě otevřenou otázkou bylo, jak se biologicky aktivní brassinosteroidy dostávají z místa své syntézy k receptoru BRI1. Většina enzymů biosyntetické dráhy je lokalizována v membránách endoplazmatického retikula, zatímco percepce hormonu probíhá prostřednictvím extracelulární domény receptorového komplexu lokalizovaného na plazmatické membráně. Přestože byly brassinosteroidy dlouho považovány za dostatečně lipofilní molekuly schopné prosté difuze přes membránu, jejich vysoký stupeň hydroxylace naznačoval, že tento proces nemusí být dostatečně efektivní.



Obrázek 22: Snížení transportu CSA přes plasmodesmata po ošetření kořene *Arabidopsis thaliana* brassinolidem (BL) nebo bikininem (BIK). Pětidenní semenáčky byly inkubovány 24 hodin v přítomnosti BL (200 nM), BIK (30 μ M) nebo DMSO (kontrola), následně byla pomocí bioortogonální reakce připravena fluorescenčně značená sonda CS-BDP-FL. Měřítka: 100 μ m. Převzato z Wang Y., 2023 [100].

Zásadní průlom přinesla naše společná práce s týmy dr. Russinové a dr. Suna publikovaná v roce 2024 [102], která identifikovala transportér ABCB19 (ATP-BINDING CASSETTE SUBFAMILY B MEMBER 19) jako první specifický exportní systém pro brassinosteroidy. Tento protein byl přitom po dlouhou dobu studován téměř výhradně v souvislosti s transportem auxinu a patřil mezi nejlépe charakterizované členy rodiny ABC transportérů zapojených do regulace jeho distribuce v rostlině [103,104]. Pomocí kombinace biochemických metod, transportních experimentů v proteolipozomech, analýz v rostlinných protoplastech (obrázek 23) a kryoelektronové mikroskopie bylo prokázáno, že ABCB19 váže biologicky aktivní brassinosteroidy a aktivně je transportuje přes plazmatickou membránu za využití energie získané hydrolýzou ATP. Strukturální studie navíc umožnila popsat vazebné místo hormonu uvnitř transportéru a objasnit molekulární princip rozpoznávání brassinosteroidů. Tento objev nejen poskytl první přímý důkaz existence specializovaného membránového systému zajišťujícího export brassinosteroidů z buňky, ale současně významně rozšířil dosavadní představy o substrátové specifitě transportéru ABCB19.



Obrázek 23: Zrychlený export CSA v *Arabidopsis* protoplastech exprimujících GFP-ABCB19, nikoli však v protoplastech exprimujících mutantní variantu GFP-ABCB19 (F59A). Fluorescenční signál byl detekován po bioortogonální reakci s azid-BDP-FL. INC – po 4h inkubaci. Měřítka: 10 μ m. Převzato Ying W., 2024 [102].

Na tuto práci poté navázala další naše studie [105] zaměřená na homologní transportér ABCB1 (ATP-BINDING CASSETTE SUBFAMILY B MEMBER 1). Využitím obdobného souboru

metod, zahrnujícího měření ATPázové aktivity, transportní experimenty s chemicky modifikovanými brassinosteroidy, click chemii a kryoelektronovou mikroskopii, bylo prokázáno, že také ABCB1 funguje jako transportér biologicky aktivních brassinosteroidů. Strukturální analýzy odhalily vazbu brassinolidu do hydrofobní kapsy transmembránové domény a popsaly konformační změny doprovázející transport hormonu. Tyto výsledky ukázaly, že export brassinosteroidů není zajišťován jediným transportérem, ale pravděpodobně skupinou příbuzných ABC transportérů, které společně přispívají k regulaci brassinosteroidní signalizace.

Současné poznatky tedy ukazují, že ačkoli brassinosteroidy nepodléhají významnému dálkovému transportu mezi orgány rostliny, jejich lokální distribuce je regulována komplexním souborem mechanismů zahrnujících prostorově organizovanou biosyntézu, mezibuněčný transport prostřednictvím plasmodesmat a aktivní export biologicky aktivních hormonů pomocí ABC transportérů. Významnou roli při objasňování těchto procesů sehrály také přístupy chemické biologie, zejména využití fluorescenčně značených ligandů, bioortogonálně modifikovaných brassinosteroidních derivátů, click chemie a moderních strukturně-biologických metod. Díky těmto nástrojům bylo možné poprvé přímo sledovat pohyb brassinosteroidů v rostlinných buňkách a detailně popsat mechanismy odpovědné za jejich transport, distribuci a percepci.

5 Závěr

Výzkum brassinosteroidů prošel během posledních desetiletí výrazným vývojem. Zatímco v počátečních fázích byla pozornost soustředěna především na izolaci nových přírodních brassinosteroidů a hledání možností jejich syntézy, současný výzkum se zaměřuje zejména na objasňování jejich biologických funkcí, signalizace a transportu. Přestože by se mohlo zdát, že význam chemie v této oblasti postupně ustupuje do pozadí, výsledky shrnuté v této práci ukazují, že tomu tak není. Chemie brassinosteroidů zůstává důležitým nástrojem nejen pro přípravu nových sloučenin, ale také pro získávání nových poznatků o jejich působení v rostlinách.

Studium syntetických analogů přineslo řadu informací o vztahu mezi strukturou brassinosteroidů a jejich biologickou aktivitou. Ukázalo se, že některé části molekuly lze poměrně výrazně modifikovat, aniž by došlo k úplné ztrátě aktivity, zatímco změny v jiných oblastech mají na biologické účinky zásadní vliv. Výsledky získané při studiu nejrůznějších syntetických analogů pomohly lépe pochopit význam jednotlivých strukturních prvků molekuly a ukázaly, které úpravy jsou z hlediska biologické aktivity přijatelné a které nikoliv. Současně se ukázalo, že biologická aktivita látky není dána pouze její schopností navázat se na receptor, ale také jejími fyzikálně-chemickými vlastnostmi a schopností dostat se v rostlině na místo svého působení. Významnou oblast představovalo také využití syntetických derivátů při studiu brassinosteroidních receptorů. Kombinace chemicky modifikovaných ligandů a vazebných experimentů umožnila podrobněji popsat, které strukturní prvky jsou důležité pro rozpoznání hormonu receptorem BRI1 i příbuznými receptory BRL1–3. Tyto studie současně ukázaly, že biologicky významnou roli nemusí hrát pouze brassinolid, ale také další přirozeně se vyskytující brassinosteroidy a některé jejich biosyntetické prekurzory. Chemické přístupy tak významně přispěly k lepšímu pochopení toho, jak rostliny brassinosteroidy rozpoznávají a jakým způsobem je receptorový systém využívá k regulaci růstu a vývoje.

Význam chemie se výrazně projevil také při studiu lokalizace a transportu brassinosteroidů. Příprava fluorescenčně značených a bioortogonálně modifikovaných derivátů umožnila poprvé přímo sledovat pohyb brassinosteroidních molekul v živých rostlinných buňkách. Díky těmto přístupům bylo možné objasnit osud receptorového komplexu po navázání ligandu, prokázat mezibuněčný transport brassinosteroidů prostřednictvím plasmodesmat a identifikovat transportéry zajišťující jejich aktivní přenos přes plazmatickou membránu. Tyto výsledky významně rozšířily dosavadní představy o distribuci brassinosteroidů v rostlinných pletivech a ukázaly, že regulace jejich koncentrace je mnohem složitější, než se dříve předpokládalo.

Lze tedy konstatovat, že chemie brassinosteroidů dnes nepředstavuje pouze prostředek pro přípravu nových sloučenin. Moderní chemické a chemicko-biologické přístupy se staly nedílnou součástí výzkumu těchto rostlinných hormonů a významně přispěly k objasnění jejich biologické aktivity, mechanismu působení i transportu. Chemie tak zůstává jedním z nejdůležitějších nástrojů, které umožňují porozumět biologii brassinosteroidů na molekulární úrovni.

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Příloha 1

Brassinosteroids: Synthesis and Biological Activities

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Brassinosteroids: synthesis and biological activities

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Abstract Brassinosteroids (BRs) are a relatively recently discovered group of phytohormones that are essential for normal plant growth and development. They participate in regulation of numerous vital physiological processes in plants, such as elongation, germination, photomorphogenesis, immunity and reproductive organ development. Structurally they are very similar to animal steroid hormones and include about 70 polyhydroxylated sterol derivatives. They are found at low levels in practically all plant organs. Recent studies have indicated that BRs have antiproliferative, anticancer, antiangiogenic, antiviral and antibacterial properties in animal cell systems, and thus have potential medical applications. Among others, BRs can inhibit replication of viruses in confluent human cell cultures, sometimes with high selectivity indexes, inducing cytotoxic effects in various types of cancer cells but not normal human cells. Thus, they include promising leads for developing potent new anticancer drugs. The aims of this article are to overview chemical characteristics, biological activities and the potential medical applications of natural BRs.

Keywords Brassinosteroids · Chemical synthesis · Plant biological activity · Antiproliferative activity · Antiviral activity

Introduction

Brassinosteroids (BRs) are a class of plant-specific steroid hormones characterized by polyhydroxylated sterol structures with significant growth-promoting activities (Clouse and Sasse 1998). They were initially reported at the start of the 1970s, when Mitchell et al. (1970, 1971) showed that ether extracts of *Brassica napus* (rape) pollen, designated “brassin”, promote stem elongation and cell division in the bean internode assay. The first BR to be isolated, from 40 kg of bee-collected rape pollen was named brassinolide (2) (Grove et al. 1979). All BRs have a 5 α -cholestane skeleton, with functional variations due to differences in orientations of oxygenated functions on the skeleton (Bajguz and Tretyn 2003).

BRs are distributed throughout the plant kingdom and have been isolated, to date, from 64 plant species including 53 angiosperms, one pteridophyte, one bryophyte and three algae (Bajguz 2011). They have been detected in all examined plant organs, including pollen, seeds, leaves, stems, roots, flowers and grains. They are also present in insect and crown galls. However, pollen and seeds are the richest sources of BRs, with contents in the 1–100 ng g⁻¹ FW range, while shoots and leaves usually have much lower

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amounts, 0.01–0.1 ng g⁻¹ FW (Bajguz 2011). BRs are involved in the regulation of many vital plant physiological activities, such as cell expansion, cell division, vegetative growth, reproduction, senescence, seed germination and stress tolerance (Clouse 2002; Bhardwaj et al. 2006; Krishna 2003). Recent studies have shown that BRs also have potent antiviral, antifungal, antiproliferative, antibacterial, neuroprotective and antiangiogenic activities in animal and human systems (Wachsman et al. 2000, 2002; Michelini et al. 2004, 2008; Malíková et al. 2008; Steigerová et al. 2010; Rárová et al. 2012).

An array of brassinosteroids with variations in C-24 alkyl substituents are synthesized from campesterol, sitosterol, and cholesterol. All three sterols are converted to large numbers of metabolites in plant cells, but only a few of the metabolites have biological activity and biosynthetic pathways starting from campesterol seem to be the most important *in planta* (Hartmann 1998). Three major pathways of BR biosynthesis starting from this precursor are known. In two of these pathways the intermediate campestanol is converted via either an “early” or “late” C-6 oxidation route, in which C-6 oxidation occurs before and after introduction of C22 and C23 vicinal hydroxyls, respectively (Fujioka and Yokota 2003). These parallel pathways converge at castasterone (1), the immediate precursor of BL. Late C-6 oxidation is more prevalent in a number of species, including *Arabidopsis* and pea (Nomura et al. 2001). The third pathway, proposed by Ohnishi et al. (2006) following analysis of BR biosynthesis in *Arabidopsis*, involves direct (campestanol-independent) conversion of early C22-hydroxylated intermediates to 3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol via C-23 hydroxylation. Proteins involved in BR signal transduction pathways have also been identified. In *Arabidopsis* BRs are perceived by the BR INTENSIFIER1 (BRI1) plasma-membrane receptor kinase and activation of BRI1/BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) kinase complexes by transphosphorylation. Subsequently, BRASSINOSTEROID INSENSITIVE 2 (BIN2) kinase is dephosphorylated and inactivated, resulting in the accumulation of unphosphorylated BRASSINAZOLE RESISTANT (BZR) transcription factors in the nucleus (Kim and Wang 2010; Li and Chory 1997; Wang et al. 2001). In this review we summarize

current knowledge about BRs’ chemistry and biological activities in plant and animal systems (Fig. 1).

Chemistry

The first syntheses of BRs were described shortly after their isolation and structural determination, when Thompson et al. (1979) published syntheses of 24-epicastasterone (24-epiCS, 3) and 24-epibrassinolide (24-epiBL, 4). Starting from commercially available ergosterol (7), these BRs were respectively obtained after nine and 12 steps, as shown in Scheme 1. The most important reactions in this scheme are isomerization and formation of i-ergosterol (9), oxidation to i-sterone (10), reduction of the 7-double bond, formation of 2,3-olefin 14, *cis*-dihydroxylation, and Baeyer–Villiger oxidation. This is still a standard strategy for preparation of 24-epibrassinosteroids and few improvements have been published. However, McMorris and Patil (1993) improved synthesis of 24-epibrassinolide (4), reducing the number of reaction steps to seven. This was done by catalyzing isomerization of i-sterone (5) to 2,3-olefin (14) using pyridinium hydrochloride and lithium bromide in one step, and performing the Baeyer–Villiger oxidation of 24-epicastasterone (3) using trifluoroperoxyacetic acid solution without prior acetylation of hydroxyl groups (in both cases with 80 % yield). If the starting material is brassicasterol 24-epibrassinolide (4) can be prepared in just five steps (Takatsuto and Ikekawa 1984). However, low availability and high price of brassicasterol limit its use as a starting material.

28-homobrassinolide (6) is prepared in a similar fashion to the synthesis of 24-epibrassinolide (4) already described. The starting material is usually commercially available stigmaterol (16), from which 28-homobrassinolide (6) can be obtained after six reaction steps (McMorris et al. 1994). Another synthetic approach, published by Sakakibara and Mori (1982), also starts from stigmaterol (16), but involves epoxidation of the 22,23-double bond in 17, followed by cleavage of the epoxide ring of 18 with hydrobromic acid and formation of bromohydrin (19). Acetylation of bromohydrin (19) and hydrolysis of bromide followed by saponification of tetraacetate (21) affords 24-homocastasterone (5) (Scheme 2).

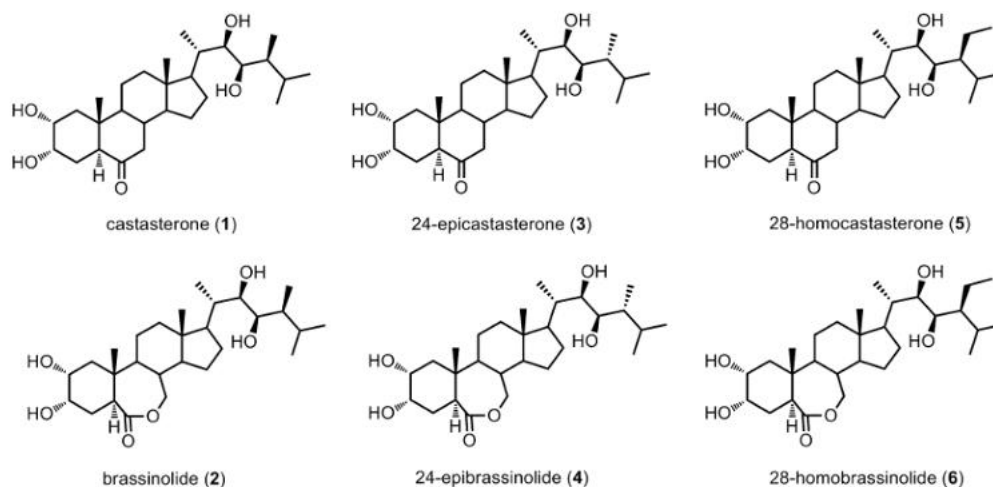


Fig. 1 Structures of representative natural brassinosteroids

24-Homobrassinolide (6) is then obtained by standard Baeyer–Villiger oxidation.

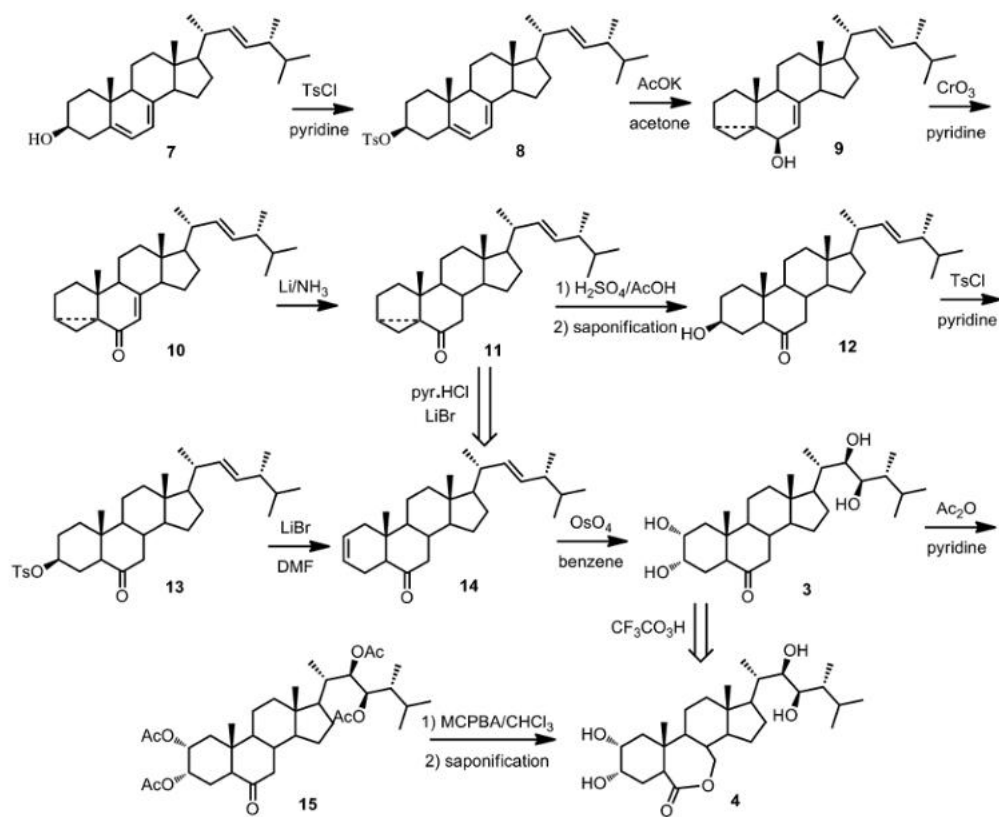
The syntheses described thus far are straightforward, but a crucial reaction that significantly influences overall yields is osmium tetroxide-catalyzed asymmetric dihydroxylation of double bonds. While dihydroxylation of the double bond on the A-ring leads only to the 2 α ,3 α -diol, dihydroxylation of the side chain double bond can afford both 22*R*,23*R*-diol (natural) and 22*S*,23*S*-diol (unnatural). The stereoselectivity of dihydroxylation mostly depends on the C-24 configuration and chiral ligand used in the reaction. It was proved that 9-*O*-(9'-phenanthryl)-dihydroquinidine is so far the best chiral ligand for Sharpless dihydroxylation ($\text{OsO}_4 + \text{K}_3[\text{Fe}(\text{CN})_6] + \text{chiral ligand}$) of 22-ene steroids (Huang et al. 1993). The effect was most evident in case of (22*E*,24*S*)-24-ethyl substituted side chain when the ratio of *R,R*- and *S,S*-diol was increased up to 8:1. Without chiral ligand this dihydroxylation gave only *S,S*-diol. In the case of (22*E*,24*R*)-24-methyl substituted side chain the same dihydroxylation procedure favors *R,R*-diol in a much higher ratio (13:1). Slow dihydroxylation of the side chain double bond can be accelerated by adding methanesulfonamide (McMorris and Patil 1993).

A completely different strategy has been developed for synthesis of the first discovered BR, brassinolide (2), and castasterone (1). This is mainly due to low

availability of starting material for the strategy used to synthesize 24-epibrassinolide (4) and 28-homobrassinolide (6). For example, crinosterol could potentially be used, but only analytical amounts that cannot be used for multistep procedures are available, although its synthesis has been described (Anastasia et al. 1983). Thus, efforts have mainly focused on preparation of a new side chain with a 24*S*-methyl group. There are many approaches for building a chain with the desired methyl group configuration. The first, published shortly after isolation of brassinolide (Fung and Siddall 1980), was based on the reaction of aldehyde 22 with lithium butyldimethyl-(*E*)-2,3-dimethylbutenylalanate as a carbon–carbon formation reagent with 46 % yield. Subsequent epoxidation of olefin 23 and cleavage of epoxide 24 affords the desired side chain (Scheme 3).

A similar procedure has also been described for preparation of 28-homobrassinolide (6) and dolicholide (Mori et al. 1984). Compound 23 can also be obtained from 20-formyl derivative 22 via three-step synthesis using 3-methyl-2-oxobutyl(triphenyl)arsonium bromide as a carbon–carbon bond formation reagent (Shen and Zhou 1990).

Another synthetic strategy is based on formation of alkyne side chain (e.g. 23) as a key intermediate for further modification. The main reagent used for its synthesis is 3-methylbut-1-ynyl lithium, prepared

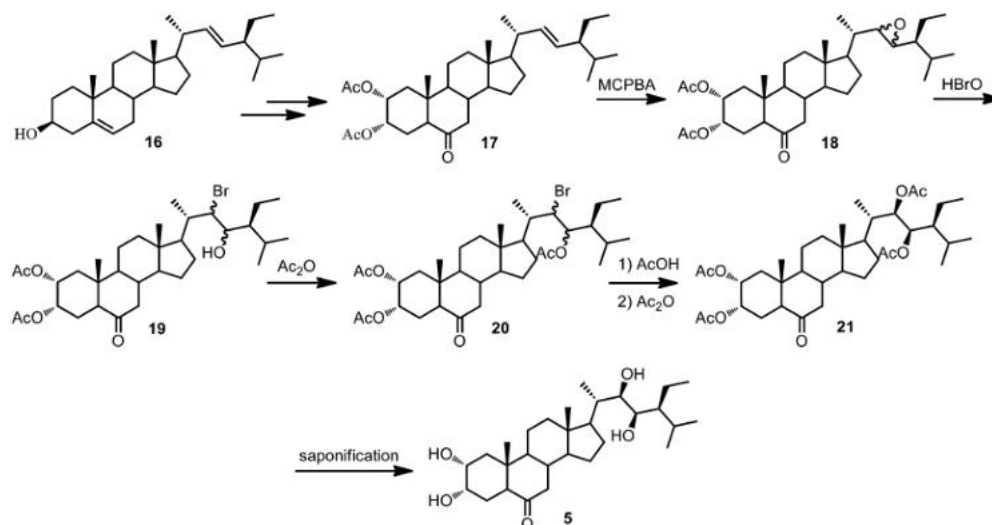


Scheme 1 Synthesis of 24-epicastasterone (**3**) and 24-epibrassinolide (**4**) from ergosterol (**7**) (double arrows indicate improved and shortened synthesis)

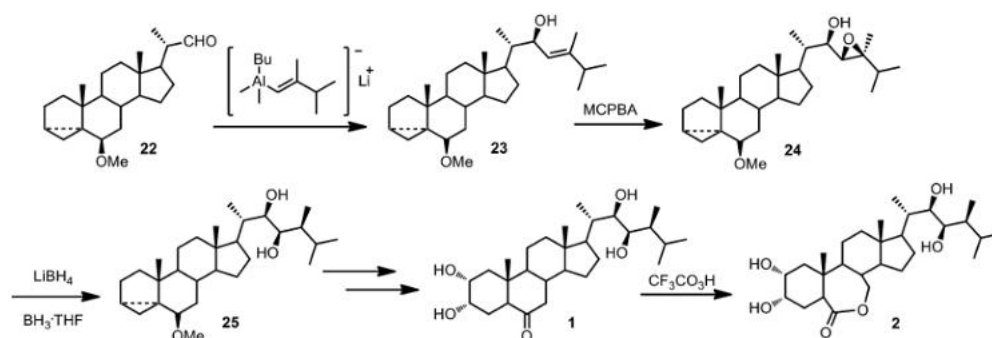
in situ from 1,1-dibromo-3-methyl-but-1-ene. Ishiguro et al. (1980) published the first alkyne side chain synthesis using this reagent.

However, such a synthesis of brassinolide (**2**) involves 22 steps and thus is very inefficient, in large part because reduction of the cyano group of **31** to methyl alone requires eight steps (Scheme 4). The same group also used this procedure for synthesizing typhasterol and several 28-norbrassinosteroids (Takatsuto et al. 1984). More efficient way was published few years later (Sakakibara and Mori 1983; Mori et al. 1984; Aburatani et al. 1985). The improvement that allowed significant reduction of reaction steps is based on opening of epoxide ring of reaction steps is based on opening of epoxide ring of **29** with trimethylaluminum directly to 24S methyl group (Scheme 4).

Although this strategy is more efficient in number of reaction steps, the overall yield is affected by non-stereoselectivity of the alkyne formation: both isomers of 22-hydroxy alkynes (**27R** and **27S**) are obtained in 1:1 ratio. This problem was subsequently solved via stereoselective sulfenate-sulfoxide transformation (Zhou and Shen 1991). The mixture of both hydroxy alkynes (**27S** and **27R**) can be hydrogenated to olefins **28S** and **28R**, which are then subjected to reaction with benzenesulfonylchloride to form sulfoxides **33S** and **33R**. Methylation with iodomethane in the presence of lithium diisopropylamide followed by cleavage of sulfoxide **34** with trimethyl phosphite affords the 22R-hydroxy-24-methyl olefin **35** and its 22S isomer in a 8.4:1 ratio with 53 % overall yield (Scheme 5).



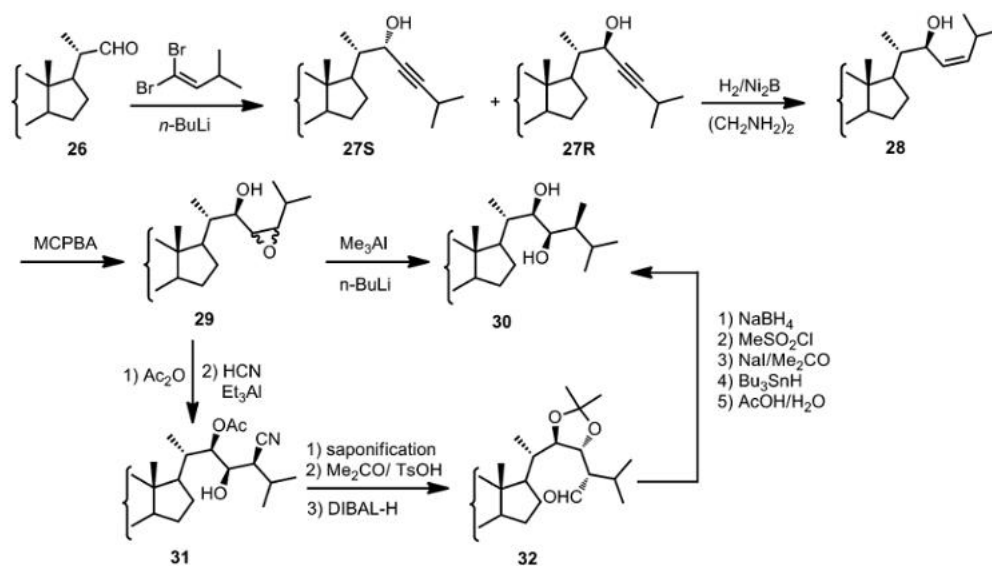
Scheme 2 Alternative synthesis of 28-homocasterone (**5**) (undesired isomers omitted for clarity)



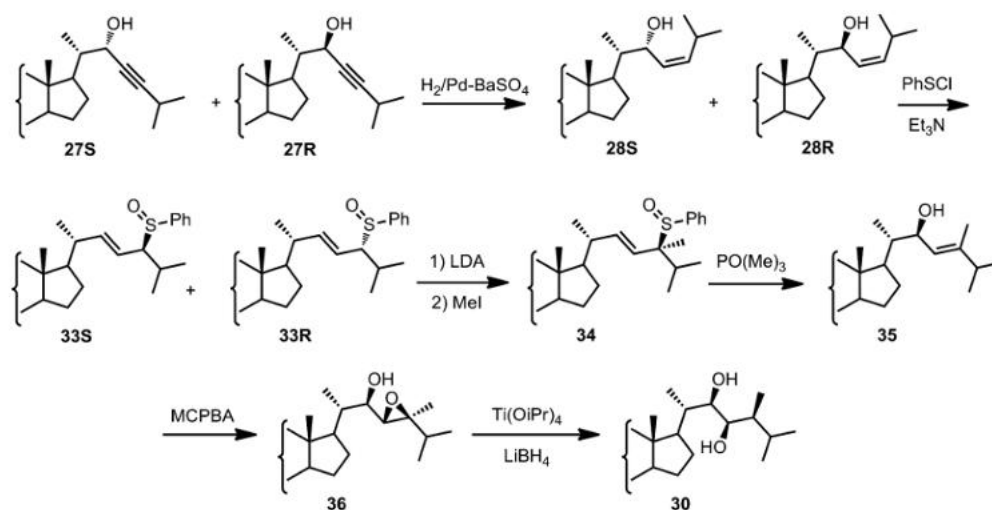
Scheme 3 Construction of brassinolide (**2**) with organoaluminum reagent

Another approach to build the side chain of brassinolide (**2**) is Julia-Lythgoe olefination with chiral sulfone (Sakakibara et al. 1982). Starting with aldehyde **26** and chiral (*S*)-2,3-dimethylbutyl-phenylsulfone this olefination can afford the side chain with desired 24*S* configuration (crinosterol side chain). Further reduction of sulfone **37** by sodium amalgam in methanol and ethyl acetate gives olefin **38**, which can be oxidized by osmium tetroxide (Scheme 6). Efficient and stereo-controlled preparation of brassinolide (**2**) can be achieved by using of 3-isopropylbut-2-enolide

(McMorris et al. 1996). Its reaction with lithium diisopropylamide followed by addition of aldehyde **26** gave hydroxybutenolide **39**. Such a lactone is hydrogenated and then reduced with lithium aluminum hydride to afford the side chain with desired configurations on all chiral carbons. Oxidation of primary alcohol **42** and further decarbonylation of aldehyde **43** with Wilkinson's catalyst removes oxygen from position 28 (Scheme 7). The overall yield of this procedure is 32 %. A shorter and more efficient way of constructing the side chain involves a similar sequence



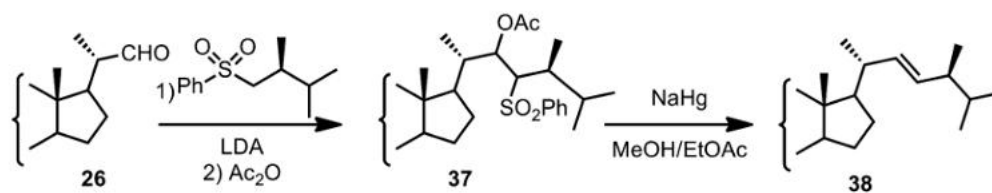
Scheme 4 Two routes for constructing side chain 30 via alkyne



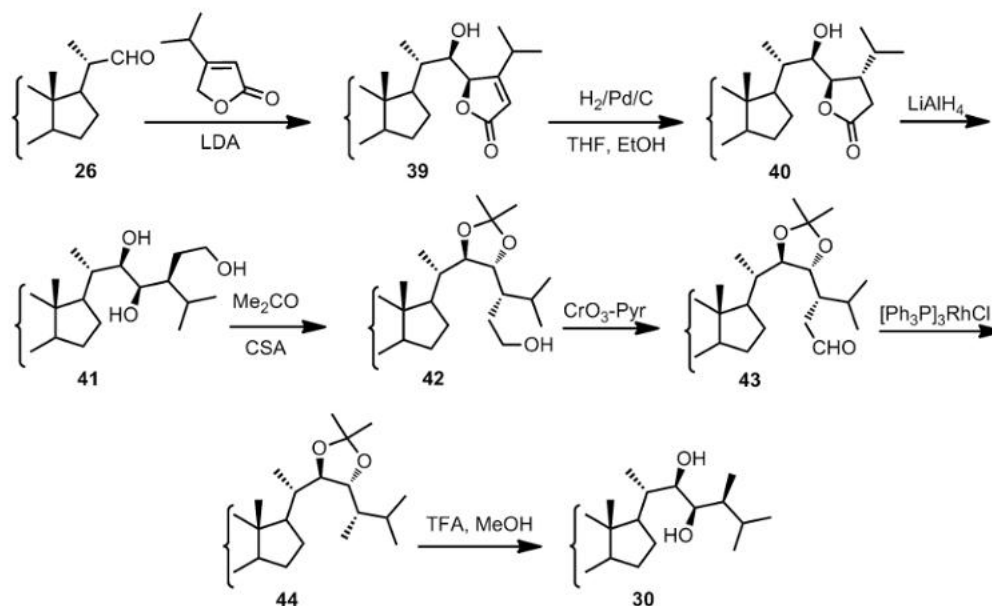
Scheme 5 Efficient construction of brassinolide side chain 30 via sulfoxide

of reactions but starts with 3,4-dimethylbut-2-enolide (Donaubauer et al. 1984). In this case, the key intermediate is 26-hydroxy derivative 47. The hydroxyl group is removed to obtain brassinolide side chain

by standard reactions for this purpose; mesylation and reduction of mesylate 49 with lithium aluminum hydride. The overall yield of this sequence is 70 % (Scheme 8).



Scheme 6 Construction of the crinosterol side chain using Julia-Lythgoe olefination



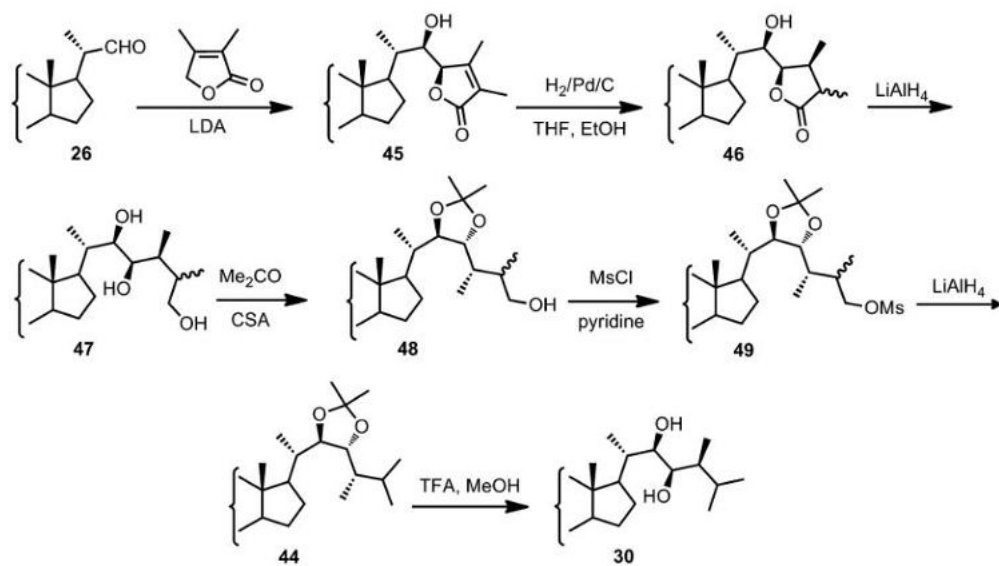
Scheme 7 Construction of brassinolide side chain using 3-isopropylbut-2-enolide

Another option for controlling the side chain stereochemistry is to use furan-2-yl lithium as a reagent (Kametani et al. 1989; Honda et al. 1990; Tsubuki et al. 1992). Stereoselective hydride reduction of 52 and subsequent 1,4-addition of the 24-methyl group to the pyranone derivative 55 are the key reaction steps of this side chain (Scheme 9).

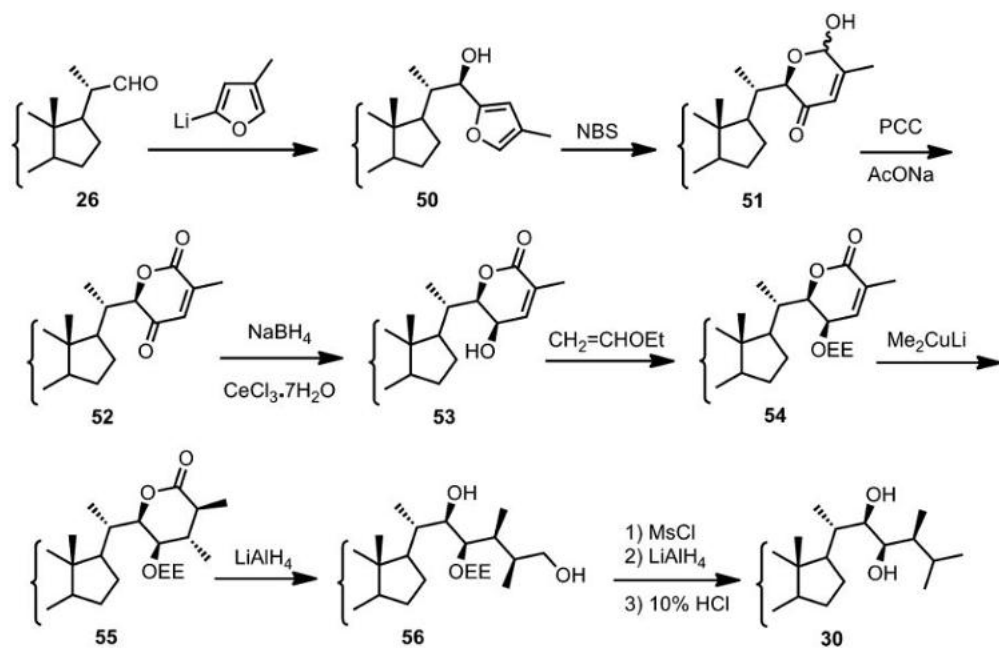
Nucleophilic addition to aldehyde 26 has also been exploited, in the most recently published construction of the brassinolide side chain (Hurski et al. 2013, 2015), which can provide brassinolide (2) in a few steps via use of chiral thioacetal (Scheme 10). Selective reduction of 23-oxoderivate 60 (cryptolide side

chain) is afforded by presence of the bulky *t*-butyldimethylsilyloxy group on neighboring carbon. Moreover, this synthesis can also afford other BR biosynthetic precursors and metabolites (e.g. cathasterone 59 and cryptolide 58).

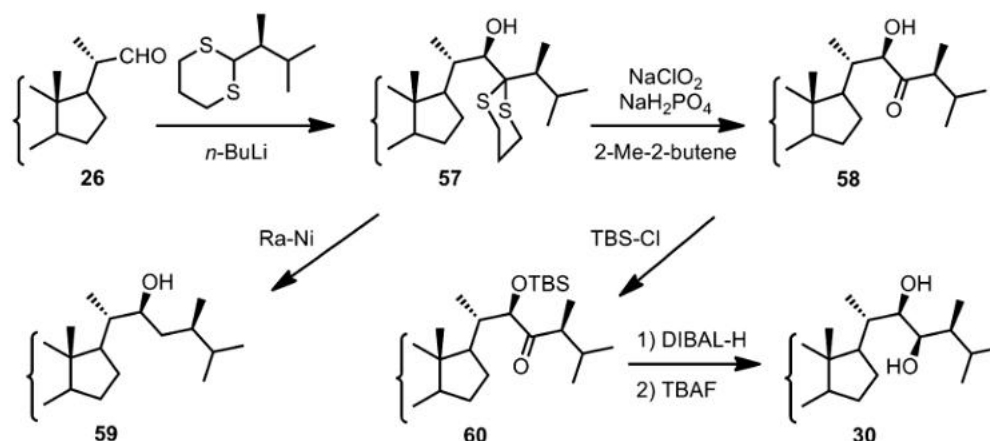
The above mentioned approaches are landmark examples of BR syntheses during the last ca. 40 years. Many other published procedures have been patterned on them and others are probably awaiting publication, highlighting the intense interest among organic chemists working on plant hormones in finding simple procedures for synthesizing BRs and their natural analogues. Reasons for this interest are described below.



Scheme 8 Construction of brassinolide side chain using 2,3-dimethylbut-2-enolide



Scheme 9 Construction of brassinolide side chain using furan-2-yl lithium



Scheme 10 Construction of the brassinolide side chain using chiral thioacetal, including cryptolide 58 and cathasterone 59 side chain

Biological activities of BRs on plants

BRs promote growth and developmental processes in all plant tissues and organs. At cellular levels, they regulate cell elongation, cell division and differentiation. At whole-plant levels, they participate in control of root and shoot development, seed germination and fertility (Kvasnica et al. 2014; Sasse 2003; Yang et al. 2011). BRs apparently co-ordinate and integrate diverse processes required for growth, partly via interactions with other hormones (Müssig 2005). In addition to their pivotal roles in plant growth and development, they appear to participate in mechanisms conferring tolerance to diverse environmental stresses, including high and low temperatures, drought, salinity, herbicidal injury, and pathogen attack (Krishna 2003).

Effects of BRs on cell elongation and division

Brassinosteroids participate in plant growth regulation by acting on both cell expansion and division. Genetic studies have confirmed that these processes are severely retarded in most BR-deficient and BR-insensitive mutants (Clouse et al. 1996). BR-deficient mutants display severe leaf phenotypic perturbations, including production of small, round leaves and short petioles (Szekeres et al. 1996; Li et al. 2001). Feeding these mutants with BRs restores the normal leaf size, by increasing both sizes and numbers of mesophyll

cells, implying that BRs promote leaf growth by enhancing both cell expansion and cell division (Nakaya et al. 2002). In rice the abaxial sclerenchyma cell number of lamina joints is closely related to leaf erectness and BR signaling tightly regulates their proliferation. Sun and co-workers identified a rice U-type cyclin *CYC U4;1* that plays a positive role in promoting leaf erectness by controlling the abaxial sclerenchyma cell proliferation (Sun et al. 2015). A rice *dwarf1* mutant, which lacks BR C-6 oxidase activity, exhibits abnormal organization and polar elongation of leaf and stem cells, leading to dramatic defects in development of many organs (Hong et al. 2002). Conversely, BR-insensitive (*BR11*) mutants have larger and longer leaf blades, and longer petioles, than wild-type counterparts, due to increases in epidermal cell numbers, suggesting that BRs solely promote cell division during leaf growth (Gonzalez et al. 2010; Oh et al. 2011). However, Zhiponova et al. (2013) found that stimulating BR signaling and BR biosynthesis respectively promoted cell proliferation and cell differentiation. Hence, while enhancing *BR11* activity led to increased numbers of dividing cells (Gonzalez et al. 2010; Oh et al. 2011), continuous exposure to the hormone resulted in a lower proportion of mitotic cells and enhanced cell expansion (Zhiponova et al. 2013; Singh and Savaldi-Goldstein 2015). Early studies with *Zinnia elegans* L. mesophyll cell cultures showed that BRs also play an important role in promoting xylem differentiation (Yamamoto et al.

1997). Notably, the BR synthesis inhibitors uniconazole and brassinazole prevent xylem differentiation in *Zinnia* cell cultures, which can be restored by treatment with BL or 28-homoBL (Iwasaki and Shibaoka 1991). BL is essential for entry into the final stage of tracheary element differentiation, where secondary wall formation and cell death occur (Iwasaki and Shibaoka 1991).

BRs regulate root growth

Several recent studies have demonstrated the presence of BRs in maize and tomato roots (Kim et al. 2000; Yokota et al. 2001). The effects of BRs on root growth are strongly dependent on the BR concentration used: low (nM) concentrations can stimulate primary root growth, while higher concentrations (μM) can inhibit it (Haubrick and Assmann 2006). 24-EpiBL (4) reportedly has inhibitory effects on root formation in mung bean (*Vigna radiata*), wheat (*Triticum aestivum*), maize (*Zea mays*), tomato (*Solanum lycopersicum*) and *Arabidopsis* seedlings (Roddick and Ikekawa 1992; Clouse et al. 1993; Guan and Roddick 1988). Two recent studies on the role of BRs in root growth have shown that BRs play a regulatory role in the control of cell-cycle progression and differentiation in the *Arabidopsis* root meristem (Gonzalez-Garcia et al. 2011; Hacham et al. 2011; Vragovic et al. 2015; Chaiwanon and Wang 2015). More specifically, analyses of BRI1 loss-of-function (*bri1-116* null) and gain-of-function (*bes1-D*) *Arabidopsis* mutants by Gonzalez-Garcia et al. (2011) showed that balanced BR signaling is needed to maintain meristem size and overall root growth, and that BR signaling is required for cell cycle progression. Hacham et al. (2011) showed that the size of the root meristem is controlled by BRI1 activity in the epidermis and the signal from the epidermis is probably transmitted by a component that differs from BZR1 and BES1/BZR2. Further, BRs' activity in regulation of root meristem size is integrated with the action of PIN auxin efflux carriers (Hacham et al. 2012), as *bri1* mutants and plants treated with the BR biosynthesis inhibitor brassinazole, had lower than wild-type PIN2 levels. Moreover, restricting BRI1 activity in the epidermis restored PIN2 expression while BRI1 activity in the inner cells had weaker effects. In addition, levels of transcripts encoding endogenous PIN2 and transgenic PIN2-GFP slightly changed in response to short BR treatment, in opposite directions to PIN2 protein levels. Thus, PIN2

appears to be regulated by BRs post-transcriptionally. Interestingly, BRs had similar effects on PIN4 accumulation in the columella, although Hacham et al. (2012) detected no apparent transcriptional perturbation of PIN 1, 3 and 7 genes in BR-deficient plants.

Effects of BRs on shoot growth

BRs have particularly strong growth-promoting effects in stems of seedlings and young plants (providing the basis for one of the most widely used BR bioassays, the second bean internode bioassay), mainly via promotion of expression of genes involved in cell elongation and wall extensibility (Horvath et al. 2003; Yang et al. 2011). Expression of the BR receptor BRI1 or a BR biosynthetic enzyme (CPD) in the epidermis, but not the vasculature, can rescue the dwarf phenotype of null mutants. BR signaling from the epidermis is not sufficient to establish normal vascular organization. Moreover, shoot growth is restricted when the epidermis is depleted of BRs and BRs act locally within a leaf (Savaldi-Goldstein et al. 2007). Ibanes et al. (2009) showed that BR signaling is required to set the number and arrangement of vascular bundles in *Arabidopsis* shoots, as BR-deficient and BR-insensitive mutants have fewer bundles, while BR signaling mutants have more than wild type stems. In *Arabidopsis*, *BRL1* and *BRL3* (but not *BRL2*) are functional genes involved in vascular tissue development (Cano-Delgado et al. 2004). Growth is also associated with increased carbohydrate demand for biosynthetic metabolism, and Goetz et al. (2000) provided evidence for a role of BR in carbohydrate allocation in hypocotyls, showing that expression of the tomato *Lin6* gene is specifically induced by BR in the 10 mm section immediately below the hypocotyl hook. Furthermore, Tanaka et al. (2003) found that BR treatment can dramatically increase the length of hypocotyl cells in light-grown plants, while application of the BR-biosynthesis inhibitor brassinazole (Brz) reduces their average length.

BRs act interactively with other plant hormones in the regulation of plant elongation, notably they have essentially additive effects with gibberellins (GA) and synergistic effects with auxin (IAA) on stem segment elongation (Unterholzner et al. 2015; Tong et al. 2014). More specifically, Katsumi (1985) showed that treating cucumber hypocotyl sections with 28-homoBL

(6) followed by IAA resulted in synergistic enhancement of auxin-induced elongation, but when the order of treatment was reversed, 28-homoBI (6) was inactive, suggesting that BRs modulate stem tissues' capacity to respond to IAA. Effects of various combinations of GAs, IAA and brassinolide (2) on the development and leaf anatomy of *Tabebuia alba* (Cham.) seedlings have also been evaluated (Ono et al. 2000). GA plus brassinolide (2) induced the highest stem and petiole growth rates of the tested combinations, accompanied by significant development of lateral buds, while BI (2) application alone stimulated petiole growth but not stem growth. The effects of varying concentrations of IAA alone or in combination with BI (2) or the cytokinin 6-benzylaminopurine (BAP), on ethylene production in *Arabidopsis* inflorescences have also been evaluated (Arteca and Arteca 2008). The cited authors found that treating inflorescences with BI (2) alone had no effect on ethylene production. However, application of BI (2) in combination with IAA increased ethylene production dramatically more than IAA alone, so co-application of these hormones had a synergistic effect on ethylene production. In addition, BI (2) reportedly promotes stomatal closure and inhibits stomatal opening in *Vicia faba* L., by inhibiting inwardly rectifying K⁺ currents of guard cell protoplasts in a similar manner to ABA (Haubrick et al. 2006). However, effects of applying BI (2) and ABA together were not additive, suggesting that these two hormones may function in interacting pathways that regulate stomatal and guard cell physiology.

Roles of BRs in reproductive organ development

Very high BR levels are found in pollen and seeds, and extensive studies have demonstrated that BR plays a significant role in regulation of reproductive development in *Arabidopsis* (Chory et al. 1991; Li et al. 2010). On the female side, BRs influence numbers of ovule primordia and, thus, numbers of seeds produced (Huang et al. 2013), while dwarf BR *Arabidopsis* mutants display male sterility, due to abnormal tapetum development and reductions in pollen number (Ye et al. 2010). Conversely, exogenous application of 24-epiBL can stimulate *Arabidopsis* pollen germination and pollen tube growth rates, and increase final pollen tube lengths, in vitro (Vogler et al. 2014). BRs may also influence branching and flower formation through

modulation of metabolic pathways and nutrient allocation patterns, then subsequently promote fertilization by stimulating filament and pollen growth (Müssig 2005). Numerous other factors are, of course, interactively influential. For example, Kesy et al. (2003) found that the short-day plant *Pharbitis nil* Choisy treated with BI (2) and CS (1) formed fewer flowers than control plants, but the degree of inhibition depended on the concentration applied, the application method and length of the inductive dark period. Nevertheless, spraying BR at flowering generally leads to a significant increase in the production of various crops (Vriet et al. 2012). For example, Symons et al. (2006) found that exogenous application of 24-epiBI (4) and brassinazole respectively promoted and delayed ripening of grape (*Vitis vinifera* L.) fruits. Similarly, Vardhini and Rao (2002) found that applying 24-epiBI (4) and 28-homoBI (6) to tomato pericarp discs promoted ripening-associated changes, including increases in lycopene and carbohydrate contents, accompanied by increases in ethylene production and reductions in chlorophyll and ascorbic acid contents.

Roles of BRs in seed development and germination

Little is known about effects of BRs on seeds' composition (protein, fat, mineral and carbohydrate contents). However, results of exogenous 24-epiBI (4) application on wheat and soybean (*Glycine max*) plants and seeds suggest that BRs significantly increase β -carotene and tocopherol contents, to varying degrees depending on the treatment method (Janeczko et al. 2009, 2010). Hayat and Ahmad (2003) found that 28-homoBL (6) treatment increased catalase, α -amylase and peroxidase levels of wheat seedlings. They also found that soaking *Lens culinaris* Medik seeds in 28-homoBI solutions resulted in seedlings with reduced root lengths and numbers of nodules, but higher than control nitrate reductase activities (Hayat and Ahmad 2003). In addition, analysis of seed development, and effects of BR on genes known to control various aspect of seed development, in BR-deficient (*det2*) and BR-insensitive (*bri1-5*) mutants by Jiang et al. (2013) demonstrated that BRs play crucial roles in regulation of the size, mass, and shape of *Arabidopsis* seeds. Their results indicate that BR increased seed size by affecting the integument, endosperm, and embryo development, and that BR-activated BZR1 directly

regulated several genes known to control seed size. They further showed that the seed shape was determined largely by BR signals from maternal tissues, while BRs produced by the embryo and endosperm seemed sufficient to increase seed size, providing evidence for a localized mode of BR action in seed development. BR applications also reportedly enhance germination of the seeds of certain parasitic angiosperms and cereals (Yamaguchi et al. 1987; Takeuchi et al. 1995).

Leubner-Metzger (2001) extended these findings by showing that BR and the gibberellin GA₄ and Bl both promote germination of *Nicotiana tabacum* L. cv. Havana 425 seeds, but by distinct signal transduction pathways. GAs and light release photodormancy via a common pathway, but BRs do not release photodormancy. Furthermore, induction of β GLU I, a molecular marker for endosperm rupture, is associated with the GA/light pathway, but not BR signaling. Instead, the BR pathway seems to promote rupture of non-dormant seeds' endosperm by enhancing the growth potential of the embryo (Leubner-Metzger 2001). Additional insights have been provided by Nomura et al. (2004, 2007), who found that a severely BR-deficient mutant of pea, *lk*, produces irregularly shaped seeds, and that Bl and CS levels peaked, together with levels of transcripts of two BR C-6-oxidase genes (*CYP85A1*, *CYP85A6*), when pea seeds were rapidly growing. In early germination stages they also observed increases in levels of CS (but not Bl) in the growing tissues, accompanied by high levels of *CYP85A1* and *CYP85A6* transcripts. However, the level of 6-deoxocathasterone decreased while levels of downstream intermediates increased. These results suggest that during germination and early growth stages CS plays a significant role, while 6-deoxocathasterone is the major BR for storage and utilization (Nomura et al. 2004). The transgenic rice plants expressing maize, rice or *Arabidopsis* genes encoding sterol C-22 hydroxylases that control BR hormone levels using a promoter that is active in only the stems, leaves and roots were created by Wu and co-workers. These plants produced more tillers and more seed than wild-type plants. The phenotypic changes brought about 15–44 % increases in grain yield per plant relative to wild-types in greenhouse and field trial (Wu et al. 2008).

Roles of BRs in stress tolerance

BRs play important roles in tolerance to diverse biotic and abiotic stresses, including drought, salt,

heat, cold, hypoxia, pesticides, heavy metals and disease (Bajguz and Hayat 2009). Drought, salinity and freeze-induced dehydration constitute direct osmotic stresses, and thus share some effects with chilling and hypoxia, which can indirectly induce osmotic stress by impairing water uptake and increasing water losses. BR can reportedly increase Indian mustard plants' tolerance of water stress, as both foliar sprays of 28-homoBl (6) and exposure to drought can raise their levels of antioxidant systems (Fariduddin et al. 2009). Application of 28-HomoBl (6) can also stimulate growth of wheat (*Triticum aestivum* L.) under drought stress, through increasing relative water contents, nitrate reductase activity, chlorophyll contents and photosynthesis rates (Sairam 1994), and these effects are stronger in drought-tolerant varieties. Further studies demonstrated that 24-epiBl (4) confers cucumber plants with hypoxia tolerance, probably by enhancing sugar supplies to hypoxic roots and shifting hypoxic metabolism from lactate fermentation to alcohol fermentation (Kang et al. 2009). Furthermore, treatment with Bl (2) enhances increases in superoxide dismutase, catalase, ascorbate peroxidase, ascorbic acid and carotenoid contents in maize seedlings subjected to water stress (Li et al. 1998). Similarly, the *Arabidopsis det2* mutant is insensitive to the negative growth effects of very low oxygen concentrations, and its enhanced oxidative stress resistance has been associated with constitutive increases in superoxide dismutase activity and catalase transcript levels, suggesting that long-term BR deficiency results in a constant in vivo physiological stress in this mutant (Cao et al. 2005). Treating tomato and rape seedlings with 24-epiBl (4) increases thermotolerance, which is associated with higher synthesis of heat shock proteins (HSPs) and expression of mitochondrial small HSPs in the leaves (Dhaubhadel et al. 2002; Kagale et al. 2007). Finally, cultures of *Chlorella vulgaris* BEIJERNICK treated with BRs and heavy metals show lower bioaccumulation of the heavy metals zinc, cadmium, lead and copper than cultures treated with the metals alone (with effects declining in the presented order of metals). Hence, BRs reduce the impact of heavy metals stress on growth of these cultures, by decreasing chlorophyll, sugar and protein losses and increasing synthesis of phytochelatin (Bajguz and Hayat 2009; Bajguz 2000, 2002).

Roles of BRs in plant immunity

Exogenously applied BRs can induce resistance to a broad spectrum of pathogens. For example, BI (2) can protect rice against blast and bacterial blight diseases, and enhance resistance of tobacco to the bacterial pathogen *Pseudomonas syringe* and fungal pathogen *Oidium* (Nakashita et al. 2003). Similarly, Kripach et al. (2000) found that cultivation in medium containing BI (2), 24-epiBI (4) and 28-homoBI (6) increased potato cuttings' resistance to viral infection during all developmental stages. 24-EpiBI can also protect barley against *Fusarium* diseases: in the absence of BR, 31 % of spikelets inoculated with *F. culmorum* by Shahin et al. (2013) displayed typical *Fusarium* head blight bleaching symptoms. In contrast, just 4 % of spikelets treated with 24-epiBI showed such symptoms and they had substantially lower losses in grain number and weight. Other authors have shown that BI treatment can increase Cucumber mosaic virus resistance in *Arabidopsis* by enhancing antioxidative enzyme activities and expression of defense-associated genes (Zhang et al. 2015). Furthermore, numerous studies have indicated that BR signaling components play essential roles in innate immunity, particularly BAK1, which besides participating in BR signaling is involved in regulation of microbe-induced cell death and interacts with various pattern recognition receptors (Kemmerling et al. 2007; Albrecht et al. 2012; Belkhadir et al. 2012).

Biological activities of BRs in human cells

Roles of BRs in plant cells and plants have been intensively examined. Much less is known about their effects on human cells. However, as reviewed in the following sections, various studies have shown that natural BRs have potentially valuable activities for medical applications, including antiviral (Wachsman et al. 2000; Wachsman and Castilla 2012), immunomodulatory and neuroprotecting activity (Michelini et al. 2008, 2013), antioxidant and neuroprotective activities in a mammalian neuronal cell line (Carange et al. 2011), and antiproliferative effects in animal cells in vitro (Franek et al. 2003; Malíková et al. 2008; Obakan et al. 2014a, b; Rárová et al. 2012; Steigerová et al. 2010, 2012; Wu and Lou 2007).

Antiviral effects of BRs

Antiviral activities of various natural BRs have been reported recently, including in vitro activities of 28-homoCS (5) and 28-homoBI (6) against herpes virus, measles and arenaviruses (Wachsman et al. 2000, 2002). Brassinolide (2) and 28-homoCS (5) also have broad antiviral activities at μM levels, against both RNA and DNA viruses, including poliovirus, HSV-1 and HSV-2, measles virus, vesicular stomatitis virus (and the arenaviruses Junin, Tacaribe and Pichinde), mediated by interference with virus protein synthesis and maturation of viral particles (Wachsman and Castilla 2012). Several series of synthetic BR analogues have also been tested for antiherpetic and other antiviral activities, and ranked in terms of inhibitory effects on viral replication in confluent, non-growing Vero cell cultures (Michelini et al. 2004; Ramírez et al. 2000; Wachsman et al. 2004). Many of the BR derivatives were active against different viruses. The compounds were also usually cytotoxic, with varying selectivity indexes, and only a few exhibited medium cytotoxicity towards Vero cells (IC_{50} values between 20 and 60 μM).

Antiproliferative activities of BRs

To date, more than 70 anticancer molecules have been derived from natural products (Newman and Cragg 2007), and many other natural products have been identified as potential sources of new anticancer drugs. The first published indications of relevant activities were stimulatory effects of 24-epiBI (4) on cultured hybridoma mouse cells, including increases in mitochondrial membrane potential and proportions of cells in the G_0/G_1 phase, and reductions in intracellular antibody levels and proportions of cells in S phase (Franek et al. 2003). Subsequently, the natural BRs 24-epiBI (4) and 28-homoCS (5) were shown to have inhibitory effects on the growth and viability of various cancer cell lines (Malíková et al. 2008). Promisingly, these BR substances affected the viability of human cancer cell lines (including CEM T-lymphoblastic leukemia, MCF7 breast carcinoma, A549 lung carcinoma, K562 chronic myeloid leukemia, RPMI 8226 multiple myeloma, HeLa cervical carcinoma, G361 malignant melanoma and HOS osteosarcoma lines) without affecting growth of normal human cells (BJ fibroblasts). Treatments with

28-homoCS (5) and 24-epiBl (4) resulted in potent, dose-dependent reductions in the viability of CEM and RPMI 8226 cells, with IC_{50} levels ranging from 13 to 50 μ M. 28-HomoCS (5) was the most active compound against CEM cells (IC_{50} 13 μ M), inducing approximately three times stronger responses than 28-homoBl (6), indicating that transformation of 6-oxo-7-oxalactone to 6-oxo functionality substantially increases the growth inhibitory activity of BRs. The presence of a 24R side chain strongly reduces BR cytotoxicity. The 24R side chain in 24-epicastosterone also reduced anticancer activity compared to castasterone. 28-HomoCS (5) and 28-homoBl (6) have an ethyl group in their side chains at C24 and are more effective than the corresponding analogues with C24 methyl groups. Since β -ecdysone, which contains 2 β ,3 β ,22 α -functionality, showed no detectable activity, the most important groups for strong cytotoxic BR effect could be a 3 α -hydroxy group, 2 α ,3 α -vicinal diol or 3 α ,4 α -vicinal diol (Malíková et al. 2008). The cited authors also found that natural BRs had varying effects on estrogen- and androgen-sensitive and insensitive breast and prostate cancer cell lines, but they were more potent towards the hormone-sensitive cell lines, possibly due to their modulation of steroid receptors. Flow cytometry-based analysis revealed that the BRs can disturb the cell cycle in breast and prostate cancer cell lines. More specifically, 28-homoCS (5) and 24-epiBl (4) blocked the cell cycle in the G₁ phase with concomitant reductions in percentages of cells in the S phase (Malíková et al. 2008). These are typical growth inhibitory effects of antiestrogens on MCF7 cells (Parl 2000). More recently, Hamdy et al. (2009) isolated a new natural BR-related metabolite, 3-keto-22-epi-28-norcastasterone, from *Cystoseira myrica* that exhibited anticancer activity against human liver (HAPG-2) and colon cancer (HCT116) cells with IC_{50} values of 5.63 and 1.16 μ M, respectively.

Furthermore, 24-epiBl (4) can induce apoptosis by activating a polyamine catabolic pathway in LNCaP and DU145 prostate cancer cells, thereby inducing accumulation of cytotoxic hydrogen peroxide and aldehydes, and LNCaP cells expressing androgen receptor (AR) are more sensitive than cells lacking functional AR (Obakan et al. 2014a). The same authors also showed that 24-epiBl (4) can induce mitochondria-mediated and caspase-dependent apoptosis, regardless

of p53 expression, in androgen-insensitive PC3 prostate cancer cells. Biosynthetic precursors of natural 24-norbrassinolide phytohormones containing low-polarity groups in the cyclic moiety of the molecule also have antiproliferative and apoptosis-inducing activities towards LNCaP human prostate adenocarcinoma cells, mediated by blockage of the cell cycle (Khrpach et al. 2012).

BRs' effects in wound healing and angiogenesis

The growth of new blood vessels in animals, angiogenesis, is essential for organ growth, growth of solid tumors, and establishment of metastasis (Folkman 1974), to meet the organs' or tumors' oxygen and nutrient supply requirements. The most important components of angiogenic tissues, the endothelial cells, could be targets for antiangiogenic therapy because they are not transformed, and easily accessible to antiangiogenic agents. Furthermore, tumor angiogenesis could be potentially targeted by novel generations of drugs (of natural or synthetic origin) that inhibit proteolytic enzymes which break down the extracellular matrix surrounding existing capillaries, and/or inhibit endothelial cell proliferation and migration, and/or enhance apoptosis of tumors' endothelial cells (Kesisis et al. 2007; Bhat and Singh 2008). Such candidate leads include various steroids with known antiangiogenic activity, among others 2-methoxyestradiol, progestin, medroxyprogesterone acetate, and glucocorticoids such as dexamethasone and cortisone (Pietras and Weinberg 2005). Several natural BRs also have effects on endothelial cells, including potential antiangiogenic activity (Rárová et al. 2012). Furthermore, two natural BRs, 24-epiBl (4) and 28-homoCS (5), reportedly inhibit growth of Human Microvascular Endothelial Cells (HMEC-1) in a dose-dependent manner, and reduce migration of Human Umbilical Vein Endothelial Cells (HUVEC). Treatment with these agents slightly reduced numbers of tubes that formed, as well as numbers of nodes, one of the parameters usually used to assess antiangiogenic activity. This antiangiogenic activity of BRs, along with their antiproliferative activity, suggests that these plant hormones and new synthetic derivatives with stronger antiangiogenic activities are potentially important leads for new anticancer drugs (Rárová et al. 2012).

BR effects on neuronal cells

Oxidative stress and apoptosis are frequently implicated in the neuronal cell damage associated with various neurodegenerative disorders, such as Parkinson's disease. BRs are known to promote stress tolerance in plants via modulation of the antioxidative enzyme cascade. Their antioxidative effects on mammalian neuronal cells have not yet been examined in detail. However, Carange et al. (2011) analyzed the ability of 24-epiBI to protect PC12 neuronal cells from oxidative stress induced by 1-methyl-4-phenylpyridinium, MPP(+), and consequent apoptosis in dopaminergic neurons. They found that 24-epiBI reduced levels of intracellular reactive oxygen species, and modulated superoxide dismutase, catalase, and glutathione peroxidase activities. The observed antioxidative properties of 24-epiBI led to the inhibition of MPP(+)-induced apoptosis by reducing DNA fragmentation, the Bax/Bcl-2 protein ratio and caspase-3 cleavage. This was the first demonstration of the potent antioxidant and neuroprotective effects of 24-epiBI in a mammalian neuronal cell line (Carange et al. 2011).

Molecular mechanism of BR action in human cells

Signaling in plants by BRs, and the resulting genomic responses, are initiated by a BR molecule binding to a receptor kinase, brassinosteroid-insensitive 1 (BRI1), localized in the plasma membrane (Kim and Wang 2010). However, there are differences between steroids' actions in animal and plant cells. In animal models, lipophilic steroids bind to steroid receptors located in either the cytosol or nucleus that diffuse through the plasma membrane. Ligand binding induces a conformational change and dimerization with another receptor that allows the ligand/receptor complex to bind to target DNA sequences and directly modify gene expression over hours or even days (Losel and Wehling 2003).

Because of the similarity between BRs and human steroids, we have studied interactions of BRs with human steroid receptors using reporter assays and a competitive binding assay. Reporter assays showed that 24-epiBI (4) is a weak antagonist of estrogen-receptor- α (ER- α). However, we found no evidence that 24-epiBI (4) and 28-homoCS (5) directly bind to ER- α and ER- β in competitive binding assays.

24-EpiBI and 28-homoCS were also found to modulate levels of PR in a dose-response manner by western blotting, although we did not observe any interaction in the cellular reporter assay for PR response. Treatment with 24-epiBI increased protein levels of PR, while 28-homoCS led to downregulation of PR protein expression using western blotting. It seems possible that there are other mode of actions, both steroid receptor-dependent and independent, similarly to what is considered for 2-methoxyestradiol, which binds to estrogen receptors. Furthermore, the action mechanism of the antiangiogenic response is probably unrelated to the receptor pathway (Rárová et al. 2012).

BR treatment has been shown to affect ER- α and ER- β localization patterns in MCF7 breast adenocarcinoma cells by Steigerová et al. (2010), who observed strong and uniform ER- α immuno-nuclear labelling in untreated MCF7 cells, and cytoplasmic speckles of ER- α immunofluorescence in MCF7 cells treated with 28-homoCS (5) or 24-epiBI (4). In contrast to ER- α , ER- β was predominantly found in the cytoplasm of untreated MCF7 cells. However, ER- β was notably re-located to the nuclei after 28-homoCS (5) treatment, whereas it was predominantly present at the periphery of the nuclei, in 24-epiBI (4)-treated cells. These changes were accompanied by down-regulation of the ERs following BR treatment (Steigerová et al. 2010). As mentioned above, these natural BRs inhibited cell growth and blocked the cell cycle at G₁ phase, with concomitant reductions in percentages of cells in the S phase. Concomitant reductions in expression of key cycling proteins—including cyclin-dependent kinases (CDKs) 2/4/6 and cyclins D₁ and E—and pRb phosphorylation were also detected during cell cycle arrest, accompanied by up-regulation of the cyclin-dependent kinase inhibitors p21^{Waf1/Cip1} and p27^{Kip1}, which inhibit cyclin/CDK complexes. BRs also induced apoptosis in both estrogen-sensitive and estrogen-insensitive breast cancer cell lines, by distinct modulation of the expression of apoptosis-related proteins (Steigerová et al. 2010). Induction of apoptosis after the treatment with BRs was confirmed by TUNEL staining and double staining with propidium iodide and acridine orange in both MCF7 and MDA-MB-468 breast cancer cell lines, but the changes in expression profiles of apoptosis-related proteins triggered by the BRs differed between the lines. In MCF7 cells, the BR treatments induced expression of the

antiapoptotic Bcl-2 and Bcl-X_L proteins, and slightly affected levels of p53 and MDM-2 proteins. In MDA-MB-468 cells cleavage of caspase-3 into fragments (part of the apoptotic cascade) 24 h after the BR treatment was followed by G₁ phase arrest and increases in the subG₁ fraction, representing apoptotic bodies. The mechanism of action of BRs in human cells is still largely unknown, but it seems that BRs may interact with one or more of the numerous steroid-binding proteins. Moreover, BRs could also induce multiple effects, both steroid receptor-dependent and independent (Steigerová et al. 2010).

Both BRs also inhibited cell growth, induced G₁ blocks and reduced expression of cyclin D₁, CDK4/6 and pRb in LNCaP androgen-sensitive prostate cancer cells, while in androgen-insensitive DU-145 prostate cancer cells they increased proportions of cells in the G₂/M phase, and down-regulated cyclins A and B₁. Changes in AR localization patterns in LNCaP cells treated with BRs were also detected immunofluorescently. Apoptosis caused by BRs was detected in both prostate cancer cell lines, but (as observed following BR treatment of breast cancer cells) changes in their expression profiles of apoptosis-related proteins differed (Steigerová et al. 2012). Effects of 24-epiB1 (4) in androgen-sensitive LNCaP prostate cancer cells, androgen-insensitive DU145 prostate cancer cells and PNT1a normal prostate epithelial cells were also examined to probe possible mechanisms of apoptotic cell death and related changes in polyamine biosynthetic and catabolic pathways. 24-EpiB1 (4) decreased intracellular polyamine levels and significantly down-regulated ornithine decarboxylase in both prostate cancer cell lines, and also modulated antizyme and antizyme inhibitor expression levels in LNCaP cells. In both cell lines, it also up-regulated expression of the catabolic enzymes spermidine/spermine N¹-acetyltransferase and polyamine oxidase. Adding specific inhibitors of these enzymes and co-treatment with 24-epiB1 (4) for 24 h decreased formation of cleaved PARP fragments in LNCaP cells (Obakan et al. 2014a). In conclusion, BRs seem to have extremely potent activities indicating that they may be valuable natural leads for novel anticancer drugs.

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Příloha 2

Synthesis of New Brassinosteroid 24-Norcholane Type Analogs Conjugated in C-3 with Benzoate Groups

Ferrer, K.; Díaz, K.; Kvasnica, M.; Olea, A. F.; Cuellar, M.; Espinoza, L.

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Article

Synthesis of New Brassinosteroid 24-Norcholane Type Analogs Conjugated in C-3 with Benzoate Groups

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Abstract: The metabolism of brassinosteroid leads to structural modifications in the ring skeleton or the side alkyl chain. The esterification and glycosylation at C-3 are the most common metabolic pathways, and it has been suggested that conjugate brassinosteroids are less active or inactive. In this way, plants regulate the content of active brassinosteroids. In this work, the synthesis of brassinosteroid 24-norcholane type analogs conjugated at C-3 with benzoate groups, carrying electron donor and electron attractant substituents on the aromatic ring, is described. Additionally, their growth-promoting activities were evaluated using the Rice Lamina Inclination Test (RLIT) and compared with that exhibited by brassinolide (used as positive control) and non-conjugated analogs. The results indicate that at the lowest tested concentrations (10^{-8} – 10^{-7} M), all analogs conjugated at C-3 exhibit similar or higher activities than brassinolide, and the diastereoisomers with *S* configuration at C-22 are the more active ones. Increasing concentration (10^{-6} M) reduces the biological activities of analogs as compared to brassinolide.

Keywords: synthesis; brassinosteroids; analogs; 24-norcholane; benzoate esters; Rice Lamina Inclination Test; conjugated in C-3

1. Introduction

Brassinosteroids (BRs) are an important group of polyhydroxylated sterol plant growth regulators in multiple developmental processes, at nanomolar to micromolar concentration, including cell division, cell elongation, vascular differentiation, reproductive development, and modulation of gene expression [1]. BRs also influence various other developmental processes such as the germination of seeds, rhizogenesis, flowering, senescence, abscission, and maturation. They also confer resistance to plants against various abiotic and biotic stresses [2–5].

Since the discovery of brassinolide (1) (Figure 1) [6], 70 BRs, among them 65 unconjugated (free) and 5 conjugated BRs, have been isolated from 60 plant species including 51 angiosperms (12 monocotyledons and 39 dicotyledons), 6 gymnosperms, 1 pteridophyte (*Equisetum arvense*), 1 bryophyte (*Marchantia polymorpha*), and chlorophyte, the alga (*Hydrodictyon reticulatum*). Thus, BRs are widely distributed in the plant kingdom, including higher and lower plants [7].

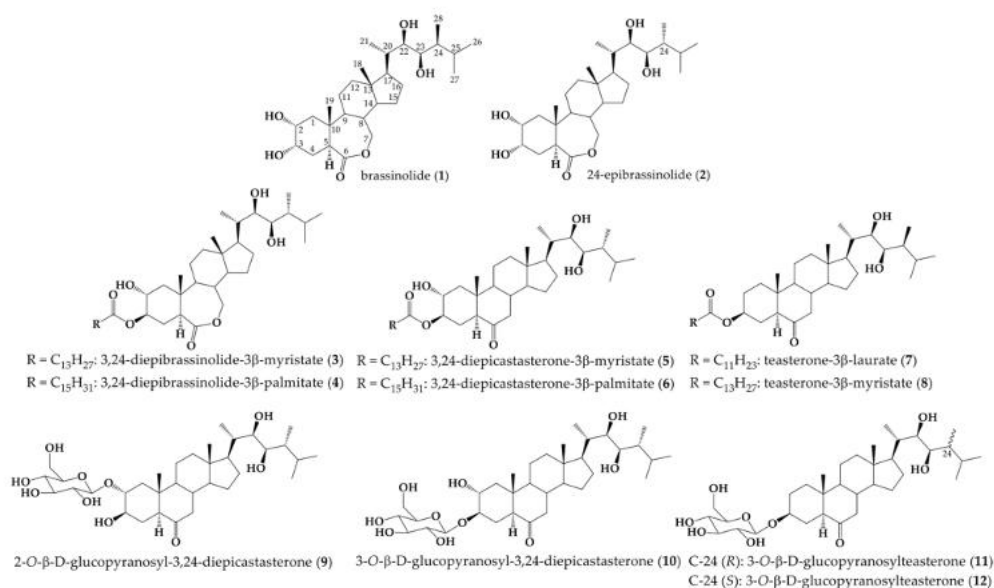


Figure 1. Structure of natural occurring brassinolide (1), 24-epibrassinolide (2), metabolites conjugated in C-3 formed by esterification (compounds 3–8), and metabolites conjugated in C-2 and C-3 formed by glycosylation (compounds 9–12).

On the other hand, a study of the miscellaneous pathways of BRs metabolism in plants reported the existence of around 19 conjugated metabolites in positions C-2, C-3, C-23, C-25, or C-26 [8]. Eight out of nineteen correspond to conjugates formed by esterification in C-3 [9–14]. The other eleven conjugated metabolites are formed by glycosylation at C-2, C-3, C-23, C-25, or C-26 [8,13–20]. Some examples of these structures are shown in Figure 1. It seems that conjugated compounds are used by plants to store inactive BRs that can be converted to active forms by de-conjugation reactions. Additionally, the natural conjugates 3, 4 (Figure 1) were synthesized from 24-epibrassinolide (2) [21].

On the other hand, a series of C-3 esterified derivatives of 24-epibrassinolide (13–15) and synthetic BRs analogs (16–19) (Figure 2) have been reported [21,22].

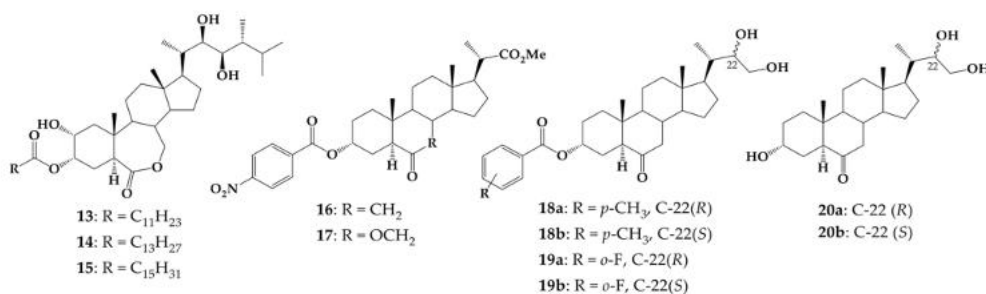


Figure 2. Structure of synthetic brassinosteroids (BRs) analogs conjugated in C-3 (compounds 13–19b) and free synthetic analogs 20a and 20b.

However, biological evaluations in the Bean Second-Internode Bioassay (BSIB) for compounds 16 and 17 indicated that these analogs are less active than 24-epibrassinolide [22]. These results are in line with previously established structure-activity relationships ob-

tained for natural BRs. These structure-activity relationship (SAR) studies have been made using BSIB and the Rice Lamina Inclination Test (RLIT) [23–25], and their main goal is to define general structural requirements for the growth-promoting activity of BRs [24,26–29]. These results have been used to guide the synthesis of BRs analogs with a variety of structural modifications but keeping those considered essential for biological activity.

Several studies have proved that synthetic BRs analogs with significant structural changes and different substituents, both in the ring and the alkyl chain, can induce similar or even higher biological effects in plants as compared to natural BRs [30–36]. Some recent reviews of the growth-promoting activity of BRs and their analogs have established novel structural requirements for the existence of biological activity [23,37–39]. For example, it has been shown that methyl ethers at C-3 are more active than **1** in the RLIT [40], whereas benzoate esters in the C-3 position were found to be less active than 24-epibrassinolide in the BSIB test [22].

In a previous *in silico* study, we have assessed the effect on activity of different groups attached to position C-3 of BRs analogs. The results suggest that bulky groups reduce the activity, whereas functionalization with electronegative and hydrophobic groups would increase it [29]. Thus, in this work, we present the synthesis of four new BR 24-norcholane type analogs conjugated with benzoate groups in C-3 (Figure 2, compounds **18a**, **18b**, **19a**, and **19b**). The aromatic ring of the benzoate group contains electron-donor and electron-withdrawing substituents. Their growth-promoting activities were evaluated using RLIT, and the results were compared with those reported for other structurally similar analogs (Figure 2, compounds **20a** and **20b**) [38,41,42].

The synthesis and evaluation of biological activity of these BRs analogs, conjugated in C-3 with benzylic esters, are studied either to get new active molecules or to elucidate if esterification could be a metabolic path for exogenous BRs.

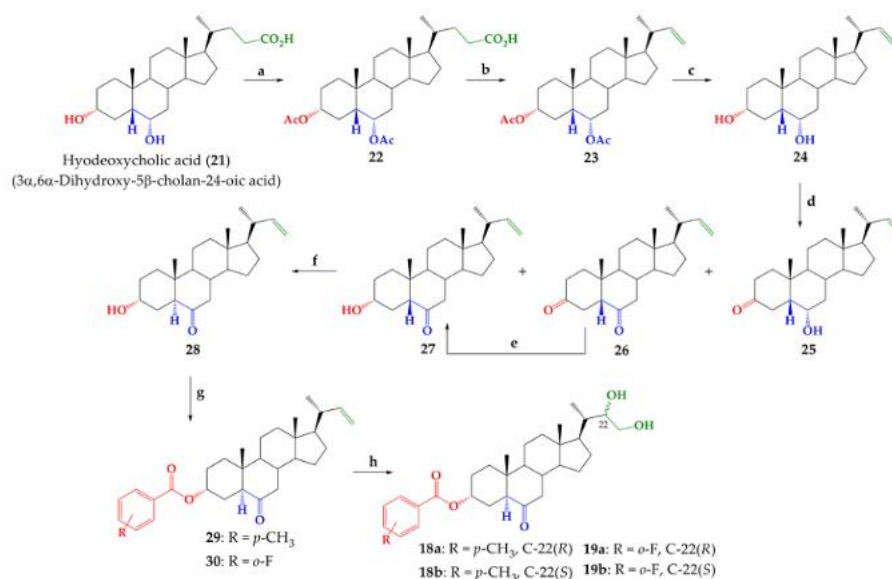
2. Results and Discussion

2.1. Chemistry

To obtain the new BR analogs conjugated in C-3 (**18a**, **18b**, **19a**, and **19b**, Figure 2), the synthetic strategy shown in Scheme 1 was developed. The synthesis of the key intermediate alkene **28** has been previously reported [43], but herein, we have introduced some modifications in the synthesis steps to increase the yields of reactions. In addition, more clear spectroscopic evidence (¹H- and ¹³C-NMR) is provided [43–45].

The standard acetylation (Ac₂O/*N,N*-dimethylaminopyridine(DMAP)/CH₂Cl₂) of hydoxycholeic acid (**21**) leads to known diacetylated derivative (**22**) in 91.1% yield (ref. 80% yield, [44,45]). In the ¹H-NMR spectrum of compound **22** (Figure S1, Supplementary Materials), the protons of both acetate groups appear at $\delta_H = 2.02$ ppm (3H, s, CH₃CO₂-C6) and 1.99 ppm (3H, s, CH₃CO₂-C3) [44,45]. While in the ¹³C-NMR spectrum (Figure S1, Supplementary Materials), the observed signals at $\delta_C = 170.56$ ppm (CH₃CO₂-C6), 170.52 ppm (CH₃CO₂-C3), 21.36 ppm (CH₃CO₂-C6), and 21.32 ppm (CH₃CO₂-C3) confirm the presence of both acetate groups.

Oxidative decarboxylation of the side chain of compound **22**, with the PhI(OAc)₂/Cu(OAc)₂ system [44,45], leads to olefin **23** in 99.6% yield (yield data were not reported by other authors). In the ¹H-NMR of compound **23** (Figure S2, Supplementary Materials), the protons H-22, H_{trans}-23, and H_{cis}-23 appear at $\delta_H = 5.65$ ppm (ddd, *J* = 17.1, 10.2 and 8.4 Hz), 4.90 ppm (dd, *J* = 17.1 and 2.0 Hz), and 4.81 ppm (dd, *J* = 10.2 and 2.0 Hz), respectively [44,45]. Meanwhile, in the ¹³C-NMR (Figure S2, Supplementary Materials), the carbons C-22 and C-23 appear at $\delta_C = 145.06$ and 111.69 ppm, respectively. These signals confirm the presence of terminal alkene.



Scheme 1. Synthesis of hydoxycholeic acid derivatives 22–30 and C-3 conjugated brassinosteroid analogues 18a, 18b, 19a, and 19b. Reagents and conditions: (a) Ac₂O/DMAP, CH₂Cl₂, rt, 48 h, 91.1% yield; (b) PhI(OAc)₂/Cu(OAc)₂, C₆H₆, reflux, 5 h, 99.6% yield; (c) K₂CO₃ (15% *p/v*, H₂O)/(CH₃)₂CO/CH₃OH, reflux, 7 h, 97.1% yield; (d) PCC/CH₂Cl₂, rt, 48 h, Column Chromatography (C.C.) separation, 25 (2.4% yield), 26 (19.1% yield), and 27 (40.2% yield); (e) NaBH₄/MeOH, 0–5 °C, 1 h, 76.3% yield; (f) HCl/CH₃OH 2.5% *v/v*, rt, 48 h, 74.8% yield; (g) *p*-CH₃C₆H₄COCl or *o*-FC₆H₄COCl/DMAP/CH₂Cl₂, rt, 2 h, 29 (87.9% yield), 3 h, 30 (56.0% yield); (h) Dihydroquinidine-Chlorobenzoate(DHQD-CLB)/CH₃SO₂NH₂, K₂CO₃/K₃[Fe(CN)₆], OsO₄/(CH₃)₃COH/H₂O, rt, 5 h, 18a/18b (1.0:1.0), 91.6% yield; 19a/19b (1.0:1.0), 80.8% yield.

The saponification of diacetate 23 with the system K₂CO₃/acetone/methanol/reflux leads to diol 24 in 97.1% yield (ref. 98% yield, [43]). Although compound 24 was previously reported, no NMR spectroscopic data were reported [43,45]. So, the observed signals in the ¹H-NMR spectrum (Figure S3, Supplementary Materials) at δ_H = 4.02–3.96 ppm (1H, m) and 3.48–3.42 ppm (1H, m) were assigned to carbinolic hydrogens H-6 and H-3, respectively (Table 1). While in the ¹³C-NMR (Figure S3, Supplementary Materials), the carbons C-6 and C-3 appear at δ_C = 67.63 and 71.72 ppm, respectively (Table 1). The assignments for the H-6 and H-3 signals were confirmed by the 2D HSQC spectrum of compound 24.

Table 1. Differences in ¹H- and ¹³C-NMR chemical shifts for H-3, H-6, C-3, and C-6 observed for compounds 24–27.

Compound	H-3 (δ_H ppm)	H-6 (δ_H ppm)	C-3 (δ_C ppm)	C-6 (δ_C ppm)
24	3.48–3.42	4.02–3.96	71.72	67.63
25	–	4.15–4.10	212.63	67.73
26	–	–	208.65	210.82
27	3.70–3.62	–	70.18	213.89

The subsequent oxidation of compound 24 with the PCC/CH₂Cl₂ system produces a mixture of three oxidation products (Scheme 1), which were efficiently separated by flash chromatographic column. So, the least polar product was identified as diketone 26 (19.1% yield), a product of intermediate polarity identified as monoketone 25 (2.4% yield), and the most polar product identified as the desired monoketone 27 (40.2% yield). Diketone 26 was previously obtained by the oxidation of glycol with Jones reagent in

95% yield [45]. Meanwhile, diketone **26** and monoketone **27** were obtained by oxidation with the PDC/CH₂Cl₂ system, with 21% and 61.7% yields, respectively [43]. The IR and ¹H-NMR spectroscopic data for compounds **26** and **27** were consistent with those reported (Figures S5 and S6, Supplementary Materials) [43,45]. However, none of these previous works reported obtaining monoketone **25**. In the ¹H-NMR spectrum of this compound (Figure S4, Supplementary Materials), the observed signal at $\delta_H = 4.15\text{--}4.10$ ppm (1H, m) was assigned to carbinolic hydrogen H-6, whereas in the ¹³C-NMR spectrum (Figure S4, Supplementary Materials), the observed signal at $\delta_C = 67.73$ ppm corresponds to C-6.

Table 1 shows the differences detected for the main signals observed in the ¹H- and ¹³C-NMR spectra of compounds **24** to **27**. All this information was confirmed by the 2D HSQC correlation spectra of compounds **25**–**27**.

Diketone **26** was conveniently converted to the desired monoketone **27** by selective reduction with NaBH₄/MeOH [43] at low temperature (0–5 °C) with 76.3% yield (step e, Scheme 1). The spectroscopic data of this compound and **27**, which was obtained by direct oxidation from **24** (step d, Scheme 1), were identical.

Then, compound **27** was easily isomerized under acid condition (2.5% *v/v* HCl/MeOH) to give the derivative **28** possessing 5 α -cholestan-6-one skeleton (74.8% yield) [43,44,46–49]. The IR, ¹H- and ¹³C-NMR spectroscopic data registered for compound **28** were consistent with those reported (Figure S7, Supplementary Materials) [43,46].

The C-3 benzylation reactions of **28** were carried out according to the methodology reported for other steroidal nuclei [41,50,51]. So, treatment of **28** with 4-methylbenzoyl chloride/DMAPI in CH₂Cl₂ and pyridine led to 4-methylbenzoate derivative **29** with 87.9%. Similarly, the reaction of **28** with 2-fluorobenzoyl chloride led to 2-fluorobenzoate derivative **30** with 56.0%. The structures of both derivatives were mainly characterized by ¹H and ¹³C spectroscopy. For derivative **29**, the presence of aromatic signals at $\delta_H = 8.00$ ppm (2H, d, *J* = 9.0 Hz) and 6.93 ppm (2H, d, *J* = 9.0 Hz) were assigned to the hydrogens HAr-2' and HAr-3', respectively, whereas the signals appearing at $\delta_C = 163.36$, 131.58, 123.35, and 113.70 ppm were assigned to the aromatic carbons C4', C2'/C6', C1', and C3'/C5' (Figure S8, Supplementary Materials). For derivative **30**, the presence of the aromatic signals at $\delta_H = 7.92$ ppm (1H, td, *J* = 7.6 and 1.8 Hz); 7.54–7.48 ppm (1H, m); 7.21 ppm (1H, td, *J* = 7.6 and 1.2 Hz), and 7.13 ppm (1H, ddd, *J* = 10.7, 7.6 and 0.9 Hz) were assigned to the hydrogens HAr-6', HAr-4', HAr-3', and HAr-5', respectively (Figure S9, Supplementary Materials). In the ¹³C-NMR spectrum (Figure S9, Supplementary Materials), the observed signals at $\delta_C = 161.92$ ppm (d, ¹*J*_{CF} = 259.1 Hz); 134.44 ppm (d, ³*J*_{CF} = 8.4 Hz); 132.32 ppm (d, ³*J*_{CF} = 0.9 Hz); 124.11 ppm (d, ⁴*J*_{CF} = 3.6 Hz); 119.38 (d, ²*J*_{CF} = 9.6 Hz); and 117.05 (d, ²*J*_{CF} = 21.6 Hz) were assigned to the aromatic carbons C2', C4', C6', C5', C1', and C3', respectively (Figure 3).

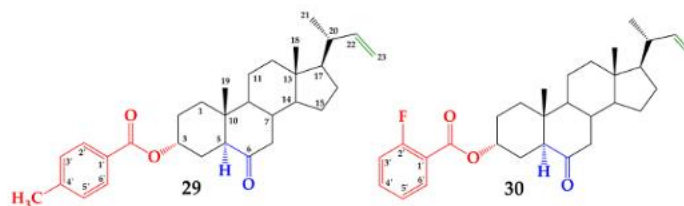


Figure 3. Structures of derivatives **29** and **30** and numbering of aromatic carbon atoms used in this study.

Recently, the synthesis of glycols C22/C23 in steroids with the shortest side chain of 24-nor-5 α -cholane type by a Sharpless dihydroxylation reaction has been reported [42]. The results showed that this type of hydroxylation leads to a mixture of C-22 glycols (*R/S*) with an approximate 1:1 ratio of both diastereomers [42]. Thus, both olefins **29** and **30** were dihydroxylated following this method and using dihydroquinidine *p*-chlorobenzoate

(DHQD-CLB) as a chiral ligand (Scheme 1) [32,42]. The Sharpless dihydroxylation of derivative **29** produced the **18a/18b** diastereoisomer mixture with a total 91.6% yield. The diastereomeric ratio of each glycol in the mixture can be established by the integration of ^1H -NMR signals assigned to the C-21 methyl group, which appear at $\delta_{\text{H}} = 0.921$ and 0.953 ppm in **18a** and **18b** diastereoisomers, respectively. Based on these NMR measurements, the relative ratio of **18a:18b** was determined as 1.0:1.0. Subsequently, the diastereoisomers mixture was separated by a semi-preparative HPLC system, allowing obtaining the analogs **18a** and **18b**.

The structure and stereochemistry at C-22 of compounds **18a** and **18b** was established by a simple comparison of ^1H - and ^{13}C -NMR spectra obtained for derivatives **20a** and **20b**, which were previously reported [41,42]. These comparisons considered chemical shifts (δ), coupling constants (J), and multiplicities of signals corresponding to H-22, H-23a, H-23b, and CH₃-21 (^1H -NMR) and chemical shifts (δ) in ^{13}C -NMR of both epimers. The main differences in these spectroscopic parameters are listed in Table 2.

Table 2. Comparison between signals of ^1H - (500.1 MHz, CDCl_3) and ^{13}C - (125.8 MHz, CDCl_3) NMR for H/C21, H/C22, and H/C23a-b, for the epimers **18a** and **18b**.

H/C Signal	Compound 18a	Compound 18b
H-21	0.921 ppm (3H, d, $J = 6.7$ Hz)	0.953 ppm (3H, d, $J = 7.0$ Hz)
H-22	3.66–3.61 ppm (1H, m)	3.51 ppm (1H, t, $J = 10.2$ Hz)
H-23a	3.80 ppm (1H, ddd, $J = 8.9, 3.3$ and 1.2 Hz)	3.83–3.76 ppm (1H, m)
H-23b	3.52 ppm (1H, dd, $J = 10.8$ and 3.3 Hz)	3.69–3.57 ppm (1H, m)
C21	12.73 ppm	13.15 ppm
C22	74.14 ppm	73.92 ppm
C23	66.16 ppm	62.54 ppm

Similarly, a Sharpless dihydroxylation of derivative **30** produced the **19a/19b** diastereoisomers mixture with a total 80.8% yield. The diastereomeric ratio of each glycol in the mixture was 1.0:1.0 (established by the integration of ^1H -NMR signals assigned to the C-21 methyl group, which appear at $\delta_{\text{H}} = 0.917$ and 0.954 ppm in **19a** and **19b** diastereoisomers, respectively). The diastereoisomers mixture was separated by semi-preparative HPLC system, allowing obtaining analogs **19a** and **19b**. Similar to the above, the main differences in spectroscopic parameters of epimers are listed in Table 3.

Table 3. Comparison between signals of ^1H - (500.1 MHz, CDCl_3) and ^{13}C - (125.8 MHz, CDCl_3) NMR for H/C21, H/C22, and H/C23a-b, for the epimers **19a** and **19b**.

H/C Signal	Compound 19a	Compound 19b
H-21	0.917 ppm (3H, d, $J = 6.4$ Hz)	0.954 ppm (3H, d, $J = 6.7$ Hz)
H-22	3.75–3.39 ppm (1H, m)	3.51 ppm (1H, t, $J = 10.2$ Hz)
H-23a	3.87–3.76 ppm (1H, m)	3.84–3.76 ppm (1H, m)
H-23b	3.75–3.39 ppm (1H, m)	3.71–3.59 ppm (1H, m)
C21	12.73 ppm	13.13 ppm
C22	75.56 ppm	73.90 ppm
C23	61.15 ppm	62.52 ppm

In summary, four new BRs 24-norcholane type analogs conjugated at the C-3 position with benzoate groups substituted with electron donor and electron-withdrawing groups in the *p*-position (compounds **18a**, **18b**, **19a** and **19b**) have been synthesized and characterized.

2.2. Biological

In this work, the activity of new BR 24-norcholane type analogs conjugated at the C-3 position was evaluated using the Rice Lamina Inclination Test. The results of this test were compared with those obtained for other free analogs of 24-norcholane type (analogs **20a** and **20b** [38]) and with brassinolide. This assay was used because of its specificity and high

sensitivity for **1** and their analogs [31,52,53]. The bending angles were measured as the difference between the induced angle produced by treatment with each compound and that found for the negative control. Results obtained for **1**, which was used as positive control, and BR analogs **18a**, **18b**, **19a**, **19b**, **20a**, and **20b** are listed in Table 4.

Table 4. Comparison between BRs C-3 conjugated 24-norcholeane and free 24-norcholeane type analogs on lamina inclination of rice seedlings.

Compounds	Bending Angle between Laminae and Sheaths (Degrees $\pm$ SD)		
	1×10^{-8} M	1×10^{-7} M	1×10^{-6} M
1 (C+)	31 $\pm$ 11	41 $\pm$ 4.5	70 $\pm$ 7.6
18a	61 $\pm$ 6.3 *	68 $\pm$ 9.6 *	46 $\pm$ 7.5 *
18b	64 $\pm$ 3.3 *	48 $\pm$ 2.9	14 $\pm$ 4.8 *
19a	43 $\pm$ 5.0 *	58 $\pm$ 2.4 *	68 $\pm$ 9.6
19b	68 $\pm$ 5.0 *	61 $\pm$ 2.5 *	30 $\pm$ 0.0 *
20a †	45 $\pm$ 9.5 *	31 $\pm$ 5.0	24 $\pm$ 5.8 *
20b †	35 $\pm$ 3.0	60 $\pm$ 3.0 *	62 $\pm$ 12
Control (C−)		7 $\pm$ 5.0	

† Data previously obtained and reported in reference [38]. Brassinolide (**1**) was used as positive control. The negative control only contained sterile distilled water. These values represent the mean $\pm$ standard deviation of two independent experiments with at least six replicates each ($n = 12$). (*) Represents experiments with a significant difference between positive control (**1**) and analog treatments at $p < 0.05$ significance level (least square differences (LSD) t -test).

Interestingly, these data clearly indicate that 24-nor-5 α -choleane type analogs conjugated at C-3 exhibit interesting growth-promoting activity. These results are in line with previous studies for other analogs of 24-norcholeane type [38,48]. All C-3 conjugated analogs exhibit higher activity than brassinolide at the lowest concentrations (1×10^{-8} and 1×10^{-7} M) (Figure 4 and Table 4).

To simplify the data analysis, we will consider the data obtained at 1×10^{-8} and 1×10^{-7} M to analyze the correlation between chemical structure and biological activity. The results indicate that at these concentrations, **18b** and **19b** are the most active in the series of conjugated analogs (**19b** was the most active at the concentration of 1×10^{-8} M, whereas **18a** was the most active at the concentration of 1×10^{-7} M) (see Table 3), and they were more active than the free analogs **20a** and **20b**. Another important effect to consider is related to the configuration on the C-22 carbon of the side chain. Thus, at the concentration of 1×10^{-8} , analogs **18a** and **19a** with C-22(R) configuration are less active than analogs **18b** and **19b** with C-22(S) configuration. However, an opposite effect is observed for analogs **20a** and **20b**. Similarly, at the concentration of 1×10^{-7} M, the analogs **19b** and **20b** with C-22(S) configuration are more active than analogs **19a** and **20a** with C-22(R) configuration. However, an opposite effect is observed for analogs **18a** and **18b**. The results observed for the pairs **18a/18b** (at 1×10^{-7} M) and **20a/20b** (at 1×10^{-8} M) would be aligned with those reported for natural occurring BRs with an intact side chain, which indicates that glycol function with C-22(R) and C-23(R) configuration appears essential for a high biological activity and are more active than those with C-22(S) and C-23(S) configuration [3,54]. However, these apparently contradictory structural effects of BRs analogs could be explained in attributed to the shorter side chains. This structural feature could give a greater rotational freedom degree.

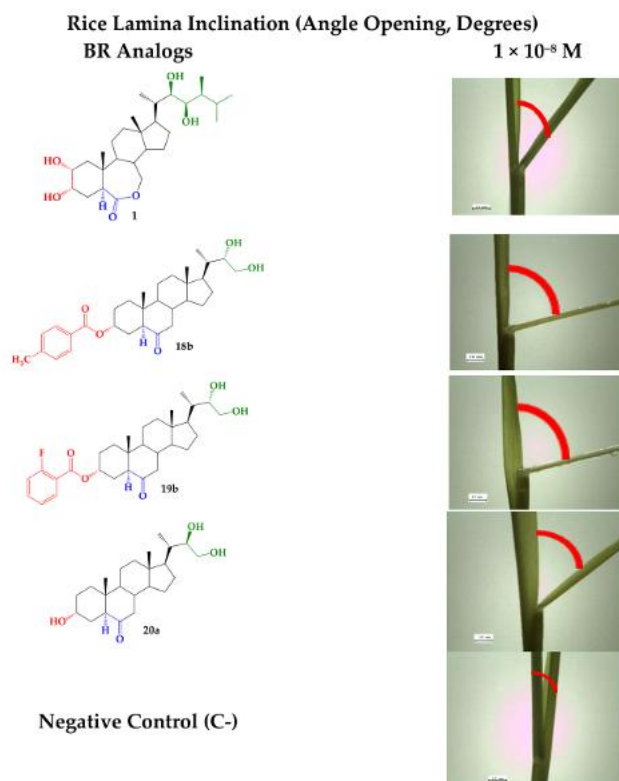


Figure 4. Rice-lamina assays using the second leaf lamina joints of excised leaf segments treated with BR analogs (18b, 19b, and 20a) at 1×10^{-8} M. Brassinolide (1) was used as positive control at the same concentrations. The negative control only contained sterile distilled water.

3. Materials and Methods

3.1. Chemistry

All reagents were purchased from commercial suppliers and used without further purification. Melting points were measured on a SMP3 apparatus (Stuart-Scientific, now Merck KGaA, Darmstadt, Germany) and are uncorrected. ^1H -, ^{13}C -, ^{13}C -DEPT-135, gs 2D HSQC, and gs 2D HMBC NMR spectra were recorded in CDCl_3 and MeOD solutions, and they are referenced to the residual peaks of CHCl_3 at $\delta = 7.26$ ppm and $\delta = 77.00$ ppm for ^1H and ^{13}C , respectively and CD_3OD at $\delta = 3.30$ ppm and $\delta = 49.00$ ppm for ^1H and ^{13}C , on an Avance 400 Digital NMR spectrometer (Bruker, Rheinstetten, Germany) operating at 400.1 MHz for ^1H and 100.6 MHz for ^{13}C , and JEOL JNM-ECA 500 NMR spectrometer (JEOL, Tokyo, Japan) operating at 500.16 MHz for ^1H , 125.77 MHz for ^{13}C , and 470.62 MHz for ^{19}F . Chemical shifts are reported in ppm and coupling constants (J) are given in Hz; multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), triplet (t), quartet (q), multiplet (m), and broad singlet (bs). IR spectra were recorded as KBr disks in a Fourier Transform Infrared (FT-IR) 6700 spectrometer (Nicolet, Thermo Scientific, San Jose, CA, USA) and frequencies are reported in cm^{-1} . High-resolution mass spectra (HRMS) were recorded in an API HRMS instrument, and the samples were dissolved in chloroform (or chloroform: methanol; 1:1; v/v , in the case of hydroxylated compounds) to a concentration of $10 \mu\text{g mL}^{-1}$. The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into

the ion source, and analyzed in full scan mode. The source of the Synapt G2-Si mass spectrometer (Waters, Manchester, UK) was operated in positive ionization mode (ASAP⁺), if not stated otherwise, at a source temperature of 120 °C. The corona needle current was kept at 5 μ A and the collision energy was kept at 4 V. The probe temperature was ramped up from 50 to 600 °C in 3 min. Data were acquired from 50 to 1000 Da with 1.0 s scan time in high-resolution mode. The data were processed using the Masslynx 4.1 software (Waters, Milford, MA, USA). A mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. For analytical TLC, silica gel 60 in a 0.25 mm layer was used, and TLC spots were detected by heating after spraying with 10% H₂SO₄ in H₂O. Chromatographic separations were carried out by conventional column on silica gel 60 (230–400 mesh) using EtOAc–hexane gradients of increasing polarity. All organic extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure, below 40 °C. The HPLC system consisted of a Waters semi-preparative HPLC system including a quaternary pump, a liquid handler, and UV-Vis and Evaporative Light Scattering Detector (ELSD) detectors. The semi-preparative column was filled with silica gel.

3.1.1. Synthesis

3 α ,6 α -Diacetoxy-5 β -cholan-24-oic acid (**22**)

To a solution of hyodeoxycholic acid (**21**) (25.4 g, 64.62 mmol) in 400 mL of CH₂Cl₂ (DCM), 150 mg of DMAP, 2 mL of pyridine, and 24.4 mL (257.6 mmol) of Ac₂O were added. The reaction mixture was kept under constant stirring and room temperature for 48 h. The end of the reaction was verified by TLC; then, the mixture was concentrated to a volume approximately 50 mL under reduced pressure. Then, EtOAc (200 mL) and 200 mL of HCl solution (1 $\times$ 10^{−5} M) were added. The organic layer was separated and washed with water (4 $\times$ 50 mL), with saline (NaCl) solution (3 $\times$ 50 mL) until pH = 5, dried over Na₂SO₄, and filtered. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH₂Cl₂ (16 mL) and chromatographed on silica gel with EtOAc/hexane (20%, 200 mL). Compound **22** (28.06 g 91.1% yield) was a colorless solid, m.p. = 109.7–110.9 °C (106–110 °C [44]). IR $\nu_{\max}$ (cm^{−1}): 3527 (O–H); 2948; 2899 and 2870 (C–H); 1738 (C=O); 1722 (C=O); 1681 (C=O); 1455 (CH₂); 1364 (CH₃); 1256 (C–O); 1242 (C–O); 1027 (C–O). ¹H-NMR (400.1 MHz, CDCl₃) (Figure S1, Supplementary Materials): δ (ppm) = 5.14–5.10 (1H, m, H-6); 4.71–4.65 (1H, m, H-3); 2.37 (1H, ddd, *J* = 15.3, 10.1 and 5.0 Hz, H-23a); 2.23 (1H, ddd, *J* = 16.0, 10.1, and 6.4 Hz, H-23b); 2.02 (3H, s, CH₃CO₂-C6); 1.99 (3H, s, CH₃CO₂-C3); 1.97–1.94 (1H, m, H-5); 0.95 (3H, s, H-19); 0.90 (3H, d, *J* = 6.4 Hz, H-21); 0.62 (3H, s, H-18). ¹³C-NMR (100.6 MHz, CDCl₃) (Figure S1, Supplementary Materials) δ (ppm) = 180.09 (C-24); 170.56 (CH₃CO₂-C6); 170.52 (CH₃CO₂-C3); 73.66 (C-3); 70.92 (C-6); 56.06 (C-14); 55.84 (C-17); 45.28 (C-9); 42.81 (C-13); 39.78 (C-5 and C-12); 35.97 (C-10); 35.18 (C-20); 34.96 (C-7); 34.54 (C-8); 31.20 (C-1); 30.93 (C-22); 30.64 (C-23); 28.01 (C-4); 26.35 (C-16); 26.15 (C-2); 24.00 (C-15); 23.19 (C-19); 21.36 (CH₃CO₂-C6); 21.32 (CH₃CO₂-C3); 20.61 (C-11); 18.17 (C-21); 11.95 (C-18).

24-Nor-5 β -cholan-22-ene-3 α ,6 α -diyl diacetate (**23**)

To a solution of **22** (2.00 g, 4.20 mmol) in dry benzene (150 mL) were added Cu(OAc)₂·H₂O (200 mg, 1.0 mmol) and pyridine (2.5 mL). Then, under reflux, PhI(OAc)₂ (7.04 g, 21.5 mmol) was added in four portions at hourly intervals. After the addition was completed, the reaction was continued for 1 h. The end of the reaction was verified by TLC, and then, the mixture was filtered, and the solvent was evaporated under reduced pressure. The crude was re-dissolved in DCM (5 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (19.8:0.2 $\rightarrow$ 15.8:4.2). The reaction was repeated 5 times under identical conditions. Compound **23** (9.0 g, 99.6% yield) was obtained as a colorless solid, m.p. = 89.0–90.9 °C (88–89 °C [45]). IR $\nu_{\max}$ (cm^{−1}): 3082 (CH=CH₂); 2940; 2887 and 2867 (C–H); 1740 (C=O); 1727 (C=O); 1633 (C=C); 1460 (CH₂); 1366 (CH₃); 1244 (C–O); 1026 (C–O); 908 (CH=CH₂). ¹H-NMR (400.1 MHz, CDCl₃) (Figure S2, Supplementary Materials): δ (ppm) = 5.65 (1H, ddd, *J* = 17.1, 10.2, and 8.4 Hz, H-22); 5.16–5.13 (1H, m, H-6); 4.90 (1H,

dd, $J = 17.1$ and 2.0 Hz, H-23a); 4.81 (1H, dd, $J = 10.2$ and 2.0 Hz, H-23b); 4.71–4.69 (1H, m, H-3); 2.07–2.03 (1H, m, H-20); 2.04 (3H, s, CH₃CO₂-C6); 2.01 (3H, s, CH₃CO₂-C3); 2.00–1.94 (1H, m, H-5); 1.02 (3H, d, $J = 6.6$ Hz, H-21); 0.97 (3H, s, H-19); 0.67 (3H, s, H-18). ¹³C-NMR (100.6 MHz, CDCl₃) (Figure S2, Supplementary Materials) δ (ppm) = 170.47 (CH₃CO₂-C6); 170.45 (CH₃CO₂-C3); 145.06 (C-22); 111.69 (C-23); 73.70 (C-3); 70.95 (C-6); 56.21 (C-14); 55.59 (C-17); 45.39 (C-9); 42.83 (C-13); 41.13 (C-20); 39.93 (C-5); 39.79 (C-12); 36.07 (C-10); 35.06 (C-1); 34.63 (C-8); 31.30 (C-7); 28.36 (C-16); 26.44 (C-2); 26.25 (C-4); 24.09 (C-15); 23.27 (C-19); 21.41 (CH₃CO₂-C6); 21.37 (CH₃CO₂-C3); 20.68 (C-11); 20.07 (C-21); 12.18 (C-18).

24-Nor-5 β -chol-22-ene-3 α ,6 α -diol (**24**)

To a solution of **23** (8.06 g, 18.71 mmol) in a mixture 1:1 of acetone/MeOH (60 mL), a 15% aqueous solution of K₂CO₃ (37.4 mmol) was added. The suspension was stirred and refluxed for 7 h. The end of the reaction was verified by TLC. Then, the solvent was removed, the residue was diluted with EtOAc (80 mL), and the mixture was washed with 3×80 mL of HCl solution (1×10^{-3} M). The organic layer was dried over Na₂SO₄ and filtered. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH₂Cl₂ (15 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (19.8:0.2 $\rightarrow$ 7.2:12.8). Compound **24** (6.3 g; 97.1% yield) was obtained as a colorless solid, m.p. = 154.4–155.2 °C (150–152 °C [45]). IR $\nu_{\max}$ (cm⁻¹): 3381 (O-H); 3088 (CH=CH₂); 2934, 2889, and 2868 (C-H); 1637 (C=C); 1462 (CH₂); 1336 (CH₃); 1269 (C-O); 1043 (C-O); 912 (CH=CH₂). ¹H-NMR (400.1 MHz, Acetone) (Figure S3, Supplementary Materials): δ (ppm) = 5.66 (1H, ddd, $J = 17.4$, 10.0, and 8.6 Hz, H-22); 4.90 (1H, dd, $J = 17.1$ and 2.0 Hz, H-23a); 4.82 (1H, dd, $J = 10.2$ and 2.0 Hz, H-23b); 4.02–3.96 (1H, m, H-6); 3.48–3.42 (1H, m, H-3); 1.02 (3H, d, $J = 6.6$ Hz, H-21); 0.91 (3H, s, H-19); 0.66 (3H, s, H-18). ¹³C-NMR (100.6 MHz, Acetone) (Figure S3, Supplementary Materials): δ (ppm) = 146.00 (C-22); 112.05 (C-23); 71.72 (C-3); 67.63 (C-6); 57.20 (C-14); 56.40 (C-17); 49.70 (C-9); 43.51 (C-13); 42.09 (C-20); 40.89 (C-5); 40.80 (C-12); 36.65 (C-1); 36.55 (C-10); 35.97 (C-7); 35.70 (C-8); 31.43 (C-4); 30.05 (C-2); 29.15 (C-16); 24.90 (C-15); 24.10 (C-19); 21.54 (C-11); 20.55 (C-21); 12.55 (C-18).

6 α -Hydroxy-24-nor-5 β -chol-22-en-3-one (**25**), 24-nor-5 β -chol-22-ene-3,6-dione (**26**) and 3 α -hydroxy-24-nor-5 β -chol-22-en-6-one (**27**)

A solution of **24** (6.0 g, 17.3 mmol) in DCM (100 mL) with 3.76 g (17.3 mmol) of Pyridinium chlorochromate (PCC) in 60 mL of DCM (added by slow drip) was slowly stirred for 48 h at room temperature. The end of the reaction was verified by TLC; then, the reaction mixture was filtered on alumina and washed with ethyl acetate (20 mL). The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH₂Cl₂ (10 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (19.8:0.2 $\rightarrow$ 8.8:11.2). Four fractions were obtained: Fraction I, 1.14 g (19.1% yield) of compound **26**; Fraction II, 0.141 g (2.4% yield) of compound **25**; Fraction III, 2.39 g (40.2% yield) of compound **27**; and Fraction IV, 3.58 g of unreacted **24**. The reaction was repeated with compound **24** twice with another 5.5 g each and **26** (2.08 g), **25** (4.28 g), and **27** (0.258 g) were obtained.

Compound **25** was obtained as a colorless solid. ¹H-NMR (400.1 MHz, CDCl₃) (Figure S4, Supplementary Materials): δ (ppm) = 5.68 (1H, ddd, $J = 17.1$, 10.2, and 8.4 Hz, H-22); 4.93 (1H, dd, $J = 17.1$ and 2.0 Hz, H-23a); 4.84 (1H, dd, $J = 10.2$ and 2.0 Hz, H-23b); 4.15–4.10 (1H, m, H-6); 2.42–2.39 (2H, m, H-4); 1.05 (3H, d, $J = 6.6$ Hz, H-21); 1.03 (3H, s, H-19); 0.72 (3H, s, H-18). ¹³C-NMR (100.6 MHz, CDCl₃) (Figure S4, Supplementary Materials): δ (ppm) = 212.63 (C-3); 145.00 (C-22); 111.77 (C-23); 67.73 (C-6); 56.17 (C-14); 55.55 (C-17); 50.16 (C-9); 42.79 (C-13); 41.15 (C-20); 40.31 (C-5); 39.74 (C-12); 37.08 (C-1); 37.06 (C-7); 36.24 (C-10); 36.02 (C-4); 34.56 (C-8); 34.40 (C-2); 28.35 (C-16); 24.17 (C-15); 22.85 (C-19); 21.08 (C-11); 20.10 (C-21); 12.23 (C-18).

Compound **26** was obtained as a colorless solid, m.p. = 177.5–178.9 °C (197–200 °C [45]). IR $\nu_{\max}$ (cm⁻¹): 3073 (CH=CH₂); 2964, 2947, 2873, and 2855 (C-H); 1716 (C=O); 1693 (C=O); 1632 (C=C); 1466 (CH₂); 1382 (CH₃); 1245 (C-O); 1216 (C-O); 908 (CH=CH₂). ¹H-NMR (400.1

MHz, CDCl₃) (Figure S5, Supplementary Materials): δ (ppm) = 5.68 (1H, ddd, J = 17.1, 10.2, and 8.4 Hz, H-22); 4.94 (1H, dd, J = 17.1 and 2.0 Hz, H-23a); 4.86 (1H, dd, J = 10.2 and 2.0 Hz, H-23b); 2.67 (1H, dd, J = 15.0 and 13.2 Hz, H-4a); 2.50 (1H, dd, J = 12.5 and 4.9 Hz, H-5); 2.42 (1H, dd, J = 14.3 and 5.3 Hz, H-1a); 2.36 (1H, dd, J = 5.2 and 1.9 Hz, H-4b); 1.07 (3H, d, J = 6.5 Hz, H-21); 0.98 (3H, s, H-19); 0.74 (3H, s, H-18). ¹³C-NMR (100.6 MHz, CDCl₃) (Figure S5, Supplementary Materials): δ (ppm) = 210.82 (C-6); 208.65 (C-3); 144.72 (C-22); 111.99 (C-23); 59.74 (C-5); 56.86 (C-14); 55.40 (C-17); 43.02 (C-13); 42.16 (C-7); 41.06 (C-20); 40.98 (C-9); 39.90 (C-4); 39.41 (C-12); 38.31 (C-10); 36.69 (C-8); 36.49 (C-1); 35.78 (C-2); 28.21 (C-16); 23.93 (C-15); 22.48 (C-19); 21.30 (C-11); 20.09 (C-21); 12.17 (C-18).

Compound 27 was obtained as a colorless solid, m.p. = 151.9–153.6 °C. IR $\nu_{\max}$ (cm^{−1}): 3288 (O-H); 3074 (CH=CH₂); 2970, 2949, and 2867 (C-H); 1702 (C=O); 1637 (C=C); 1458 (CH₂); 1379 (CH₃); 1248 (C-O); 1064 (C-O); 912 (CH=CH₂). ¹H-NMR (400.1 MHz, CDCl₃) (Figure S6, Supplementary Materials): δ (ppm) = 5.67 (1H, ddd, J = 17.1, 10.1, and 8.4 Hz, H-22); 4.93 (1H, dd, J = 17.1 and 2.0 Hz, H-23a); 4.85 (1H, dd, J = 10.1 and 2.0 Hz, H-23b); 3.70–3.62 (1H, m, H-3); 2.20–2.18 (2H, m, H-7); 2.14 (1H, dd, J = 12.1 and 5.1 Hz, H-5); 1.05 (3H, d, J = 6.6 Hz, H-21); 0.86 (3H, s, H-19); 0.70 (3H, s, H-18). ¹³C-NMR (100.6 MHz, CDCl₃) (Figure S6, Supplementary Materials): δ (ppm) = 213.89 (C-6); 144.89 (C-22); 111.87 (C-23); 70.18 (C-3); 59.41 (C-5); 56.89 (C-14); 55.41 (C-17); 43.04 (C-13); 42.93 (C-7); 41.11 (C-20); 40.07 (C-9); 39.53 (C-12); 37.99 (C-10); 37.07 (C-8); 34.86 (C-1); 34.38 (C-4); 29.86 (C-2); 28.26 (C-16); 23.99 (C-15); 23.17 (C-19); 20.84 (C-11); 20.08 (C-21); 12.15 (C-18).

3 α -Hydroxy-24-nor-5 β -chol-22-en-6-one (27) from 24-nor-5 β -chol-22-ene-3,6-dione (26)

A solution of compound 26 (3.22 g, 9.4 mmol) was prepared in 100 mL of 1:1 MeOH/THF mixture. This solution was placed in a bath of ice-water between 0 and 5 °C. Subsequently, 117.9 mg (3.15 mmol) of NaBH₄ were added in four portions (approximately 29.5 mg each) maintaining the temperature and with slow stirring. The end of the reaction was verified by TLC, 10 mL of acetone, and subsequently, 5 mL of HCl 2.5% were added, maintaining the reaction temperature. The reaction mixture was concentrated by evaporation under reduced pressure to a volume of about 15 mL, and then, EtOAc (50 mL) was added. The organic layer was washed with saturated solution of NaHCO₃ (20 mL) and water (2 $\times$ 30 mL), dried over anhydrous MgSO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (9.8:0.2 $\rightarrow$ 5.8:4.2). Two fractions were obtained: Fraction I, 0.753 g of unreacted compound 26; Fraction II, 2.47 g (76.3% yield) of compound 27. The melting point and spectroscopic properties (¹H- and ¹³C-NMR) of compounds 26 and 27 were identical to those reported above for the direct oxidation of compound 24.

3 α -Hydroxy-24-nor-5 α -chol-22-en-6-one (28)

Compound 27 (6.08 g, 17.65 mmol) was dissolved in 100 mL of 2.5% *v/v* HCl-MeOH at room temperature and constant agitation for 48 h. The end of the reaction was verified by TLC. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in 60 mL of EtOAc. The organic layer was washed with saturated solution of NaHCO₃ (2 $\times$ 15 mL) and water (2 $\times$ 30 mL), dried over MgSO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (5 mL) and chromatographed on silica gel with PE/EtOAc mixtures of increasing polarity (9.8:0.2 $\rightarrow$ 4.0:6.0). Two fractions were obtained: Fraction I, 4.55 g (74.8% yield) of compound 28 and Fraction II, 1.15 g of unreacted compound 27. Compound 28 was obtained as a colorless solid, m.p. = 169.9–170.9 °C. ¹H-NMR (400.1 MHz, CDCl₃) (Figure S7, Supplementary Materials): δ (ppm) = 5.68 (1H, ddd, J = 17.0, 10.1, and 8.3 Hz, H-22); 4.93 (1H, dd, J = 17.0 and 2.0 Hz, H-23a); 4.84 (1H, dd, J = 10.1 and 2.0 Hz, H-23b); 4.20–4.17 (1H, m, H-3); 2.74 (1H, t, J = 7.9 Hz, H-5); 2.32 (1H, dd, J = 13.1 and 4.5 Hz, H-7 α); 2.15–1.97 (3H, m, H-20, H-12 α , and H-7 β); 1.06 (3H, d, J = 6.6 Hz, H-21); 0.75 (3H, s, H-19); 0.71 (3H, s, H-18). ¹³C-NMR (100.6 MHz, CDCl₃) (Figure S7, Supplementary Materials): δ (ppm) =

212.67 (C-6); 144.96 (C-22); 111.81 (C-23); 65.47 (C-3); 56.84 (C-14); 55.37 (C-17); 53.88 (C-9); 51.69 (C-5); 46.86 (C-7); 42.96 (C-13); 41.57 (C-10); 41.12 (C-20); 39.41 (C-12); 37.98 (C-8); 31.69 (C-1); 28.19 (C-2 and C-4); 27.71 (C-16); 23.93 (C-15); 21.07 (C-11); 20.07 (C-21); 12.32 (C-19); 12.20 (C-18). HRMS (API+) (Figure S14, Supplementary Materials): m/z calculated for $C_{23}H_{37}O_2$ ($[M + H]^+$) 345.2794; found 345.2795.

6-Oxo-24-nor-5 α -chol-22-en-3 α -yl 4-methylbenzoate (29)

A solution of compound **28** (150 mg, 0.44 mmol) and DMAP (44 mg, 0.36 mmol) was prepared in 3 mL of anhydrous pyridine. To this solution, 4-methylbenzoyl chloride 174 μ L (1.32 mmol) was added by slow dripping, and the reaction was maintained at room temperature with constant stirring for 2 h. The end of the reaction was verified by TLC. After completion of the reaction, 3 mL of hot water was added. After an additional 20 min of stirring, the mixture was extracted with EtOAc (20 mL) and washed successively with saturated $NaHCO_3$ solution (2×10 mL) and water (2×10 mL), dried over anhydrous $MgSO_4$, and filtered. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH_2Cl_2 (5 mL) and chromatographed on silica gel with EtOAc/cyclohexane (1.0:19) mixture. Compound **29** (117 mg, 87.9% yield) was obtained as a colorless solid, m.p. = 133.3–135.2 $^{\circ}C$. 1H -NMR (500.16 MHz, $CDCl_3$) (Figure S8, Supplementary Materials): δ (ppm) = 7.90 (2H, d, J = 8.0 Hz, HAr-2'); 7.24 (2H, d, J = 8.0 Hz, HAr-3'); 5.65 (1H, ddd, J = 17.1, 10.1 and 8.6 Hz, H-22); 5.36–5.34 (1H, m, H-3); 4.91 (1H, ddd, J = 17.1, 1.8 and 0.9 Hz, H-23a); 4.82 (1H, dd, J = 10.1 and 1.8 Hz, H-23b); 2.66 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.41 (3H, s, CH_3 -Ar); 2.32 (1H, dd, J = 13.1 and 4.6 Hz, H-7 α); 1.03 (3H, d, J = 6.7 Hz, H-21); 0.786 (3H, s, H-19); 0.695 (3H, s, H-18). ^{13}C -NMR (125.77 MHz, $CDCl_3$) (Figure S8, Supplementary Materials): δ (ppm) = 211.92 (C-6); 165.78 (CO_2 -Ar); 145.01 (C-22); 143.61 (C4'-Ar); 129.60 (C2'-Ar and C6'-Ar); 129.18 (C3'-Ar and C5'-Ar); 128.18 (C1'-Ar); 111.95 (C-23); 69.37 (C-3); 56.86 (C-14); 55.50 (C-17); 54.09 (C-9); 53.01 (C-5); 46.85 (C-7); 43.05 (C-13); 41.48 (C-10); 41.24 (C-20); 39.44 (C-12); 38.06 (C-8); 32.81 (C-1); 28.31 (C-16); 25.57 (C-2); 25.25 (C-4); 24.02 (C-15); 21.79 (CH_3 -Ar); 21.22 (C-11); 20.16 (C-21); 12.57 (C-19); 12.31 (C-18). HRMS (API+) (Figure S15, Supplementary Materials): m/z calculated for $C_{31}H_{43}O_3$ ($[M + H]^+$) 463.3212; found 463.3212.

6-Oxo-24-nor-5 α -chol-22-en-3 α -yl 2-fluorobenzoate (30)

A solution of compound **29** (120 mg, 0.348 mmol) and DMAP (35 mg, 0.286 mmol) was prepared in 3 mL of anhydrous pyridine. To this solution, 2-fluorobenzoyl chloride 123.5 μ L (1.05 mmol) was added by slow dripping, and the reaction was maintained at room temperature with constant stirring for 3 h. The end of the reaction was verified by TLC. After completion of the reaction, 3 mL of hot water was added. After an additional 20 min of stirring, the mixture was extracted with EtOAc (20 mL) and washed successively with saturated $NaHCO_3$ solution (2×10 mL) and water (2×10 mL), dried over anhydrous $MgSO_4$, and filtered. The solvent was evaporated under reduced pressure, and the crude was re-dissolved in CH_2Cl_2 (5 mL) and chromatographed on silica gel with EtOAc/cyclohexane (1.0:19) mixture. Compound **30** (91 mg, 56.0% yield) was obtained as a colorless solid, m.p. = 120.6–120.8 $^{\circ}C$. 1H -NMR (500.16 MHz, $CDCl_3$) (Figure S9, Supplementary Materials): δ (ppm) = 7.92 (1H, td, J = 7.6 and 1.8 Hz, HAr-6'); 7.54–7.48 (1H, m, HAr-4'); 7.21 (1H, td, J = 7.6 and 1.2 Hz, HAr-3'); 7.13 (1H, ddd, J = 10.7, 7.6 and 0.9 Hz, HAr-5'); 5.65 (1H, ddd, J = 17.1, 10.1, and 8.6 Hz, H-22); 5.42–5.39 (1H, m, H-3); 4.90 (1H, ddd, J = 17.1, 1.8, and 0.9 Hz, H-23a); 4.82 (1H, dd, J = 10.1 and 1.8 Hz, H-23b); 2.71 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.31 (1H, dd, J = 13.1 and 4.6 Hz, H-7 α); 1.03 (3H, d, J = 6.7 Hz, H-21); 0.781 (3H, s, H-19); 0.692 (3H, s, H-18). ^{13}C -NMR (125.77 MHz, $CDCl_3$) (Figure S9, Supplementary Materials): δ (ppm) = 212.04 (C-6); 163.85 (d, $^3J_{CF}$ = 3.6 Hz, CO_2 -Ar); 161.92 (d, $^1J_{CF}$ = 259.1 Hz, C2'-Ar); 145.02 (C-22); 134.44 (d, $^3J_{CF}$ = 8.4 Hz, C4'-Ar); 132.32 (d, $^3J_{CF}$ = 0.9 Hz, C6'-Ar); 124.11 (d, $^4J_{CF}$ = 3.6 Hz, C5'-Ar); 119.38 (d, $^2J_{CF}$ = 9.6 Hz, C1'-Ar); 117.05 (d, $^2J_{CF}$ = 21.6 Hz, C3'-Ar); 111.93 (C-23); 70.34 (C-3); 56.82 (C-14); 55.50 (C-17); 53.99 (C-9); 52.79 (C-5); 46.84 (C-7); 43.05 (C-13); 41.42 (C-10); 41.23 (C-20); 39.43

(C-12); 38.06 (C-8); 32.59 (C-1); 28.30 (C-16); 25.51 (C-2); 25.22 (C-4); 24.01 (C-15); 21.21 (C-11); 20.17 (C-21); 12.63 (C-19); 12.30 (C-18). ^{19}F -NMR (470.62 MHz, CDCl_3) δ (ppm) = 108.90 (s, 1F). HRMS (API+) (Figure S16, Supplementary Materials): m/z calculated for $\text{C}_{30}\text{H}_{40}\text{O}_3\text{F}$ ($[\text{M} + \text{H}]^+$) 467.2961; found 467.2962.

(22R)-22,23-Dihydroxy-6-oxo-24-nor-5 α -cholan-3 α -yl 4-methylbenzoate (**18a**) and (22S)-22,23-dihydroxy-6-oxo-24-nor-5 α -cholan-3 α -yl 4-methylbenzoate (**18b**)

To a mixture of *t*-butanol/water (10 mL, 1:1 *v/v*) and alkene **29** (100 mg, 0.22 mmol), DHQD-CLB (20.1 mg; 0.043 mmol), $\text{CH}_3\text{SO}_2\text{NH}_2$ (41.12 mg; 0.43 mmol), K_2CO_3 (179.2 mg; 1.3 mmol), and $\text{K}_3[\text{Fe}(\text{CN})_6]$ (427.0 mg; 1.3 mmol) were added; then, the mixture was homogenized by magnetic stirring for 10 min. Later, 100 μL of OsO_4 solution (1.0 g, 0.562 mmol in 20 mL of *t*-butanol) were added, and the mixture reaction was stirred at room temperature for 5 h. The end of the reaction was verified by TLC; then, H_2O (10 mL) and a saturated solution of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (2 mL) were added. The mixture was stirred for another 20 min. Later, it was extracted with EtOAc (2×35 mL) and washed with water (2×35 mL), and both organic phases were combined, dried over MgSO_4 , and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH_2Cl_2 (1.0 mL) and chromatographed on silica gel with EtOAc/cyclohexane (16:4) mixture. A mixture of **18a/18b** = 1.0/1.0 was obtained (93 mg; 91.6% yield). Separation by HPLC of an analytical sample allowed the separation and obtaining of the pure compounds **18a** and **18b**.

Compound **18a** was obtained as a colorless solid, m.p. = 74.1 ± 4.6 °C. ^1H -NMR (500.16 MHz, CDCl_3) (Figure S10, Supplementary Materials): δ (ppm) = 7.90 (2H, d, J = 8.3 Hz, HAr-2'); 7.24 (2H, d, J = 8.3 Hz, HAr-3'); 5.36–5.34 (1H, m, H-3); 3.80 (1H, ddd, J = 8.9, 3.3, and 1.2 Hz, H-23a); 3.66–3.61 (1H, m, H-22); 3.52 (1H, dd, J = 10.8 and 3.3 Hz, H-23b); 2.66 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.41 (3H, s, CH_3 -Ar); 2.32 (1H, dd, J = 13.1 and 4.6 Hz, H-7 α); 0.921 (3H, d, J = 6.7 Hz, H-21); 0.783 (3H, s, H-19); 0.681 (3H, s, H-18). ^{13}C -NMR (125.77 MHz, CDCl_3) (Figure S10, Supplementary Materials): δ (ppm) = 211.91 (C-6); 165.82 (CO_2 -Ar); 143.65 (C4'-Ar); 129.61 (C2'-Ar, C6'-Ar); 129.20 (C3'-Ar, C5'-Ar); 128.15 (C1'-Ar); 74.14 (C-22); 69.36 (C-3); 66.16 (C-23); 56.71 (C-14); 53.96 (C-17); 53.00 (C-9); 52.47 (C-5); 46.79 (C-7); 42.94 (C-13); 41.46 (C-10); 39.53 (C-20); 38.09 (C-12); 37.97 (C-8); 32.78 (C-1); 27.72 (C-16); 25.55 (C-2); 25.24 (C-4); 23.95 (C-15); 21.78 (CH_3 -Ar); 21.22 (C-11); 12.73 (C-21); 12.55 (C-19); 12.00 (C-18). HRMS (API+) (Figure S17, Supplementary Materials): calculated for $\text{C}_{31}\text{H}_{45}\text{O}_5$ ($[\text{M} + \text{H}]^+$) 497.3267, found 497.3262.

Compound **18b** was obtained as a colorless solid, m.p. = 83.0 ± 5.0 °C. ^1H -NMR (500.16 MHz, CDCl_3) (Figure S11, Supplementary Materials): δ (ppm) = 7.89 (2H, d, J = 8.2 Hz, HAr-2'); 7.23 (2H, d, J = 8.2 Hz, HAr-3'); 5.36–5.34 (1H, m, H-3); 3.83–3.76 (1H, m, H-23a); 3.69–3.57 (1H, m, H-23b); 3.51 (1H, t, J = 10.2 Hz, H-22); 2.65 (1H, dd, J = 12.5 and 3.1 Hz, H-5); 2.41 (3H, s, CH_3 -Ar); 2.32 (1H, dd, J = 13.1 and 4.6 Hz, H-7 α); 0.953 (3H, d, J = 7.0 Hz, H-21); 0.778 (3H, s, H-19); 0.679 (3H, s, H-18). ^{13}C -NMR (125.77 MHz, CDCl_3) (Figure S11, Supplementary Materials): δ (ppm) = 211.82 (C-6); 165.79 (CO_2 -Ar); 143.65 (C4'-Ar); 129.60 (C2'-Ar and C6'-Ar); 129.18 (C3'-Ar and C5'-Ar); 128.16 (C1'-Ar); 73.92 (C-22); 69.36 (C-3); 62.54 (C-23); 56.42 (C-14); 54.06 (C-17); 53.02 (C-9); 52.92 (C-5); 46.79 (C-7); 43.48 (C-13); 41.43 (C-10); 40.15 (C-20); 39.47 (C-12); 38.04 (C-8); 32.81 (C-1); 27.47 (C-16); 25.55 (C-2); 25.23 (C-4); 24.12 (C-15); 21.78 (CH_3 -Ar); 21.21 (C-11); 13.15 (C-21); 12.55 (C-19); 11.85 (C-18). HRMS (API+) (Figure S18, Supplementary Materials): calculated for $\text{C}_{31}\text{H}_{45}\text{O}_5$ ($[\text{M} + \text{H}]^+$) 497.3267, found 497.3265.

(22R)-22,23-Dihydroxy-6-oxo-24-nor-5 α -cholan-3 α -yl 2-fluorobenzoate (**19a**) and (22S)-22,23-dihydroxy-6-oxo-24-nor-5 α -cholan-3 α -yl 2-fluorobenzoate (**19b**)

To a mixture of *t*-butanol/water (8 mL; 1:1 *v/v*), alkene **29** (75 mg; 0.161 mmol), DHQD-CLB (15.9 mg; 0.0343 mmol), $\text{CH}_3\text{SO}_2\text{NH}_2$ (32.61 mg; 0.343 mmol), K_2CO_3 (142.2 mg; 1.03 mmol), and $\text{K}_3[\text{Fe}(\text{CN})_6]$ (338.7 mg; 1.03 mmol) were added; then, the mixture was homogenized by magnetic stirring for 10 min. Later, 100 μL of OsO_4 solution (1.0 g, 0.562 mmol in 20 mL of *t*-butanol) were added, and the mixture reaction was stirred at

room temperature for 5 h. The end of the reaction was verified by TLC; then, H₂O (10 mL) and a saturated solution of Na₂S₂O₃·5H₂O (2 mL) were added. The mixture was stirred for another 20 min. Later, it was extracted with EtOAc (2 × 35 mL) and washed with water (2 × 35 mL), and both organic phases were combined, dried over MgSO₄, and filtered. The solvent was evaporated under reduced pressure. The crude was re-dissolved in CH₂Cl₂ (1.0 mL) and chromatographed on silica gel with an EtOAc/cyclohexane (16:4) mixture. A mixture of **19a**/**19b** = 1.0/1.0 was obtained (65 mg; 80.8% yield). The separation by HPLC of an analytical sample allowed the separation and obtaining of the pure compounds **19a** and **19b**.

Compound **19a** was obtained as a colorless solid, m.p. = 56.9 ± 4.6 °C. ¹H-NMR (500.16 MHz, CDCl₃) (Figure S12, Supplementary Materials): δ (ppm) = 7.92 (1H, td, *J* = 7.6 and 1.8 Hz, HAr-6'); 7.56–7.48 (1H, m, HAr-4'); 7.21 (1H, td, *J* = 7.6 and 1.2 Hz, HAr-3'); 7.13 (1H, ddd, *J* = 10.7, 7.6 and 0.9 Hz, HAr-5'); 5.40–5.39 (1H, m, H-3); 3.87–3.76 (1H, m, H-23a); 3.75–3.39 (2H, m, H-22, H-23b); 2.71 (1H, dd, *J* = 12.5 and 3.1 Hz, H-5); 2.31 (1H, dd, *J* = 13.1 and 4.6 Hz, H-7α); 0.917 (3H, d, *J* = 6.4 Hz, H-21); 0.776 (3H, s, H-19); 0.676 (3H, s, H-18). ¹³C-NMR (125.77 MHz, CDCl₃) (Figure S12, Supplementary Materials): δ (ppm) = 212.12 (C-6); 163.89 (d, ³*J*_{CF} = 3.6 Hz, CO₂-Ar); 161.93 (d, ¹*J*_{CF} = 259.1 Hz, C2'-Ar); 134.49 (d, ³*J*_{CF} = 8.4 Hz, C4'-Ar); 132.31 (d, ³*J*_{CF} = 0.9 Hz, C6'-Ar); 124.13 (d, ⁴*J*_{CF} = 3.6 Hz, C5'-Ar); 117.17 (d, ²*J*_{CF} = 9.6 Hz, C1'-Ar); 116.99 (d, ²*J*_{CF} = 21.6 Hz, C3'-Ar); 75.56 (C-22); 70.33 (C-3); 61.15 (C-23); 56.67 (C-14); 53.85 (C-17); 52.79 (C-9); 52.45 (C-5); 46.78 (C-7); 43.47 (C-13); 42.93 (C-10); 41.41 (C-20); 39.51 (C-12); 38.09 (C-8); 32.55 (C-1); 27.72 (C-16); 25.49 (C-2); 25.20 (C-4); 23.94 (C-15); 21.21 (C-11); 12.73 (C-21); 12.61 (C-19); 12.00 (C-18). ¹⁹F-NMR (470.62 MHz, CDCl₃) δ (ppm) = −108.91 (s, 1F). HRMS (API+) (Figure S19, Supplementary Materials): calculated for C₃₀H₄₂O₅F ([M + H]⁺) 501.3016, found 501.3015.

Compound **19b** was obtained as a colorless solid, m.p. = 75.4 ± 1.5 °C. ¹H-NMR (500.16 MHz, CDCl₃) (Figure S13, Supplementary Materials): δ (ppm) = 7.92 (1H, td, *J* = 7.6 and 1.8 Hz, HAr-6'); 7.54–7.49 (1H, m, HAr-4'); 7.21 (1H, td, *J* = 7.6 and 1.2 Hz, HAr-3'); 7.13 (1H, ddd, *J* = 10.7, 7.6, and 0.9 Hz, HAr-5'); 5.40–5.39 (1H, m, H-3); 3.84–3.76 (1H, m, H-23a); 3.71–3.69 (1H, m, H-23b); 3.51 (1H, t, *J* = 10.2 Hz, H-22); 2.70 (1H, dd, *J* = 12.5 and 3.1 Hz, H-5); 2.31 (1H, dd, *J* = 13.1 and 4.6 Hz, H-7α); 0.954 (3H, d, *J* = 6.7 Hz, H-21); 0.777 (3H, s, H-19); 0.681 (3H, s, H-18). ¹³C-NMR (125.77 MHz, CDCl₃) (Figure S13, Supplementary Materials): δ (ppm) = 211.97 (C-6); 163.89 (d, ³*J*_{CF} = 3.6 Hz, CO₂-Ar); 160.87 (d, ¹*J*_{CF} = 259.1 Hz, C2'-Ar); 134.48 (d, ³*J*_{CF} = 8.4 Hz, C4'-Ar); 132.34 (d, ³*J*_{CF} = 0.9 Hz, C6'-Ar); 124.13 (d, ⁴*J*_{CF} = 3.6 Hz, C5'-Ar); 118.37 (d, ²*J*_{CF} = 9.6 Hz, C1'-Ar); 117.04 (d, ²*J*_{CF} = 21.6 Hz, C3'-Ar); 73.90 (C-22); 70.32 (C-3); 62.52 (C-23); 56.37 (C-14); 53.95 (C-17); 52.90 (C-9); 52.79 (C-5); 46.78 (C-7); 43.48 (C-13); 41.38 (C-10); 40.15 (C-20); 39.46 (C-12); 38.04 (C-8); 32.57 (C-1); 27.47 (C-16); 25.48 (C-2); 25.20 (C-4); 24.11 (C-15); 21.19 (C-11); 13.13 (C-21); 12.62 (C-19); 11.85 (C-18). ¹⁹F-NMR (470.62 MHz, CDCl₃) δ (ppm) = −108.90 (s, 1F). HRMS (API+) (Figure S20, Supplementary Materials): calculated for C₃₀H₄₂O₅F ([M + H]⁺) 501.3016, found 501.3014.

3.2. Biological

Rice Lamina Inclination Test (RLIT)

The biological activity of the growth of the compounds was evaluated by the rice lamina inclination test [55,56], according to a previously described procedure [38], and using the same a Zafiro cultivar (*Oryza sativa*) provided by the Institute of Agricultural Research (INIA-Quilamapu-Chile) as previous studies.

The seeds were sown and cultivated until the seedlings presenting the second internode of the rice blade were selected for cutting. Six segments per treatment were incubated in Petri dishes containing 60 mL of distilled water, and the amount of test compound (BRs analogs **18a**, **18b**, **19a**, **19b**, **20a**, and **20b** and positive control (**1**)) needed to reach final concentrations equal to 1 × 10^{−8} M; 1 × 10^{−7} M; and 1 × 10^{−6} M. The negative control only contained sterile distilled water. All treatments were incubated by 48 h at 25 °C in

darkness, and the angles developed between the blade and the sheath were measured. Each experiment was performed by duplicate.

Results were expressed as mean $\pm$ standard deviation (SD) using twelve angle measurements. Statistical analysis was done using a statistical package Excel by applying mean values using one-way ANOVA with the post-hoc least square differences (LSD) test to determine if there was a significant difference between the positive control and the treatments. A *P* value of less than 0.05 was considered significant.

4. Conclusions

Brassinosteroid 24-norcholane type analogs conjugated at C-3 and configurations *S* and *R* on the C-22 carbon of the side chain have been synthesized and characterized. The synthesis uses hydoxycholeic acid as the starting material, and epimers with different configuration at C-22 are obtained. These epimers have been separated, and their growth-promoting activity was measured using RLIT. The results show that the esterification of BRs analog at C-3 has no effect on the biological activity of synthetic analogs. This suggest that reducing activity by esterification at C-3 requires a long chain carboxylic acid. In addition, the presence of a hydroxyl group at C-3 is not an essential structural feature for activity. This result confirms previous SAR where it has been proposed that activity is not determined by the presence or absence of specific groups in the BR structure.

Supplementary Materials: The following are available online, Figure S1: NMR spectra of 3 α ,6 α -diacetoxo-5 β -cholan-24-oic acid (22), Figure S2: NMR spectra of 24-nor-5 β -cholan-22-ene-3 α ,6 α -diyl diacetate (23), Figure S3: NMR spectra of 24-nor-5 β -chol-22-ene-3 α ,6 α -diol (24), Figure S4: NMR spectra of 6 α -hydroxy-24-nor-5 β -chol-22-en-3-one (25), Figure S5: NMR spectra of 24-nor-5 β -chol-22-ene-3,6-dione (26), Figure S6: NMR spectra of 3 α -hydroxy-24-nor-5 β -chol-22-en-6-one (27), Figure S7: NMR spectra of 3 α -hydroxy-24-nor-5 β -chol-22-en-6-one (28), Figure S8: NMR spectra of 6-oxo-24-nor-5 β -chol-22-en-3 α -yl 4-methylbenzoate (29), Figure S9: NMR spectra of 6-oxo-24-nor-5 β -chol-22-en-3 α -yl 2-fluorobenzoate (30), Figure S10: NMR spectra of (22*R*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 4-methylbenzoate (18*a*), Figure S11: NMR spectra of (22*S*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 4-methylbenzoate (18*b*), Figure S12: NMR spectra of (22*R*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 2-fluorobenzoate (19*a*), Figure S13: NMR spectra of (22*S*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 2-fluorobenzoate (19*b*), Figure S14: HRMS (API⁺) spectrum of 3 α -hydroxy-24-nor-5 β -chol-22-en-6-one (28), Figure S15: HRMS (API⁺) spectrum of 6-oxo-24-nor-5 β -chol-22-en-3 α -yl 4-methylbenzoate (29), Figure S16: HRMS (API⁺) spectrum of 6-oxo-24-nor-5 β -chol-22-en-3 α -yl 2-fluorobenzoate (30), Figure S17: HRMS (API⁺) spectrum of (22*R*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 4-methylbenzoate (18*a*), Figure S18: HRMS (API⁺) spectrum of (22*S*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 4-methylbenzoate (18*b*), Figure S19: HRMS (API⁺) spectrum of (22*R*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 2-fluorobenzoate (19*a*), Figure S20: HRMS (API⁺) spectrum of (22*S*)-22,23-dihydroxy-6-oxo-24-nor-5 β -cholan-3 α -yl 2-fluorobenzoate (19*b*).

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Příloha 3

Synthesis and Biological Activity of Brassinosteroid Analogues with a Nitrogen-Containing Side Chain

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Synthesis and Biological Activity of Brassinosteroid Analogues with a Nitrogen-Containing Side Chain

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Abstract: Brassinosteroids are a class of plant hormones that regulate a broad range of physiological processes such as plant growth, development and immunity, including the suppression of biotic and abiotic stresses. In this paper, we report the synthesis of new brassinosteroid analogues with a nitrogen-containing side chain and their biological activity on *Arabidopsis thaliana*. Based on molecular docking experiments, two groups of brassinosteroid analogues were prepared with short and long side chains in order to study the impact of side chain length on plants. The derivatives with a short side chain were prepared with amide, amine and ammonium functional groups. The derivatives with a long side chain were synthesized using amide and ammonium functional groups. A total of 25 new brassinosteroid analogues were prepared. All 25 compounds were tested in an Arabidopsis root sensitivity bioassay and cytotoxicity screening. The synthesized substances showed no significant inhibitory activity compared to natural 24-epibrassinolide. In contrast, in low concentration, several compounds (8a, 8b, 8e, 16e, 22a and 22e) showed interesting growth-promoting activity. The cytotoxicity assay showed no toxicity of the prepared compounds on cancer and normal cell lines.

Keywords: brassinosteroid; organic synthesis; nitrogen-containing steroid; plant bioassay; cytotoxicity

1. Introduction

Brassinosteroids (BRs, Figure 1) are a group of plant steroid hormones that regulate many important aspects of plant growth and development, such as cell division, elongation and differentiation, pollen tube growth, seed germination, regulation of gene expression, enzyme activation and photosynthesis [1–3]. They also induce tolerance against a wide range of biotic and abiotic stresses, such as water or drought, temperature, oxidative stresses, high salinity and different environmental pollutants [4,5]. At the molecular level, BRs modify the pathway of enzymes and change the gene expression when plants are exposed to stress. Plants perceive brassinosteroids at the cell membrane, using the membrane-integral receptor kinase brassinosteroid insensitive 1 (BRI1) and its co-receptor BAK1 [6–9]. The encoded protein, BRI1, is a member of a large family of plant LRR (leucine-rich repeat) receptor-like kinases, characterized by an extracellular LRR domain, a single-pass transmembrane segment and a cytoplasmic kinase domain. BRI1 has been established as an authentic brassinosteroid receptor through genetic and biochemical investigations [10]. Moreover, recent studies have shown that natural BRs and their synthetic analogues have potential application not only in agriculture, but also in medicine due to their antiviral [11,12], immunomodulatory and neuroprotective activities [13–15], and anti-proliferative effects on animal cells in vitro [16–20].

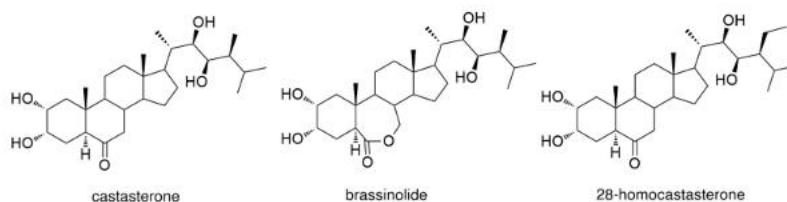


Figure 1. Structure of the most common natural brassinosteroids.

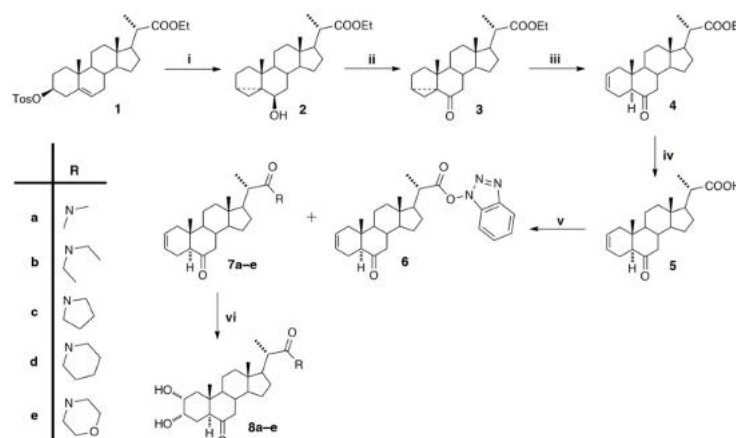
Since the first BR isolation and structural identification, chemists began to synthesize new brassinosteroid analogues with different modifications of the sterol skeleton and tested them on various biological activities, especially in plant bioassays. Modification of the side chain is one of the main aims of synthesis. Such modifications include shortening or prolonging the side chain, oxidation or reduction of oxygen functional groups, incorporation of heteroatoms other than oxygen (mostly nitrogen and halogens) and modification of newly synthesized functional groups (e.g., acetylation, methylation, etc.) [21–28]. Preparation of derivatives with a nitrogen-containing side chain has not been at the forefront of the interest of steroidal chemists. Few examples have been published so far. Back et al. described the synthesis and biological activities of 25-azabassinolide. Unfortunately, the rice lamina inclination biotest showed no biological activity [29]. Several BR analogues with nitrogen-containing heterocycles (e.g., isoxazoline, oxazole, thiazole and pyridine) were synthesized by Wendeborn's group [25]. Other analogues with nitrogen groups in the side chain were prepared mostly for other chemical modifications, e.g., azide derivatives for photoaffinity labeling [30] or activated esters for conjugation reactions [31,32].

the aim of this study was to synthesize new BRs analogues with nitrogen functional groups (amides, amines and ammonium salts) in the side chain and study their biological properties. the synthesis was also based on molecular docking. It uses computational methods for prediction of interaction between receptors and ligands. It is a tool for predicting the first results during the preparation of new ligands. In our study, we were interested mostly in tertiary amides and amines to avoid the presence of hydrogen directly attached to nitrogen (with the exception of hydrochloride salts of tertiary amines whose existence is pH-dependent) and because they have an additional hydrogen bond acceptor than standard BRs hydroxy groups. the plant growth-promoting activity of synthetic analogues was assayed using the Arabidopsis root sensitivity bioassay. Further, the cytotoxic activity of all compounds was tested on one normal and three cancer cell lines.

2. Results and Discussion

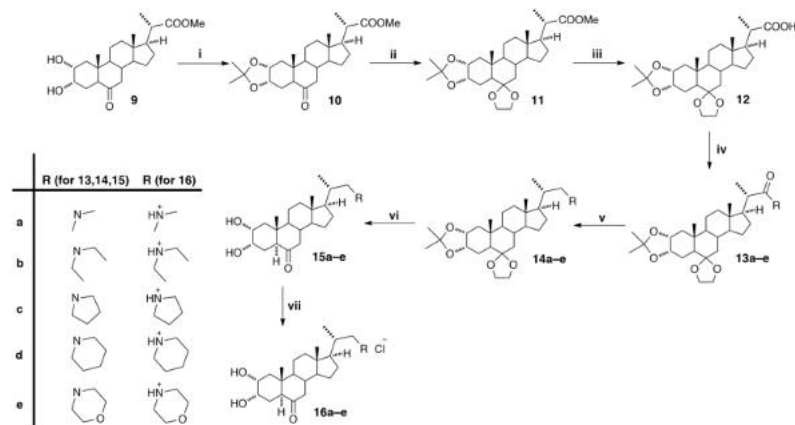
2.1. Chemistry

the synthesis of all nitrogen-containing compounds was divided into three groups—short chain amides (Scheme 1), short chain amines and ammonium chlorides (Scheme 2) and long chain derivatives (Scheme 3). the synthesis of amides with a short side chain started with the known tosylate **1** [33]. Other reaction steps correspond to standard brassinosteroid synthesis [3]. Firstly, hydroxy ester **2** is formed from tosylate, followed by oxidation with Jones reagent to oxo-ester **3**. This ketone undergoes rearrangement to olefin **4**. Hydrolysis of the ester group afforded carboxylic acid **5** that is suitable for amide formation. Five different secondary amines were used for the amidation reaction—dimethylamine, diethylamine, pyrrolidine, piperidine and morpholine. In all five cases, olefinic amides **7a–e** were obtained in very good yield. However, activated ester **5** can also be isolated after 4–5 h of reaction. As the last reaction, we used Upjohn dihydroxylation to prepare five dihydroxy amides, **8a–e**.



Scheme 1. Synthesis of short side chain amides: (i) AcOK, acetone, H₂O, reflux.; (ii) Jones reagent, acetone, 0 °C; (iii) LiBr, PPTS, dimethylacetamide, reflux; (iv) KOH, EtOH, H₂O, reflux; (v) amine, HOBt (hydroxybenzotriazole), EDC, DMF, 40 °C; (vi) OsO₄, *N*-Me-Mf-*N*-oxide, THF, acetone, H₂O, r.t.

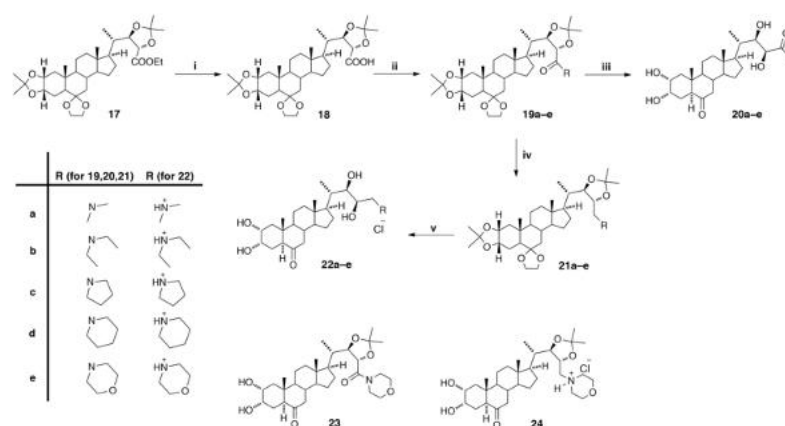
Due to the reduction step in the synthesis of amine analogues, the previous reaction strategy cannot be used here. It is necessary to protect both the hydroxy groups and ketone. Thus, we started with the known dihydroxy ester **9** [34]. It was firstly protected on hydroxy groups with acetone (compound **10**), followed by protection of the ketone with ethylene glycol (compound **11**). Fully protected ester was then hydrolyzed to acid **12** which is used for amide formation. Amides **13a–e** were then subjected to reduction with lithium aluminum hydride to obtain amines **14a–e**. Subsequent deprotection with basic work-up afforded dihydroxy amines **15a–e**, whose treatment with hydrochloric acid formed ammonium hydrochloride **16a–e**.



Scheme 2. Synthesis of short side chain amines and ammonium salts: (i) TsOH, acetone, r.t.; (ii) 2,2-diMe-1,3-dioxolane, TsOH, 90 °C; (iii) KOH, *i*-PrOH, H₂O, reflux, then HCl; (iv) amine, HOBt, EDC, DMF, 40 °C; (v) LiAlH₄, dioxane, reflux; (vi) HCl, THF, H₂O, 45 °C, then NaHCO₃; (vii) HCl, MeOH, H₂O, r.t.

For the preparation of long side chain analogues, we used the known fully protected ester **17** [35]. Hydrolysis of the ester and condensation of acid **18** with amines afforded five

fully protected amides, **19a–e**. Firstly, all amides were deprotected to form tetrahydroxy amides **20a–e**. Another portion of amides was used in lithium aluminum hydride reduction to prepare amines **21a–e**. After their acidic deprotection, we obtained ammonium chlorides **22a–e**. the deprotection on the side chain must be carried out at 45 °C. Lower temperature (below 30 °C) led only to partial deprotection on the A-ring and isolation of amide **23** and ammonium chloride **24** with the still protected side chain.



Scheme 3. Synthesis of long side chain derivatives: (i) KOH, MeOH, H₂O, reflux, then HCl; (ii) amine, HOBt, EDC, DMF, 40 °C; (iii) HCl, THF, H₂O, 45 °C, then NaHCO₃; (iv) LiAlH₄, dioxane, reflux; (v) HCl, THF, H₂O, 45 °C.

2.2. Molecular Docking

Molecular docking is a useful tool to understand the pose and energetics of a protein-ligand complex. the binding site of BRI1 is located on the surface of the receptor ectodomain as a nonpolar cavity lined by nonpolar amino acids. Brassinolide fits into the cavity via its nonpolar side and displays its hydroxyl groups towards the solvent and protein partners [26]. Due to this reason, we used only tertiary amides and amines. Especially in the case of long side chain derivatives, the presence of a new hydrogen bond donor can affect their pose in the protein cavity. Molecular docking predicted similar or better binding energies than for brassinolide for compounds **8c**, **8d**, **8e**, **15d**, **20c**, **22c** and **22d** (see Table S1). This implies that derivatives with nitrogen groups in the side chain should bind within the BRI1 cavity at least as easily as brassinolide itself. Based on molecular docking, these were also candidates for showing similar binding experimentally. For poses of all nitrogen-containing derivatives used for the plant biotest, see Figures S1–S20 in the Supplementary Material.

2.3. Biology

Several bioassays such as the first bean internode, root growth and rice lamina inclination have been developed to evaluate the growth-promoting activity of BR derivatives [36]. In this work, the activity of new BR analogues was evaluated using the Arabidopsis root sensitivity test because of its simplicity and high sensitivity for BRs. [27,28]. the characteristic effect of exogenously applied BRs on light-grown Arabidopsis is dose-dependent—BRs in higher concentrations cause inhibition of root growth; vice versa, low concentrations are stimulating [37–39]. the results obtained for 24-epiBL, which was used as a positive control, and the BR derivatives are shown in Figure 2, Figure 3 and Figure S21. the most active BR (24-epiBL) inhibited Arabidopsis root growth in 1 nM and higher concentrations. Most of the newly synthesized BR derivatives were not active, and only the long side chain derivatives **20a** and **20c** show a weak inhibitory effect in the concentration range, which was

not confirmed using the higher tested concentration of 100 nM (Figure 3 and Figure S21). Compound 22b shows the same effect only at low concentrations; on the contrary, compound 8a shows weak inhibition up to the highest tested concentration of 100 nM (see Figure S21). On the other hand, in low concentrations (0.1 and 1 nM), a slight elongation of roots was observed for derivatives with a short side chain (8a, 8b, 8e and 16e) compared to the control. A similar trend was shown for dimethylammonium hydrochloride **22a** and morpholinium hydrochloride **22e** with a long side chain. For numerical values of the average, SD and *p*-value, see Table S2.

the structure–activity relationship of brassinosteroids has been studied in detail in recent years and it has been postulated that the (2 α ,3 α)- and (22R,23R)-vicinal diol moieties are required for optimum bioactivity and 7-oxalactone BRs have stronger biological activity than 6-oxo types, whereas B-ring non-oxidized BRs reveal no activity in biological tests [25,40,41]. Recently, several research groups prepared BR synthetic analogues with various nitrogen modifications in the side chain. Wendeborn and co-workers [25] synthesized BR derivatives where the isoxazoline ring was introduced to replace the (22,23)-vicinal diols and showed no statistically significant activity in the bean second internode elongation bioassay. As well as 25-azabassinolide, it proved to be completely inactive at all doses studied in the rice leaf lamina inclination assay [29]. the low biological activity of BR analogues with the nitrogen-containing side chain is probably due to the lower BRI-1 receptor affinity.

the effect of the prepared BRs derivatives on the viability (in Calcein AM assays) of BJ human fibroblasts (as an example of a “normal” cell line) and human cancer cell lines of various histopathological origins, including: T-lymphoblastic leukemia CEM, breast carcinoma MCF7 and cervical carcinoma cell line HeLa, was also studied. Cells of all these lines were exposed to six 3-fold dilutions of each drug for 72 h prior to determination of cell survival. the IC₅₀ (concentration leading to 50% inhibition of viability) values obtained from the Calcein AM cytotoxicity assay were calculated. 28-Homocastasterone was used as a positive control, which is the most potent natural brassinosteroid towards CEM cells (IC₅₀ 13 μ M [42]). All tested BR nitrogen-containing derivatives had no detectable activity, even when tested in concentrations of up to 50 μ M. No BRs derivative-mediated loss of viability was observed in the BJ fibroblasts (see Table S3).

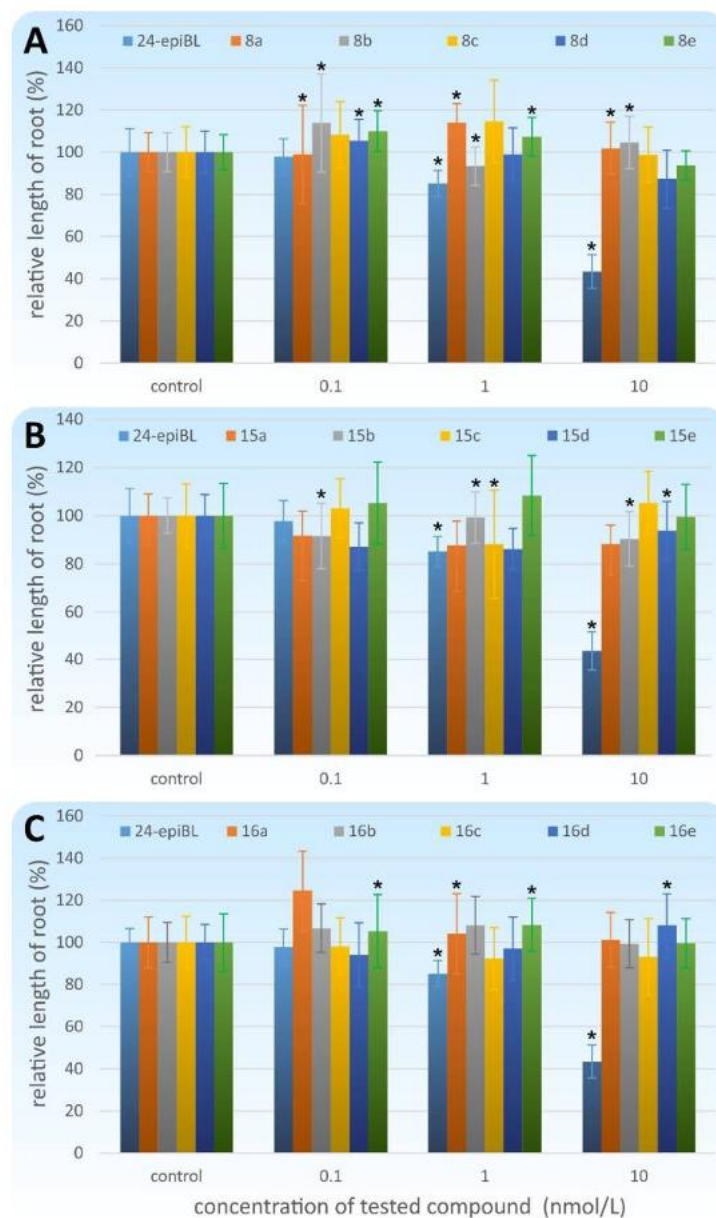


Figure 2. Effect of selected brassinosteroid derivatives on the inhibition of *Arabidopsis* root length. Seven-day-old *Arabidopsis thaliana* seedlings (Columbia ecotype, Col-0) were treated with DMSO as control/24epiBL/BR short side chain derivatives; (A)—amides, (B)—amines, (C)—ammonium hydrochlorides. For each treatment, more than 15 seedlings were analyzed in two biological repeats. Error bars represent SD. Asterisks represent significant changes (*t*-test); * represents *p*-value < 0.05, in comparison to control.

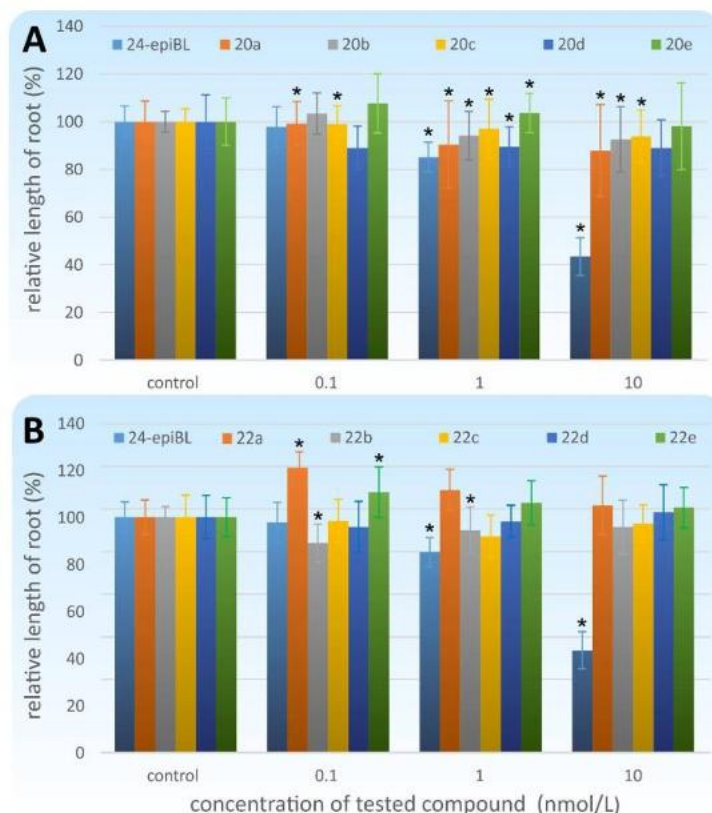


Figure 3. Effect of selected brassinosteroid derivatives on the inhibition of Arabidopsis root length. Seven-day-old Arabidopsis thaliana seedlings (Columbia ecotype, Col-0) were treated with DMSO as control/24epiBL/BR long side chain derivatives; (A)—amides, (B)—ammonium hydrochlorides. For each treatment, more than 15 seedlings were analyzed in two biological repeats. Error bars represent SD. Asterisks represent significant changes (*t*-test); * represents *p*-value < 0.05, in comparison to control.

3. Materials and Methods

3.1. Materials and Instruments

the melting points (Mp) were determined on a Stuart SMP30 instrument (Bibby Scientific Ltd., Staffordshire, UK). Elemental analyses were performed using an EA 1108 elemental analyzer (Fison Instruments, Glasgow, UK); the values (C, H, N) agreed with the calculated values within acceptable limits. the NMR spectra were taken on a JEOL JNM-ECA 500 (JEOL, Tokyo, Japan; ^1H , 500 MHz; ^{13}C , 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. ^1H NMR and ^{13}C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, ^1H δ = 0 ppm) or solvents: CDCl_3 (^1H δ = 7.26 ppm, ^{13}C δ = 77.00 ppm), CD_3OD (^1H δ = 3.31 ppm, ^{13}C δ = 49.00 ppm), $\text{DMSO}-d_6$ (^1H δ = 2.46 ppm, ^{13}C δ = 40.00 ppm) or D_2O (^1H δ = 4.79 ppm). Chemical shifts are given in ppm (δ -scale) and coupling constants (J) are given in Hz. All values were obtained by first-order analysis. the most common abbreviations for NMR signals were used: s—singlet, d—doublet, t—triplet, q—quartet, m—multiplet, b—broad (and combinations thereof, e.g., dd—doublet of doublet, bs—broad singlet, etc.). For API HRMS analysis, the samples were dissolved in chloroform (or chlo-

roform/methanol; 1:1; *v/v*, in the case of hydroxylated compounds) to a concentration $10 \mu\text{g}\cdot\text{mL}^{-1}$. the ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into the ion source and analyzed in full scan mode. the source of the Synapt G2-Si Mass Spectrometer (Waters, Manchester, UK) was operated in positive ionization mode (ASAP+) and, if not stated otherwise, at a source temperature of 120°C . the Corona needle current was kept at $5 \mu\text{A}$ and the collision energy was kept at a value of 4. the probe temperature was ramped up from 50 to 600°C in 3 min. Data were acquired from 50 to 1000 Da with 1.0 s scan time in high-resolution mode and processed using the Masslynx 4.1 software (Waters, Manchester, UK). Mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were not purified.

3.2. Chemistry

Ethyl (2*S*)-6 β -hydroxy-3 α ,5-cyclo-5 α -pregnane-20-carboxylate (2)

Potassium acetate (5.4 g; 55 mmol) was added to a solution of tosylate **1** (3.0 g; 5.54 mmol) in acetone (140 mL) and water (40 mL). the reaction was stirred under reflux for 7 h. Then, excess of acetone was removed under reduced pressure and the residual suspension was dissolved in diethyl ether and extracted twice with water. Organic solvents were dried with sodium sulfate and removed under reduced pressure. the crude product was purified on silica gel (mobile phase—30% ethyl acetate in cyclohexane) to afford hydroxyl ester **2** (2.1 g; 97%) as a white solid.

Mp 59 – 60°C . ^1H NMR (CDCl_3) δ 0.29 (dd, 3H, $J = 8.1$ and 4.5 Hz); 0.53 (t, 1H, $J = 4.5$ Hz, H-4 β); 0.74 (s, 3H, CH_3); 1.06 (s, 3H, CH_3); 1.19 (d, 3H, $J = 6.7$ Hz, CH_3); 1.25 (t, 3H, $J = 7.0$ Hz, CH_2CH_3); 1.93 (dt, 1H, $J = 12.5$ and 4.0 Hz); 2.41 (dq, 1H, $J = 10.6$ and 8.0 Hz, H-20); 3.26 (t, 1H, $J = 2.9$ Hz, H-6 α); 4.07–4.16 (m, 2H, CH_2CH_3). ^{13}C NMR δ 11.57, 12.37, 14.25, 17.10, 20.20, 22.60, 24.22, 24.26, 24.98, 27.19, 29.85, 33.17, 37.03, 38.85, 39.92, 42.61, 42.78, 42.86, 47.57, 52.97, 56.00, 59.96, 73.68, 177.00. HRMS: (API+) calculated for $\text{C}_{24}\text{H}_{39}\text{O}_3$ ($[\text{M} + \text{H}]^+$) 375.2899, found 375.2899.

Ethyl (2*S*)-6-oxo-3 α ,5-cyclo-5 α -pregnane-20-carboxylate (3)

Jones reagent (10 mL) was added to a solution of hydroxyl ester **2** (2 g; 5.13 mmol) in acetone (150 mL) at 0°C . the reaction was stirred at 0°C for an additional 20 min and then quenched with isopropanol (20 mL). Excess of acetone was removed under reduced pressure. the suspension was diluted with diethyl ether and extracted twice with water. Organic solvents were dried with sodium sulfate and removed under reduced pressure. the crude product was purified on silica gel (mobile phase—10% ethyl acetate in cyclohexane) to afford oxoester **3** (1.7 g; 85%) as a white solid.

Mp 86 – 87°C . ^1H NMR (CDCl_3) δ 0.73 (t, 1H, $J = 4.9$ Hz, H-4 β); 0.74 (s, 3H, CH_3); 1.01 (s, 3H, CH_3); 1.20 (d, 3H, $J = 6.7$ Hz, CH_3); 1.25 (t, 3H, $J = 7.0$ Hz, CH_2CH_3); 1.89–1.95 (m, 2H); 1.99 (t, 1H, $J = 12.6$ and 3.5 Hz); 2.38–2.45 (m, 2H, H-7 β , H-20); 4.07–4.16 (m, 2H, CH_2CH_3). ^{13}C NMR δ 11.65, 12.20, 14.24, 17.08, 19.65, 22.76, 24.10, 25.85, 27.04, 33.44, 34.72, 35.34, 39.45, 42.53, 42.75, 44.67, 45.98, 46.28, 46.70, 52.72, 56.48, 60.04, 176.75, 209.47. HRMS: (API+) calculated for $\text{C}_{24}\text{H}_{37}\text{O}_3$ ($[\text{M} + \text{H}]^+$) 373.2743, found 373.2744.

Ethyl (2*S*)-6-oxo-5 α -pregn-2-ene-20-carboxylate (4)

Lithium bromide (165 mg; 1.9 mmol) and pyridinium *p*-toluenesulfonate (165 mg; 0.66 mmol) were added to a solution of oxoester **3** (1.65 g; 4.2 mmol) in dimethylacetamide (60 mL). the reaction mixture was stirred under reflux for 5 h. Dimethylacetamide was then removed under reduced pressure. the crude mixture was diluted in diethyl ether and extracted twice with water. Organic solvents were dried with sodium sulfate and removed under reduced pressure. the crude product was purified on silica gel (mobile phase—10% ethyl acetate in cyclohexane) to afford unsaturated ester **4** (1.45 g; 88%) as a white solid.

Mp 118–120 °C. ^1H NMR (CDCl_3) δ 0.70 (s, 3H, CH_3); 0.72 (s, 3H, CH_3); 1.20 (d, 3H, $J = 6.7$ Hz, CH_3); 1.25 (t, 3H, $J = 7.0$ Hz, CH_2CH_3); 1.71–1.79 (m, 2H); 1.97–2.05 (m, 4H); 2.26 (m, 1H); 2.34–2.36 (m, 2H); 2.41 (dq, 1H, $J = 10.4$ and 7.0 Hz, H-20); 4.07–4.16 (m, 2H, CH_2CH_3); 5.57, 5.69 (both m, 1H, H-2 and H-3). ^{13}C NMR δ 12.11, 13.48, 14.24, 17.07, 21.03, 21.68, 23.99, 26.92, 37.61, 39.23, 39.29, 39.99, 42.51, 42.84, 46.89, 52.73, 53.28, 53.78, 56.25, 60.04, 124.45, 124.93, 176.74, 211.81. HRMS: (API+) calculated for $\text{C}_{24}\text{H}_{37}\text{O}_3$ ($[\text{M} + \text{H}]^+$) 373.2743, found 373.2745.

(20S)-6-oxo-5 α -pregn-2-ene-20-carboxylic acid (**5**)

Potassium hydroxide (1.0 g; 18 mmol) was added to a solution of ester **4** (1.4 g; 3.63 mmol) in ethanol (100 mL) and water (5 mL). the reaction mixture was stirred under reflux for 8 h. the excess ethanol was then removed under reduced pressure. the suspension was diluted with ethyl acetate and extracted with 5% aqueous hydrochloric acid and twice with water. Organic solvents were dried with sodium sulfate and evaporated under reduced pressure. the crude product was purified on silica gel (mobile phase—50–80% ethyl acetate in cyclohexane) to afford acid **5** (1.18 g; 91%) as a white solid. All data correspond to the data published [34].

General procedure for amide formation from acids **5**, **12** and **18**

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.1 equivalents) was added to a solution of either acid **5** (100 mg; 0.28 mmol), acid **12** (200 mg; 0.43 mmol) or acid **18** (200 mg; 0.36 mmol), secondary amine (1.5 equivalents) and 1-hydroxybenzotriazole hydrate (1.1 equivalents) in dimethylformamide (10 mL). the reaction mixture was stirred at 40 °C for 12 h. Then, it was diluted with ethyl acetate and extracted twice with water. the combined organic fractions were dried with sodium sulfate and evaporated under reduced pressure. the crude product was purified on silica gel (mobile phase at each experiment).

1H-benzotriazol-1-yl (20S)-6-oxo-5 α -pregn-2-ene-20-carboxylate (**6**)

Compound **6** can be obtained as a white solid intermediate (quant. yield) in amide formation according to the general procedure after 5 h at room temperature.

Mp > 160 °C (decomp.). ^1H NMR (CDCl_3) δ 0.75 (s, 3H, CH_3); 0.84 (s, 3H, CH_3); 1.55 (d, 3H, $J = 6.9$ Hz, CH_3); 1.78–1.91 (m, 2H); 2.00–2.13 (m, 4H); 2.28 (m, 1H); 2.34–2.42 (m, 2H); 2.96 (m, 1H, H-20); 5.59, 5.71 (both m, 1H, H-2 and H-3); 7.38 (dt, 1H, $J = 8.5$ and 0.9 Hz, H_{Ar}); 7.44 (ddd, 1H, $J = 8.5$, 7.1 and 0.9 Hz, H_{Ar}); 7.56 (ddd, 1H, $J = 8.5$, 7.1 and 0.9 Hz, H_{Ar}); 8.08 (dt, 1H, $J = 8.5$ and 0.9 Hz, H_{Ar}). ^{13}C NMR δ 12.23, 13.52, 17.17, 21.05, 21.68, 24.12, 27.37, 37.58, 39.21, 39.29, 39.96, 40.38, 43.31, 46.79, 52.37, 53.20, 53.80, 56.15, 108.10, 120.58, 124.37, 124.78, 124.97, 128.50, 128.71, 143.48, 172.22, 211.48. HRMS: (API+) calculated for $\text{C}_{28}\text{H}_{36}\text{N}_3\text{O}_3$ ($[\text{M} + \text{H}]^+$) 462.2757, found 462.2758.

N,N-dimethyl-(20S)-6-oxo-5 α -pregn-2-ene-20-carboxamide (**7a**)

the general procedure for amide formation with acid **5** and dimethylamine and chromatography on silica gel (isopropanol/cyclohexane—1/9) yielded 79 mg (76%) of the title compound **7a** as a colorless oil:

^1H NMR (CDCl_3) δ 0.73 (s, 3H, CH_3); 0.74 (s, 3H, CH_3); 1.15 (d, 3H, $J = 6.7$ Hz, CH_3); 1.75 (dtd, 1H, $J = 12.4$, 10.8 and 4.3 Hz); 1.81–1.89 (m, 2H); 1.97–2.05 (m, 4H); 2.27 (m, 1H); 2.33–2.39 (m, 2H); 2.96 (m, 1H, H-20); 2.76 (dq, 1H, $J = 9.8$ and 6.7 Hz, H-20); 3.01 (bs, 6H, $2 \times \text{NCH}_3$); 5.58, 5.69 (both m, 1H, H-2 and H-3). ^{13}C NMR δ 12.35, 13.48, 17.04, 21.05, 21.67, 23.97, 27.35, 37.66 ($2 \times \text{C}$), 37.68, 39.25, 39.29 ($2 \times \text{C}$), 40.05, 42.62, 46.92, 52.88, 53.24, 53.76, 56.12, 124.47, 124.90, 176.56, 211.97. HRMS: (API+) calculated for $\text{C}_{24}\text{H}_{38}\text{NO}_2$ ($[\text{M} + \text{H}]^+$) 372.2903, found 372.2903.

N,N-diethyl-(20S)-6-oxo-5 α -pregn-2-ene-20-carboxamide (**7b**)

the general procedure for amide formation with acid **5** and diethylamine and chromatography on silica gel (isopropanol/cyclohexane—1/19) yielded 87 mg (78%) of the title compound **7b** as a colorless oil:

^1H NMR (CDCl_3) δ 0.72 (s, 3H, CH_3); 0.73 (s, 3H, CH_3); 1.10 (t, 3H, $J = 7.1$ Hz, CH_3); 1.16 (d, 3H, $J = 6.7$ Hz, CH_3); 1.19 (t, 3H, $J = 7.1$ Hz, CH_3); 1.74 (m, 1H); 1.80–1.90 (m, 2H); 1.97–2.05 (m, 4H); 2.26 (m, 1H); 2.34 (dd, 1H, $J = 13.3$ and 4.1 Hz); 2.37 (dd, 1H, $J = 11.1$ and 4.9 Hz); 2.64 (dq, 1H, $J = 9.7$ and 6.6 Hz, H-20); 3.22–3.33 (m, 2H, $2 \times \text{NCH}$); 3.37–3.51 (m, 2H, $2 \times \text{NCH}$); 5.58, 5.69 (both m, 1H, H-2 and H-3). ^{13}C NMR δ 12.43, 13.01, 13.50, 14.94, 17.69, 21.08, 21.68, 23.96, 27.10, 37.53, 37.72, 39.28, 39.30, 40.07, 40.48, 42.11, 42.61, 46.92, 53.04, 53.26, 53.77, 56.12, 124.49, 124.91, 175.71, 212.02. HRMS: (API+) calculated for $\text{C}_{26}\text{H}_{42}\text{NO}_2$ ($[\text{M} + \text{H}]^+$) 400.3216, found 400.3215.

1-((20S)-6-oxo-5 α -pregn-2-ene-20-carbonyl)pyrrolidine (7c)

the general procedure for amide formation with acid 5 and pyrrolidine and chromatography on silica gel (isopropanol/cyclohexane—1/19) yielded 91 mg (82%) of the title compound 7c as a colorless oil:

^1H NMR (CDCl_3) δ 0.71 (s, 6H, $2 \times \text{CH}_3$); 1.16 (d, 3H, $J = 6.7$ Hz, CH_3); 1.74 (m, 1H); 1.81–1.89 (m, 4H); 1.92–2.05 (m, 6H); 2.25 (m, 1H); 2.34 (dd, 1H, $J = 13.1$ and 4.3 Hz); 2.38 (dd, 1H, $J = 11.0$ and 4.9 Hz); 2.55 (dq, 1H, $J = 9.8$ and 6.7 Hz, H-20); 3.43–3.55 (m, 4H, $4 \times \text{NCH}$); 5.57, 5.69 (both m, 1H, H-2 and H-3). ^{13}C NMR δ 12.38, 13.48, 16.92, 21.05, 21.67, 23.97, 24.35, 26.09, 27.25, 37.71, 39.20, 39.28, 40.06, 40.29, 42.58, 45.56, 46.79, 46.92, 52.71, 53.24, 53.75, 56.10, 124.48, 124.89, 175.05, 212.01. HRMS: (API+) calculated for $\text{C}_{26}\text{H}_{40}\text{NO}_2$ ($[\text{M} + \text{H}]^+$) 398.3059, found 398.3062.

1-((20S)-6-oxo-5 α -pregn-2-ene-20-carbonyl)piperidine (7d)

the general procedure for amide formation with acid 5 and piperidine and chromatography on silica gel (isopropanol/cyclohexane—1/19) yielded 99 mg (86%) of the title compound 7d as a colorless oil:

^1H NMR (CDCl_3) δ 0.720 (s, 3H, CH_3); 0.724 (s, 3H, CH_3); 1.15 (d, 3H, $J = 6.7$ Hz, CH_3); 1.75 (dtd, 1H, $J = 12.5$, 10.7 and 4.3 Hz); 1.82–1.91 (m, 2H); 1.98–2.05 (m, 4H); 2.26 (m, 1H); 2.35 (dd, 1H, $J = 13.1$ and 4.3 Hz); 2.37 (dd, 1H, $J = 11.0$ and 4.9 Hz); 2.76 (dq, 1H, $J = 9.7$ and 6.7 Hz, H-20); 3.46–3.68 (m, 4H, $4 \times \text{NCH}$); 5.58, 5.69 (both m, 1H, H-2 and H-3). ^{13}C NMR δ 12.33, 13.50, 17.43, 21.06, 21.68, 24.01, 24.71, 25.88 (vbs), 26.89 (vbs, $2 \times \text{C}$), 27.62, 37.70, 39.27, 39.30, 40.07, 42.67, 42.92 (vbs), 46.81 (vbs), 46.93, 52.74, 53.26, 53.77, 56.17, 124.49, 124.91, 174.79, 212.02. HRMS: (API+) calculated for $\text{C}_{27}\text{H}_{42}\text{NO}_2$ ($[\text{M} + \text{H}]^+$) 412.3216, found 412.3216.

4-((20S)-6-oxo-5 α -pregn-2-ene-20-carbonyl)morpholine (7e)

the general procedure for amide formation with acid 5 and morpholine and chromatography on silica gel (isopropanol/cyclohexane—1/19) yielded 97 mg (84%) of the title compound 7e as a colorless oil:

^1H NMR (CDCl_3) δ 0.718 (s, 3H, CH_3); 0.720 (s, 3H, CH_3); 1.16 (d, 3H, $J = 6.7$ Hz, CH_3); 1.75 (dtd, 1H, $J = 12.4$, 10.6 and 4.2 Hz); 1.82–1.91 (m, 2H); 1.98–2.05 (m, 4H); 2.26 (m, 1H); 2.35 (dd, 1H, $J = 13.1$ and 4.3 Hz); 2.38 (dd, 1H, $J = 11.0$ and 4.6 Hz); 2.70 (dq, 1H, $J = 10.0$ and 6.8 Hz, H-20); 3.54–3.69 (m, 8H, $8 \times \text{Hmorpholine}$); 5.57, 5.69 (both m, 1H, H-2 and H-3). ^{13}C NMR δ 12.44, 13.61, 17.47, 21.16, 21.79, 24.11, 27.74, 37.41 (bs), 37.77, 39.37, 39.41, 40.16, 42.15 (bs), 42.81, 46.40 (bs), 47.01, 52.80, 53.34, 53.89, 56.24, 66.97 (bs), 67.18 (bs), 124.57, 125.03, 175.26, 212.01. HRMS: (API+) calculated for $\text{C}_{26}\text{H}_{40}\text{NO}_3$ ($[\text{M} + \text{H}]^+$) 414.3008, found 414.3012.

General procedure for Upjohn dihydroxylation

A solution (50 mg/mL) of osmium tetroxide in *t*-butanol (0.1 mL; 0.02 mmol) was added to a solution of unsaturated amide (~0.15 mmol) and *N*-methylmorpholine-*N*-oxide (88 mg; 0.75 mmol) in acetone (4 mL), tetrahydrofuran (4 mL) and water (0.5 mL). the reaction mixture was stirred for 12 h at room temperature. A saturated aqueous solution of sodium sulfite (2 mL) was then added, and the mixture was stirred for an additional 30 min. the reaction was diluted with ethyl acetate and extracted twice with water. the organic phase was dried with sodium sulfate and solvents were evaporated

under reduced pressure. the crude product was purified on silica gel (mobile phase at each experiment).

N,N-dimethyl-(20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-carboxamide (**8a**)

the general procedure for dihydroxylation with amide **7a** and chromatography on silica gel (isopropanol/ethyl acetate—1/19) yielded 52 mg (85%) of the title compound **8a** as a white solid:

Mp > 230 °C (decomp.). ¹H NMR (CDCl₃) δ 0.68 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 1.10 (d, 3H, *J* = 6.7 Hz, CH₃); 1.86 (dt, 1H, *J* = 15.2 and 3.4 Hz); 1.94–2.02 (m, 2H); 2.24 (dd, 1H, *J* = 13.1 and 4.6 Hz); 2.67 (dd, 1H, *J* = 12.5 and 3.1 Hz); 2.73 (dq, 1H, *J* = 9.7 and 6.8 Hz, H-20); 2.91 (s, 3H, NCH₃); 3.05 (s, 3H, NCH₃); 3.67 (m, 1H, Σ *J* = 19.6 Hz, H-2 β); 3.98 (m, 1H, H-3 β). ¹³C NMR δ 12.36, 13.43, 16.87, 21.06, 23.89, 26.21, 27.20, 35.66, 37.61 (2 $\times$ C), 37.62, 39.13, 39.93, 42.56, 42.71, 46.59, 50.70, 52.78, 53.54, 55.98, 68.01, 68.06, 176.98, 212.76. HRMS: (API+) calculated for C₂₄H₄₀NO₄ ([M + H]⁺) 406.2957, found 406.2957. Anal. Calcd. for C₂₄H₃₉NO₄: C, 71.07; H, 9.69; N, 3.45. Found: C, 71.02; H, 9.73; N, 3.42%.

N,N-diethyl-(20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-carboxamide (**8b**)

the general procedure for dihydroxylation with amide **7b** and chromatography on silica gel (isopropanol/ethyl acetate—1/19) yielded 57 mg (88%) of the title compound **8b** as a white solid:

Mp > 200 °C (decomp.). ¹H NMR (CDCl₃) δ 0.72 (s, 3H, CH₃); 0.78 (s, 3H, CH₃); 1.10 (bt, 3H, *J* = 6.7 Hz, CH₃); 1.16 (d, 3H, *J* = 6.7 Hz, CH₃); 1.20 (bt, 3H, *J* = 6.7 Hz, CH₃); 1.93 (dt, 1H, *J* = 15.0 and 3.4 Hz); 1.98–2.05 (m, 2H); 2.30 (dd, 1H, *J* = 13.1 and 4.6 Hz); 2.64 (dq, 1H, *J* = 9.9 and 6.8 Hz, H-20); 2.73 (dd, 1H, *J* = 12.7 and 2.9 Hz); 3.24–3.32 (m, 2H, NCH₂); 3.39–3.47 (m, 2H, NCH₂); 3.75 (m, 1H, Σ *J* = 19.6 Hz, H-2 β); 4.08 (m, 1H, H-3 β). ¹³C NMR δ 12.52, 12.93, 13.57, 14.86, 17.59, 21.19, 23.97, 26.31, 27.05, 37.59, 37.67, 39.34, 40.31, 40.82 (bs), 42.33 (bs), 42.67, 42.80, 46.76, 50.85, 52.96, 53.85, 56.21, 68.43, 68.50, 176.14, 212.06. HRMS: (API+) calculated for C₂₆H₄₄NO₄ ([M + H]⁺) 434.3270, found 434.3270. Anal. Calcd. for C₂₆H₄₃NO₄: C, 72.02; H, 10.00; N, 3.23. Found: C, 71.95; H, 10.06; N, 3.20%.

1-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-carbonyl)pyrrolidine (**8c**)

the general procedure for dihydroxylation with amide **7c** and chromatography on silica gel (isopropanol/ethyl acetate—1/19) yielded 55 mg (85%) of the title compound **8c** as a white solid:

Mp > 180 °C (decomp.). ¹H NMR (CDCl₃) δ 0.71 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 1.16 (d, 3H, *J* = 6.7 Hz, CH₃); 1.92 (dt, 1H, *J* = 15.1 and 3.3 Hz); 1.93–2.03 (m, 4H); 2.29 (dd, 1H, *J* = 13.1 and 4.6 Hz); 2.55 (dq, 1H, *J* = 9.8 and 6.5 Hz, H-20); 2.73 (dd, 1H, *J* = 12.5 and 2.8 Hz); 3.43–3.55 (m, 4H, 2 $\times$ NCH₂); 3.75 (m, 1H, Σ *J* = 19.6 Hz, H-2 β); 4.07 (m, 1H, H-3 β). ¹³C NMR δ 12.46, 13.54, 16.81, 21.17, 23.98, 24.29, 26.02, 26.31, 27.20, 37.67, 39.30, 40.27, 40.33, 42.66, 42.78, 45.84, 46.74, 47.02, 50.84, 52.73, 53.84, 56.22, 68.41, 68.48, 175.45, 212.12. HRMS: (API+) calculated for C₂₆H₄₂NO₄ ([M + H]⁺) 432.3114, found 432.3116. Anal. Calcd. for C₂₆H₄₁NO₄: C, 72.35; H, 9.57; N, 3.25. Found: C, 72.29; H, 9.61; N, 3.22%.

1-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-carbonyl)piperidine (**8d**)

the general procedure for dihydroxylation with amide **7d** and chromatography on silica gel (isopropanol/ethyl acetate—1/19) yielded 58 mg (87%) of the title compound **8d** as a white solid:

Mp > 230 °C (decomp.). ¹H NMR (CDCl₃) δ 0.71 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 1.14 (d, 3H, *J* = 6.7 Hz, CH₃); 1.93 (dt, 1H, *J* = 15.1 and 3.5 Hz); 1.98–2.04 (m, 2H); 2.29 (dd, 1H, *J* = 13.1 and 4.6 Hz); 2.73 (dd, 1H, *J* = 12.7 and 2.9 Hz); 2.76 (dq, 1H, *J* = 9.8 and 6.7 Hz, H-20); 3.48–3.60 (m, 4H, 2 $\times$ NCH₂); 3.74 (m, 1H, Σ *J* = 19.6 Hz, H-2 β); 4.07 (m, 1H, H-3 β). ¹³C NMR δ 12.41, 13.56, 17.37, 21.17, 24.01, 24.61, 26.29, ~26.76 (vbm 3 $\times$ C), 37.64, 39.35, 40.30, 42.67, 42.83, 43.02 (vbs), 46.76, 46.88 (vbs), 50.84, 52.77, 53.26, 53.85, 56.28, 68.41, 68.44, 175.03, 212.08. HRMS: (API+) calculated for C₂₇H₄₄NO₄ ([M + H]⁺) 446.3270, found

446.3271. Anal. Calcd. for $C_{27}H_{43}NO_4$: C, 72.77; H, 9.73; N, 3.14. Found: C, 72.75; H, 9.78; N, 3.10%.

4-((2*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-carbonyl)morpholine (**8e**)

the general procedure for dihydroxylation with amide **7e** and chromatography on silica gel (isopropanol/ethyl acetate—1/9) yielded 56 mg (84%) of the title compound **8e** as a white solid:

Mp > 270 °C (decomp.). 1H NMR ($CDCl_3$ +MeOD) δ 0.68 (s, 3H, CH_3); 0.73 (s, 3H, CH_3); 1.13 (d, 3H, J = 6.7 Hz, CH_3); 1.87 (dt, 1H, J = 15.3 and 3.5 Hz); 1.95–2.03 (m 2H); 2.26 (dd, 1H, J = 13.1 and 4.6 Hz); 2.65–2.71 (m, 2H); 3.54–3.68 (m, 8H, $8 \times H_{morpholine}$); 3.69 (m, 1H, H-2 β); 3.99 (m, 1H, H-3 β). ^{13}C NMR δ 12.34, 13.46, 17.22, 21.06, 23.92, 26.22, 27.51, 37.23 (bs), 37.59, 39.13, 39.97, 42.05 (bs), 42.54, 42.79, 46.26 (bs), 46.58, 50.69, 52.58, 53.54, 56.00, 66.77 (bs), 66.97 (bs), 68.00, 68.07, 175.38, 212.52. HRMS: (API+) calculated for $C_{26}H_{42}NO_5$ ($[M + H]^+$) 448.3063, found 448.3063. Anal. Calcd. for $C_{26}H_{41}NO_5$: C, 69.77; H, 9.23; N, 3.13. Found: C, 69.72; H, 9.25; N, 3.09%.

Methyl (2*S*)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carboxylate (**11**)

p-Toluensulfonic acid (50 mg; 0.29 mmol) was added to a solution of ester **9** (1.0 g; 2.55 mmol) in dry acetone (100 mL). the reaction mixture was stirred at room temperature for 5 h. the excess acetone was removed under reduced pressure and the suspension was diluted in ethyl acetate and extracted with a saturated solution of sodium bicarbonate and twice with water. the organic phase was dried over sodium sulfate and solvents were evaporated under reduced pressure. Partially protected ester **10** was dissolved in 2,2-dimethyl-1,3-dioxolan (20 mL) and p-toluensulfonic acid (10 mg; 0.06 mmol) was added. the reaction mixture was stirred at 90 °C for 10 h. the mixture was diluted with ethyl acetate and extracted with a saturated solution of sodium bicarbonate and twice with water. the organic phase was dried over sodium sulfate and solvents were evaporated under reduced pressure. the crude product was purified on silica gel (mobile phase—30% ethyl acetate in cyclohexane) to afford protected methyl ester **11** (888 mg; 74% after 2 steps) as a colorless oil.

1H NMR ($DMSO-d_6$) δ 0.62 (s, 3H, CH_3); 0.76 (s, 3H, CH_3); 1.10 (d, 3H, J = 6.7 Hz, CH_3); 1.21 (s, 3H, CH_3); 1.35 (s, 3H, CH_3); 1.62–1.71 (m, 3H); 1.79 (dd, 1H, J = 12.5 and 7.0 Hz); 1.86 (m, 1H); 1.96 (m, 1H); 2.32 (dq, 1H, J = 10.3 and 6.9 Hz, H-20); 3.55 (s, 3H, CH_3); 3.67 (m, 1H, CH_2O); 3.80–3.91 (m, 3H, CH_2O); 4.01 (m, 1H, H-2 β); 4.18 (m, 1H, H-3 β). ^{13}C NMR δ 11.83, 13.18, 16.91, 20.34, 21.46, 23.87, 26.65, 26.74, 28.65, 32.53, 37.61, 38.82, 40.64, 41.83, 42.09, 42.44, 45.12, 51.21, 52.16, 52.65, 54.81, 63.72, 65.18, 72.04, 72.27, 106.80, 108.86, 176.27. HRMS: (API+) calculated for $C_{28}H_{45}O_6$ ($[M + H]^+$) 477.3216, found 477.3220.

(2*S*)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carboxylic acid (**12**)

Potassium hydroxide (500 mg; 8.9 mmol) was added to a solution of methyl ester **11** (850 mg; 1.79 mmol) in isopropanol (80 mL) and water (10 mL). the reaction mixture was stirred under reflux for 4 h. After cooling to room temperature, the excess of isopropanol was removed under reduced pressure and the residue was diluted in ethyl acetate and extracted with 5% aqueous solution of citric acid and twice with water. the organic phase was dried over sodium sulfate and solvents were evaporated under reduced pressure to obtain acid **12** (795 mg; 96%) in adequate purity for other reactions.

1H NMR ($DMSO-d_6$) δ 0.62 (s, 3H, CH_3); 0.77 (s, 3H, CH_3); 1.09 (d, 3H, J = 6.9 Hz, CH_3); 1.22 (s, 3H, CH_3); 1.36 (s, 3H, CH_3); 1.62–1.74 (m, 3H); 1.79 (dd, 1H, J = 12.7 and 6.9 Hz); 1.87 (m, 1H); 1.97 (m, 1H); 2.20 (dq, 1H, J = 10.4 and 6.8 Hz, H-20); 3.67 (m, 1H, CH_2O); 3.80–3.91 (m, 3H, CH_2O); 4.01 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β); 11.94 (bs, 1H, COOH). ^{13}C NMR δ 11.84, 13.15, 17.02, 20.32, 21.42, 23.90, 26.60, 26.95, 28.60, 32.48, 37.57, 38.92,

40.60, 41.95, 42.10, 42.40, 45.08, 52.15, 52.42, 54.87, 63.67, 65.13, 72.00, 72.22, 106.74, 108.82, 177.40. HRMS: (API+) calculated for $C_{27}H_{43}O_6$ ($[M + H]^+$) 463.3060, found 463.3059.

N,N-dimethyl-(20*S*)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carboxamide (**13a**)

the general procedure for amide formation with acid **12** and dimethylamine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 180 mg (85%) of the title compound **13a** as a colorless oil:

1H NMR (DMSO- d_6) δ 0.67 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.98 (d, 3H, J = 6.7 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.62–1.74 (m, 3H); 1.79 (dd, 1H, J = 12.7 and 6.9 Hz); 1.88 (m, 1H); 1.97 (m, 1H); 2.72 (m, 1H, H-20); 2.78 (s, 3H, CH₃); 3.00 (s, 3H, CH₃); 3.67 (m, 1H, CH₂O); 3.80–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ^{13}C NMR δ 12.12, 13.16, 17.02, 20.34, 21.42, 23.87, 26.36, 26.62, 27.00, 28.61, 32.47, 35.02, 37.04, 37.57, 39.08, 40.64, 41.76, 42.40, 45.07, 52.18, 52.91, 54.90, 63.65, 65.15, 72.02, 72.23, 106.75, 108.86, 175.35. HRMS: (API+) calculated for $C_{29}H_{48}NO_5$ ($[M + H]^+$) 490.3532, found 490.3534.

N,N-diethyl-(20*S*)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carboxamide (**13b**)

the general procedure for amide formation with acid **12** and diethylamine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 186 mg (83%) of the title compound **13b** as a colorless oil:

1H NMR (DMSO- d_6) δ 0.67 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.97 (t, 3H, J = 7.0 Hz, CH₃); 1.00 (d, 3H, J = 6.7 Hz, CH₃); 1.10 (t, 3H, J = 7.0 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.62–1.74 (m, 3H); 1.80 (dd, 1H, J = 12.7 and 6.9 Hz); 1.89 (m, 1H); 1.97 (m, 1H); 2.58 (m, 1H, H-20); 3.13 (m, 1H, NCH); 3.24–3.40 (m, 3H, 3 $\times$ NCH); 3.67 (m, 1H, CH₂O); 3.80–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ^{13}C NMR δ 12.14, 12.97, 13.17, 14.94, 17.65, 20.35, 21.42, 23.78, 26.35, 26.61, 26.76, 28.60, 32.47, 36.70, 37.56, 39.09, 40.65, 41.52, 41.75, 42.39, 45.06, 52.18, 53.02, 54.87, 63.65, 65.15, 72.01, 72.22, 106.74, 108.86, 174.50. HRMS: (API+) calculated for $C_{31}H_{52}NO_5$ ($[M + H]^+$) 518.3845, found 518.3849.

1-((20*S*)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carbonyl)pyrrolidine (**13c**)

the general procedure for amide formation with acid **12** and pyrrolidine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 196 mg (88%) of the title compound **13c** as a colorless oil:

1H NMR (DMSO- d_6) δ 0.66 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 1.00 (d, 3H, J = 6.7 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.73–1.81 (m, 3H); 1.82–1.89 (m, 3H); 1.97 (m, 1H); 2.51 (m, 1H, H-20); 3.21–3.24 (m, 2H, 2 $\times$ NCH); 3.40–3.46 (m, 2H, 2 $\times$ NCH); 3.67 (m, 1H, CH₂O); 3.80–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ^{13}C NMR δ 12.18, 13.20, 16.93, 20.38, 21.46, 23.89, 23.96 (2 $\times$ C), 25.73, 26.65, 26.92, 28.65, 32.53, 37.61, 39.09, 40.68, 41.76, 42.43, 45.11, 45.25, 46.22, 52.22, 52.81, 54.91, 63.69, 65.19, 72.05, 72.27, 106.80, 108.89, 173.81. HRMS: (API+) calculated for $C_{31}H_{50}NO_5$ ($[M + H]^+$) 516.3689, found 516.3690.

1-((20*S*)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carbonyl)piperidine (**13d**)

the general procedure for amide formation with acid **12** and piperidine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 192 mg (84%) of the title compound **13d** as a colorless oil:

1H NMR (DMSO- d_6) δ 0.66 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.99 (d, 3H, J = 6.7 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.64–1.74 (m, 3H); 1.79 (dd, 1H, J = 12.5 and 6.7 Hz); 1.89 (m, 1H); 1.97 (m, 1H); 2.72 (m, 1H, H-20); 3.39–3.50 (m, 4H, 4 $\times$ NCH); 3.67 (m, 1H, CH₂O); 3.80–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ^{13}C NMR δ 12.09, 13.17, 17.40, 20.34, 21.44, 23.89, 24.24, 25.55, 26.56, 26.63, 27.31, 28.62, 32.47, 37.58, 39.10, 39.27, 40.67, 41.81, 42.07, 42.40, 45.09, 46.11, 52.19, 52.75, 54.94, 63.67, 65.17, 72.03, 72.24,

106.76, 108.87, 173.64. HRMS: (API+) calculated for $C_{32}H_{51}NO_5$ ($[M + H]^+$) 530.3845, found 530.3846.

4-((20S)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-carbonyl)morpholine (**13e**)

the general procedure for amide formation with acid **12** and morpholine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 193 mg (84%) of the title compound **13b** as a colorless oil:

1H NMR (DMSO- d_6 , 45 °C) δ 0.68 (s, 3H, CH₃); 0.78 (s, 3H, CH₃); 1.02 (d, 3H, J = 6.7 Hz, CH₃); 1.23 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.61–1.75 (m, 5H); 1.80 (dd, 1H, J = 12.5 and 7.0 Hz); 1.89 (m, 1H); 1.98 (m, 1H); 2.73 (m, 1H, H-20); 3.44–3.56 (m, 8H, 8 $\times$ Hmo_{morpholine}); 3.68 (m, 1H, CH₂O); 3.81–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ^{13}C NMR δ 12.10, 13.18, 17.29, 20.35, 21.45, 23.92, 26.64, 27.21, 28.64 (2 $\times$ C), 32.49, 37.60, 39.10, 40.68, 41.59, 41.88, 42.42, 45.11, 45.76, 52.20, 52.71, 54.92, 63.69, 65.19, 66.32, 66.48, 72.04, 72.26, 106.79, 108.88, 174.22. HRMS: (API+) calculated for $C_{31}H_{50}NO_6$ ($[M + H]^+$) 532.3638, found 532.3639.

General procedure for reduction of amides with lithium aluminum hydride

An amount of 1M solution of lithium aluminum hydride in diethyl ether (1.0 mL; 1.0 mmol) was added dropwise to a solution of amide (150 mg) in dry dioxane (10 mL). the reaction mixture was stirred under reflux for 3 h. After cooling, a saturated aqueous solution of sodium potassium tartrate (1 mL) was added, and the mixture was stirred for an additional 10 min. Then, it was diluted with ethyl acetate and extracted twice with water. the organic phase was dried with sodium sulfate and solvents were evaporated under reduced pressure. the crude product was purified on silica gel (mobile phase at each experiment).

N,N-dimethyl-((20S)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-methyl)amine (**14a**)

the general procedure for amide reduction with amide **13a** (150 mg; 0.31 mmol) and chromatography on silica gel (2–4% methanol in ammonia chloroform) yielded 106 mg (73%) of the title compound **14a** as a colorless oil:

1H NMR (DMSO- d_6 , 55 °C) δ 0.66 (s, 3H, CH₃); 0.79 (s, 3H, CH₃); 0.96 (d, 3H, J = 6.5 Hz, CH₃); 1.23 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.64–1.75 (m, 3H); 1.76 (m, 1H); 1.81 (dd, 1H, J = 12.5 and 7.0 Hz); 1.94–2.01 (m, 2H); 2.19 (bs, 6H, 2 $\times$ CH₃); 3.69 (m, 1H, CH₂O); 3.81–3.91 (m, 3H, CH₂O); 4.03 (m, 1H, H-2 β); 4.20 (m, 1H, H-3 β). ^{13}C NMR δ 11.86, 13.16, 17.59, 20.34, 21.42, 24.00, 26.61, 27.65, 28.62, 32.47, 34.02 (vb), 37.56, 39.07, 40.65, 42.34, 42.42, 45.08, 45.33 (vb, 2 $\times$ C), 52.20, 54.54 (b), 54.98, 59.78, 63.67, 65.14, 72.01, 72.23, 106.74, 108.86. HRMS: (API+) calculated for $C_{29}H_{50}NO_4$ ($[M + H]^+$) 476.3740, found 476.3742.

N,N-diethyl-((20S)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-methyl)amine (**14b**)

the general procedure for amide reduction with amide **13b** (150 mg; 0.29 mmol) and chromatography on silica gel (2–4% methanol in ammonia chloroform) yielded 104 mg (71%) of the title compound **14b** as a colorless oil:

1H NMR (DMSO- d_6 , 55 °C) δ 0.64 (s, 3H, CH₃); 0.75 (s, 3H, CH₃); 0.89–1.00 (m, 9H, 3 $\times$ CH₃); 1.61–1.72 (m, 3H); 1.78 (dd, 1H, J = 12.7 and 6.9 Hz); 1.80 (m, 1H); 1.91–1.98 (m, 2H); 2.50–3.10 (vbs, 6H, 6 $\times$ NCH), 3.66 (m, 1H, CH₂O); 3.78–3.88 (m, 3H, CH₂O); 3.99 (m, 1H, H-2 β); 4.17 (m, 1H, H-3 β). ^{13}C NMR δ 11.76, 13.15, 14.55 (vb, 2 $\times$ C), 17.65 (b), 20.32, 21.42, 23.90 (b), 26.61, 27.59 (b), 28.61, 32.47, 37.55, 39.08, 40.63, 42.34, 42.40, 45.06, 51.97 (vb, 2 $\times$ C), 52.11, 55.01, 63.22 (vb, C), 63.66, 65.15, 71.99, 72.22, 106.74, 108.84, 2 $\times$ C n. f. HRMS: (API+) calculated for $C_{31}H_{54}NO_4$ ($[M + H]^+$) 504.4053, found 504.4050.

1-((20S)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-methyl)pyrrolidine (**14c**)

the general procedure for amide reduction with amide **13c** (150 mg; 0.29 mmol) and chromatography on silica gel (2–4% methanol in ammonia chloroform) yielded 110 mg (75%) of the title compound **14c** as a colorless oil:

^1H NMR (DMSO- d_6) δ 0.65 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 1.04 (d, 3H, J = 6.7 Hz, CH₃); 1.21 (s, 3H, CH₃); 1.35 (s, 3H, CH₃); 1.62–1.72 (m, 3H); 1.73–1.81 (m, 2H); 1.91–1.98 (m, 2H); 2.66–3.14 (vbs, 6H, 6 $\times$ NCH), 3.67 (m, 1H, CH₂O); 3.80–3.84 (m, 2H, CH₂O); 3.89 (m, 1H, CH₂O); 4.01 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ^{13}C NMR δ 11.81, 13.19, 17.29, 20.38, 21.45, 22.66 (2 $\times$ C), 23.90, 26.65, 27.53, 28.66, 32.52, 37.60, 39.08, 40.65, 42.37, 42.46, 45.12, 52.14, 53.66 (vb, 2 $\times$ C), 55.02, 60.49 (vb), 63.73, 65.19, 72.04, 72.27, 106.80, 108.88, 2 $\times$ C n. f. HRMS: (API+) calculated for C₃₁H₅₂NO₄ ([M + H]⁺) 502.3896, found 502.3895.

1-((20S)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-methyl)pyrrolidine (**14d**)

the general procedure for amide reduction with amide **13d** (150 mg; 0.28 mmol) and chromatography on silica gel (2–4% methanol in ammonia chloroform) yielded 107 mg (73%) of the title compound **14d** as a colorless oil:

^1H NMR (DMSO- d_6) δ 0.67 (s, 3H, CH₃); 0.79 (s, 3H, CH₃); 1.04 (d, 3H, J = 6.7 Hz, CH₃); 1.23 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.64–1.83 (m, 5H); 1.93–2.01 (m, 2H); 2.65–3.00 (vbm, 4H, 4 $\times$ NCH), 3.14–3.55 (vbs, 2H, 2 $\times$ NCH), 3.70 (m, 1H, CH₂O); 3.81–3.92 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.20 (m, 1H, H-3 β). ^{13}C NMR δ 11.76, 13.14, 17.67, 20.30, 21.41, 23.24, 23.90 (vb, 2 $\times$ C), 26.60, 27.52, 28.61, 32.46, 37.55, 39.08, 40.60, 42.35, 42.39, 45.05, 52.08, 54.62 (vb, 2 $\times$ C), 54.96, 60.23 (vb), 63.66, 65.12, 71.97, 72.20, 106.73, 108.82, 3 $\times$ C n. f. HRMS: (API+) calculated for C₃₂H₅₄NO₄ ([M + H]⁺) 516.4053, found 516.4055.

4-((20S)-2 α ,3 α -(1-methylethylidene)dioxy-6,6-ethylenedioxy-5 α -pregnane-20-methyl)morpholine (**14e**)

the general procedure for amide reduction with amide **13e** (150 mg; 0.28 mmol) and chromatography on silica gel (2–4% methanol in ammonia chloroform) yielded 115 mg (79%) of the title compound **14e** as a colorless oil:

^1H NMR (DMSO- d_6 , 50 °C) δ 0.65 (s, 3H, CH₃); 0.78 (s, 3H, CH₃); 0.97 (d, 3H, J = 6.7 Hz, CH₃); 1.23 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.63–1.72 (m, 3H); 1.78–1.82 (m, 2H); 1.89–2.00 (m, 3H); 2.13–2.19 (m, 3H, 3 $\times$ NCH); 2.38–2.42 (m, 2H, 2 $\times$ NCH); 3.51–3.59 (m, 4H, 2 $\times$ CH₂O_{morpholine}); 3.69 (m, 1H, CH₂O); 3.81–3.91 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.20 (m, 1H, H-3 β). ^{13}C NMR δ 11.67, 12.92, 17.72, 20.16, 21.29, 23.82, 26.17, 27.40, 28.38, 32.32, 32.99, 37.38, 38.97, 40.51, 42.21, 42.32, 44.97, 52.19, 53.80 (2 $\times$ C), 54.59, 54.86, 63.43, 64.47, 64.95, 66.14 (2 $\times$ C), 71.85, 72.08, 106.53, 108.72. HRMS: (API+) calculated for C₃₁H₅₂NO₅ ([M + H]⁺) 518.3845, found 518.3849.

General procedure for deprotection of short side chain amine ketals

A 5% aqueous solution of hydrochloric acid (1 mL) was added to a solution of ketals **14a–e** (80 mg) in tetrahydrofuran (5 mL) and the mixture was stirred at 45 °C for 2 h. After cooling, the saturated solution of sodium bicarbonate (2 mL) was added, and the reaction was stirred at room temperature for an additional 15 min. the mixture was diluted with ethyl acetate and extracted twice with water. the organic phase was dried with sodium sulfate and solvents were evaporated under reduced pressure. the crude product was washed with diethylether to obtain a pure product (no chromatography needed).

(20S)-20-(N,N-dimethylaminomethyl)-2 α ,3 α -dihydroxy-5 α -pregnane-6-one (**15a**)

the general procedure for deprotection with ketal **14a** (80 mg; 0.17 mmol) yielded 57 mg (88%) of the title compound **15a** as an off-white solid:

Mp 219–221 °C; >240 (decomp.). ^1H NMR (DMSO- d_6) δ 0.64 (s, 3H, CH₃); 0.65 (s, 3H, CH₃); 0.96 (d, 3H, J = 6.5 Hz, CH₃); 1.67 (m, 1H); 1.79 (m, 1H); 1.97–2.13 (m, 3H); 2.19 (vbs, 6H, 2 $\times$ NCH₃); 2.60 (dd, 1H, J = 12.2 and 3.4 Hz); 3.46 (m, 1H, H-2 β); 3.75 (m, 1H, H-3 β); 4.20 (d, 1H, J = 2.4 Hz, OH); 4.34 (d, 1H, J = 6.1 Hz, OH). ^{13}C NMR δ 11.90, 13.36, 17.55, 20.78, 23.67, 26.84, 27.57, 33.92 (vb), 36.98, 38.97, 41.84, 42.81, 45.22 (vb, 2 $\times$ C), 46.00, 50.34, 52.91,

54.36 (vb), 55.61, 65.00 (vb), 67.11, 67.49, 211.50, 1 × C n. f. HRMS: (API+) calculated for C₂₄H₄₂NO₃ ([M + H]⁺) 392.3165, found 392.3164. Anal. Calcd. for C₂₄H₄₁NO₃: C, 73.61; H, 10.55; N, 3.58. Found: C, 73.57; H, 10.59; N, 3.57%.

(20S)-20-(N,N-diethylaminomethyl)-2α,3α-dihydroxy-5α-pregnane-6-one (**15b**)

the general procedure for deprotection with ketal **14b** (80 mg; 0.16 mmol) yielded 56 mg (85%) of the title compound **15b** as an off-white solid:

Mp > 190 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.64 (s, 3H, CH₃); 0.65 (s, 3H, CH₃); 0.94–1.08 (m, 9H, 3 × CH₃); 1.67 (m, 1H); 1.83 (m, 1H); 1.97–2.13 (m, 3H); 2.37 (vbs, 2H, 2 × NCH); 2.60 (dd, 1H, *J* = 12.1 and 3.2 Hz); 2.61 (vbs, 2H, 2 × NCH); 3.46 (m, 1H, H-2β); 3.76 (m, 1H, H-3β); 4.21 (bs, 1H, OH); 4.35 (bs, 1H, OH). ¹³C NMR δ 11.88, 13.36, 17.80, 19.08 (vb, 2 × C), 20.77, 23.69, 26.84, 27.62, 34.43 (vb), 37.00, 38.95, 41.84, 42.80, 46.01, 46.93 (vb, 2 × C), 50.33, 52.91, 54.34 (vb), 55.65, 58.55 (vb), 67.11, 67.49, 211.52, 1 × C n. f. HRMS: (API+) calculated for C₂₆H₄₆NO₃ ([M + H]⁺) 420.3478, found 420.3476. Anal. Calcd. for C₂₆H₄₅NO₃: C, 74.42; H, 10.81; N, 3.34. Found: C, 74.39; H, 10.86; N, 3.31%.

(20S)-20-(pyrrolidin-1-ylmethyl)-2α,3α-dihydroxy-5α-pregnane-6-one (**15c**)

the general procedure for deprotection with ketal **14c** (80 mg; 0.16 mmol) yielded 58 mg (88%) of the title compound **15c** as an off-white solid:

Mp > 240 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.64 (s, 3H, CH₃); 0.65 (s, 3H, CH₃); 1.01 (d, 3H, *J* = 6.5 Hz, CH₃); 1.63–1.84 (m, 6H); 1.98–2.13 (m, 3H); 2.25–2.75 (vbm, 4H, 4 × NCH); 2.60 (dd, 1H, *J* = 12.1 and 3.5 Hz); 3.46 (m, 1H, H-2β); 3.73 (m, 1H, H-3β); 4.21 (d, 1H, *J* = 2.4 Hz, OH); 4.34 (d, 1H, *J* = 6.1 Hz, OH). ¹³C NMR δ 11.85, 13.36, 17.69, 20.77, 22.94 (vb, 2 × C), 23.63, 26.83, 27.59, 35.06 (vb), 36.94, 38.93, 41.82, 42.75, 45.98, 50.33, 52.88, 54.11 (vb, 3 × C), 55.61, 61.35 (vb), 67.11, 67.48, 211.48, 1 × C n. f. HRMS: (API+) calculated for C₂₆H₄₄NO₃ ([M + H]⁺) 418.3321, found 418.3322. Anal. Calcd. for C₂₆H₄₃NO₃: C, 74.77; H, 10.38; N, 3.35. Found: C, 74.74; H, 10.42; N, 3.30%.

(20S)-20-(piperidin-1-ylmethyl)-2α,3α-dihydroxy-5α-pregnane-6-one (**15d**)

the general procedure for deprotection with ketal **14d** (80 mg; 0.16 mmol) yielded 57 mg (85%) of the title compound **15d** as an off-white solid:

Mp > 210 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.64 (s, 6H, 2 × CH₃); 0.96 (d, 3H, *J* = 6.5 Hz, CH₃); 1.66 (m, 1H); 1.79 (m, 1H); 1.92–2.13 (m, 5H, incl. 2 × NCH); 2.10–2.33 (m, 2H, 2 × NCH); 2.43–2.62 (m, 2H, 2 × NCH); 2.60 (dd, 1H, *J* = 12.1 and 3.5 Hz); 3.47 (m, 1H, H-2β); 3.75 (m, 1H, H-3β); 4.20 (d, 1H, *J* = 2.1 Hz, OH); 4.34 (d, 1H, *J* = 5.8 Hz, OH). ¹³C NMR δ 11.89, 13.36, 17.90, 20.77, 23.69, 23.93 (vb), 25.32 (b, 2 × C), 26.84, 27.58, 33.40 (vb), 36.99, 38.98, 41.84, 42.82, 46.01, 50.34, 52.91, 54.52 (vb, 2 × C), 55.62, 64.52 (vb), 67.11, 67.49, 211.51, 1 × C n. f. HRMS: (API+) calculated for C₂₇H₄₆NO₃ ([M + H]⁺) 432.3478, found 432.3474. Anal. Calcd. for C₂₆H₄₃NO₃: C, 74.77; H, 10.38; N, 3.35. Found: C, 74.74; H, 10.42; N, 3.30%.

(20S)-20-(morpholin-4-ylmethyl)-2α,3α-dihydroxy-5α-pregnane-6-one (**15e**)

the general procedure for deprotection with ketal **14e** (80 mg; 0.15 mmol) and chromatography on silica gel yielded 57 mg (86%) of the title compound **15e** as an off-white solid:

Mp > 210 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.64 (s, 6H, 2 × CH₃); 0.96 (d, 3H, *J* = 6.5 Hz, CH₃); 1.66 (m, 1H); 1.80 (m, 1H); 1.92 (dd, 1H, *J* = 11.9 and 10.5 Hz); 1.98–2.19 (m, 7H, incl. 4 × NCH); 2.39 (vbs, 2H, 2 × NCH); 2.59 (dd, 1H, *J* = 12.2 and 3.4 Hz); 3.46 (m, 1H, H-2β); 3.50–3.58 (m, 4H, 2 × CH₂O_{morpholine}); 3.75 (m, 1H, H-3β); 4.20 (d, 1H, *J* = 2.4 Hz, OH); 4.33 (d, 1H, *J* = 6.1 Hz, OH). ¹³C NMR δ 11.90, 13.35, 17.82, 20.76, 23.70, 26.83, 27.62, 33.11, 36.98, 38.95, 41.82, 42.82, 46.01, 50.33, 52.92, 53.92 (b, 2 × C), 54.56, 55.61, 64.55, 66.30 (2 × C), 67.10, 67.47, 211.48. HRMS: (API+) calculated for C₂₆H₄₄NO₄ ([M + H]⁺) 434.3270, found 434.3267. Anal. Calcd. for C₂₆H₄₃NO₄: C, 72.02; H, 10.00; N, 3.23. Found: C, 71.98; H, 10.04; N, 3.22%.

General procedure for preparation of aminium chlorides

A 5% aqueous solution of hydrochloric acid (0.5 mL) was added to a solution of amine (20 mg; ~0.05 mmol) in methanol (2 mL). the mixture was shaken, evaporated to dryness and washed with diethyl ether. the prepared salts obtained in quantitative yield had sufficient purity.

N,N-dimethyl-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-methyl)aminium chloride (**16a**)

Mp > 290 °C (decomp.). ¹H NMR (D₂O) δ 0.67 (s, 3H, CH₃); 0.70 (s, 3H, CH₃); 1.00 (d, 3H, J = 6.7 Hz, CH₃); 1.75–1.83 (m, 2H); 1.88 (m, 1H); 1.99 (m, 1H); 2.17–2.20 (m, 2H); 2.68 (dd, 1H, J = 11.1 and 4.9 Hz); 2.77 (s, 3H, NCH₃); 2.83 (s, 3H, NCH₃); 2.87 (dd, 1H, J = 12.9 and 11.3 Hz, H-22a); 3.04 (dd, 1H, J = 12.9 and 3.2 Hz, H-22b); 3.73 (m, 1H, ΣJ = 20.2 Hz, H-2 β); 3.98 (m, 1H, H-3 β). ¹³C NMR δ 11.22, 12.72, 15.69, 20.79, 23.43, 26.01, 27.23, 32.23, 38.03, 38.79, 38.92, 40.88, 43.04, 43.09, 45.18, 46.11, 50.87, 52.98, 53.00, 55.55, 63.62, 67.72, 68.11, 219.52. HRMS: (API+) calculated for C₂₄H₄₂NO₃ ([M – Cl]⁺) 392.3165, found 392.3164. Anal. Calcd. for C₂₄H₄₂ClNO₃: C, 67.34; H, 9.89; N, 3.27. Found: C, 67.28; H, 9.96; N, 3.23%.

N,N-diethyl-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-methyl)aminium chloride (**16b**)

Mp > 270 °C (decomp.). ¹H NMR (CD₃OD) δ 0.77 (s, 3H, CH₃); 0.80 (s, 3H, CH₃); 1.15 (d, 3H, J = 6.5 Hz, CH₃); 1.87–2.00 (m, 2H); 2.09–2.15 (m, 2H); 2.21 (dd, 1H, J = 13.1 and 4.9 Hz); 2.73 (dd, 1H, J = 12.4 and 3.2 Hz); 2.89 (dd, 1H, J = 13.3 and 11.2 Hz, H-22a); 3.08 (dd, 1H, J = 13.3 and 2.4 Hz, H-22b); 3.15–3.22 (m, 2H, 2 $\times$ NCH); 3.25–3.34 (m, 2H, 2 $\times$ NCH); 3.66 (m, 1H, ΣJ = 19.9 Hz, H-2 β); 3.95 (m, 1H, H-3 β). ¹³C NMR δ 8.41 (vb), 9.55 (vb), 12.51, 14.01, 17.69, 22.44, 25.07, 27.99, 29.03, 34.30, 39.16, 40.72, 41.10, 43.68, 44.71, 47.50, 47.77 (vb), 50.73 (vb), 52.22, 55.03, 55.14, 57.61, 59.51, 69.23, 69.61, 214.89. HRMS: (API+) calculated for C₂₆H₄₆NO₃ ([M – Cl]⁺) 420.3478, found 420.3480. Anal. Calcd. for C₂₆H₄₆ClNO₃: C, 68.47; H, 10.17; N, 3.07. Found: C, 68.40; H, 10.23; N, 3.02%.

1-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-methyl)pyrrolidin-1-ium chloride (**16c**)

Mp > 260 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.61 (s, 3H, CH₃); 0.63 (s, 3H, CH₃); 1.06 (d, 3H, J = 6.5 Hz, CH₃); 1.83–1.97 (m, 5H); 2.00 (dd, 1H, J = 13.1 and 4.9 Hz); 2.08 (bt, 1H, J = 12.3 Hz); 2.57 (dd, 1H, J = 11.9 and 3.4 Hz); 2.86–3.00 (m, 4H, 4 $\times$ NCH); 3.43 (m, 1H, H-2 β); 3.46–3.53 (m, 2H, 2 $\times$ NCH); 3.72 (m, 1H, H-3 β); 4.18 (d, 1H, J = 2.4 Hz, OH); 4.32 (d, 1H, J = 6.1 Hz, OH); 9.62 (bs, 1H, NH). ¹³C NMR δ 11.79, 13.36, 17.26, 20.78, 22.41, 22.69, 23.49, 26.84, 27.39, 33.51, 36.93, 38.85, 41.81, 42.78, 45.91, 50.34, 52.11, 52.80, 53.23, 55.50, 55.61, 60.15, 67.11, 67.49, 211.46, 1 $\times$ C n. f. HRMS: (API+) calculated for C₂₆H₄₄NO₃ ([M – Cl]⁺) 418.3321, found 418.3321. Anal. Calcd. for C₂₆H₄₄ClNO₃: C, 68.77; H, 9.77; N, 3.08. Found: C, 68.71; H, 9.83; N, 3.03%.

1-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-methyl)piperidin-1-ium chloride (**16d**)

Mp > 280 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.65 (s, 3H, CH₃); 0.67 (s, 3H, CH₃); 1.08 (d, 3H, J = 6.5 Hz, CH₃); 1.83–1.93 (m, 2H); 1.99 (m, 1H); 2.04 (dd, 1H, J = 13.1 and 4.9 Hz); 2.11 (bt, 1H, J = 12.4 Hz); 2.60 (dd, 1H, J = 12.1 and 3.5 Hz); 2.73–2.80 (m, 2H, 2 $\times$ NCH); 2.89–2.97 (m, 2H, 2 $\times$ NCH); 3.29–3.34 (m, 2H, 2 $\times$ NCH); 3.46 (m, 1H, H-2 β); 3.76 (m, 1H, H-3 β); 9.25 (bs, 1H, NH). ¹³C NMR δ 11.78, 13.36, 17.87, 20.76, 21.39, 22.01, 22.04, 23.48, 26.84, 27.41, 31.50, 36.93, 38.85, 41.81, 42.81, 45.91, 50.34, 50.76, 52.77, 53.33, 54.64, 55.62, 61.99, 67.11, 67.49, 211.47, 1 $\times$ C n. f. HRMS: (API+) calculated for C₂₇H₄₆NO₃ ([M – Cl]⁺) 432.3478, found 432.3475. Anal. Calcd. for C₂₇H₄₆ClNO₃: C, 69.28; H, 9.90; N, 2.99. Found: C, 69.20; H, 9.98; N, 2.95%.

4-((20*S*)-2 α ,3 α -dihydroxy-6-oxo-5 α -pregnane-20-methyl)morpholin-4-ium chloride (**16e**)

Mp > 240 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.62 (s, 3H, CH₃); 0.64 (s, 3H, CH₃); 1.03 (d, 3H, J = 6.5 Hz, CH₃); 1.65 (m, 1H); 1.76 (m, 1H); 1.84 (m, 1H); 1.96 (m, 1H); 2.03 (dd, 1H, J = 13.1 and 4.9 Hz); 2.08 (bt, 1H, J = 12.5 Hz); 2.58 (dd, 1H, J = 12.4 and 3.5 Hz); 2.83 (dd, 1H, J = 12.9 and 11.0 Hz, NCH); 2.96 (td, 1H, J = 12.2 and 3.8 Hz, NCH) 3.02 (dd, 1H,

$J = 12.9$ and 2.3 Hz, NCH); 3.13 (td, 1H, $J = 12.2$ and 3.8 Hz, NCH); 3.30 (bd, 1H, $J = 11.6$ Hz, NCH); 3.43–3.48 (m, 2H, H-2 β , NCH); 3.70 (m, 1H, OCH_{morph}); 3.75–3.81 (m, 2H, H-3 β and OCH_{morph}); 3.88–3.93 (m, 2H, 2 $\times$ OCH_{morph}). ^{13}C NMR δ 12.15, 13.75, 17.63, 21.19, 23.90, 27.12, 27.69, 31.43, 37.48, 40.23, 42.34, 43.28, 46.34, 50.38, 50.79, 53.20, 53.49, 53.73, 55.96, 62.65, 63.34, 63.46, 67.44, 67.79, 212.71, 1 $\times$ C n. f. HRMS: (API+) calculated for $\text{C}_{26}\text{H}_{44}\text{NO}_4$ ($[\text{M} - \text{Cl}]^+$) 434.3270, found 434.3272. Anal. Calcd. for $\text{C}_{26}\text{H}_{44}\text{ClNO}_4$: C, 66.43; H, 9.43; N, 2.98. Found: C, 66.38; H, 9.48; N, 2.95%.

(2*S*,22*R*,23*S*)-2 α ,3 α ,22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-oic acid (**18**)

Potassium hydroxide (570 mg; 10.18 mmol) was added to a solution of ethyl ester **17** (1.2 g; 2.03 mmol) in methanol (80 mL) and water (10 mL). the reaction mixture was stirred under reflux for 1 h. After cooling to room temperature, the excess of methanol was removed under reduced pressure, and the residue was diluted in ethyl acetate and extracted with 5% aqueous solution of citric acid and twice with water. the organic phase was dried over sodium sulfate and solvents were evaporated under reduced pressure to obtain acid **18** (795 mg; 96%) as a colorless oil in adequate purity for other reactions.

^1H NMR (DMSO- d_6) δ 0.61 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 0.89 (d, 3H, $J = 6.5$ Hz, CH₃); 1.21 (s, 3H, CH₃); 1.26 (s, 3H, CH₃); 1.33 (s, 3H, CH₃); 1.35 (s, 3H, CH₃); 1.76–1.86 (m, 2H); 1.91 (m, 1H); 1.96 (m, 1H); 3.67 (m, 1H, CH₂O); 3.78–3.90 (m, 3H, CH₂O); 4.01 (m, 1H, H-2 β); 4.11–4.14 (m, 2H, H-22, H-23); 4.18 (m, 1H, H-3 β). HRMS: (API-) calculated for $\text{C}_{32}\text{H}_{49}\text{O}_8$ ($[\text{M} - \text{H}]^-$) 561.3427, found 561.3426.

N,N-dimethyl-(2*S*,22*R*,23*S*)-2 α ,3 α ,22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-amide (**19a**)

the general procedure for amide formation with acid **18** and dimethylamine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 191 mg (91%) of the title compound **19a** as a colorless oil:

^1H NMR (DMSO- d_6) δ 0.60 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.91 (d, 3H, $J = 6.7$ Hz, CH₃); 1.22 (s, 3H, CH₃); 1.23 (s, 3H, CH₃); 1.33 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.62–1.74 (m, 3H); 1.77–1.86 (m, 2H); 1.91 (td, 1H, $J = 11.9$ and 3.2 Hz); 1.97 (m, 1H); 2.83 (s, 3H, NCH₃); 3.04 (s, 3H, NCH₃); 3.67 (m, 1H, CH₂O); 3.81–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β); 4.33 (d, 1H, $J = 7.6$ Hz, H-23); 4.53 (dd, 1H, $J = 7.6$ and 1.1 Hz, H-22). ^{13}C NMR δ 11.54, 12.61, 13.12, 20.36, 21.42, 23.73, 25.98, 26.60, 26.70, 27.14, 28.62, 32.54, 35.07, 35.55, 36.48, 37.55, 40.65, 41.93, 42.40, 45.08, 52.09, 53.23, 55.03, 63.64, 65.14, 72.00, 72.22, 73.65, 78.97, 106.73, 108.61, 108.85, 168.43, 1 $\times$ C n. f. HRMS: (API+) calculated for $\text{C}_{34}\text{H}_{56}\text{NO}_7$ ($[\text{M} + \text{H}]^+$) 590.4057, found 590.4061.

N,N-diethyl-(2*S*,22*R*,23*S*)-2 α ,3 α ,22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-amide (**19b**)

the general procedure for amide formation with acid **18** and diethylamine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 193 mg (88%) of the title compound **19b** as a colorless oil:

^1H NMR (DMSO- d_6) δ 0.60 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.91 (d, 3H, $J = 6.7$ Hz, CH₃); 1.01 (t, 3H, $J = 7.0$ Hz, CH₃); 1.11 (t, 3H, $J = 7.0$ Hz, CH₃); 1.218 (s, 3H, CH₃); 1.221 (s, 3H, CH₃); 1.33 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.62–1.74 (m, 3H); 1.77–1.86 (m, 2H); 1.91 (td, 1H, $J = 11.9$ and 3.1 Hz); 1.97 (m, 1H); 3.18–3.41 (m, 4H, 2 $\times$ NCH₂); 3.67 (m, 1H, CH₂O); 3.81–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β); 4.27 (d, 1H, $J = 7.2$ Hz, H-23); 4.53 (dd, 1H, $J = 7.2$ and 1.2 Hz, H-22). ^{13}C NMR δ 11.59, 12.65, 12.71, 13.17, 14.39, 20.40, 21.46, 23.77, 25.84, 26.65, 26.72, 27.25, 28.66, 32.58, 35.92, 37.60, 39.04, 39.57, 40.68, 41.07, 41.98, 42.45, 45.12, 52.12, 53.23, 55.07, 63.69, 65.18, 72.04, 72.27, 74.04, 79.09, 106.78, 108.70, 108.88, 168.14. HRMS: (API+) calculated for $\text{C}_{36}\text{H}_{60}\text{NO}_7$ ($[\text{M} + \text{H}]^+$) 618.4370, found 618.4375.

1-((20*S*,22*R*,23*S*)-2 α ,3 α ;22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-oyl) pyrrolidine (**19c**)

the general procedure for amide formation with acid **18** and pyrrolidine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 195 mg (89%) of the title compound **19c** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.60 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 0.91 (d, 3H, *J* = 6.7 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.24 (s, 3H, CH₃); 1.32 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.62–1.72 (m, 3H); 1.73–1.88 (m, 6H); 1.91 (td, 1H, *J* = 11.8 and 3.1 Hz); 1.97 (m, 1H); 3.25–3.33 (m, 2H, NCH₂); 3.54 (t, 2H, *J* = 6.7 Hz, NCH₂); 3.67 (m, 1H, CH₂O); 3.81–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β); 4.24 (d, 1H, *J* = 7.4 Hz, H-23); 4.49 (dd, 1H, *J* = 7.4 and 1.1 Hz, H-22). ¹³C NMR δ 11.56, 12.62, 13.14, 20.38, 21.43, 23.46, 23.73, 25.64, 25.92, 26.61, 26.70, 27.19, 28.63, 32.55, 35.76, 37.56, 40.65, 41.94, 42.41, 45.09, 45.83, 45.94, 52.10, 53.18, 55.04, 63.65, 65.15, 72.01, 72.23, 75.15, 79.03, 106.74, 108.75, 108.86, 167.32, 1 $\times$ C n. f. HRMS: (API+) calculated for C₃₆H₅₈NO₇ ([M + H]⁺) 616.4213, found 616.4218.

1-((20*S*,22*R*,23*S*)-2 α ,3 α ;22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-oyl) piperidine (**19d**)

the general procedure for amide formation with acid **18** and piperidine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 188 mg (84%) of the title compound **19d** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.60 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.90 (d, 3H, *J* = 6.7 Hz, CH₃); 1.22 (s, 6H, 2 $\times$ CH₃); 1.33 (s, 3H, CH₃); 1.36 (s, 3H, CH₃); 1.62–1.74 (m, 3H); 1.77–1.86 (m, 2H); 1.91 (m, 1H); 1.97 (m, 1H); 3.41–3.60 (m, 4H, 2 $\times$ NCH₂); 3.67 (m, 1H, CH₂O); 3.81–3.90 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β); 4.32 (d, 1H, *J* = 7.3 Hz, H-23); 4.58 (dd, 1H, *J* = 7.3 and 0.9 Hz, H-22). ¹³C NMR δ 11.56, 12.65, 13.13, 20.38, 21.42, 23.74, 24.05, 25.35, 25.91, 26.12, 26.61, 26.69, 27.18, 28.62, 32.54, 35.85, 37.56, 40.65, 41.95, 42.41, 42.48, 45.09, 45.86, 52.11, 53.23, 55.04, 63.64, 65.14, 72.01, 72.23, 73.84, 78.89, 106.74, 108.60, 108.86, 167.02, 1 $\times$ C n. f. HRMS: (API+) calculated for C₃₇H₆₀NO₇ ([M + H]⁺) 630.4370, found 630.4370.

4-((20*S*,22*R*,23*S*)-2 α ,3 α ;22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-oyl) morpholine (**19e**)

the general procedure for amide formation with acid **18** and morpholine and chromatography on silica gel (40–60% ethyl acetate in cyclohexane) yielded 191 mg (85%) of the title compound **19e** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.62 (s, 3H, CH₃); 0.78 (s, 3H, CH₃); 0.92 (d, 3H, *J* = 6.7 Hz, CH₃); 1.23 (s, 3H, CH₃); 1.24 (s, 3H, CH₃); 1.34 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.63–1.75 (m, 3H); 1.78–1.87 (m, 2H); 1.92 (td, 1H, *J* = 11.7 and 2.9 Hz); 1.99 (m, 1H); 3.43–3.63 (m, 8H, 8 $\times$ H_{morph}); 3.68 (m, 1H, CH₂O); 3.81–3.91 (m, 3H, CH₂O); 4.02 (m, 1H, H-2 β); 4.20 (m, 1H, H-3 β); 4.36 (d, 1H, *J* = 7.3 Hz, H-23); 4.58 (dd, 1H, *J* = 7.3 and 1.2 Hz, H-22). ¹³C NMR δ 11.60, 12.65, 13.15, 20.39, 21.46, 23.77, 25.92, 26.64, 26.67, 27.20, 28.65, 32.58, 35.87, 37.59, 40.68, 41.99, 42.05, 42.44, 45.12, 45.66, 52.13, 53.24, 55.07, 63.68, 65.18, 66.09, 66.32, 72.04, 72.26, 73.69, 78.85, 106.78, 108.81, 108.88, 167.63, 1 $\times$ C n. f. HRMS: (API+) calculated for C₃₆H₅₈NO₈ ([M – Cl]⁺) 632.4162, found 632.4168.

General procedure for deprotection of long side chain amide ketals

A 5% aqueous solution of hydrochloric acid (1 mL) was added to a solution of ketals **19a–e** (20 mg) in tetrahydrofuran (5 mL) and the mixture was stirred at 45 °C for 2 h. After cooling, the mixture was diluted with ethyl acetate and extracted twice with water. The organic phase was dried with sodium sulfate and solvents were evaporated under reduced pressure. The crude product was purified on silica gel (mobile phase at each experiment).

N,N-dimethyl-1-((20*S*,22*R*,23*S*)-2 α ,3 α ;22,23-tetrahydroxy-6-oxo-5 α -cholan-24-amide (**20a**)

the general procedure for amide deprotection with ketal **19a** (50 mg; 0.08 mmol) and chromatography on silica gel (5–10% isopropanol in chloroform) yielded 14 mg (86%) of the title compound **20a** as an off-white solid:

Mp > 210 °C (decomp.). ¹H NMR (CD₃OD+CDCl₃) δ 0.65 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.99 (d, 3H, *J* = 6.7 Hz, CH₃); 1.75–1.82 (m, 2H); 1.95 (m, 1H); 1.99–2.05 (m, 2H); 2.23 (dd, 1H, *J* = 13.1 and 4.6 Hz); 2.68 (dd, 1H, *J* = 12.4 and 2.9 Hz); 2.95 (s, 3H, NCH₃); 3.08 (s, 3H, NCH₃); 3.64 (m, 1H, H-2β); 3.77 (dd, 1H, *J* = 5.7 and 1.2 Hz, H-22); 3.94 (m, 1H, H-3β); 4.32 (d, 1H, *J* = 5.7 Hz, H-23). ¹³C NMR δ 12.33, 13.45, 14.00, 21.90, 24.57, 27.14, 28.42, 36.41, 37.56, 38.67, 39.89, 40.14, 40.47, 43.38, 43.53, 47.30, 51.65, 53.34, 54.42, 57.31, 68.10, 68.90, 71.50, 73.91, 173.67, 214.81. HRMS: (API+) calculated for C₂₆H₄₄NO₆ ([M + H]⁺) 466.3169, found 466.3172. Anal. Calcd. for C₂₆H₄₃NO₆: C, 67.07; H, 9.31; N, 3.01. Found: C, 68.40; H, 10.23; N, 3.02%.

N,N-diethyl-(20*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-amide (**20b**)

the general procedure for amide deprotection with ketal **19b** (50 mg; 0.08 mmol) and chromatography on silica gel (5–10% isopropanol in chloroform) yielded 14 mg (88%) of the title compound **20b** as an off-white solid:

Mp > 160 °C (decomp.). ¹H NMR (CD₃OD+CDCl₃) δ 0.64 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.99 (d, 3H, *J* = 6.7 Hz, CH₃); 1.10 (t, 3H, *J* = 7.2 Hz, CH₃); 1.22 (t, 3H, *J* = 7.2 Hz, CH₃); 1.94 (m, 1H); 1.99–2.05 (m, 2H); 2.23 (dd, 1H, *J* = 13.1 and 4.6 Hz); 2.68 (dd, 1H, *J* = 12.5 and 2.8 Hz); 3.27 (m, 1H, NCH); 3.37–3.42 (m, 2H, 2×NCH); 3.47 (m, 1H, NCH); 3.64 (m, 1H, H-2β); 3.76 (d, 1H, *J* = 6.2 Hz, H-22); 3.94 (m, 1H, H-3β); 4.24 (d, 1H, *J* = 6.2 Hz, H-23). ¹³C NMR δ 12.27, 13.01, 13.61, 13.95, 14.78, 21.81, 24.49, 27.03, 28.29, 38.58, 39.21, 40.03, 40.38, 41.22, 42.50, 43.30, 43.46, 47.23, 51.56, 53.27, 54.32, 57.22, 68.57, 68.80, 71.21, 74.44, 172.76, 214.71. HRMS: (API+) calculated for C₂₈H₄₈NO₆ ([M + H]⁺) 494.3482, found 494.3481. Anal. Calcd. for C₂₈H₄₇NO₆: C, 68.12; H, 9.60; N, 2.84. Found: C, 68.06; H, 9.66; N, 2.80%.

1-((20*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-oyl)pyrrolidine (**20c**)

the general procedure for amide deprotection with ketal **19c** (50 mg; 0.08 mmol) and chromatography on silica gel (5–10% isopropanol in chloroform) yielded 13 mg (83%) of the title compound **20c** as an off-white solid:

Mp > 200 °C (decomp.). ¹H NMR (CD₃OD+CDCl₃) δ 0.69 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 1.00 (d, 3H, *J* = 6.7 Hz, CH₃); 1.84–2.07 (m, 6H); 2.10 (bt, 1H, *J* = 12.5 Hz); 2.22 (dd, 1H, *J* = 12.8 and 4.8 Hz); 2.78 (dd, 1H, *J* = 12.5 and 3.1 Hz); 3.38–3.48 (m, 2H, 2×NCH); 3.56 (m, 1H, NCH); 3.64–3.71 (m, 2H, H-2β, NCH); 3.83 (d, 1H, *J* = 7.1 Hz, H-22); 3.95 (m, 1H, H-3β); 4.22 (d, 1H, *J* = 7.1 Hz, H-23). ¹³C NMR δ 12.40, 13.93, 14.02, 22.48, 25.04 (2×C); 27.23, 27.97, 28.81, 39.31, 39.55, 40.95, 41.11, 43.76, 44.07, 47.45, 47.63, 47.98, 52.21, 54.12, 55.16, 58.01, 69.26, 69.90, 74.01, 74.82, 173.02, 215.17. HRMS: (API+) calculated for C₂₈H₄₆NO₆ ([M + H]⁺) 492.3325, found 492.3325. Anal. Calcd. for C₂₈H₄₅NO₆: C, 68.40; H, 9.23; N, 2.85. Found: C, 68.35; H, 9.30; N, 2.81%.

1-((20*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-oyl)piperidine (**20d**)

the general procedure for amide deprotection with ketal **19d** (50 mg; 0.08 mmol) and chromatography on silica gel (5–10% isopropanol in chloroform) yielded 14 mg (85%) of the title compound **20d** as an off-white solid:

Mp > 200 °C (decomp.). ¹H NMR (DMSO-*d*₆) δ 0.58 (s, 3H, CH₃); 0.63 (s, 3H, CH₃); 0.87 (d, 3H, *J* = 6.5 Hz, CH₃); 1.80–1.86 (m, 2H); 1.94 (m, 1H); 2.03 (dd, 1H, *J* = 13.0 and 4.7 Hz); 2.1 (m, 1H); 2.60 (dd, 1H, *J* = 12.1 and 3.2 Hz); 3.34–3.52 (m, 5H, H-2β, 2×NCH₂); 3.60 (d, 1H, *J* = 6.8 Hz, H-22); 3.75 (bs, 1H, H-3β); 4.16–4.21 (m, 2H, H-23, OH); 4.34 (bd, 1H, *J* = 5.9 Hz, OH); 4.37 (bd, 1H, *J* = 4.6 Hz, OH); 4.94 (bd, 1H, *J* = 4.9 Hz, OH). ¹³C NMR δ 11.73, 13.00, 13.37, 20.82, 23.48, 24.08, 25.45, 26.20, 26.85, 27.21, 29.03, 37.08, 37.90, 41.85, 42.21, 42.40, 45.69, 46.01, 50.33, 52.23, 52.88, 55.98, 67.11, 67.49, 71.47, 72.48, 170.11, 211.61, 1×C n. f. HRMS: (API+) calculated for C₂₉H₄₈NO₆ ([M + H]⁺) 506.3482, found 506.3479. Anal. Calcd. for C₂₉H₄₇NO₆: C, 68.88; H, 9.37; N, 2.77. Found: C, 68.82; H, 9.42; N, 2.75%.

4-((20S,22R,23S)-2 α ,3 α ,22,23-tetrahydroxy-6-oxo-5 α -cholan-24-oyl)morpholine (**20e**)

the general procedure for amide deprotection with ketal **19e** (50 mg; 0.08 mmol) and chromatography on silica gel (5–10% isopropanol in chloroform) yielded 14 mg (87%) of the title compound **20e** as an off-white solid:

Mp > 210 °C (decomp.). ¹H NMR (CD₃OD+CDCl₃) δ 0.66 (s, 3H, CH₃); 0.74 (s, 3H, CH₃); 0.98 (d, 3H, J = 6.7 Hz, CH₃); 1.76–1.81 (m, 2H); 1.96 (m, 1H); 2.00–2.06 (m, 2H); 2.23 (dd, 1H, J = 13.0 and 4.6 Hz); 2.69 (dd, 1H, J = 12.4 and 2.9 Hz); 3.56–3.69 (m, 9H, H-2 β , 8 $\times$ H_{morph}); 3.78 (dd, 1H, J = 6.2 and 0.9 Hz, H-22); 3.95 (m, 1H, H-3 β); 4.30 (d, 1H, J = 6.2 Hz, H-23). ¹³C NMR δ 12.42, 13.42, 14.01, 21.95, 24.62, 27.20, 28.49, 30.39, 38.72, 39.45, 40.20, 40.51, 43.41, 43.46, 43.59, 48.95, 47.33, 51.70, 53.35, 54.47, 57.37, 67.48 (2 $\times$ C), 68.69, 68.94, 72.08, 74.14, 172.27, 214.87. HRMS: (API+) calculated for C₂₈H₄₆NO₇ ([M + H]⁺) 508.3274, found 508.3272. Anal. Calcd. for C₂₈H₄₅NO₇: C, 66.25; H, 8.93; N, 2.76. Found: C, 66.20; H, 8.99; N, 2.73%.

4-((20S,22R,23S)-2 α ,3 α -dihydroxy-22,23-(1-methylethylidene)dioxy-6-oxo-5 α -cholan-24-oyl)morpholine (**23**)

the general procedure for amide deprotection (at room temperature) with ketal **19e** (50 mg; 0.08 mmol) and chromatography on silica gel (isopropanol/ethyl acetate—1/19) yielded 31 mg (72%) of the title compound **23** as a colorless oil. the product was obtained during optimization of the reaction condition.

¹H NMR (DMSO-*d*₆, 45 °C) δ 0.63 (s, 3H, CH₃); 0.65 (s, 3H, CH₃); 0.93 (d, 3H, J = 6.7 Hz, CH₃); 1.25 (s, 3H, CH₃); 1.34 (s, 3H, CH₃); 1.68 (qd, 1H, J = 10.9 and 5.7 Hz); 1.87 (m, 1H); 1.97 (m, 1H); 2.03–2.11 (m, 2H); 2.61 (dd, 1H, J = 12.4 and 3.5 Hz); 3.44–3.64 (m, 9H, H-2 β , 8 $\times$ H_{morph}); 3.77 (m, 1H, H-3 β); 4.06 (bd, 1H, J = 2.4 Hz, OH); 4.21 (bd, 1H, J = 6.1 Hz, OH); 4.37 (d, 1H, J = 7.2 Hz, H-23); 4.59 (dd, 1H, J = 7.2 and 1.4 Hz, H-22). ¹³C NMR δ 11.61, 12.63, 13.35, 20.82, 23.39, 25.92, 26.63, 26.83, 27.09, 31.31, 35.93, 37.09, 38.91, 41.83, 42.06, 42.45, 45.65, 46.02, 50.34, 52.88, 53.08, 55.72, 66.08, 66.29, 66.98, 67.51, 73.77, 78.85, 108.82, 167.65, 211.50. HRMS: (API+) calculated for C₃₁H₅₀NO₇ ([M + H]⁺) 548.3587, found 548.3586.

N,N-dimethyl-((20S,22R,23S)-2 α ,3 α ;22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-yl)amine (**21a**)

the general procedure for amide reduction with amide **19a** (150 mg; 0.25 mmol) and chromatography on silica gel (0–5% methanol in ethyl acetate) yielded 114 mg (78%) of the title compound **21a** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.59 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.87 (d, 3H, J = 6.5 Hz, CH₃); 1.18 (s, 3H, CH₃); 1.22 (s, 3H, CH₃); 1.23 (s, 3H, CH₃); 1.32 (s, 3H, CH₃); 1.58–1.70 (m, 3H); 1.74–1.82 (m, 2H); 1.86–1.94 (m, 2H); 2.13 (s, 6H, 2 $\times$ NCH₃); 2.24 (dd, 1H, J = 12.9 and 6.7 Hz, H-24a); 2.34 (dd, 1H, J = 12.9 and 3.4 Hz, H-24b); 3.61–3.66 (m, 2H, CH₂O, H-22); 3.72–3.87 (m, 4H, 3 $\times$ CH₂O, H-23); 3.99 (m, 1H, H-2 β); 4.15 (m, 1H, H-3 β). ¹³C NMR δ 11.45, 12.53, 13.04, 20.29, 21.34, 23.62, 26.46, 26.93, 26.96, 27.25, 28.47, 32.42, 35.14, 37.47, 40.53, 41.89, 42.32, 44.96, 45.26 (b, 2 $\times$ C), 52.18, 52.92, 55.12, 61.27, 63.55, 65.03, 71.99, 72.19, 74.76 (b), 80.64, 106.63, 107.74, 108.81, 1 $\times$ C n. f. HRMS: (API+) calculated for C₃₄H₅₈NO₆ ([M + H]⁺) 576.4264, found 576.4369.

N,N-diethyl-((20S,22R,23S)-2 α ,3 α ;22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5 α -cholan-24-yl)amine (**21b**)

the general procedure for amide reduction with amide **19b** (150 mg; 0.24 mmol) and chromatography on silica gel (0–5% methanol in ethyl acetate) yielded 110 mg (75%) of the title compound **21b** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.62 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 0.90 (d, 3H, J = 6.7 Hz, CH₃); 0.92–0.99 (m, 6H, 2 $\times$ CH₃); 1.22 (s, 3H, CH₃); 1.27 (s, 6H, 2 $\times$ CH₃); 1.36 (s, 3H, CH₃); 1.60–1.74 (m, 4H); 1.77–1.85 (m, 2H); 1.90–1.98 (m, 2H); 2.34–2.67 (vb m, 6H, 6 $\times$ NCH₃); 3.67 (m, 1H, CH₂O); 3.72 (d, 1H, J = 8.3 Hz, H-22); 3.80–3.86 (m, 3H, 2 $\times$ CH₂O, H-23); 3.89 (m, 1H, CH₂O); 4.02 (m, 1H, H-2 β); 4.19 (m, 1H, H-3 β). ¹³C NMR δ 11.53, 11.74 (vb, 2 $\times$ C),

12.64, 13.15, 20.40, 21.44, 23.70, 26.62, 27.04, 27.14, 27.19, 28.64, 32.58, 35.22, 37.56, 39.11, 40.65, 41.95, 42.42, 45.11, 47.07, 52.14, 53.18, 55.09, 55.67 (vb), 63.67, 65.15, 72.01, 72.23, 74.84 (vb) 81.34 (vb), 106.75, 107.32 (vb), 108.87, 1×C n. f. HRMS: (API+) calculated for C₃₆H₆₂NO₆ ([M + H]⁺) 604.4577, found 604.4579.

1-((20S,22R,23S)-2α,3α,22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5α-cholan-24-yl) pyrrolidine (**21c**)

the general procedure for amide reduction with amide **19c** (150 mg; 0.24 mmol) and chromatography on silica gel (0–5% methanol in ethyl acetate) yielded 113 mg (77%) of the title compound **21c** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.62 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 0.91 (d, 3H, *J* = 6.7 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.28 (s, 6H, 2×CH₃); 1.35 (s, 3H, CH₃); 1.62–1.74 (m, 7H); 1.76–1.84 (m, 2H); 1.89–1.99 (m, 2H); 2.55–2.71 (vb m, 6H, 6×NCH); 3.65–3.69 (m, 2H, CH₂O, H-22); 3.80–3.90 (m, 4H, 3×CH₂O, H-23); 4.01 (m, 1H, H-2β); 4.19 (m, 1H, H-3β). ¹³C NMR δ 11.51, 12.70, 13.14, 20.38, 21.43, 23.01 (2×C); 23.70, 26.61, 27.04, 27.14, 27.27, 28.63, 32.55, 35.17, 37.56, 40.65, 41.95, 42.42, 45.10, 52.11, 53.11, 54.13 (2×C), 55.07, 63.66, 65.15, 72.00, 72.23, 75.46, 80.77, 106.74, 107.82 (vb), 108.86, 2×C n. f. HRMS: (API+) calculated for C₃₆H₆₀NO₆ ([M + H]⁺) 602.4421, found 602.4426.

1-((20S,22R,23S)-2α,3α,22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5α-cholan-24-yl) piperidine (**21d**)

the general procedure for amide reduction with amide **19d** (150 mg; 0.24 mmol) and chromatography on silica gel (0–5% methanol in ethyl acetate) yielded 106 mg (72%) of the title compound **21d** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.65 (s, 3H, CH₃); 0.78 (s, 3H, CH₃); 0.91 (d, 3H, *J* = 6.7 Hz, CH₃); 1.23 (s, 3H, CH₃); 1.268 (s, 3H, CH₃); 1.274 (s, 3H, CH₃); 1.37 (s, 3H, CH₃); 1.61–1.75 (m, 4H); 1.79–1.87 (m, 2H); 1.91–2.01 (m, 2H); 2.33–2.50 (vb m, 6H, 6×NCH); 3.67–3.72 (m, 2H, CH₂O, H-22); 3.77–3.91 (m, 4H, 3×CH₂O, H-23); 4.02 (m, 1H, H-2β); 4.20 (m, 1H, H-3β). ¹³C NMR δ 11.55, 12.74, 13.15, 20.39, 21.43, 23.75, 25.50 (2×C), 26.62, 27.10, 27.18, 27.26, 28.63, 32.57, 35.20, 37.56, 40.65, 41.98, 42.43, 45.11, 52.13, 53.20, 54.73 (b, 2×C), 55.10, 61.34 (vb), 63.67, 65.14, 72.02, 72.24, 74.80 (vb), 81.34 (b), 106.75, 107.52, 108.87, 1×C n. f. HRMS: (API+) calculated for C₃₇H₆₂NO₆ ([M + H]⁺) 616.4577, found 616.4578.

4-((20S,22R,23S)-2α,3α,22,23-bis((1-methylethylidene)dioxy)-6,6-ethylenedioxy-5α-cholan-24-yl) morpholine (**21e**)

the general procedure for amide reduction with amide **19e** (150 mg; 0.24 mmol) and chromatography on silica gel (0–5% methanol in ethyl acetate) yielded 109 mg (74%) of the title compound **21e** as a colorless oil:

¹H NMR (DMSO-*d*₆) δ 0.64 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 0.90 (d, 3H, *J* = 6.7 Hz, CH₃); 1.22 (s, 3H, CH₃); 1.26 (s, 6H, 2×CH₃); 1.36 (s, 3H, CH₃); 1.62–1.74 (m, 3H); 1.78–1.85 (m, 2H); 1.90–1.99 (m, 2H); 2.37–2.45 (m, 6H, 6×NCH); 3.52–3.55 (m, 4H, 4×CH₂O morph); 3.65–3.70 (m, 2H, CH₂O, H-22); 3.80–3.91 (m, 4H, 3×CH₂O, H-23); 4.02 (m, 1H, H-2β); 4.19 (m, 1H, H-3β). ¹³C NMR δ 11.55, 12.75, 13.15, 20.39, 21.44, 23.74, 26.62, 27.12, 27.18, 27.32, 28.64, 32.57, 35.22, 37.57, 40.65, 41.98, 42.42, 45.11, 52.13, 53.21, 54.04 (2×C), 55.10, 60.99, 63.67, 65.16, 66.23 (2×C), 72.02, 72.24, 75.05, 81.06, 106.75, 107.58, 108.87, 1×C n. f. HRMS: (API+) calculated for C₃₆H₆₀NO₇ ([M + H]⁺) 618.4370, found 618.4376.

General procedure for deprotection of long side chain amine ketals

A 5% aqueous solution of hydrochloric acid (1 mL) was added to a solution of ketals **21a–e** (20 mg; 0.03 mmol) in tetrahydrofuran (5 mL) and the mixture was stirred at 45 °C for 2 h. During the reaction, white crystals appeared. After cooling, solvents were evaporated under reduced pressure and the crude product was washed twice with ethyl acetate. the prepared salts obtained in quantitative yield had sufficient purity.

N,N-dimethyl-((20S,22R,23S)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-yl)aminium chloride (**22a**)

Mp > 270 °C (decomp.). ¹H NMR (CD₃OD+D₂O) δ 0.62 (s, 3H, CH₃); 0.67 (s, 3H, CH₃); 0.87 (d, 3H, J = 6.4 Hz, CH₃); 1.85 (m, 1H); 1.95 (m, 1H); 2.07–2.16 (m, 2H); 2.65 (dd, 1H, J = 11.9 and 2.8 Hz); 3.00 (bt, 1H, J = 12.5 Hz, H-24a); 3.10 (dd, 1H, J = 12.5 and 2.9 Hz, H-24b); 3.38 (bd, 1H, J = 7.0 Hz, H-22); 3.64 (m, 1H, H-2β); 3.84 (ddd, 1H, J = 10.7, 7.5, and 2.9 Hz, H-23); 3.92 (m, 1H, H-3β). ¹³C NMR δ 12.30, 12.89, 13.90, 22.17, 24.69, 27.45, 28.47, 39.00, 39.31, 40.46 (2×C), 42.01, 43.82, 44.00, 45.89, 47.39, 52.10, 53.22, 54.56, 57.44, 60.28, 68.34, 69.00, 69.33, 75.87, 218.18. HRMS: (API+) calculated for C₂₆H₄₆NO₅ ([M – Cl]⁺) 452.3376, found 452.3377. Anal. Calcd. for C₂₆H₄₆ClNO₅: C, 63.98; H, 9.50; N, 2.87. Found: C, 63.95; H, 9.55; N, 2.85%.

N,N-diethyl-((2*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-yl)aminium chloride (**22b**)

Mp > 250 °C (decomp.). ¹H NMR (CD₃OD) δ 0.75 (s, 3H, CH₃); 0.77 (s, 3H, CH₃); 1.00 (d, 3H, J = 6.7 Hz, CH₃); 1.33 (t, 3H, J = 7.3 Hz, CH₃); 1.34 (t, 3H, J = 7.3 Hz, CH₃); 1.99 (m, 1H); 2.05–2.14 (m, 2H); 2.22 (dd, 1H, J = 13.1 and 4.9 Hz); 2.73 (dd, 1H, J = 12.4 and 3.2 Hz); 3.05 (dd, 1H, J = 13.2 and 11.0 Hz, H-24a); 3.17 (dd, 1H, J = 13.2 and 3.2 Hz, H-24b); 3.21–3.37 (m, 4H, 4×NCH); 3.44 (dd, 1H, J = 6.7 and 1.4 Hz, H-22); 3.66 (ddd, 1H, J = 11.8, 4.8, and 3.2 Hz, H-2β); 3.87 (ddd, 1H, J = 11.1, 6.7, and 3.1 Hz, H-23); 3.95 (m, 1H, H-3β). ¹³C NMR δ 8.90, 9.39, 12.50, 13.20, 14.02, 22.51, 25.05, 27.99, 28.78, 39.33, 39.78, 41.04, 41.12, 43.76, 44.11, 47.61, 47.87, 50.30, 52.23, 53.72, 55.16, 55.35, 58.01, 68.59, 69.26, 69.63, 75.77, 215.17. HRMS: (API+) calculated for C₂₈H₅₀NO₅ ([M – Cl]⁺) 480.3689, found 480.3689. Anal. Calcd. for C₂₈H₅₀ClNO₅: C, 65.15; H, 9.76; N, 2.71. Found: C, 65.09; H, 9.81; N, 2.68%.

1-((2*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-yl)pyrrolidinium chloride (**22c**)

Mp > 250 °C (decomp.). ¹H NMR (CD₃OD+D₂O) δ 0.63 (s, 3H, CH₃); 0.67 (s, 3H, CH₃); 0.88 (d, 3H, J = 6.4 Hz, CH₃); 1.86 (m, 1H); 1.93–1.99 (m, 3H); 2.05–2.16 (m, 3H); 2.66 (dd, 1H, J = 12.4 and 3.0 Hz); 2.97–3.15 (m, 4H, 4×NCH); 3.39 (bd, 1H, J = 7.0 Hz, H-22); 3.58–3.66 (m, 3H, 2×NCH, H-2β); 3.81 (m, H-23); 3.92 (m, 1H, H-3β). ¹³C NMR δ 12.32, 12.95, 13.90, 22.19, 23.78, 23.96, 24.72, 27.49, 28.51, 39.13, 39.31, 40.52, 43.85, 43.98, 47.39, 49.78, 52.12, 53.30, 54.08, 54.61, 56.83, 57.50, 58.04, 69.03, 69.37, 69.73, 75.79, 217.93. HRMS: (API+) calculated for C₂₈H₄₈NO₅ ([M – Cl]⁺) 478.3532, found 478.3535. Anal. Calcd. for C₂₈H₄₈ClNO₅: C, 65.41; H, 9.41; N, 2.72. Found: C, 65.36; H, 9.48; N, 2.69%.

1-((2*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-yl)piperidinium chloride (**22d**)

Mp > 250 °C (decomp.). ¹H NMR (CD₃OD) δ 0.74 (s, 3H, CH₃); 0.76 (s, 3H, CH₃); 0.99 (d, 3H, J = 6.7 Hz, CH₃); 1.93–2.02 (m, 2H); 2.05–2.14 (m, 2H); 2.20 (dd, 1H, J = 13.4 and 4.8 Hz); 2.73 (dd, 1H, J = 12.2 and 3.1 Hz); 2.92 (td, 1H, J = 12.0 and 2.9 Hz, NCH); 3.01 (dd, 1H, J = 12.9 and 11.5 Hz, H-24a); 3.05 (td, 1H, J = 12.0 and 4.4 Hz, NCH); 3.15 (dd, 1H, J = 12.9 and 2.4 Hz, H-24b); 3.41 (dd, 1H, J = 6.9 and 1.4 Hz, H-22); 3.49 (bd, 1H, J = 11.9 Hz, NCH); 3.62–3.68 (m, 2H, NCH, H-2β); 3.92 (m, 1H, H-23); 3.95 (m, 1H, H-3β). ¹³C NMR δ 12.48, 13.05, 14.01, 22.50, 22.89, 24.03, 24.08, 25.03, 27.99, 28.80, 39.32, 39.68, 41.03, 41.12, 43.76, 44.11, 47.60, 52.24, 52.74, 53.64, 55.16, 56.36, 57.96, 60.09, 67.88, 69.26, 69.62, 75.86, 215.24. HRMS: (API+) calculated for C₂₈H₄₈NO₆ ([M – Cl]⁺) 494.3482, found 494.3485. Anal. Calcd. for C₂₈H₅₀ClNO₅: C, 65.95; H, 9.54; N, 2.65. Found: C, 65.89; H, 9.60; N, 2.63%.

4-((2*S*,22*R*,23*S*)-2α,3α,22,23-tetrahydroxy-6-oxo-5α-cholan-24-yl)morpholinium chloride (**22e**)

Mp > 270 °C (decomp.). ¹H NMR (CD₃OD+D₂O) δ 0.59 (s, 3H, CH₃); 0.64 (s, 3H, CH₃); 0.84 (d, 3H, J = 6.7 Hz, CH₃); 1.71 (qd, 1H, J = 10.3 and 7.2 Hz); 1.79 (m, 1H); 1.92 (m, 1H); 2.07–2.13 (m, 2H); 2.63 (dd, 1H, J = 11.6 and 4.3 Hz); 3.03 (dd, 1H, J = 13.0 and 11.3 Hz, H-24a); 3.08 (m, 1H, NCH); 3.14 (dd, 1H, J = 13.0 and 3.1 Hz, H-24b); 3.18 (m, 1H, NCH); 3.38 (dd, 1H, J = 6.9 and 1.2 Hz, H-22); 3.39 (m, 1H, NCH); 3.52 (bd, 1H, J = 12.2 Hz, NCH); 3.64 (m, 1H, H-2β); 3.73–3.81 (m, 2H, CH-O); 3.89–3.94 (m, 2H, H-3β, H-23); 3.96–4.02 (m,

2H, CH-O). ^{13}C NMR δ 12.25, 12.84, 13.85, 22.05, 24.56, 27.31, 28.37, 38.79, 39.28, 40.27, 40.30, 43.73, 44.09, 47.32, 51.57, 52.05, 53.11, 54.37, 54.53, 57.26, 59.87, 64.66 ($2\times\text{C}$), 67.44, 68.90, 69.26, 75.89, 219.27. HRMS: (API+) calculated for $\text{C}_{28}\text{H}_{48}\text{NO}_6$ ($[\text{M} - \text{Cl}]^+$) 494.3482, found 494.3482. Anal. Calcd. for $\text{C}_{28}\text{H}_{48}\text{ClNO}_6$: C, 63.44; H, 9.13; N, 2.64. Found: C, 63.40; H, 9.18; N, 2.61%.

4-((20S,22R,23S)-2 α ,3 α -dihydroxy-22,23-(1-methylethylidene)dioxy-6-oxo-5 α -cholan-24-yl) morpholinium chloride (**24**)

the product was obtained during optimization of the reaction condition (deprotection of long side chain amine ketals at room temperature) as white crystals.

Mp > 210 °C (decomp.). ^1H NMR (D_2O) δ 0.63 (s, 3H, CH_3); 0.70 (s, 3H, CH_3); 0.95 (d, 3H, $J = 6.7$ Hz, CH_3); 1.37 (s, 3H, CH_3); 1.38 (s, 3H, CH_3); 1.77–1.90 (m, 2H); 1.98 (m, 1H); 2.20 (d, 2H, $J = 8.3$ Hz); 2.69 (dd, 1H, $J = 11.2$ and 5.0 Hz); 3.10–3.63 (vb, 4H, $4\times\text{NCH}$); 3.30 (dd, 1H, $J = 13.6$ and 1.9 Hz, H-24a); 3.35 (dd, 1H, $J = 13.6$ and 9.5 Hz, H-24b); 3.73 (m, 1H, H-2 β); 3.78–4.13 (vb, 4H, $4\times\text{CH-O}$); 3.94 (d, 1H, $J = 8.3$ Hz, H-22); 3.98 (m, 1H, H-3 β); 4.22 (m, 1H, H-23). ^{13}C NMR δ 10.91, 12.09, 12.72, 20.82, 23.32, 25.93, 25.94, 26.02, 27.21, 35.11, 38.17, 38.85, 38.92, 42.75, 43.09, 46.14, 50.88, 52.77 ($2\times\text{C}$), 53.01 ($2\times\text{C}$), 55.78, 59.34, 63.64 ($2\times\text{C}$), 67.74, 68.12, 71.64, 81.16, 110.75, 219.66. HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{52}\text{NO}_6$ ($[\text{M} - \text{Cl}]^+$) 534.3795, found 534.3796.

3.3. Plant Bioassay

the biological activity of the new BRs derivatives was tested by an Arabidopsis sensitivity bioassay which was performed according to [28]. Briefly, seedlings of *Arabidopsis thaliana* L. (Columbia ecotype, Col-0) were stratified for 2 days at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar at 22 °C in a 16 h/8 h light–dark cycle. the plates contained concentrations of 24-epiBL (as positive control) and BR derivatives ranging from 0.1 to 10 nM, respectively 100 nM (for selected derivatives only). After 7 days, roots were straightened on solid media plates and scanned with an Epson high-resolution scanner. the entire root length was measured with ImageJ (<http://rsbweb.nih.gov/ij/>). For each treatment, more than 15 seedlings were analyzed in two biological repeats, and p-values were calculated with the two-tailed Student t-test using Excel software.

3.4. Cell Cultures and Cytotoxicity Assay

the screening cell lines: T-lymphoblastic leukemia CEM; breast carcinoma MCF7 (estrogen-sensitive); cervical carcinoma cell line HeLa; and human foreskin fibroblasts BJ, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Sigma, St. Louis, MO, USA). Media used were supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1% penicillin–streptomycin. the cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

Cells were subcultured twice or three times a week using the standard trypsinization procedure. Cytotoxicity in cancer cell lines after 72 h was determined as described earlier [43]. Control cultures were treated with DMSO alone, and the final concentration of DMSO in the mixture never exceeded 0.6%. Six serial 3-fold dilutions of the test substances were added at time zero in 20 μL aliquots to the microtiter plate wells and the highest final concentration in the wells was 50 μM .

3.5. Molecular Docking

We used AutoDock Vina 1.05 [44] in this study of Brassinosteroid receptor BRI1 (PDBID: 3RGZ). Datasets of 3D structures of brassinolide derivatives were prepared using Marvin 15.1.5.0, a software used for the drawing, displaying and characterization of chemical structures, substructures and reactions. AutoDock Tools [45] (ADT) was used for preparation of input data for molecular docking.

4. Conclusions

In this study, 25 brassinosteroid analogues with a nitrogen-containing side chain were synthesized based on molecular docking and tested using an Arabidopsis sensitivity bioassay and Calcein AM cytotoxicity assay. New analogues were divided into two groups—short side chain BRs and long side chain BRs. As a nitrogen-containing functional group, tertiary amides, tertiary amines and ammonium chlorides were prepared. The synthesized substances showed no significant inhibitory activity compared to natural 24-epibrassinolide. In contrast, several compounds showed interesting growth-promoting activity. The cytotoxicity assay showed no toxicity of the prepared compounds on cancer or normal cell lines. Unfortunately, the results of the molecular docking, which proved the good binding affinity of the tested compounds to the BRI1 receptor, were not confirmed by plant experiments. However, the very low or no biological activity of nitrogen derivatives can also be caused by different reasons other than low affinity to the receptor, e.g., by an unknown mechanism that prevents transport of these nitrogen compounds to the receptor. Based on these observations, it is possible to state that the presence of an amide or amine in the side chain of BRs analogues significantly reduces the biological activity in plants.

Supplementary Materials: Supplementary materials can be found at <https://www.mdpi.com/1422-0067/22/1/155/s1>. The following are available online. Table S1: Resulting binding free energies obtained for molecular docking experiments. Figures S1–S20: Poses of brassinolide and new nitrogen analogues within the BRI1 binding site. Figure S21: Plant bioassay with concentration of 100 nmol/L. Table S2: Data for Figure 2, Figure 3 and Figure S21. Table S3: Cytotoxicity data. NMR/HR-MS spectra of all new compounds.

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Příloha 4

Analytical Methods for Brassinosteroid Analysis: Recent Advances and Applications

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Analytical Methods for Brassinosteroid Analysis: Recent Advances and Applications

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Brassinosteroids (BRs) are plant steroidal hormones that play crucial roles in plant growth and development. Accurate quantification of BRs in plant tissues is essential for understanding their biological functions. This study presents a comprehensive overview of the latest methods used for the quantification of BRs in plants. We discuss the principles, advantages and limitations of various analytical techniques, including immunoassays, gas chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry that are used for the detection and quantification of BRs from complex plant matrixes. We also explore the use of isotopically labeled internal standards to improve the accuracy and reliability of BR quantification.

Keywords: Brassinosteroids • Chemical synthesis
• Chromatography • Immunoassays • Mass spectrometry
• Quantification

Introduction

Brassinosteroids (BRs) are a class of polyhydroxysteroids that have been recognized as a sixth class of plant hormones. They are structurally related to animal and insect steroid hormones. BRs were first explored during the 1970s when Mitchell with coworkers reported promotion in stem elongation and cell division by the treatment of organic extracts of rapeseed (*Brassica napus* L.) pollen (Mitchell et al. 1970). Brassinolide (BL) was the first BR to be isolated in 1979 (Grove et al. 1979). Since its discovery, over 70 BR compounds have been isolated from plants.

BRs have been found in all plant organs, including pollen, anthers, seeds, leaves, stems, roots, flowers and grains. They have also been detected in insect and crown galls. In general, pollen and immature seeds are particularly rich sources of BRs (1–100 ng/g fresh weight), while concentrations in vegetative tissues are very low (about 0.1 ng/g fresh weight) compared to those of other plant hormones (Bajguz and Tretyn 2003). All natural BRs are characterized by their 5 α -cholestane skeleton, and their structural differences are due to the type and

orientation of the oxygen functions on rings A and B (Fig. 1A). BRs are divided into 7-oxalactone, 6-oxo and 6-deoxo according to the arrangement on ring B. They can be further classified as C27, C28 or C29 BRs depending on the alkyl substitution at C-24 in the side chain. Furthermore, their conjugates with sugars or fatty acids have also been identified in plants (Bajguz 2011).

The structural diversity of BRs can affect their behavior in different purification and quantification methods. For instance, sometimes 6-oxo BRs show activity similar to 7-oxalactone compounds, while non-oxidized BRs exhibit essentially little activity in certain bioassays (Manghwar et al. 2022). This implies that the chemical structure of BRs can influence their detectability in bioassays, which are often used for their quantification (Kanwar et al. 2017). Moreover, the presence of conjugates and the variability in the alkyl substitution on the side chain can complicate the purification process, as these structural differences can affect the solubility and other physical and chemical properties of BRs. Therefore, the chemical structure of BRs plays a crucial role in their purification and quantification from plant material (Hou et al. 2017). If complications arise when using BR conjugates, a viable alternative is to replace them with deuterium-labeled BRs. The introduction of deuterium into the BR structure offers a significant advantage: unlike conjugates, this modification leaves the physical properties essential for liquid chromatography–mass spectrometry (LC–MS) analysis unchanged.

This review presents a detailed discussion of the analytical methods used for BR extraction, purification and quantification. It describes the principles, advantages, limitations, recent advances and applications of various techniques, including chromatographic, spectrometric and immunochemical methods.

Immunochemical Methods

BRs are crucial for plant growth and development, but their identification and quantification in plant tissues can be a time-consuming and laborious process. Traditional chromatographic methods of the BR analysis can be lengthy and labor-intensive

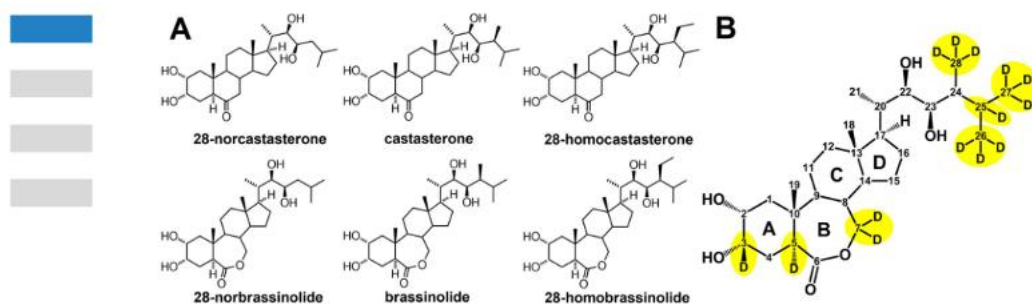


Fig. 1 Structures of brassinosteroids. (A) Examples of natural brassinosteroids with 27, 28 and 29 carbons. (B) Structure of brassinolide with marked positions into which deuterium atoms have been introduced.

and require sophisticated equipment. From the historical point of view, immunoassays provide a good alternative due to the simplicity of the equipment and measuring procedure (Khripach et al. 2011). The immunochemical approach involves the use of antibodies that can specifically bind to BRs, allowing their detection and quantification (Khripach et al. 2011).

Polyclonal antibody-based methods

Polyclonal antibodies are a heterogeneous mixture of antibodies that recognize different epitopes on the same antigen. They are produced by injecting an antigen into an animal, which then produces a series of antibodies against the antigen. Yokota et al. (1990) elucidated a robust radioimmunoassay (RIA) method for the quantification of BRs. To generate an antiserum against castasterone (CS), a rabbit was immunized with CS carboxymethylloxime conjugated to bovine serum albumin. The assay demonstrated detection limits for CS and BL of ~0.3 pmol, exceeding the sensitivity of the rice bioassay. The challenge in achieving increased sensitivity is the increased background signal due to nonspecific binding of BRs to the test tubes. Investigation of cross-reactivity with 27 compounds, including both natural and synthetic BRs, demonstrated the specificity of the antiserum in recognizing different functional groups. Despite a notable exception where BRs with a 24-methylene group showed low cross-reactivity, it is noteworthy that the functional recognition observed in the RIA is consistent with the potent biological activities established by structure–activity relationship studies. This RIA system proved to be effective in the analysis of endogenous BRs in seeds and stems of *Phaseolus vulgaris* (Yokota et al. 1990). In addition, the antiserum derived from this study was instrumental in localizing BRs in pollen tissue by immunocytochemistry (Taylor et al. 1993). Swaczynová et al. (2007) subsequently improved the enzyme-linked immunosorbent assay (ELISA) method by using selective polyclonal antibodies against 24-epicastasterone (24-epiCS). This polyclonal antibody was able to bind to BL and 24-epibrassinolide (24-epiBL), but did not react with other plant sterols. The ELISA method was used to determine BRs in the tissues of *B. napus* and

Arabidopsis thaliana. In addition, good agreement was found between the results obtained by the ELISA method and the high performance liquid chromatography–mass spectrometry (HPLC–MS) approach developed simultaneously (Swaczynová et al. 2007).

In recent years, three ELISA tests based on polyclonal antibodies have been developed. First, an immunochemical system for the determination of 28-homobrassinolide and 28-homocastasterone was showed by Khripach et al. (2008a). In the second case, the antibodies of the test system specifically bound to steroids containing 2 α ,3 α -diol, 22R,23R-diol and a 7-membered 7-oxalactone B-ring (B-lactone-BR), but they had low (2–8%) cross-reactivity with the BR 6-keto series (Khripach et al. 2008b). Therefore, Pradko et al. (2015) developed a system specific for the quantification of 6-keto BRs using the conjugate of 28-norcastasterone with bovine serum albumin or horse radish peroxidase.

Monoclonal antibody-based methods

In contrast, monoclonal antibodies are a homogeneous population of identical antibodies that recognize a single epitope on an antigen. They are produced by fusing a single B cell with a cancerous myeloma cell to create a hybridoma cell line that produces identical antibodies. Horgen et al. (1984) produced monoclonal antibodies directed against a synthetic analog of BL non-covalently linked to a carrier protein. Hybridoma clones were derived from CAF1 mice, and the resulting antibodies were used in an ELISA to study the distribution of BL in *B. napus* tissues. Despite the successful generation of antibodies, they showed considerable cross-reactivity (35–48%) with prominent plant sterols such as sitosterol, ergosterol and stigmasterol. This substantial cross-reactivity rendered the antibodies unsuitable for the analysis of BRs due to the lack of specificity for the target compounds. Anti-BR antibodies can also be used in immunoaffinity chromatography (IAC) for rapid and specific purification of BRs before the analytical methods. Using a specific monoclonal antibody prepared against BR, Oklestkova et al. (2017) developed an immunoaffinity

extraction to rapidly and selectively isolate endogenous BRs containing $2\alpha,3\alpha$ -diol within the steroid molecule's ring A. Immunoaffinity chromatography was shown to significantly reduce the matrix effect, thereby increasing the selectivity and sensitivity of subsequent ultra high performance liquid chromatography (UHPLC)–MS/MS analysis. The combination of IAC and UHPLC–MS/MS is used to detect endogenous BRs in tissue extracts of *Zea mays*, *A. thaliana*, *Humulus lupulus* and *B. napus* (Oklestkova et al. 2017). This method has also been used to elucidate the effect of BRs on root growth and development (Vukašinović et al. 2021), where it was found that high levels of BRs have an effect on root cell elongation, whereas lower levels promote cell division. Therefore, changes in the levels of these hormones along the root are probably used to tell the cells whether they should still divide or start to elongate.

Chromatographic and Mass Spectrometric Methods

Isotopically (deuterium) labeled internal standards of BRs

The use of deuterated BRs as internal standards represents an important approach that enables the precision of quantitative measurements in plant hormone research. This approach not only guarantees the reproducibility and reliability of quantitative assessments but also ensures an accurate representation of BR concentration levels in complex biological samples. Furthermore, the incorporation of deuterium into the steroidal framework provides numerous advantages over derivatization. The primary benefit lies in maintaining the same values of key physical properties with natural BRs, such as stability, polarity, solubility and retention time. Considering the significance of deuterated BRs for the HPLC–MS analysis, numerous strategies for their synthesis have been developed (Patil et al. 2015) to introduce deuterium atom(s) to several positions of the BR skeleton (Fig. 1B).

Due to the importance of deuterium in increasing the efficiency of the LC–MS analysis of BRs, derivatives containing three or more deuterium atoms were deliberately synthesized. This enrichment with a higher number of deuterium atoms serves to optimize the analytical accuracy of identification and quantification of BRs using the LC–MS analysis. The chemical synthesis of deuterated BRs involved multi-step reactions. A successful approach to introducing three or more deuterium atoms into the structure of a BR involved the construction of the entire side chain. This strategic method uses low-molecular weight substances containing deuterium atoms in the methyl group(s). An alternative way is sequential reduction of the carboxy group to the methyl group with lithium aluminum deuteride. This is thereby increasing the overall efficiency of the synthesis process and the final content of deuterium atoms in the structure. This strategic approach facilitated the synthesis of derivatives featuring three, six, or even up to seven deuterium atoms exclusively positioned at the C-25 (one atom), C-26 (three atoms),

C-27 (three atoms) and C-28 (three atoms) sites of the side chain (Takatsuto and Ikekawa 1986a, 1986b, 1986c, Khripach et al. 2002a, 2002b, 2012a, 2012b, Antonchick et al. 2004). While the synthetic preparation of the BR derivatives with more than seven deuterium atoms is theoretically feasible, such compounds have not been synthesized to date.

When incorporating deuterium onto steroid rings, the presence of functional groups, particularly oxygen-based ones, becomes essential. For the B-ring, a straightforward exchange of hydrogens for deuterium on the alpha carbons adjacent to the 6-oxo group is achievable, catalyzed by both acids and bases. This method enables the preparation of the derivatives with one deuterium in the C-5 position and two in the C-7 position (Allevi et al. 1988, Kolbe et al. 1992). The maximum number of deuterium atoms introduced into the BR structure by such an exchange was seven. Kolbe and his coworkers thus prepared $[2,2,4,4,5,7,7\text{-}^2\text{H}_7]$ -3-dehydro-24-epiteasterone with deuterium atoms on both A and B rings (Kolbe et al. 1998). However, it turned out that the resulting product had only 20% isotopic purity and contained other less deuterated analogs. The main drawback in using such derivatives lies in the potential re-exchange of deuterium for hydrogen during the chromatographic analysis, particularly when the mobile phase involves acids or bases. To address this concern, oxidizing the deuterated ketones to lactones effectively resolves the issue, especially in the context of two deuterium atoms positioned at the C-7 site. This modification significantly diminishes the likelihood of their exchange with hydrogen. An alternative procedure was employed for the synthesis of 24-epibrassinolide, ensuring the incorporation of two deuterium atoms exclusively at the C-7 position (Khripach et al. 2007). In contrast to the relative ease of introducing deuterium onto the B-ring, deuteration reactions in the case of the A-ring are less common. Marek and coworkers published a stereoselective reduction of chlorocarbonates for the specific deuteration of the 3β -position (Marek et al. 2012, 2015). Through this method, they successfully synthesized deuterated forms of both 24-epicastasterone and 24-epibrassinolide. However, it is worth noting that the presence of just one deuterium atom, as aforementioned, may not be optimal for the chromatographic analysis.

The choice of the deuteration site depends only on the complexity of the synthesis and the possibility of introducing more deuterium atoms. If the deuteration site is not affected by an undesired reaction during the chromatographic analysis (exchange of hydrogen for deuterium in addition to the carbonyl group), the choice of deuterium position does not affect the analysis.

It should be emphasized that the aforementioned deuteration methods and the preparation of BRs with deuterium incorporated into the skeleton are particularly suitable for LC–MS analyses. In the case of gas chromatography–mass spectrometry (GC–MS) analyses, derivatization of hydroxyl groups is required to increase the volatility. In this case, deuteration can be achieved using synthesized or commercially available deuterium-labeled derivatization reagents (see later).

Derivatization-based LC–MS methods

Liquid chromatography–mass spectrometry (LC–MS) is becoming increasingly popular as a valuable technique for BR analysis. Its advantages include increased analytical sensitivity, improved selectivity and the ability to quantitatively detect multiple analytes in a single run.

Given the complexity and diversity of BRs, LC–MS can effectively separate these compounds and provide detailed information about their structure and quantity. Increasing the sensitivity and selectivity of detection can also be influenced by the choice of an appropriate extraction, purification or derivatization protocol (Liu et al. 2022). The following overview of LC–MS methods requiring derivatization is further subdivided according to the extraction/purification protocols chosen.

Two-dimensional solid-phase extraction. Five BRs with high biological activity—24-epibrassinolide (24-epiBL), 24-epiCS, 6-deoxo-24-epicastasterone, teasterone and typhasterol (TY)—were selected as target analytes for the analysis using online two-dimensional solid-phase extraction (2D μ SPE) on column derivatization (OCD) coupled with HPLC–MS/MS (Wu et al. 2013). C18 sorbent was first combined with phenyl boronic acid sorbent to form a 2D μ SPE column, thereby increasing the extraction selectivity. Then, after BRs were enriched on C18 sorbent, OCD was carried out with *m*-aminophenylboronic acid as the derivatization reagent increasing MS/MS sensitivity. This online method showed high selectivity (matrix effect, 80–124%), good repeatability (<10%), low limits of detection (LOD) (about 1 pg) and short analysis time (30 min) (Wu et al. 2013).

Polymer monolith microextraction. In 2020, Wang and coworkers presented an online method for quantifying endogenous BR in plant tissues. This method involved the integration of polymer monolith microextraction, in situ derivatization and liquid chromatography–mass spectrometry (LC–MS) analysis. They modified an anionic hydrophilic interaction liquid chromatography monolithic column with 2-methyl-4-phenylaminomethylphenylboronic acid (2-methyl-4-PAMBA) through ion exchange interactions. They used this modified column as a boronate affinity medium to selectively capture, purify and derivatize BRs in a crude plant extract. The derivatization process involved the reaction of the boronic acid group of 2-methyl-4-PAMBA with the *cis*-diol group of BR. The BR derivatives were easily separated by breaking the ion exchange interactions using an acidic elution solvent. The entire process, including extraction and LC–MS analysis, was automated and completed within 50 min. The limits of detection ranged from 0.10 to 1.29 pg/ml. Finally, the proposed method was used for quantification of endogenous BL, CS, TY, 28-norbrassinolide (28-norBL) and 28-homobrassinolide in milligram plant samples including *Oryza sativa* L., *P. vulgaris* L., *Vigna unguiculata* and *A. thaliana* flowers (Wang et al. 2020).

Magnetic particle-based extraction. Magnetic solid-phase extraction (MSPE) is one option for BRs extraction based on

magnetic or magnetizable nanoparticles as sorbents (Wierucka and Biziuk 2014, Ding et al. 2016). Due to their magnetic properties, the sorbents easily clump together and disperse within the sample solution when an external magnetic field is applied/removed. This process simplifies the separation between the solution and the sorbents. Ding et al. (2014) created a method that integrates BR magnetic extraction and in situ derivatization together (MSPE-ISD). BRs were first extracted onto TiO₂-coated magnetic hollow mesoporous silica sphere through hydrophilic interaction. The BR-adsorbed TiO₂-coated magnetic hollow mesoporous silica sphere was then employed as a 'microreactor' for the derivatization of BRs with 4-(*N,N*-dimethylamino)phenylboronic acid (DMAPBA). This reduced the sample pretreatment process to 10 min. The BR derivatives are released from the magnetic sorbents using water and then analyzed by UPLC–electrospray ionization tandem mass spectrometry (ESI–MS/MS) (Ding et al. 2014).

In 2016, BRs were extracted from plant samples using mQuEChERS and polymer (4-vinylphenylboronic acid-co-ethylene glycol dimethacrylate)-coated Fe₃O₄@SiO₂ as sequential MSPE sorbents (Ding et al. 2016). After coating the plant sample on p(4-VPBA-co-EGDMA)–Fe₃O₄@SiO₂, 4-(*N,N*-dimethylamino)phenylboronic acid (4-DMAPBA) solution was employed for the in situ desorption/derivatization of BRs through a transesterification reaction, and finally, the resulting solution was subjected to LC–MS/MS for BR quantification. BRs (BL, CS, TY, 28-norCS and 28-norBL) were quantified only in a single flower or flower bud of *B. napus* L. using the proposed method (Ding et al. 2016).

Another possibility of using MSPE is the method based on boronate affinity magnetic nanoparticles, which allows direct fishing of BRs from plant matrixes (Xin et al. 2018). A boronate affinity magnetic nanoparticle solid phase extraction (SPE) method combined with UPLC–MS/MS was used to determine BRs in rice and *Arabidopsis* model plants. The levels of BL, CS, TY and 6-deoxo-CS in WS-2 wild-type and BRi1-5 mutants of *A. thaliana*, as well as the CS content ratio of different organs of the rice mutant M107 to its wild-type Nipponbare, were compared (Xin et al. 2018).

Boronate affinity solid-phase extraction. Yu et al. (2018) developed a highly sensitive and selective strategy for BR profiling by stable isotope labeling liquid chromatography and multiple reaction monitoring scan mass spectrometry (SIL–LC–MRM–MS) analysis. They used a pair of stable isotope labeling agents, 4-phenylaminomethyl-benzeneboronic acid (4-PAMBA) and [²H₅]-4-phenylaminomethyl-benzeneboronic acids (4-PAMBA-²H₅), which reacted with C22-, C23-*cis*-diol on a BR molecule. The BRs subsequently generated two characteristic collision-induced dissociation (CID) neutral losses, which are used to establish multiple reaction monitoring (MRM)–based detection and screening. Using this SIL–LC–MRM–MS strategy, 13 BRs were successfully quantified in different tissues of rape flower (Yu et al. 2018).

Xin et al. (2013a) developed a rapid two-step SPE extraction based on mixed-mode anion exchange and cation exchange

cartridges, which combine ion exchange and reversed-phase mechanisms. To enhance the MS response, the BRs were subsequently derivatized with 3-(dimethylamino)-phenylboronic acid (DMAPBA). As a result, a hybrid strategy combining UPLC–MRM³–MS analysis with isotope dilution-based quantification was used to profile BL, CS, teasterone and TY in 1 g of samples of rice and *Arabidopsis* and to describe the distribution of BRs in different tissues of these model plants (Xin et al. 2013a). To optimize and reduce the matrix effect, an additional step was added to the purification protocol. Boronate affinity magnetic nanoparticles (BA-MNPs) were synthesized, which specifically recognize and adsorb neutral interferents (mainly saccharides) via their affinity for boronate (Xin et al. 2013b). Although the addition of the BA-MNP treatment to the original protocol (Xin et al. 2013a) extended the overall purification time by ~2 h, the sensitivity of the method was significantly increased (e.g. 5-fold for 24-epiBL, Xin et al. 2013b). Three years later, the method was further improved by performing BR screening on UPLC–QTrap MS and UPLC–QToF MS systems. This enabled the identification of a completely new BR precursor—6-deoxo-28-homotyphasterol—in rice panicles (Xin et al. 2016).

The method, based on pipette tip solid-phase followed by the use of a quaternary ammonium derivatization reagent, enabled a significant reduction of the amount of plant material to submilligram levels (Deng et al. 2016). The authors adopted the PT-SPE purification procedure with C18 sorbent BR isolation and purification. Subsequently, they used 4-boronic-N,N,N-trimethylbenzenammonium iodide (BTBA) as a chemical derivatization agent, which led to an increase in the ionization efficiency of BRs and thus a significant enhancement of the MS response. Thanks to this method (PT-SPE-UPLC–MS/MS), it was possible to quantify 24-epiBL in only 0.5 mg of FW rice leaves (Deng et al. 2016).

Other use of quaternary ammonium boronates was described by Luo et al. (2018). They used a hybrid approach that combines LC–MS with an integrated sample pretreatment method based on strong cation exchange and boronate affinity. First, the plant extract was subjected to solid-phase boronate affinity labeling. This was followed by desorption and salt phase transition extraction (SPTE) for further purification. The integration of labeling and extraction, desorption and SPTE significantly increased the rapidity, sensitivity and selectivity of this method. The LOD for six BRs ranged from 1.4 to 2.8 pg/ml (Luo et al. 2018). However, in *Arabidopsis* shoots, the authors were only able to detect one BR, 6-deoxocasterone, which is weaker, e.g. compared to the method described by Yu et al. (2019) (Table 1). They synthesized a new derivatizing agent called 2-methyl-4-phenylaminomethylphenylboronic acid (2-methyl-4-PAMBA). The 2-methyl-4-PAMBA derivatives with BR showed significantly better stability in the aqueous solvent environment, especially for the C27-BR derivatives (28-norCS and 28-norBL). A subsequent method based on LC–MRM–MS and assisted labeling of 2-methyl-4-PAMBA and 2-methyl-4-PAMBA-[2HS] allowed the detection of endogenous C27-, C28-

and C29-BRs in milligram plant samples, e.g. also in *Arabidopsis* shoots (Yu et al. 2019, Table 1).

It is also possible to use rhodamine B-boronic acid (RhB-BA) to derivatize BRs (An et al. 2020), and the LC–HV-p-MRM–MS method was proposed using a RhB-BA derivatization for the rapid determination of BRs in sample plants. The HV-p-MRM strategy was suggested based on the idea that the precursor ion of BR remains stable in collision cells and is less likely to fragment at high CID voltage. Meanwhile, coexisting impurities that are easily fragmented will break down into smaller ions, thus improving the signal-to-noise ratio of the analytes. The LOD ranged from 1.49 to 6.56 pg/ml (An et al. 2020).

A novel approach to the extraction of BRs described by Chen et al. (2018) is based on in situ the extraction/derivatization/desorption method coupled with UPLC–MS. First, the thiol-functionalized was linked to 4-mercaptophenylboronic acid (4-MPBA) via a disulfide bond. 4-MPBA selectively captured BRs from the plant extract by in situ derivatization to form BR-MPBA derivatives. The reducing agent dithiothreitol was then used to cleave the disulfide bond, allowing the BR-MPBA derivatives to be eluted from the sorbent and finally subjected to LC–MS analysis (Chen et al. 2018). Unfortunately, the starting weight of plant material for this type of analysis is relatively high (100 mg) compared to the other BR analyses mentioned.

Dispersive solid-phase extraction. Another option for BR analysis published in 2019 is dispersive matrix solid-phase extraction (DMSPE) coupled with high-performance liquid chromatography–tandem mass spectrometry. The DMSPE method involves the following steps: solid samples are first combined with a solid sorbent (called as dispersant) in a microcentrifuge tube. The mixture is then centrifuged after the addition of an extraction solvent and detergents, which form another type of sorbent. In essence, DMSPE combines mechanical grinding of the sample and sorbent with efficient cleaning by centrifugation (Li et al. 2019). The authors described three different protocols of DMSPE for different plant matrix (for oilseed rape and for *A. thaliana*). After DMSPE, BRs were derivatized with BTBA and subsequently analyzed by HPLC–MS/MS. This method was also used to geographically profile the endogenous BRs of oilseed rape across various provinces in the Yangtze River Basin (Li et al. 2019).

Non-derivatization LC–MS methods

Most of the methods mentioned earlier require the derivatization of BRs before their LC–MS analysis, mainly to improve their detection limits. However, it turns out that derivatization may not be the key to successfully detect BR, when the plant matrix contains a high level of interfering substances causing a huge chemical background (Tarkowska et al. 2014). Therefore, Tarkowska et al. (2016) developed an extremely sensitive LC–MS method capable of profiling up to 22 inherent BRs. The method achieved detection limits ranging from 0.05 to 40 pg and eliminated the need for derivatization. Extraction of BRs with 60%

Table 1 Endogenous BR concentrations (pg/g FW) obtained in *Arabidopsis* shoots or seeds by various chromatographic and mass spectrometric methods

	BRs content (pg/g FW)				
		LC-MS			GC-MS
	Oklestkova et al. (2017)	Li et al. (2019)	Yu et al. (2019)	Luo et al. (2018)	Fujioka et al. (1998)
BL	40 ± 20	n.d.	4.34 ± 0.54	n.d.	1,860
28-norBL	50 ± 20	n.d.	n.d.	n.d.	n.d.
CS	n.d.	n.d.	2.34 ± 0.18	n.d.	440
24-epiCS	n.d.	210 ± 30	n.d.	n.d.	n.d.
28-norCS	n.d.	n.d.	2.20 ± 0.12	n.d.	n.d.
6-deoxoCS	n.d.	n.d.	n.d.	1,510 ± 70	1,570
TY	40 ± 20	n.d.	0.16 ± 0.07	n.d.	1,340

Abbreviations: 6-deoxoCS, 6-deoxocasterone; 24-epiCS, 24-epicasterone; n.d., not detected; 28-norCS, 28-norcastasterone.

acetonitrile was followed by a two-step purification by SPE and finally the UHPLC analysis coupled with ESI-MS/MS (Tarkowska et al. 2016). The described method proved to be sufficiently sensitive for quantification of not only biologically active BRs themselves but also their biosynthetic precursors and metabolite. A sensitive method for the BR analysis with a very low detection limit (0.010–0.070 pg/ml) was described by Huo et al. (2021). They extracted BRs by online solid-phase extraction using the boronic acid-functionalized Scholl coupling microporous polymer (SMP). The polymer was synthesized by Scholl coupling and then Schiff's reaction with 4-formylphenylboronic acid to form boronic acid-functionalized B-SMP. This B-SMP was then packed into a SPE cartridge and coupled to HPLC-MS/MS and successfully used to analyze BL, 24-epiBL and 28-homobrassinolide in 15 plant-derived foodstuffs (Huo et al. 2021).

Gas chromatography–mass spectrometry

BRs can also be analyzed by gas chromatography, which uses an inert or unreactive gas (a carrier gas), as a mobile phase. Since BRs are not volatile compounds, they must be derivatized prior to the GC-MS analysis, for which methylboronic acid in pyridine is most often used. Derivatization can be also carried out by methyl boronation of the side chain followed by trimethylsilylation of C-3-hydroxy groups (Takatsuto 1994, Kanwar et al. 2017).

GC-MS of BRs experienced its greatest development in the 80s and 90s of the last century when, e.g. some biosynthetic precursors of BL were identified using this method (Schmidt et al. 1993, Abe et al. 1995, Takatsuto et al. 1999). In recent years, the GC-MS analysis has been used to describe the biosynthetic relationship between C28- and C29-BRs in rice (*Oryza sativa*) seedlings (Joo et al. 2015) and also helped to reveal the CS phosphorylation process, as an important conjugation process for maintaining the homeostatic level of active BRs in *A. thaliana* and *Lycopersicon esculentum* plants (Kim et al. 2015). One of the drawbacks of GC-MS is that it requires a greater amount of plant material for extractions compared to LC-MS methods. For instance, Takatsuto et al. (1989) used 2.9 kg of sunflower pollen to identify BL, CS and 28-nor CS in *Helianthus*

annuus L., while Yokota and coworkers used 900 g of wheat grain to identify 3-dehydrocosterone and 3,6-diketobrassinosteroids (Yokota et al. 1994). Although it has gradually been possible to reduce the amount of plant material required to gram quantities (e.g. 10 g of *Arabidopsis* seeds, Fujioka et al. 1998, Table 1.), this is still incomparable to the milligram and sub-milligram weights of plant material needed for LC-MS analysis (Table 2).

Other Methods

In the last decade, complex quantitative proteomic analyses have been increasingly used to analyze the function of phytohormones. Two-dimensional difference gel electrophoresis coupled to mass spectrometry was used to identify BR-regulated proteins in *Arabidopsis* (Deng et al. 2007). This technique identified 42 BR-regulated proteins predicted to play a potential role in BR regulation of specific cellular processes such as signaling, cytoskeletal rearrangement, vesicle trafficking and phytohormone and vitamin biosynthesis (Deng et al. 2007). The regulatory role of BRs in rice seed germination was elucidated using the iTRAQ-based quantitative proteomic analysis (isobaric tags for relative and absolute quantification), which identified more than 800 BR-responsive proteins. These results not only significantly enriched the BR-regulated protein database in rice but also provided new insights into the regulatory role of BRs in rice seed germination (Li et al. 2016). Quantitative proteomics is an essential part of the toolbox for studying regulatory processes in plants, such as growth and development (Yan et al. 2022).

While liquid chromatography–mass spectrometry (LC-MS) remains a powerful analytical tool widely used in plant research for biomolecule profiling and quantification, it is limited by the spatial distribution of target molecules within samples. The homogenization process during sample preparation removes spatial information. Mass spectrometric imaging coupled with desorption electrospray ionization mass spectrometry (DESI) offers a solution. DESI enables label-free, multiplexed and objective measurements of molecular targets directly from complex surfaces. By analyzing samples without prior homogenization, researchers gain spatially resolved molecular insights

Table 2 Overview of LC–MS methods for the analysis of BRs developed in the last 10 years

Pretreatment	Detection	LOD (pg/ml)	Amount of plant tissues (mg)	Plant tissues	Reference
Derivatization methods					
mSiO ₂ -SS-PBA nanofiber	UPLC–MS	0.21–0.62	100	<i>Humulus lupulus</i> , <i>A. thaliana</i> , <i>O. sativa</i>	Chen et al. 2018
2-Methyl-4-PAMBA	LC–MRM-MS	0.1–1.68	1	<i>Oryza sativa</i> , <i>A. thaliana</i> , <i>B. napus</i>	Yu et al. 2019
BA-MNPS	UPLC–MS/MS	8.6–34.8	10	<i>Oryza sativa</i> , <i>A. thaliana</i>	Xin et al. 2018
RhB-CID (HV-P-MRM)	LC–MS	1.49–6.56	10	<i>Brassica napus</i>	An et al. 2020
Solid-phase boronate affinity labeling-SPTE	UPLC–ESI–MS/MS	1.4–2.8	10–20	<i>Oryza sativa</i> , <i>A. thaliana</i> , <i>B. napus</i>	Luo et al. 2018
mQuEChERS-4-DMAPBA	UPLC–MS/MS	0.27–1.29	100	<i>Oryza sativa</i> , <i>B. napus</i>	Ding et al. 2016
Online 2D _i SPE-OCED	HPLC–MS/MS	1.4–6.6	225	<i>Lycopersicon esculentum</i>	Wu et al. 2013
4-PAMBA + d ₅ -4-PAMBA	SIL–LC–MRM-MS	Not specified	50	<i>Brassica napus</i>	Yu et al. 2018
Online PMME-ISD (2-methyl-4-PAMBA)	LC–MS	0.1–1.29	1	<i>Oryza sativa</i> , <i>P. vulgaris</i> , <i>V. unguiculata</i> , <i>A. thaliana</i>	Wang et al. 2020
PT-SPE-BTBA	UPLC–MS/MS	0.013–0.042	0.5–5	<i>Oryza sativa</i>	Deng et al. 2016
DMSPE-BTBA	HPLC–MS/MS	1.38–6.75	0.5–2	<i>Arabidopsis thaliana</i> , <i>B. napus</i>	Li et al. 2019
Non-derivatization methods					
Two-step SPE	UPLC–ESI–MS/MS	0.05–10	50	<i>Brassica napus</i>	Tarkowska et al. 2016
SPE-IAC	UPLC–ESI–MS/MS	1–50	50	<i>Zea mays</i> , <i>A. thaliana</i> , <i>H. lupulus</i> , <i>P. vulgaris</i>	Oklestkova et al. 2017
Online-SPE	HPLC–MS/MS	0.01–0.07	1,000	Various fruits and vegetables	Huo et al. 2021

(Wiseman et al. 2008). For example, Zhang et al. (2021) used DESI–mass spectrometric imaging to study the distribution and abundance variations of plant hormones (jasmonic acid, auxins and abscisic acid), their precursors and metabolites in wounded leaves and leaf-print TLC plates. Their results not only revealed distinct levels of phytohormones between wounded and unwounded leaves (as detected by LC–MS/MS analysis) but also highlighted heterogeneous distributions and strong correlations within wounded regions. Anticipated future applications include the extension of this method to other phytohormone groups, including BRs.

Concluding Remarks

In summary, the BR analysis has made great strides in recent years, particularly in the area of sample pretreatment and the sensitivity and precision of the analysis itself. Sophisticated methods for BR purification and subsequent derivatization have significantly reduced the amount of plant material required (down to submicrogram levels) and the overall analysis time (summarized in Table 2). Technological advances in the development of chromatographic and spectrometric instrumentation for the final quantification of BRs have also allowed the detection limits, for LC–MS to be reduced to 0.01 pg/ml (Huo et al. 2021). Further advances in the BR analysis are expected to contribute to a better understanding of the role of BRs in plant development and a detailed description of the branched BR biosynthetic pathway.

Data Availability

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Příloha 5

Design, Synthesis and Biological Activities of New Brassinosteroid Analogues with a Phenyl Group in the Side Chain

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Design, synthesis and biological activities of new brassinosteroid analogues with a phenyl group in the side chain†

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We have prepared and studied a series of new brassinosteroid derivatives with a *p*-substituted phenyl group in the side chain. To obtain the best comparison between molecular docking and biological activities both types of brassinosteroids were synthesized; 6-ketones, 10 examples, and B-lactones, 8 examples. The phenyl group was introduced into the steroid skeleton by Horner–Wadsworth–Emmons. The docking studies were carried out using AutoDock Vina 1.05. Plant biological activities were established using different brassinosteroid bioassays in comparison with natural brassinosteroids. Differences in the production of the plant hormone ethylene were also observed in etiolated pea seedlings after treatment with new brassinosteroids. The most active compounds were lactone **8f** and 6-oxo derivatives **8c** and **9c**, their biological activities were comparable or even better than naturally occurring brassinolide. Finally the cytotoxicity of the new derivatives was studied using human normal and cancer cell lines.

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Introduction

Brassinosteroids (BRs, Fig. 1) are a class of plant steroid hormones, which are now known to be essential for many aspects of plant growth and development, such as cell division, elongation and differentiation, pollen tube growth, seed germination, regulation of gene expression, enzyme activation and photosynthesis.^{1–3} They are also involved in defense against a wide range of biotic and abiotic stresses, such as water, temperature, oxidative stresses and high salinity.^{4,5} Moreover, recent studies have shown that natural BRs have potential

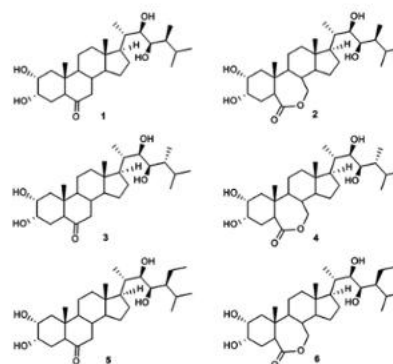


Fig. 1 Structures of the most common natural brassinosteroids; castasterone (1), brassinolide (2), 24-epicastasterone (3), 24-epibrassinolide (4), 28-homocastasterone (5), 28-homobrassinolide (6).

application in medicine due to their antiviral,^{6,7} immunomodulatory and neuroprotective activities,^{8,9} and anti-proliferative effects in animal cells *in vitro*.^{10–15} In contrast to the mammalian nuclear steroid receptors, BRs are perceived at the cell surface by the transmembrane receptor complex formed by the receptor kinase BRI1 and its co-receptor BAK1.^{16–19} The BRI1 receptor has a binding site for BRs located in the extracellular ectodomain. There, the nonpolar side of BRs fits into a highly

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nonpolar cavity of the receptor cleft, and the hydroxyl groups of BRs are exposed to the solvent or towards interactions with BAK1 or SERK1.^{18,20} This structural knowledge, formed the basis of the idea of the brassinosteroid side chain modifications using a nonpolar group such as the phenyl group. Moreover, some analogues containing cycloalkyl substituents at C-24 (replacing the isopropyl group of brassinolide 1) exhibited significant activity in the rice lamina inclination biotest, where the compound with the cyclohexyl group, showed the lowest activity, whereas the cyclopentyl analogue was comparably active as brassinolide in this bioassay.^{21,22}

The aim of this study was to synthesize new brassinosteroid derivatives with a *p*-substituted phenyl group in the side chain and study their biological properties. The phenyl group was chosen owing to its successful molecular docking into the active site of BRI1 using AutoDock Vina. Some compounds showed marked interactions with the BRI1 receptor. The biological activities of the newly prepared derivatives were confirmed by a plant bioassay (pea inhibition biotest, Arabidopsis root and hypocotyl sensitivity bioassay and BES-1 dephosphorylation assay). Their cytotoxic activities were studied using human normal and cancer cell lines.

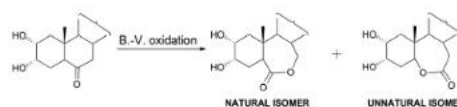
Results and discussion

Chemistry

For the preparation of the desired phenyl analogues of brassinosteroids, we set out from the known aldehyde **7** ((2*S*)-6,6-ethylenedioxy-5 α -pregn-2-en-20-carbaldehyde) prepared according to a published procedure.²³ With this aldehyde Horner-Wadsworth-Emmons (HWE) reaction was then carried out with different commercially available *p*-substituted benzylphosphonates. Based on the character of the BRI1 non-polar part of the cavity for the side chain, we used these substituents in the *para* position: fluorine, chlorine, bromine, iodine, nitro, methyl, methoxy, nitrile, and isopropyl. Benzyl triphenylphosphonium chloride was used for preparation of non-substituted aryl analogues.

Despite the standard use of sodium hydride as a base in the HWE reaction, we observed isomerization of the methyl group on C-20 (see the ESI† for detailed reaction conditions and analysis). Using this base along with steric hindrance of the reaction site for bulky aryl groups, the reaction time increased and this led to enolization of the aldehyde. The aryl group of phosphonates used is crucial for this epimerization as it was not observed when smaller stabilizing groups were used (e.g. COOR, CN).²⁴ Using *n*-butyllithium instead solved the problem and only the desired aryl dienes **8a–17a** with 22*E* configuration were obtained. In almost all the cases, the reaction gave products in good yields (80–90%). Only compound **12a** was prepared in lower yield (65%) due to the presence of reactive iodine.

Further hydrolysis of the ketal group gave aryl dienons **8b–17b**, which were subjected to dihydroxylation in almost quantitative yield. Simultaneous Sharpless dihydroxylation of both



Scheme 1 Baeyer-Villiger oxidation of the B-ring showing formation of natural and unnatural isomeric lactones.

double bonds was used to minimize the formation of unnatural configurations of 22 and 23 hydroxy groups. As a chiral ligand, we used hydroquinidine 4-chlorobenzoate. The reaction rate was increased by addition of methanesulfonamide.²⁵ Without methanesulfonamide, the reaction took more than 48 hours. Such reaction conditions allowed us to isolate only the desired 22*R*,23*R*-isomers **8c–17c** in good yields (75–83%). The correct configuration was determined according to a published alternative preparation of **8c**.²⁶ The unnatural isomers (22*S*,23*S*) were formed only in trace amounts and therefore not isolated. Dihydroxylation of the double bond on the A-ring is stereocontrolled by A-ring conformation and the presence of a methyl group on C10. The attack of the reagent is always from the bottom side of the molecule and thus only 2 α ,3 α -diol was detected.

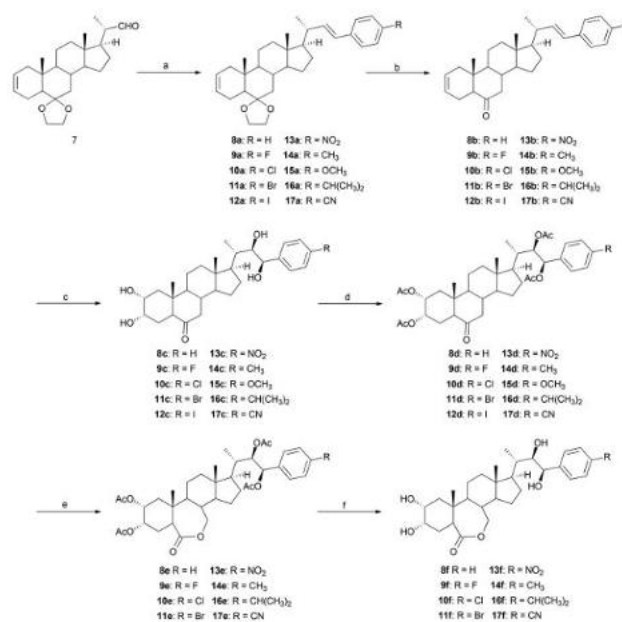
As brassinosteroids with lactone in the B-ring are known to show a higher biological activity than the corresponding 6-ketones, we decided to prepare them as well. The direct Baeyer-Villiger oxidation of tetrahydroxy-ketones with freshly prepared trifluoroperoxyacetic acid led to both isomers in a ratio of approx. 10–15 : 1 (with the natural isomer favoured). However, these mixtures were inseparable even by HPLC (Scheme 1).

For this reason, we had to prepare tetraacetates **8d–17d** first and carry out the oxidation reaction on these. This “detour” allowed us to prepare and easily separate the required lactones **8e–11e**, **13e**, **14e**, **16e**, and **17e** in good yields (79–88%). Unfortunately, two aryl-ketones **12d** and **15d** were unstable during the reaction and only a mixture of products was obtained in both cases. The instability of the iodophenyl derivative **12d** was predictable owing to the presence of easily oxidizable iodine. Due to the presence of the activating group, the methoxyphenyl derivative **15d** is more nucleophilic on the benzene ring than other derivatives and thus also undergoes the electrophilic aromatic substitution reaction (substitution with trifluoroacetate). These two reactions also failed using 3-chloroperoxybenzoic acid.

The last reaction step was basic hydrolysis of tetraacetoxy-lactones to the corresponding tetrahydroxy-lactones **8f–11f**, **13f**, **14f**, **16f**, and **17f** in almost quantitative yields. The reaction steps can be seen in Scheme 2. All compounds were characterized by NMR, IR and MS techniques. Compounds used for biological testing were also characterized for purity by elemental analysis and melting point.

Molecular docking

Molecular docking is a useful tool for understanding the pose and energetics of a protein-ligand complex. The binding site



Scheme 2 Synthesis of brassinosteroid phenyl analogues: (a) benzyltriphenylphosphonium bromide or diethyl arylphosphonates, *n*-BuLi/THF; (b) 5% HCl/THF; (c) OsO₄, CH₃SO₂NH₂, K₃[Fe(CN)₆], K₂CO₃, hydroquinidine 4-chlorobenzoate/*t*-BuOH, H₂O; (d) Ac₂O/pyridine; (e) trifluoroperoxyacetic acid/CH₂Cl₂; (f) NaOH/THF, H₂O.

of BRI1 is located on the surface of the receptor ectodomain as a nonpolar cleft lined by nonpolar aromatic and aliphatic residues (I540, I563, W564, Y599, Y642, M657, F681, I682, I706), whereas hydroxyl groups form the cleft ridge (Y597, Y599, Y642, S647). Brassinolide fits into the cleft *via* its nonpolar side and displays its hydroxyl groups towards the solvent and protein partners (Fig. 2).

Since there is a space left in the cleft around the brassinolide chain, we were running molecular docking of BR derivatives with a phenyl ring on the tail replacing the 1',2'-dimethylpropyl moiety of brassinolide. Molecular docking

predicted similar or better binding energies than for brassinolide for compounds **8c**, **8f**, **9c**, **9f**, **10c**, **10f**, and **14f** (Fig. 3). This implies that derivatives with a phenyl ring on the tail or a phenyl ring with small groups such as fluorine, chlorine or methyl should be accommodated within the BRI1 cleft at least as easily as brassinolide itself and hence they were the best

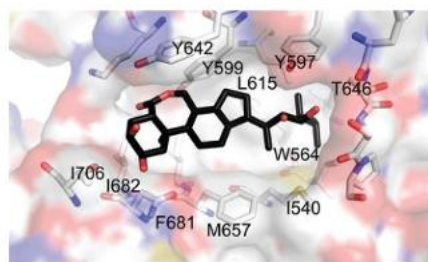


Fig. 2 3D view of brassinolide with amino acids around the active site.

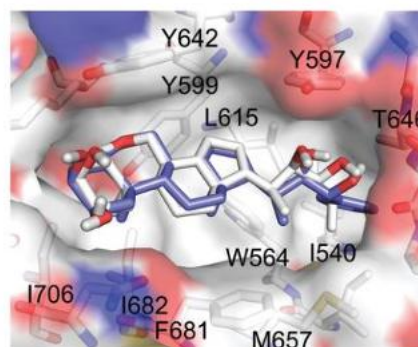


Fig. 3 Pose of **8f** within the BRI1 binding site. The structure of brassinolide and BRI1 binding sites are shown in white, and the structure of **8f** is shown in blue.

candidates for showing similar binding experimentally. On the other hand, derivatives with larger groups did not fit well into the cleft and the docked pose often revealed the tail out of the cleft completely (for the pose of all compounds see the ESI†). In all docking cases, lactones showed better binding energies than 6-ketones due to a better fit to cavity, close to Y599. The best compound to emerge from molecular docking was compound **8f**.

Biology

The response of plant tissues to applied BRs varies with BR concentration. In most cases, low concentration induces elongation and curvature as a result of cell division and cell elongation. Most BR bioassays are based on this effect.²⁷ However, there are other ways of regulating growth by BRs. For example, BRs inhibit the growth of etiolated pea seedlings at high concentration and this is probably caused by increased ethylene production. Ethylene effects alteration of the normal planes of cell growth. Radial swelling or abnormal radial expansion of the stem, such as that seen in the response of etiolated pea seedlings to ethylene application, results from inhibited elongation, increased radial expansion and probably also accounts for leaf epinasty.²⁸ These effects are known as the "triple response" of etiolated seedlings to ethylene.²⁹

The IC_{50} values obtained from the pea inhibition biotest are summarized in Table 1. The most active lactones were **8f**, **9f**, **11f** and **14f** (IC_{50} 2.56×10^{-6} – 1.4×10^{-6} mol L⁻¹) whereas brassinolide (BL), the most active natural BR used as a positive control, was about ten times less active (IC_{50} 2.2×10^{-5} mol L⁻¹). 6-Oxoderivatives **8c** and **9c** were also active in this bioassay. Their IC_{50} values were comparable with active lactones (Table 1). Compounds **15c**, **13f** and **16f** showed no inhibition of etiolated pea plants. Dose response curves for the most active BR derivatives are shown in Fig. 4.

The levels of ethylene were measured in cultivation vessels during the incubation of etiolated pea plants after treatment

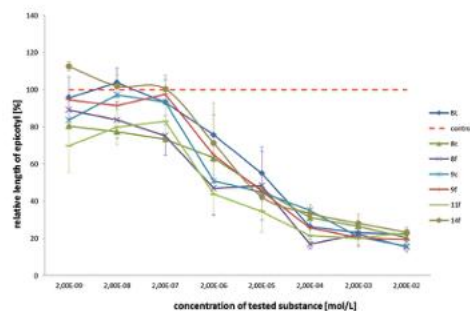


Fig. 4 Effect of selected brassinosteroid derivatives on the inhibition of etiolated pea seedlings. Error bars represent S.D.

with different BR derivatives (Fig. 5). The highest concentration of ethylene (235 and 224 nL L⁻¹) was determined after 24 h treatment with **8c** and **14f** compared to 206 nL L⁻¹ for BL treatment. However, the level of this gaseous plant hormone produced by untreated control pea plants was found to be significantly lower (about 80 nL L⁻¹, Fig. 5). Artea *et al.*³⁰ also observed stimulated ethylene production in etiolated mung bean segments 4 hours after treatment with 1 μ M BL and this increased production became greater over the following 20 h.

The most potent compounds (**8c**, **8f** and **9c**) were tested in Arabidopsis brassinosteroid sensitivity and BES-1 dephosphorylation bioassays. The potency of these compounds was as follows: BL $\geq$ **8f** $>$ **9c** $>$ **8c** in Arabidopsis sensitivity bioassays. The effects of the tested compounds on the Arabidopsis roots and hypocotyls are shown in the ESI (Fig. S3 and S4†). Compound **8f** significantly increased dephosphorylation of BES1 (Fig. 6), which is an important transcription factor in the BR signalling pathway. Altogether, these results confirm that the biological activity of compound **8f** is comparable with natural BR-brassinolide.

The antiproliferative activity of the prepared brassinosteroid derivatives was tested using several models of normal and

Table 1 IC_{50} (mol L⁻¹) values obtained from the pea inhibition biotest

Compound	IC_{50} (mol L ⁻¹)
Brassinolide	$2.2 \times 10^{-5} \pm 2 \times 10^{-6}$
8c	$2.5 \times 10^{-6} \pm 3 \times 10^{-7}$
8f	$1.8 \times 10^{-6} \pm 5 \times 10^{-8}$
9c	$2.0 \times 10^{-6} \pm 3 \times 10^{-7}$
9f	$2.6 \times 10^{-6} \pm 8 \times 10^{-8}$
10c	$1.8 \times 10^{-5} \pm 3 \times 10^{-6}$
10f	$2.3 \times 10^{-5} \pm 2 \times 10^{-6}$
11c	$2.7 \times 10^{-4} \pm 2 \times 10^{-5}$
11f	$1.7 \times 10^{-6} \pm 3 \times 10^{-7}$
12c	$2.1 \times 10^{-2} \pm 4 \times 10^{-3}$
13c	$4.0 \times 10^{-2} \pm 5 \times 10^{-4}$
13f	No inhibition
14c	$1.8 \times 10^{-4} \pm 7 \times 10^{-6}$
14f	$1.4 \times 10^{-6} \pm 2 \times 10^{-7}$
15c	No inhibition
16c	$2.1 \times 10^{-4} \pm 5 \times 10^{-6}$
16f	No inhibition
17c	$2.0 \times 10^{-3} \pm 6 \times 10^{-5}$
17f	$2.1 \times 10^{-4} \pm 5 \times 10^{-6}$

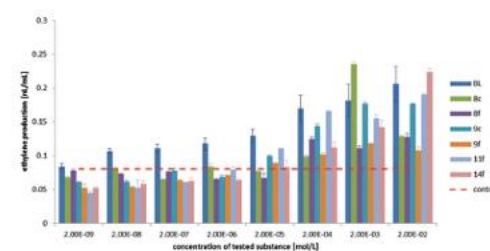


Fig. 5 Effect of selected brassinosteroid derivatives on ethylene production (nL mL⁻¹) in etiolated pea seedlings determined by GC-FID 24 h after ventilation. Error bars represent S.D.

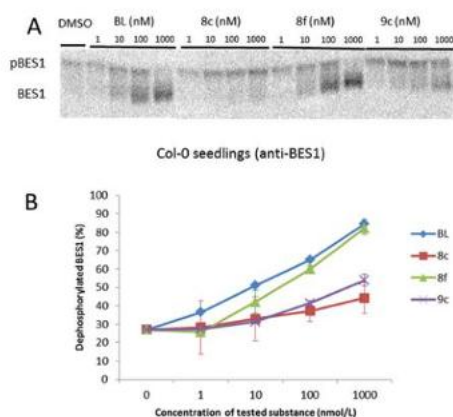


Fig. 6 (A) Immunoblot analysis of BES1 in *Arabidopsis thaliana* (Col-0) seedlings showing dephosphorylation of BES1 after BR treatment. (B) Graph shows the percentage of dephosphorylated BES1 relative to the total BES1 detected in Arabidopsis. The data are the average of two biological repeats. Error bars indicate S.E.M. of pBES1 and phosphorylated BES1.

cancer cell lines. We compared the *in vitro* cytotoxic activity of selected analogues against human foreskin fibroblasts (BJ) and cancer cell lines of various histopathological origins, including T-lymphoblastic leukemia CEM, breast carcinoma (MCF7) and cervical carcinoma (HeLa). Cells were exposed to six 3-fold dilutions of each drug for 72 h prior to determination of cell survival. The IC_{50} (concentration leading to 50% inhibition of viability) values obtained from the calcein AM cytotoxicity assay are presented in the ESI (Table S2†). Most tested BR analogues had no detectable cytotoxic activity, even when tested in concentrations of up to 50 μ M. Only compounds **10f**, **11f** and **13f** showed moderate cytotoxic activity against CEM and HeLa cell lines (IC_{50} around 35 μ M). No BR derivative mediated loss of viability was observed in the BJ fibroblasts. 24-Epibrassinolide was used as a control. It is a natural brassinosteroid with modest cytotoxicity against CEM cells (IC_{50} 44 μ M).¹⁵

Experimental

General methods

The melting points were determined on a Stuart SMP30 instrument (Bibby Scientific Ltd, UK). Elemental analyses were performed using an EA 1108 elemental analyzer (Fison Instruments); the values (C, H, N) agreed with the calculated values within acceptable limits. The infrared spectra were recorded on a Thermo Scientific Nicolet spectrometer iZ10 using the ATR technique. The wave numbers are given in cm^{-1} . The NMR spectra were taken on a JEOL JNM-ECA 500

(JEOL, Tokyo, Japan; 1H , 500 MHz; ^{13}C , 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. 1H NMR and ^{13}C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, 1H δ = 0 ppm) or solvents: $CDCl_3$ (1H δ = 7.26 ppm, ^{13}C δ = 77.00 ppm) or $DMSO-d_6$ (1H δ = 2.46 ppm, ^{13}C δ = 40.00 ppm). Chemical shifts are given in ppm (δ -scale), coupling constants (J) in Hz. All values were obtained by first-order analysis. For API HRMS analysis, the samples were dissolved in chloroform (or chloroform:methanol; 1:1; v/v, in the case of hydroxylated compounds) to a concentration of 10 μ g mL^{-1} . The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into the ion source and analysed in full scan mode. The source of the Synapt G2-Si mass spectrometer (Waters, Manchester, UK) was operated in positive ionisation mode (ASAP+), if not stated otherwise, at a source temperature of 120 $^{\circ}C$. The corona needle current was kept at 5 μ A and the collision energy at 4 V. The probe temperature was ramped up from 50 $^{\circ}C$ to 600 $^{\circ}C$ in 3 minutes. Data were acquired from 50 to 1000 Da with 1.0 s scan time in high resolution mode. The data were processed using the Masslynx 4.1 software (Waters). A mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. The HPLC system consisted of a Waters semi-preparative HPLC system including a quaternary pump, a liquid handler, and UV-VIS and ELSD detectors. The semi-preparative column was filled with silica gel. Reagents and solvents were purchased from Sigma-Aldrich and were not purified. For experimental procedures and data for compounds of series b, d, e, and f, see the ESI†.

(22E)-6,6-Ethylenedioxy-23-phenyl-24-nor-5 α -chola-2,22-diene (8a). To a suspension of benzyl triphenylphosphonium chloride (0.81 mmol) in dried THF (10 mL) was added *n*-BuLi (1.6 M solution in *n*-hexane, 0.81 mmol) at 0 $^{\circ}C$ and stirred for 1 h. To the resultant red solution was added a solution of aldehyde **7** (200 mg, 0.54 mmol) in THF (10 mL) and stirred for 4 h at 25 $^{\circ}C$. The reaction mixture was quenched with water and extracted with Et_2O (2×10 mL). The combined organic fractions were washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the volatiles under reduced pressure followed by column chromatography on silica gel (Et_2O /cyclohexane = 1/19) gave 211 mg (88%) of the title compound **8a** as a colorless oil: IR ν (cm^{-1}) 2933, 1655, 1598. 1H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.68–1.74 (m, 2H), 1.78 (m, 2H), 1.93–2.03 (m, 3H), 2.09 (m, 1H), 2.26 (m, 1H, ΣJ = 37.6 Hz), 3.78 (m, 1H, ΣJ = 24.1 Hz, OCH), 3.88–3.99 (m, 3H, 3 $\times$ OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.06 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.29 (d, 1H, J = 15.7 Hz, H-23), 7.17 (m, 1H, Ar-H), 7.25–7.34 (m, 4H, 4 $\times$ Ar-H). ^{13}C NMR δ 12.25 (CH_3), 13.60 (CH_3), 20.41 (CH_3), 20.87 (CH_2), 21.43 (CH_2), 24.15 (CH_2), 28.36 (CH_2), 33.35 (CH), 35.91 (C), 39.68 (CH_2), 40.49 (CH), 41.20 (CH_2), 41.24 (CH_2), 42.65 (C), 48.08 (CH), 53.50 (CH), 55.93 (CH), 56.00 (CH), 64.08 (CH_2), 65.56 (CH_2), 110.02 (C), 124.80 (CH), 125.70 (CH), 125.90 (2 $\times$ CH), 126.64 (CH), 127.19 (CH), 128.43

(2 × CH), 137.30 (CH), 138.08 (C). HRMS: (API⁺) calculated for C₃₁H₄₃O₂ ([M + H]⁺) 447.3263, Found 447.3266.

General procedure for the Wadsworth–Horner–Emmons (WHE) reaction

To a suspension of substituted diethyl phenylphosphonate (1.08 mmol) in dried THF (10 mL) was added *n*-BuLi (500 µL, 1.6 M solution in *n*-hexane, 0.81 mmol) at 0 °C and stirred for 1 h. To the resultant yellow solution was added a solution of aldehyde **7** (200 mg, 0.54 mmol) in THF (10 mL) and stirred for 4 h at room temperature. The reaction mixture was quenched with water and extracted with Et₂O (2 × 10 mL). The combined organic fractions were washed with brine and dried over anhydrous magnesium sulfate. Evaporation of the volatiles under reduced pressure followed by column chromatography on silica gel gave the desired product.

(22E)-6,6-Ethylenedioxy-23-(4-fluorophenyl)-24-nor-5α-chola-2,22-diene (9a). The general procedure for the WHE reaction with diethyl 4-fluorophenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) yielded 221 mg (89%) of the title compound **9a** as a colorless oil: IR ν (cm⁻¹) 2933, 1654, 1599. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, *J* = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.23 (m, 1H, ΣJ = 37.6 Hz), 3.77 (q, 1H, *J* = 6.8 Hz, OCH), 3.87–3.99 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 5.97 (dd, 1H, *J* = 15.6, *J'* = 8.9 Hz, H-22), 6.25 (d, 1H, *J* = 15.6 Hz, H-23), 6.94–6.98 (m, 2H, 2 × Ar-H), 7.25–7.28 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.21 (CH₃), 13.57 (CH₃), 20.36 (CH₃), 20.86 (CH₂), 21.42 (CH₂), 24.12 (CH₂), 28.37 (CH₂), 33.34 (CH), 35.90 (C), 39.68 (CH₂), 40.45 (CH), 41.20 (CH₂), 41.24 (CH₂), 42.63 (C), 48.08 (CH), 53.50 (CH), 55.92 (CH), 56.00 (CH), 64.07 (CH₂), 65.55 (CH₂), 109.99 (C), 115.22 (d, *J* = 21.6 Hz, 2 × CH), 124.77 (CH), 125.69 (CH), 126.04 (CH), 127.25 (d, *J* = 7.2 Hz, 2 × CH), 134.18 (d, *J* = 3.6 Hz, C), 137.01 (d, *J* = 2.4 Hz, CH), 161.77 (d, *J* = 244.7 Hz, C). ¹⁹F NMR {¹H} δ -116.36 (s, 1F). HRMS: (API⁺) calculated for C₃₁H₄₂FO₂ ([M + H]⁺) 465.3169, Found 465.3170.

(22E)-6,6-Ethylenedioxy-23-(4-chlorophenyl)-24-nor-5α-chola-2,22-diene (10a). The general procedure for the WHE reaction with diethyl 4-chlorophenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) yielded 225 mg (87%) of the title compound **10a** as a colorless oil: IR ν (cm⁻¹) 2933, 1656, 1593. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, *J* = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.25 (m, 1H, ΣJ = 37.6 Hz), 3.78 (q, 1H, *J* = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.04 (dd, 1H, *J* = 15.7, *J'* = 8.9 Hz, H-22), 6.25 (d, 1H, *J* = 15.7 Hz, H-23), 7.24 (m, 2H, 2 × Ar-H), 7.26 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.24 (CH₃), 13.60 (CH₃), 20.30 (CH₃), 20.86 (CH₂), 21.43 (CH₂), 24.14 (CH₂), 28.36 (CH₂), 33.35 (CH), 35.91 (C), 39.68 (CH₂), 40.50 (CH), 41.21 (CH₂), 41.24 (CH₂), 42.67 (C), 48.08 (CH), 53.48 (CH), 55.85 (CH), 55.98 (CH), 64.09 (CH₂), 65.58 (CH₂), 110.01 (C), 124.79 (CH), 125.70 (CH), 126.06 (CH), 127.11 (2 × CH), 128.53 (2 × CH), 132.13 (C), 136.56 (C), 138.02 (CH). HRMS: (API⁺) calculated for C₃₁H₄₂ClO₂ ([M + H]⁺) 481.2873, Found 481.2878.

(22E)-6,6-Ethylenedioxy-23-(4-bromophenyl)-24-nor-5α-chola-2,22-diene (11a). The general procedure for the WHE reaction with diethyl 4-bromophenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 231 mg (82%) of the title compound **11a** as a colorless oil: IR ν (cm⁻¹) 2933, 1656, 1595. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, *J* = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.25 (m, 1H, ΣJ = 37.6 Hz), 3.78 (q, 1H, *J* = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.05 (dd, 1H, *J* = 15.7, *J'* = 8.7 Hz, H-22), 6.23 (d, 1H, *J* = 15.7 Hz, H-23), 7.18 (m, 2H, 2 × Ar-H), 7.39 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.23 (CH₃), 13.59 (CH₃), 20.25 (CH₃), 20.85 (CH₂), 21.42 (CH₂), 24.13 (CH₂), 28.35 (CH₂), 33.34 (CH), 35.90 (C), 39.68 (CH₂), 40.51 (CH), 41.20 (CH₂), 41.23 (CH₂), 42.67 (C), 48.07 (CH), 53.47 (CH), 55.82 (CH), 55.97 (CH), 64.08 (CH₂), 65.57 (CH₂), 110.00 (C), 120.21 (C), 124.78 (CH), 125.69 (CH), 126.10 (CH), 127.46 (2 × CH), 131.46 (2 × CH), 137.01 (C), 138.15 (CH). HRMS: (API⁺) calculated for C₃₁H₄₂BrO₂ ([M + H]⁺) 525.2368, Found 525.2370.

(22E)-6,6-Ethylenedioxy-23-(4-iodophenyl)-24-nor-5α-chola-2,22-diene (12a). The general procedure for the WHE reaction with diethyl 4-iodophenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 200 mg (65%) of the title compound **12a** as a colorless oil: IR ν (cm⁻¹) 2933, 1656, 1595. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, *J* = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.24 (m, 1H, ΣJ = 37.1 Hz), 3.78 (q, 1H, *J* = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.06 (dd, 1H, *J* = 15.7, *J'* = 8.7 Hz, H-22), 6.21 (d, 1H, *J* = 15.7 Hz, H-23), 7.06 (m, 2H, 2 × Ar-H), 7.59 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.23 (CH₃), 13.59 (CH₃), 20.24 (CH₃), 20.85 (CH₂), 21.42 (CH₂), 24.13 (CH₂), 28.33 (CH₂), 33.34 (CH), 35.90 (C), 39.67 (CH₂), 40.50 (CH), 41.20 (CH₂), 41.23 (CH₂), 42.67 (C), 48.07 (CH), 53.47 (CH), 55.81 (CH), 55.96 (CH), 64.08 (CH₂), 65.57 (CH₂), 91.52 (C), 110.00 (C), 124.78 (CH), 125.70 (CH), 126.20 (CH), 127.75 (2 × CH), 137.43 (2 × CH), 137.60 (C), 138.29 (CH). HRMS: (API⁺) calculated for C₃₁H₄₂IO₂ ([M + H]⁺) 573.2229, Found 573.2230.

(22E)-6,6-Ethylenedioxy-23-(4-nitrophenyl)-24-nor-5α-chola-2,22-diene (13a). The general procedure for the WHE reaction with diethyl 4-nitrophenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 230 mg (87%) of the title compound **13a** as a colorless oil: IR ν (cm⁻¹) 2930, 1646, 1594, 1510, 1346. ¹H NMR (CDCl₃) δ 0.76, 0.89 (both s, 3H, CH₃), 1.15 (d, 3H, *J* = 6.5 Hz, CH₃), 1.72 (m, 2H), 1.78 (m, 2H), 1.92–2.04 (m, 3H), 2.09 (m, 1H), 2.32 (m, 1H, ΣJ = 37.0 Hz), 3.78 (q, 1H, *J* = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.28 (dd, 1H, *J* = 15.7, *J'* = 8.6 Hz, H-22), 6.37 (d, 1H, *J* = 15.7 Hz, H-23), 7.43 (m, 2H, 2 × Ar-H), 8.15 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.26 (CH₃), 13.59 (CH₃), 20.02 (CH₃), 20.85 (CH₂), 21.42 (CH₂), 24.14 (CH₂), 28.32 (CH₂), 33.34 (CH), 35.90 (C), 39.68 (CH₂), 40.75 (CH), 41.21 (CH₂), 41.23 (CH₂), 42.79 (C), 48.08 (CH), 53.46 (CH), 55.63 (CH), 55.92 (CH), 64.09 (CH₂), 65.58 (CH₂),

109.97 (C), 123.94 (2 × CH), 124.74 (CH), 125.70 (2 × CH), 126.31 (2 × CH), 142.50 (CH), 144.67 (C), 146.31 (C). HRMS: (API+) calculated for C₃₁H₄₂NO₄ ([M + H]⁺) 492.3114, Found 492.3118.

(22E)-6,6-Ethylenedioxy-23-(4-methylphenyl)-24-nor-5α-chola-2,22-diene (14a). The general procedure for the WHE reaction with diethyl 4-methylphenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 200 mg (81%) of the title compound **14a** as a colorless oil: IR ν (cm⁻¹) 2934, 1656, 1595. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, J = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.77 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.23 (m, 1H, ΣJ = 37.2 Hz), 2.31 (s, 3H, CH₃), 3.77 (q, 1H, J = 6.5 Hz, OCH), 3.87–3.98 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.00 (dd, 1H, J = 15.6, J' = 8.9 Hz, H-22), 6.26 (d, 1H, J = 15.6 Hz, H-23), 7.08 (d, 2H, J = 7.9 Hz, 2 × Ar-H), 7.21 (d, 2H, J = 7.9 Hz, 2 × Ar-H). ¹³C NMR δ 12.20 (CH₃), 13.56 (CH₃), 20.42 (CH₃), 20.84 (CH₂), 21.07 (CH₃), 21.40 (CH₂), 24.11 (CH₂), 28.32 (CH₂), 33.32 (CH), 35.87 (C), 39.66 (CH₂), 40.42 (CH), 41.16 (CH₂), 41.21 (CH₂), 42.58 (C), 48.04 (CH), 53.47 (CH), 55.96 (2 × CH), 64.03 (CH₂), 65.53 (CH₂), 109.97 (C), 124.77 (CH), 125.67 (CH), 125.76 (2 × CH), 126.96 (CH), 129.08 (2 × CH), 135.24 (C), 136.25 (CH), one aromatic C not detected. HRMS: (API+) calculated for C₃₂H₄₅O₂ ([M + H]⁺) 461.3420, Found 461.3422.

(22E)-6,6-Ethylenedioxy-23-(4-methoxyphenyl)-24-nor-5α-chola-2,22-diene (15a). The general procedure for the WHE reaction with diethyl 4-methoxyphenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 203 mg (80%) of the title compound **15a** as a colorless oil: IR ν (cm⁻¹) 2934, 1656, 1594. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, J = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.92–2.03 (m, 3H), 2.09 (m, 1H), 2.22 (m, 1H, ΣJ = 37.4 Hz), 3.77 (q, 1H, J = 6.7 Hz, OCH), 3.79 (s, 3H, OCH₃), 3.87–3.99 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 5.91 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.24 (d, 1H, J = 15.7 Hz, H-23), 6.82 (m, 2H, 2 × Ar-H), 7.25 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.21 (CH₃), 13.58 (CH₃), 20.50 (CH₃), 20.85 (CH₂), 21.41 (CH₂), 24.12 (CH₂), 28.38 (CH₂), 33.33 (CH), 35.88 (C), 39.67 (CH₂), 40.43 (CH), 41.18 (CH₂), 41.22 (CH₂), 42.58 (C), 48.06 (CH), 53.48 (CH), 55.24 (CH₃), 55.99 (CH), 56.02 (CH), 64.05 (CH₂), 65.54 (CH₂), 109.99 (C), 113.83 (2 × CH), 124.78 (CH), 125.68 (CH), 126.48 (CH), 126.92 (2 × CH), 130.87 (C), 135.23 (CH), 158.48 (C). HRMS: (API+) calculated for C₃₂H₄₅O₃ ([M + H]⁺) 477.3369, Found 477.3371.

(22E)-6,6-Ethylenedioxy-23-(4-isopropylphenyl)-24-nor-5α-chola-2,22-diene (16a). The general procedure for the WHE reaction with diethyl 4-isopropylphenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 231 mg (88%) of the title compound **16a** as a colorless oil: IR ν (cm⁻¹) 2935, 1656, 1593. ¹H NMR (CDCl₃) δ 0.74, 0.89 (both s, 3H, CH₃), 1.11 (d, 3H, J = 6.7 Hz, CH₃), 1.229, 1.231 (both d, 3H, J = 7.0 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.93–2.03 (m, 3H), 2.09 (m, 1H), 2.24 (m, 1H, ΣJ = 37.6 Hz), 2.87 (septet, 1H, J = 7.0 Hz, CH(CH₃)₂), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2),

6.01 (dd, 1H, J = 15.8, J' = 8.6 Hz, H-22), 6.27 (d, 1H, J = 15.8 Hz, H-23), 7.15 (d, 2H, J = 8.3 Hz, 2 × Ar-H), 7.26 (d, 2H, J = 8.3 Hz, 2 × Ar-H). ¹³C NMR δ 12.24 (CH₃), 13.60 (CH₃), 20.49 (CH₃), 20.86 (CH₂), 21.43 (CH₂), 23.97 (2 × CH₃), 24.14 (CH₂), 28.35 (CH₂), 33.35 (CH), 33.79 (CH), 35.91 (C), 39.68 (CH₂), 40.50 (CH), 41.19 (CH₂), 41.24 (CH₂), 42.61 (C), 48.07 (CH), 53.49 (CH), 55.97 (CH), 56.00 (CH), 64.07 (CH₂), 65.56 (CH₂), 110.02 (C), 124.81 (CH), 125.69 (CH), 125.86 (2 × CH), 126.50 (2 × CH), 126.97 (CH), 135.69 (C), 136.44 (CH), 147.43 (C). HRMS: (API+) calculated for C₃₄H₄₉O₂ ([M + H]⁺) 489.3733, Found 489.3736.

(22E)-6,6-Ethylenedioxy-23-(4-cyanophenyl)-24-nor-5α-chola-2,22-diene (17a). The general procedure for the WHE reaction with diethyl 4-cyanophenylphosphonate and chromatography on silica (Et₂O/cyclohexane – 1/19) afforded 220 mg (87%) of the title compound **17a** as an amorphous solid: IR ν (cm⁻¹) 2933, 2224, 1646, 1604. ¹H NMR (CDCl₃) δ 0.75, 0.89 (both s, 3H, CH₃), 1.13 (d, 3H, J = 6.7 Hz, CH₃), 1.71 (m, 2H), 1.78 (m, 2H), 1.93–2.03 (m, 3H), 2.09 (m, 1H), 2.30 (m, 1H, ΣJ = 37.2 Hz), 3.78 (q, 1H, J = 6.8 Hz, OCH), 3.88–4.00 (m, 3H, 3 × OCH), 5.54 (m, 1H, H-3), 5.66 (m, 1H, H-2), 6.21 (dd, 1H, J = 15.7, J' = 8.6 Hz, H-22), 6.31 (d, 1H, J = 15.7 Hz, H-23), 7.39 (m, 2H, 2 × Ar-H), 7.56 (m, 2H, 2 × Ar-H). ¹³C NMR δ 12.25 (CH₃), 13.59 (CH₃), 20.07 (CH₃), 20.84 (CH₂), 21.42 (CH₂), 24.13 (CH₂), 28.32 (CH₂), 33.34 (CH), 35.90 (C), 39.67 (CH₂), 40.66 (CH), 41.20 (CH₂), 41.22 (CH₂), 42.76 (C), 48.08 (CH), 53.45 (CH), 55.65 (CH), 55.92 (CH), 64.09 (CH₂), 65.58 (CH₂), 109.77 (C), 109.97 (C), 119.21 (C), 124.75 (CH), 125.70 (CH), 125.99 (CH), 126.36 (2 × CH), 132.30 (2 × CH), 141.46 (CH), 142.62 (C). HRMS: (API+) calculated for C₃₂H₄₃NO₂ ([M + H]⁺) 472.3216, Found 472.3218.

General procedure for dihydroxylation of dienes

To a solution of diene (160 mg), hydroquinidine 4-chlorobenzoate (45 mg; 0.097 mmol), methanesulfonamide (65 mg; 0.68 mmol), potassium carbonate (280 mg; 2.03 mmol), and potassium ferricyanide (700 mg; 2.13 mmol) in the mixture of *t*-butanol and water (15 mL; 1:1 v/v) was added 0.2 mL of osmium tetroxide in *t*-butanol (1 g per 20 mL; 0.039 mmol). The reaction mixture was stirred at room temperature for 24 h. A saturated solution of sodium sulfite (3 mL) was then added. After an additional 30 minutes of stirring, the reaction mixture was diluted with ethyl acetate (30 mL) and extracted with water (2 × 20 mL). The combined organic fractions were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Column chromatography on silica gel gave the desired product.

(22R,23R)-2α,3α,22,23-Tetrahydroxy-23-phenyl-24-nor-5α-cholan-6-one (8c). The general procedure for dihydroxylation of **8b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 144 mg (77%) of the title compound **8c** as a white solid: m. p. 268–270 °C (EtOH), IR ν (cm⁻¹) 3344, 2940, 1708, 1496. ¹H NMR (DMSO-*d*₆) δ 0.30, 0.59 (both s, 3H, CH₃), 0.86 (d, 3H, J = 6.4 Hz, CH₃), 1.80 (m, 1H), 1.86 (m, 1H), 1.97 (dd, 1H, J = 13.1, J' = 4.6 Hz), 2.07 (t, 1H, J = 12.6 Hz), 2.58 (dd, 1H, J = 12.1, J' = 3.2 Hz), 3.44 (m, 1H), 3.48 (dd, 1H, J = 8.4, J' =

4.4 Hz), 3.74 (m, 1H), 4.19 (d, 1H, $J = 2.8$ Hz, OH), 4.32 (d, 1H, $J = 6.1$ Hz, OH), 4.35 (dd, 1H, $J = 8.6$, $J' = 3.9$ Hz), 4.51 (d, 1H, $J = 4.3$ Hz, OH), 5.14 (d, 1H, $J = 3.9$ Hz, OH), 7.22–7.27 (m, 3H), 7.31 (m, 2H). ^{13}C NMR δ 11.39 (CH₃), 12.47 (CH₃), 13.36 (CH₃), 20.79 (CH₂), 23.28 (CH₂), 26.82 (CH₂), 27.22 (CH₂), 36.29 (CH), 37.02 (CH), 39.14 (C), 41.84 (C), 42.03 (CH₂), 45.95 (CH₂), 50.28 (CH), 51.88 (CH), 52.85 (CH), 55.95 (CH), 67.10 (CH), 67.49 (CH), 75.16 (CH), 76.30 (CH), 127.01 (2 $\times$ CH), 127.21 (CH), 128.07 (2 $\times$ CH), 143.28 (C), 211.57 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API+) calculated for C₂₉H₄₃O₅ ([M + H]⁺) 471.3110, Found 471.3108. Anal. Calcd for C₂₉H₄₃O₅: C, 74.01; H, 8.99. Found: C, 73.95; H, 9.06%.

(22R,23R)-2 α ,3 α ,22,23-Tetrahydroxy-23-(4-fluorophenyl)-24-nor-5 α -cholan-6-one (9c). The general procedure for dihydroxylation of **9b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 149 mg (80%) of the title compound **9c** as a white solid: m. p. 277–279 °C (EtOH), IR ν (cm⁻¹) 3251, 2937, 1709, 1607, 1513. ^1H NMR (DMSO-*d*₆) δ 0.33, 0.60 (both s, 3H, CH₃), 0.84 (d, 3H, $J = 6.7$ Hz, CH₃), 1.80 (m, 1H), 1.85 (m, 1H), 1.98 (dd, 1H, $J = 13.0$, $J' = 4.8$ Hz), 2.07 (t, 1H, $J = 12.6$ Hz), 2.58 (dd, 1H, $J = 12.2$, $J' = 3.1$ Hz), 3.42–3.47 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, $J = 2.4$ Hz, OH), 4.32 (d, 1H, $J = 6.1$ Hz, OH), 4.37 (dd, 1H, $J = 8.7$, $J' = 3.5$ Hz), 4.54 (d, 1H, $J = 4.3$ Hz, OH), 5.14 (d, 1H, $J = 4.0$ Hz, OH), 7.14 (m, 2H), 7.30 (m, 2H). ^{13}C NMR δ 11.42 (CH₃), 12.44 (CH₃), 13.38 (CH₃), 20.80 (CH₂), 23.30 (CH₂), 26.84 (CH₂), 27.28 (CH₂), 36.35 (CH), 37.04 (CH), 39.15 (C), 41.86 (C), 42.06 (CH₂), 45.97 (CH₂), 50.31 (CH), 51.89 (CH), 52.88 (CH), 55.97 (CH), 67.11 (CH), 67.51 (CH), 74.40 (CH), 76.34 (CH), 114.84 (d, $J = 21.6$ Hz, 2 $\times$ CH), 128.86 (d, $J = 8.4$ Hz, 2 $\times$ CH), 139.58 (d, $J = 2.4$ Hz, C), 161.26 (d, $J = 242.3$ Hz, C), 211.62 (C). One CH₂ covered by a DMSO multiplet. ^{19}F NMR (^1H) δ -115.37 (s, 1F). HRMS: (API+) calculated for C₂₉H₄₂FO₅ ([M + H]⁺) 489.3016, Found 489.3017. Anal. Calcd for C₂₉H₄₁FO₅: C, 71.28; H, 8.46. Found: C, 71.88; H, 8.55%.

(22R,23R)-2 α ,3 α ,22,23-Tetrahydroxy-23-(4-chlorophenyl)-24-nor-5 α -cholan-6-one (10c). The general procedure for dihydroxylation of **10b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 152 mg (82%) of the title compound **10c** as a white solid: m. p. 251–253 °C (EtOH), IR ν (cm⁻¹) 3220, 2940, 1712, 1598, 1494. ^1H NMR (DMSO-*d*₆) δ 0.34, 0.60 (both s, 3H, CH₃), 0.85 (d, 3H, $J = 6.7$ Hz, CH₃), 1.80 (m, 1H), 1.85 (m, 1H), 1.98 (dd, 1H, $J = 13.0$, $J' = 4.8$ Hz), 2.07 (t, 1H, $J = 12.6$ Hz), 2.58 (dd, 1H, $J = 12.2$, $J' = 3.4$ Hz), 3.42–3.48 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, $J = 2.4$ Hz, OH), 4.33 (d, 1H, $J = 6.1$ Hz, OH), 4.37 (dd, 1H, $J = 8.4$, $J' = 3.8$ Hz), 4.58 (d, 1H, $J = 4.6$ Hz, OH), 5.20 (d, 1H, $J = 3.8$ Hz, OH), 7.29 (m, 2H), 7.37 (m, 2H). ^{13}C NMR δ 11.46 (CH₃), 12.49 (CH₃), 13.38 (CH₃), 20.80 (CH₂), 23.30 (CH₂), 26.84 (CH₂), 27.29 (CH₂), 36.47 (CH), 37.05 (CH), 39.15 (C), 41.87 (C), 42.07 (CH₂), 45.98 (CH₂), 50.31 (CH), 51.89 (CH), 52.89 (CH), 55.97 (CH), 67.12 (CH), 67.51 (CH), 74.44 (CH), 76.21 (CH), 128.10 (2 $\times$ CH), 128.87 (2 $\times$ CH), 131.52 (C), 142.41 (C), 211.62 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API+) calculated for C₂₉H₄₂ClO₅ ([M + H]⁺) 505.2721, Found 505.2723. Anal. Calcd for C₂₉H₄₁ClO₅: C, 68.96; H, 8.18. Found: C, 68.90; H, 8.28%.

(22R,23R)-2 α ,3 α ,22,23-Tetrahydroxy-23-(4-bromophenyl)-24-nor-5 α -cholan-6-one (11c). The general procedure for dihydroxylation of **11b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 146 mg (80%) of the title compound **11c** as a white solid: m. p. 247–249 °C (i-PrOH), IR ν (cm⁻¹) 3237, 2941, 1709, 1600, 1490. ^1H NMR (DMSO-*d*₆) δ 0.34, 0.60 (both s, 3H, CH₃), 0.84 (d, 3H, $J = 6.7$ Hz, CH₃), 1.79 (m, 1H), 1.87 (m, 1H), 1.98 (dd, 1H, $J = 13.0$, $J' = 4.6$ Hz), 2.07 (t, 1H, $J = 12.5$ Hz), 2.58 (dd, 1H, $J = 12.2$, $J' = 3.1$ Hz), 3.42–3.47 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, $J = 2.8$ Hz, OH), 4.33 (d, 1H, $J = 6.1$ Hz, OH), 4.36 (dd, 1H, $J = 8.6$, $J' = 3.8$ Hz), 4.58 (d, 1H, $J = 4.3$ Hz, OH), 5.20 (d, 1H, $J = 3.8$ Hz, OH), 7.23 (d, 2H, $J = 8.6$ Hz, 2 $\times$ Ar-H), 7.51 (d, 2H, $J = 8.6$ Hz, 2 $\times$ Ar-H). ^{13}C NMR δ 11.49 (CH₃), 12.50 (CH₃), 13.38 (CH₃), 20.81 (CH₂), 23.31 (CH₂), 26.84 (CH₂), 27.30 (CH₂), 36.50 (CH), 37.06 (CH), 39.15 (C), 41.87 (C), 42.08 (CH₂), 45.99 (CH₂), 50.32 (CH), 51.89 (CH), 52.89 (CH), 55.98 (CH), 67.13 (CH), 67.52 (CH), 74.51 (CH), 76.16 (CH), 120.08 (C), 129.26 (2 $\times$ CH), 131.01 (2 $\times$ CH), 142.83 (C), 211.62 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API+) calculated for C₂₉H₄₂⁷⁹BrO₅ ([M + H]⁺) 549.2216, Found 549.2216. Anal. Calcd for C₂₉H₄₁BrO₅: C, 63.38; H, 7.52. Found: C, 63.29; H, 7.55%.

(22R,23R)-2 α ,3 α ,22,23-Tetrahydroxy-23-(4-iodophenyl)-24-nor-5 α -cholan-6-one (12c). The general procedure for dihydroxylation of **12b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 150 mg (83%) of the title compound **12c** as a white solid: m. p. 253–255 °C (EtOH), IR ν (cm⁻¹) 3193, 2943, 1710, 1590. ^1H NMR (DMSO-*d*₆) δ 0.35, 0.60 (both s, 3H, CH₃), 0.84 (d, 3H, $J = 6.7$ Hz, CH₃), 1.79 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, $J = 13.2$, $J' = 4.9$ Hz), 2.07 (t, 1H, $J = 12.5$ Hz), 2.58 (dd, 1H, $J = 12.2$, $J' = 3.4$ Hz), 3.41–3.48 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, $J = 2.8$ Hz, OH), 4.32 (d, 1H, $J = 6.1$ Hz, OH), 4.34 (dd, 1H, $J = 8.6$, $J' = 3.7$ Hz), 4.56 (d, 1H, $J = 4.3$ Hz, OH), 5.18 (d, 1H, $J = 3.8$ Hz, OH), 7.09 (m, 2H, 2 $\times$ Ar-H), 7.67 (m, 2H, 2 $\times$ Ar-H). ^{13}C NMR δ 11.49 (CH₃), 12.48 (CH₃), 13.36 (CH₃), 20.80 (CH₂), 23.29 (CH₂), 26.83 (CH₂), 27.28 (CH₂), 36.51 (CH), 37.03 (CH), 39.14 (C), 41.85 (C), 42.05 (CH₂), 45.96 (CH₂), 50.30 (CH), 51.87 (CH), 52.86 (CH), 55.95 (CH), 67.11 (CH), 67.49 (CH), 74.60 (CH), 76.07 (CH), 92.95 (C), 129.40 (2 $\times$ CH), 136.83 (2 $\times$ CH), 143.19 (C), 211.57 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API+) calculated for C₂₉H₄₂IO₅ ([M + H]⁺) 597.2077, Found 597.2076. Anal. Calcd for C₂₉H₄₁IO₅: C, 58.39; H, 6.93. Found: C, 58.32; H, 7.01%.

(22R,23R)-2 α ,3 α ,22,23-Tetrahydroxy-23-(4-nitrophenyl)-24-nor-5 α -cholan-6-one (13c). The general procedure for dihydroxylation of **13b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 144 mg (78%) of the title compound **13c** as a white solid: m. p. 244–246 °C (EtOH), IR ν (cm⁻¹) 3180, 2940, 1711, 1605, 1523, 1349. ^1H NMR (DMSO-*d*₆) δ 0.34, 0.59 (both s, 3H, CH₃), 0.87 (d, 3H, $J = 6.7$ Hz, CH₃), 1.80 (m, 1H), 1.86 (m, 1H), 1.97 (dd, 1H, $J = 13.2$, $J' = 4.6$ Hz), 2.06 (t, 1H, $J = 12.5$ Hz), 2.58 (dd, 1H, $J = 12.2$, $J' = 3.4$ Hz), 3.45 (m, 1H), 3.50 (m, 1H), 3.74 (m, 1H), 4.19 (d, 1H, $J = 2.8$ Hz, OH), 4.33 (d, 1H, $J = 6.1$ Hz, OH), 4.53 (dd, 1H, $J = 7.9$, $J' = 3.5$ Hz), 4.74 (d, 1H, $J = 4.6$ Hz, OH), 5.47 (d, 1H, $J = 3.5$ Hz, OH), 7.57 (m, 2H), 8.19 (m, 2H). ^{13}C NMR δ 11.46 (CH₃), 12.64 (CH₃), 13.37 (CH₃),

20.80 (CH₂), 23.30 (CH₂), 26.83 (CH₂), 27.32 (CH₂), 36.77 (CH), 37.03 (CH), 39.14 (C), 41.86 (C), 42.09 (CH₂), 45.97 (CH₂), 50.31 (CH), 51.92 (CH), 52.87 (CH), 55.95 (CH), 67.11 (CH), 67.51 (CH), 74.46 (CH), 76.09 (CH), 123.31 (2 × CH), 128.25 (2 × CH), 146.62 (C), 151.53 (C), 211.61 (C). One CH₂ covered by a DMSO multiplet. HRMS: (ESI⁺) calculated for C₂₉H₄₁NO₇ ([M]⁺) 515.2883, Found 515.2888. Anal. Calcd for C₂₉H₄₁NO₇: C, 67.55; H, 8.01. Found: C, 67.45; H, 8.09%.

(22R,23R)-2α,3α,22,23-Tetrahydroxy-23-(4-methylphenyl)-24-nor-5α-cholan-6-one (14c). The general procedure for dihydroxylation of **14b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 145 mg (78%) of the title compound **14c** as a white solid: m. p. 271–272 °C (EtOH), IR ν (cm⁻¹) 3215, 2937, 1710, 1610, 1516. ¹H NMR (DMSO-d₆) δ 0.32, 0.59 (both s, 3H, CH₃), 0.84 (d, 3H, *J* = 6.7 Hz, CH₃), 1.80 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, *J* = 13.2, *J'* = 4.6 Hz), 2.07 (t, 1H, *J* = 12.5 Hz), 2.27 (s, 3H, CH₃), 2.58 (dd, 1H, *J* = 12.2, *J'* = 3.4 Hz), 3.42–3.48 (m, 2H), 3.74 (m, 1H), 4.19 (d, 1H, *J* = 2.8 Hz, OH), 4.31 (dd, 1H, *J* = 8.6, *J'* = 3.8 Hz), 4.34 (d, 1H, *J* = 6.1 Hz, OH), 4.46 (d, 1H, *J* = 4.4 Hz, OH), 5.00 (d, 1H, *J* = 3.8 Hz, OH), 7.10–7.15 (m, 4H). ¹³C NMR δ 11.50 (CH₃), 12.44 (CH₃), 13.40 (CH₃), 20.83 (CH₂, CH₂), 23.32 (CH₂), 26.85 (CH₂), 27.28 (CH₂), 36.38 (CH), 37.07 (CH), 39.15 (C), 41.89 (C), 42.07 (CH₂), 46.00 (CH₂), 50.33 (CH), 51.90 (CH), 52.91 (CH), 56.00 (CH), 67.14 (CH), 67.52 (CH), 74.96 (CH), 76.36 (CH), 126.99 (2 × CH), 128.71 (2 × CH), 136.15 (C), 140.20 (C), 211.68 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API⁺) calculated for C₃₀H₄₅O₅ ([M + H]⁺) 485.3267, Found 485.3270. Anal. Calcd for C₃₀H₄₅O₅: C, 74.34; H, 9.15. Found: C, 74.30; H, 9.20%.

(22R,23R)-2α,3α,22,23-Tetrahydroxy-23-(4-methoxyphenyl)-24-nor-5α-cholan-6-one (15c). The general procedure for dihydroxylation of **15b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 150 mg (81%) of the title compound **15c** as a white solid: m. p. 242–244 °C (EtOH), IR ν (cm⁻¹) 3197, 2934, 1710, 1616, 1513. ¹H NMR (DMSO-d₆) δ 0.33, 0.60 (both s, 3H, CH₃), 0.84 (d, 3H, *J* = 6.7 Hz, CH₃), 1.80 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, *J* = 13.2, *J'* = 4.6 Hz), 2.07 (t, 1H, *J* = 12.7 Hz), 2.58 (dd, 1H, *J* = 12.2, *J'* = 3.4 Hz), 3.42–3.49 (m, 2H), 3.72 (s, 3H, CH₃), 3.74 (m, 1H), 4.18 (d, 1H, *J* = 2.5 Hz, OH), 4.30 (dd, 1H, *J* = 8.6, *J'* = 3.8 Hz), 4.32 (d, 1H, *J* = 6.4 Hz, OH), 4.44 (d, 1H, *J* = 4.1 Hz, OH), 4.96 (d, 1H, *J* = 3.8 Hz, OH), 6.87 (m, 2H), 7.17 (m, 2H). ¹³C NMR δ 11.48 (CH₃), 12.38 (CH₃), 13.37 (CH₃), 20.80 (CH₂), 23.30 (CH₂), 26.83 (CH₂), 27.26 (CH₂), 36.31 (CH), 37.04 (CH), 39.14 (C), 41.86 (C), 42.04 (CH₂), 45.97 (CH₂), 50.30 (CH), 51.89 (CH), 52.88 (CH), 54.97 (CH₃), 55.98 (CH), 67.11 (CH), 67.50 (CH), 74.59 (CH), 76.29 (CH), 113.43 (2 × CH), 128.12 (2 × CH), 135.17 (C), 158.29 (C), 211.59 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API⁺) calculated for C₃₀H₄₅O₆ ([M + H]⁺) 501.3218, Found 501.3216. Anal. Calcd for C₃₀H₄₅O₆: C, 71.97; H, 8.86. Found: C, 71.91; H, 8.97%.

(22R,23R)-2α,3α,22,23-Tetrahydroxy-23-(4-isopropylphenyl)-24-nor-5α-cholan-6-one (16c). The general procedure for dihydroxylation of **16b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 146 mg (79%) of the title compound **16c** as a white solid: m. p. 247–249 °C (EtOH), IR ν (cm⁻¹) 3215, 2938, 1710, 1617, 1513. ¹H NMR (DMSO-d₆) δ 0.31, 0.59 (both s, 3H,

CH₃), 0.84 (d, 3H, *J* = 6.7 Hz, CH₃), 1.17 (d, 6H, *J* = 6.9 Hz, 2 × CH₃), 1.77–1.86 (m, 2H), 1.98 (dd, 1H, *J* = 13.2, *J'* = 4.6 Hz), 2.07 (t, 1H, *J* = 12.5 Hz), 2.58 (dd, 1H, *J* = 12.1, *J'* = 3.2 Hz), 2.86 (septet, 1H, *J* = 6.9 Hz), 3.44 (m, 1H), 3.50 (dt, 1H, *J* = 8.3, *J'* = 4.1 Hz), 3.74 (m, 1H), 4.19 (d, 1H, *J* = 2.8 Hz, OH), 4.31 (dd, 1H, *J* = 8.6, *J'* = 3.8 Hz), 4.33 (d, 1H, *J* = 6.1 Hz, OH), 4.44 (d, 1H, *J* = 4.0 Hz, OH), 5.00 (d, 1H, *J* = 3.8 Hz, OH), 7.17 (s, 4H). ¹³C NMR δ 11.46 (CH₃), 12.50 (CH₃), 13.37 (CH₃), 20.80 (CH₂), 23.32 (CH₂), 23.92 (CH₃), 24.02 (CH₃), 26.85 (CH₂), 27.20 (CH₂), 33.11 (CH), 36.33 (CH), 37.05 (CH), 39.14 (C), 41.87 (C), 42.07 (CH₂), 45.98 (CH₂), 50.32 (CH), 51.97 (CH), 52.89 (CH), 55.96 (CH), 67.12 (CH), 67.52 (CH), 75.05 (CH), 76.18 (CH), 125.97 (2 × CH), 127.04 (2 × CH), 140.66 (C), 147.21 (C), 211.62 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API⁺) calculated for C₃₂H₄₉O₅ ([M + H]⁺) 513.3580, Found 513.3585. Anal. Calcd for C₃₂H₄₉O₅: C, 74.96; H, 9.44. Found: C, 74.89; H, 9.51%.

(22R,23R)-2α,3α,22,23-Tetrahydroxy-23-(4-cyanophenyl)-24-nor-5α-cholan-6-one (17c). The general procedure for dihydroxylation of **17b** and chromatography on silica (MeOH/CHCl₃ – 1/16) afforded 139 mg (75%) of the title compound **17c** as a white solid: m. p. 260–262 °C (EtOH), IR ν (cm⁻¹) 3198, 2936, 2230, 1706, 1611, 1510. ¹H NMR (DMSO-d₆) δ 0.33, 0.59 (both s, 3H, CH₃), 0.85 (d, 3H, *J* = 6.7 Hz, CH₃), 1.74–1.88 (m, 2H), 1.98 (dd, 1H, *J* = 13.2, *J'* = 4.8 Hz), 2.06 (t, 1H, *J* = 12.5 Hz), 2.57 (dd, 1H, *J* = 12.2, *J'* = 3.4 Hz), 3.46–3.50 (m, 2H), 3.74 (m, 1H), 4.20 (d, 1H, *J* = 2.4 Hz, OH), 4.34 (d, 1H, *J* = 6.1 Hz, OH), 4.46 (dd, 1H, *J* = 8.1, *J'* = 4.3 Hz), 4.69 (d, 1H, *J* = 4.3 Hz, OH), 5.04 (d, 1H, *J* = 4.0 Hz, OH), 7.48 (d, 2H, *J* = 8.3 Hz, 2 × Ar-H), 7.79 (d, 2H, *J* = 8.3 Hz, 2 × Ar-H). ¹³C NMR δ 11.47 (CH₃), 12.64 (CH₃), 13.41 (CH₃), 20.83 (CH₂), 23.33 (CH₂), 26.87 (CH₂), 27.33 (CH₂), 36.71 (CH), 37.08 (CH), 39.14 (C), 41.89 (C), 42.11 (CH₂), 46.00 (CH₂), 50.35 (CH), 51.94 (CH), 52.91 (CH), 55.98 (CH), 67.15 (CH), 67.54 (CH), 74.74 (CH), 76.12 (CH), 109.86 (C), 119.03 (C), 128.05 (2 × CH), 132.14 (2 × CH), 149.34 (C), 211.68 (C). One CH₂ covered by a DMSO multiplet. HRMS: (API⁺) calculated for C₃₀H₄₁NO₅ ([M + H]⁺) 496.3063, Found 496.3064. Anal. Calcd for C₃₀H₄₁NO₅: C, 72.70; H, 8.34. Found: C, 72.69; H, 8.39%.

Molecular docking

Docking was performed to predict the conformation and energy ranking between the BR11 receptor (PDB ID: 3RGZ) and the steroid molecule. The docking studies were carried out using AutoDock Vina 1.05.³¹ All 3D structures of BR11 ligands were obtained with Marvin 5.10.3³² software, which can be used for drawing, displaying and characterization of chemical structures, substructures and reactions. Ligands were prepared as derivatives of the natural ligand brassinolide (BLD). Polar hydrogens were added to all ligands and proteins with the AutoDock Tools (ADT)³⁶ program prior to docking with the Autodock Vina program. A grid box with a size of 40 Å was centered on the active site of the protein. The exhaustiveness parameter was set to 20 (default 8). After docking, we compared the docked ligand with brassinolide crystal-like poses and the best crystal-like poses of each ligand were analyzed.

The pea inhibition biotest

Pea seedlings (*Pisum arvense* L. sort Arvica) germinating for 2 days were selected for uniformity from a large population and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7). After 24 h in a dark cultivation room (24 °C, humidity 75%) the seedlings were treated with different amounts of tested compounds in 5 µl fractionated lanolin. The substances were applied as microdrops to the scar left after the removal of the bract. The control plants were treated with lanolin alone. At least seven plants were used for each experiment and the assays were repeated at least three times. The inhibition of etiolated pea stems was measured after 4 days and the difference in length between the treated and control plants provided a measure of activity. For each treatment, 8 seedlings were analyzed in two biological replicates. The mean values were subjected to the statistical analysis using the Student's *t* test.

Determination of ethylene production

For measurement of ethylene production, pea seedlings (8 plants/tested amount of substance) were placed in a 0.5 L glass container for 24 h in the dark. One milliliter of head-space gas from the chamber was withdrawn for each measurement and injected into a gas chromatograph (Agilent Technologies, GC Systems) equipped with a flame ionic detector (FID) and a capillary column (HP-AL/S stationary phase, 15 µm, i.d. = 0.535). The chromatographic analytical parameters were as follows: column temperature: 150 °C; detector temperature: 220 °C; and helium was used as a carrier gas. The area under the resultant peak (*y*-axis) versus sensitivity (*x*-axis; nL mL⁻¹) represented a quantitative measure of ethylene concentration. The measurements were done in triplicate and data were statistically analyzed using the Student's *t* test.

Arabidopsis brassinosteroid sensitivity assays

Arabidopsis thaliana L. (Heyhn.) (Columbia ecotype, Col-0; referred to as *Arabidopsis*) seedlings were stratified for 2 d at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of BL (Fuji Chemical Industries) and BR derivatives at 22 °C in a 16 h/8 h light-dark cycle for 5 d. For the hypocotyl assay, after stratification, the plants were exposed to light for 6 h and grown in the dark for 5 days. Roots and hypocotyls were then straightened on solid media plates, scanned with an Epson high-resolution scanner and the entire root and hypocotyl length measured with ImageJ (<http://rsbweb.nih.gov/ij/>). For each treatment, more than 25 seedlings were analyzed in two biological repeats. *P* values were calculated with a two-tailed Student *t*-test using Excel software.

BES-1 dephosphorylation assay

For BES1 dephosphorylation studies, thirty to sixty 5-day-old *Arabidopsis* seedlings grown on BL and new BR derivatives in continuous light were used. DMSO was used as the control solvent. The protein extraction and western blot analysis were

carried out as previously described.³³ Endogenous BES1 was detected using rabbit polyclonal anti-BES1 antibodies (1:1000)³⁴ and HRP-conjugated anti-rabbit antibodies (1:10 000; NA934, GE Healthcare). Signals were detected using ECL (ECL plus, GE Healthcare).

Cell cultures

The screening cell lines: T-lymphoblastic leukemia CEM; breast carcinoma MCF7 (estrogen-sensitive); cervical carcinoma cell line HeLa; and human foreskin fibroblasts BJ were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma, MO, USA). Media used were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO₂ in a humid environment. The cells were subcultured twice or thrice a week using the standard trypsinization procedure.

Calcein AM cytotoxicity assay

Suspensions with approximately 1.0×10^5 cells per mL were distributed in 96-well microtiter plates and after 24 h of stabilization the BR analogues tested were added at the desired concentrations in DMSO. Control cultures were treated with DMSO alone, and the final concentration of DMSO in the reaction mixture never exceeded 0.6%. In most cases, six serial 3-fold dilutions of the test substances were added at time zero in 20 µl aliquots to the microtiter plate wells and the highest final concentration in the wells was 50 µM. After incubation for 72 h, calcein AM solution (2 µM, Molecular Probes) was added and the cells were incubated for an additional hour. The fluorescence of the viable cells was then quantified using a Fluoroskan Ascent instrument (LabSystems, Finland). The percentage of the surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from the control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC₅₀ values, the concentrations of the respective compounds that were lethal to 50% of the tumor cells, were calculated.

Conclusions

Several novel brassinosteroid 23-phenyl analogues were synthesized based on molecular docking into the BRI1 receptor. The introduction of a phenyl group with no or small non-polar substituents (fluorine, chlorine, methyl) resulted in new compounds with plant growth promoting activities comparable with natural brassinosteroids. The results of biological screenings showed that molecular docking into BRI1 is a powerful tool for prediction and design of new compounds with strong brassinosteroid activities. New active compounds might be good candidates for potential application in agriculture to improve growth and yield or to increase the resistance of plants against various biotic and abiotic stresses. Recent

progress in the chemical synthesis also leads to overcoming economic restrictions, which are currently major constraints for using BRs at a large scale in the field.

Author contribution

M. Kvasnica – chemical synthesis; J. Oklestkova, P. Kofinková, J. Mikulik, M. Strnad, Q. Lu, E. Russinova – screening of biological activities on plants; L. Rárová – screening of cytotoxicity; M. Budesinsky, T. Beres – analysis of compounds prepared; V. Bazgier, K. Berka – molecular docking.

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Příloha 6

Synthesis of Novel Aryl Brassinosteroids through Alkene Cross-Metathesis and Preliminary Biological Study

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Synthesis of novel aryl brassinosteroids through alkene cross-metathesis and preliminary biological study



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ABSTRACT

A series of phenyl analogues of brassinosteroids was prepared via alkene cross-metathesis using commercially available styrenes and 24-nor-5 α -chola-2,22-dien-6-one. All derivatives were successfully docked into the active site of BR11 using AutoDock Vina. Plant growth promoting activity was measured using the pea inhibition biotest and *Arabidopsis* root sensitivity assay and then was compared with naturally occurring brassinosteroids. Differences in the production of plant hormone ethylene were also observed in etiolated pea seedlings after treatment with the new and also five known brassinosteroid phenyl analogues. Antiproliferative activity was also studied using normal human fibroblast and human cancer cell lines.

1. Introduction

Brassinosteroids (BRs, Fig. 1) represent a large group of plant steroids with more than 70 structurally and functionally related compounds [1]. BRs have been found at low concentrations throughout the plant kingdom, widely distributed in higher and lower plants, and have been detected in various plant parts such as pollen, seeds, leaves, stems, roots, and flowers. They are essential for many aspects of plant growth and development, such as cell division, elongation and differentiation, pollen tube growth, seed germination, regulation of gene expression, enzyme activation and photosynthesis [2–5]. At the molecular level, BRs change the gene expression and the metabolism of nucleic acids and proteins. BRs have structures similar to those of animal steroid hormone. Unlike animals, plants perceive steroids at cell membrane, using the membrane-integral receptor kinase brassinosteroid insensitive 1 (BR1) [6–8]. The encoded protein, BR1, belongs to a large family of plant LRR (leucine-rich repeat) receptor-like kinases, characterized by an extracellular LRR domain, a single-pass transmembrane segment and a cytoplasmic kinase domain. BR1 has been established as an authentic brassinosteroid receptor by genetic and biochemical investigations [9]. Crystal structures of BR1 in both free (PDB ID: 3RIZ, 3RGX), and brassinolide-bound (PDB ID: 3RJ0, 3BRZ), forms are available, following

independent X-ray diffraction structural determinations by two groups [9,10]. The structure of the ligand-binding domain resembles a superhelix of 25 twisted LRRs. A 70-amino acid island domain between LRRs 21 and 22 folds back into the interior of the superhelix, creating a surface pocket where the brassinosteroids bind. These recently published structures of *Arabidopsis thaliana* BR1 enable the rational design of brassinosteroid-like antagonists and agonists. Recent studies [11,12] have indicated that molecular docking is a powerful tool to predict how effective incorporation of different functional groups into brassinosteroid skeleton is and to design new types of BRs with biological activities comparable to natural BRs [11].

The aim of our study is related to the synthesis of new aryl analogues of BRs by alkene cross-metathesis and to study of their biological properties. Alkene cross-metathesis was chosen for preparation of all aryl analogues as an efficient method for construction of the new side chains using different commercially available substituted styrenes. The biological activities of newly prepared derivatives were evaluated using plant bioassays (pea inhibition biotest and *Arabidopsis* root sensitivity bioassay) and Calcein AM cytotoxicity assay. All derivative structures were subjected to docking studies using AutoDock Vina [13] in order to analyze the results with theoretical studies.

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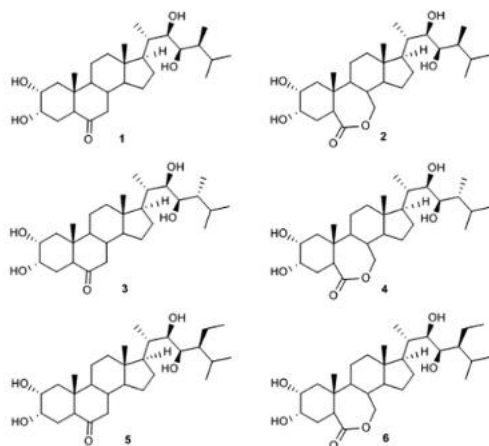


Fig. 1. Structures of most common natural brassinosteroids: castasterone (1), brassinolide (2), 24-epicastasterone (3), 24-epibrassinolide (4), 28-homocastasterone (5), 28-homobraslinolide (6).

2. Experimental

2.1. General methods

The melting points were determined on a Stuart SMP30 instrument (Bibby Scientific Ltd., UK). Elemental analyses were performed using an EA 1108 elemental analyzer (Fison Instruments); the values (C, H, N) agreed with the calculated values within acceptable limits. The infrared spectra were recorded on a Thermo Scientific Nicolet spectrometer iZ10 using the ATR technique. The wave numbers are given in cm^{-1} . The NMR spectra were taken on a JEOL JNM-ECA 500 (JEOL, Tokyo, Japan; ^1H , 500 MHz; ^{13}C , 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. ^1H NMR and ^{13}C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, ^1H δ = 0 ppm) or solvents: CDCl_3 (^1H δ = 7.26 ppm, ^{13}C δ = 77.00 ppm) or $\text{DMSO}-d_6$ (^1H δ = 2.46 ppm, ^{13}C δ = 40.00 ppm). Chemical shifts are given in ppm (δ -scale), coupling constants (J) in Hz. All values were obtained by first-order analysis. For API HRMS analysis, the samples were dissolved in chloroform (or chloroform: methanol; 1:1; v/v, in case of hydroxylated compounds) to a concentration $10 \mu\text{g}\cdot\text{mL}^{-1}$. The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample solution, placed into the ion source and analysed in fullscan mode. The source of the Synapt G2-Si Mass Spectrometer (Waters, Manchester, UK) was operated in positive ionisation mode (ASAP+), if not stated otherwise, at source temperature of 120°C . The Corona needle current was kept at $5 \mu\text{A}$ and the collision energy at value 4. The probe temperature was ramped up from 50°C to 600°C in 3 min. Data were acquired from 50 to 1000 Da with 1.0 s scan time in High Resolution Mode. The data were processed using the Masslynx 4.1 software (Waters). Mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. The HPLC system consisted of a Waters semi-preparative HPLC system including quaternary pump, liquid handler, UV–VIS and ELSD detectors. The semi preparative column was filled with silica gel. Reagents and solvents were purchased from Sigma–Aldrich and were not purified.

2.1.1. General procedure for cross metathesis

Hoveyda-Grubbs 2nd generation catalyst (19 mg; 0.03 mmol) was added to a solution of dien **7** (100 mg; 0.31 mmol) and styrene

(2.48 mmol) in dichloroethane (5 mL). The reaction mixture was heated at 80°C for 5 h. Then, another portion of H-G catalyst (19 mg; 0.03 mmol) was added and the reaction mixture was heated at 80°C for additional 5 h. Then, the solvent was evaporated and crude solid was purified by column chromatography on silica gel (mobile phase – 3% ethyl acetate in cyclohexane, R_f of products 0.18–0.25). In some cases, stated in each experiment, HPLC had to be used due to very close retention time of product and starting material (mobile phase – 0.5% ethyl acetate in cyclohexane).

2.1.2. (22E)-23-phenyl-24-nor-5 α -chola-2,22-dien-6-one (**8a**)

The general procedure with styrene afforded 120 mg (81%) of the title compound **8a** as a colorless oil. ^1H NMR (CDCl_3) δ 0.72, 0.74 (both s, 3H, CH_3), 1.14 (d, 3H, J = 6.4 Hz, CH_3), 1.69–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, J = 12.6, J' = 3.3 Hz), 2.23–2.31 (m, 2H), 2.34–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.06 (dd, 1H, J = 15.9, J' = 8.9 Hz, H-22), 6.30 (d, 1H, J = 15.9 Hz, H-23), 7.19 (m, 1H, Ar-H), 7.27–7.35 (m, 4H, 4 $\times$ Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.38 (C-21), 21.10, 21.70, 23.92, 28.21, 37.68, 39.35, 39.37, 40.04, 40.39, 42.92, 46.96, 53.40, 53.83, 55.83, 56.75, 124.49 (C-3), 124.95 (C-2), 125.92 (2 $\times$ C), 126.72, 127.42 (C-23), 128.45 (2 $\times$ C), 136.94 (C-22), 137.98, 211.98 (C-6). Spectral data in agreement with literature [11].

2.1.3. (22E)-23-(2-fluorophenyl)-24-nor-5 α -chola-2,22-dien-6-one (**8b**)

The general procedure with *o*-fluorostyrene afforded 84 mg (65%) of the title compound **9a** as a colorless oil: IR ν (cm^{-1}) 2930, 1702, 1655, 1593, 1560, 965. ^1H NMR (CDCl_3) δ 0.73, 0.75 (both s, 3H, CH_3), 1.15 (d, 3H, J = 6.7 Hz, CH_3), 1.72–1.81 (m, 2H), 1.97–2.06 (m, 4H), 2.08 (dt, 1H, J = 12.6, J' = 3.5 Hz), 2.24–2.33 (m, 2H), 2.34–2.38 (m, 2H), 5.58 (m, 1H, H-3), 5.70 (m, 1H, H-2), 6.14 (dd, 1H, J = 15.9, J' = 8.6 Hz, H-22), 6.47 (d, 1H, J = 15.9 Hz, H-23), 7.01 (ddd, J = 10.9, J' = 7.8, J'' = 0.9 Hz, Ar-H), 7.07 (td, 1H, J = 7.8, J' = 1.2 Hz, Ar-H), 7.16 (m, 1H, Ar-H), 7.41 (td, 1H, J = 7.8, J' = 1.8 Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.48 (C-19), 20.27 (C-21), 21.08, 21.70, 23.91, 28.17, 37.65, 39.32, 39.35, 40.01, 40.76, 42.92, 46.93, 53.37, 53.80, 55.68, 56.71, 115.56 (d, J = 22.8 Hz), 119.79 (d, J = 3.6 Hz), 123.91 (d, J = 3.6 Hz), 124.48 (C-3), 124.94 (C-2), 125.62 (d, J = 13.2 Hz), 126.92 (d, J = 3.6 Hz), 127.86 (d, J = 8.4 Hz), 139.50 (d, J = 3.6 Hz), 159.94 (d, J = 248.3 Hz, C-F), 211.90 (C-6). ^{19}F NMR (^1H) δ –118.83 (s, 1F). HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{38}\text{FO}$ ($[\text{M} + \text{H}]^+$) 421.2907, Found 421.2910.

2.1.4. (22E)-23-(3-fluorophenyl)-24-nor-5 α -chola-2,22-dien-6-one (**8c**)

The general procedure with *m*-fluorostyrene afforded 94 mg (73%) of the title compound **10a** as a colorless oil: IR ν (cm^{-1}) 2933, 1705, 1656, 1593, 1560, 966. ^1H NMR (CDCl_3) δ 0.73, 0.74 (both s, 3H, CH_3), 1.14 (d, 3H, J = 6.7 Hz, CH_3), 1.71–1.80 (m, 2H), 1.97–2.04 (m, 4H), 2.07 (dt, 1H, J = 12.5, J' = 3.2 Hz), 2.23–2.31 (m, 2H), 2.34–2.38 (m, 2H), 5.58 (m, 1H, H-3), 5.70 (m, 1H, H-2), 6.08 (dd, 1H, J = 15.7, J' = 8.7 Hz, H-22), 6.28 (d, 1H, J = 15.7 Hz, H-23), 6.88 (td, 1H, J = 8.3, J' = 2.6 Hz, Ar-H), 7.03 (m, 1H, Ar-H), 7.08 (b d, 1H, J = 8.3 Hz, Ar-H), 7.24 (td, 1H, J = 7.8, J' = 6.1 Hz, Ar-H). ^{13}C NMR δ 12.16 (C-18), 13.46 (C-19), 20.22 (C-21), 21.06, 21.68, 23.87, 28.16, 37.62, 39.30, 39.32, 39.98, 40.30, 42.90, 46.90, 53.33, 53.77, 55.67, 56.66, 112.25 (d, J = 21.6 Hz), 113.44 (d, J = 21.6 Hz), 121.81 (d, J = 2.4 Hz), 124.45 (C-3), 124.92 (C-2), 126.46 (d, J = 2.4 Hz), 129.79 (d, J = 8.4 Hz), 138.28, 140.35 (d, J = 7.2 Hz), 163.08 (d, J = 244.7 Hz, C-F), 211.82 (C-6). ^{19}F NMR (^1H) δ –113.78 (s, 1F). HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{38}\text{FO}$ ($[\text{M} + \text{H}]^+$) 421.2907, Found 421.2910.

2.1.5. (22E)-23-(4-fluorophenyl)-24-nor-5 α -chola-2,22-dien-6-one (**8d**)

The general procedure with *p*-fluorostyrene afforded 96 mg (75%) of the title compound **8** as a colorless oil. ^1H NMR (CDCl_3) δ 0.72, 0.73 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.7 Hz, CH_3), 1.71–1.80 (m, 2H),

1.96–2.04 (m, 4H), 2.08 (dt, 1H, $J = 12.5$, $J' = 3.2$ Hz), 2.22–2.31 (m, 2H), 2.33–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 5.97 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22), 6.26 (d, 1H, $J = 15.7$ Hz, H-23), 6.97 (m, 2H, 2 × Ar-H), 7.27 (m, 2H, 2 × Ar-H). ^{13}C NMR δ 12.17 (C-18), 13.48 (C-19), 20.35 (C-21), 21.08, 21.69, 23.88, 28.21, 37.65, 39.32, 39.34, 40.01, 40.33, 42.89, 46.92, 53.36, 53.80, 55.79, 56.71, 115.25 (d, $J = 20.4$ Hz, 2 × C), 124.47 (C-3), 124.93 (C-2), 126.25 (C-23), 127.28 (d, $J = 8.4$ Hz, 2 × C), 134.07 (d, $J = 3.6$ Hz), 136.65 (d, $J = 2.4$ Hz, C-22), 161.79 (d, $J = 244.7$ Hz, C-F), 211.93 (C-6). Spectral data in agreement with literature [11].

2.1.6. (22E)-23-(2-chlorophenyl)-24-nor-5 α -chola-2,22-dien-6-one (8e)

The general procedure with *o*-chlorostyrene afforded 86 mg (64%) of the title compound **11a** as a colorless oil: IR ν (cm^{-1}) 2941, 1701, 1655, 1593, 1563, 964. ^1H NMR (CDCl_3) δ 0.73, 0.75 (both s, 3H, CH_3); 1.16 (d, 3H, $J = 6.7$ Hz, CH_3); 1.71–1.81 (m, 2H); 1.96–2.04 (m, 4H); 2.08 (dt, 1H, $J = 12.5$, $J' = 3.2$ Hz); 2.22–2.33 (m, 2H); 2.34–2.38 (m, 2H); 5.58 (m, 1H, H-3); 5.70 (m, 1H, H-2); 6.04 (dd, 1H, $J = 15.6$, $J' = 8.9$ Hz, H-22); 6.69 (d, 1H, $J = 15.9$ Hz, H-23); 7.13 (m, 1H, $\Sigma J = 16.8$ Hz, Ar-H); 7.19 (m, 1H, $\Sigma J = 15.9$ Hz, Ar-H); 7.33 (dd, 1H, $J = 7.9$, $J' = 1.2$ Hz, Ar-H); 7.47 (dd, 1H, $J = 7.9$, $J' = 1.5$ Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.46 (C-19), 20.26 (C-21), 21.07, 21.68, 23.91, 28.17, 37.62, 39.30, 39.32, 39.98, 40.51, 42.93, 46.91, 53.34, 53.77, 55.63, 56.68, 123.80, 124.46 (C-3), 124.91 (C-2), 126.53, 126.64, 127.73, 129.52, 132.58, 136.00, 139.72, 211.87 (C-6). HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{38}\text{ClO}$ ($[\text{M} + \text{H}]^+$) 437.2611, Found 437.2614.

2.1.7. (22E)-23-(3-chlorophenyl)-24-nor-5 α -chola-2,22-dien-6-one (8f)

The general procedure with *m*-chlorostyrene afforded 94 mg (70%) of the title compound **12a** as a colorless oil: IR ν (cm^{-1}) 2944, 1701, 1654, 1593, 1562, 964. ^1H NMR (CDCl_3) δ 0.72, 0.73 (both s, 3H, CH_3); 1.13 (d, 3H, $J = 6.7$ Hz, CH_3); 1.70–1.80 (m, 2H); 1.96–2.05 (m, 4H); 2.07 (dt, 1H, $J = 12.5$, $J' = 3.2$ Hz); 2.22–2.31 (m, 2H); 2.33–2.37 (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.07 (dd, 1H, $J = 15.9$, $J' = 8.9$ Hz, H-22); 6.25 (d, 1H, $J = 15.9$ Hz, H-23); 7.14–7.22 (m, 3H, 3 × Ar-H); 7.31 (t, 1H, $J = 1.7$ Hz, Ar-H). ^{13}C NMR δ 12.18 (C-18), 13.48 (C-19), 20.24 (C-21), 21.08, 21.69, 23.89, 28.19, 37.64, 39.31, 39.33, 40.01, 40.39, 42.92, 46.92, 53.34, 53.79, 55.66, 56.68, 124.23, 124.47 (C-3), 124.94 (C-2), 126.22, 126.63, 127.74, 129.63, 134.36, 138.47, 139.85, 211.92 (C-6). HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{38}\text{ClO}$ ($[\text{M} + \text{H}]^+$) 437.2611, Found 437.2615.

2.1.8. (22E)-23-(4-chlorophenyl)-24-nor-5 α -chola-2,22-dien-6-one (8g)

The general procedure with *p*-chlorostyrene afforded 100 mg (75%) of the title compound **8** as a colorless oil. ^1H NMR (CDCl_3) δ 0.72, 0.73 (both s, 3H, CH_3), 1.13 (d, 3H, $J = 6.7$ Hz, CH_3), 1.70–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, $J = 12.5$, $J' = 3.4$ Hz), 2.22–2.30 (m, 2H), 2.34–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.03 (dd, 1H, $J = 15.9$, $J' = 8.9$ Hz, H-22), 6.27 (d, 1H, $J = 15.9$ Hz, H-23), 7.24 (s, 4H, 4 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.27 (C-21), 21.09, 21.70, 23.91, 28.21, 37.66, 39.33, 39.36, 40.04, 40.39, 42.93, 46.94, 53.37, 53.82, 55.75, 56.72, 124.49 (C-3), 124.96 (C-2), 126.30 (C-23), 127.12 (2 × C), 128.55 (2 × C), 132.22, 136.45, 137.64 (C-22), 211.96 (C-6). Spectral data in agreement with literature [11].

2.1.9. (22E)-23-(2-bromophenyl)-24-nor-5 α -chola-2,22-dien-6-one (8h)

The general procedure with *o*-bromostyrene afforded 87 mg (59%) of the title compound **13a** as a colorless oil: IR ν (cm^{-1}) 2944, 1699, 1654, 1593, 1564, 964. ^1H NMR (CDCl_3) δ 0.72, 0.75 (both s, 3H, CH_3); 1.15 (d, 3H, $J = 6.7$ Hz, CH_3); 1.71–1.81 (m, 2H); 1.96–2.04 (m, 4H); 2.08 (dt, 1H, $J = 12.5$, $J' = 3.1$ Hz); 2.22–2.38 (m, 4H); 5.57 (m, 1H, H-3); 5.69 (m, 1H, H-2); 5.99 (dd, 1H, $J = 15.6$, $J' = 8.9$ Hz, H-22); 6.63 (d, 1H, $J = 15.6$ Hz, H-23); 7.05 (m, 1H, $\Sigma J = 16.8$ Hz, Ar-H); 7.23 (m, 1H, $\Sigma J = 16.2$ Hz, Ar-H); 7.45 (dd, 1H, $J = 7.6$, $J' = 1.5$ Hz, Ar-H); 7.52 (m, 1H, $\Sigma J = 9.2$ Hz, Ar-H). ^{13}C NMR δ 12.24 (C-18), 13.51

(C-19), 20.28 (C-21), 21.10, 21.70, 23.96, 28.21, 37.66, 39.34, 39.36, 40.03, 40.47, 42.98, 46.95, 53.39, 53.83, 55.66, 56.72, 123.26, 124.50 (C-3), 124.96 (C-2), 126.50, 126.81, 127.33, 128.06, 132.77, 137.80, 139.93, 211.97 (C-6). HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{38}^{79}\text{BrO}$ ($[\text{M} + \text{H}]^+$) 481.2106, Found 481.2107.

2.1.10. (22E)-23-(3-bromophenyl)-24-nor-5 α -chola-2,22-dien-6-one (8i)

The general procedure with *m*-bromostyrene afforded 97 mg (66%) of the title compound **14a** as a colorless oil: IR ν (cm^{-1}) 2944, 1700, 1655, 1588, 1560, 964. ^1H NMR (CDCl_3) δ 0.72, 0.73 (both s, 3H, CH_3); 1.13 (d, 3H, $J = 6.7$ Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.06 (dt, 1H, $J = 12.5$, $J' = 3.4$ Hz); 2.22–2.31 (m, 2H); 2.34–2.37 (m, 2H); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.06 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22); 6.23 (d, 1H, $J = 15.7$ Hz, H-23); 7.14 (t, 1H, $J = 7.8$ Hz, Ar-H); 7.22 (m, 1H, Ar-H); 7.30 (m, 1H, Ar-H); 7.47 (t, 1H, $J = 1.7$ Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.25 (C-21), 21.09, 21.70, 23.91, 28.20, 37.66, 39.34, 39.36, 40.03, 40.41, 42.95, 46.94, 53.37, 53.82, 55.69, 56.71, 122.69, 124.48 (C-3), 124.69, 124.96 (C-2), 126.15, 128.70, 129.56, 129.95, 138.55, 140.17, 211.92 (C-6). HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{38}^{79}\text{BrO}$ ($[\text{M} + \text{H}]^+$) 481.2106, Found 481.2108.

2.1.11. (22E)-23-(4-bromophenyl)-24-nor-5 α -chola-2,22-dien-6-one (8j)

The general procedure with *p*-bromostyrene afforded 107 mg (73%) of the title compound **8** as a colorless oil. ^1H NMR (CDCl_3) δ 0.72, 0.73 (both s, 3H, CH_3), 1.13 (d, 3H, $J = 6.7$ Hz, CH_3), 1.70–1.79 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, $J = 12.5$, $J' = 3.3$ Hz), 2.22–2.30 (m, 2H), 2.33–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.05 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22), 6.24 (d, 1H, $J = 15.7$ Hz, H-23), 7.18 (m, 2H, 2 × Ar-H), 7.39 (m, 2H, 2 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.49 (C-19), 20.24 (C-21), 21.08, 21.70, 23.90, 28.19, 37.65, 39.32, 39.35, 40.02, 40.40, 42.92, 46.93, 53.36, 53.81, 55.71, 56.70, 120.30, 124.47 (C-3), 124.95 (C-2), 126.33 (C-23), 127.47 (2 × C), 131.48 (2 × C), 136.89, 137.77 (C-22), 211.92 (C-6). Spectral data in agreement with literature [11].

2.1.12. (22E)-23-(2-methylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8k)

The general procedure with *o*-methylstyrene afforded 83 mg (65%) of the title compound **15a** as a colorless oil: IR ν (cm^{-1}) 2932, 1701, 1653, 1593, 1563, 964. ^1H NMR (CDCl_3) δ 0.72, 0.74 (both s, 3H, CH_3); 1.14 (d, 3H, $J = 6.7$ Hz, CH_3); 1.71–1.80 (m, 2H); 1.96–2.04 (m, 4H); 2.07 (dt, 1H, $J = 12.6$, $J' = 3.6$ Hz); 2.22–2.31 (m, 2H); 2.32 (s, 3H, Ar- CH_3); 2.33–2.37 (m, 2H); 5.57 (m, 1H, H-3); 5.69 (m, 1H, $J = 15.6$ Hz, H-2); 5.89 (dd, 1H, $J = 15.6$, $J' = 8.9$ Hz, H-22); 6.49 (d, 1H, $J = 15.6$ Hz, H-23); 7.10–7.15 (m, 3H, 3 × Ar-H); 7.37 (d, 1H, $J = 7.3$ Hz, Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.48 (C-19), 19.58 (Ar- CH_3), 20.53 (C-21), 21.08, 21.69, 23.92, 28.30, 37.65, 39.31, 39.34, 40.01, 40.72, 42.87, 46.93, 53.35, 53.78, 55.71, 56.73, 124.48 (C-3), 124.93 (C-2), 125.28, 125.45, 125.92, 126.67, 130.06, 134.94, 137.15, 138.43, 211.98 (C-6). HRMS: (API+) calculated for $\text{C}_{30}\text{H}_{41}\text{O}$ ($[\text{M} + \text{H}]^+$) 417.3157, Found 417.3160.

2.1.13. (22E)-23-(3-methylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8l)

The general procedure with *m*-methylstyrene afforded 88 mg (69%) of the title compound **16a** as a colorless oil: IR ν (cm^{-1}) 2933, 1700, 1653, 1593, 1562, 964. ^1H NMR (CDCl_3) δ 0.71, 0.73 (both s, 3H, CH_3); 1.12 (d, 3H, $J = 6.7$ Hz, CH_3); 1.71–1.79 (m, 2H); 1.95–2.04 (m, 4H); 2.06 (dt, 1H, $J = 12.6$, $J' = 3.5$ Hz); 2.20–2.30 (m, 2H); 2.32 (s, 3H, Ar- CH_3); 2.33–2.37 (m, 2H); 5.57 (m, 1H, H-3); 5.68 (m, 1H, H-2); 6.04 (dd, 1H, $J = 15.9$, $J' = 8.9$ Hz, H-22); 6.27 (d, 1H, $J = 15.9$ Hz, H-23); 7.00 (d, v, $J = 7.3$ Hz, Ar-H); 7.11–7.19 (m, 3H, 3 × Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.49 (C-19), 20.40 (C-21), 21.09, 21.40 (Ar- CH_3), 21.70, 23.90, 28.20, 37.66, 39.32, 39.35, 40.03, 40.40, 42.88, 46.94, 53.37, 53.80, 55.82, 56.73, 123.09, 124.49 (C-3), 124.94 (C-2), 126.60, 127.45, 127.51, 128.36, 136.73, 137.89, 137.95, 211.99 (C-6). HRMS: (API+) calculated for $\text{C}_{30}\text{H}_{41}\text{O}$ ($[\text{M} + \text{H}]^+$) 417.3157, Found 417.3161.

2.1.14. (22E)-23-(4-methylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8m)

The general procedure with *p*-methylstyrene afforded 91 mg (71%) of the title compound **8** as a colorless oil. ^1H NMR (CDCl_3) δ 0.72, 0.73 (both s, 3H, CH_3), 1.12 (d, 3H, $J = 6.7$ Hz, CH_3), 1.70–1.79 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, $J = 12.5$, $J' = 3.4$ Hz), 2.22–2.30 (m, 2H), 2.32 (s, 3H, CH_3), 2.33–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.00 (dd, 1H, $J = 15.9$, $J' = 8.9$ Hz, H-22), 6.27 (d, 1H, $J = 15.9$ Hz, H-23), 7.09 (d, 2H, $J = 7.9$ Hz, $2 \times \text{Ar-H}$), 7.22 (d, 2H, $J = 7.9$ Hz, $2 \times \text{Ar-H}$). ^{13}C NMR δ 12.19 (C-18), 13.49 (C-19), 20.42 (C-21), 21.09 (CH_2 , Ar- CH_3), 21.70, 23.90, 28.19, 37.68, 39.34, 39.36, 40.04, 40.34, 42.88, 46.95, 53.39, 53.81, 55.89, 56.75, 124.50 (C-3), 124.95 (C-2), 125.80 ($2 \times \text{C}$), 127.21 (C-23), 129.13 ($2 \times \text{C}$), 135.18, 135.94 (C-22), 136.40, 212.00 (C-6). Spectral data in agreement with literature [11].

2.1.15. (22E)-23-(2-trifluoromethylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8n)

The general procedure with *o*-trifluoromethylstyrene afforded 82 mg (57%) of the title compound **17a** as a colorless oil: IR ν (cm^{-1}) 2942, 1701, 1653, 1593, 1560, 1128, 964. ^1H NMR (CDCl_3) δ 0.73, 0.76 (both s, 3H, CH_3), 1.15 (d, 3H, $J = 6.7$ Hz, CH_3), 1.71–1.81 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, $J = 12.6$, $J' = 3.6$ Hz), 2.23–2.31 (m, 2H), 2.31–2.38 (m, 2H), 5.58 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.00 (dd, 1H, $J = 15.6$, $J' = 8.9$ Hz, H-22); 6.68 (qd, 1H, $J = 15.6$, $J' = 2.1$ Hz, H-23); 7.29 (t, 1H, $J = 7.7$ Hz, Ar-H); 7.46 (t, 1H, $J = 7.7$ Hz, Ar-H); 7.55 (d, 1H, $J = 7.7$ Hz, Ar-H); 7.60 (d, 1H, $J = 7.7$ Hz, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.48 (C-19), 20.17 (C-21), 21.08, 21.70, 23.94, 28.02, 37.63, 39.32 ($2 \times \text{C}$), 40.00, 40.59, 42.95, 46.92, 53.37, 53.80, 55.68, 56.69, 123.72, 124.38 (q, $J = 273.5$ Hz), 124.47 (C-3), 124.93 (C-2), 125.58 (q, $J = 6.0$ Hz), 126.44, 126.92 (q, $J = 30.0$ Hz), 127.28, 131.65, 137.26, 141.19, 211.90 (C-6). ^{19}F NMR (^1H) δ -59.56 (s, 1F). HRMS: (API+) calculated for $\text{C}_{30}\text{H}_{38}\text{F}_3\text{O}$ ([M+H] $^+$) 471.2875, Found 471.2876.

2.1.16. (22E)-23-(3-trifluoromethylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8o)

The general procedure with *m*-trifluoromethylstyrene afforded 95 mg (66%) of the title compound **18** as a colorless oil: IR ν (cm^{-1}) 2940, 1701, 1655, 1593, 1561, 1124, 964. ^1H NMR (CDCl_3) δ 0.72, 0.74 (both s, 3H, CH_3), 1.14 (d, 3H, $J = 6.7$ Hz, CH_3), 1.71–1.80 (m, 2H), 1.97–2.05 (m, 4H), 2.07 (dt, 1H, $J = 12.6$, $J' = 3.3$ Hz), 2.22–2.32 (m, 2H), 2.34–2.37 (m, 2H), 5.58 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.14 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22); 6.34 (d, 1H, $J = 15.7$ Hz, H-23); 7.37–7.44 (m, 2H, $2 \times \text{Ar-H}$); 7.48 (d, 1H, $J = 7.3$ Hz, Ar-H), 7.56 (b s, 1H, Ar-H). ^{13}C NMR δ 12.19 (C-18), 13.50 (C-19), 20.24 (C-21), 21.09, 21.70, 23.91, 28.23, 37.66, 39.36, 39.37, 40.03, 40.48, 42.96, 46.93, 53.37, 53.82, 55.64, 56.71, 122.52 (q, $J = 3.6$ Hz), 123.26 (q, $J = 3.6$ Hz), 124.19 (q, $J = 272.3$ Hz), 124.48 (C-3), 124.96 (C-2), 126.27, 128.85, 129.14, 130.81 (q, $J = 32.4$ Hz), 138.71, 138.90, 211.92 (C-6). ^{19}F NMR (^1H) δ -62.59 (s, 1F). HRMS: (API+) calculated for $\text{C}_{30}\text{H}_{38}\text{F}_3\text{O}$ ([M+H] $^+$) 471.2875, Found 471.2879.

2.1.17. (22E)-23-(4-trifluoromethylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8p)

The general procedure with *p*-trifluoromethylstyrene afforded 102 mg (71%) of the title compound **19a** as a colorless oil: IR ν (cm^{-1}) 2940, 1702, 1653, 1593, 1562, 1127, 963. ^1H NMR (CDCl_3) δ 0.72, 0.74 (both s, 3H, CH_3), 1.15 (d, 3H, $J = 6.7$ Hz, CH_3), 1.71–1.81 (m, 2H), 1.96–2.05 (m, 4H), 2.07 (dt, 1H, $J = 12.5$, $J' = 3.4$ Hz), 2.21–2.31 (m, 2H), 2.34–2.37 (m, 2H), 5.58 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.17 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22); 6.34 (d, 1H, $J = 15.7$ Hz, H-23); 7.41 (d, 2H, $J = 8.4$ Hz, $2 \times \text{Ar-H}$); 7.53 (d, 2H, $J = 8.4$ Hz, $2 \times \text{Ar-H}$). ^{13}C NMR δ 12.21 (C-18), 13.51 (C-19), 20.19 (C-21), 21.10, 21.70, 23.92, 28.19, 37.67, 39.34, 39.37, 40.04, 40.49, 42.99, 46.94, 53.38, 53.83, 55.85, 56.70, 124.28 (q, $J = 271.5$ Hz), 124.48 (C-3), 124.97 (C-

2), 125.38, 125.40 (q, $J = 3.6$ Hz, $2 \times \text{C}$), 126.04 ($2 \times \text{C}$), 126.35, 128.56 (q, $J = 32.4$ Hz), 139.66, 211.91 (C-6). ^{19}F NMR (^1H) δ -62.25 (s, 1F). HRMS: (API+) calculated for $\text{C}_{30}\text{H}_{38}\text{F}_3\text{O}$ ([M+H] $^+$) 471.2875, Found 471.2879.

2.1.18. (22E)-23-(2,4-dimethylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8q)

The general procedure with 2,4-dimethylstyrene and purification on HPLC afforded 81 mg (61%) of the title compound **20a** as a colorless oil: IR ν (cm^{-1}) 2935, 1701, 1652, 1593, 1560, 964. ^1H NMR (CDCl_3) δ 0.72, 0.74 (both s, 3H, CH_3), 1.13 (d, 3H, $J = 6.7$ Hz, CH_3), 1.71–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.07 (dt, 1H, $J = 12.5$, $J' = 3.4$ Hz), 2.22–2.37 (m, 4H), 2.28 (s, 6H, $2 \times \text{Ar-CH}_3$), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 5.85 (dd, 1H, $J = 15.6$, $J' = 8.9$ Hz, H-22); 6.45 (d, 1H, $J = 15.6$ Hz, H-23); 6.94 (s, 1H, Ar-H); 6.95 (d, 1H, $J = 8.3$ Hz, Ar-H); 7.27 (d, 1H, $J = 8.3$ Hz, Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.48 (C-19), 19.76 (Ar- CH_3), 20.57 (C-21), 20.97 (Ar- CH_3), 21.08, 21.70, 23.92, 28.28, 37.66, 39.32, 39.35, 40.02, 40.68, 42.85, 46.93, 53.37, 53.79, 55.78, 56.75, 124.48 (C-3), 124.93 (C-2), 125.09, 125.38, 126.64, 130.84, 134.29, 134.77, 136.28, 137.58, 211.98 (C-6). HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{43}\text{O}$ ([M+H] $^+$) 431.3314, Found 431.3318.

2.1.19. (22E)-23-(2,5-dimethylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8r)

The general procedure with 2,5-dimethylstyrene and purification on HPLC afforded 77 mg (58%) of the title compound **21a** as a colorless oil: IR ν (cm^{-1}) 2931, 1699, 1653, 1593, 1560, 963. ^1H NMR (CDCl_3) δ 0.72, 0.74 (both s, 3H, CH_3), 1.13 (d, 3H, $J = 6.7$ Hz, CH_3), 1.71–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.08 (dt, 1H, $J = 12.5$, $J' = 3.3$ Hz), 2.22–2.31 (m, 2H), 2.27, 2.30 (both s, 3H, Ar- CH_3), 2.32–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 5.88 (dd, 1H, $J = 15.6$, $J' = 8.9$ Hz, H-22); 6.46 (d, 1H, $J = 15.6$ Hz, H-23); 6.92 (dd, 1H, $J = 7.6$, $J' = 1.2$ Hz, Ar-H); 7.00 (d, 1H, $J = 7.6$ Hz, Ar-H); 7.18 (b s, 1H, Ar-H). ^{13}C NMR δ 12.20 (C-18), 13.48 (C-19), 19.36 (Ar- CH_3), 20.57 (C-21), 21.00 (Ar- CH_3), 21.09, 21.70, 23.92, 28.31, 37.66, 39.33, 39.37, 40.02, 40.73, 42.87, 46.93, 53.37, 53.80, 55.74, 56.77, 124.48 (C-3), 124.94 (C-2), 125.37, 126.07, 127.43, 130.01, 131.89, 135.23, 136.88, 138.12, 211.95 (C-6). HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{43}\text{O}$ ([M+H] $^+$) 431.3314, Found 431.3318.

2.1.20. (22E)-23-(4-*t*-butylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8s)

The general procedure with *p*-*t*-butylstyrene and purification on HPLC afforded 84 mg (60%) of the title compound **22a** as a colorless oil: IR ν (cm^{-1}) 2939, 1702, 1651, 1593, 1564, 1372, 963. ^1H NMR (CDCl_3) δ 0.73, 0.74 (both s, 3H, CH_3), 1.13 (d, 3H, $J = 6.7$ Hz, CH_3), 1.32 (s, 9H, *t*-Bu), 1.71–1.80 (m, 2H), 1.97–2.05 (m, 4H), 2.08 (dt, 1H, $J = 12.7$, $J' = 3.4$ Hz), 2.23–2.32 (m, 2H), 2.35–2.38 (m, 2H), 5.59 (m, 1H, H-3), 5.70 (m, 1H, H-2), 6.03 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22); 6.30 (d, 1H, $J = 15.7$ Hz, H-23); 7.27–7.29 (m, 2H, $2 \times \text{Ar-H}$); 7.32–7.34 (m, 2H, $2 \times \text{Ar-H}$). ^{13}C NMR δ 12.18 (C-18), 13.48 (C-19), 20.46 (C-21), 21.08, 21.70, 23.90, 28.17, 31.28 ($3 \times \text{C}$, *t*-Bu), 37.66, 39.32, 39.35, 40.02, 40.39, 42.86, 46.94, 53.38, 53.80, 55.84, 56.74, 124.48 (C-3), 124.94 (C-2), 125.35 ($2 \times \text{C}$), 125.60 ($2 \times \text{C}$), 127.09, 135.17, 136.17, 149.72, 211.97 (C-6). HRMS: (API+) calculated for $\text{C}_{33}\text{H}_{47}\text{O}$ ([M+H] $^+$) 459.3627, Found 459.3629.

2.1.21. (22E)-23-(4-phenylphenyl)-24-nor-5 α -chola-2,22-dien-6-one (8t)

The general procedure with *p*-phenylstyrene afforded 47 mg (32%) of the title compound **23a** as a colorless oil: IR ν (cm^{-1}) 2949, 1700, 1654, 1593, 1565, 1465, 960. ^1H NMR (CDCl_3) δ 0.72, 0.75 (both s, 3H, CH_3), 1.15 (d, 3H, $J = 6.7$ Hz, CH_3), 1.71–1.80 (m, 2H), 1.96–2.04 (m, 4H), 2.08 (dt, 1H, $J = 12.5$, $J' = 3.1$ Hz), 2.22–2.31 (m, 2H), 2.33–2.37 (m, 2H), 5.57 (m, 1H, H-3), 5.69 (m, 1H, H-2), 6.11 (dd, 1H, $J = 15.7$, $J' = 8.7$ Hz, H-22); 6.34 (d, 1H, $J = 15.7$ Hz, H-23); 7.33 (m, 1H, Ar-H); 7.39–7.44 (m, 4H, $4 \times \text{Ar-H}$); 7.52–7.54 (m, 2H, $2 \times \text{Ar-H}$); 7.57–7.60 (m, 2H, $2 \times \text{Ar-H}$). ^{13}C NMR δ 12.20 (C-18), 13.50 (C-19),

20.39 (C-21), 21.10, 21.70, 23.92, 28.21, 37.68, 39.34, 39.37, 40.04, 40.47, 42.93, 46.95, 53.39, 53.82, 55.84, 56.74, 124.50 (C-3), 124.96 (C-2), 126.32 (2 × C), 126.86 (2 × C), 126.98, 127.12, 127.16 (2 × C), 128.73 (2 × C), 137.04, 137.15, 139.51, 140.83, 211.98 (C-6). HRMS: (API+) calculated for $C_{35}H_{43}O$ ([M+H]⁺) 479.3314, Found 479.3317.

2.1.22. (22E)-23-(2-isopropoxyphenyl)-24-nor-5α-cholan-2,22-dien-6-one (9)

The general procedure with different *bis-ortho substituted* styrene or without styrene afforded 20 mg (14%) of the title compound **9** as a colorless oil: IR ν (cm⁻¹) 2940, 1701, 1653, 1591, 1561, 963. ¹H NMR (CDCl₃) δ 0.72, 0.74 (both s, 3H, CH₃); 1.14 (d, 3H, *J* = 6.7 Hz, CH₃); 1.34 (d, 3H, *J* = 6.1 Hz, CH(CH₃)₂); 1.35 (d, 3H, *J* = 6.1 Hz, CH(CH₃)₂); 1.71–1.81 (m, 2H); 1.96–2.03 (m, 4H); 2.08 (dt, 1H, *J* = 12.5, *J'* = 3.2 Hz); 2.22–2.31 (m, 2H); 2.33–2.37 (m, 2H); 4.49 (septet, 1H, *J* = 6.1 Hz, OCH(CH₃)₂); 5.58 (m, 1H, H-3); 5.69 (m, 1H, H-2); 6.03 (dd, 1H, *J* = 15.9, *J'* = 8.9 Hz, H-22); 6.62 (d, 1H, *J* = 15.9 Hz, H-23); 6.85–6.90 (m, 2H); 7.13 (m, 1H); 7.40 (dd, 1H, *J* = 7.6, *J'* = 1.5 Hz). ¹³C NMR δ 12.21 (C-18), 13.50 (C-19), 20.51 (C-21), 21.10, 21.70, 22.25 (2 × C, CH(CH₃)₂), 23.94, 28.21, 37.69, 39.34, 39.37, 40.05, 40.70, 42.89, 46.97, 53.41, 53.82, 55.94, 56.77, 71.04 (OCH(CH₃)₂), 114.70, 120.71, 122.32, 124.51 (C-3), 124.94 (C-2), 126.31, 127.49, 128.43, 137.13, 154.79, 212.05 (C-6). HRMS: (API+) calculated for $C_{32}H_{45}O_2$ ([M+H]⁺) 461.3420, Found 461.3423.

2.2. General procedure for dihydroxylation of dienes

To a solution of diene (0.2 mmol); hydroquinidine 4-chlorobenzoate (23 mg; 0.049 mmol); methansulfonamide (33 mg; 0.35 mmol); potassium carbonate (140 mg; 1.01 mmol); and potassium ferricyanide (350 mg; 1.06 mmol) in the mixture of *t*-butanol and water (15 mL; 1:1 v/v) was added 0.1 mL of osmium tetroxide in *t*-butanol (1 g/20 mL; 0.02 mmol). Reaction mixture was stirred at room temperature for 24 h. A saturated solution of sodium sulfite (3 mL) was then added. After an additional 30 min of stirring, the reaction mixture was diluted with ethyl acetate (30 mL) and extracted with water (2 × 20 mL). The combined organic fractions were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Column chromatography on silica gel (mobile phase – MeOH/CHCl₃ – 1/16) gave the desired product.

Compounds **10a** (m. p. 269–271 °C, EtOH), **10d** (m. p. 276–278 °C, EtOH), **10g** (m. p. 251–253 °C, EtOH), **10j** (m. p. 248–249 °C, *i*-PrOH), and **10m** (m. p. 271–273 °C, EtOH) were prepared according to general procedure which is also described in literature [11]. All data for these compounds are in agreement with same literature.

2.2.1. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2-fluorophenyl)-24-nor-5α-cholan-6-one (10b)

The general procedure for dihydroxylation of **8b** (60 mg; 0.14 mmol) afforded 54 mg (77%) of the title compound **10b** as a white solid: m. p. 288–290 °C (EtOH); IR ν (cm⁻¹) 3348 vb, 2941, 1710, 1620, 1587, 1492, 757. ¹H NMR (DMSO-*d*₆) δ 0.34 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.86 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.80 (m, 1H); 1.87 (m, 1H); 1.98 (m, 1H); 2.07 (t, 1H, *J* = 12.6 Hz); 2.58 (d, 1H, *J* = 12.4 Hz); 3.44 (m, 1H, H-2); 3.58 (m, 1H, H-3); 3.74 (m, 1H, H-22); 4.18 (br s, 1H, OH); 4.31 (d, 1H, *J* = 5.8 Hz, OH); 4.62 (d, 1H, *J* = 1.8 Hz, OH); 4.66 (d, 1H, *J* = 8.3 Hz, H-23); 5.25 (br s, 1H, OH); 7.12 (m, 1H); 7.18 (m, 1H); 7.29 (m, 1H); 7.38 (m, 1H). ¹³C NMR δ 11.32 (C-18), 12.51 (C-19), 13.30 (C-21), 20.73, 23.27, 26.73, 27.21, 36.77, 36.98, 39.06, 40.10, 41.79, 42.00, 45.94, 50.25, 51.89, 52.86, 55.93, 67.08 (C-2), 67.45 (C-3), 68.48 (C-23), 75.65 (C-22), 114.92 (d, *J* = 22.8 Hz), 124.31 (d, *J* = 3.6 Hz), 128.82 (d, *J* = 3.6 Hz), 128.87, 130.01 (d, *J* = 14.2 Hz), 159.61 (d, *J* = 243.5 Hz, C-F), 211.39 (C-6). ¹⁹F NMR {¹H} δ –118.49 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{42}FO_5$ ([M+H]⁺) 489.3016, Found 489.3017. Anal. Calcd for $C_{29}H_{41}FO_5$: C, 71.28; H, 8.46. Found: C, 71.30; H, 8.45%.

2.2.2. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(3-fluorophenyl)-24-nor-5α-cholan-6-one (10c)

The general procedure for dihydroxylation of **8c** (60 mg; 0.14 mmol) afforded 59 mg (85%) of the title compound **10c** as a white solid: m. p. 278–279 °C (EtOH); IR ν (cm⁻¹) 3334 vb, 2939, 1710, 1615, 1591, 1461, 763. ¹H NMR (DMSO-*d*₆) δ 0.34 (H-18), 0.59 (H-19) (both s, 3H, CH₃); 0.86 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.80 (m, 1H); 1.86 (m, 1H); 1.98 (dd, 1H, *J* = 12.8, *J'* = 4.4 Hz); 2.07 (t, 1H, *J* = 12.8 Hz); 2.57 (dd, 1H, *J* = 12.0, *J'* = 3.0 Hz); 3.43–3.48 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.18 (d, 1H, *J* = 2.4 Hz, OH); 4.32 (d, 1H, *J* = 6.1 Hz, OH); 4.39 (dd, 1H, *J* = 7.9, *J'* = 3.4 Hz, H-23); 4.57 (d, 1H, *J* = 4.3 Hz, OH); 5.24 (d, 1H, *J* = 4.0 Hz, OH); 7.04–7.12 (m, 3H); 7.35 (m, 1H). ¹³C NMR δ 11.38 (C-18), 12.53 (C-19), 13.35 (C-21), 20.78, 23.28, 26.81, 27.25, 36.48, 37.01, 39.08, 40.09, 41.83, 42.05, 45.94, 50.28, 51.88, 52.85, 55.94, 67.09 (C-2), 67.48 (C-3), 74.56 (C-23), 76.17 (C-22), 113.46 (d, *J* = 21.6 Hz), 113.88 (d, *J* = 21.6 Hz), 123.05, 129.96 (d, *J* = 8.4 Hz), 146.53 (d, *J* = 7.2 Hz), 162.04 (d, *J* = 242.3 Hz, C-F), 211.54 (C-6). ¹⁹F NMR {¹H} δ –113.41 (s, 1F). HRMS: (API+) calculated for $C_{29}H_{41}FO_5$ ([M+H]⁺) 489.3016, Found 489.3017. Anal. Calcd for $C_{29}H_{41}FO_5$: C, 71.28; H, 8.46. Found: C, 71.26; H, 8.49%.

2.2.3. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2-chlorophenyl)-24-nor-5α-cholan-6-one (10e)

The general procedure for dihydroxylation of **8e** (60 mg; 0.14 mmol) afforded 54 mg (78%) of the title compound **10e** as a white solid: m. p. 290–292 °C (EtOH); IR ν (cm⁻¹) 3307 vb, 2936, 1693, 1595, 1572, 1463, 760. ¹H NMR (DMSO-*d*₆) δ 0.38 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.93 (d, 3H, *J* = 6.7 Hz, H-21, CH₃); 1.79 (m, 1H); 1.89 (m, 1H); 1.98 (dd, 1H, *J* = 13.4, *J'* = 4.7 Hz); 2.07 (t, 1H, *J* = 12.3 Hz); 2.58 (dd, 1H, *J* = 12.3, *J'* = 3.4 Hz); 3.45 (m, 1H, H-2); 3.58 (bd, 1H, *J* = 8.0 Hz, H-3); 3.74 (m, 1H, H-22); 4.18 (bd, 1H, *J* = 2.4 Hz, OH); 4.31 (d, 1H, *J* = 6.1 Hz, OH); 4.58 (bd, 1H, *J* = 3.4 Hz, H-23); 4.79 (d, 1H, *J* = 7.3 Hz, OH); 5.28 (bs, 1H, OH); 7.26 (m, 1H); 7.34 (td, 1H, *J* = 7.5, *J'* = 1.2 Hz); 7.39 (dd, 1H, *J* = 7.9, *J'* = 1.2 Hz); 7.44 (dd, 1H, *J* = 7.8, *J'* = 1.7 Hz). ¹³C NMR δ 11.37 (C-18), 13.36 (C-19), 13.39 (C-21), 20.76, 23.33, 26.81, 27.40, 37.01, 37.36, 39.04, 40.10, 41.82, 42.08, 45.93, 50.26, 52.13, 52.84, 55.93, 67.09 (C-2), 67.47 (C-3), 71.00 (C-23), 75.66 (C-22), 127.19, 128.65, 128.92, 129.13, 131.92, 141.13, 211.54 (C-6). HRMS: (API+) calculated for $C_{29}H_{42}ClO_5$ ([M+H]⁺) 505.2721, Found 505.2720. Anal. Calcd for $C_{29}H_{41}ClO_5$: C, 68.96; H, 8.18. Found: C, 68.93; H, 8.19%.

2.2.4. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(3-chlorophenyl)-24-nor-5α-cholan-6-one (10f)

The general procedure for dihydroxylation of **8f** (60 mg; 0.14 mmol) afforded 56 mg (80%) of the title compound **10f** as a white solid: m. p. 269–270 °C (EtOH); IR ν (cm⁻¹) 3257 vb, 2936, 1709, 1600, 1576, 1461, 781, 699. ¹H NMR (DMSO-*d*₆) δ 0.35 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.86 (d, 3H, *J* = 6.7 Hz, H-21, CH₃); 1.79 (m, 1H); 1.87 (m, 1H); 1.96 (dd, 1H, *J* = 13.4, *J'* = 4.6 Hz); 2.07 (t, 1H, *J* = 12.3 Hz); 2.58 (dd, 1H, *J* = 12.3, *J'* = 3.2 Hz); 3.43–3.46 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.18 (bd, 1H, *J* = 2.1 Hz, OH); 4.33 (d, 1H, *J* = 5.8 Hz, OH); 4.38 (bd, 1H, *J* = 8.3 Hz, H-23); 4.59 (d, 1H, *J* = 3.4 Hz, OH); 5.26 (bs, 1H, OH); 7.23 (m, 1H); 7.29–7.31 (m, 2H); 7.35 (t, 1H, *J* = 7.6 Hz). ¹³C NMR δ 11.44 (C-18), 12.58 (C-19), 13.39 (C-21), 20.81, 23.33, 26.85, 27.31, 36.59, 37.06, 39.12, 40.11, 41.88, 42.09, 45.98, 50.33, 51.93, 52.90, 55.98, 67.13 (C-2), 67.52 (C-3), 74.56 (C-23), 76.16 (C-22), 125.73, 126.77, 127.12, 130.01, 132.75, 146.12, 211.64 (C-6). HRMS: (API+) calculated for $C_{29}H_{42}ClO_5$ ([M+H]⁺) 505.2721, Found 505.2716. Anal. Calcd for $C_{29}H_{41}ClO_5$: C, 68.96; H, 8.18. Found: C, 68.95; H, 8.23%.

2.2.5. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2-bromophenyl)-24-nor-5α-cholan-6-one (10h)

The general procedure for dihydroxylation of **8h** (60 mg;

0.13 mmol) afforded 55 mg (81%) of the title compound **10h** as a white solid: m. p. 288–289 °C (EtOH); IR ν (cm⁻¹) 3313 vb, 2944, 1699, 1593, 1564, 1471, 786, 682. ¹H NMR (DMSO-*d*₆) δ 0.39 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.95 (d, 3H, *J* = 6.7 Hz, H-21, CH₃); 1.78 (m, 1H); 1.89 (m, 1H); 1.98 (dd, 1H, *J* = 13.1, *J'* = 4.6 Hz); 2.07 (t, 1H, *J* = 12.3 Hz); 2.58 (dd, 1H, *J* = 12.3, *J'* = 3.2 Hz); 3.46 (m, 1H, H-2); 3.58 (bd, 1H, *J* = 7.0 Hz, H-3); 3.74 (m, 1H, H-22); 4.20 (d, 1H, *J* = 1.8 Hz, OH); 4.34 (d, 1H, *J* = 5.8 Hz, OH); 4.58 (m, 1H, H-23); 4.73 (d, 1H, *J* = 7.0 Hz, OH); 5.31 (bs, 1H, OH); 7.18 (m, 1H); 7.38 (m, 1H); 7.42 (dd, 1H, *J* = 7.9, *J'* = 1.8 Hz); 7.55 (dd, 1H, *J* = 7.9, *J'* = 1.2 Hz). ¹³C NMR δ 11.45 (C-18), 13.40 (C-19), 13.69 (C-21), 20.81, 23.40, 26.86, 27.47, 37.07, 37.60, 39.10, 40.10, 41.89, 42.15, 45.98, 50.34, 52.22, 52.89, 55.97, 67.13 (C-2), 67.52 (C-3), 73.47 (C-23), 75.65 (C-22), 122.72, 127.76, 129.08, 129.46, 132.26, 142.83, 211.68 (C-6). HRMS: (API+) calculated for C₂₉H₄₂⁷⁹BrO₅ ([M+H]⁺) 549.2216, Found 549.2211. Anal. Calcd for C₂₉H₄₁BrO₅: C, 63.38; H, 7.52. Found: C, 63.35; H, 7.56%.

2.2.6. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(3-bromophenyl)-24-nor-5a-cholan-6-one (10i)

The general procedure for dihydroxylation of **8i** (60 mg; 0.13 mmol) afforded 54 mg (79%) of the title compound **10i** as a white solid: m. p. 264–265 °C (EtOH); IR ν (cm⁻¹) 3242 vb, 2935, 1709, 1596, 1572, 1460, 782, 700. ¹H NMR (DMSO-*d*₆) δ 0.35 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.86 (d, 3H, *J* = 6.7 Hz, H-21, CH₃); 1.79 (m, 1H); 1.87 (m, 1H); 1.98 (dd, 1H, *J* = 13.1, *J'* = 4.8 Hz); 2.07 (t, 1H, *J* = 12.3 Hz); 2.58 (dd, 1H, *J* = 12.3, *J'* = 3.2 Hz); 3.42–3.46 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (bd, 1H, *J* = 5.5 Hz, OH); 4.37 (bd, 1H, *J* = 8.3 Hz, H-23); 4.59 (bs, 1H, OH); 5.26 (bs, 1H, OH); 7.26–7.30 (m, 2H); 7.43 (m, 1H); 7.45 (m, 1H). ¹³C NMR δ 11.44 (C-18), 12.58 (C-19), 13.38 (C-21), 20.80, 23.32, 26.84, 27.30, 36.60, 37.05, 39.11, 40.10, 41.87, 42.09, 45.97, 50.32, 51.93, 52.89, 55.97, 67.12 (C-2), 67.51 (C-3), 74.52 (C-23), 76.15 (C-22), 121.39, 126.10, 129.69, 130.00, 130.33, 146.38, 211.62 (C-6). HRMS: (API+) calculated for C₂₉H₄₂⁷⁹BrO₅ ([M+H]⁺) 549.2216, Found 549.2211. Anal. Calcd for C₂₉H₄₁BrO₅: C, 63.38; H, 7.52. Found: C, 63.32; H, 7.59%.

2.2.7. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2-methylphenyl)-24-nor-5a-cholan-6-one (10k)

The general procedure for dihydroxylation of **8k** (60 mg; 0.14 mmol) afforded 53 mg (76%) of the title compound **10k** as a white solid: m. p. 269–271 °C (EtOH); IR ν (cm⁻¹) 3300 vb, 2942, 1706, 1631, 1604, 1461, 981, 759. ¹H NMR (DMSO-*d*₆) δ 0.29 (H-18), 0.55 (H-19) (both s, 3H, CH₃); 0.81 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.76 (m, 1H); 1.83 (m, 1H); 1.97 (dd, 1H, *J* = 13.1, *J'* = 4.9 Hz); 2.02 (t, 1H, *J* = 12.3 Hz); 2.26 (s, 3H, CH₃); 2.58 (dd, 1H, *J* = 12.3, *J'* = 3.2 Hz); 3.40 (m, 1H, H-2); 3.59 (dd, 1H, *J* = 8.1, *J'* = 2.6 Hz, H-3); 3.69 (m, 1H, H-22); 4.13 (d, 1H, *J* = 2.1 Hz, OH); 4.27 (d, 1H, *J* = 5.8 Hz, OH); 4.44 (d, 1H, *J* = 4.0 Hz, OH); 4.52 (dd, 1H, *J* = 8.3, *J'* = 1.8 Hz, H-23); 4.94 (d, 1H, *J* = 3.4 Hz, OH); 7.06–7.08 (m, 2H); 7.10 (m, 1H); 7.20 (d, 1H, *J* = 7.3 Hz). ¹³C NMR δ 11.42 (C-18), 13.25 (C-19), 13.37 (C-21), 19.32 (Ar-CH₃), 20.79, 23.35, 26.83, 27.35, 36.79, 37.04, 39.07, 40.10, 41.86, 42.09, 45.96, 50.31, 52.21, 52.87, 55.95, 67.11 (C-2), 67.50 (C-3), 71.41 (C-23), 75.50 (C-22), 125.74, 126.82, 127.38, 130.15, 135.06, 141.42, 211.61 (C-6). HRMS: (API+) calculated for C₃₀H₄₅O₅ ([M+H]⁺) 485.3267, Found 485.3268. Anal. Calcd for C₃₀H₄₄O₅: C, 74.34; H, 9.15. Found: C, 74.29; H, 9.19%.

2.2.8. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(3-methylphenyl)-24-nor-5a-cholan-6-one (10l)

The general procedure for dihydroxylation of **8l** (60 mg; 0.14 mmol) afforded 55 mg (79%) of the title compound **10l** as a white solid: m. p. 276–278 °C (EtOH); IR ν (cm⁻¹) 3315 vb, 2938, 1708, 1611, 1593, 1460, 988, 707. ¹H NMR (DMSO-*d*₆) δ 0.32 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.85 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.80 (m, 1H); 1.87 (m, 1H); 1.98 (dd, 1H, *J* = 13.2, *J'* = 4.9 Hz); 2.07 (t, 1H, *J* = 12.6 Hz);

2.28 (s, 3H, CH₃); 2.58 (dd, 1H, *J* = 12.1, *J'* = 3.2 Hz); 3.44–3.49 (m, 2H, H-2, H-3); 3.74 (m, 1H, H-22); 4.18 (d, 1H, *J* = 2.1 Hz, OH); 4.30–4.33 (m, 2H, H-23, OH); 4.46 (d, 1H, *J* = 4.0 Hz, OH); 5.03 (d, 1H, *J* = 3.4 Hz, OH); 7.03–7.05 (m, 2H); 7.07 (s, 1H); 7.19 (t, 1H, *J* = 7.5 Hz). ¹³C NMR δ 11.45 (C-18), 12.51 (C-19), 13.37 (C-21), 18.59 (Ar-CH₃), 20.80, 23.32, 26.83, 27.23, 36.35, 37.04, 39.12, 40.10, 41.86, 42.08, 45.97, 50.31, 51.91, 52.88, 55.97, 67.11 (C-2), 67.50 (C-3), 75.15 (C-23), 76.21 (C-22), 124.15, 127.67, 127.83, 127.94, 136.99, 143.20, 211.62 (C-6). HRMS: (API+) calculated for C₃₀H₄₅O₅ ([M+H]⁺) 485.3267, Found 485.3269. Anal. Calcd for C₃₀H₄₄O₅: C, 74.34; H, 9.15. Found: C, 74.31; H, 9.16%.

2.2.9. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2-trifluoromethylphenyl)-24-nor-5a-cholan-6-one (10n)

The general procedure for dihydroxylation of **8n** (60 mg; 0.13 mmol) afforded 50 mg (73%) of the title compound **10n** as a white solid: m. p. 268–270 °C (EtOH); IR ν (cm⁻¹) 3418 vb, 2939, 1701, 1613, 1589, 1315, 1114, 772. ¹H NMR (DMSO-*d*₆) δ 0.38 (H-18), 0.60 (H-19) (both s, 3H, CH₃); 0.87 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.82 (m, 1H); 1.88 (m, 1H); 1.99 (dd, 1H, *J* = 13.1, *J'* = 4.6 Hz); 2.07 (t, 1H, *J* = 12.6 Hz); 2.58 (dd, 1H, *J* = 12.2, *J'* = 3.1 Hz); 3.45 (m, 1H, H-2); 3.66 (m, 1H, H-3); 3.74 (bs, 1H, H-22); 4.18 (d, 1H, *J* = 2.1 Hz, OH); 4.31 (d, 1H, *J* = 6.1 Hz, OH); 4.63 (d, 1H, *J* = 4.3 Hz, H-23); 4.70 (d, 1H, *J* = 4.6 Hz, OH); 5.32 (bs, 1H, OH); 7.46 (m, 1H); 7.65–7.70 (m, 3H). ¹³C NMR δ 11.31 (C-18), 12.83 (C-19), 13.35 (C-21), 20.75, 23.34, 26.81, 27.38, 37.00, 37.77, 39.04, 40.10, 41.82, 42.11, 45.91, 50.28, 52.32, 52.84, 55.91, 67.09 (C-2), 67.47 (C-3), 69.76 (C-23), 75.19 (C-22), 124.49 (q, *J* = 274.7 Hz, CF₃), 126.26 (q, *J* = 28.8 Hz, CF₃), 125.21 (q, *J* = 5.0 Hz), 127.59, 129.38, 132.39, 142.56, 211.54 (C-6). ¹⁹F NMR (¹H) δ -55.95 (s, 1F). HRMS: (API+) calculated for C₃₀H₄₂F₃O₅ ([M+H]⁺) 539.2984, Found 539.2986. Anal. Calcd for C₃₀H₄₁F₃O₅: C, 66.89; H, 7.67. Found: C, 66.88; H, 7.69%.

2.2.10. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(3-trifluoromethylphenyl)-24-nor-5a-cholan-6-one (10o)

The general procedure for dihydroxylation of **8o** (60 mg; 0.13 mmol) afforded 53 mg (77%) of the title compound **10o** as a white solid: m. p. 243–245 °C (EtOH); IR ν (cm⁻¹) 3257 vb, 2934, 1712, 1618, 1457, 1329, 1113, 706. ¹H NMR (DMSO-*d*₆) δ 0.32 (H-18), 0.59 (H-19) (both s, 3H, CH₃); 0.86 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.79 (m, 1H); 1.86 (m, 1H); 1.97 (dd, 1H, *J* = 13.1, *J'* = 4.6 Hz); 2.07 (t, 1H, *J* = 12.6 Hz); 2.57 (dd, 1H, *J* = 12.2, *J'* = 3.3 Hz); 3.42–3.49 (m, 2H, H-2, H-3); 3.74 (bs, 1H, H-22); 4.19 (d, 1H, *J* = 1.8 Hz, OH); 4.33 (d, 1H, *J* = 5.5 Hz, OH); 4.50 (d, 1H, *J* = 7.9 Hz, OH); 4.65 (bs, 1H, H-23); 5.35 (bs, 1H, OH); 7.54–7.62 (m, 4H). ¹³C NMR δ 11.30 (C-18), 12.59 (C-19), 13.36 (C-21), 20.78, 23.30, 26.83, 27.22, 36.54, 37.01, 39.08, 40.10, 41.84, 42.09, 45.94, 50.31, 51.93, 52.86, 55.94, 67.11 (C-2), 67.50 (C-3), 74.52 (C-23), 76.23 (C-22), 123.38 (q, *J* = 4.0 Hz), 123.84 (q, *J* = 4.0 Hz), 124.37 (q, *J* = 272.3 Hz, CF₃), 128.77 (q, *J* = 31.8 Hz), 129.14, 131.12, 145.01, 211.59 (C-6). ¹⁹F NMR (¹H) δ -60.97 (s, 1F). HRMS: (API+) calculated for C₃₀H₄₂F₃O₅ ([M+H]⁺) 539.2984, Found 539.2985. Anal. Calcd for C₃₀H₄₁F₃O₅: C, 66.89; H, 7.67. Found: C, 66.85; H, 7.71%.

2.2.11. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(4-trifluoromethylphenyl)-24-nor-5a-cholan-6-one (10p)

The general procedure for dihydroxylation of **8p** (60 mg; 0.13 mmol) afforded 54 mg (78%) of the title compound **10p** as a white solid: m. p. 265–266 °C (EtOH); IR ν (cm⁻¹) 3224 vb, 2937, 1710, 1621, 1461, 1331, 1121, 838. ¹H NMR (DMSO-*d*₆) δ 0.33 (H-18), 0.59 (H-19) (both s, 3H, CH₃); 0.86 (d, 3H, *J* = 6.4 Hz, H-21, CH₃); 1.80 (m, 1H); 1.86 (m, 1H); 1.98 (dd, 1H, *J* = 13.1, *J'* = 4.6 Hz); 2.07 (t, 1H, *J* = 12.6 Hz); 2.58 (dd, 1H, *J* = 12.2, *J'* = 3.1 Hz); 3.44 (m, 1H, H-2); 3.50 (d, 1H, *J* = 7.6 Hz, H-3); 3.74 (bs, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (d, 1H, *J* = 4.6 Hz, OH); 4.47 (d, 1H, *J* = 8.3 Hz, H-23); 4.66 (bs, 1H, OH); 5.34 (bs, 1H, OH); 7.50 (d, 2H, *J* = 8.1 Hz); 7.68 (d, 2H,

$J = 8.1$ Hz). ^{13}C NMR δ 11.41 (C-18), 12.57 (C-19), 13.36 (C-21), 20.80, 23.29, 26.83, 27.26, 36.61, 37.03, 39.11, 40.10, 41.85, 42.07, 45.96, 50.31, 51.92, 52.87, 55.95, 67.11 (C-2), 67.50 (C-3), 74.68 (C-23), 76.10 (C-22), 124.40 (q, $J = 271.9$ Hz, CF_3), 124.96 (q, $2 \times \text{C}$, $J = 3.6$ Hz), 127.70 (q, $J = 31.2$ Hz), 127.80 ($2 \times \text{C}$), 148.29, 211.59 (C-6). ^{19}F NMR (^1H) δ -60.59 (s, 1F). HRMS: (API+) calculated for $\text{C}_{30}\text{H}_{42}\text{F}_3\text{O}_5$ ($[\text{M}+\text{H}]^+$) 539.2984, Found 539.2986. Anal. Calcd for $\text{C}_{30}\text{H}_{41}\text{F}_3\text{O}_5$: C, 66.89; H, 7.67. Found: C, 66.86; H, 7.70%.

2.2.12. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2,4-dimethylphenyl)-24-nor-5a-cholan-6-one (10q)

The general procedure for dihydroxylation of **8q** (60 mg; 0.14 mmol) afforded 52 mg (75%) of the title compound **10q** as a white solid: m. p. 253–255 °C (EtOH); IR ν (cm^{-1}) 3350 vb, 2943, 1693, 1617, 1502, 1458, 987, 817. ^1H NMR ($\text{DMSO}-d_6$) δ 0.35 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.85 (d, 3H, $J = 6.4$ Hz, H-21, CH_3); 1.78–1.88 (m, 2H); 1.98 (dd, 1H, $J = 13.1$, $J' = 4.6$ Hz); 2.07 (t, 1H, $J = 12.5$ Hz); 2.22 (s, 3H, Ar- CH_3); 2.26 (s, 3H, Ar- CH_3); 2.58 (dd, 1H, $J = 12.1$, $J' = 3.0$ Hz); 3.44 (m, 1H, H-2); 3.63 (dd, 1H, $J = 8.3$ Hz, H-3); 3.74 (m, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (bs, 1H, OH); 4.46 (bs, 1H, OH); 4.52 (d, 1H, $J = 8.3$ Hz, H-23); 4.89 (bs, 1H, OH); 6.93 (s, 1H); 6.96 (d, 1H, $J = 7.9$ Hz); 7.12 (d, 1H, $J = 7.9$ Hz). ^{13}C NMR δ 11.50 (C-18), 13.13 (C-19), 13.38 (C-21), 19.25 (Ar- CH_3), 20.65 (Ar- CH_3), 20.80, 23.36, 26.84, 27.36, 36.79, 37.05, 39.09, 40.10, 41.87, 42.09, 45.98, 50.31, 52.17, 52.88, 55.97, 67.12 (C-2), 67.51 (C-3), 71.26 (C-23), 75.44 (C-22), 126.41, 127.40, 130.84, 134.94, 135.62, 138.30, 211.64 (C-6). HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{47}\text{O}_5$ ($[\text{M}+\text{H}]^+$) 499.3423, Found 499.3426. Anal. Calcd for $\text{C}_{31}\text{H}_{46}\text{O}_5$: C, 74.66; H, 9.30. Found: C, 74.63; H, 9.32%.

2.2.13. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(2,5-dimethylphenyl)-24-nor-5a-cholan-6-one (10r)

The general procedure for dihydroxylation of **8r** (60 mg; 0.14 mmol) afforded 49 mg (71%) of the title compound **10r** as a white solid: m. p. 281–283 °C (EtOH); IR ν (cm^{-1}) 3332 vb, 2933, 1707, 1616, 1506, 1461, 985. ^1H NMR ($\text{DMSO}-d_6$) δ 0.36 (H-18), 0.60 (H-19) (both s, 3H, CH_3); 0.86 (d, 3H, $J = 6.4$ Hz, H-21, CH_3); 1.82 (m, 1H); 1.87 (m, 1H); 1.98 (dd, 1H, $J = 13.1$, $J' = 4.6$ Hz); 2.07 (t, 1H, $J = 12.6$ Hz); 2.23 (s, 3H, Ar- CH_3); 2.24 (s, 3H, Ar- CH_3); 2.58 (dd, 1H, $J = 12.0$, $J' = 3.1$ Hz); 3.45 (m, 1H, H-2); 3.61 (bd, 1H, $J = 7.9$ Hz, H-3); 3.74 (bs, 1H, H-22); 4.20 (bs, 1H, OH); 4.34 (d, 1H, $J = 5.2$ Hz, OH); 4.47 (bs, 1H, OH); 4.52 (d, 1H, $J = 7.9$ Hz, H-23); 4.94 (bs, 1H, OH); 6.92 (dd, 1H, $J = 7.6$, $J' = 1.2$ Hz); 6.99 (d, 1H, $J = 7.6$ Hz); 7.06 (bs, 1H). ^{13}C NMR δ 11.47 (C-18), 13.29 (C-19), 13.40 (C-21), 18.92 (Ar- CH_3), 20.81, 20.85 (Ar- CH_3), 23.42, 26.86, 27.32, 36.91, 37.08, 39.10, 40.11, 41.88, 42.13, 45.98, 50.34, 52.25, 52.91, 55.98, 67.14 (C-2), 67.52 (C-3), 71.45 (C-23), 75.53 (C-22), 127.46, 127.99, 130.08, 131.91, 134.34, 141.26, 211.68 (C-6). HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{47}\text{O}_5$ ($[\text{M}+\text{H}]^+$) 499.3423, Found 499.3425. Anal. Calcd for $\text{C}_{31}\text{H}_{46}\text{O}_5$: C, 74.66; H, 9.30. Found: C, 74.61; H, 9.33%.

2.2.14. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(4-*t*-butylphenyl)-24-nor-5a-cholan-6-one (10s)

The general procedure for dihydroxylation of **8s** (60 mg; 0.13 mmol) afforded 53 mg (77%) of the title compound **10s** as a white solid: m. p. 255–256 °C (EtOH); IR ν (cm^{-1}) 3234 vb, 2938, 1709, 1616, 1512, 1461, 1084, 991, 831. ^1H NMR ($\text{DMSO}-d_6$) δ 0.32 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.85 (d, 3H, $J = 6.4$ Hz, H-21, CH_3); 1.26 (s, 3H, *t*-Bu); 1.77–1.88 (m, 2H); 1.98 (dd, 1H, $J = 13.1$, $J' = 4.6$ Hz); 2.07 (t, 1H, $J = 12.6$ Hz); 2.58 (dd, 1H, $J = 12.3$, $J' = 3.4$ Hz); 3.44 (m, 1H, H-2); 3.51 (d, 1H, $J = 8.3$ Hz, H-3); 3.74 (m, 1H, H-22); 4.20 (vb s, 2H, $2 \times \text{OH}$); 4.32 (d, 1H, $J = 8.3$ Hz, H-23); 4.42 (vb s, 1H, OH); 4.99 (vb s, 1H, OH); 7.18 (d, 2H, $J = 8.5$ Hz); 7.33 (d, 2H, $J = 8.5$ Hz). ^{13}C NMR δ 11.44 (C-18), 12.48 (C-19), 13.33 (C-21), 20.77, 23.29, 26.81, 27.13, 31.19 ($3 \times \text{C}$, *t*-Bu), 34.21, 36.33, 37.00, 39.10, 40.10, 41.81, 42.03, 45.94, 50.27, 51.95, 52.84, 55.92, 67.08 (C-

2), 67.47 (C-3), 74.95 (C-23), 76.05 (C-22), 124.72 ($2 \times \text{C}$), 126.75 ($2 \times \text{C}$), 140.23, 149.41, 211.53 (C-6). HRMS: (API+) calculated for $\text{C}_{33}\text{H}_{51}\text{O}_5$ ($[\text{M}+\text{H}]^+$) 527.3736, Found 527.3734. Anal. Calcd for $\text{C}_{33}\text{H}_{50}\text{O}_5$: C, 75.25; H, 9.57. Found: C, 75.22; H, 9.61%.

2.2.15. (22R, 23R)-2a,3a,22,23-tetrahydroxy-23-(4-phenylphenyl)-24-nor-5a-cholan-6-one (10t)

The general procedure for dihydroxylation of **8t** (40 mg; 0.08 mmol) afforded 33 mg (72%) of the title compound **10t** as a white solid: m. p. 251–253 °C (EtOH); IR ν (cm^{-1}) 3328 vb, 2955, 1705, 1643, 1600, 1580, 1465, 967, 763. ^1H NMR ($\text{DMSO}-d_6$) δ 0.34 (H-18), 0.59 (H-19) (both s, 3H, CH_3); 0.89 (d, 3H, $J = 6.4$ Hz, H-21, CH_3); 1.78–1.89 (m, 2H); 1.97 (dd, 1H, $J = 13.1$, $J' = 4.6$ Hz); 2.07 (t, 1H, $J = 12.6$ Hz); 2.58 (dd, 1H, $J = 12.1$, $J' = 3.0$ Hz); 3.44 (m, 1H, H-2); 3.54 (d, 1H, $J = 8.6$ Hz, H-3); 3.74 (bs, 1H, H-22); 4.19 (bs, 1H, OH); 4.33 (bs, 1H, OH); 4.41 (d, 1H, $J = 8.9$ Hz, H-23); 4.55 (bs, 1H, OH); 5.15 (bs, 1H, OH); 7.33–7.37 (m, 3H); 7.45 (t, 2H, $J = 7.6$ Hz); 7.64 (d, 2H, $J = 8.3$ Hz); 7.68 (t, 2H, $J = 8.3$ Hz). ^{13}C NMR δ 11.49 (C-18), 12.52 (C-19), 13.35 (C-21), 20.79, 23.29, 26.81, 27.27, 36.52, 37.02, 39.12, 40.09, 41.83, 42.05, 45.95, 50.28, 51.92, 52.86, 55.95, 67.10 (C-2), 67.48 (C-3), 74.87 (C-23), 76.17 (C-22), 126.26 ($2 \times \text{C}$), 126.51 ($2 \times \text{C}$), 127.38, 127.67 ($2 \times \text{C}$), 128.96 ($2 \times \text{C}$), 138.74, 139.80, 142.58, 211.57 (C-6). HRMS: (API+) calculated for $\text{C}_{35}\text{H}_{47}\text{O}_5$ ($[\text{M}+\text{H}]^+$) 547.3423, Found 547.3421. Anal. Calcd for $\text{C}_{35}\text{H}_{46}\text{O}_5$: C, 76.89; H, 8.48. Found: C, 76.85; H, 8.53%.

3. Molecular docking

Docking was performed prediction of conformation and energy ranking between BRI1 receptor (PDBID: 3RGZ) and. Docking studies was carried out using AutoDock Vina 1.05 [13]. All 3D structures of BRI1 ligands were prepared with Marvin 5.10.3 [10], software which can be used for drawing, displaying and characterization of chemical structure, substructures and reactions. Ligands were prepared as derivatives of natural ligand brassinolide (BLD). Polar hydrogens were added to all ligands and proteins with the AutoDock Tools (ADT) [14] program prior to docking with Autodock Vina program. Grid box with size of 40 Å were centered on active site of protein. Exhaustiveness parameter was set to 20 (default 8). After docking we compared docked ligand with brassinolide crystal-like poses and the best crystal-like poses of each ligand were analyzed.

4. The pea inhibition biotest

Pea seedlings (*Pisum arvense* L. sort Arvica) germinating for 2 days were selected for uniformity from a large population and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7) After 24 h in a dark cultivation room (24 °C, humidity 75%) the seedlings were treated with different amounts of tested compounds in 5 μl fractionated lanolin. The substances were applied as microdrops to the scar left after the removal of bract. The control plants were treated with lanolin alone. At least seven plants were used for each experiment and the assays were repeated at least three times. The inhibition of etiolated pea stems were measured after 4 days and the difference in length between treated and control plants provided a measure of activity. For each treatment, 8 seedlings were analyzed in two biological replicates. The mean values were subjected to the statistical analysis using the Student's *t* test.

5. Determination of ethylene production

For measurement of ethylene production, pea seedlings (8 plants/ tested amount of substance) were placed in a 0.5 L glass container for 24 h in the dark. One milliliter of headspace gas from the chamber was withdrawn for each measurement and injected into a gas chromatograph (Agilent Technologies, GC System, USA) equipped with a flame

Table 1
Isolated yields of products in alkene cross-metathesis.

Entry	Ar	Yield (%)	Entry	Ar	Yield (%)
a		81	l		69
b		65	m		71
c		73	n		57
d		75	o		66
e		64	p		71
f		70	q		61
g		75	r		58
h		59	s		60
i		66	t		32
j		73	u		na
k		65	v		na

na – compound not isolated.

ionic detector (FID) and a capillary column (HP-AL/S stationary phase, 15 lm, i.d. = 0.535). The chromatographic analytical parameters were as follows: column temperature: 150 °C; detector temperature: 220 °C;

and helium was used as carrier gas. The area under the resultant peak (y-axis) versus sensitivity (x-axis; nL.mL⁻¹) was representing a quantitative measure of ethylene concentration. The measurements were done in triplicates and data were statistically analyzed using the Student's *t* test.

6. Arabidopsis brassinosteroid sensitivity assay

Arabidopsis thaliana L. (Heyhn.) (Columbia ecotype, Col-0; referred to Arabidopsis) seedlings were stratified for 2 d at 4 °C and germinated on vertical half-strength Murashige and Skoog (1% w/v sucrose) agar plates with different concentrations of 24-epiBL and BR derivatives at 22 °C in a 16 h/8 h light-dark cycle for 5 d. Roots were then straightened on solid media plates, scanned with an Epson high-resolution scanner and the entire root length measured with ImageJ (<http://rsbweb.nih.gov/ij/>). For each treatment, more than 15 seedlings were analyzed in two biological repeats. *P* values were calculated with a two-tailed Student *t*-test using Excel software.

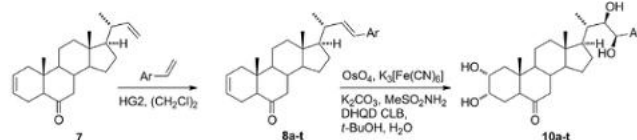
7. Cytotoxicity

Calcein AM cytotoxicity assay was performed exactly as described earlier [11].

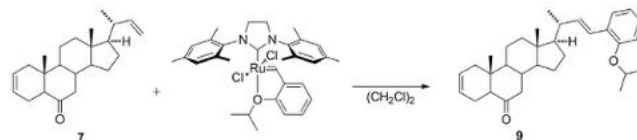
8. Results and discussion

8.1. Chemistry

For the preparation of above mentioned compounds we started with the known [15] 24-nor-5 α -chola-2,22-dien-6-one (7). With this compound cross-metathesis were carried out using twenty commercially available styrenes substituted with fluorine, chlorine, bromine, trifluoromethyl, alkyls and phenyl group. The reaction was catalyzed by Hoveyda-Grubbs second generation catalyst in refluxing dichloroethane. This catalyst has proven to be efficient for different steroid side chain cross-metatheses [15–18]. Due to the fact that compound 7 and used styrenes belongs to the group of type I olefins (non-, meta- and para- substituted styrenes) and type II olefins (ortho- substituted styrenes) if Hoveyda-Grubbs second generation catalyst is used [19], the reactions proceed in two steps. Firstly, the homodimerization of styrene with strong release of ethylene takes place followed by the secondary cross-metathesis of steroid and *in-situ* formed stilbene. This is the main reason of long reaction time. In most cases, the cross-metathesis gave the corresponding 23-aryl products in good isolated yields 60–80% (see Scheme 1). Lower yield is typical for ortho-substituted styrenes as their homodimers are sparingly consumable during secondary cross-metathesis. In the case of 4-phenylstyrene the low yield (32%) is caused by formation of poorly soluble 4,4'-diphenylstilbene



Scheme 1. Preparation of arylidenones and aryl-tetraols. For Ar see Table 1.



Scheme 2. Formation of isopropoxy derivative 9 if bis-ortho substituted or no styrenes are used in the reaction.

Table 2
IC₅₀ (mol/L) values obtained from the pea inhibition biotest. Compounds **10a**, **10d**, **10g**, **10j**, and **10m** are presented for comparison; the results were published [11].

Compound	IC ₅₀ (mol/L)
24-epibrassinolide	1.66×10^{-5}
10f	1.80×10^{-5}
10b	2.15×10^{-5}
10n	2.30×10^{-5}
10a	2.52×10^{-6}
10d	2.0×10^{-6}
10g	1.8×10^{-5}
10j	2.7×10^{-4}
10m	1.8×10^{-4}

during homodimerization step. No desired products were observed if bis-*ortho* substituted styrenes were used. In these cases we were able to isolate only very small amount of 2-isopropoxyphenyl derivative **9** as a product of reaction between steroid olefin and Hoveyda-Grubbs catalyst (see Scheme 2). This was probably caused by steric hindrance near steroidal terminal double bond. All products **8a–t** were obtained as *trans* double bond isomers as indicated by NMR analysis.

Next, Sharpless simultaneous dihydroxylation of both double bonds was used to minimize formation of unnatural configuration of 22 and 23 hydroxy groups. Hydroquinidine 4-chlorobenzoate was used as chiral ligand. The reaction rate was increased by addition of methanesulfonamide [20]. Such reaction conditions led only to the desired 22*R*,23*R*-isomers **10a–t** in very good yields (75–83%).

All compounds were characterized by NMR, IR and MS techniques together with elemental analysis for all tetraols. Compounds **10a**, **10d**, **10g**, **10j**, and **10m** were also prepared by different synthetic strategy [11] (see Table 1).

8.2. Biological activity and docking

Biological activity of new BRs derivatives was monitored by pea inhibition biotest. This test is based on that BRs inhibit the growth of etiolated seedlings at high concentration and the inhibition is probably caused by ethylene production which is mediated by BRs. Dose response curves for all prepared BRs derivatives are shown in SFig. 1. The IC₅₀ values obtained from the pea inhibition biotest are summarized in Table 2. The most active BRs derivatives were **10f**, **10b**, and **10n** (IC₅₀ 1.8×10^{-5} – 2.3×10^{-5} mol.L⁻¹) compared to 24-epibrassinolide (IC₅₀ 1.6×10^{-5} mol.L⁻¹), used as a positive control (Fig. 2).

The crosstalk of BRs and ethylene regulates various aspects of plant growth and development. Hansen et al. [21] showed that exogenously

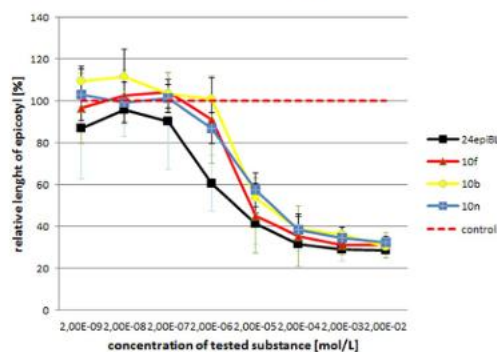


Fig. 2. Effect of selected brassinosteroid derivatives on the inhibition of etiolated pea seedlings. Error bars represent s.d.

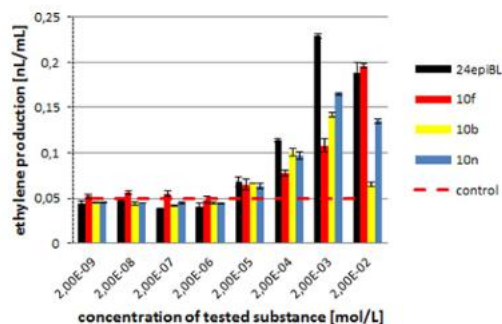


Fig. 3. Effect of selected brassinosteroid derivatives on ethylene production (nL.L⁻¹) in etiolated pea seedlings determined by GC-FID 24 h after ventilation. Error bars represent s.d.

applied BRs stimulate ethylene production by stabilizing the ACS protein. When low levels of ethylene are applied to etiolated pea seedlings, the characteristic triple response can be observed: inhibition of stem elongation, radial swelling of the stem and the absence of a normal geotropic response [22]. Production of ethylene was measured in cultivation vessels during the incubation of etiolated pea plants after treatment of different BR derivatives (SFig. 2). The high concentrations of ethylene (196, 142 and 165 nL.L⁻¹) were determined after treatment of **10f**, **10b** and **10n** and compared to 229 nL.L⁻¹ for 24-epiBL treatment. While level of this gaseous plant hormone produced by untreated control pea plants, was found to be significantly lower (about 60 nL.L⁻¹, Fig. 3).

BRs derivatives were further assessed for biological activity based on their inhibitory effect on Arabidopsis root growth [23]. The effects of compounds **10f**, **10b** and **10n** on the Arabidopsis roots are shown in Fig. 4. Results of all prepared analogues are summarized in SFig. 3.

Molecular docking into BR11 receptor shows that several compounds binds with very high binding energy. In some cases the binding energy was even better than for naturally occurring brassinolide (-10.6 kcal/mol) [11] (see SI for molecular docking of all new compounds). The high binding affinity of compounds **10f** (-11.0 kcal/mol), **10b** (-10.7 kcal/mol), and **10n** (-11.9 kcal/mol) also proves the good biological activities of these analogues (Fig. 5).

Relationship between biological activity and substitution pattern in the phenyl group shows that no substitution or substitution with one small group (fluorine, chlorine) led to promising compounds with high

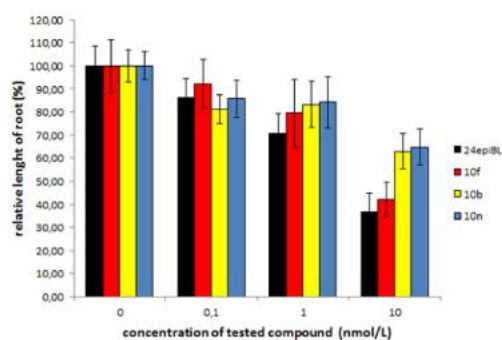


Fig. 4. Effect of selected brassinosteroid derivatives on the inhibition of Arabidopsis root length. 5 days old *Arabidopsis thaliana* seedlings (Columbia ecotype, Col-0) were treated by DMSO/24epiBL/BR analogues. For each treatment more than 25 seedlings were analyzed in two biological repeats.

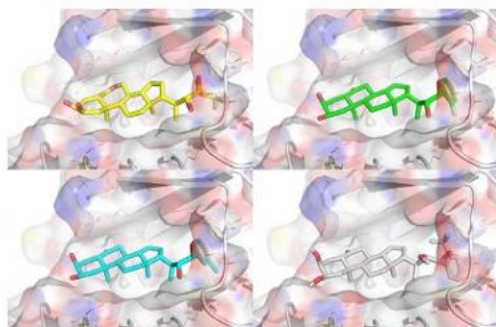


Fig. 5. Poses of 24-epibrassinolide (yellow) and three new analogues of brassinosteroids, 10b (green), 10f (cyan), and 10n (grey) within BRI1 binding site.

plant activities. The most active are compounds 10a (no substitution), 10b and 10c (fluorine in *ortho* and *para* position), and 10f (chlorine in *meta* position). On the other hand, substitution with bulky groups (phenyl, *t*-butyl, methyl or trifluoromethyl) or substitution with more than one group (e.g. 10q and 10r) causes significant decrease or complete loss of plant activity. The only exception from bulky groups is compound 10n which showed good activity in two of the three assays. This may be explained by smaller interaction of substituents in *ortho* position with receptor cavity – the group can be oriented out of the cavity.

Antiproliferative activity of BRs derivatives was screened towards various tumor cell lines and normal cells, including T-lymphoblastic leukemia CEM, breast carcinoma (MCF7) and cervical carcinoma (HeLa) and human foreskin fibroblasts (BJ). All tested BR analogues had no detectable cytotoxic activity, even when tested in concentrations up to 50 μ M (data not shown).

9. Conclusions

Overall 15 novel and 5 known phenyl analogues of brassinosteroid were synthesized *via* alkene cross metathesis. The metathesis showed as an effective method for preparation of new brassinosteroid derivatives with plant growth promoting activities comparable with natural brassinosteroids. The results of biological screenings showed that molecular docking into BRI1 is a powerful tool for prediction and design of new compounds with strong brassinosteroid activities. Very potent brassinosteroids analogues prepared by this synthesis can be also used for potential application in agriculture to improve growth and yield or to increase the resistance of plants against various biotic and abiotic stresses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.steroids.2017.08.010>.

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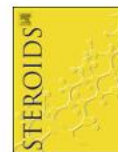
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Příloha 7

Synthesis, Characterization and Antiproliferative Activity of Seco Analogues of Brassinosteroids

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Steroids 2019, 146, 1-13



Synthesis, characterization and antiproliferative activity of seco analogues of brassinosteroids

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ABSTRACT

Synthesis and structure–activity relationship analysis of a two groups of 2,3-seco analogues of brassinosteroids (BRs) were performed to examine their antiproliferative activities. Two steroid skeletons were chosen for the preparation of seco analogues – cholestane and stigmastane. The synthetic strategy consists of multistep reactions and detailed analysis of compounds prepared. We have discovered unpublished behaviour of 2,3-seco-2,3-dihydroxy-6-ketones leading to formation of intramolecular ketal with two new steroidal rings. Their reaction intermediates were also characterized in some cases. All compounds prepared were fully characterized with NMR and MS techniques. Most of compounds were tested for *in vitro* cytotoxicity on three cancer cell lines (CEM, MCF7, and HeLa) and normal human fibroblasts (BJ). It was discovered that some seco analogues caused apoptosis in cancer cells. The most promising seco derivative **28** proved to have high therapeutic index.

1. Introduction

Brassinosteroids (BRs) represent a large group of plant steroids that include > 70 structurally and functionally related compounds [1–4]. The most important are castasterone and brassinolide and their 24-epimers and 28-homoanalogues (see Fig. 1). BRs have been found in a wide range of plant species, including higher and lower plants, and have been detected in various plant parts such as seeds, pollen, roots, leaves, stems and flowers. They demonstrate various kinds of regulatory action on the growth and development of plants, such as cell division and stimulation of cell enlargement, improvement of the biomass formation, yield and quality of seeds, and plant adaptability [5–9]. They also protect plants from stresses, such as drought, chilling, salinity, lack of water and nutrients etc. [10,11]. At the molecular level, BRs change the gene expression and metabolism of nucleic acids and proteins [12]. BRs have structures similar to those of animal steroid hormones. In addition to their extensively studied activity on plants, they also act as antiproliferative and antiangiogenic agents in human cancer and/or endothelial cells with influence on steroid receptors.

Even so, knowledge of the effects of BRs and their synthetic analogues in animal cells is still limited: increase in the mitochondrial membrane potential or fraction of cells in the G₀/G₁ phase and decrease in S-phase cells by 24-epibrassinolide (24-epiBL) in cultured hybridoma mouse cells, were shown at concentrations 10^{−13} and 10^{−12} mol/l [13]. Natural brassinosteroids caused apoptosis in breast cancer cells [14], in prostate cancer cells [15,16], and in colon cancer cells [17]. An inhibitory effect of the natural BRs, 24-epiBL and 28-homocastasterone (28-homoCS), on the growth and viability of different normal and cancer cell lines has been reported [18]. A cytotoxic effect of natural BRs was found in cancer cells but not in untransformed human fibroblasts. Evidently, BRs can induce different responses in cancer cells and in normal fibroblasts. Natural BRs have been also shown to cause cell cycle arrest and apoptosis of human breast cancer cells irrespective of estrogen status [18].

We showed that BRs inhibited cell growth and induced block in the G₁-phase of the cell cycle. In breast carcinoma cell line MCF7, estrogen receptor-α (ER-α) immunoreactivity was uniformly present in the cell nucleus, while in cells treated with 28-homoCS or 24-epiBL (IC₅₀, 24 h),

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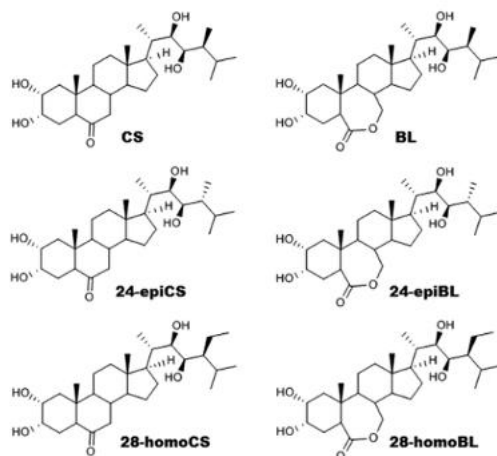


Fig. 1. Natural brassinosteroids (CS – castasterone, BL – brassinolide).

cytoplasmic speckles of ER- α immunofluorescence appeared [14]. After treatment with 28-homoCS, re-localization of estrogen receptor- β (ER- β) to nuclei was evident, while in 24-epiBL-treated cells, ER- β was found predominantly at the periphery of the nuclei. These changes were accompanied by down-regulation of ERs after the treatment. Application of BR to MDA-MB-468 cells resulted not only in G₁-phase arrest but also in increase of the subG₁ fraction containing apoptotic bodies. In both cell lines, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining, however, confirmed the possible induction of apoptosis by BRs. The expression of apoptosis related proteins was differently modulated by BRs for each cell line [14]. Some of the cholestane derivatives structurally related to BRs, were found to have strong cytotoxic activity in several human normal and cancer cell lines. The presence of a 3-hydroxy, 2,3-vicinal diol or 3,4-vicinal diol moiety were found to be necessary for optimum biological activity, as well as a six-membered B ring. According to the profiling of all steroid receptors in both agonist and antagonist mode, the majority of the cholestanes were weakly active or inactive compared to the natural ligands. Two active cholestane derivatives were shown to strongly influence cell viability, proliferation, cell cycle distribution, apoptosis and molecular pathways responsible for these processes in hormone-sensitive/insensitive (MCF7/MDA-MB-468) breast cancer cell lines [19]. In this article we describe the synthesis and antiproliferative activity of steroids with two primary hydroxyl groups instead of steroids with standard vicinal diols on the A ring.

2. Experimental

2.1. General methods

The NMR spectra were taken on a JEOL JNM-ECA 500 (JEOL, Tokyo, Japan; ¹H, 500 MHz; ¹³C, 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. ¹H NMR and ¹³C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, ¹H δ = 0 ppm) or solvents: CDCl₃ (¹H δ = 7.26 ppm, ¹³C δ = 77.00 ppm) or DMSO-*d*₆ (¹H δ = 2.46 ppm, ¹³C δ = 40.00 ppm). Chemical shifts are given in ppm (δ -scale), coupling constants (J) in Hz. All values were obtained by first-order analysis. For API HRMS analysis, the samples were dissolved in chloroform (or chloroform:methanol; 1:1; v/v, in the case of hydroxylated compounds) to a concentration 10 μ g·mL⁻¹. The ASAP (Atmospheric Solids Analysis Probe) was dipped into the sample

solution, placed into the ion source and analysed in fullscan mode. The source of the Synapt G2-Si Mass Spectrometer (Waters, Manchester, UK) was operated in positive ionisation mode (ASAP+) and, if not stated otherwise, at source temperature of 120 °C. The Corona needle current was kept at 5 μ A and the collision energy at value 4. The probe temperature was ramped up from 50 °C to 600 °C in 3 min. Data were acquired from 50 to 1000 Da with 1.0 s scan time in High Resolution Mode and processed using the Masslynx 4.1 software (Waters). Mass accuracy of 1 ppm or less was achieved with the described instrumentation for all compounds. Merck silica gel Kieselgel 60 (230–400 mesh) was used for column chromatography. The HPLC system consisted of a Waters semi-preparative HPLC system including quaternary pump, liquid handler, UV-VIS and ELSD detectors. The semi preparative column was filled with silica gel. Reagents and solvents were purchased from Sigma–Aldrich and were not purified.

2.2. Work-up methods

Method A refers to pouring the reaction mixture into H₂O, extraction of the product with ethyl acetate or diethylether, washing the organic layer successively with H₂O followed by drying over MgSO₄, filtration, and evaporation of the filtrate under reduced pressure. Analytical samples were dried over P₂O₅ under diminished pressure.

Method B refers to pouring the reaction mixture into diluted HCl (1:10), extraction of the product with ethyl acetate or CHCl₃, washing the organic layer successively with H₂O followed by drying over MgSO₄, filtration, and evaporation of the filtrate under reduced pressure. Analytical samples were dried over P₂O₅ under diminished pressure.

Method C refers to pouring the reaction mixture into saturated aqueous solution of NaHCO₃, extraction of the product with ethyl acetate, washing the organic layer successively with H₂O followed by drying over MgSO₄, filtration, and evaporation of the filtrate under reduced pressure. Analytical samples were dried over P₂O₅ under diminished pressure.

2.3. General procedure for Upjohn dihydroxylation of 2,3-double bond

N-methylmorpholine-*N*-oxide (4.0 mmol) was added to a solution of steroidal olefin (1.0 mmol) in tetrahydrofuran (20 mL), acetone (20 mL), and water (2 mL). A solution of osmium tetroxide (0.2 mmol) in *t*-butanol (1 mL) was then added dropwise. The reaction was stirred at room temperature for 10–15 h until no starting material was detected on TLC plates. The reaction is stopped by addition of a saturated aqueous solution of sodium sulfite (2 mL). The work-up (method A) and column chromatography afforded both diols, major 2 α ,3 α -diol and minor 2 β ,3 β -diol.

2.4. General procedure for ketal formation

Ethyleneglycol (1 mL; 18 mmol) and *p*-toluenesulfonic acid (9 mg; 0.05 mmol) was added to a solution of steroidal ketone (0.5 mmol) in toluene (15 mL). The solution was refluxed with a Dean-Stark apparatus to remove water. After 4 h, the mixture was worked up (method C). Column chromatography on silica afforded the desired ketal.

2.5. General procedure for ketal deprotection

5% aqueous solution of hydrochloric acid (1 mL) was added to a solution of ketal (0.7 mmol) in tetrahydrofuran (10 mL). The mixture was stirred at 40 °C for 2 h. Work-up (method A) and column chromatography on silica yielded the desired ketone.

2.6. General procedure for acetylation

Acetic anhydride (2 mL) was added to a solution of steroidal alcohol

(1 mmol) in pyridine (10 mL). The reaction mixture was stirred at room temperature or at 60 °C (see in each experiment) for 12 h. The reaction was quenched by addition of methanol (3 mL) and stirred for an additional 10 min. Work-up (method B) and column chromatography on silica yielded the desired acetate.

2.7. General procedure for hydrolysis of acetates

5.3 M solution of sodium methoxide in methanol (1 mL) was added to a solution of steroidal acetate (0.7 mmol) in dry tetrahydrofuran (20 mL). The reaction was stirred at room temperature for 30 min. 5% aqueous solution of hydrochloric acid (1 mL) was then added. Work-up (method A) and column chromatography on silica gave the desired alcohol.

2.8. General procedure for oxidative cleavage of 2,3-diols

Sodium periodate supported on silica gel (3 g; 25 wt%) was added to a solution of diol (2 mmol) in dichloromethane (10 mL). The reaction was stirred vigorously for 3 h. The silica gel was then filtered off and dichloromethane evaporated under reduced pressure. The prepared dialdehydes have sufficient purity and can be used for other purposes without purification.

2.9. General procedure for aldehyde reduction

1 M solution of lithium aluminium hydride in diethylether (2.5 mL) was added to a solution of steroidal dialdehyde (1 mmol) in anhydrous tetrahydrofuran (10 mL). The reaction mixture was refluxed for 5 h. The reaction was then cooled and a saturated solution of potassium sodium tartrate (3 mL) was added dropwise. The work-up method (A) and column chromatography yielded the desired 2,3-secodiol.

2.10. General procedure for baeyer–villiger oxidation

A solution of trifluoroperoxyacetic acid, freshly prepared from trifluoroacetic anhydride (0.45 mL, 3.2 mmol) and hydrogen peroxide (30%; 0.1 mL) in dichloromethane (3 mL), was added to a solution of 6-oxocompound (0.40 mmol) in dichloromethane (8 mL). After standing at room temperature for 5 h, the reaction mixture was poured into water and taken up in chloroform (50 mL). The chloroform extract was washed with a saturated solution of sodium bicarbonate and sodium sulfite, water, and dried over anhydrous sodium sulfate. Solvents were evaporated under reduced pressure and products were separated and purified by column chromatography.

2.11. 6,6-Ethylenedioxy-5 α -cholest-2-ene (2)

The general procedure for ketal formation with olefin **1** [20] (2 g, 5.2 mmol) afforded compound **2** (2.1 g; 94%) as colorless oil. ¹H NMR (CDCl₃) δ 0.81 (s, 3H, CH₃); 0.793, 0.797 (both d, 3H, J = 6.8 Hz, 2 $\times$ CH₃); 0.81 (s, 3H, CH₃); 0.84 (d, 3H, J = 6.7 Hz, CH₃); 1.64 (dd, 1H, J = 11.1 and 5.1 Hz); 1.68–1.78 (m, 3H); 1.84–1.96 (m, 3H); 2.02 (m, 1H); 3.71 (m, 1H, H_{ketal}); 3.81–3.93 (m, 3H, 3 $\times$ H_{ketal}); 5.47 (m, 1H, H-2); 5.59 (m, 1H, H-3). ¹³C NMR δ 11.97, 13.59, 18.67, 20.86, 21.43, 22.55, 22.81, 23.79, 24.16, 28.00, 28.13, 33.35, 35.76, 35.89, 36.14, 39.49, 39.78, 41.22, 41.24, 42.52, 48.08, 53.48, 55.97, 56.17, 64.08, 65.56, 110.09, 124.84, 125.68. HRMS: (API+) calculated for C₂₉H₄₈O₂ ([M+H]⁺) 429.3733, Found 429.3733.

2.12. Upjohn dihydroxylation of ketal 2

The general procedure for Upjohn dihydroxylation with ketal **2** (2.3 g; 5.37 mmol) and column chromatography (mobile phase – ethyl acetate:cyclohexane 2:3) afforded two products as amorphous solid; minor 2 β ,3 β -diol **3a** (202 mg; 8%) and major 2 α ,3 α -diol **3b** (2.1 g;

85%). An analytical sample of both diols was also purified on HPLC (ethyl acetate:cyclohexane 1:1).

6,6-Ethylenedioxy-5 α -cholestan-2 β ,3 β -diol (**3a**): ¹H NMR (CDCl₃) δ 0.67 (s, 3H, CH₃); 0.864, 0.868 (both d, 3H, J = 6.7 Hz, 2 $\times$ CH₃); 0.91 (d, 3H, J = 6.5 Hz, CH₃); 1.13 (s, 3H, CH₃); 1.99 (dt, 1H, J = 12.6 and 3.5 Hz); 2.07 (dd, 1H, J = 14.7 and 2.8 Hz); 3.63 (dt, 1H, J = 11.3 and 4.3 Hz; H-3 α); 3.80 (m, 1H, H_{ketal}); 3.90–3.95 (m, 2H, 2 $\times$ H_{ketal}); 3.96–4.02 (m, 2H, H-2 α , H_{ketal}). ¹³C NMR δ 12.05, 16.64, 18.63, 21.10, 22.54, 22.81, 23.68, 23.78, 24.16, 28.00, 28.13, 32.84, 35.74, 36.12, 36.67, 39.48, 39.75, 41.24, 42.60, 44.34, 51.15, 54.51, 55.91, 56.15, 64.25, 65.51, 69.69, 72.68, 109.56. HRMS: (API+) calculated for C₂₉H₅₀O₄ ([M+H]⁺) 462.3787, Found 462.3789.

6,6-Ethylenedioxy-5 α -cholestan-2 α ,3 α -diol (**3b**): ¹H NMR (CDCl₃) δ 0.67 (s, 3H, CH₃); 0.866, 0.871 (both d, 3H, J = 6.7 Hz, 2 $\times$ CH₃); 0.91 (d, 3H, J = 6.5 Hz, CH₃); 0.94 (s, 3H, CH₃); 1.71 (dd, 1H, J = 12.1 and 4.7 Hz); 1.76 (dd, 1H, J = 13.1 and 4.0 Hz); 1.85–1.94 (m, 5H); 2.00 (dt, 1H, J = 12.5 and 3.4 Hz); 3.73–3.79 (m, 2H, H-3 β , H_{ketal}); 3.89–3.95 (m, 3H, 3 $\times$ H_{ketal}); 4.06 (q, 1H, J = 3.0 Hz, H-2 β). ¹³C NMR δ 12.03, 14.51, 18.65, 20.74, 22.55, 22.81, 23.80, 24.15, 25.86, 28.00, 28.15, 33.22, 35.75, 36.12, 38.44, 39.49, 39.67, 41.14, 42.22, 42.56, 44.29, 53.44, 55.92, 56.10, 64.13, 65.39, 68.75, 68.94, 109.98. HRMS: (API+) calculated for C₂₉H₅₀O₄ ([M+H]⁺) 462.3787, Found 462.3787.

2.13. 6,6-Ethylenedioxy-2,3-seco-5 α -cholestan-2,3-dial (4)

The general procedure for oxidative cleavage with diols **3a** and **3b** (650 mg; 1.4 mmol) yielded the desired dialdehyde **4** (613 mg; 95%) as a viscous oil. ¹H NMR (CDCl₃) δ 0.69 (s, 3H, CH₃); 0.869, 0.873 (both d, 3H, J = 6.7 Hz, 2 $\times$ CH₃); 0.91 (d, 3H, J = 6.6 Hz, CH₃); 0.93 (s, 3H, CH₃); 1.82 (dd, 1H, J = 13.2 and 3.4 Hz); 1.85 (m, 1H); 2.04 (dt, 1H, J = 12.8 and 3.5 Hz); 2.23 (dd, 1H, J = 15.9 and 2.3 Hz, H-5 α); 2.30 (dd, 1H, J = 15.5 and 3.2 Hz, H-1 α); 2.45 (ddd, 1H, J = 15.9, 10.3 and 5.2 Hz, H-4 α); 2.58 (dd, 1H, J = 15.5 and 2.6 Hz, H-1 β); 2.63 (dd, 1H, J = 10.3 and 2.3 Hz, H-4 β); 3.59 (q, 1H, J = 7.1 Hz, H_{ketal}); 3.78 (td, 1H, J = 7.1 and 5.7 Hz, H_{ketal}); 3.92–4.01 (m, 2H, 2 $\times$ H_{ketal}); 9.52 (d, 1H, J = 5.2 Hz, H-3); 9.87 (bt, 1H, J = 3.0 Hz, H-2). ¹³C NMR δ 11.91, 17.43, 18.62, 22.09, 22.53, 22.79, 23.79, 24.17, 26.90, 27.99, 28.11, 33.01, 35.73, 36.09, 38.90, 39.49, 40.00, 41.99, 42.41, 46.05, 48.76, 50.46, 55.71, 56.10, 62.44, 65.38, 109.73, 200.65, 202.46. HRMS: (API+) calculated for C₂₉H₄₈O₄ ([M+H]⁺) 461.3631, Found 461.3632.

2.14. 6,6-Ethylenedioxy-2,3-seco-5 α -cholestan-2,3-diol (5)

The general procedure for aldehyde reduction with dialdehyde **4** (500 mg; 1.09 mmol) and column chromatography (mobile phase – ethyl acetate:cyclohexane 7:3) yielded diol **5** (453 mg; 90%) as a waxy solid. ¹H NMR (DMSO-*d*₆) δ 0.61 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.837, 0.842 (both d, 3H, J = 6.7 Hz, 2 $\times$ CH₃); 0.88 (d, 3H, J = 6.7 Hz, CH₃); 1.68 (dd, 1H, J = 13.1 and 3.4 Hz); 1.76 (m, 1H); 1.91 (m, 1H); 3.46 (m, 1H); 3.30–3.41 (m, 3H); 3.69 (q, 1H, J = 7.1 Hz, H_{ketal}); 3.80 (td, 1H, J = 7.1 and 4.7 Hz, H_{ketal}); 3.86 (q, 1H, J = 7.1 Hz, H_{ketal}); 3.96 (td, 1H, J = 7.1 and 4.7 Hz, H_{ketal}); 4.24 (bs, 1H, OH); 4.32 (bt, 1H, J = 4.6 Hz, OH). ¹³C NMR δ 11.74, 17.88, 18.54, 20.80, 21.20, 22.44, 22.70, 23.17, 23.86, 27.42, 27.78, 27.87, 32.59, 35.18, 35.62, 38.95, 39.11, 39.61, 40.06, 42.00, 45.48, 46.94, 55.40, 55.63, 56.22, 62.51, 62.82, 64.96, 110.76. HRMS: (API+) calculated for C₂₉H₅₁O₃ [(M–H₂O)+H]⁺ 447.3838, Found 447.3834.

2.15. Ketal deprotection of diol 5

The general procedure for ketal deprotection with diol **5** (100 mg; 0.22 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 1:9) yielded unsaturated ether **6a** (46 mg; 53%). Unsaturated ether **6a** was not stable in CDCl₃ and changed to ketal **6b** spontaneously within several hours.

3,6-epoxy-2,3-secocholest-5(6)-en-2-ol (**6a**): ^1H NMR (CDCl_3) δ 0.68 (s, 3H, CH_3); 0.870, 0.874 (both d, 3H, $J = 6.7$ Hz, $2 \times \text{CH}_3$); 0.92 (d, 3H, $J = 6.5$ Hz, CH_3); 0.93 (s, 3H, CH_3); 1.78 (ddd, 1H, $J = 14.1$, 8.8 and 5.8 Hz); 1.84 (m, 1H); 2.00–2.05 (m, 2H); 2.49–2.63 (m, 2H, H-4a, H-4b); 3.53 (td, 1H, $J = 9.8$ and 5.8 Hz, H-2a); 3.62 (td, 1H, $J = 9.8$ and 5.5 Hz, H-2b); 4.23 (dt, 1H, $J = 10.4$ and 8.7 Hz, H-4a); 4.32 (ddd, 1H, $J = 10.4$, 8.8 and 6.7 Hz, H-4b). ^{13}C NMR δ 11.78, 18.68, 21.22, 22.54, 22.68, 22.80, 23.77, 24.31, 28.00, 28.11, 28.92, 29.08, 33.09, 35.73, 36.11, 36.68, 39.48, 39.49, 39.56, 42.41, 43.83, 56.09, 56.62, 60.05, 68.85, 112.18, 150.79. HRMS: (API+) calculated for $\text{C}_{27}\text{H}_{47}\text{O}_2$ ($[\text{M} + \text{H}]^+$) 403.3576, Found 403.3575.

2,6:3,6-Diepoxy-2,3-seco-5a-cholestane (**6b**): ^1H NMR (CDCl_3) δ 0.67 (s, 3H, CH_3); 0.858, 0.863 (both d, 3H, $J = 6.6$ Hz, $2 \times \text{CH}_3$); 0.90 (d, 3H, $J = 6.5$ Hz, CH_3); 0.94 (s, 3H, CH_3); 1.92 (dd, 1H, $J = 12.0$ and 7.2 Hz, H-5); 1.99 (m, 1H, H-4a); 2.01 (m, 1H, H-12 α); 2.03 (dd, 1H, $J = 14.8$ and 6.2 Hz, H-7a); 3.78 (m, 2H, H-2a and H-2b); 3.96 (q, 1H, $J = 8.0$ Hz, H-3a); 4.10 (ddd, 1H, $J = 9.6$, 8.0 and 2.3 Hz, H-3b). ^{13}C NMR δ 12.39, 18.60, 22.21, 22.54, 22.80, 23.70, 23.76, 23.80, 24.94, 27.99, 28.22, 32.65, 34.31, 35.19, 35.20, 35.72, 36.09, 39.46, 40.64, 42.78, 46.70, 46.98, 56.12, 56.35, 59.75, 68.22, 105.18. HRMS: (API+) calculated for $\text{C}_{27}\text{H}_{47}\text{O}_2$ ($[\text{M} + \text{H}]^+$) 403.3576, Found 403.3579.

2.16. 6,6-Ethylenedioxy-2,3-seco-5a-cholestane-2,3-diyl diacetate (**7**)

The general procedure for acetylation with diol **5** (300 mg; 0.65 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 3:17) afforded the desired diacetate **7** (322 mg; 91%) as a viscous oil. ^1H NMR (CDCl_3) δ 0.67 (s, 3H, CH_3); 0.83 (s, 3H, CH_3); 0.864, 0.868 (both d, 3H, $J = 6.7$ Hz, $2 \times \text{CH}_3$); 0.91 (d, 3H, $J = 6.5$ Hz, CH_3); 2.04, 2.05 (both s, 3H, $2 \times \text{CH}_3$); 3.82 (m, 1H, H_{ketal}); 3.88 (m, 1H, H_{ketal}); 3.97 (q, 1H, $J = 7.0$ Hz, H_{ketal}); 4.01–4.16 (m, 4H, $2 \times \text{H}-2$, H-3a, H_{ketal}); 4.29 (ddd, 1H, $J = 10.5$, 8.2 and 4.9 Hz, H-3b). ^{13}C NMR δ 11.93, 17.56, 18.80, 21.05, 21.08, 21.59, 22.54, 22.81, 23.74, 23.79, 24.18, 27.99, 28.13, 32.89, 34.97, 35.74, 36.09, 39.48, 39.69, 40.20, 40.22, 42.38, 45.70, 47.64, 55.92, 56.14, 60.78, 63.13, 65.32, 66.01, 111.28, 171.19, 171.23. HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{53}\text{O}_4$ ($[(\text{M} - \text{AcOH}) + \text{H}]^+$) 489.3944, Found 489.3947.

2.17. Deprotection of diacetate **7**

The general procedure for ketal deprotection with acetate **7** (300 mg; 0.55 mmol) and column chromatography on silica gel afforded two products; firstly, mobile phase (ethyl acetate:cyclohexane 3:17) eluted major acetate **8a** (234 mg; 85%) and then (ethyl acetate:cyclohexane 7:17) eluted minor monoacetate **8b** (20 mg; 8%), both as a viscous oil.

6-Oxo-2,3-seco-5a-cholestane-2,3-diyl diacetate (**8a**): ^1H NMR (CDCl_3) δ 0.65 (s, 3H, CH_3); 0.69 (s, 3H, CH_3); 0.867, 0.872 (both d, 3H, $J = 6.7$ Hz, $2 \times \text{CH}_3$); 0.91 (d, 3H, $J = 6.7$ Hz, CH_3); 1.97 (t, 1H, $J = 12.8$ Hz, H-7a); 2.04, 2.07 (both s, 3H, $2 \times \text{CH}_3$); 2.36 (dd, 1H, $J = 12.5$ and 4.3 Hz, H-7b); 2.45 (bd, 1H, $J = 10.7$ Hz, H-5a); 3.87 (ddd, 1H, $J = 10.9$, 9.4 and 5.0 Hz, H-3a); 4.14–4.21 (m, 2H); 4.11 (m, 1H). ^{13}C NMR δ 11.87, 17.55, 18.57, 20.95, 21.02, 21.90, 22.12, 22.53, 22.80, 23.81, 23.96, 27.98, 28.01, 34.21, 35.68, 36.02, 37.90, 39.43, 39.47, 42.79, 44.05, 47.35, 48.17, 53.52, 56.12, 56.82, 59.97, 63.68, 171.13 ($2 \times \text{C}$), 210.97. HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{49}\text{O}_4$ ($[(\text{M} - \text{AcOH}) + \text{H}]^+$) 445.3682, Found 445.3678.

2-Hydroxy-6-oxo-2,3-seco-5a-cholestane-3-yl acetate (**8b**): ^1H NMR (CDCl_3) δ 0.65 (s, 3H, CH_3); 0.66 (s, 3H, CH_3); 0.867, 0.872 (both d, 3H, $J = 6.7$ Hz, $2 \times \text{CH}_3$); 0.91 (d, 3H, $J = 6.7$ Hz, CH_3); 1.96 (t, 1H, $J = 12.7$ Hz, H-7a); 2.04 (s, 3H, CH_3); 2.05 (m, 1H); 2.13 (m, 1H, H-4a); 2.34 (dd, 1H, $J = 12.7$ and 4.1 Hz, H-7b); 2.53 (bd, 1H, $J = 10.4$ Hz, H-5a); 3.69–3.78 (m, 2H, H-2a, H-2b); 3.91 (ddd, 1H, $J = 10.9$, 8.6 and 6.0 Hz, H-3a); 4.13 (ddd, 1H, $J = 10.9$, 6.6 and 4.7 Hz, H-3b). ^{13}C NMR δ 11.86, 17.64, 18.58, 21.02, 21.96, 22.15, 22.52, 22.78, 23.77, 23.95, 27.96, 28.00, 35.65, 36.02, 37.85, 38.65,

39.42, 39.47, 42.80, 44.10, 47.34, 48.04, 53.89, 56.09, 56.82, 58.06, 64.07, 171.26, 211.40. HRMS: (API+) calculated for $\text{C}_{27}\text{H}_{47}\text{O}_2$ ($[(\text{M} - \text{AcOH}) + \text{H}]^+$) 403.3576, Found 403.3578.

2.18. Baeyer-Villiger oxidation of acetate **8a**

The general procedure for Baeyer-Villiger oxidation with acetate **8a** (200 mg; 0.40 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 1:4) yielded two products as a viscous oil; minor lactone **9a** (55 mg; 27%) and major lactone **9b** (132 mg; 64%).

7a-Homo-7-oxa-6-oxo-2,3-seco-5a-cholestane-2,3-diyl diacetate (**9a**): ^1H NMR (CDCl_3) δ 0.69 (s, 3H, CH_3); 0.863, 0.868 (both d, 3H, $J = 6.7$ Hz, $2 \times \text{CH}_3$); 0.876 (s, 3H, CH_3); 0.90 (d, 3H, $J = 6.5$ Hz, CH_3); 1.94 (m, 1H); 2.03 (m, 1H); 2.05, 2.07 (both s, 3H, $2 \times \text{CH}_3$); 2.18 (m, 1H, $\Sigma J = 34.2$ Hz); 2.99 (dd, 1H, $J = 11.9$ and 1.8 Hz, H-5a); 3.91–3.97 (m, 2H, H-2a, H-2b); 4.01 (dd, 1H, $J = 12.5$ and 9.8 Hz, H-7a β); 4.09 (m, 1H, H-3a); 4.12 (dd, 1H, $J = 12.5$ and 1.8 Hz, H-7a α); 4.26 (ddd, 1H, $J = 11.0$, 5.7 and 4.4 Hz, H-3b). ^{13}C NMR δ 11.81, 18.49, 19.30, 20.94, 22.52, 22.77, 22.88, 23.76, 24.73, 26.70, 27.96 ($2 \times \text{C}$), 29.68, 35.08, 35.68, 35.93, 38.40, 38.99, 39.39, 39.56, 42.64, 45.12, 51.54, 52.12, 56.04, 59.66, 63.31, 70.41, 171.07, 171.11, 175.10. HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{49}\text{O}_4$ ($[(\text{M} - \text{AcOH}) + \text{H}]^+$) 461.3631, Found 461.3630.

7a-Homo-6-oxa-7-oxo-2,3-seco-5a-cholestane-2,3-diyl diacetate (**9b**): ^1H NMR (CDCl_3) δ 0.69 (s, 3H, CH_3); 0.862, 0.864 (both d, 3H, $J = 6.7$ Hz, $2 \times \text{CH}_3$); 0.893 (s, 3H, CH_3); 0.894 (d, 3H, $J = 6.7$ Hz, CH_3); 1.86 (m, 1H); 1.91–2.02 (m, 3H); 2.06, 2.07 (both s, 3H, $2 \times \text{CH}_3$); 2.39 (dd, 1H, $J = 12.9$ and 11.9 Hz, H-7a β); 2.53 (bd, 1H, $J = 12.9$ Hz, H-7a α); 3.92 (td, 1H, $J = 10.7$ and 5.7 Hz, H-2a); 4.07 (td, 1H, $J = 10.7$ and 5.8 Hz, H-2b); 4.16 (ddd, 1H, $J = 10.9$, 9.1 and 5.2 Hz, H-3a); 4.32 (ddd, 1H, $J = 10.9$, 6.2 and 4.1 Hz, H-3b); 4.44 (dd, 1H, $J = 11.0$ and 2.1 Hz, H-5a). ^{13}C NMR δ 11.79, 16.13, 18.48, 20.91, 20.93, 22.51, 22.76, 22.91, 23.75, 25.21, 27.58, 27.94, 29.45, 34.57, 35.29, 35.64, 35.87, 37.62, 39.38, 39.60, 41.59, 42.62, 51.82, 55.63, 56.40, 59.39, 61.27, 80.94, 170.98 ($2 \times \text{C}$), 174.37. HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{49}\text{O}_4$ ($[(\text{M} - \text{AcOH}) + \text{H}]^+$) 461.3631, Found 461.3629.

2.19. 7a-Homo-2,3-dihydroxy-6-oxa-2,3-seco-5a-cholestan-7-one (**10**)

The general procedure for hydrolysis of acetates with lactone **9b** (100 mg; 0.19 mmol) yielded the desired dihydroxylactone **10** (75 mg; 89%) as a white crystal (MeOH). ^1H NMR (CDCl_3) δ 0.70 (s, 3H, CH_3); 0.866, 0.870 (both d, 3H, $J = 6.6$ Hz, $2 \times \text{CH}_3$); 0.88 (s, 3H, CH_3); 0.90 (d, 3H, $J = 6.7$ Hz, CH_3); 1.82–1.90 (m, 3H); 1.99–2.08 (m, 2H); 2.48–2.51 (m, 2H, H-7a β , H-7a α); 3.61 (m, 1H); 3.68 (m, 1H); 3.75–3.84 (m, 2H); 4.64 (dd, 1H, $J = 11.0$ and 1.8 Hz, H-5a). ^{13}C NMR δ 11.82, 16.23, 18.53, 22.52, 22.78, 22.99, 23.76, 25.27, 27.61, 27.97, 30.28, 32.70, 34.60, 35.66, 35.91, 37.77, 39.28, 39.41, 39.68, 41.61, 42.66, 51.69, 55.70, 56.40, 59.23, 81.73, 174.27. HRMS: (API+) calculated for $\text{C}_{27}\text{H}_{49}\text{O}_4$ ($[\text{M} + \text{H}]^+$) 437.3631, Found 437.3627.

2.20. Hydrolysis of acetate **9a**

The general procedure for hydrolysis of acetates with lactone **9a** (45 mg; 0.09 mmol) yielded the unexpected dihydroxylactone **11** (11 mg; 29%) as a solid lyophilized from *t*-BuOH. ^1H NMR (CDCl_3) δ 0.68 (s, 3H, CH_3); 0.862, 0.867 (both d, 3H, $J = 6.6$ Hz, $2 \times \text{CH}_3$); 0.90 (d, 3H, $J = 6.7$ Hz, CH_3); 1.19 (s, 3H, CH_3); 1.84–2.04 (m, 4H); 2.89 (bd, 1H, $J = 10.1$ Hz, H-5a); 3.66 (dd, 1H, $J = 11.6$ and 1.8 Hz, H-7a); 3.77 (t, 2H, $J = 5.8$ Hz, $2 \times \text{H}-3$); 3.87 (dd, 1H, $J = 11.6$ and 2.1 Hz, H-7b); 4.38 (ddd, 1H, $J = 11.6$, 9.5 and 5.2 Hz, H-2a); 4.38 (ddd, 1H, $J = 11.6$, 6.2 and 4.9 Hz, H-2b). ^{13}C NMR δ 11.79, 18.48, 22.52, 22.76, 23.73, 23.91, 24.66, 25.21, 27.60, 27.87, 27.95, 30.96, 35.79, 35.92, 38.69, 39.42, 39.73, 40.48, 42.05, 43.73, 46.39, 51.49, 56.27, 61.62,

62.29, 65.95, 177.01. HRMS: (API+) calculated for $C_{27}H_{40}O_4$ ([M+H]⁺) 437.3631, Found 437.3632.

2.21. 6-Ethylenedioxy-5 α -stigmasta-2,22-diene (13)

The general procedure for ketal formation with olefin 12 [21] (2.0 g, 4.9 mmol) yielded compound 13 (2.13 g; 94%) as a colorless oil. ¹H NMR (CDCl₃) δ 0.71 (s, 3H, CH₃); 0.80 (d, 3H, J = 6.7 Hz, CH₃); 0.81 (t, 3H, J = 7.3 Hz, CH₃); 0.85 (d, 3H, J = 6.7 Hz, CH₃); 0.89 (s, 3H, CH₃); 1.02 (d, 3H, J = 6.5 Hz, CH₃); 1.76–1.81 (m, 2H); 1.93–2.13 (m, 5H); 3.78 (m, 1H, H_{ketal}); 3.89–4.01 (m, 3H, 3 $\times$ H_{ketal}); 5.02 (dd, 1H, J = 15.3 and 8.7 Hz, H-23); 5.15 (dd, 1H, J = 15.3 and 8.6 Hz, H-22); 5.55 (m, 1H, H-2); 5.66 (m, 1H, H-3). ¹³C NMR δ 12.16, 12.24, 13.59, 18.98, 20.85, 21.08, 21.21, 21.44, 24.21, 25.38, 28.81, 31.86, 33.35, 35.90, 39.68, 40.50, 41.22, 41.24, 42.41, 48.10, 51.22, 53.51, 55.97, 56.07, 64.09, 65.57, 110.07, 124.82, 125.68, 129.24, 138.31. HRMS: (API+) calculated for $C_{31}H_{51}O_2$ ([M+H]⁺) 455.3889, Found 455.3892.

2.22. Upjohn dihydroxylation of ketal 13

The general procedure for Upjohn dihydroxylation with ketal 13 (2.0 g; 4.4 mmol) and column chromatography (mobile phase – ethyl acetate:cyclohexane 2:3) afforded two products as a waxy solid; minor 2 β ,3 β -diol 14a (258 mg; 12%) and major 2 α ,3 α -diol 14b (1.79 g; 83%).

6,6-Ethylenedioxy-5 α -stigmast-22-ene-2 β ,3 β -diol (14a): ¹H NMR (CDCl₃) δ 0.69 (s, 3H, CH₃); 0.798 (d, 3H, J = 6.7 Hz, CH₃); 0.805 (t, 3H, J = 7.3 Hz, CH₃); 0.85 (d, 3H, J = 6.7 Hz, CH₃); 1.01 (d, 3H, J = 6.6 Hz, CH₃); 1.14 (s, 3H, CH₃); 1.73 (dd, 1H, J = 13.1 and 4.0 Hz); 1.79 (m, 1H); 1.95–2.05 (m, 4H); 2.07 (dd, 1H, J = 14.5 and 2.9 Hz); 3.63 (m, 1H, ΣJ = 20.2 Hz, H-3 α); 3.80 (m, 1H, H_{ketal}); 3.91–3.96 (m, 2H, 2 $\times$ H_{ketal}); 3.97–4.02 (m, 2H, H-2 α , H_{ketal}); 5.01 (dd, 1H, J = 15.3 and 8.8 Hz, H-23); 5.14 (dd, 1H, J = 15.3 and 8.7 Hz, H-22). ¹³C NMR δ 12.24 (2 $\times$ C), 16.64, 18.98, 21.08, 21.17, 23.70, 24.22, 25.38, 28.82, 31.85, 32.84, 36.69, 39.65, 40.47, 41.24, 42.50, 44.34, 51.18, 51.21, 54.53, 55.95, 56.02, 64.26, 65.52, 69.70, 72.68, 109.55, 129.29, 138.24. HRMS: (API+) calculated for $C_{31}H_{53}O_4$ ([M+H]⁺) 489.3944, Found 489.3942.

6,6-Ethylenedioxy-5 α -stigmast-22-ene-2 α ,3 α -diol (14b): ¹H NMR (CDCl₃) δ 0.69 (s, 3H, CH₃); 0.80 (d, 3H, J = 6.7 Hz, CH₃); 0.81 (t, 3H, J = 7.3 Hz, CH₃); 0.85 (d, 3H, J = 6.7 Hz, CH₃); 0.94 (d, 3H, J = 6.6 Hz, CH₃); 1.02 (s, 3H, CH₃); 1.69–1.78 (m, 2H); 1.85–1.93 (m, 2H); 1.95–2.06 (m, 2H); 3.72–3.78 (m, 2H, H-3 α , H_{ketal}); 3.87–3.95 (m, 3H, 3 $\times$ H_{ketal}); 4.05 (m, 1H, H-2 α); 5.01 (dd, 1H, J = 15.3 and 8.7 Hz, H-23); 5.15 (dd, 1H, J = 15.3 and 8.6 Hz, H-22). ¹³C NMR δ 12.22, 12.24, 14.50, 18.98, 20.73, 21.08, 21.21, 24.22, 25.38, 25.86, 28.82, 31.85, 33.21, 38.44, 39.57, 40.50, 41.13, 42.20, 42.45, 44.29, 51.20, 53.47, 55.88, 56.01, 64.13, 65.39, 68.73, 68.91, 110.00, 129.28, 138.24. HRMS: (API+) calculated for $C_{31}H_{53}O_4$ ([M+H]⁺) 489.3944, Found 489.3944.

2.23. 6-Ethylenedioxy-2,3-seco-5 α -stigmast-22-ene-2,3-dial (15)

The general procedure for oxidative cleavage with diols 14a and 14b (1.5 g; 3.07 mmol) afforded the desired dialdehyde 4 (1.39 g; 93%) as a viscous oil. ¹H NMR (CDCl₃) δ 0.70 (s, 3H, CH₃); 0.798 (d, 3H, J = 6.6 Hz, CH₃); 0.805 (t, 3H, J = 7.2 Hz, CH₃); 0.85 (d, 3H, J = 6.6 Hz, CH₃); 0.93 (s, 3H, CH₃); 1.01 (d, 3H, J = 6.9 Hz, CH₃); 1.81 (dd, 1H, J = 13.2 and 3.4 Hz); 1.99–2.08 (m, 2H); 2.23 (dd, 1H, J = 15.9 and 2.4 Hz, H-5 α); 2.30 (dd, 1H, J = 15.5 and 3.2 Hz, H-1 α); 2.44 (ddd, 1H, J = 15.9, 10.3 and 5.3 Hz, H-4 α); 2.58 (dd, 1H, J = 15.1 and 2.8 Hz, H-1 β); 2.63 (dd, 1H, J = 10.3 and 2.3 Hz, H-4 β); 3.59 (q, 1H, J = 7.1 Hz, H_{ketal}); 3.77 (m, 1H, H_{ketal}); 3.92–4.00 (m, 2H, 2 $\times$ H_{ketal}); 5.03 (dd, 1H, J = 15.3 and 8.6 Hz, H-23); 5.15 (dd, 1H, J = 15.3 and 8.6 Hz, H-22); 9.51 (d, 1H, J = 5.2 Hz, H-3); 9.87 (bt, 1H, J = 3.0 Hz, H-2). ¹³C NMR δ 12.10, 12.22, 17.44, 19.00, 21.06, 21.17,

22.08, 24.23, 25.36, 28.75, 31.85, 33.01, 38.90, 39.39, 40.00, 40.41, 42.01, 42.31, 46.07, 48.78, 50.47, 51.22, 55.81, 55.91, 62.45, 65.39, 109.72, 129.52, 138.04, 200.68, 202.49. HRMS: (API+) calculated for $C_{31}H_{51}O_4$ ([M+H]⁺) 487.3787, Found 487.3790.

2.24. 6,6-Ethylenedioxy-2,3-seco-5 α -stigmast-22-ene-2,3-diol (16)

The general procedure for aldehyde reduction with dialdehyde 15 (1.0 g; 2.06 mmol) and filtration through silica gel in ethyl acetate afforded diol 16 (887 mg; 88%) as a waxy solid. ¹H NMR (DMSO-*d*₆) δ 0.63 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.769 (d, 3H, J = 6.4 Hz, CH₃); 0.772 (t, 3H, J = 7.3 Hz, CH₃); 0.82 (d, 3H, J = 6.4 Hz, CH₃); 0.98 (d, 3H, J = 6.7 Hz, CH₃); 1.62 (m, 1H); 1.67 (dd, 1H, J = 13.1 and 3.4 Hz); 1.90 (m, 1H); 2.01 (m, 1H); 3.29–3.41 (m, 3H, 3 $\times$ CH-OH); 3.47 (td, 1H, J = 9.5 and 4.9 Hz, CH-OH); 3.69 (q, 1H, J = 7.0 Hz, H_{ketal}); 3.80 (td, 1H, J = 7.0 and 4.7 Hz, H_{ketal}); 3.86 (q, 1H, J = 7.0 Hz, H_{ketal}); 3.96 (td, 1H, J = 7.0 and 4.7 Hz, H_{ketal}); 4.24 (bs, 1H, OH); 4.32 (bs, 1H, J = 4.6 Hz, OH); 5.01 (dd, 1H, J = 15.1 and 8.9 Hz, H-23); 5.14 (dd, 1H, J = 15.1 and 8.6 Hz, H-22). ¹³C NMR δ 11.90, 12.16, 17.87, 18.88, 20.97, 21.12, 21.17, 23.85, 24.88, 27.87, 28.49, 31.34, 32.58, 39.07, 39.42, 39.99, 40.09, 41.88, 45.51, 46.97, 48.61, 50.59, 55.39, 55.49, 62.50 (2 $\times$ C), 62.85, 64.99, 110.76, 128.81, 138.08. HRMS: (API+) calculated for $C_{31}H_{55}O_4$ ([M+H]⁺) 491.4100, Found 491.4096.

2.25. Ketal deprotection of diol 16

The general procedure for ketal deprotection with diol 16 (200 mg; 0.41 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 1:9) afforded intramolecular ketal 17 (113 mg; 65%) as a waxy solid.

2,6:3,6-Diepoxy-2,3-seco-5 α -stigmast-22-ene (17): ¹H NMR (CDCl₃) δ 0.70 (s, 3H, CH₃); 0.796 (d, 3H, J = 6.6 Hz, CH₃); 0.806 (t, 3H, J = 7.4 Hz, CH₃); 0.85 (d, 3H, J = 6.6 Hz, CH₃); 0.95 (s, 3H, CH₃); 1.01 (d, 3H, J = 6.6 Hz, CH₃); 1.93 (m, 1H); 1.98–2.07 (m, 4H); 3.79 (m, 1H, H-2 α); 3.80 (bd, 1H, J = 7.4 Hz, H-2 β); 3.97 (q, 1H, J = 8.0 Hz, H-3); 4.11 (ddd, 1H, J = 9.6, 8.2, and 2.4 Hz, H-3 β); 5.02 (dd, 1H, J = 15.2 and 8.9 Hz, H-23); 5.14 (dd, 1H, J = 15.2 and 8.7 Hz, H-22). ¹³C NMR δ 12.24, 12.57, 18.95, 21.08, 21.10, 22.20, 23.71, 23.82, 24.89, 25.41, 28.96, 31.86, 32.65, 34.28, 35.17 (2 $\times$ C), 40.49 (2 $\times$ C), 42.65, 46.69, 46.98, 51.22, 55.89, 56.45, 59.75, 68.22, 105.12, 129.39, 138.15. HRMS: (API+) calculated for $C_{29}H_{49}O_2$ ([M+H]⁺) 429.3733, Found 429.3736.

2.26. Sharpless dihydroxylation of diol 16

Osmium tetroxide in *t*-butanol (0.3 mL; 1 g/20 mL; 0.06 mmol) was added to a solution of diol 16 (600 mg; 1.2 mmol); hydroquinidine 4-chlorobenzoate (140 mg; 0.3 mmol); methanesulfonamide (285 mg; 3.0 mmol); potassium carbonate (830 mg; 6.0 mmol); and potassium ferricyanide (2.0 g; 6.0 mmol) in a mixture of *t*-butanol and water (40 mL; 1:1 v/v). The reaction mixture was stirred at room temperature for 7 days. A saturated solution of sodium sulfite (5 mL) was then added. After an additional 30 min of stirring, the reaction mixture was diluted with ethyl acetate (70 mL) and extracted with water (2 $\times$ 70 mL). The combined organic fractions were dried over anhydrous magnesium sulfate and evaporated under reduced pressure. Column chromatography on silica gel (mobile phase – methanol:chloroform – 1:19) gave the two main products; tetraol 18a (115 mg; 18%) and tetraol 18b (194 mg; 30%) in a ratio 18a:18b 2:3.

(22S,23S)-6,6-Ethylenedioxy-2,3-seco-5 α -stigmastane-2 β ,3 β ,22,23-tetraol (18a): ¹H NMR (DMSO-*d*₆) δ 0.63 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.78 (d, 3H, J = 6.7 Hz, CH₃); 0.87 (t, 3H, J = 7.5 Hz, CH₃); 0.89 (d, 3H, J = 6.7 Hz, CH₃); 0.93 (d, 3H, J = 6.7 Hz, CH₃); 1.67 (dd, 1H, J = 12.8 and 3.4 Hz); 1.69 (m, 1H); 1.94 (m, 1H); 2.06 (td, 1H, J = 6.9 and 2.4 Hz); 3.30–3.51 (m, 6H); 3.69 (q, 1H, J = 7.0 Hz, H_{ketal}); 3.75 (d, 1H, J = 7.6 Hz, sec-OH); 3.80 (m, 1H, H_{ketal}); 3.85 (q, 1H,

$J = 7.0$ Hz, H_{ketal}); 3.96 (m, 1H, H_{ketal}); 4.20 (d, 1H, $J = 6.4$ Hz, sec-OH); 4.25 (b t, 1H, $J = 5.0$ Hz, prim-OH); 4.34 (b t, 1H, $J = 5.2$ Hz, prim-OH). ^{13}C NMR δ 11.68, 14.29, 14.44, 17.54, 17.90, 18.27, 21.25, 21.94, 24.17, 26.23, 27.56, 27.89, 29.07, 32.66, 39.62, 40.08, 42.04, 42.49, 45.52, 46.95, 48.78, 52.26, 55.10, 56.27, 62.54 ($2 \times \text{C}$), 62.89, 65.00, 69.49, 71.05, 110.82. HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{57}\text{O}_6$ ($[\text{M} + \text{H}]^+$) 525.4155, Found 525.4153.

(22R,23R)-6,6-Ethylenedioxy-2,3-seco-5 α -stigmastane-2 β ,3 β ,22,23-tetraol (**18b**): ^1H NMR (DMSO- d_6) δ 0.63 (s, 3H, CH_3); 0.75 (s, 3H, CH_3); 0.81 (d, 3H, $J = 6.7$ Hz, CH_3); 0.884 (t, 3H, $J = 7.5$ Hz, CH_3); 0.891 (d, 3H, $J = 6.7$ Hz, CH_3); 0.90 (d, 3H, $J = 6.7$ Hz, CH_3); 1.70 (dd, 1H, $J = 12.8$ and 3.4 Hz); 1.76 (dq, 1H, $J = 13.3$ and 6.7 Hz); 1.85–1.94 (m, 2H); 3.31–3.42 (m, 4H); 3.46–3.52 (m, 2H); 3.71 (q, 1H, $J = 7.0$ Hz, H_{ketal}); 3.81 (d, 1H, $J = 4.0$ Hz, sec-OH); 3.82 (m, 1H, H_{ketal}); 3.88 (q, 1H, $J = 7.0$ Hz, H_{ketal}); 3.97 (m, 1H, H_{ketal}); 4.06 (d, 1H, $J = 4.6$ Hz, sec-OH); 4.12 (t, 1H, $J = 5.0$ Hz, prim-OH); 4.19 (t, 1H, $J = 5.2$ Hz, prim-OH). ^{13}C NMR δ 11.65, 12.12, 13.54, 17.91, 18.85, 19.76, 21.12, 21.29, 23.75, 27.30, 27.89, 28.62, 29.07, 31.34, 32.72, 36.95, 40.08, 41.74, 45.54, 46.05, 47.02, 52.23, 55.59, 56.28, 62.55 ($2 \times \text{C}$), 62.86, 65.03, 71.17, 73.11, 110.81. HRMS: (API+) calculated for $\text{C}_{31}\text{H}_{57}\text{O}_6$ ($[\text{M} + \text{H}]^+$) 525.4155, Found 525.4150.

2.27. (22R,23R)-2 α ,3 α ,22,23-Tetrahydroxy-2,3-seco-5 α -stigmastane-6-one (**19**)

5% aqueous solution of hydrochloric acid (0.25 mL) was added to a solution of ketal **18b** (50 mg; 0.1 mmol) in tetrahydrofuran (5 mL). The mixture was stirred at room temperature for 1 h. Work-up (method A) and evaporation of ethyl acetate in 25 bath afforded the desired tetrahydroxyketone **19** (43 mg; 94%) in 85–90% purity (based on ^1H NMR). Purification on silica or crystallization always led to decomposition and formation of several less polar compounds. ^1H NMR (DMSO- d_6) δ 0.54 (s, 3H, CH_3); 0.61 (s, 3H, CH_3); 0.80 (d, 3H, $J = 6.4$ Hz, CH_3); 0.871 (t, 3H, $J = 7.5$ Hz, CH_3); 0.878 (d, 3H, $J = 6.4$ Hz, CH_3); 0.89 (d, 3H, $J = 6.6$ Hz, CH_3); 1.86–1.87 (m, 4H); 2.06 (m, 1H, H-5 α); 3.13 (m, 1H); 3.38 (m, 1H); 3.45–3.50 (m, 4H); 3.95 (d, 1H, $J = 4.3$ Hz, sec-OH); 4.22 (d, 1H, $J = 4.9$ Hz, sec-OH); 4.32 (t, 1H, $J = 5.0$ Hz, prim-OH); 4.38 (t, 1H, $J = 5.3$ Hz, prim-OH). ^{13}C NMR δ 11.56, 12.05, 13.51, 17.55, 18.80, 19.70, 21.07, 22.57, 23.44, 25.60, 27.14, 28.60, 31.31, 36.82, 37.55, 38.74, 42.08, 43.41, 46.00, 46.82, 47.16, 52.13, 52.48, 55.71, 56.15, 59.80, 71.15, 73.04, 211.41. HRMS: (API+) calculated for $\text{C}_{29}\text{H}_{53}\text{O}_5$ ($[\text{M} + \text{H}]^+$) 481.3893, Found 481.3893.

2.28. (22R,23R)-6-Oxo-3 α ,5-cyclo-5 α -stigmastane-22,23-diyl diacetate (**21**)

The general procedure for acetylation with diol **20** [22] (2.0 g; 4.5 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 1:9) afforded the desired diacetate **21** (2.23 g; 94%) as a viscous oil. ^1H NMR (CDCl_3) δ 0.72 (t, 1H, $J = 4.9$ Hz, H-4 β); 0.74 (s, 3H, CH_3); 0.84 (d, 3H, $J = 6.7$ Hz, CH_3); 0.97 (d, 3H, $J = 6.7$ Hz, CH_3); 0.98 (t, 3H, $J = 7.5$ Hz, CH_3); 1.00 (s, 3H, CH_3); 1.04 (d, 3H, $J = 6.7$ Hz, CH_3); 1.97 (s, 3H, CH_3); 2.02 (s, 3H, CH_3); 1.98–2.10 (m, 3H); 2.43 (dd, 1H, $J = 15.1$ and 2.9 Hz, H-7 β); 5.18 (bd, 1H, $J = 9.3$ Hz, H-22); 5.31 (dd, 1H, $J = 9.3$ and 1.2 Hz, H-23). ^{13}C NMR δ 11.61, 11.77, 12.83, 13.05, 18.58, 19.54, 19.66, 20.87, 20.96, 21.10, 22.81, 23.91, 25.86, 28.14, 28.24, 33.44, 34.77, 35.31, 36.94, 39.59, 42.54, 44.64, 45.69, 45.95, 46.26, 46.70, 52.47, 56.84, 72.80, 75.56, 170.40, 170.55, 209.55. HRMS: (API+) calculated for $\text{C}_{33}\text{H}_{53}\text{O}_5$ ($[\text{M} + \text{H}]^+$) 529.3893, Found 529.3896.

2.29. (22R,23R)-6-Oxo-5 α -stigmast-2-ene-22,23-diyl diacetate (**22**)

Pyridinium *p*-toluenesulfonate (200 mg; 0.8 mmol) and lithium bromide (220 mg; 2.5 mmol) were added to a solution of diacetate **21** (2.1 g; 4.0 mmol) in dimethylacetamide (25 mL). The reaction mixture

was heated at 170 °C for 6 h. Then, it was diluted with ethyl acetate and worked up (method A). The crude product was purified on silica gel (mobile phase – ethyl acetate:cyclohexane 1:9) to afford desired olefin **22** (1.89 g; 90%) as waxy solid. ^1H NMR (CDCl_3) δ 0.63 (s, 3H, CH_3); δ 0.64 (s, 3H, CH_3); 0.77 (d, 3H, $J = 6.9$ Hz, CH_3); 0.90 (d, 3H, $J = 6.9$ Hz, CH_3); 0.91 (t, 3H, $J = 7.4$ Hz, CH_3); 0.97 (d, 3H, $J = 6.8$ Hz, CH_3); 1.90 (s, 3H, CH_3); 1.95 (s, 3H, CH_3); 1.87–2.03 (m, 5H, $2 \times \text{H-1}$, H-4 α , H-7 α , H-16 α); 2.18 (m, 1H, H-4 β); 2.25–2.30 (m, 2H, H-5 α , H-7 β); 5.11 (dd, 1H, $J = 9.3$ and 1.1 Hz, H-22); 5.31 (dd, 1H, $J = 9.3$ and 1.4 Hz, H-23); 5.50 (m, 1H, H-2); 5.61 (m, 1H, H-3). ^{13}C NMR δ 11.67, 12.81, 13.04, 13.49, 18.59, 19.55, 20.86, 20.94, 21.08 ($2 \times \text{C}$), 21.69, 23.81, 28.03, 28.26, 36.95, 37.68, 39.34, 39.40, 39.97, 42.65, 45.73, 46.85, 52.53, 53.33, 53.79, 56.63, 72.86, 75.57, 124.45, 124.97, 170.36, 170.54, 211.82. HRMS: (API+) calculated for $\text{C}_{33}\text{H}_{53}\text{O}_5$ ($[\text{M} + \text{H}]^+$) 529.3893, Found 529.3896.

2.30. (22R,23R)-6,6-Ethylenedioxy-5 α -stigmast-2-ene-22,23-diyl diacetate (**23**)

The general procedure for ketal formation with diacetate **22** (1.80 g, 3.4 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 1:9) afforded compound **23** (1.81 g; 93%) as a colorless oil. ^1H NMR (CDCl_3) δ 0.71 (s, 3H, CH_3); 0.85 (d, 3H, $J = 6.8$ Hz, CH_3); 0.89 (s, 3H, CH_3); 0.97 (d, 3H, $J = 6.8$ Hz, CH_3); 0.99 (t, 3H, $J = 7.5$ Hz, CH_3); 1.03 (d, 3H, $J = 6.7$ Hz, CH_3); 1.69–1.79 (m, 4H); 1.97 (s, 3H, CH_3); 2.02 (s, 3H, CH_3); 1.92–2.04 (m, 4H); 2.08 (m, 1H, H-4 β); 3.78 (m, 1H, H_{ketal}); 3.90–4.01 (m, 3H, $3 \times H_{\text{ketal}}$); 5.18 (dd, 1H, $J = 9.3$ and 1.1 Hz, H-22); 5.31 (dd, 1H, $J = 9.3$ and 1.4 Hz, H-23); 5.54 (m, 1H, H-2); 5.66 (m, 1H, H-3). ^{13}C NMR δ 11.71, 12.81, 13.02, 13.60, 18.60, 19.55, 20.83, 20.89, 20.96, 21.10, 21.41, 24.02, 26.89, 28.13, 28.22, 33.39, 35.85, 36.95, 39.68, 41.09, 41.23, 45.66, 48.03, 52.55, 53.37, 55.85, 64.10, 65.57, 72.90, 75.66, 109.97, 124.74, 125.69, 170.60 ($2 \times \text{C}$). HRMS: (API+) calculated for $\text{C}_{35}\text{H}_{57}\text{O}_6$ ($[\text{M} + \text{H}]^+$) 573.4155, Found 573.4160.

2.31. Upjohn dihydroxylation of ketone **22**

The general procedure for Upjohn dihydroxylation with ketone **22** (100 mg; 0.19 mmol) and column chromatography (mobile phase – ethyl acetate:cyclohexane 3:2) afforded a mixture of two products (minor 2 β ,3 β -diol **24a** and major 2 α ,3 α -diol **24b**) as a solid in an overall yield 97 mg (92%) and a ratio 2 β ,3 β :2 α ,3 α – 1:9. An analytical sample of mixture was used for HPLC (mobile phase – ethyl acetate:cyclohexane 3:2) to separate and analyze both isomers.

(22R,23R)-2 β ,3 β -dihydroxy-6-oxo-5 α -stigmastane-22,23-diyl diacetate (**24a**): ^1H NMR (CDCl_3) δ 0.69 (s, 3H, CH_3); 0.84 (d, 3H, $J = 6.9$ Hz, CH_3); 0.96 (d, 3H, $J = 6.7$ Hz, CH_3); 0.977 (t, 3H, $J = 7.5$ Hz, CH_3); 0.979 (s, 3H, CH_3); 1.03 (d, 3H, $J = 6.7$ Hz, CH_3); 1.97 (s, 3H, CH_3); 2.02 (s, 3H, CH_3); 2.12 (dd, 1H, $J = 12.5$ and 2.8 Hz, H-7 α); 2.20 (dd, 1H, $J = 12.5$ and 2.8 Hz, H-7 β); 2.30 (dd, 1H, $J = 13.4$ and 4.6 Hz, H-5 α); 3.63 (m, 1H, H-2 α); 4.03 (m, 1H, H-3 α); 5.17 (dd, 1H, $J = 9.4$ and 0.9 Hz, H-22); 5.30 (dd, 1H, $J = 9.4$ and 1.4 Hz, H-23). ^{13}C NMR δ 11.77, 12.79, 13.03, 15.22, 18.56, 19.54, 20.88, 20.95, 21.09, 21.49, 23.81, 24.19, 28.04, 28.21, 36.92, 37.20, 39.39, 40.51, 42.34, 42.82, 45.68, 46.31, 52.50, 54.58, 56.50, 57.07, 69.05, 71.75, 72.79, 75.59, 170.39, 170.59, 210.48. HRMS: (API+) calculated for $\text{C}_{33}\text{H}_{55}\text{O}_7$ ($[\text{M} + \text{H}]^+$) 545.3948, Found 545.3950.

(22R,23R)-2 α ,3 α -dihydroxy-6-oxo-5 α -stigmastane-22,23-diyl diacetate (**24b**): ^1H NMR (CDCl_3) δ 0.68 (s, 3H, CH_3); 0.75 (s, 3H, CH_3); 0.84 (d, 3H, $J = 6.7$ Hz, CH_3); 0.96 (d, 3H, $J = 6.7$ Hz, CH_3); 0.98 (t, 3H, $J = 7.5$ Hz, CH_3); 1.03 (d, 3H, $J = 6.7$ Hz, CH_3); 1.97 (s, 3H, CH_3); 2.03 (s, 3H, CH_3); 1.91 (dt, 1H, $J = 15.0$ and 3.4 Hz, H-4 α); 1.95–2.03 (m, 2H, H-7 α , H-16 α); 2.28 (dd, 1H, $J = 13.3$ and 4.4 Hz, H-7 β); 2.67 (dd, 1H, $J = 9.4$ and 1.4 Hz, H-5 α); 3.76 (m, 1H, $\Sigma J = 19.9$ Hz, H-2 β); 4.04 (m, 1H, H-3 β); 5.17 (dd, 1H, $J = 9.5$ and 0.9 Hz, H-22); 5.30 (dd, 1H, $J = 9.5$ and 1.2 Hz, H-23). ^{13}C NMR δ 11.71, 12.78, 13.01, 13.51,

18.54, 19.50, 20.86, 20.92, 21.07, 21.10, 23.78, 26.25, 26.86, 27.99, 28.19, 36.87, 37.62, 39.25, 40.10, 42.74, 45.67, 46.58, 50.65, 52.42, 53.55, 56.45, 68.20, 68.31, 72.80, 75.51, 170.44, 170.59, 212.08. HRMS: (API+) calculated for $C_{33}H_{55}O_7$ $[(M+H)^+]$ 545.3948, Found 545.3949.

2.32. Upjohn dihydroxylation of ketal **23**

The general procedure for Upjohn dihydroxylation with ketal **23** (1.7 g; 3.0 mmol) and column chromatography (mobile phase – ethyl acetate:cyclohexane 3:2) afforded a mixture of two products (minor 2 β ,3 β -diol **25a** and major 2 α ,3 α -diol **25b**) as a solid in an overall yield 1.66 g (92%) and a ratio 2 β ,3 β :2 α ,3 α – 1:10. Analytical sample of mixture was used for HPLC (mobile phase – ethyl acetate:cyclohexane 3:2) to separate and analyze both isomers.

(22R,23R)-2 β ,3 β -dihydroxy-6,6-ethylenedioxy-5 α -stigmastane-22,23-diyl diacetate (**25a**): 1H NMR ($CDCl_3$) δ 0.69 (s, 3H, CH_3); 0.84 (d, 3H, J = 6.9 Hz, CH_3); 0.97 (d, 3H, J = 6.9 Hz, CH_3); 0.98 (t, 3H, J = 7.5 Hz, CH_3); 1.02 (d, 3H, J = 6.7 Hz, CH_3); 1.13 (s, 3H, CH_3); 1.97 (s, 3H, CH_3); 1.98 (m, 1H); 2.02 (s, 3H, CH_3); 2.07 (dd, 1H, J = 14.4 and 2.4 Hz); 3.63 (m, 1H, H-3 α); 3.80 (m, 1H, H_{ketal}); 3.90–3.95 (m, 2H, 2 $\times$ H_{ketal}); 3.96–4.02 (m, 2H, H-2 α , H_{ketal}); 5.17 (d, 1H, J = 9.1 Hz, H-22); 5.30 (d, 1H, J = 9.1 Hz, H-23). ^{13}C NMR δ 11.78, 12.80, 13.01, 16.63, 18.58, 19.54, 20.89, 20.95, 21.07, 21.09, 23.64, 24.03, 26.88, 28.13, 28.21, 32.87, 36.63, 36.93, 39.64, 41.12, 42.48, 45.65, 51.11, 52.54, 54.39, 55.79, 64.28, 65.52, 69.64, 72.62, 72.87, 75.65, 109.45, 170.40, 170.63. HRMS: (API+) calculated for $C_{35}H_{59}O_8$ $[(M+H)^+]$ 607.4210, Found 607.4211.

(22R,23R)-2 α ,3 α -dihydroxy-6,6-ethylenedioxy-5 α -stigmastane-22,23-diyl diacetate (**25b**): 1H NMR ($CDCl_3$) δ 0.68 (s, 3H, CH_3); 0.83 (d, 3H, J = 6.9 Hz, CH_3); 0.93 (s, 3H, CH_3); 0.96 (d, 3H, J = 6.9 Hz, CH_3); 0.97 (t, 3H, J = 7.5 Hz, CH_3); 1.01 (d, 3H, J = 6.7 Hz, CH_3); 1.86 (dd, 1H, J = 12.7 and 2.9 Hz); 1.91 (dt, 1H, J = 14.1 and 3.1 Hz); 1.96 (s, 3H, CH_3); 2.02 (s, 3H, CH_3); 3.72–3.79 (m, 2H, H-3 β , H_{ketal}); 3.86–3.94 (m, 3H, 3 $\times$ H_{ketal}); 4.05 (bd, 1H, J = 2.8 Hz, H-2 β); 5.17 (dd, 1H, J = 9.3 and 0.9 Hz, H-22); 5.29 (dd, 1H, J = 9.3 and 1.7 Hz, H-23). ^{13}C NMR δ 11.76, 12.79, 13.01, 14.50, 18.58, 19.52, 20.67, 20.89, 20.93, 21.08, 24.02, 25.84, 26.87, 28.11, 28.20, 33.24, 36.90, 39.54, 40.97, 42.13, 42.42, 44.23, 45.85, 52.48, 53.29, 55.75, 64.12, 65.38, 68.66, 68.85, 72.90, 75.60, 109.91, 170.42, 170.64. HRMS: (API+) calculated for $C_{35}H_{59}O_8$ $[(M+H)^+]$ 607.4210, Found 607.4212.

2.33. (22R,23R)-2,3,6-Trioxo-2,3-seco-5 α -stigmastane-22,23-diyl diacetate (**26**)

The general procedure for oxidative cleavage with a mixture of diols **24a** and **24b** (70 mg; 0.12 mmol) afforded desired dialdehyde **26** (62 mg; 89%) as a viscous oil. The product was lyophilized from benzene. 1H NMR ($CDCl_3$) δ 0.69 (s, 3H, CH_3); 0.76 (s, 3H, CH_3); 0.84 (d, 3H, J = 6.7 Hz, CH_3); 0.96 (d, 3H, J = 6.9 Hz, CH_3); 0.98 (t, 3H, J = 7.5 Hz, CH_3); 1.03 (d, 3H, J = 6.7 Hz, CH_3); 1.84 (m, 1H); 1.97 (s, 3H, CH_3); 2.03 (s, 3H, CH_3); 2.08 (m, 1H, H-7 α); 2.26 (dd, 1H, J = 16.5 and 1.8 Hz, H-1a); 2.31 (dd, 1H, J = 17.6 and 3.2 Hz, H-4a); 2.41 (dd, 1H, J = 13.1 and 4.3 Hz, H-7 β); 2.63 (dd, 1H, J = 16.5 and 2.8 Hz, H-1b); 3.08 (ddd, 1H, J = 17.6, 9.7 and 1.2 Hz, H-4b); 3.44 (dd, 1H, J = 9.7 and 2.8 Hz, H-5 α); 5.17 (dd, 1H, J = 9.3 and 0.9 Hz, H-22); 5.30 (dd, 1H, J = 9.3 and 1.2 Hz, H-23); 9.79 (bd, 1H, J ~ 1.0 Hz, H-3); 9.83 (bt, 1H, J ~ 2.4 Hz, H-2). ^{13}C NMR δ 11.59, 12.78, 13.03, 17.48, 18.56, 19.52, 20.85, 20.94, 21.08, 22.20, 23.77, 27.97, 28.20, 36.87, 37.06, 37.86, 39.03, 42.53, 44.11, 45.69, 46.31, 48.33, 49.36, 52.16, 52.36, 56.35, 72.73, 75.38, 170.37, 170.55, 200.18, 200.92, 209.00. HRMS: (API+) calculated for $C_{33}H_{53}O_7$ $[(M+H)^+]$ 561.3791, Found 561.3790.

2.34. (22R,23R)-6,6-Ethylenedioxy-2,3-dioxo-2,3-seco-5 α -stigmastane-22,23-diyl diacetate (**27**)

The general procedure for oxidative cleavage with mixture of diols **25a** and **25b** (1.50 g; 2.48 mmol) afforded desired dialdehyde **27** (1.36 mg; 91%) as a viscous oil. The product was lyophilized from benzene. 1H NMR ($CDCl_3$) δ 0.71 (s, 3H, CH_3); 0.85 (d, 3H, J = 6.9 Hz, CH_3); 0.93 (s, 3H, CH_3); 0.97 (d, 3H, J = 6.9 Hz, CH_3); 0.98 (t, 3H, J = 7.5 Hz, CH_3); 1.03 (d, 3H, J = 6.7 Hz, CH_3); 1.80 (dd, 1H, J = 13.1 and 3.7 Hz); 1.97 (s, 3H, CH_3); 2.03 (s, 3H, CH_3); 2.23 (dd, 1H, J = 15.9 and 2.1 Hz, H-5 α); 2.31 (dd, 1H, J = 15.5 and 3.1 Hz, H-1a); 2.45 (ddd, 1H, J = 15.9, 10.3 and 5.5 Hz, H-4a); 2.57 (dd, 1H, J = 15.5 and 2.9 Hz, H-1b); 2.64 (dd, 1H, J = 10.3 and 2.3 Hz, H-4b); 3.59 (q, 1H, J = 7.2 Hz, H_{ketal}); 3.78 (td, 1H, J = 7.2, J = 5.8 Hz, H_{ketal}); 3.92–4.01 (m, 2H, 2 $\times$ H_{ketal}); 5.18 (dd, 1H, J = 9.3 and 0.9 Hz, H-22); 5.31 (dd, 1H, J = 9.3 and 1.4 Hz, H-23); 9.52 (d, 1H, J = 5.5 Hz, H-3); 9.86 (bt, 1H, J ~ 3.0 Hz, H-2). ^{13}C NMR δ 11.65, 12.80, 13.04, 17.44, 18.60, 19.54, 20.87, 20.95, 21.08, 21.98, 24.02, 28.09, 28.23, 33.01, 36.92, 38.87, 39.35, 39.86, 41.90, 42.26, 45.69, 45.91, 48.57, 50.39, 52.45, 55.58, 62.45, 65.38, 72.83, 75.51, 109.60, 170.37, 170.61, 200.58, 202.36. HRMS: (API+) calculated for $C_{35}H_{57}O_8$ $[(M+H)^+]$ 605.4053, Found 605.4055.

2.35. (22R,23R)-6,6-Ethylenedioxy-2,3-seco-5 α -stigmastane-2,3,22,23-tetraol (**18b**)

The general procedure for aldehyde reduction with dialdehyde **27** (1.0 g; 1.66 mmol) and column chromatography (mobile phase – methanol:chloroform – 1:19) afforded diol **18b** (754 mg; 87%) as an amorphous solid. All data correspond to diol **18b** prepared in previous experiment by Sharpless dihydroxylation.

2.36. (22R,23R)-6,6-Ethylenedioxy-2,3-seco-5 α -stigmastane-2,3,22,23-tetraol tetraacetate (**28**)

The general procedure for acetylation with diol **18b** (600 mg; 1.15 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 3:7) afforded desired diacetate **28** (752 mg; 95%) as a viscous oil. 1H NMR ($CDCl_3$) δ 0.69 (s, 3H, CH_3); 0.83 (s, 3H, CH_3); 0.84 (d, 3H, J = 6.7 Hz, CH_3); 0.97 (d, 3H, J = 6.9 Hz, CH_3); 0.98 (t, 3H, J = 7.5 Hz, CH_3); 1.02 (d, 3H, J = 6.7 Hz, CH_3); 1.97 (s, 3H, CH_3); 2.03 (s, 3H, CH_3); 2.038 (s, 3H, CH_3); 2.042 (s, 3H, CH_3); 3.82 (q, 1H, J = 7.1 Hz, H_{ketal}); 3.88 (td, 1H, J = 7.1 and 5.0 Hz, H_{ketal}); 3.97 (q, 1H, J = 7.1 Hz, H_{ketal}); 4.00–4.16 (m, 4H, 2 $\times$ H-2, H-3a, H_{ketal}); 4.29 (ddd, 1H, J = 10.5, 8.0 and 4.9 Hz, H-3b); 5.17 (dd, 1H, J = 9.4 and 0.9 Hz, H-22); 5.30 (dd, 1H, J = 9.4 and 1.4 Hz, H-23). ^{13}C NMR δ 11.68, 12.78, 13.03, 17.56, 18.60, 19.54, 20.88, 20.95, 21.04, 21.09, 21.55, 23.34, 23.76, 24.03, 28.11, 28.23, 32.93, 34.99, 36.92, 39.58, 40.10, 40.15, 42.27, 45.67, 45.71, 47.55, 52.50, 55.83, 60.68, 63.16, 65.33, 65.97, 72.87, 75.58, 111.18, 170.37, 170.62, 171.17 (2 $\times$ C). HRMS: (API+) calculated for $C_{37}H_{61}O_8$ $[(M-AcOH)+H]^+$ 633.4366, Found 633.4367.

2.37. Ketal deprotection of tetraacetate **28**

The general procedure for ketal deprotection with tetraacetate **28** (700 mg; 1.01 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 3:7) afforded one main product – ketone **29a** (472 mg; 72%) and four minor compounds – triacetate **29b** (75 mg; 12%), unsaturated ether **29c** (21 mg; 3.5%), and chloride **29d** (44 mg; 7%).

(22R,23R)-6-oxo-2,3-seco-5 α -stigmastane-2,3,22,23-tetraol tetraacetate (**29a**): 1H NMR ($CDCl_3$) δ 0.676 (s, 3H, CH_3); 0.682 (s, 3H, CH_3); 0.84 (d, 3H, J = 6.9 Hz, CH_3); 0.96 (d, 3H, J = 6.9 Hz, CH_3); 0.98 (t, 3H, J = 7.5 Hz, CH_3); 1.02 (d, 3H, J = 6.7 Hz, CH_3); 1.85 (ddd, 1H, J = 15.1, 9.6 and 5.7 Hz); 1.94 (m, 1H, H-7 α); 1.97 (s, 3H, CH_3); 2.026

(s, 3H, CH₃); 2.034 (s, 3H, CH₃); 2.06 (s, 3H, CH₃); 2.15 (m, 1H, H-4a); 2.35 (dd, 1H, *J* = 12.5 and 4.0 Hz, H-7β); 2.43 (bd, 1H, *J* = 10.7 Hz, H-5α); 3.87 (ddd, 1H, *J* = 10.9, 9.3 and 5.2 Hz, H-3a); 4.07 (td, 1H, *J* = 10.3 and 5.7 Hz); 4.14–4.20 (m, 2H); 5.17 (dd, 1H, *J* = 9.3 and 0.9 Hz, H-22); 5.30 (dd, 1H, *J* = 9.3 and 1.2 Hz, H-23). ¹³C NMR δ 11.61, 12.76, 13.02, 17.53, 18.55, 19.52, 20.85, 20.93 (2 × C), 20.99, 21.08, 21.85, 22.11, 23.80, 28.00, 28.20, 34.21, 36.89, 37.82, 39.34, 42.62, 43.96, 45.68, 47.17, 48.07, 52.48, 53.51, 56.69, 59.85, 63.63, 72.74, 75.45, 170.36, 170.56, 171.08 (2 × C), 210.69. HRMS: (API+) calculated for C₃₅H₅₇O₇ [(M–AcOH)+H]⁺ 589.4104, Found 589.4102.

(22*R*,23*R*)-2-hydroxy-6-oxo-2,3-seco-5α-stigmastane-3,22,23-triyl triacetate (**29b**): ¹H NMR (CDCl₃) δ 0.65 (s, 3H, CH₃); 0.67 (s, 3H, CH₃); 0.83 (d, 3H, *J* = 6.9 Hz, CH₃); 0.96 (d, 3H, *J* = 6.9 Hz, CH₃); 0.97 (t, 3H, *J* = 7.5 Hz, CH₃); 1.02 (d, 3H, *J* = 6.7 Hz, CH₃); 1.94 (m, 1H, H-7α); 1.96 (s, 3H, CH₃); 2.01 (s, 3H, CH₃); 2.03 (s, 3H, CH₃); 2.11 (m, 1H, H-4a); 2.33 (dd, 1H, *J* = 12.5 and 4.0 Hz, H-7β); 2.52 (bd, 1H, *J* = 10.4 Hz, H-5α); 3.68–3.76 (m, 2H, H-2a, H-2b); 3.89 (ddd, 1H, *J* = 10.9, 8.6 and 6.1 Hz, H-3a); 4.12 (ddd, 1H, *J* = 10.9, 6.7 and 4.6 Hz, H-3b); 5.17 (dd, 1H, *J* = 9.3 and *J* = 0.8 Hz, H-22); 5.30 (dd, 1H, *J* = 9.3 and 1.2 Hz, H-23). ¹³C NMR δ 11.59, 12.75, 13.01, 17.62, 18.53, 19.50, 20.83, 20.91, 20.99, 21.06, 21.90, 22.11, 23.80, 27.98, 28.18, 36.87, 37.81, 38.58, 39.35, 42.62, 44.05, 45.65, 47.16, 47.93, 52.46, 53.83, 56.67, 57.99, 64.01, 72.76, 75.48, 170.38, 170.55, 171.25, 211.17. HRMS: (API+) calculated for C₃₅H₅₇O₇ [(M–H₂O)+H]⁺ 589.4104, Found 589.4107.

(22*R*,23*R*)-3,6-epoxy-2,3-seco-5α-stigmastane-5(6)-ene-2,22,23-triyl triacetate (**29c**): ¹H NMR (CDCl₃) δ 0.70 (s, 3H, CH₃); 0.84 (d, 3H, *J* = 6.9 Hz, CH₃); 0.93 (s, 3H, CH₃); 0.97 (d, 3H, *J* = 6.9 Hz, CH₃); 0.98 (t, 3H, *J* = 7.5 Hz, CH₃); 1.03 (d, 3H, *J* = 6.7 Hz, CH₃); 1.83 (ddd, 1H, *J* = 14.0, 9.7 and 6.3 Hz, H-1a); 1.97 (s, 3H, CH₃); 2.02 (s, 3H, CH₃); 2.03 (s, 3H, CH₃); 1.98–2.06 (m, 3H); 2.45–2.64 (m, 2H, 2 × H-4); 3.90 (td, 1H, *J* = 10.3 and 5.0 Hz, H-2a); 3.98 (td, 1H, *J* = 10.3 and 6.6 Hz, H-2b); 4.22–4.33 (m, 2H, 2 × H-3); 5.18 (bd, 1H, *J* = 9.3 Hz, H-22); 5.30 (dd, 1H, *J* = 9.3 and 1.2 Hz, H-23). ¹³C NMR δ 11.55, 12.83, 13.05, 18.59, 19.54, 20.87, 20.96, 21.10, 21.12, 22.52, 24.18, 26.88, 28.09, 28.24, 28.77, 28.89, 33.08, 35.30, 36.78, 36.91, 39.38, 42.26, 43.71, 45.68, 52.49, 56.47, 61.93, 68.86, 72.88, 75.58, 111.28, 151.02, 170.40, 170.57, 171.18. HRMS: (API+) calculated for C₃₅H₅₇O₇ [(M+H)⁺] 589.4104, Found 589.4103.

(22*R*,23*R*)-3-chloro-6-oxo-2,3-seco-5α-stigmastane-2,22,23-triyl triacetate (**29d**): ¹H NMR (CDCl₃) δ 0.67 (s, 3H, CH₃); 0.69 (s, 3H, CH₃); 0.84 (d, 3H, *J* = 6.9 Hz, CH₃); 0.96 (d, 3H, *J* = 6.9 Hz, CH₃); 0.98 (t, 3H, *J* = 7.5 Hz, CH₃); 1.02 (d, 3H, *J* = 6.7 Hz, CH₃); 1.83 (ddd, 1H, *J* = 14.7, 9.7 and 5.5 Hz, H-1a); 1.94–2.03 (m, 3H); 1.97 (s, 3H, CH₃); 2.03 (s, 3H, CH₃); 2.06 (s, 3H, CH₃); 2.29 (m, 1H, H-4a); 2.34 (dd, 1H, *J* = 12.5 and 4.0 Hz, H-7β); 2.63 (bd, 1H, *J* = 10.7 Hz, H-5α); 3.41 (td, 1H, *J* = 10.5 and 4.7 Hz, H-3a); 3.72 (m, 1H, H-3b); 4.12 (td, 1H, *J* = 10.3 and 5.7 Hz, H-2a); 4.23 (td, 1H, *J* = 10.3 and 5.8 Hz, H-2b); 5.17 (bd, 1H, *J* = 9.2 Hz, H-22); 5.30 (bd, 1H, *J* = 9.2 Hz, H-23). ¹³C NMR δ 11.63, 12.77, 13.04, 17.75, 18.57, 19.54, 20.87, 20.95, 21.04, 21.09, 21.79, 23.82, 26.11, 28.00, 28.22, 34.28, 36.90, 37.85, 39.30, 42.63, 44.01, 44.53, 45.69, 47.28, 48.07, 52.48, 53.80, 56.68, 59.85, 72.76, 75.46, 170.37, 170.59, 171.12, 211.02. HRMS: (API+) calculated for C₃₃H₅₃O₅Cl [(M–AcOH)+H]⁺ 565.3660, Found 565.3659.

2.38. Baeyer-Villiger oxidation of tetraacetate **29a**

The general procedure for Baeyer-Villiger oxidation with tetraacetate **29a** (400 mg; 0.62 mmol) and column chromatography on silica gel (mobile phase – ethyl acetate:cyclohexane 3:7) afforded two products as viscous oil; minor lactone **30a** (98 mg; 24%) and major lactone **30b** (270 mg; 66%).

(22*R*,23*R*)-7α-Homo-7-oxa-6-oxo-2,3-seco-5α-stigmastane-2,3,22,23-tetraol tetraacetate (**30a**): ¹H NMR (CDCl₃) δ 0.72 (s, 3H, CH₃); 0.84 (d, 3H, *J* = 6.7 Hz, CH₃); 0.88 (s, 3H, CH₃); 0.96 (d, 3H, *J* = 6.7 Hz, CH₃);

0.98 (t, 3H, *J* = 7.5 Hz, CH₃); 1.02 (d, 3H, *J* = 6.7 Hz, CH₃); 1.92 (m, 1H); 1.97 (s, 3H, CH₃); 2.02 (s, 3H, CH₃); 2.05 (s, 3H, CH₃); 2.06 (s, 3H, CH₃); 2.18 (m, 1H); 2.97 (dd, 1H, *J* = 11.9 and 1.8 Hz, H-5α); 3.88–3.98 (m, 2H, H-3a, H-2a); 4.00 (dd, 1H, *J* = 13.0 and 10.2 Hz, H-7aβ); 4.07–4.12 (m, 2H, H-3b, H-7aa); 4.25 (m, 1H, Σ*J* = 20.8 Hz, H-2b); 5.15 (dd, 1H, *J* = 9.3 and 0.9 Hz, H-22); 5.30 (dd, 1H, *J* = 9.3 and 1.2 Hz, H-23). ¹³C NMR δ 11.56, 12.66, 13.01, 18.51, 19.29, 19.49, 20.77, 20.88, 20.90, 21.04, 22.82, 24.54, 26.66, 27.95, 28.17, 29.63, 35.04, 36.94, 38.30, 38.98, 39.43, 42.48, 45.02, 45.85, 51.49, 52.05, 52.38, 59.53, 63.22, 70.13, 72.65, 75.34, 170.32, 170.52, 171.00, 171.05, 174.93. HRMS: (API+) calculated for C₃₅H₅₇O₈ [(M–AcOH)+H]⁺ 605.4053, Found 605.4055.

(22*R*,23*R*)-7α-Homo-6-oxa-7-oxo-2,3-seco-5α-stigmastane-2,3,22,23-tetraol tetraacetate (**30b**): ¹H NMR (CDCl₃) δ 0.72 (s, 3H, CH₃); 0.84 (d, 3H, *J* = 6.7 Hz, CH₃); 0.89 (s, 3H, CH₃); 0.96 (d, 3H, *J* = 6.7 Hz, CH₃); 0.99 (t, 3H, *J* = 7.5 Hz, CH₃); 1.01 (d, 3H, *J* = 6.7 Hz, CH₃); 1.93 (m, 1H); 1.97 (s, 3H, CH₃); 2.02 (s, 3H, CH₃); 2.055 (s, 3H, CH₃); 2.061 (s, 3H, CH₃); 2.38 (t, 1H, *J* = 12.5 Hz, H-7aa); 2.51 (bd, 1H, *J* = 12.5 Hz, H-7aβ); 3.90 (td, 1H, *J* = 11.0 and 5.8 Hz, H-3a); 4.07 (td, 1H, *J* = 10.8 and 5.5 Hz, H-2a); 4.16 (ddd, 1H, *J* = 11.0, 9.1 and 4.9 Hz, H-3b); 4.32 (ddd, 1H, *J* = 10.8, 6.2 and 4.1 Hz, H-2b); 4.43 (dd, 1H, *J* = 11.0 and 1.8 Hz, H-5α); 5.16 (dd, 1H, *J* = 9.3 and 0.8 Hz, H-22); 5.30 (dd, 1H, *J* = 9.3 and 1.2 Hz, H-23). ¹³C NMR δ 11.50, 12.61, 12.93, 16.10, 18.47, 19.45, 20.74, 20.84, 20.86, 21.00, 22.82, 24.99, 26.80, 27.53, 28.11, 29.39, 34.51, 35.23, 36.88, 37.42, 39.45, 41.53, 42.42, 45.58, 51.68, 52.69, 55.52, 59.26, 61.18, 72.59, 75.24, 80.83, 170.29, 170.45, 170.81, 170.90, 174.09. HRMS: (API+) calculated for C₃₅H₅₇O₈ [(M–AcOH)+H]⁺ 605.4053, Found 605.4056.

2.39. Hydrolysis of acetate **30b**

The general procedure for hydrolysis of acetates with lactone **30b** (150 mg; 0.23 mmol) afforded minor diacetate **31a** (20 mg; 15%) as a viscous oil and major tetrahydroxylactone **31b** (91 mg; 81%) as white crystals (MeOH).

(22*R*,23*R*)-2,3-dihydroxy-7α-homo-6-oxa-7-oxo-2,3-seco-5α-stigmastane-2,2,23-diyl diacetate (**31a**): ¹H NMR (CDCl₃) δ 0.72 (s, 3H, CH₃); 0.84 (d, 3H, *J* = 6.7 Hz, CH₃); 0.88 (s, 3H, CH₃); 0.96 (d, 3H, *J* = 6.7 Hz, CH₃); 0.99 (t, 3H, *J* = 7.5 Hz, CH₃); 1.01 (d, 3H, *J* = 6.7 Hz, CH₃); 1.97 (s, 3H, CH₃); 2.02 (s, 3H, CH₃); 2.43–2.50 (m, 2H, H-7aa, H-7aβ); 3.59–3.71 (m, 2H); 3.76–3.84 (m, 2H); 4.63 (dd, 1H, *J* = 11.0 and 1.8 Hz, H-5α); 5.16 (dd, 1H, *J* = 9.3 and 0.8 Hz, H-22); 5.29 (dd, 1H, *J* = 9.3 and 1.3 Hz, H-23). ¹³C NMR δ 11.59, 12.73, 13.01, 16.26, 18.56, 19.54, 20.87, 20.95, 21.09, 22.98, 25.12, 27.63, 28.19, 32.70, 34.65, 36.95, 37.64, 39.26, 39.59, 41.65, 42.54, 45.67, 51.66, 52.77, 55.62, 57.68, 59.32, 72.73, 75.40, 81.72, 170.41, 170.56, 175.03. HRMS: (API+) calculated for C₃₃H₅₇O₈ [(M+H)⁺] 581.4053, Found 581.4050.

(22*R*,23*R*)-2,3,22,23-tetrahydroxy-7α-homo-6-oxa-2,3-seco-5α-stigmastane-7-one (**31b**): ¹H NMR (DMSO-*d*₆) δ 0.65 (s, 3H, CH₃); 0.73 (s, 3H, CH₃); 0.81 (d, 3H, *J* = 6.7 Hz, CH₃); 0.885 (t, 3H, *J* = 7.5 Hz, CH₃); 0.889 (d, 3H, *J* = 6.7 Hz, CH₃); 0.90 (d, 3H, *J* = 6.7 Hz, CH₃); 1.89–1.93 (m, 2H); 2.19 (bd, 1H, *J* = 13.3 Hz, H-7aβ); 2.61 (t, 1H, *J* = 13.3 Hz, H-7aa); 3.33–3.53 (m, 6H, 2 × H-2, 2 × H-3, H-22, H-23); 3.83 (d, 1H, *J* = 4.3 Hz, sec-OH); 4.09 (d, 1H, *J* = 4.6 Hz, sec-OH); 4.33 (t, 1H, *J* = 5.0 Hz, prim-OH); 4.47 (t, 1H, *J* = 5.3 Hz, prim-OH); 4.55 (dd, 1H, *J* = 11.2 and 1.7 Hz, H-5α). ¹³C NMR δ 11.32, 11.85, 13.39, 15.83, 18.61, 19.61, 20.80, 22.37, 24.63, 26.63, 28.60, 32.58, 34.20, 36.80, 37.17, 39.31, 39.72, 40.75, 41.81, 46.15, 50.46, 52.39, 54.84, 55.32, 57.26, 71.20, 72.86, 80.34, 174.02. HRMS: (API+) calculated for C₂₉H₅₃O₈ [(M+H)⁺] 497.3842, Found 497.3845.

2.40. Cell cultures

The screening human cancer cell lines: T-lymphoblastic leukaemia CEM, cervix epitheloid carcinoma HeLa, and breast carcinoma MCF7

(estrogen-sensitive), all of which were obtained from the European Collection of Authenticated Cell Cultures (ECACC, London, UK). Human foreskin fibroblasts (BJ) were purchased from the American Type Culture Collection (Manassas, VA, USA). CEM was cultured in RPMI 1640 medium while all other cells in DMEM medium. L-glutamine, penicillin and streptomycin, Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 and resazurin were purchased from Sigma Aldrich. Fetal bovine serum (FBS) was purchased from HyClone (GE Healthcare, Logan, UT, USA). All media used in this study were supplemented with 2 mM L-glutamine, 1% penicillin–streptomycin and 10% fetal bovine serum. Standard cell culture conditions (37 °C, 5% CO₂ and a humid environment) were utilized for cultivation of all cell lines. Using the standard trypsinisation procedure, cells were subcultured two or three times a week.

2.41. Cytotoxicity assay

Cytotoxicity in cancer cell lines after 72 h was determined as described earlier [23]. Control cultures were treated with DMSO alone, and the final concentration of DMSO in the reaction mixture never exceeded 0.6%. Six serial 3-fold dilutions of the test substances were added at time zero in 20 µL aliquots to the microtiter plate wells and the highest final concentration in the wells was 50 µM. The data shown in Table 1 are means ± standard deviation (SD) obtained from three independent experiments performed in triplicate.

2.42. SDS–polyacrylamide gel electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting was performed as described earlier [23]. Apoptotic proteins were detected in HeLa cells after 48 h of treatment with seco analogues. The protein expression in treated cells was compared to that observed in untreated controls.

Table 1
Cytotoxicity of seco analogues in three cancer cell lines and normal human fibroblasts after 72 h. Compounds not shown were inactive.

Compound	IC ₅₀ (µM)			
	CEM	HeLa	MCF7	BJ
3a	10.7 ± 3.6	12.1 ± 1.4	14.6 ± 1.1	7.5 ± 0.1
3b	9.0 ± 0.5	9.9 ± 0.4	18.1 ± 1.3	11.8 ± 1.0
9a	40.3 ± 0.1	30.1 ± 1.1	34.8 ± 3.4	18.3 ± 1.1
9b	31.3 ± 1.3	28.3 ± 3.7	22.0 ± 9.3	17.6 ± 2.3
10	30.0 ± 2.3	31.5 ± 0.7	35.2 ± 9.1	39.0 ± 9.1
14a	32.0 ± 5.4	34.7 ± 0.6	47.0 ± 0.6	33.8 ± 1.3
14b	11.9 ± 2.4	7.6 ± 0.1	11.0 ± 0.4	8.9 ± 1.1
15	3.2 ± 0.4	7.5 ± 0.1	9.3 ± 0.0	5.8 ± 1.1
18a	>50	36.5 ± 0.1	43.7 ± 4.4	>50
18b	27.6 ± 4.7	16.9 ± 1.4	8.7 ± 0.6	44.0 ± 0.4
19	38.0 ± 2.1	32.9 ± 2.6	34.8 ± 4.9	>50
24a	26.6 ± 3.0	29.3 ± 0.4	18.2 ± 1.8	45.6 ± 0.2
24b	28.0 ± 1.9	17.4 ± 0.4	15.2 ± 2.1	18.5 ± 3.0
25a	13.6 ± 1.6	10.0 ± 1.3	27.1 ± 3.5	18.5 ± 2.0
25b	11.4 ± 0.0	13.8 ± 1.6	17.8 ± 0.0	23.4 ± 1.3
26	3.1 ± 0.5	8.1 ± 0.5	11.7 ± 0.7	4.4 ± 0.1
27	0.8 ± 0.2	2.9 ± 0.1	3.6 ± 0.1	1.6 ± 0.0
28	37.4 ± 6.9	6.8 ± 0.2	2.8 ± 0.1	>50
29a	26.3 ± 4.7	7.9 ± 0.3	7.4 ± 0.2	41.3 ± 2.6
29b	26.6 ± 7.9	21.6 ± 4.5	19.6 ± 3.7	43.1 ± 3.6
30a	21.7 ± 3.9	10.8 ± 2.1	10.0 ± 3.2	>50
30b	20.6 ± 7.5	11.1 ± 3.4	10.2 ± 0.4	20.1 ± 6.3
31a	10.8 ± 1.1	27.8 ± 2.2	19.2 ± 2.1	26.8 ± 0.2
28-homoCS	13.2 ± 2.8	> 50	> 50	> 50
cholesterol	> 50	> 50	> 50	> 50

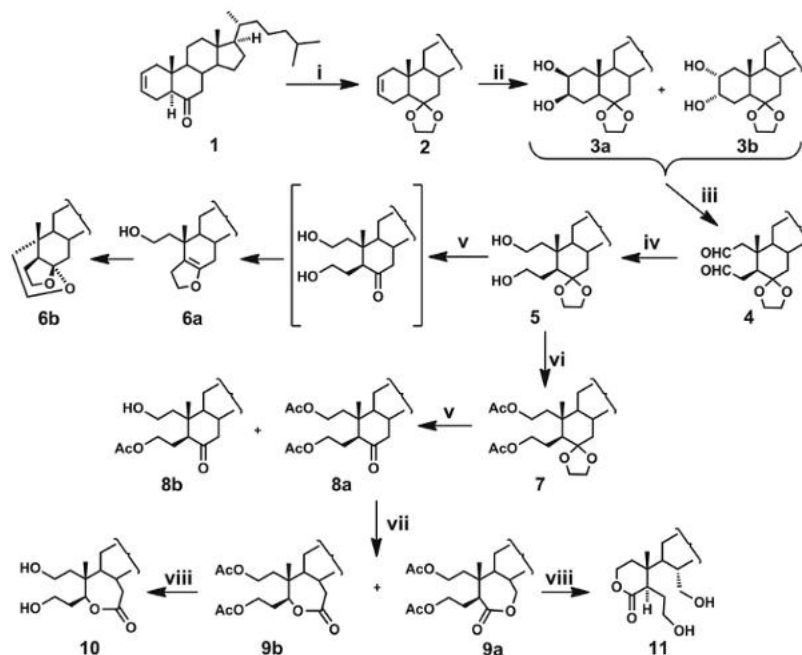
3. Results and discussion

3.1. Chemistry

According to our synthetic strategy (scheme 1) we prepared 5α-cholest-2-en-6-one (1) as the first starting compound.[20,21] Due to the planned reduction of aldehydes, the 6-oxo group had to be protected with ethylene glycol. This protected ketal 2 was then used for Upjohn dihydroxylation of the double bond using osmium tetroxide and N-methylmorpholine-N-oxide. We obtained two isomers of diol; minor 2β,3β-diol 3a and major 2α,3α-diol 3b in a ratio 3a:3b 9:1 (this ratio is similar for other Upjohn dihydroxylations). Determination of both isomers is based on published results with the same reaction.[24,25] The dihydroxylation from α side is always preferred due to steric hindrance of the 19-methyl group. Fortunately, as the next step was oxidative cleavage of diols with sodium periodate on silica, it was not necessary to separate the mixture. However, in this case the separation of both product was done. The reaction of both isomers only led to one seco-dialdehyde 4. Preparation of dialdehyde 4 can also be done in one step from protected olefin 2 using ozone. Unfortunately, the reaction is too fast and it is difficult to control the reaction rate and to avoid over-oxidation. For this reason the yield of ozone oxidation varied from 30 to 60 percent. Dialdehyde 4 was then reduced with lithium aluminium hydride to the corresponding seco-diol 5. Attempts to use sodium borohydride as a reduction reagent only led to a complex mixture of unstable products, probably hemiacetals. One of the most surprising reactions of the synthetic strategy was acidic hydrolysis of ketal 5. While the TLC monitoring showed the formation of desired polar dihydroxyketone in an approximate yield of 60–70%, all attempts to isolate it failed. We were only able to isolate a product of intramolecular cyclization 6a. This unsaturated ether was stabilized by formation of a new five-membered ring. Moreover, this compound was unstable over time (ten and more hours in CDCl₃ solution and several days in solid state) and converted to intramolecular ketal 6b, which was more stabilized by formation of an additional six-membered ring. While the formation of 2,3-seco-2,3-dihydroxyketone was not successful we proceeded to prepare lactone B-ring analogues. Firstly, the ketal 5 was acetylated to acetate 7 to prevent any possible cyclization followed by acidic hydrolysis of its ketal group. This time we obtained the desired diacetate 8a for the most part, with the minor presence of monoacetate 8b. The minor product proved that the free 3-hydroxy group was essential for starting the cyclization, while the free 2-hydroxy group did not lead to the formation of a new ring. The next step was Bayer-Villiger oxidation (Fig. 2) of diacetate 8a with *in-situ* prepared trifluoroperoxyacetic acid. Unlike the same reaction in the presence of ring A where oxidation occurs mostly between carbon 6 and 7 (natural isomers), in the case of A-seco analogues the oxygen is incorporated between carbon 5 and 6 (unnatural isomers). Similarly, the unnatural isomer is the main product if no hydroxyl group is on the A ring.[26]

Thus, we obtained lactone 9b as a major product and lactone 9a as a minor product. The last step was hydrolysis of both acetates. The hydrolysis of lactone 9b afforded dihydroxylactone 10 as expected. On the other hand, hydrolysis of lactone 9a led to a new lactone 11 as the major product of the mixture formed. This can be explained by formation of a more stable six-membered lactone between 2-hydroxyl and carboxyl groups instead of the standard seven-membered lactone. However, since only a small yield of compound 11 was obtained (29%) this is only our hypothesis based on the only product isolated. No other compounds were isolated from this reaction. Compound 11 showed also some instability as we detected new compounds on TLC after several hours in different solutions (CHCl₃, ethyl acetate, acetone).

Based on the previous results with the cholestane skeleton, we carried out the same reaction procedure with stigmastane one (scheme 2). The first strategy was based on preparation of A-secodiol followed by Sharpless dihydroxylation to form 2,3-seco-28-homocasterone. This time we prepared the known 5α-stigmast-2,22-dien-6-one (12)



Scheme 1. Reagents and conditions. (i) $(\text{CH}_3\text{OH})_2$, TsOH , PhMe , refl.; (ii) OsO_4 , NMO , THF , acetone, r.t.; (iii) NaIO_4 , silica, CH_2Cl_2 , r.t.; (iv) LiAlH_4 , THF , refl.; (v) HCl , THF , H_2O , 40°C ; (vi) Ac_2O , pyridine, r.t.; (vii) $\text{CF}_3\text{CO}_2\text{H}$, CH_2Cl_2 , r.t.; (viii) MeONa , MeOH , THF , r.t..

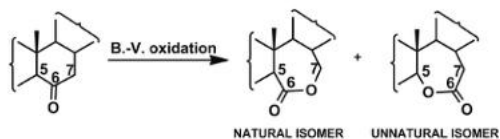
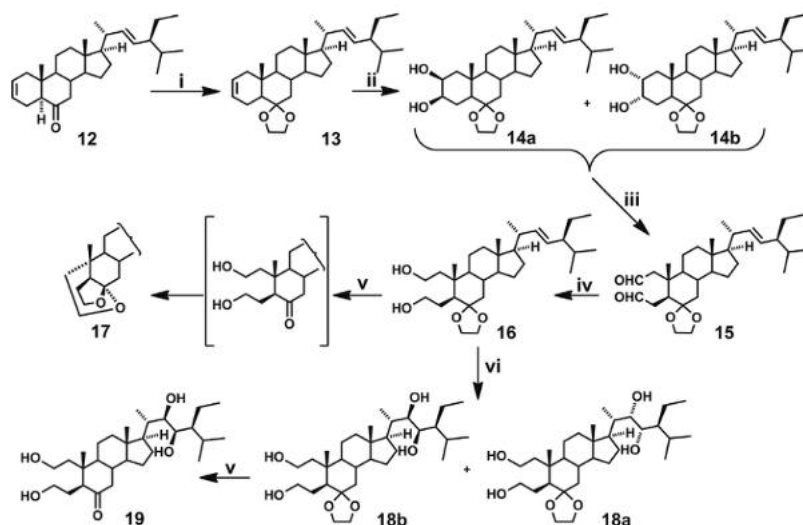


Fig. 2. General scheme for Bayer-Villiger oxidation of 6-oxo steroids.

[21] as a starting material. Preparation of ketal **13**, mixture of diols **14a** and **14b**, dialdehyde **15**, and diol **16** was straightforward and corresponded with cholestane analogues. The acidic hydrolysis of ketal **16** also led to a non-polar compound. Unlike the cholestane analogue this time we obtained only intramolecular ketal **17** with no corresponding cyclic enol ether. Due to the spontaneous cyclization we carried out the Sharpless dihydroxylation with protected diol **16**. The presence of the 24-ethyl group reduced the rate of dihydroxylation of the side chain significantly. This resulted in a complex mixture of products with low yield of desired tetraols **18a** and **18b**. A product with a configuration 22*S*,23*S* **18a** was obtained in 18% yield while a product with the natural configuration 22*R*,23*R* **18b** was obtained in 30% yield. Determination of both isomers was based on analogous Sharpless asymmetric dihydroxylation.[27,28] In general, the standard of *S,S*-isomer of stigmastane series can also be obtained by Upjohn dihydroxylation as the only product.[29,30] The mild acidic hydrolysis of **18b** led to 2,3-seco-28-homocasterone (**19**) which was not purified by either chromatography or crystallization. It resulted in approx. 85–90% of purity (based on NMR data), however this was the only case where we were able to prepare such a dihydroxyketone. Even though we were able to synthesize the desired seco-28-homocasterone (**19**) the low overall yield caused us to plan a different strategy for the preparation of B-

lactone analogues starting with the already dihydroxylated side chain.

(22*R*,23*R*)-22,23-Dihydroxy-3 α ,5-cyclo-5 α -stigmastan-6-one (**20**) [22] was used as a starting material for the last reaction strategy (scheme 3). First, we acetylated compound **20** to avoid future oxidative cleavage of diol on the side chain. Diacetate **21** was then treated with pyridinium *p*-toluenesulfonate and lithium bromide to form olefin **22** followed by ketone protection with ethyleneglycol to prepare ketal **23**. We then first used unprotected olefin **22** for Upjohn dihydroxylation to form dihydroxyketones **24a** (2 β ,3 β) and **24b** (2 α ,3 α). Oxidative diol cleavage of this mixture with sodium periodate afforded trioxo derivative **26**. This compound was prepared for biological testing only and not for further reactions. Afterwards, we continued with protected olefin **23**. Upjohn dihydroxylation followed by oxidative cleavage of diols yielded the desired dialdehyde **27**. Reduction of this compound with lithium aluminium hydride led to preparation of tetraol **18b** as both acetates were reduced as well. Other reactions were similar to those used for the cholestane skeleton. Firstly we prepared tetraacetate **28** followed by acidic hydrolysis of the ketal group. This time we obtained not only the major oxo-tetraacetate **29a**, but also other three minor products of partial hydrolysis. Formation of triacetate **29b** was the same as for cholestane analogues **8b**. On the other hand, we obtained a product of the partial hydrolysis on the 3-hydroxyl group as well. While triacetate **29c** corresponds to cyclic ether **6a** of the cholestane skeleton, formation of chloride **29d** was observed for the first time. The latter can be explained by opening of the ether cycle of **29c** with hydrogen chloride during acidic hydrolysis. The following reactions were similar to those used with the cholestane skeleton – Bayer-Villiger oxidation of **29a** for preparation of lactone **30a** and **30b** and basic hydrolysis of **30b** to form minor diacetate **31a** and major tetraol **31b**. Minor diacetate **31a** can be isolated for steric reasons on the side chain, depending on reaction time. Unlike the hydrolysis of lactone **9b**,



Scheme 2. Reagents and conditions. (i) $(\text{CH}_3\text{OH})_2$, TsOH, PhMe, refl.; (ii) OsO_4 , NMO, THF, acetone, r.t.; (iii) NaIO_4 , silica, CH_2Cl_2 , r.t.; (iv) LiAlH_4 , THF, refl.; (v) HCl , THF, H_2O , 40 °C; (vi) OsO_4 , HQ-4ClBz, $\text{K}_3[\text{Fe}(\text{CN})_6]$, K_2CO_3 , MeSO_2NH_2 , *t*-BuOH, r.t..

we failed to isolate any product of the hydrolysis of lactone **30a**. In this case, the instability of the product was so high that any purification procedure only led to another mixture of products. All new compounds were characterized by ^1H and ^{13}C NMR and by mass spectrometry. Compounds with new structural motif (e.g. **6b**, **8b**, **11**, **17**, and **29b–29d**) were also analyzed by 2D NMR techniques (COSY, TOCSY, HSQC, HMBC, and NOESY) to determine the correct structure. For graphical presentation of important cross-peaks of chosen structural motifs see [supp. information](#).

3.2. Cytotoxicity

We investigated the effects of seco analogues of 28-homobrainsteroids on the viability of different cancer cell lines and normal human fibroblasts. We evaluated the cytotoxic properties of seco analogues using T-lymphoblastic leukaemia CEM cells, human cervical carcinoma cell line HeLa, the human breast cancer cell line MCF7, and normal human skin fibroblasts BJ. Cells were exposed to six 3-fold dilutions of each drug for 72 h prior to determination of cell survival. The IC_{50} values were obtained from the resazurin cytotoxicity assay.

Eight of the synthesized and tested seco analogues were significantly cytotoxic ($\text{IC}_{50} < 10 \mu\text{M}$) against at least one of the studied cancer cell lines – CEM, MCF7 or HeLa (Table 1). Four compounds were active in human malignant melanoma CEM cells; seven compounds had highly cytotoxic effects on cervical carcinoma HeLa cells, and five compounds were cytotoxic towards breast carcinoma MCF7 cells. Six analogues were cytotoxic ($\text{IC}_{50} < 10 \mu\text{M}$) against at least two of three tested cancer cell lines; two compounds were active against all three tested cancer cell lines. **17**, **18a**, **19**, **28**, **30a**, and **31b** had no effect on the viability of normal human BJ fibroblasts even at the highest tested concentration of $50 \mu\text{M}$ which suggests high therapeutic index. A very interesting compound **28** inhibited the growth of tumor cells in micro molar range (e.g. IC_{50} in MCF7 cells $2.8 \mu\text{M} \pm 0.1$), but not normal human fibroblasts. The most promising substances (**26**, **27**, **28**, **29a**, **30a** and **30b**) were chosen for following assays to identify possible mechanisms of action.

3.3. Seco analogues caused apoptosis in cancer cells

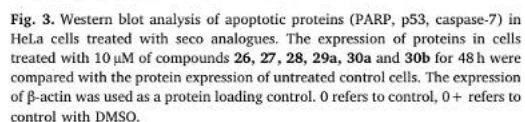
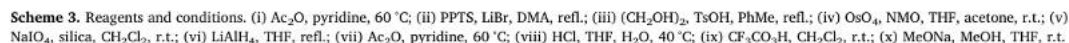
The anti-apoptotic properties of seco analogues on cervical carcinoma cells HeLa were investigated. Cells were treated with $10 \mu\text{M}$ of six substances (**26**, **27**, **28**, **29a**, **30a** and **30b**) for 48 h. The cells were then harvested, lysed and analyzed by western blotting.

Apoptotic proteins in HeLa cells treated with seco analogues were detected using western blotting. Executioner proteases caspase-3 and 7 cleaved poly-(ADP-ribose) polymerase (PARP) and caused subsequent DNA degradation and apoptotic death.[31] The treatment with $10 \mu\text{M}$ **26** and **27** showed degradation of caspase-7 into cleaved fragments (part of the apoptotic cascade) in HeLa cells (Fig. 3). A final step of caspase activation, PARP cleavage, was observed in HeLa cells treated with **26** and **27** (Fig. 3). Due to the absence of caspase-7 fragment in cells treated with **28**, this substance probably resulted in weaker induction of apoptosis than **26** and **27**. In contrast, strong induction of tumor suppressor protein p53 was observed after treatment with $10 \mu\text{M}$ **28** for 48 h in HeLa. The negative regulator of the cell division cycle p53 protein is inactive in 30% of all breast cancer cells. DNA damage, stress or activation by oncogenes, upregulate tumor suppressor p53 levels which then prevent the cells from entering the S phase of the cell cycle or induce apoptosis.[32]

4. Conclusion

Forty-two novel steroid compounds were prepared in a multi-step synthesis. Analysis of several compounds showed the formation of interesting steroid skeletons that have never been described before (e.g. **6a**, **6b**, **11**). Discovery of cyclization of 2,3-seco-2,3-dihydroxy-6-ketone could lead to the preparation of a new series of steroids with possible biological activities.

Twenty-five seco analogues of 28-homobrainsteroids were tested in cell viability assay in different cancer cell lines and normal human fibroblasts. Eight of the synthesized and tested seco analogues were significantly cytotoxic ($\text{IC}_{50} < 10 \mu\text{M}$) against at least one of the studied cancer cell lines. **17**, **18a**, **19**, **28**, **30a**, and **31b** did not affect the viability of normal human BJ fibroblasts even at the highest tested concentration of $50 \mu\text{M}$ which suggests high therapeutic index. A very



interesting seco-tetraacetate **28** inhibited the growth of tumor cells in micro molar range (e.g. IC₅₀ in MCF7 cells 2.8 μM ± 0.1), but not normal human fibroblasts. These seco analogues have interesting cytotoxic activities on cancer cell lines and most of them without affecting the growth of normal cells. The high therapeutic index is a major advantage in their potential use as precursors for new antiproliferative drugs. Regarding the structure activity relationship study, we can conclude that compounds with the best therapeutic index are compounds with acetates rather than compounds with free hydroxyl groups. The lowest therapeutic index and high toxicity was found in the case of all dialdehydes.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.steroids.2019.03.004>.

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Příloha 8

A mechanistic framework for the recognition of chemically diverse brassinosteroids by BRI1-family receptor kinases

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A mechanistic framework for the recognition of chemically diverse brassinosteroids by BRI1-family receptor kinases

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Brassinosteroids (BRs) are chemically diverse plant steroid hormones produced via a branched biosynthetic pathway. The potent BR brassinolide is sensed by the membrane receptor kinase BRI1 and a SERK co-receptor, but the physiological functions of other abundant BRs remain to be characterized. Here we present quantitative binding kinetics for 4 *Arabidopsis thaliana* BR receptors and 15 BRs, which define the key chemical features required for high-affinity receptor binding, ligand positioning and co-receptor recognition. BRI1, BRL1 and BRL3 share overlapping ligand preferences, whereas BRL2 binds C₂₈ BRs with moderate affinity. Structural analyses of BR-bound BRI1 and BRL3 ectodomains combined with extensive in vitro and in vivo mutagenesis studies reveal a high structural plasticity of the hormone-binding pocket. Functional assays using structure-based BR agonists and antagonists uncover that BR receptor–co-receptor signalling complexes can recognize chemically diverse BRs, introducing an additional, intriguing layer of BR signalling regulation.

Brassinosteroids (BRs) are a class of lipophilic, polyhydroxylated steroid hormones first isolated from rapeseed pollen and later recognized for their growth-promoting roles in plants¹. BRs are synthesized via three main biosynthetic branches, producing C₂₇, C₂₈ or C₂₉ compounds. While all share a steroidal ring core, they differ in the structure of their aliphatic side chains² (Fig. 1a). The C₂₇ branch, derived from cholesterol, yields 28-norbrassinolide^{3,4}, whereas the C₂₉ branch, from sitosterol, produces 28-homocastasterone and 28-homobrassinolide (Fig. 1b). The C₂₈ pathway originates from campesterol and leads to brassinolide, one of the most bioactive BRs² (Fig. 1b). The C₂₈ pathway includes two routes: early and late C₂₂ oxidation (Fig. 1b). In the early pathway, DWARF4 (DWF4), a cytochrome P450 monooxygenase, converts campesterol to (22S)-22-hydroxycampesterol^{5,6}. Further steps involve the P450 enzymes CPD^{7,8} and ROT3⁹, and the steroid-5 α -reductase DET2

(ref. 10–12), producing 6-deoxocastasterone (Fig. 1b). The enzymes BR6ox1 and BR6ox2 then convert this intermediate to castasterone¹³, and BR6ox2 can further catalyse formation of a seven-membered lactone ring from the C₆ ketone, yielding brassinolide¹³ (Fig. 1b).

In the late C₂₂ oxidation pathway, campestanol is processed into cathasterone and typhasterol¹⁴ (Fig. 1b). BR6ox2 can convert typhasterol into 2-deoxybrassinolide¹⁵, or alternatively, typhasterol may be converted to castasterone via secasterone, a 2,3-epoxy intermediate¹⁶ (Fig. 1b). The different branches of BR biosynthesis are likely interconnected². BR homeostasis is regulated by several catabolic enzymes, including the P450 enzyme BAS1 (ref. 17), the UDP-glycosyltransferase UGT73C5 (ref. 18) and sulfotransferases¹⁹.

BR biosynthesis occurs, at least in part, within the endoplasmic reticulum^{20,21}. Local BR transport is facilitated via plasmodesmata²²,

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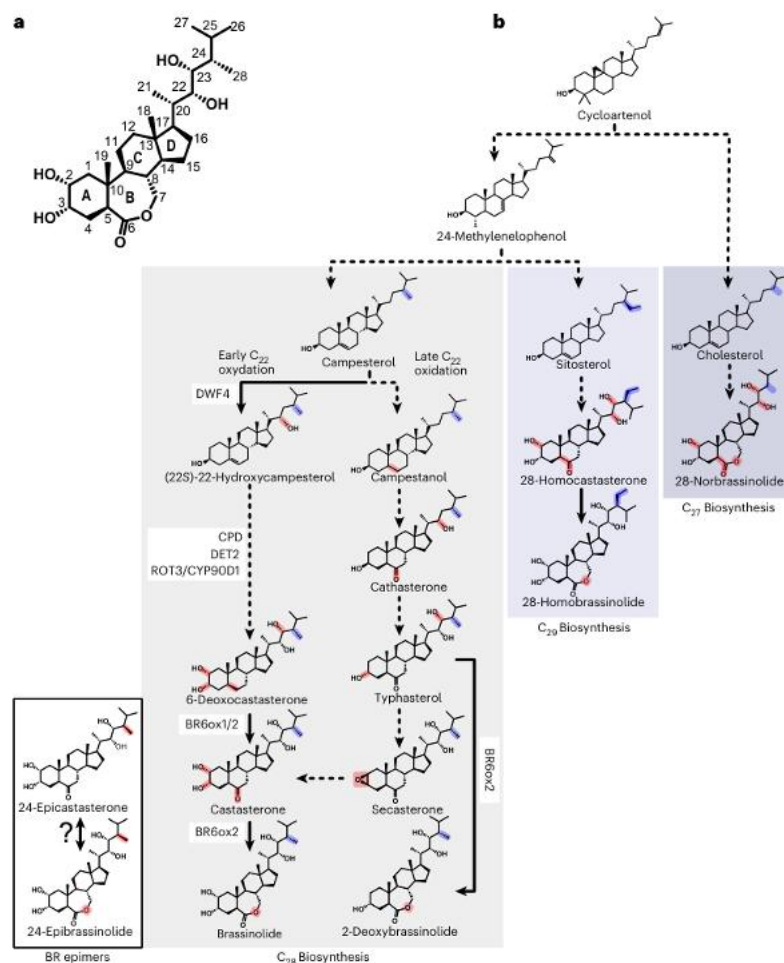


Fig. 1 | The branched architecture of the BR biosynthesis pathway. a, Chemical structure of brassinolide with labelled steroidal rings (letters) and carbon atoms (numbers). **b,** Schematic representation of the BR biosynthesis pathway, highlighting the relative positions of the BRs discussed in this study. The C₂₈, C₂₉ and C₂₇ branches are shaded from light to dark grey, respectively. Key chemical

differences between branches are indicated in blue, while red marks chemical modifications along each biosynthetic route. Solid lines denote single-step enzymatic reactions; dotted lines indicate multi-step conversions. Known enzymes involved in the canonical C₂₈ pathway are shown alongside.

The ATP-binding cassette transporters ABCB1 and ABCB19 can translocate BRs across the plasma membrane into the apoplast²³, in which BR perception takes place²⁴.

BRs are perceived by the plasma membrane-localized receptor kinases BRASSINOSTEROID INSENSITIVE 1 (BRI1)^{24–28}, BRL1^{29–31} and BRL3³², which share a conserved extracellular leucine-rich repeat (LRR) ligand-binding domain. BRI1 is broadly expressed, and *bri1* loss-of-function mutants show a characteristic dwarf phenotype²⁵. BRL1 and BRL3 are present in vascular tissues and can rescue the growth defects of the weak *bri1-301* allele³², when expressed from the *BRI1* promoter²⁹. Expression of the sequence-related receptor kinase BRL2 could not rescue the *bri1-301* mutant phenotype²⁹, or a *bri1 bri1 bri3* triple mutant³³. BRL2 is also known as VASCULAR HIGHWAY1 (VH1) and has been implicated in leaf vein patterning³⁴.

BRs bind to a steroid-binding pocket formed by the LRR core and an ~70-amino-acid island domain within BRI1 or BRL1^{26,27,31}. BR binding enables BRI1 to recruit SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs) to form an active signalling complex^{26,35,36}. In the absence of BRs, SERKs are sequestered by BAK1-INTERACTING RECEPTOR-LIKE KINASE (BIR) pseudokinases^{37,38}. Assembly of the BRI1–BR–SERK complex activates the cytoplasmic kinase domains³⁹, triggering a downstream signalling cascade that leads to dephosphorylation of the BR transcription factors BES1 and BZR1^{40,41}.

Bioassays with synthetic, exogenously applied BRs have been performed across various plant species. In *Arabidopsis*, nanomolar levels of brassinolide promote hypocotyl elongation in both wild-type and *det2* mutant seedlings⁴². Brassinolide or 24-epibrassinolide applied at high nanomolar to micromolar concentrations inhibits root growth⁴³,

whereas low concentrations—high picomolar to low nanomolar—of 24-epibrassinolide or 24-epicastasterone stimulate root growth⁴⁴.

Initial BR-binding assays used microsomal fractions from transgenic *Arabidopsis* expressing BRII-GFP and [³H]-brassinolide, yielding estimated dissociation constants (K_d) of ~10–15 nM for brassinolide and 40–75 nM for castasterone²⁴. In the same assay, BRI1 and BRL3 bound brassinolide with K_d values of ~5 nM and ~50 nM, respectively, while BRL2 showed no detectable binding²⁹. Grating-coupled interferometry (GCI) quantified binding of unlabelled brassinolide to BRII with a K_d of ~10 nM (ref. 28). Brassinolide-bound BRII enables the binding of the SERK3/BAK1 LRR ectodomain, with K_d values of ~0.2–1 μ M, as determined by GCI and isothermal titration calorimetry^{28,36}. Mutation of Gly644 to Asp (*bri1-6/bri1-119*)⁴⁵ in the BRII island domain²⁶ reduces BR binding in vitro^{28,46}. The corresponding *bri1-6* allele shows intermediate growth defects⁴⁵. Mutation of neighbouring Gly643 to Glu in the gain-of-function BRII^{ind1} allele⁴⁷ enhances the structural stability of the island domain²⁶. Five simultaneous point mutations in the BRII BR-binding pocket were required to completely abolish brassinolide binding in vitro and to mimic the severe growth phenotype of *bri1* null alleles in vivo⁴⁶.

The natural occurrence of different BRs has been characterized using high-performance liquid chromatography coupled with gas chromatography–mass spectrometry. In *Arabidopsis* shoots, castasterone, 6-deoxycastasterone and typhasterol were identified^{11,48,49}. Like in other species, brassinolide accumulates more prominently in *Arabidopsis* seeds⁵⁰. Brassinolide, the most bioactive BR², is widely considered the primary signalling ligand for BRII, BRI1 and BRL3. Consequently, previous structural and biochemical investigations have focused on brassinolide^{24,26–29,31,36,46}. Given the chemical diversity of BRs from the C₂₇, C₂₈ and C₂₉ biosynthetic branches that accumulate in plants, we set out to systematically characterize their binding kinetics to the ectodomains of the *Arabidopsis thaliana* BR receptors BRII, BRI1, BRL2 and BRL3. By integrating these measurements with structural and mutational analyses of the BRII-binding pocket—both in vitro and in planta—as well as targeted BR bioassays, we define the ligand-binding specificities of each receptor and uncover the chemical features in BRs required for receptor binding and co-receptor recruitment to form an active signalling complex.

Results

BRII, BRI1 and BRL3 bind chemically diverse BRs with high affinity

We obtained the LRR ectodomains of BRII, BRI1 and BRL3 by secreted expression in baculovirus-infected insect cells, as described^{26,28,46}. Quantitative GCI kinetic binding assays confirmed^{28,29} that BRII, BRI1 and BRL3 bind brassinolide with nanomolar affinity (Fig. 2a, Extended Data Figs. 1 and 2). We then determined a crystal structure of the BRL3 ectodomain in complex with brassinolide at 2.45 Å resolution (Fig. 2b, Supplementary Figs. 1 and 2 and Supplementary Table 1). BRII (ref. 26,27), BRI1 (ref. 31) and BRL3 share a highly conserved ligand-binding pocket (root mean square deviation, r.m.s.d., is ~0.7 Å comparing 180 corresponding C_α atoms; Supplementary Fig. 1a), formed by conserved hydrophobic residues along the inner LRR surface and island domain (Fig. 2b). The brassinolide C₂₃ hydroxyl forms a hydrogen bond with a main chain amino group in the island domain of all three receptors (Fig. 2b and Supplementary Fig. 1a). Few receptor-specific polar contacts with the ligand are observed. In BRII, Tyr597 forms hydrogen bonds with the brassinolide C₂₂ and C₂₃ hydroxyls via a water molecule (Fig. 2b), whereas in BRI1 and BRL3, Tyr597 is replaced by phenylalanine. In the BRL3 complex, Arg588 (corresponding to Lys601 in BRII) and Tyr627 form hydrogen bonds with the C₆ hydroxyl group of brassinolide (Fig. 2b). BRL3 Gln666 (Phe681 in BRII) coordinates the C₂ hydroxyl via a water molecule (Fig. 2b). Additional water molecules that could potentially be involved in polar contacts between the receptor and the ligand may not be resolved in our medium-resolution

complex structures (Supplementary Fig. 2 and Supplementary Table 1). Taken together, BRII, BRI1 and BRL3 share a structurally conserved BR-binding pocket with limited receptor-specific features, and all bind brassinolide with high affinity.

Next, we quantified the interaction of BRII, BRI1 and BRL3 with 12 natural BRs from the three biosynthetic branches, along with the synthetic compounds 3-deoxycastasterone, 22-deoxycastasterone and 23-deoxycastasterone (Fig. 2c,d). BRII showed binding constants ranging from low micromolar (red in K_d/K_i plots, Fig. 2c) to low nanomolar (blue in Fig. 2c, Extended Data Figs. 1 and 2). Low-affinity ligands include cathasterone (an early C₂₈ precursor), 24-epibrassinolide and 24-epicastasterone (late C₂₈ branch), and the C₂₉ products 28-homocastasterone and 28-homobrassinolide (Figs. 1b and 2c,d). 28-Norbrassinolide (C₂₇ branch) as well as brassinolide and castasterone (late C₂₈ branch) represent high-affinity ligands (Figs. 1b and 2c,d). Additional high-affinity binders include typhasterol, secasterone and 2-deoxybrassinolide (late C₂₂ oxidation), as well as 6-deoxycastasterone (early C₂₃ oxidation), all within the C₂₈ branch (Figs. 1b and 2c,d). BRI1 and BRL3 share highly similar ligand-binding specificity profiles with BRII (Fig. 2c, Extended Data Figs. 1 and 2), consistent with their structurally conserved BR-binding pockets (Fig. 2b and Supplementary Fig. 1) and their ability to rescue *bri1-301* mutant phenotypes²⁹.

We next sought to define the chemical features of BRs that contribute to high-affinity receptor binding. Side chain modifications—such as the C₂₄ ethyl substitution in 28-homocastasterone or 28-homobrassinolide (Fig. 1a,b)—were associated with ~10- to 30-fold and ~10- to 35-fold reductions in binding affinity, respectively, compared to castasterone and brassinolide (Fig. 2c,d). Similarly, the brassinolide / castasterone enantiomers 24-epibrassinolide and 24-epicastasterone (Fig. 1a,b) showed ~10- to 20-fold and ~10- to 40-fold reduced affinities, respectively (Fig. 2c,d). The absence of a hydroxyl group at C₂₃—as in cathasterone—correlated with weak receptor binding (Figs. 1b and 2c,d).

To further assess the role of side chain hydroxylation, we synthesized 23-deoxycastasterone (Supplementary Note 1). 23-Deoxycastasterone bound BRII with a K_d of ~3 μ M, representing a 100-fold reduction compared to castasterone (Fig. 2c,d). By contrast, 22-deoxycastasterone—lacking the C₂₂ hydroxyl group present in castasterone and brassinolide (Fig. 1b)—still bound BRII with medium to high affinity (K_d ≈ 200 nM; Fig. 2c,d). These results are consistent with our structural data showing that the C₂₃, but not the C₂₂, hydroxyl group forms conserved hydrogen bonds with the island domains of BRII, BRI1 and BRL3 (Fig. 2b and Supplementary Fig. 1a).

Ring modifications were generally well tolerated by the BRII, BRI1 and BRL3 ectodomains (Fig. 2c,d). Lactonization of the B-ring had minimal impact on binding, as brassinolide and castasterone showed nearly identical K_d (Fig. 2c,d). Although C₆ oxidation is a key biosynthetic step towards brassinolide^{13,31}, 6-deoxycastasterone bound all three receptors with high affinity (Fig. 2c,d). Supporting this, the complex structures of BRII, BRI1 and BRL3 show no conserved interactions with the C₆ ketone^{26,27,31} (Fig. 2b and Supplementary Fig. 1a). The 2 α and 3 α hydroxyl groups in the A-ring also appear dispensable for receptor binding, as ligands lacking either—such as 2-deoxybrassinolide, 3-deoxycastasterone, typhasterol and secasterone—bound BRII, BRI1 and BRL3 with nanomolar affinity (Fig. 2c,d).

Overall, our quantitative binding data show that BR receptors recognize a surprisingly broad range of structurally diverse BRs with high affinity. The configuration and size of the aliphatic side chain, particularly the presence of a C₂₃ hydroxyl group and the correct C₂₄ stereochemistry, drive high-affinity receptor binding.

High structural plasticity of the hormone-binding pocket enables BRII/BRL3 to sense different BRs

To clarify the structural basis of ligand-binding specificity in BR receptors, we co-crystallized the BRII ectodomain with various BRs. Crystals

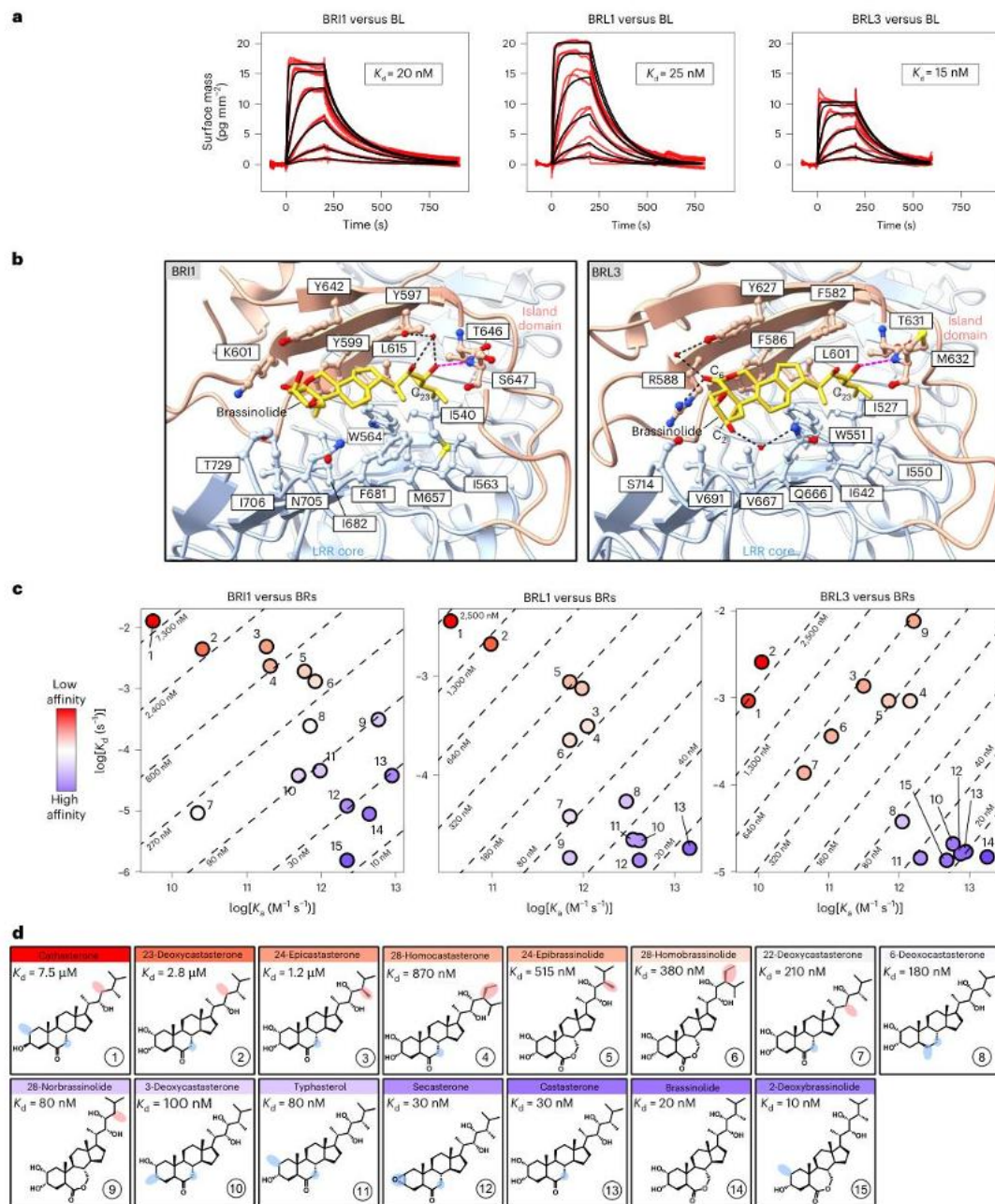


Fig. 2 | BRI1, BRL1 and BRL3 bind chemically diverse BRs. **a**, GCI analysis of ligand-binding kinetics between BRI1, BRL1, BRL3 and brassinolide. Shown are sensorgrams ($n = 2$, red) with global fits (black) and equilibrium K_d indicated. **b**, BR-binding pockets of BRI1 (left; PDB ID, 3RJO) and BRL3 (right) shown as ribbon diagrams. LRRs are shown in blue, island domains in red and brassinolide in yellow. Pocket residues are shown as ball and sticks, hydrogen bonds as dotted lines.

The conserved C_{21} -OH hydrogen bond is highlighted in purple. **c**, Isoaffinity plots of association (K_a) and dissociation (K_d) rates for receptor–BR pairs. Each point ($n = 2$) reflects a global fit; diagonal lines denote K_d . High- and low-affinity interactions are in blue and red, respectively. **d**, Structures of tested BRs, numbered as in **c**, with BRI1 K_d values. Red highlights indicate modifications that reduce binding, while blue highlights indicate modifications that have a minimal effect.

grown with cathasterone, 24-epicastasterone or 28-homocastasterone showed no interpretable difference in electron density in the BR-binding pocket, while a BRII–6-deoxocastasterone complex yielded poorly diffracting crystals. A 2.1 Å structure of the BRII–castasterone complex revealed a ligand conformation nearly identical to that of brassinolide (Fig. 3a,b, Supplementary Fig. 2 and Supplementary Table 1), consistent with its high binding affinity (Fig. 2c,d) and confirming that B-ring lactonization is not essential for receptor recognition. Similarly, a 2.7 Å structure of the BRII–typhasterol complex showed that the absence of a C₂ hydroxyl group does not affect ligand coordination by the receptor (Fig. 3a,b and Supplementary Fig. 2). By contrast, the 2.4 Å BRII–24-epibrassinolide complex structure showed weak electron density for the BR side chain, consistent with its reduced binding affinity (Figs. 2c,d and 3a,b and Supplementary Fig. 2). Finally, the 2.4 Å BRII–28-homobrassinolide complex revealed that the receptor can in principle accommodate a C₂₄ ethyl substitution in its binding pocket (Fig. 3a,b and Supplementary Fig. 2).

We next resolved the crystal structures of the BRL3 ectodomain bound to castasterone and typhasterol at 2.6 Å and 2.3 Å, respectively. In both structures, the ligands adopt conformations closely resembling that of brassinolide (Fig. 3a,b and Supplementary Fig. 2). A 3.2 Å structure of the BRL3–6-deoxocastasterone complex indicates that, despite lacking B-ring modifications, the ligand is accommodated in the binding pocket, albeit in a shifted conformation (Fig. 3a,b and Supplementary Fig. 2). In all BR receptor–ligand complexes analysed, the BR C₂₃ hydroxyl group consistently forms a conserved hydrogen bond with the main chain amino group of Ser647 in BRII or Met632 in BRL1/BRL3 (Fig. 3a). We note that, given the moderate resolution of several datasets (~2.5–3.2 Å), the level of detail observable in the electron density maps inherently limits the precision with which the ligand orientation can be defined.

Several catabolic enzymes contribute to BR homeostasis, including BAS1, which hydroxylates side chain C₂₆ (ref. 17), UGT73C5, which glucosylates the hydroxyl group at C₂₃ (ref. 18), and ST1/ST4a, which sulfonates the C₂₂ hydroxyl¹⁹. Structural analysis of our receptor–ligand complexes indicates that such tail modifications are not compatible with the BR-binding pocket (Supplementary Fig. 3). Together, the BRII and BRL3 ligand-bound structures rationalize the BR binding kinetics data, emphasizing the importance of the BR side chain for high-affinity receptor binding, while showing that modifications to the steroid ring core are generally tolerated.

To evaluate the physiological relevance of our biochemical and structural experiments, we selected ligands representing high-, medium- and low-affinity binders for root growth inhibition assays, as previously described⁴³. Brassinolide ($K_d \approx 20$ nM) and 28-norbrassinolide

($K_d \approx 80$ nM) were tested as high-affinity binders, 28-homobrassinolide ($K_d \approx 380$ nM) as medium-affinity and 23-deoxycastasterone ($K_d \approx 2,800$ nM) as a low-affinity binder (Fig. 2d). Root growth inhibition assays were performed using each compound at a final concentration of 100 nM. A strong trend for increasing root length with increasing K_d was observed in treated wild-type seedlings and a weaker trend in the *bsk3-1* control (Fig. 3c), suggesting that in vivo BR bioactivities correlate well with their receptor binding affinity in vitro. Even the low-affinity binder 23-deoxycastasterone significantly inhibited root growth of wild-type seedlings at a concentration of 100 nM (Fig. 3c).

We next correlated in vitro BR receptor binding affinities with BR levels in planta. We quantified BR levels in 10-day-old *Arabidopsis* plants using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) (Methods). In whole wild-type seedlings, brassinolide was detected at low levels (~0.6 pmol per gram fresh weight (FW)), while castasterone levels were ~20-fold higher (Fig. 3d). 28-Norbrassinolide (~0.9 pmol g⁻¹ FW) and 28-homobrassinolide (~0.8 pmol g⁻¹ FW) were also present at low concentrations, whereas 28-homocastasterone (~80 pmol g⁻¹ FW) and typhasterol (~5.0 pmol g⁻¹ FW) were abundant (Fig. 3d). Correlating these endogenous BR levels with their respective in vitro binding affinities (Fig. 2c,d) suggests that, in addition to brassinolide, other BRs—such as castasterone, 28-norbrassinolide and 28-homocastasterone—may act as physiologically relevant ligands for BRII, BRL1 and BRL3.

Constitutive activation or inactivation of BRII modulates BR homeostasis

We next compared BR levels in wild-type, *bri1* null⁴⁷ and oBIR3-iBRII (ref. 52) constitutively active seedlings (Supplementary Table 2). In the *bri1* mutant, brassinolide, castasterone, typhasterol and 28-homo-brassinolide levels were elevated by 2- to 3-fold, respectively, compared to wild type (Fig. 3d), as previously described⁴⁵. This aligns with earlier findings that BR metabolic and catabolic gene expression is altered in *bri1* loss-of-function mutants⁵³, partly mediated by the BR-regulated transcription factor BZR1^{40,54}. In oBIR3-iBRII seedlings, which show constitutive BR responses⁵², brassinolide, castasterone and 28-norbrassinolide levels were reduced compared to wild type (Fig. 3d).

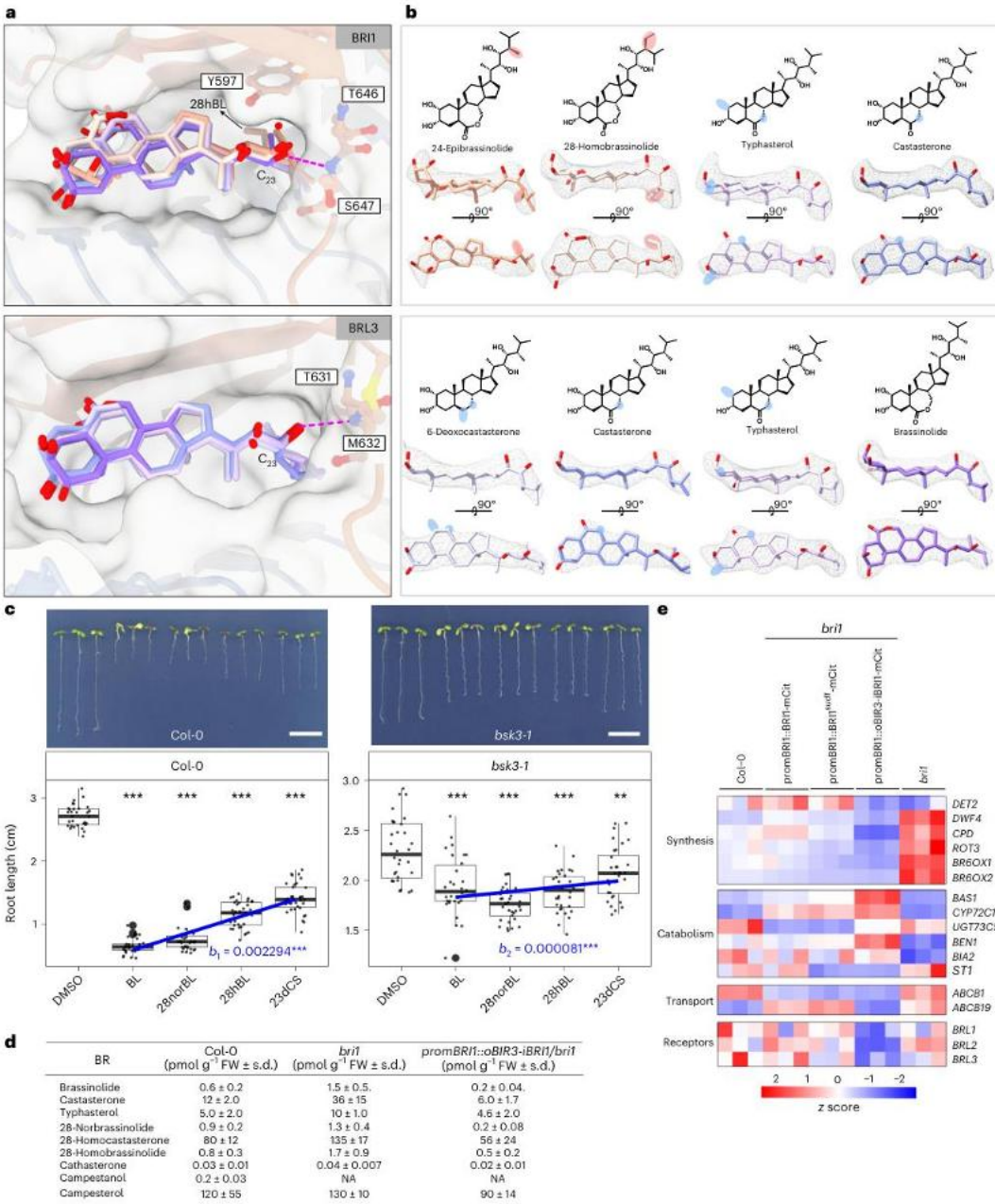
To further investigate, we performed RNA sequencing (RNA-seq) on untreated 10-day-old Col-0 seedlings, a *bri1* null mutant⁴⁷ and in *bri1* complementation lines expressing either a wild-type BRII receptor⁴⁷, the BRII^{wild} gain-of-function allele^{36,47} or the constitutively active, ligand-independent oBIR3-iBRII receptor chimera⁵². We found many BR biosynthesis genes to be mildly induced in *bri1* mutant seedlings and

Fig. 3 | The hormone-binding pocket can accommodate structurally diverse BRs. a, Structural superposition of different BRII–BR (top, r.m.s.d. of 0.4 Å comparing 186 corresponding C_α atoms) and BRL3–BR complexes (bottom, r.m.s.d. of 0.3 Å comparing 190 corresponding C_α atoms). The receptors are shown as a combined ribbon diagram (LRR domain in light blue, island domain in light red) and molecular surface views. BRs are in bonds representation colour coded according to their receptor binding affinities, ranging from low (light red) to high affinity (purple; BRII, brassinolide complex PDB ID, 3RJO). Dashed lines highlight key polar interactions among the ligand and the receptor-binding pockets. A conserved hydrogen bond between the ligand and the island domains of BRII, BRL1 and BRL3 is marked in light orange. **b**, Structures of BRs (in bonds representation, colour coded as in **a** with BRII (top) or BRL3 (bottom) and including final mF_o–DF_c omit electron density maps (grey mesh, contoured at 4 σ). The respective chemical structures are shown above, with modifications relative to brassinolide marked in red (for changes that reduce binding affinity) or blue (neutral). **c**, Root growth inhibition assay of 7-day-old Col-0 wild-type and *bsk3-1*¹⁷ mutant seedlings ($n = 30$, individual seedlings per condition) grown on ¹⁸MS plates supplemented with the indicated BR (BL, brassinolide; 28norBL, 28-norbrassinolide; 28hBL, 28-homobrassinolide; 23dCS, 23-deoxycastasterone;

0.1% (v/v) DMSO as control) at a final concentration of 100 nM (scale bars, 1 cm). Quantifications are shown as box plots (centre line, median; box, interquartile range (IQR); whiskers, lowest/highest data point within 1.5 IQR of the lower/upper quartile, raw data shown as grey dots). Multiple comparisons of the different treatments versus the DMSO control were performed using a two-sided Dunnett⁴⁹ test as implemented in the R package multcomp⁵⁰ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). The relationships between the estimated K_d for each ligand and root length were tested using a two-sided Tukey trend test^{50,52}. The blue line represents the linear trend with slope (b) and the associated test P value (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). The exact P values associated to b_1 and b_2 are 0.0000000 and 0.0000170, respectively. The experiment was repeated three times with similar results. **d**, BR quantification in 10-day-old Col-0, *bri1* (GABI_134E10)⁴⁷ and *promBRII::oBIR3-iBRII/bri1* (ref. 52) seedlings (FW, fresh weight; NA, not available). **e**, RNA-seq derived expression profile heat map of BR biosynthesis, catabolism, transport and receptor genes in 10-day-old seedlings in untreated BR signalling mutants and the Col-0 wild type. Expression values were transformed into z scores based on the normalized counts; three biological replicates are shown in three horizontal rows; the colour scale indicates expression level (red, up-regulation; blue, down-regulation).

to be repressed in the constitutively active oBIR3-iBRII²³ line (Fig. 3e). BR biosynthesis genes were up-regulated in *bri1* and down-regulated in oBIR3-iBRI1 seedlings (Fig. 3e). Conversely, several BR catabolic genes were repressed in *bri1* and induced in oBIR3-iBRI1 plants (Fig. 3e). ABCB1 and ABCB19, which function as BR transporters required for

signalling²³, also showed differential expression in *bri1* and oBIR3-iBRI1 seedlings (Fig. 3e). *BRL1*, *BRL2* and *BRL3* transcript levels were reduced in oBIR3-iBRI1 and partially induced in *bri1* (Fig. 3e). Expression of a wild-type BRI1 receptor restored gene expression in *bri1* mutants to wild-type levels (Fig. 3e). The gain-of-function allele *BRI1^{ind}* mimicked



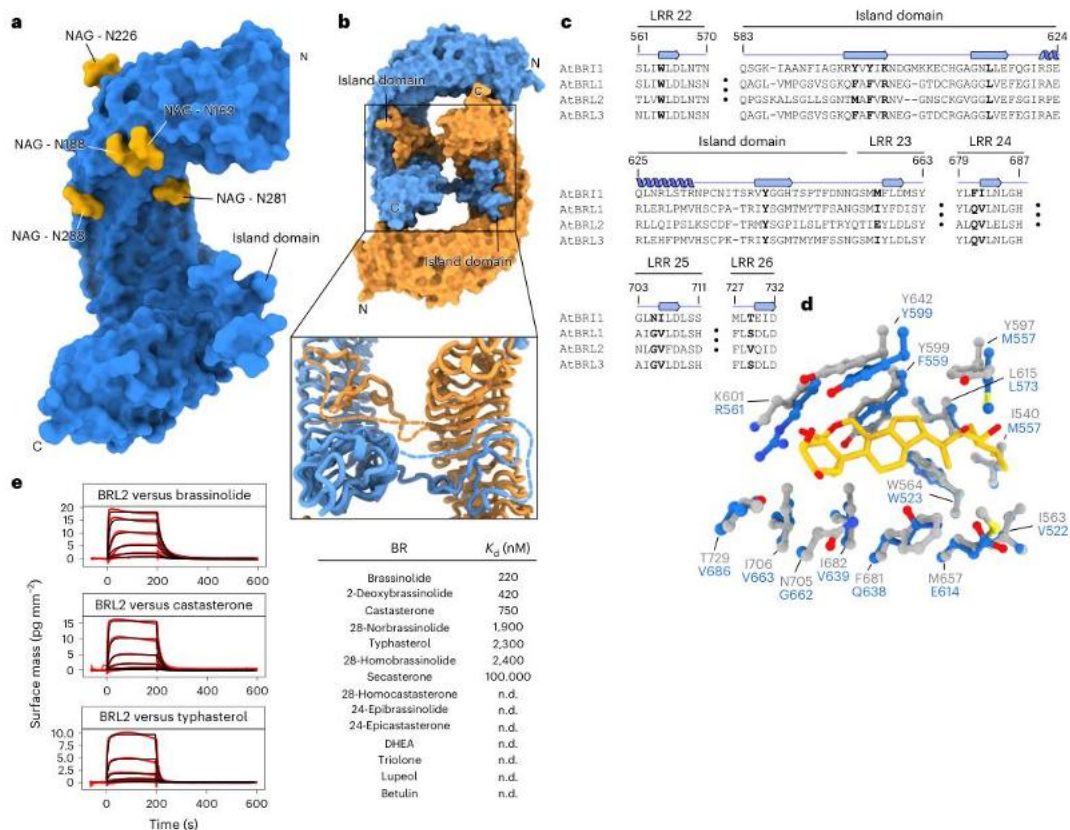


Fig. 4 | BRL2 harbours a conserved ligand-binding pocket and binds BRs with medium affinity. **a**, Crystal structure of the apo BRL2 ectodomain. Shown is a molecular surface view with the LRR and island domains in blue and N-glycans in yellow. **b**, BRL2 forms a domain-swapped crystallographic dimer. Shown are two BRL2 molecules in the asymmetric unit of the monoclinic crystal form (in blue and light orange, respectively), with the island domain of each molecule binding to the LRR domain of its neighbour. The island domains are partially disordered, indicated by dotted lines (inset). **c**, Structure-based multiple sequence alignment of steroid-binding pockets of BRII, BRL1, BRL2 and BRL3. Residues involved in ligand coordination are highlighted in bold-face (residue and secondary

structure numbering according to BRII). **d**, Structural superposition of the BRII ectodomain (ribbon diagram, in grey; PDB ID, 3RJO) with the AlphaFold2 model of the apo BRL2 ectodomain (in blue, AlphaFoldDB-ID, AF-Q9ZPS9-F1; r.m.s.d. of -0.8 Å comparing 163 C α atoms). Brassinolide is in bonds representation; interacting residues are shown as ball and sticks. **e**, GCI-derived binding kinetics of the BRL2 ectodomain versus brassinolide, castasterone and typhasterol. Red traces represent experimental sensorgrams ($n = 2$), and black lines show the global fits. A K_d table of the BRL2 ectodomain versus different BRs, non-BR steroids and triterpenoids is shown alongside (n.d., no binding detected).

the effects of the constitutively active oBIR3-iBRII receptor chimera, albeit with less pronounced effects (Fig. 3e). Together, these experiments indicate that BRII signalling activity shapes BR homeostasis by modulating the expression of BR metabolic, catabolic and transport genes.

BRL2 binds BRs with medium affinity in vitro

The structural flexibility of the BR-binding pockets in BRII, BRL1 and BRL3, along with altered BRL2 expression in BRII signalling mutants (Fig. 3e), prompted us to re-examine BRL2's ligand-binding properties. BRII and BRL2 share ~40% sequence identity in their extracellular domains. We expressed and purified the BRL2 ectodomain from insect cells and solved its apo crystal structure to 2.6 Å resolution in two independent crystal forms (Methods and Supplementary Table 1). The N-glycosylated BRL2 ectodomain adopts a superhelical architecture

with 21 LRRs (compared to 25 in BRII) with a more open conformation (Fig. 4a and Supplementary Fig. 4a). BRL2's island domain is partially disordered and participates in a domain-swapped crystallographic dimer (Fig. 4b and Supplementary Fig. 4b).

The ligand-binding pocket of BRL2 shows a high degree of sequence and structural similarity with the BR-binding sites of BRII, BRL1 and BRL3 (Fig. 4c,d and Supplementary Fig. 4c). Using GCI assays, we found that BRL2 can bind BRs, but it has a distinct ligand-binding profile compared to BRII, BRL1 and BRL3 (Figs. 2c,d and 4e). BRL2 bound brassinolide and castasterone with K_d values of ~200 nM and ~400 nM, respectively (Fig. 4e, Extended Data Fig. 3 and Supplementary Table 5), 10- to 30-fold weaker than BRII (Fig. 2c,d). Unlike BRII, which binds typhasterol and 28-norbrassinolide with nanomolar affinity, BRL2 bound these ligands only weakly (Fig. 4e). BRL2 showed no detectable binding to 28-homocastasterone, 24-epicasterone,

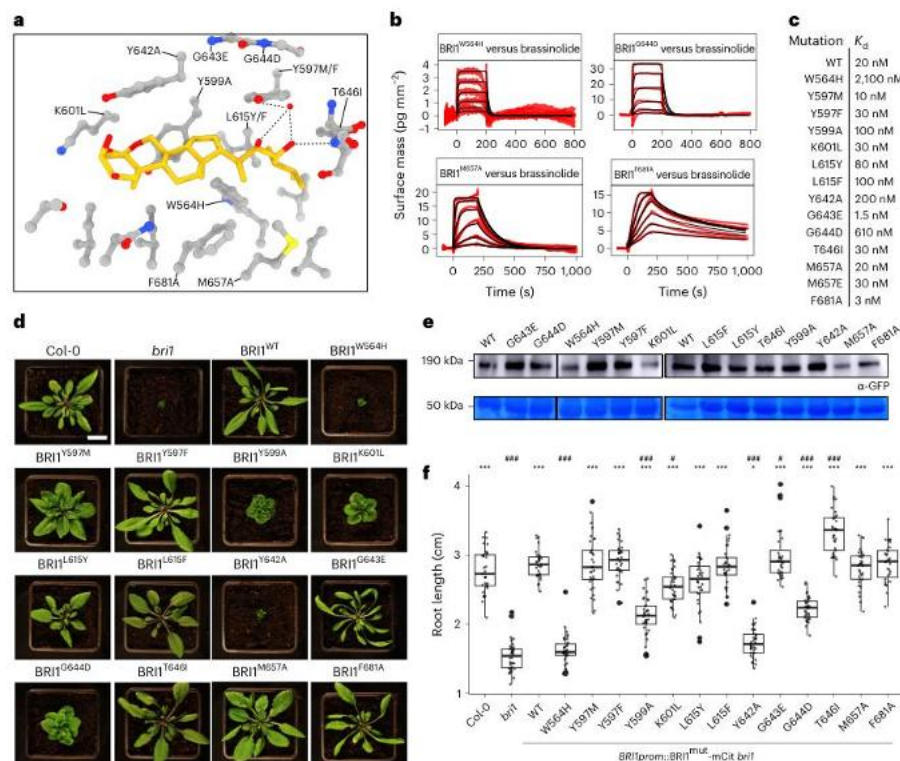


Fig. 5 | A conserved aromatic residue in the hormone-binding pocket is critical for BR perception. a, Coordination of brassinolide (in bonds representation, in yellow, PDB ID, 3RJ0) in the BRI1 steroid-binding pocket. Residues in contact with the ligand and used for the mutational analysis are displayed as ball and sticks; hydrogen bonds are shown as dotted lines. **b**, GCI-derived binding kinetics for BRI1^{W564H}, BRI1^{G644D}, BRI1^{M657A} and BRI1^{F681A} versus brassinolide. Red traces represent experimental data ($n = 2$), black lines show the global fits. **c**, K_d table for BRI1 point mutants versus brassinolide. **d**, Rosette phenotypes of representative 5-week-old transgenic T3 *BRI1prom::BRI1*-Citrine^{mut} complementation lines in *bri1* null¹ background compared to the Col-0 wild-type control (scale bar, 2 cm).

e, Quantification of BRI1^{mut}-mCitrine (theoretical molecular mass of ~158.6 kDa) protein levels by western blot from 10-day-old seedling protein extracts, immuno-blotted with an anti-GFP antibody. ReadyBlue-stained RubisCO is shown below as input loading control. **f**, Root length phenotypes of 8-day-old seedlings ($n = 30$) shown as box plots (centre line, median; box, IQR; whiskers, lowest/highest data point within 1.5 IQR of the lower/upper quartile; raw data shown as grey dots). Multiple comparison of the different treatments versus the indicated controls were performed using a two-sided Dunnett³⁹ test as implemented in the R package multcomp⁴⁰ (multiple comparison against *bri1*: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; against Col-0: † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$).

24-epibrassinolide, dehydroepiandrosterone, triolone, lupeol or betulin (Fig. 4e). These observed differences in the ligand-binding specificity between BRI1, BRL1 and BRL3, and BRL2 correlate with amino acid changes in the BRL2 BR-binding pocket (Fig. 4c). Tyr597 and Met657 in BRI1 correspond to Met557 and Glu614 in BRL2, respectively (Fig. 4d). The corresponding BRI1^{M657E} and BRI1^{Y597M/Y599F/M657E} mutant isoforms show moderate to severe growth phenotypes and reduced BR signalling responses¹⁶. Overall, BRL2 contains a structurally conserved BR-binding pocket and can bind selected BRs in vitro with mid-nanomolar to micromolar affinity.

VH1/BRL2 has been characterized as a vascular receptor kinase involved in leaf vein patterning^{34,35}. The previously described *bri1*2-1 mutant³⁵ showed wild-type-like responses in BR hypocotyl growth and BES1 dephosphorylation assays (Supplementary Fig. 5a,b). In our hands, the leaf vein patterns of *bri1*2-1 plants were not significantly different from the Col-0 wild type (Supplementary Fig. 5c). Transcriptional *BRL2prom::NLS-3xmVenus* and translational *BRL2prom::BRL2*-mCitrine reporter lines confirmed the expression of BRL2 in vascular tissues

across different developmental stages (Supplementary Fig. 5d,e and Supplementary Table 3). We generated a second *bri1*2-2 loss-of-function allele using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) gene editing (Supplementary Fig. 5f), which like *bri1*2-1 was indistinguishable from wild type (Supplementary Fig. 5g). We have previously reported that receptor chimeras, in which the extracellular and transmembrane domains of BIR3 (oBIR3) are fused to the cytoplasmic kinase domain of a SERK-dependent receptor kinase (iRK), trigger constitutive, ligand-independent signalling responses^{38,52}. A *BRL2prom::oBIR3-iBRL2* chimera in Col-0 wild-type background had elongated, slender petioles and curly leaves, similar to BRI1^{ind} gain-of-function mutants^{24,47} (Supplementary Fig. 5g and Supplementary Table 3). However, the rosette phenotypes were milder than those of *BRI1prom::oBIR3-iBRI1* chimeras⁵² (Supplementary Fig. 5g). These experiments suggest that BRL2 may have the capacity to initiate a SERK-dependent signalling cascade, yet its physiological ligand and physiological function remain to be characterized.

A mutational map of the BR-binding pocket rationalizes the specificity and plasticity of BR receptors

Given the high structural conservation of the BR-binding site in BRII, BRL2, BRL1 and BRL3 (Supplementary Figs. 1 and 6a), we examined the role of individual pocket-lining residues in BR perception. On the basis of our BRII–BR complex structures (Fig. 3a), we mutated 11 residues in the LRR core and island domain (Fig. 5a and Supplementary Table 3) and obtained brassinolide binding kinetics for each mutant ectodomain (Fig. 5b,c and Extended Data Fig. 4). All mutants behaved as well-folded monomers in solution (Supplementary Fig. 6b).

Consistent with the BR-binding site's structural plasticity, mutation of the conserved Thr646, Tyr597 or Lys601 from the island domain had no detectable effect on brassinolide binding (Fig. 5a–c). Substitution of Tyr599 or Tyr642 with alanine moderately reduced binding ($K_d \approx 100$ nM and ~ 200 nM, respectively), and replacing the invariant Leu615 with phenylalanine or tyrosine likewise had little effect on BR binding (Fig. 5a–c). By contrast, mutation of Trp564 to His caused an ~ 100 -fold drop in BR binding ($K_d \approx 2$ μ M) (Fig. 5a–c). Mutating Met657 (which corresponds to Glu614 in BRL2; Fig. 4c,d) to either alanine or glutamate did not affect brassinolide binding (Fig. 5a–c). A moderate growth phenotype has been previously reported for the BRII^{M657E} mutant⁴⁶. These findings underscore the presence of a conserved BR-binding site in BRII/BRL1/BRL3 and in BRL2 (Fig. 4d). As previously shown⁴⁶, the *bri1-6*⁴⁵ mutation (Gly644 to Asp) strongly reduces brassinolide binding (Fig. 5a–c). By contrast, the neighbouring Gly643 to Glu mutation in the BRII^{int} gain-of-function allele⁴⁷ stabilizes the island domain¹⁶ and enhances brassinolide binding by ~ 13 -fold compared to wild type (Fig. 5a–c). Similarly, mutating LRR core Phe681 to alanine increases binding by ~ 7 -fold. These results further highlight the BR-binding site's structural plasticity and identify Trp564 from the LRR core as essential for ligand recognition.

To assess the functional impact of these mutations, we expressed mutant BRII receptors under the native BRII promoter in a *bri1* null background (Fig. 5d). T3 generation transgenic lines with wild-type-like BRII expression (Fig. 5e and Supplementary Fig. 6c) and proper plasma membrane localization (Supplementary Fig. 6d) were selected. High-affinity mutants (Tyr597 to Phe, Leu615 to Phe, Thr646 to Ile) restored wild-type rosette and root growth phenotypes (Fig. 5d–f) and BR responses (Supplementary Fig. 6e,f and Supplementary Table 4). Intermediate phenotypes were observed in lines with Tyr597 to Met, Leu615 to Tyr, or Met657 to Ala, consistent with their moderate in vitro binding affinities (Fig. 5a–c). The Gly644 to Asp mutant showed medium-to-strong defects, while Trp564 to His, which showed

~ 100 -fold reduced binding, phenocopied the *bri1* null at both seedling and adult stages (Fig. 5d–f).

Conversely, Gly643 to Glu and Phe681 to Ala conferred gain-of-function rosette phenotypes, matching their enhanced binding affinities compared to wild type (Fig. 5a–d). Three mutants (Tyr599 to Ala, Lys601 to Leu, Tyr642 to Ala) showed severe growth phenotypes despite retaining strong brassinolide binding in vitro (Fig. 5a–f; see below). Overall, we observed a strong correlation between the in vitro ligand-binding affinities and the in vivo growth phenotypes of BRII receptor mutants that target amino acids involved in ligand coordination. Our analysis revealed novel loss-of-function (Trp564-His) and gain-of-function (Phe681-Ala) alleles, which can be used to precisely control BRII activity in planta.

BR antagonists target the interaction of BR receptors with SERK co-receptors

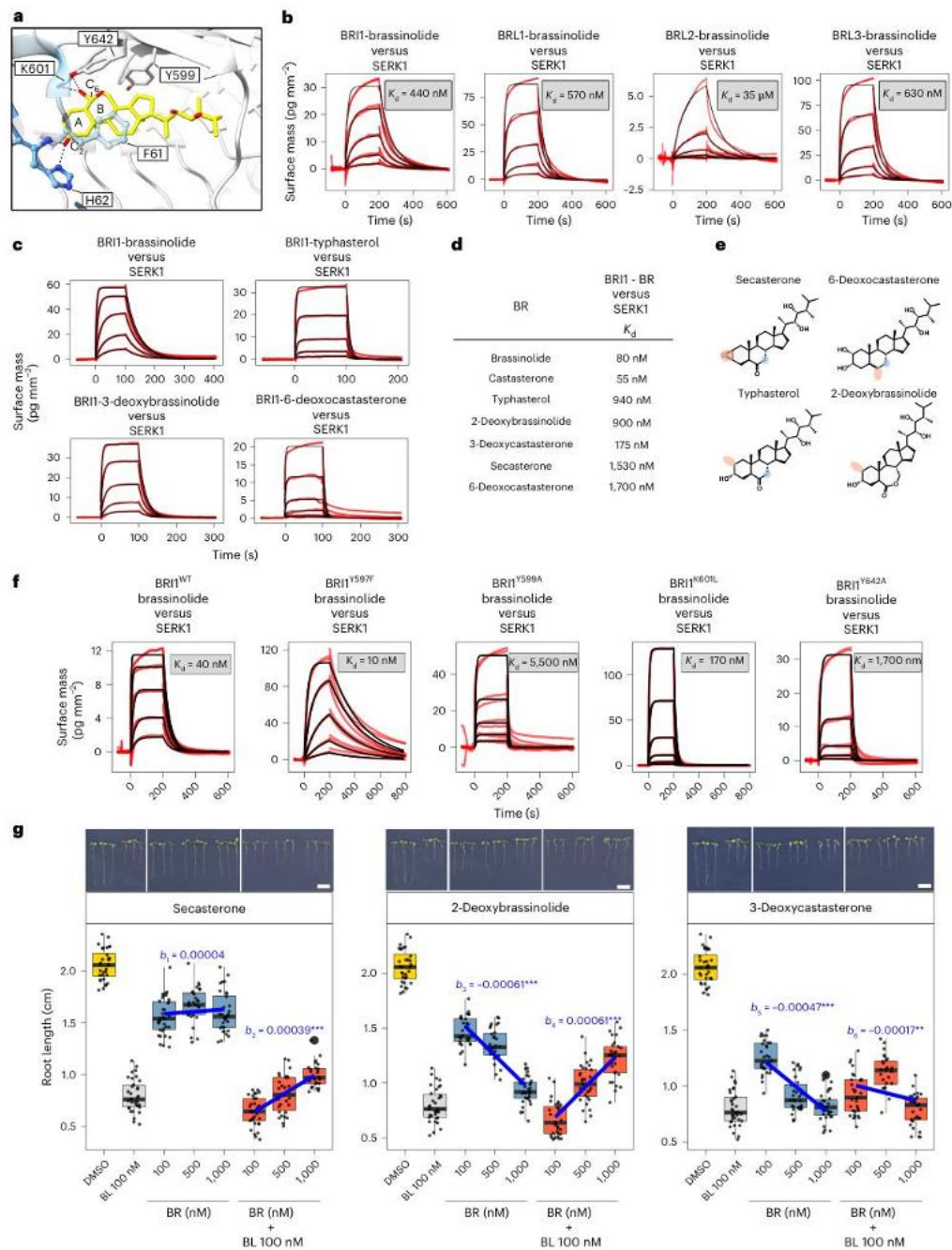
Previously, we demonstrated that the BR-binding site encompasses the LRR core and island domain of BRII, and the LRR ectodomain of a SERK co-receptor^{26,28,36} (Fig. 6a). SERK1 Phe61 stacks with the A/B rings of brassinolide, while His62 forms a conserved hydrogen bond with the C₃ hydroxyl group of the ligand³⁶ (Fig. 6a). Mutating the corresponding Phe60 and His61 in SERK3/BAK1 to alanine abolishes both co-receptor interaction in vitro and BR signalling in planta²⁸.

To test whether this activation mechanism is conserved across BR receptors, we immobilized BRII, BRL1, BRL2 and BRL3 ectodomains on a GCI chip and examined SERK1 binding in the presence of 100 nM brassinolide. BRL1 and BRL3, like BRII, bound SERK1 with high affinity, while BRL2 showed much weaker SERK1 binding ($K_d \approx 35$ μ M) (Fig. 6b and Extended Data Fig. 5).

We next quantified the interaction of SERK1 with BRII in the presence of different BRs. We used an avi-tagged BRII ectodomain to minimize steric hindrance between the GCI chip and the SERK1 co-receptor (Methods) and measured K_d of ~ 100 nM for SERK1 association to either brassinolide- or castasterone-bound BRII (Fig. 6c,d and Extended Data Fig. 5). However, SERK1 binding was ~ 15 -fold weaker with typhasterol, when compared to castasterone-bound BRII (Fig. 6c,d), despite these ligands having similar receptor binding affinities (Fig. 2c,d). This reveals that the C₂ hydroxyl coordinated by SERK1 His62 is key for receptor–co-receptor complex formation (Fig. 6a,e). Similarly, 2-deoxybrassinolide reduced SERK1 binding ~ 10 -fold, while 3-deoxycastasterone, lacking the C₃ hydroxyl, still supported high-affinity SERK1 binding (Fig. 6c–e). Secasterone—with a bulky 2,3- α -epoxy group—impaired complex formation, as did 6-deoxycastasterone, despite possessing both C₂ and C₃ hydroxyls

Fig. 6 | BR C₂ hydroxylation and C₃ oxidation are critical for SERK co-receptor binding. **a**, Close-up view of the SERK1-bound (in blue) BRII (in grey)—brassinolide (in bonds representation, in yellow) complex (PDB ID, 4LSX). Receptor and co-receptor amino acids involved in BR-binding are highlighted as ball and sticks in grey and blue, respectively. Polar interactions between the brassinolide C₂ hydroxyl group and SERK1 His62, and between the C₃ oxygen and BRII Tyr642 or Lys601 are shown as dotted lines (in black). **b**, GCI binding kinetics of amine-coupled and brassinolide-bound BRII, BRL1, BRL2 and BRL3 ectodomains versus SERK1 in the presence of a constant concentration of 1 μ M BL. Shown are sensorgrams with red traces indicating experimental data and black lines representing global fits ($n = 1$). Binding affinities (K_d) are reported alongside. **c**, Representative GCI traces showing the interaction between SERK1 and the biotinylated, avi-tagged BRII ectodomain in the presence of either brassinolide, typhasterol, 3-deoxybrassinolide or 6-deoxycastasterone. **d**, Table summaries of the receptor–BR–co-receptor interaction K_d . **e**, Chemical structures of selected BRs tested in **c** and **d**. Structural modifications relative to brassinolide that reduce co-receptor binding are highlighted in red. Modifications that have little effect on complex formation are shown in blue. **f**, GCI binding kinetics of amine-coupled wild-type BRII, BRII^{T597A}, BRII^{T599A}, BRII^{L615Y} and BRII^{T642A} ectodomains versus SERK1 in the presence of a high,

saturation concentration of brassinolide. Shown are sensorgrams with red traces indicating experimental data and black lines representing global fits ($n = 2$). Binding affinities (K_d) are reported alongside. **g**, BR seedling root growth inhibition antagonist assay ($n = 30$, individual seedlings per condition). Brassinolide (BL) at a fixed concentration of 100 nM was used in the different treatments, versus 0.02% (v/v) DMSO as control (in yellow). The antagonist candidate compounds secasterone, 2-deoxybrassinolide and 3-deoxycastasterone were tested for their ability to inhibit seedling root growth by themselves (in blue) or to reverse BL-induced root growth inhibition (in orange). Shown are boxplots with centre lines (medians); box limits indicate the 25th and 75th percentiles; whiskers extend to the full data range. The relationship between antagonist concentration and root length were tested using a two-sided Tukey trend test^{92,93}. The blue lines represent the linear trend with slope b (negative values indicate agonistic behaviour; positive values indicate antagonistic behaviour) and the associated test P value ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). The exact P values are 0.4943212 (b_1), 0.0000000 (b_2), 0.0000000 (b_3), 0.0000000 (b_4), 0.0000000 (b_5) and 0.0032034 (b_6). Representative seedling images are shown above (scale bars, 0.5 cm). The DMSO and BL 100 nM controls are the same in each panel, indicated by a white vertical line. The experiment was repeated three times with similar results.



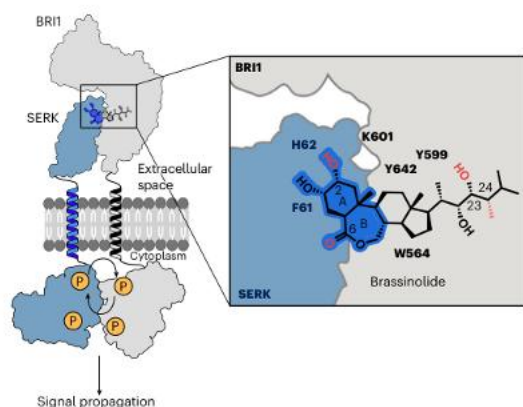


Fig. 7 | Strong receptor binding, correct positioning within the BR binding site and interaction with the co-receptor are essential for BR bioactivity. Schematic overview of BR-induced receptor-co-receptor complex formation and activation. A BR ligand binds to the BR11 receptor ectodomain (in grey), creating a binding surface for a SERK co-receptor kinase (in blue). This brings the kinase domains of receptor and co-receptor in close proximity, leading to trans-phosphorylation and intracellular signalling. The BR-binding pocket is formed by the receptor (in grey) and co-receptor (in blue). Chemical features of the BR ligand (in black) involved in co-receptor binding are highlighted in dark blue; key carbon atoms critical for receptor or co-receptor interaction are numbered, and chemical features required for receptor and/or co-receptor binding are shown in red. The approximate positions of residues critically involved in BR binding, positioning and co-receptor binding are indicated.

(Fig. 6c–e), likely because of its distorted binding mode in the BR pocket (Fig. 3a,b).

Our data identify the BR C_2 hydroxyl as essential for SERK recruitment. While C_2 oxidation on the B-ring is not required for receptor binding, its coordination by BR11 island domain residues Tyr599, Tyr642 and Lys601 may help orient the ligand for optimal co-receptor binding (Fig. 6a,c–e). Indeed, SERK1 binding is partially or severely compromised in the BR11^{Y599A}, BR11^{K601L} and BR11^{Y642A} mutants in the presence of high, saturating concentrations of brassinolide (Fig. 6f). These results rationalize the dwarf phenotypes of the BR11 Tyr599 to Ala, Lys601 to Leu and Tyr642 to Ala mutants (Figs. 5d–f and 6a), despite their ability to bind brassinolide with high affinity (Fig. 5c).

We hypothesize that BRs that bind to BR11 with high affinity but prevent SERK association may act as receptor antagonists. We performed seedling root growth inhibition assays in the presence of 100 nM brassinolide and with different antagonist candidates from our biochemical screen (Fig. 6d) and found that secosterone applied on its own did not trigger root growth inhibition in a concentration-dependent manner (Fig. 6g). However, applying increasing concentrations of secosterone significantly reversed brassinolide-induced root growth inhibition (Fig. 6g). 2-Deoxybrassinolide acted as a weak BR agonist when applied on its own but had a significant, dose-dependent antagonistic effect on brassinolide-induced root growth inhibition (Fig. 6g). By contrast, 3-deoxycasterone acted as a weak BR agonist in both the absence and presence of brassinolide in the assay (Fig. 6g). We conclude from these experiments that BRs lacking a C_2 but not C_3 hydroxyl group behave as receptor antagonists (Fig. 6a,c–e,g). A bulky head group, such as in secosterone, is also effective in blocking receptor activation in vitro and in planta (Fig. 6a,c–e,g). Taken together, BRs that inhibit BR11–SERK1 complex formation (Fig. 6c–e) in vitro without compromising isolated receptor binding (Fig. 2c,d) can partially reverse brassinolide-induced root growth inhibition in a dose-dependent

manner (Fig. 6g), underscoring the importance of A-ring features in BR receptor activation^{28,36}.

Discussion

Genetic and metabolite studies have revealed a complex BR biosynthetic network in plants^{2,14}. In this study, we provide quantitative binding kinetics for 15 distinct BRs from this network, evaluated against the four BR receptors in *Arabidopsis*. Like other plant hormones^{56,57}, BRs act as molecular glues promoting the association of receptor and co-receptor. Our work now defines the chemical features required for high-affinity receptor binding and co-receptor recruitment. The most unexpected finding is that hydroxylation at C_{23} and the C_{24} stereochemistry in the BR aliphatic tail are critical determinants for high-affinity receptor binding (Fig. 2b–d) and BR bioactivity (Figs. 3c and 7). Consistent with this, C_{23} hydroxylase mutants show severe dwarfism⁶³. A second key finding from our analysis is that improper ligand positioning within the steroid-binding pocket can be accommodated by the isolated receptor, yet becomes detrimental for co-receptor recruitment. For instance, the absence of a C_6 ketone is tolerated by BR11 and BRL3 (Fig. 2b–d) but significantly compromises high-affinity binding to SERK1 (Figs. 6c–f and 7). By contrast, the absence of a B-ring lactone still allows for high-affinity receptor and co-receptor association (Figs. 2b–d and 6c,d), in agreement with the moderate growth phenotype of the *br6ox2* mutant^{33,51}. C_2 hydroxylation is only essential for SERK recognition^{38,36} (Fig. 6c–e) but not for receptor binding (Figs. 2b–d and 3a,b), providing a mechanistic basis for known BR antagonists^{58,59} (Fig. 7). In planta, 2-deoxybrassinolide and secosterone act as receptor antagonists (Fig. 6e,g), supporting the idea that these abundant⁴⁸ biosynthetic intermediates may modulate BR signalling by competing with agonists for receptor binding (Figs. 1b and 6g). This could prevent formation of active BR signalling complexes, in a similar way to the known BIR receptor pseudo-kinases^{37,38}.

Mutational analysis of the BR-binding pocket in BR11 reveals that the receptor can tolerate various missense mutations (Figs. 5 and 7), in agreement with previous mutational studies^{46,60}. We identified a single point mutation, Trp564His, that results in a strong BR loss-of-function phenotype. This finding shows that BR11 can be effectively inactivated by a single amino acid substitution, rather than requiring the combination of multiple mutations as previously reported⁴⁶. Two additional residues (Tyr599 and Tyr642) are critically involved in the proper positioning of the ligand in the BR-binding pocket, and we report a novel gain-of-function allele (Phe681Ala) at the entry of the steroid-binding pocket (Figs. 5d–f and 7). The structural plasticity of the hormone-binding pocket supports the receptor's capacity to recognize chemically diverse BRs, while providing sufficient selectivity to discriminate between BRs, other phytosterols and triterpenoids. The BR-binding site is conserved among the four *Arabidopsis* receptors and their orthologues in crop species, implying that BR receptors in crops likely share a similar BR ligand-binding spectrum (Supplementary Fig. 5a).

On the basis of binding profiles (Figs. 2c,d and 6c,d), bioactivity data and in vivo BR levels^{18,48,50} (Fig. 3d), we propose that brassinolide and castasterone (C_{28}), 28-norbrassinolide (C_{27}) and possibly 28-homocasterone (C_{29}) may act as physiological BR ligands in *Arabidopsis*, but it is presently unknown whether and how these metabolites reach the cell wall compartment to interact with the ligand-binding domains of BR receptors. Feedback regulation of BR biosynthesis likely fine-tunes these BR levels (Fig. 3e). Given that BR biosynthetic enzymes have specific expression domains^{51,61}, different BR ligands may be perceived by the broadly expressed BR11 receptor in specific tissues and organs. Such a broad ligand spectrum has been previously reported for other small-molecule⁶² and peptide⁶³ hormone receptors in plants. Our quantitative biochemical assays also suggest that BRL2 can bind BRs with medium affinity (Fig. 4e) and can weakly interact with SERK co-receptors (Fig. 6b). We note that our in vitro binding assays with the

isolated LRR domains probably underestimate the actual BR binding affinities of intact BRI1/BRL1–SERK receptor complexes embedded in the plasma membrane, as plants can respond to BRs applied externally at picomolar to low nanomolar concentrations^{44,51}.

Taken together, our work defines the chemical determinants for a small-molecule-based molecular glue mechanism allowing formation of an active receptor–steroid ligand–co-receptor signalling complex. Distinct chemical features in BRs required for (1) high-affinity receptor binding, (2) proper ligand orientation in the receptor hormone-binding pocket and (3) high-affinity co-receptor binding (Fig. 7) have now been defined and can be exploited for rational BR agonist and antagonist design.

Methods

Protein expression and purification

The ectodomains from BRI1 (<https://www.uniprot.org> UniProt-ID Q22476, residues 1–788), BRL1 (Q92WC8, 1–770), BRL2 (Q9ZPS9, 1–752), BRL3 (Q9LJF3, 1–770) and SERK1 (Q94AG2, 23–270) ectodomains were amplified from *A. thaliana* complementary DNA (ecotype Col-0). BRI1, BRL1, BRL2 and BRL3 were cloned in vector pBB2 providing tobacco etch virus (TEV) protease cleavable C-terminal Twin-StrepII and 9xHis affinity tags. SERK1 was cloned in the same pBB2 vector containing an N-terminal azurocidin signal peptide^{56,64}. For GCI assays, AtBRI1 was also cloned in vector pBB2 containing a C-terminal Avitag followed by TEV cleavable C-terminal Twin-StrepII and 10xHis affinity tags.

All proteins were expressed in *Trichoplusia ni* (Tnao38 cells, Ximbio) using the baculovirus system⁶⁵. Tnao cells in SF900 medium (Gibco) were grown to a density of 2.0×10^6 cells per ml and subsequently infected with 10 ml of P2 virus. Cells were then incubated for 24 h at 28 °C and for an additional 48 h at 21 °C while shaking at 110 r.p.m. Cells were then pelleted at 4,000g for 20 min, and the supernatant containing the secreted protein was filtrated using 0.45 µm filters (Durapore Merck). The cleared medium was then loaded onto an Ni²⁺ metal affinity column (HisTrap Excel 5 ml, Cytiva) equilibrated in buffer A (50 mM KPi pH 7.8, 250 mM NaCl, 1 mM β-mercaptoethanol). The column was washed with 25 ml of buffer A and subsequently eluted onto a Strep-Tactin XT 4flow 5 ml column (IBA) in buffer A supplemented with 250 mM imidazole (pH 8.0). The Strep-Tactin affinity column was further washed with 25 ml buffer (20 mM Tris pH 8.0, 250 mM NaCl and 1 mM EDTA), and the protein was eluted from the Strep-Tactin column with buffer C (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM biotin, 1x BTX, IBA). For protein crystallization, the affinity purified proteins were incubated overnight at 4 °C with TEV protease using a molar receptor/protease ratio of ~10:1. The tag-free protein was separated from the tag and from TEV protease by a second Ni²⁺ affinity chromatography step. Finally, the untagged protein was applied to a HiLoad Superdex 200 16/200 pg column (Cytiva) equilibrated in 20 mM sodium citrate pH 5.0, 150 mM NaCl. Fractions containing the purified protein were concentrated and stored at –80 °C.

Receptor biotinylation for GCI was achieved by incubating Avi-tagged BRI1 with His-tagged BirA⁶⁶. Briefly, BRI1 at a final concentration of 20 µM was incubated for 1 h at 30 °C with BirA, biotin, ATP and MgCl₂ at final concentrations of 85 µM, 150 µM, 2 mM and 5 mM, respectively. The BirA enzyme was subsequently removed using a Ni²⁺ affinity chromatography step. Biotinylated BRI1 was concentrated and loaded onto a HiLoad Superdex 200 16/200 pg column (Cytiva) equilibrated in 20 mM sodium citrate pH 5.0, 150 mM NaCl.

Chemical synthesis and analysis of BRs

The chemical synthesis of typhasterol, 3-deoxycastasterone and 2-deoxybrassinolide are described in Supplementary Note 1.

GCI

GCI experiments were performed using the Creoptix WAVE system (Malvern Panalytical). Binding of BRs to the isolated BRI1, BRL1, BRL2

and BRL3 ectodomains was measured using 4PCH WAVEchips. Borate buffer (100 mM sodium borate, pH 9.0, 1 M NaCl, Xantec) injections were used to condition the chips. For the interaction screening of BRI1, BRL1, BRL2, BRL3 or BRI1^{mut} with BRs, the purified ectodomains were immobilized using standard amine coupling. Chips were activated using a 1:1 mix of 400 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and 100 mM N-hydroxysuccinimide (Xantec). Protein ligands (in 10 mM sodium acetate, pH 5.0) were injected to the desired chip surface at the concentration indicated in Supplementary Table 5. To minimize non-specific binding of analytes, a second injection of BSA (0.5% (w/v) in 10 mM sodium acetate, pH 5.0) was used to passivate the surface. Finally, the chip was then quenched by injecting 1 M ethanolamine. All kinetic analyses were performed at room temperature in citrate buffer (20 mM sodium citrate pH 5.0, 150 mM NaCl) supplemented with 0.05% (v/v) Tween and 0.025% (v/v) dimethyl sulfoxide (DMSO) to reduce unspecific binding of the respective BR analyte. Analyte concentrations were varied in 1:3 dilution series. All experiments were done in duplicates, blank injections were used for double referencing, and DMSO calibration was performed for bulk correction.

Binding of the co-receptor SERK1 to brassinolide-bound wild-type and mutant receptors was done by amine-coupling the BRI1, BRL1, BRL2 and BRL3 ectodomains onto 4PCP WAVEchips and using the purified SERK1 ectodomain as analyte in 20 mM sodium citrate pH 5.0, 150 mM NaCl) supplemented with 0.05% (v/v) Tween and 0.025% (v/v) DMSO and brassinolide to a final concentration of 1 µM. Binding of SERK1 to BRI1 in the presence of different BRs was performed by immobilizing Avi-tagged and biotinylated BRI1 by neutravidin capturing. After chip activation, neutravidin (100 µg ml⁻¹ in 10 mM sodium acetate, pH 5.0) was injected. To passivate and quench the chip surface, a further injection of 0.5% (w/v) BSA followed by an injection of 1 M ethanolamine was performed, respectively. Biotinylated BRI1 (100 µg ml⁻¹ in citrate buffer) was then injected to the desired surface concentration (Supplementary Table 5), followed by an injection of biotinylated 0.5% (w/v) BSA to passivate the surface. All the binding assays between BRI1 and SERK1 were done at room temperature in citrate buffer supplemented with 0.05% Tween and the respective BR at a final concentration of 1 µM. SERK1 injections were done in 1:3 dilution series. Immobilized receptor densities and analyte concentrations for all experiments are reported in Supplementary Table 5.

Data analysis was performed with the Creoptix WAVEcontrol software (version 4.7.2), and data were fitted using one-to-one binding models or mass transport model, using bulk correction. Plotting and data representation were performed using R (<https://www.r-project.org>, version 4.1.2).

Crystallization and data collection

BRL3 (in 20 mM citric acid pH 5.0, 250 mM NaCl) at a concentration of ~1 mg ml⁻¹ was incubated with the respective BR (10 mM stock solution in 100% (v/v) DMSO) to a final concentration of 50 µM for 4 h at room temperature and then concentrated to ~6–7 mg ml⁻¹ using a Amicon Ultra concentrator (Merck Millipore) with a molecular weight cut-off of 30 kDa. Triclinic crystals developed in hanging drops composed of 1.0 µl of protein solution and 1.0 µl of crystallization buffer (23–25% (w/v) PEG 3,350, 0.2 M Li₂SO₄, 0.1 M citric acid pH 4.0) suspended over 1.0 ml of the latter as reservoir solution. Crystals were cryoprotected by serial transfer into crystallization buffer containing ethylene glycol to a final concentration of 25% (v/v) and snap frozen in liquid N₂. BRI1 (in 20 mM citric acid pH 5.0, 250 mM NaCl)–BR (at a final concentration of 50 µM) complex crystals developed in the same crystallization condition. Crystals of apo BRL2 were obtained using micro-seeding protocols in hanging drops containing 1.0 µl of protein solution (BRL2 at 6 mg ml⁻¹ in 20 mM citric acid pH 5.0, 250 mM NaCl) and 1.0 µl of crystallization buffer (23% (w/v) PEG 3,350, 0.2 M MgCl₂·6H₂O, 0.1 M Tris pH 9.0) over 1 ml of reservoir solution. Tetragonal crystals were cryoprotected by

serial transfer in crystallization buffer supplemented with 20% (v/v) glycerol. A second, monoclinic crystal form for BRL2 developed in 22% (w/v) PEG 3,350, 0.2 M Na citrate, 0.1 M Bis-Tris pH 7.0 and in the presence of 50 μ M brassinolide. Datasets were collected at beam line X06DA of the Swiss Light Source, Villigen, Switzerland; the monoclinic BRL2 dataset was collected at beam line ID30B of the European Synchrotron Radiation Facility, Grenoble, France (Supplementary Table 1). Data processing and scaling was done with XDS⁶⁷ (version June 2024).

Crystallographic structure solution and refinement

The structures of the BRL3–brassinolide complex and of apo BRL2 were solved by molecular replacement in PHASER (version 2.8.3)⁶⁸, with BRI1 ectodomain structure³⁶ (PDB ID, 3RJ0) as search model. For BRL3, the solution revealed a dimer in the asymmetric unit, closely resembling the previously reported BRL1 dimer³¹. The BRL2 structure was resolved at 2.6 Å resolution in space group $P4_12_12_1$, containing a single molecule per asymmetric unit, and in space group $C2$ with two molecules per asymmetric unit, respectively (Supplementary Table 1). Crystals of the BRI1–BR complex shared the same crystal form as the previously described apo BRI1 (ref. 26). Restraints for the BR ligand were generated from SMILES strings using phenix.elbow (version 1.21)⁶⁹ or JLigand (version 3.21)⁷⁰. Ligands were fit manually into $mF_o - DF_c$ omit maps, and ligand occupancies were set to 1. All structures were completed through iterative cycles of manual model building in COOT (version 0.9.8)⁷¹ and restrained TLS refinement in phenix.refine (version 1.21)⁷². Analysis of the refined structures with phenix.molprobity (version 4.5.2)⁷³ revealed good to excellent stereochemistry. Final omit maps of the different BR ligands were generated with phenix.polder (version 1.21)⁷⁴. Data collection and refinement statistics are provided in Supplementary Table 1. Structural figures were prepared with ChimeraX (version 1.10)⁷⁵, and chemical structures were drawn with PubChem sketcher (version 2.4)⁷⁶.

Plant material

A. thaliana ecotype Col-0, transfer DNA insertion mutants *bsk3-1* (SALK_096500C)⁷⁷, *bri1* null (GABI_134E10)⁴⁵ and *bri2-1* (SALK_016024)⁵⁵ were obtained from the Nottingham *Arabidopsis* Stock Center, alongside the previously reported *BRI1prom::BRI1*-mCitrine / *bri1* and *BRI1prom::BRI1^{mut1}*-mCitrine / *bri1³⁶* and *BRI1prom::oBIR3-iBRI1*-mCitrine / *bri1³²* transgenic lines. Genotyping primers for the mutants are listed in Supplementary Table 2.

Molecular cloning and generation of transgenic lines

To generate BRL2 expression reporter lines, a 3.6 kb fragment upstream of the BRL2 ORF was cloned into the entry vector pUC57-L4-EcoRV-XbaI-BamHI_R1 via Gibson assembly to produce L4-BRL2prom-R1 (ref. 78). Multi-site Gateway cloning (Thermo Fisher Scientific) with Gateway LR reaction (11791020; Thermo Fisher Scientific) was used to assemble the fragments L4-BRL2prom-R1, L1-NLS-3xmVenus-L2 (ref. 79) and R2-tHSP18.2-L3 (ref. 80) into the destination vector pB7m34GW (ref. 81) to generate *BRL2prom::NLS-3xmVenus*. The coding sequence of BRL2 was introduced into *pEN-L1(MCS)-L2* (ref. 78) by Gibson assembly and subsequently combined with L4-BRL2prom-R1 and mCitrine-pDONR P2R P3 in destination vector pB7m34GW (ref. 81) to produce the translational reporter construct *BRL2prom::BRL2-mCitrine*. The BRL2 chimera (*BRL2prom::oBIR3-iBRL2-mCitrine*) were generated using a construct in which the N-terminal part of AtBIR3 (residues 1–246, containing the native signal peptide, extracellular domain and transmembrane helix) and the C-terminal region of BRL2 (residues 779–1,143, comprising the juxtamembrane and kinase domains) were joined using two-fragment Gibson assembly, as previously described³². For the BRI1 complementary assays, the BRI1 coding sequence lacking a stop codon was cloned into entry vector pDONR221 (ref. 36) using the Gateway BP reaction (11789020; Thermo Fisher Scientific). The single

point mutations were introduced by the site-directed mutagenesis by overlap extension⁸² on the entry vector. A 1,690 bp upstream fragment of BRI1 (*BRI1prom*) and a mCitrine coding sequence with the stop codon was cloned into pDONR P4-P1R and pDONR P2R-P3, respectively, as previously reported⁸². The final binary vectors *BRI1prom::BRI1mu*-mCitrine were generated by the multi-site Gateway technology (Thermo Fisher Scientific) with destination vector pB7m34GW (ref. 81). Cloning primers are listed in Supplementary Table 3.

The *bri2-2* allele was generated using a CRISPR/Cas9 (ref. 83) system⁸⁴. Two 20 bp single-guide RNA target sequences, designed using CRISPR-P 2.0 (ref. 85), were cloned into shuttle vectors pEF004/005 via mutagenesis PCR⁸⁶. These cassettes were subsequently assembled into the pBay-Bar-CRMCherry vector using Gibson assembly, and the final construct was transformed into Col-0 wild-type plants using the floral dip method⁸⁷. Primary transformants (T1) were selected on half-strength Murashige and Skoog (^{1/2}MS) medium containing 15 mg l⁻¹ phosphinothricin; Cas9-free mutant lines (T2/T3) were identified by screening for loss of the mCherry marker (Leica Stereo Microscope MSV269).

All constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and transgenic lines were generated by floral dip⁸⁷. Transformants were selected on ^{1/2}MS media containing 1% (w/v) sucrose, 0.8% (w/v) agar and 15 mg l⁻¹ phosphinothricin.

BR root growth inhibition and competition assays

For BR-mediated root growth inhibition assays^{43,44}, surface-sterilized seeds were stratified at 4 °C for 2 days and plated on ^{1/2}MS media plates containing 1% (w/v) sucrose and 0.8% (w/v) agar, supplemented with 100 nM of the indicated BR from 10 mM stock solutions prepared in 100% (v/v) DMSO (Carl Roth) or with 0.01% (v/v) DMSO as control. The previously reported *bsk3-1* T-DNA insertion mutant (SALK_096500C)⁷⁷ was used as negative control. For the BR antagonist competition assays, 100 nM brassinolide and 100, 500 or 1,000 nM of antagonist candidate were added to ^{1/2}MS plates containing 1% (w/v) sucrose and 0.8% (w/v) agar. DMSO to a final concentration of 0.02% (v/v) was used as control treatment. In all cases, plates were moved to a Percival (SE-41AR3; CLF PlantClimatics, settings: 70% humidity, 16 h light period at 22 °C with 250 μ mol m⁻² s⁻¹ light intensity and 8 h dark period at 18 °C), vertically placed for 7 days. The plates were scanned using a flat-bed scanner (Epson perfection V600 Photo, Epson). The primary root length of 30–35 individual seedlings was then determined using Fiji (version 1.54)⁸⁸.

Statistical analysis

Multiple comparisons of different treatments versus control(s) in one-way layouts were performed using a two-sided Dunnett⁸⁹ test as implemented in the R package multcomp (version 1.4-28)⁹⁰. The relationship between root length and K_d were tested using a Tukey trend test^{91,92}, that is, a multiple regression model approach using arithmetic, ordinal or logarithmic scores. We chose the linear regression model and represented the corresponding slope b (positive slopes were interpreted as antagonistic; negative slopes as agonistic) and associated P values. Data were plotted in R⁹³ (version 4.2.2); the associated raw data can be found in Supplementary Tables 6–8.

BR in vivo quantification

The extraction and quantification of endogenous BRs were carried out as previously described⁹⁴. In brief, five biological replicates, each consisting of approximately 10 mg FW of plant material, were extracted using ice-cold 60% (v/v) acetonitrile. To each sample, 25 pmol of deuterium-labelled BR internal standards (Olchemim) were added. After a 12 h incubation at 4 °C, samples were centrifuged, and the resulting supernatants were purified using 50 mg Discovery DPA-6S solid-phase extraction cartridges (Supelco). The eluates were then evaporated to dryness and reconstituted in 40 μ l of methanol.

BR quantification was performed using UHPLC–MS/MS, using the ACQUITY UPLC-Class System (Waters) and the Xevo TQ-S triple quadrupole mass spectrometer (Waters MS Technologies), using the previously reported UHPLC–MS/MS parameters^{94,95}.

RNA-seq experiments

Ten-day-old *Arabidopsis* plants grown on ^{1/2}MS media plates containing 1% (w/v) sucrose and 0.8% (w/v) agar were collected. Fifteen plants were pooled per individual biological replicate, and all samples were ground. RNA was extracted using a RNeasy Plant Mini Kit (74904; Qiagen), and total RNA concentration was estimated using a Qubit 4 Fluorometer (Q33238; Thermo Fisher Scientific) and a 2100 Bioanalyzer system (Agilent Technologies). Library preparation and sequencing (100 bp single-end with Novaseq 6000 sequencing system; Illumina) was performed at the iGE3 Genomic Platform at the Faculty of Medicine, University of Geneva, Switzerland (<https://ige3.genomics.unige.ch/>). Data analysis was conducted within the Galaxy EU platform (<https://usegalaxy.eu/>) using the following tools: quality control and adaptor trimming by MultiQC (version 1.34)⁹⁶, reads mapping to the reference genome (*Arabidopsis* TAIR10 reference genome from TAIR; <https://www.arabidopsis.org/>) by HISAT2 (version 2.1.0)⁹⁷, transcript assembly and fragments per kilobase of transcript per million mapped reads estimates by Cufflinks (version 2.2.1.2)⁹⁸, reads counting by FeatureCounts (version 2.0.8)⁹⁹ and differential gene expression analysis by DESeq2 (version 2.11.40.7)¹⁰⁰. Visualization was done using SRplot¹⁰¹ (<https://www.bioinformatics.com.cn/>), the corresponding raw data are summarized in Supplementary Table 9.

Confocal microscopy

To detect BRL2 promoter activity with *BRL2prom::NLS-3xmVenus* (in T2 generation) in the early vasculature of embryos, SCR1 Renaissance 2200 staining was performed according to the protocol^{84,102}. The embryos were dissected from the siliques in the flowering 5-week-old plants under a Leica S6D stereo microscope (Leica S6D) and incubated in an SR2200 solution containing 0.1% (v/v) SR2200, 1% (v/v) DMSO, 0.5% (v/v) Triton X-100, 5% (v/v) glycerol and 4% (v/v) paraformaldehyde in phosphate-buffered saline buffer (pH 8.0) for 5 min. Then, the embryos were washed once with H₂O and mounted in 5% (v/v) glycerol for imaging. The cell wall signal stained by SR2200 was obtained using a 405 nm laser and a detection wavelength ranging from 415 to 475 nm. To image roots, propidium iodide (PI) staining was used to label the cell wall. The primary root of 10-day-old seedlings previously grown on ^{1/2}MS plates containing 1% (w/v) sucrose and 0.8% (w/v) agar was incubated in a 10 mg l⁻¹ PI solution for 10 min. Then, it was washed three times with water and incubated in a 5% (v/v) glycerol solution as the imaging buffer. PI fluorescence was detected from 571 nm to 656 nm following a 561 nm wavelength laser excitation. The mVenus signal was detected using a laser with a 514 nm wavelength laser for excitation and detectors with an emission range between 517 and 570 nm. To detect the mCitrine signal in the roots of T2 generation *BRL1prom::BRL1^{mut}-mCitrine* complementation lines, primary root tips from 10-day-old seedlings growing on ^{1/2}MS plates containing 1% (w/v) sucrose and 0.8% (w/v) agar were sampled. The mCitrine signal was detected by a laser with 514 nm wavelength for excitation; detectors recorded emission between 517 nm and 570 nm. All imaging was performed with a LSM 780 NLO microscope (Zeiss) at the Photonic Bioimaging Center, University of Geneva, Switzerland, and data were analysed using ZEN 2.0 blue edition and Fiji⁹⁸.

Analysis of leaf vein patterning

Leaf vein patterns were analysed as described³⁵. Cotyledons from 2-week-old seedlings grown on ^{1/2}MS plates containing 1% (w/v) sucrose and 0.8% (w/v) agar were dissected and cleared in 95% ethanol, hydrated in an ethanol dilution series to water and mounted on slides

in 10% (v/v) glycerol for imaging on a Nikon binocular microscope (Nikon SMZ18).

Hypocotyl growth assay

Surface-sterilized Col-0 wild-type and *brl2-1* mutant⁵⁵ seeds were cold-stratified at 4 °C for 48 h and sown on ^{1/2}MS medium containing 0.8% (w/v) agar. The growth medium was supplemented with either 1 μM brassinazole¹⁰³ prepared from a 10 mM stock solution in 100% DMSO or 0.1% (v/v) DMSO as control. After a 1 h light treatment to promote germination, the plates were wrapped in aluminium foil and maintained in complete darkness at 22 °C for 5 days. Seedlings were then scanned using a flat-bed scanner (Epson Perfection V600 Photo scanner, Epson).

BES1 and BRL1-mCitrine immunoblotting

Arabidopsis seeds (Col-0 wild type, *brl1* (ref. 47), *brl2-1* (ref. 55) and T3 generation *BRL1prom::BRL1^{mut}-mCitrine* complementation lines) were surface-sterilized and grown on ^{1/2}MS plates containing 1% (w/v) sucrose and 0.8% (w/v) agar for 10 or 14 days, respectively. Around 250 mg of tissue samples was collected for each line in 1.5 ml tubes containing glass beads and immediately frozen with liquid nitrogen. The samples were ground with Silamat S6 (Ivolar Vivodent) and then mixed with extraction buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1× cComplete EDTA-free protease inhibitor cocktail (11873580001; Roche), 1× plant protease inhibitor cocktail (P9599; Sigma-Aldrich) and 1× PhosStop (4906845001; Roche)). After protein solubilization for 30 min on ice, cell debris was removed by centrifugation (14,000g, 2 × 15 min, 4 °C), and the supernatant of each sample was transferred to a fresh tube. Protein concentration was determined using Pierce BCA Protein Assay Kit (23225; Thermo Fisher Scientific). For BRL1-mCitrine fusion protein detection, an anti-GFP antibody coupled to horseradish peroxidase (anti-GFP-HRP, 130-091-833; Miltenyi Biotec) at a dilution of 1:5,000 was applied. Alternatively, BRL1 was detected using a primary BRL1 antibody¹⁰⁴ (1:5,000 dilution) and a secondary anti-rabbit IgG-peroxidase antibody produced in goat (1:10,000 dilution, A6154; Sigma-Aldrich). BES1 dephosphorylation levels were estimated using a primary anti-BES1 antibody⁴¹ at a dilution of 1:2,000 and a secondary anti-rabbit IgG-peroxidase antibody produced in goat (1:10,000 dilution, A6154; Sigma-Aldrich). SuperSignal West Pico PLUS Chemiluminescent Substrate (34577; Thermo Fisher Scientific) was used for chemiluminescence detection on an Amersham Imager 680 (GE). ReadyBlue Protein Gel Stain (RSB-1L; Sigma-Aldrich) was used for the total protein input detection.

Analytical size-exclusion chromatography

The oligomeric state of recombinantly expressed and purified wild-type and point-mutant BRL1 ectodomains was characterized using analytical size-exclusion chromatography; 500 μl sample at 1.0 mg ml⁻¹ was loaded onto a Superdex 200 Increase 10/300 GL column (Cytiva) pre-equilibrated in 20 mM citrate buffer (pH 5.0), with 150 mM NaCl. Protein elution at a flow rate of 0.7 ml min⁻¹ was monitored by UV absorption at 280 nm.

Quantitative real-time PCR

Expression of the BR marker genes CPD and DWF4 was estimated by qRT-PCR, using -30 individual 8-day-old seedlings per each T3 generation transgenic line. Samples were homogenized by Silamat S6 (Ivolar Vivodent), and RNA was extracted using a RNeasy Plant Mini Kit (74904; Qiagen). Subsequently, cDNA synthesis was performed with the SuperScript II Reverse Transcriptase (18064014; Invitrogen), following the manufacturer's protocol. Quantitative real-time PCR was conducted on a QuantStudio 5 thermo-cycler (Applied Biosystems) using SYBR Select Master Mix for CFX (4472942; Applied Biosystems). The relative expression (*n* = 3) was quantified with the 2^{-ΔΔCt} method.

ACT2 was used as the internal reference gene. qPCR primers are included in Supplementary Table 4.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Raw RNA-seq reads have been deposited with the NCBI Sequence Read Archive with identifier [PRJNA1299306](https://www.ncbi.nlm.nih.gov/sra/PRJNA1299306) (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1299306>). BRI1, BRL2 and BRL3 complex structures have been deposited with the Protein Data Bank (<http://www.rcsb.org>) with accession numbers **9S80** (BRL3, brassinolide), **9S87** (BRI1, castasterone), **9S85** (BRI1, typhasterol), **9S8V** (BRI1, 24-epibrassinolide), **9S8Z** (BRI1, 28-homobrassinolide), **9S90** (BRL3, castasterone), **9S96** (BRL3, typhasterol), **9S9A** (BRL3, 6-deoxycastasterone), **9S9C** (apo BRL2, tetragonal crystal form) and **9U0G** (apo BRL2, monoclinic crystal form). Source data are provided with this paper.

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Author contributions

A.C. and M.H. conceived the project. A.C. expressed and purified proteins, performed GCI assays and crystallized proteins. H.C. generated transgenic lines with the help of L.B., performed plant phenotyping and BR response assays, confocal microscopy, transcriptomics and western blotting. M.K. and K.F. synthesized BRs. U.H. crystallized the BRL3–brassinolide complex. J.O. quantified endogenous BR levels. A.C., U.H. and M.H. solved and refined structures. A.C., H.C., M.K., J.O., L.A.H., M.S. and M.H. analysed data. A.C. and M.H. wrote the manuscript, with input from the other authors. M.H. and M.S. acquired funding, and M.H. supervised the project.

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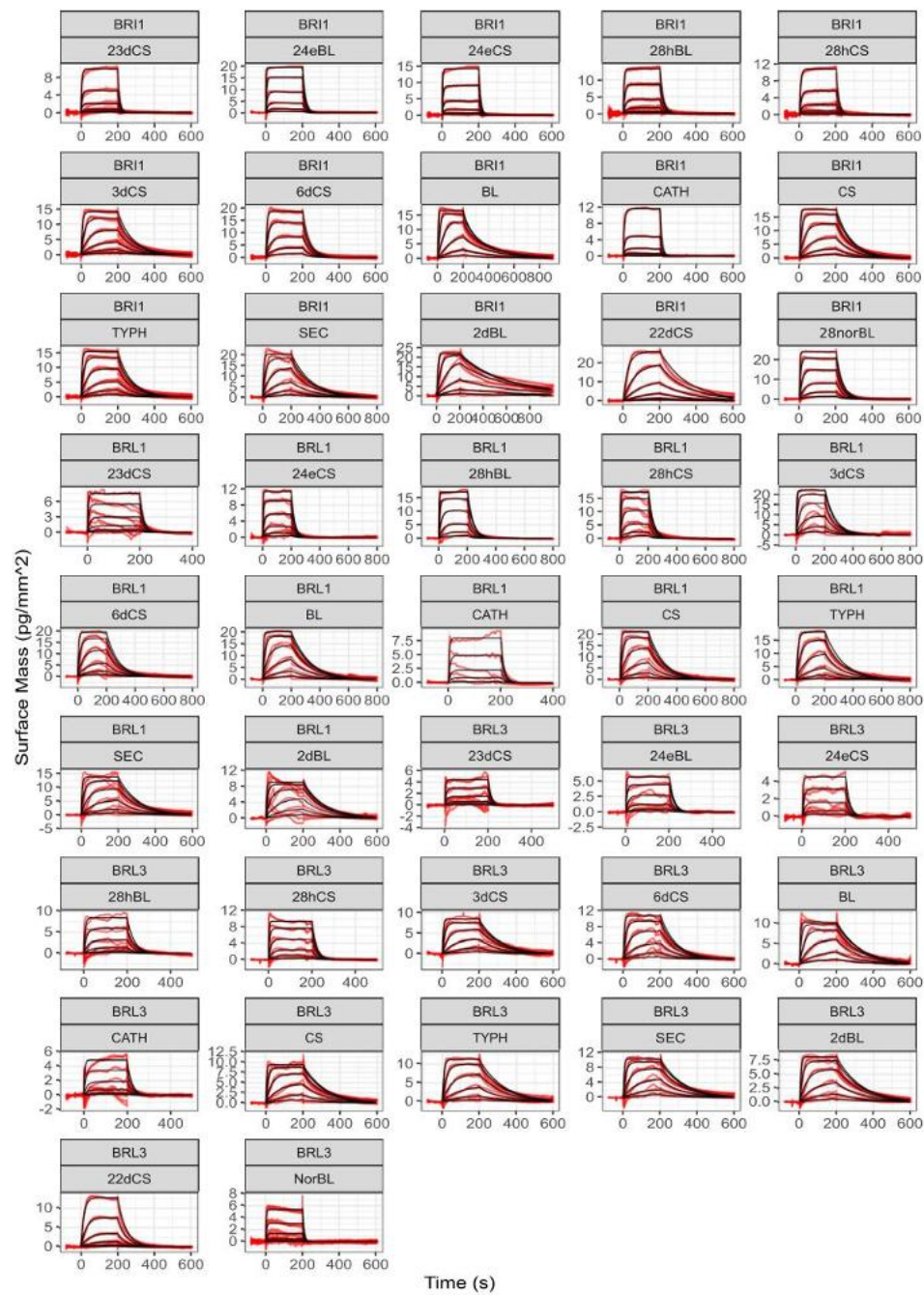
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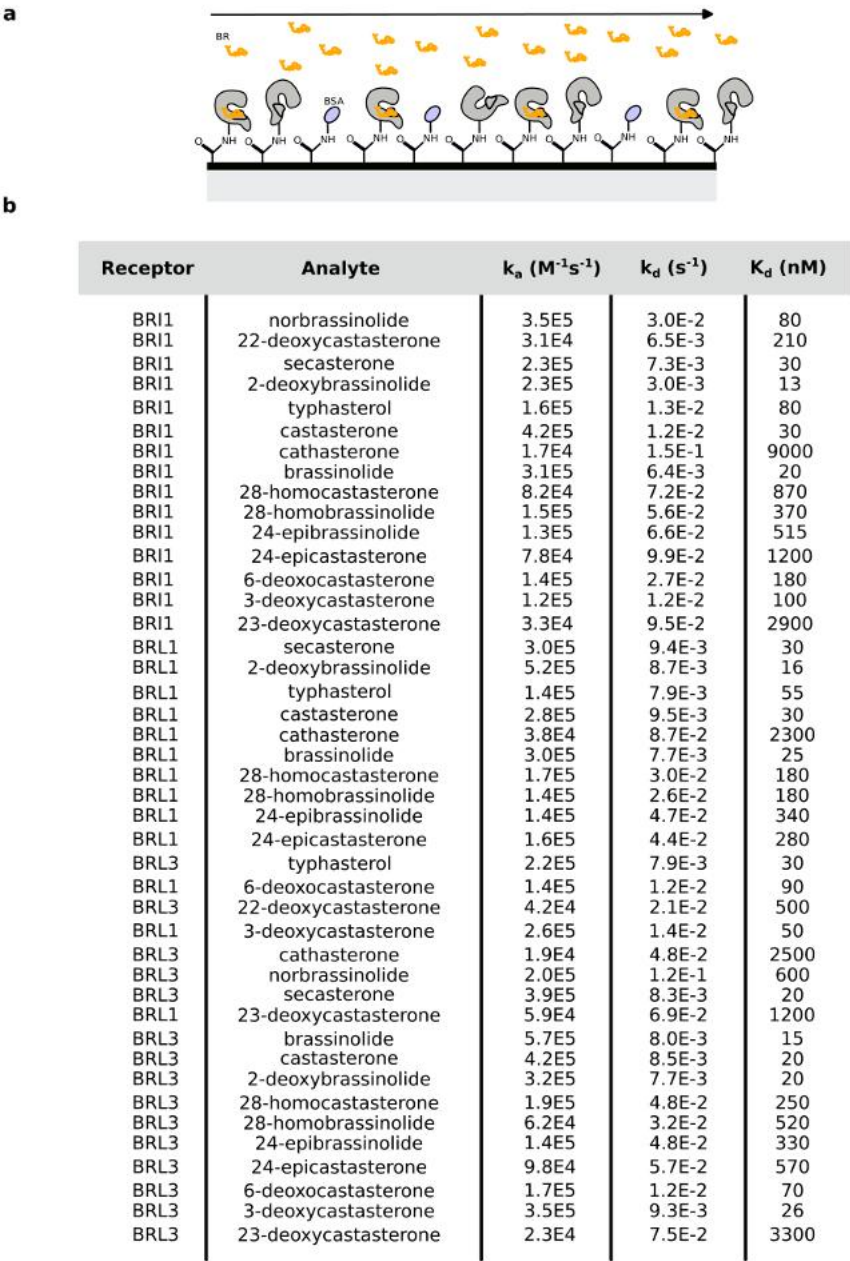
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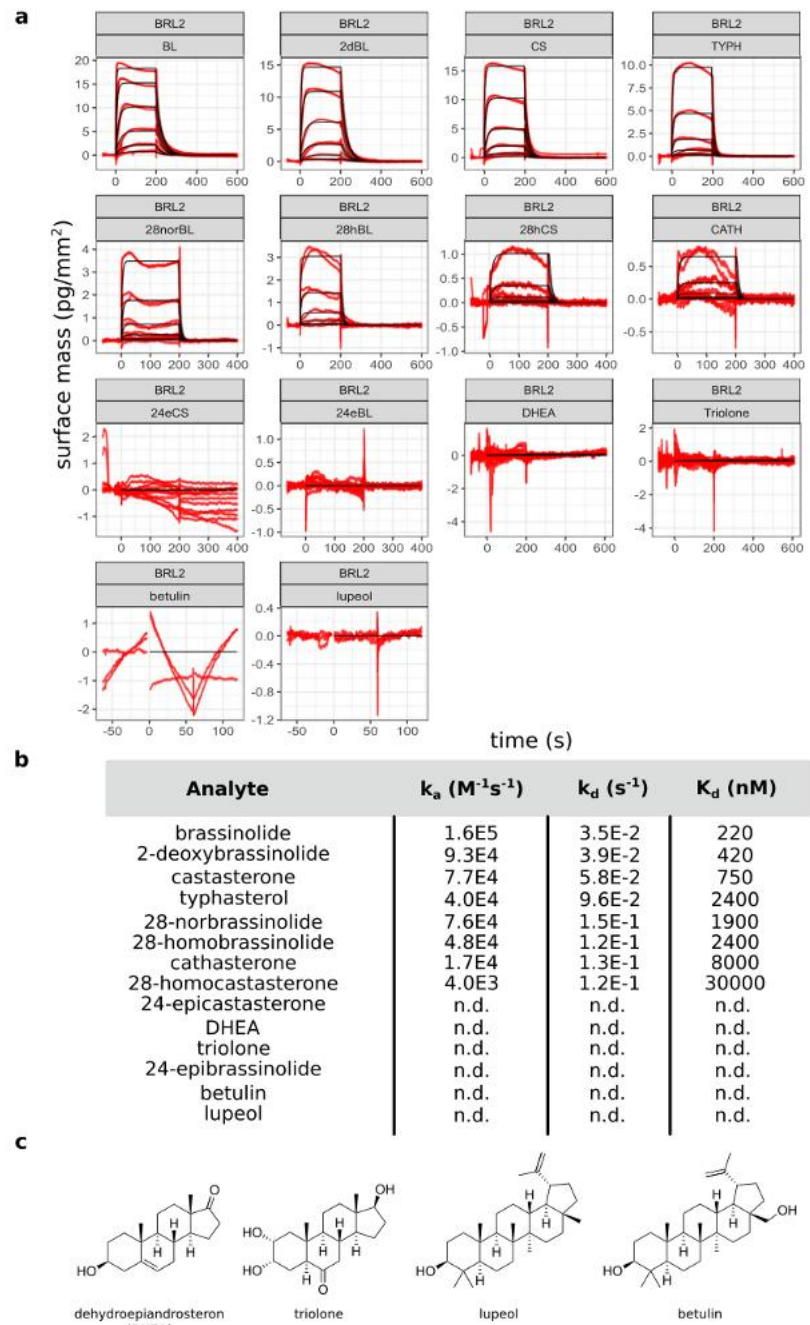
Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Complete set of GCI sensorgrams for the experiments described in Fig. 2a, c, d. Grating Coupling Interferometry (GCI)-derived binding kinetics for BRI1, BRL1, and BRL3 in response to different BRs. Raw sensorgrams are shown with experimental traces in red, and global fits as black lines. CATH: cathasterone, 23dCS: 23-deoxycastasterone, 24eCS: 24-epicastasterone, 28hCS:

28-homocastasterone, 24eBL: 24-epibrassinolide, 28hBL: 28-homobrassinolide, 22dCS: 22-deoxycastasterone, 6dCS: 6-deoxocastasterone, NorBL: 28-norbrassinolide, 3dCS: 3-deoxycastasterone, TYPH: typhasterol, CS: castasterone, SEC: secasterone, 2dBL: 2-deoxybrassinolide, BL: brassinolide.



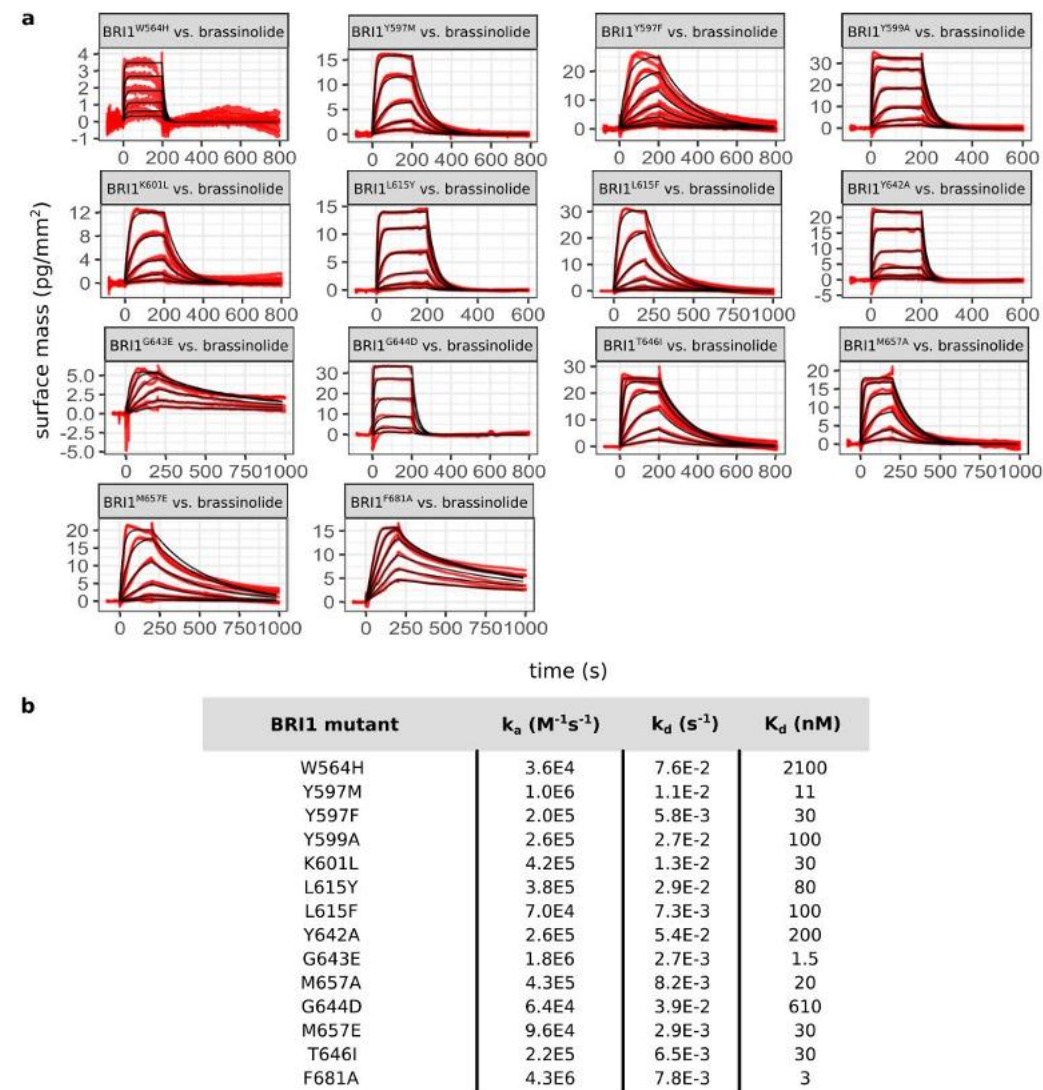
Extended Data Fig. 2 | Complete set of GCI kinetic parameters for the experiments described in Fig. 2a, c, d. a, Schematic illustration of the Grating Coupling Interferometry (GCI) setup used to screen receptor - brassinosteroid interactions. **b**, Kinetic parameters obtained for BR11, BRL1, and BRL3 in response to the 15 BRs analyzed in this study.



Extended Data Fig. 3 | See next page for caption.

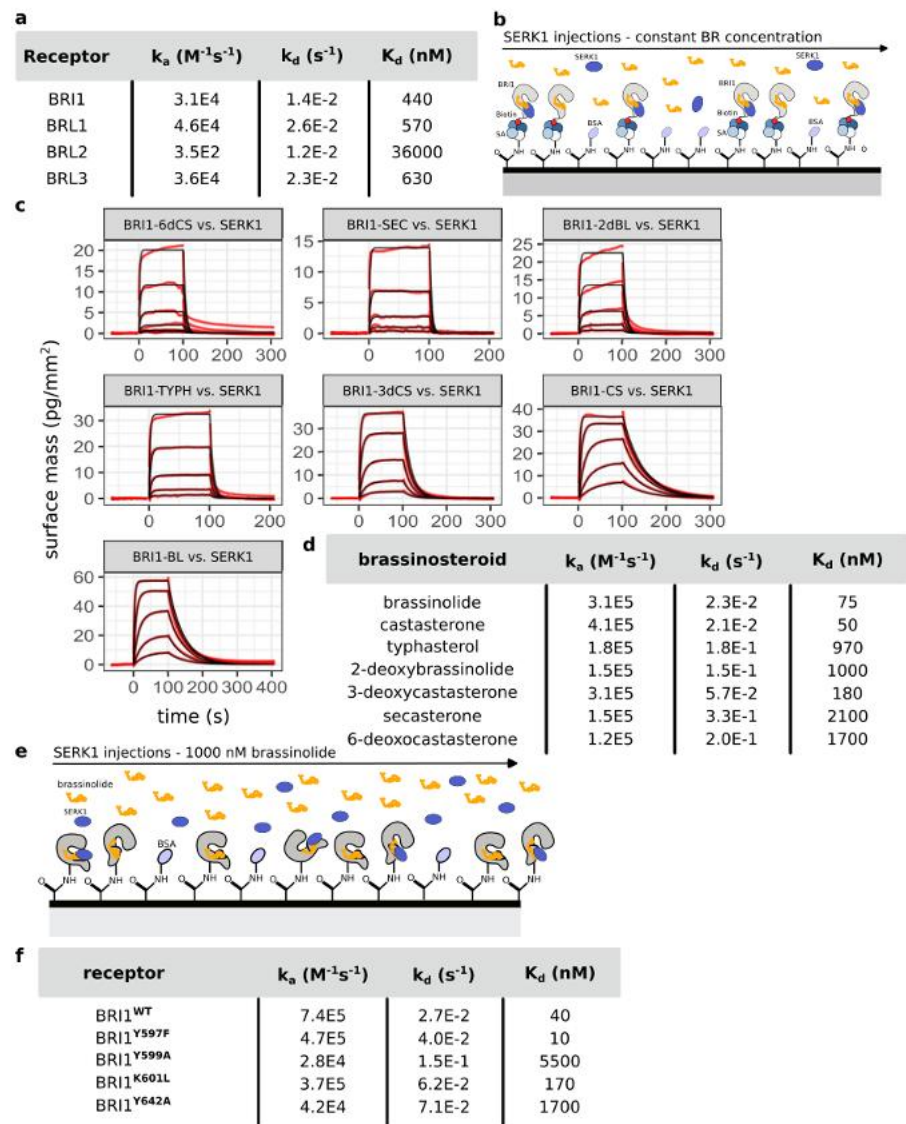
Extended Data Fig. 3 | Complete set of GCI sensorgrams and derived kinetic parameters for the experiments described in Fig. 4e. a, Grating Coupling Interferometry (GCI)-derived binding kinetics for BRL2 in response to different BRs and other plant steroids. Raw sensorgrams are shown with experimental traces in red ($n=2$), and global fits as black lines (n.d., no detectable binding). BL: brassinolide, 2dBL: 2-deoxybrassinolide, CS: castasterone, TYPH:

typhasterol, 28norBL: 28-norbrassinolide, 28hBL: 28-homobrassinolide, 28hCS: 28-homocastasterone, CATH: cathasterone, 24eCS: 24-epicastasterone, 24eBL: 24-epibrassinolide, DHEA: dehydroepiandrosterone. **b,** Kinetic parameters obtained for BRL2 in response to the different BR and non-BR ligand candidates analyzed in this study. **c,** Chemical structures of dehydroepiandrosterone (DHEA), triolone, lupeol and betulin.



Extended Data Fig. 4 | Complete set of GCI sensorgrams and derived kinetic parameters for the experiments described in Fig. 5b. c. a. Grating Coupling Interferometry (GCI)-derived binding kinetics for different BRI1 ectodomains carrying point mutations in the BR-binding pocket vs. brassinolide.

Raw sensorgrams are shown with experimental traces in red, and global fits as black lines. **b.** Table summaries of kinetic parameters obtained for the experiments shown in **a.**



Extended Data Fig. 5 | Complete set of GCI sensorgrams and derived kinetic parameters for experiments shown in Fig. 6b–f. a. Table summaries of kinetic parameters obtained for the experiments shown in Fig. 6b. **b.** Schematic overview of the GCI receptor capturing and SERK1 binding assay. For Avi-tag based coupling, streptavidin (SA, shown as a tetramer) was immobilized using the amine-coupling method on the GCI chip (black line). Next, the biotinylated avi-tagged BR receptor ectodomain was captured by streptavidin, followed by passivation and quenching of the surface with BSA (in light blue). Then, in the presence of a constant concentration of brassinolide in the analyte buffer (in yellow), SERK1 (in dark blue) association and dissociation at different protein concentrations was performed. **c.** Grating Coupling Interferometry (GCI)-derived binding kinetics for the avi-tagged BRI1 ectodomain vs. different antagonist candidate ligands including 6dCS: 6-deoxocastasterone,

SEC: secastasterone, TYPH: typhasterol, 3dCS: 3-deoxycastasterone, CS: castasterone, BL: brassinolide. Raw sensorgrams are shown with experimental traces in red, and global fits as black lines. **d.** Table summaries of kinetic parameters obtained for the experiments shown in Fig. 6c, d. **e.** Schematic overview of the GCI receptor capturing and SERK1 binding assay to analyze the effect of BR binding site missense mutations in BRI1 for SERK1 co-receptor binding (related to Fig. 6f). BRI1^{WT}, BRI1^{Y597F}, BRI1^{Y599A}, BRI1^{K601L} and BRI1^{Y642A} were immobilized by amine-coupling on the GCI chip (black line). SERK1 binding to the receptors (in gray) was then quantified in the presence of a constant, high concentration of brassinolide (in yellow) in the running buffer and by injecting the co-receptor at different concentrations (in blue). **f.** Table summaries of kinetic parameters obtained for the experiments shown in Fig. 6f.

Příloha 9

Local Brassinosteroid Biosynthesis Enables Optimal Root Growth

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Local brassinosteroid biosynthesis enables optimal root growth

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Brassinosteroid (BR) hormones are indispensable for root growth and control both cell division and cell elongation through the establishment of an increasing signalling gradient along the longitudinal root axis. Because of their limited mobility, the importance of BR distribution in achieving a signalling maximum is largely overlooked. Expression pattern analysis of all known BR biosynthetic enzymes revealed that not all cells in the *Arabidopsis thaliana* root possess full biosynthetic machinery, and that completion of biosynthesis relies on cell-to-cell movement of hormone precursors. We demonstrate that BR biosynthesis is largely restricted to the root elongation zone, where it overlaps with BR signalling maxima. Moreover, optimal root growth requires hormone concentrations to be low in the meristem and high in the root elongation zone, attributable to increased biosynthesis. Our finding that spatiotemporal regulation of hormone synthesis results in local hormone accumulation provides a paradigm for hormone-driven organ growth in the absence of long-distance hormone transport in plants.

Brassinosteroids (BRs) are a group of steroidal phytohormones essential for plant growth and development¹. Mutants deficient in or insensitive to BRs exhibit a range of growth defects, including dwarfism^{2,3}, photomorphogenesis in the dark⁴, altered stomatal development^{5,6} and reduced male fertility⁷. Because of their importance in plant development, BRs have attracted much research attention in the past two decades and, therefore, BR signalling is one of the best-characterized signal transduction pathways in *Arabidopsis thaliana*⁸. The BR signalling cascade starts with a direct binding of a BR ligand to the extracellular domain of the plasma membrane-localized receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1), or its close homologues BRI1-LIKE1 (BRL1) and BRL3 (refs. ^{9,10}). Subsequently, transphosphorylation events between the kinase domains of BRI1 and its co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family occur and fully activate the receptor complex¹¹. Further phosphorylation–dephosphorylation steps lead to dephosphorylation of the key transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and BR INSENSITIVE EMS SUPPRESSOR1 (BES1)/BZR2 and their translocation into the nucleus¹², where they either stimulate or repress thousands of specific genes required for optimal BR responses¹³.

Brassinosteroids are derived from campesterol, which is converted to brassinolide (BL), the most potent and final product of the BR biosynthetic pathway, through a series of reactions including reduction, hydroxylation, epimerization and oxidation¹. Extensive research by means of feeding experiments with labelled intermediates¹⁴ has established a biosynthetic grid with multiple possibilities by which BL could be synthesized. Nevertheless, evidence on the enzymatic substrate specificity and intermediate content analysis of the mutants^{15,16} made it possible to propose a succession of biosynthetic steps and positioning of the enzymes in a somewhat linear order (Fig. 1a)¹⁷. With the exception of DE-ETIOLATED2

(DET2), which is a steroid 5 α -reductase¹⁸, all known enzymes of the BR biosynthetic pathway belong to the family of cytochrome P450 (CYP450) mono-oxygenases¹.

Hormone levels at any given site are determined by the relative rates of de novo synthesis and catabolism, conjugate synthesis and hydrolysis and, finally, transport from the site of synthesis to the site of action¹⁹. Studies on BR transport in different plant species have demonstrated the absence of hormonal organ-to-organ mobility²⁰, thus reinforcing the importance of the regulation of biosynthesis and catabolism rates through transcriptional feedback loops¹. Increased transcript levels of BR biosynthetic genes are usually correlated with young, developing tissues and higher BR content^{21–23}. Nonetheless, a high-resolution spatiotemporal map of BR biosynthesis is still lacking.

The *Arabidopsis* root has been used extensively as a model organ for BR signalling²⁴ and plant organ growth studies²⁵. The apically positioned meristem is a region of actively dividing cells that gradually, through longitudinal expansion, leave the meristem region and enter the division-free elongation zone where they reach their mature size. Meristem size and, thus, the number of growing cells are critical parameters for optimal root growth²⁶. Because BRs regulate cell division²⁷ and control cell elongation²⁸ they are of paramount importance in determination of the root meristem size. Elevated BR signalling results in premature elongation and differentiation of meristematic cells and has a negative effect on meristem size and root growth²⁹, possibly due to the existing BR signalling gradient along the longitudinal axis of root tips, with a peak in the elongation zone³⁰. However, the factors that determine the BR signalling gradient in *Arabidopsis* root remain elusive.

Here, through localization studies, we show that not every root cell type produces all BR biosynthetic enzymes and that completion of the BR biosynthetic pathway probably relies on cell-to-cell movement of hormone precursors. Moreover, most BR biosynthetic

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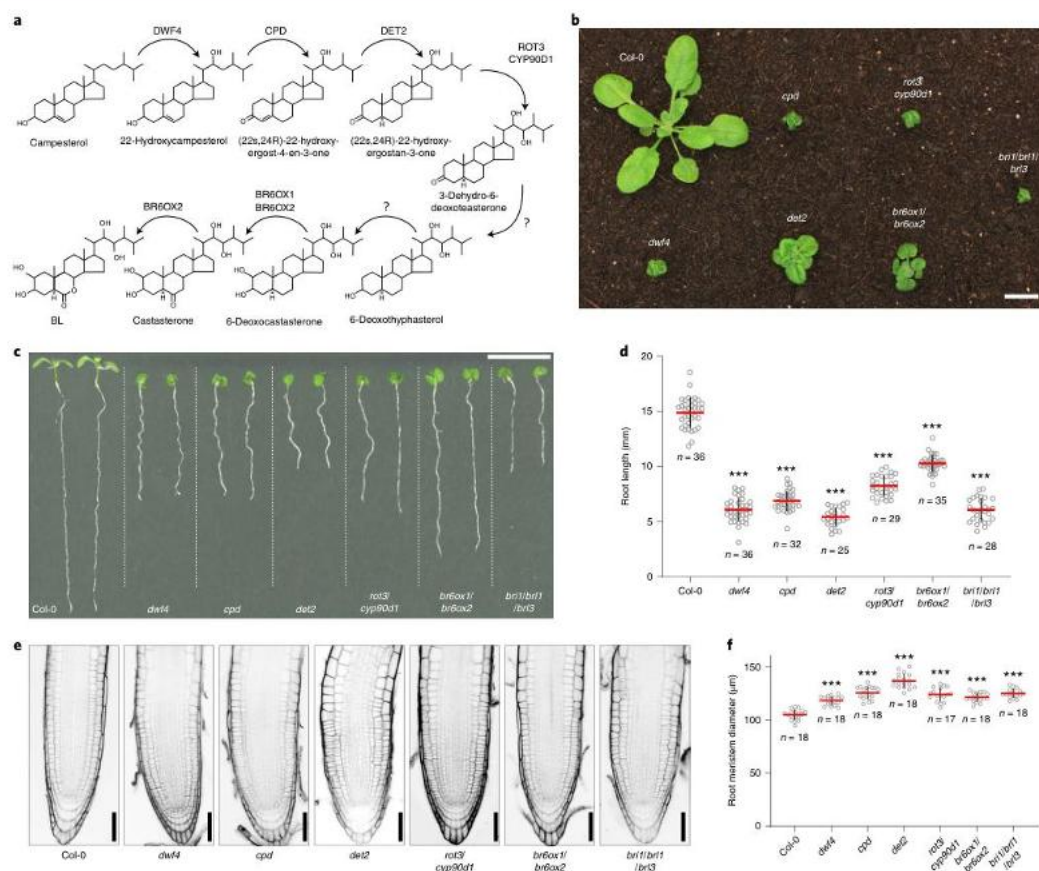


Fig. 1 | BR biosynthetic mutants are dwarfs with reduced root growth. a, Simplified BR biosynthetic pathway with all known enzymes and their presumed positions within the pathway. **b**, Dwarf phenotypes with compact rosettes of 3-week-old *dwf4*, *cpd*, *det2*, *rot3/cyp90d1* and *br6ox1/br6ox2* mutants defective in BR biosynthetic enzymes and the *brl1/brl1/brl3* triple mutant defective in BR signalling. **c**, Nine-day-old BR biosynthetic and signalling mutants with roots shorter than those of the wild-type (Col-0) control. **d**, Quantification of the root length of BR biosynthetic and signalling mutants shown in **c**. Six-day-old plants were transferred to fresh medium and root growth was measured after 3 days. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. *n* = number of roots analysed. **e**, Increased root diameter of BR biosynthetic and signalling mutants. Six-day-old roots were stained with propidium iodide. **f**, Quantification of root meristem diameter shown in **e**. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. *n* = number of roots analysed. **d,f**, Significant differences between mutants and the Col-0 control were determined by one-way ANOVA and Dunnett's multiple comparison tests. ****P* < 0.001. The *P* value versus the Col-0 control for *dwf4* is <0.0001, *cpd* <0.0001, *det2* <0.0001, *rot3/cyp90d1* <0.0001, *br6ox1/br6ox2* <0.0001 and *brl1/brl1/brl3* <0.0001. All experiments were repeated independently three times, with similar results. Scale bars, 1 cm (**b,c**) and 50 μ m (**e**).

enzymes reach their expression maxima in the root elongation zone to ensure the high hormone levels required for BR signalling maintenance. Thus, local BR biosynthesis plays a crucial role in the formation of the signalling gradient along the longitudinal root axis, enabling optimal meristem activity and timely cell elongation.

Results

BR biosynthetic enzymes are not expressed in every cell type of *Arabidopsis* root. *Arabidopsis* mutants defective in BR biosynthesis and signalling display severe phenotypic defects that include a compact rosette with dark-green, curled leaves and short roots of

increased diameter (Fig. 1b–f). To gain insight into the importance of BR synthesis and distribution for the growth and morphology of *Arabidopsis* root, we carried out localization studies for all known enzymes in the pathway (Fig. 2a). Genomic fragments of each BR biosynthetic gene, including at least 2,000-base-pair (bp) promoter regions upstream from the start codon, were fused with the coding sequence of green fluorescent protein (GFP) and introduced into the corresponding mutant backgrounds. All tested protein fusions were functional, because they complemented the mutant phenotypes and their expression patterns were identical in multiple, independent transgenic lines (Extended Data Fig. 1a,b). First, we examined the

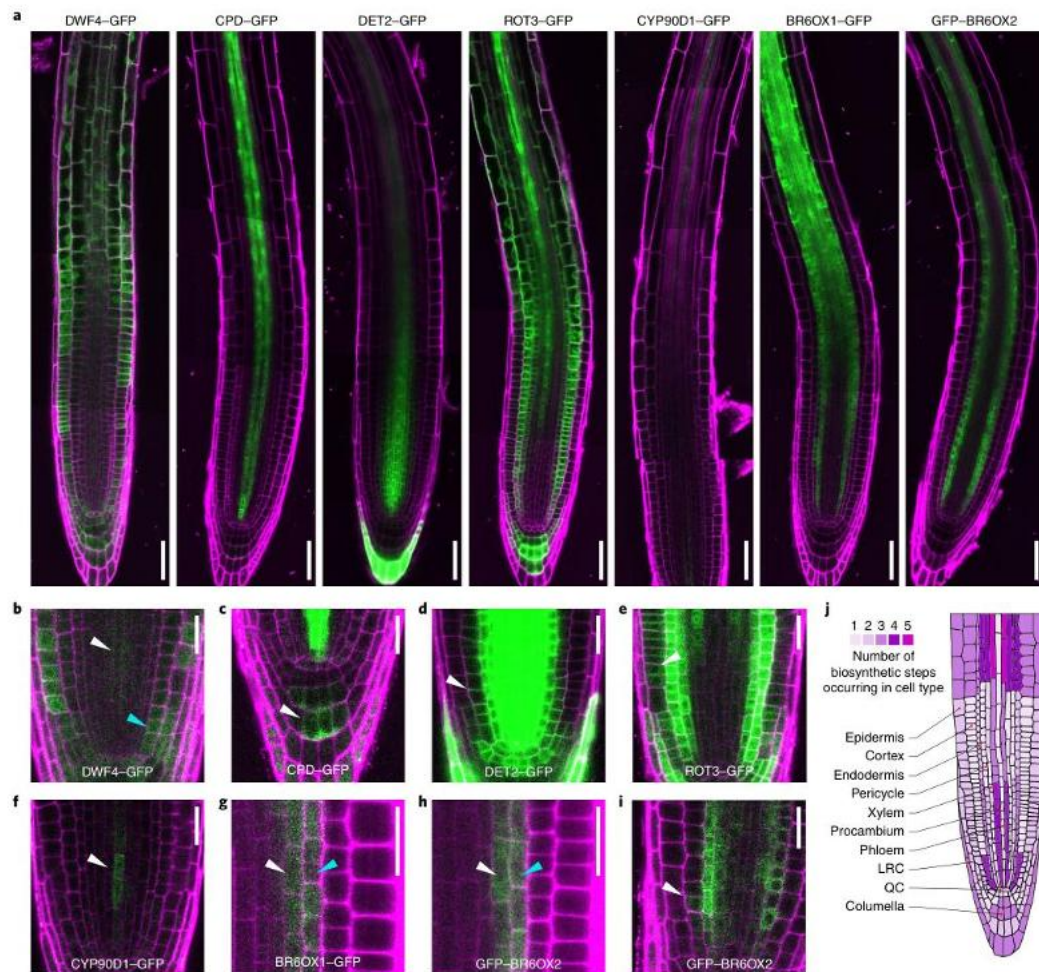


Fig. 2 | Expression patterns of BR biosynthetic enzymes in the *Arabidopsis* root. a, Roots tips of 6-day-old seedlings expressing DWF4-GFP, CPD-GFP, DET2-GFP, ROT3-GFP, CYP90D1-GFP, BR6OX1-GFP and GFP-BR6OX2 under the control of their native promoters and expressed in the respective mutants. **b**, Weak DWF4-GFP signal in procambial (white arrowhead) and cortical cells (blue arrowhead). **c**, Limited expression of CPD-GFP in the central columella region (white arrowhead). **d**, Extended DET2-GFP expression at the very tip of the root, including young cortical cells (white arrowhead). **e**, Occurrence of ROT3-GFP signal in the epidermis (white arrowhead) in addition to dominant expression in the cortex. **f**, Weak CYP90D1-GFP expression in procambial cells in the root tip near the quiescent centre (white arrowhead). **g,h**, BR6OX1-GFP (**g**) and GFP-BR6OX2 (**h**) expression limited to the pericycle (white arrowheads) and endodermis (blue arrowheads). **i**, GFP-BR6OX2 expression in young cortical cells. **b–h**, GFP signal brightness and contrast were adjusted to enhance visibility in the cell types of interest. All transgenic lines were imaged in >3 independent experiments. Representative images are shown. **j**, Schematic representation of the *Arabidopsis* root tip with colour coding denoting the number of BR biosynthetic steps occurring in individual cell types. Only procambial cells in the elongation zone of the root express all BR biosynthetic enzymes. LRC, lateral root cap. Scale bars, 50 μ m (**a**) and 20 μ m (**b–i**). Roots were stained with propidium iodide.

enzyme expression patterns in the *Arabidopsis* root meristem. The expression of CPD-GFP, DET2-GFP, BR6OX1-GFP and GFP-BR6OX2 was confined to the inner tissues of the root apical meristem (Fig. 2a). Expression of CPD-GFP occurred in procambial and central columella cells (Fig. 2a,c), whereas BR6OX1-GFP and GFP-BR6OX2 were expressed mainly in the endodermis and pericycle (Fig. 2a,g,h). A weak GFP-BR6OX2 signal was observed in young

cortical cells (Fig. 2i). The expression of DWF4-GFP and DET2-GFP in the apical meristem was somewhat broader and included additional cell layers for DET2-GFP (Fig. 2a,d) and a few cell types for DWF4-GFP, with an expression maximum in the epidermis (Fig. 2a,b). Treatment with the BR biosynthesis inhibitor brassinazole (BRZ)³⁰ strongly increased DWF4-GFP expression levels, but only in the epidermis, whereas treatment with the most active BR,

BL, completely abolished the GFP signal (Extended Data Fig. 1c). The expression domain of ROT3 and CYP90D1 was more widespread and incorporated procambial, cortical and columella cells (Fig. 2a,e,f). Gene expression reporter lines in which the promoters of BR biosynthetic genes were fused to the N-terminal nuclear localization sequence (NLS)-GFP showed largely similar expression patterns (Extended Data Fig. 1d,e).

Our localization studies indicate that most cells in the *Arabidopsis* root meristem do not possess all the enzymes needed to complete the BR biosynthetic pathway (Fig. 2j), implying that intermediates have to be exchanged between cells to finalize hormone synthesis. Additionally, we demonstrate that feedback inhibition via BR signalling is a powerful mechanism for maintenance of optimal expression levels of BR biosynthetic genes, but it is not the mechanism that determines the tissue specificity of BR enzyme expression.

BRs can move over short distances in the *Arabidopsis* root. Although BRs are not transported over long distances²⁰, the spatially restricted expression of BR biosynthetic enzymes in the *Arabidopsis* root (Fig. 2a), in contrast to the broad expression domain of the BRI1 receptor²¹, implies an obligatory BR movement. To test this hypothesis we used the *CPD* gene, which has the most restricted expression domain in the root meristem (procambial cells) of all BR biosynthetic genes, and expressed it ectopically in endodermal cell files by means of the endodermis-specific *SCARECROW* (*SCR*) promoter²² (Fig. 3a). Ectopic expression of *CPD-mCHERRY* in the endodermis complemented root growth defects of the *cpd* mutant (Fig. 3b,c) and restored the normal root meristem architecture (Fig. 3d,e and Extended Data Fig. 2a,b). Restoration of BR signalling in endodermal cell files has previously been shown to be insufficient to rescue root growth defects of the *bri1* signalling mutant^{20,33}. Because the mutants *bri1* and *cpd* have a similar phenotype due to lack of BR signalling and BR hormones, respectively, our findings demonstrate that restoration of BR biosynthesis, but not of its signalling, in endodermal cell files can complement the *cpd* mutant phenotype, probably because of the mobile nature of the hormone or its intermediates.

To further test our hormone mobility hypothesis, we blocked BR movement in the root by employing the cytochrome P450

monooxygenase PHYB-4 ACTIVATION-TAGGED SUPPRESSOR1 (BAS1) that catalyses C-26 hydroxylation of BRs and inactivates them (Fig. 3f)³⁴. BAS1 is expressed in the outer tissues of the root (lateral root cap and epidermis)³⁵, and its overexpression results in typical BR-related phenotypes (Extended Data Fig. 2c,d)³⁵. To inactivate BRs that are either synthesized or pass through this centrally positioned tissue, we ectopically expressed BAS1-GFP in the endodermis in an inducible and constitutive manner (Fig. 3g and Extended Data Fig. 2j). Inducible or constitutive expression of BAS1-GFP under the *SCR* promoter hampered root growth and altered root meristem architecture in transgenic plants, in a manner typical for BR-deficient mutants (Fig. 3h,i and Extended Data Fig. 2e-h,j-n). The decreased pool of dephosphorylated BES1 in the excised roots of oestradiol-treated *pSCR-XVE:BAS1-GFP* plants provided additional proof for decreased BR signalling in these lines (Extended Data Fig. 2i).

These findings indicate that BR intermediates and, possibly, the bioactive BRs, BL and castasterone, could be exchanged between neighbouring root cells.

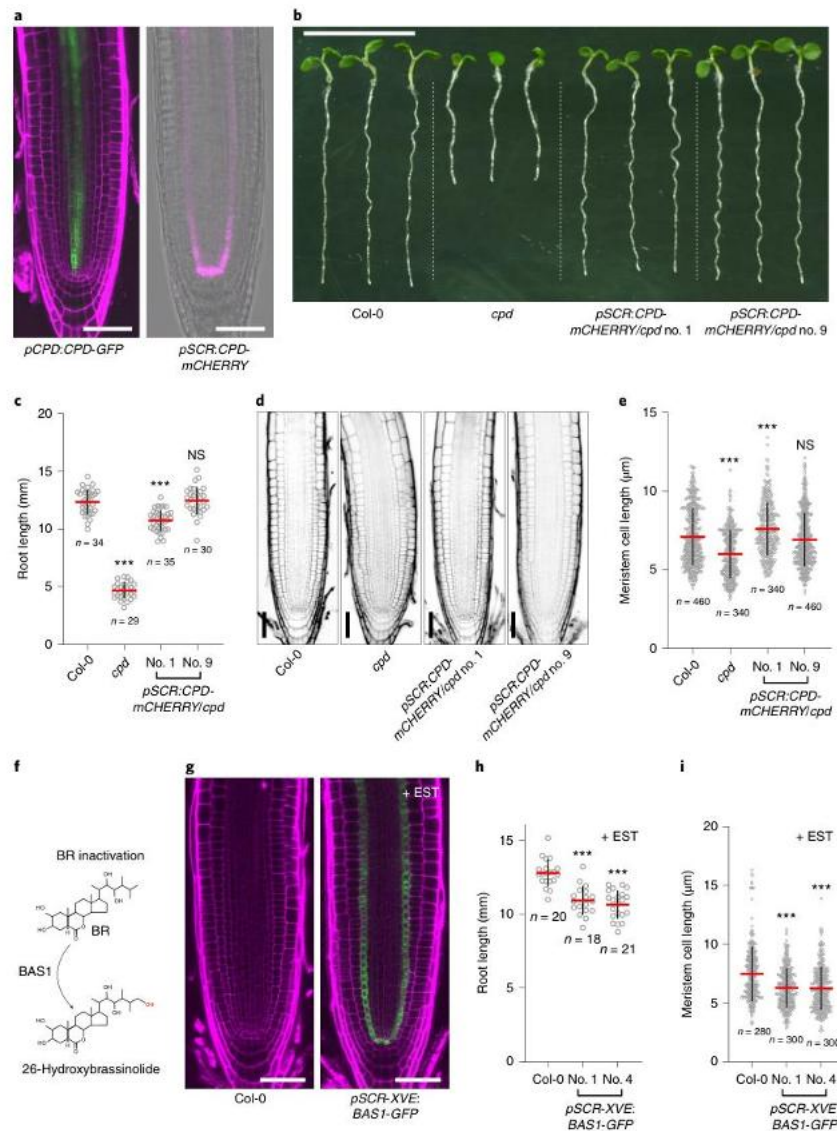
BR biosynthetic gene expression and BR signalling maxima overlap in the root elongation zone. While examining localization of BR biosynthetic enzymes, we noticed that DWF4-GFP expression expanded in the elongation zone and included all cell types (Fig. 4a, Extended Data Fig. 3a and Supplementary Video 1). Similarly, BR6OX1-GFP expression, although still very prominent in the inner tissues, increased and included epidermal and cortical cells of the elongation zone (Extended Data Fig. 3a). Finally, expression of ROT3-GFP that was mostly confined to cortical cells in the apical meristem also extended to epidermal cells and peaked in the stele of the elongation zone (Fig. 4a, Extended Data Fig. 3a and Supplementary Video 2). Besides expansion of expression domains, the majority of transgenic lines—except for DET2-GFP and GFP-BR6OX2—showed a clear increase in expression levels along the longitudinal root axis, with maxima in the elongation zone. This was very prominent in the case of *CPD-GFP* (Fig. 4a, Extended Data Fig. 3b and Supplementary Video 3). Surprisingly, when BR6OX2 was tagged with GFP at the C-terminus and expressed

Fig. 3 | BRs can move over short distances in the *Arabidopsis* root. **a**, *CPD-GFP* expression restricted to procambial cells of *Arabidopsis* root when expressed under its native promoter (left). Ectopic expression of *CPD-mCHERRY* from the endodermis-specific *SCR* promoter in the *cpd* mutant (right). **b**, Six-day-old *cpd* mutant seedlings expressing *pSCR:CPD-mCHERRY*. Two independent lines are shown. **c**, Quantification of the root length of the lines shown in **b**. Six-day-old plants were transferred to fresh medium and root growth was measured after 3 days. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. Significant differences between transgenic lines and the Col-0 control were determined by one-way ANOVA and Dunnett's multiple comparisons tests. *** P < 0.001, ** P < 0.01, * P < 0.05. The P value versus the Col-0 control for *cpd* is <0.0001, line no. 1 is <0.0001 and line no. 9 is 0.9382. The experiment was repeated independently more than three times, with similar results. **d**, Root meristem architecture of *Arabidopsis* wild-type (Col-0), *cpd* and transgenic lines expressing *pSCR:CPD-mCHERRY* in the *cpd* mutant. **e**, Quantification of cortical meristem cell length (first 20 cells of individual roots) of lines expressing *pSCR:CPD-mCHERRY* in the *cpd* mutant. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of cells analysed. Numbers of individual roots used in the experiment were 24, 17, 17 and 23 for Col-0, *cpd*, line no. 1 and line no. 9, respectively. Significant differences between transgenic lines and Col-0 were determined by one-way ANOVA and Dunnett's multiple comparison tests. *** P < 0.001. The P value versus the Col-0 control for *cpd* is <0.0001, line no. 1 is 0.0001 and line no. 9 is 0.2382. The experiment was repeated independently more than three times, with similar results. **f**, BAS1 catalysing the conversion of BRs to inactive C-26 hydroxylated forms. **g**, Root tips of 7-day-old Col-0 and *pSCR-XVE:BAS1-GFP* seedlings grown in the presence of 10 μ M β -oestradiol (EST) for 3 days. BAS1 expression was limited to the endodermal cell layer after induction. **h**, Quantification of the root length of two independent lines expressing *pSCR-XVE:BAS1-GFP*. Four-day-old plants were transferred to fresh medium containing 10 μ M β -oestradiol, and primary root length was measured after 3 days. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. Significant differences between transgenic lines and Col-0 were determined by one-way ANOVA and Dunnett's multiple comparison tests. *** P < 0.001. The P value versus Col-0 for line nos. 1 and 4 is <0.0001 and <0.0001, respectively. The experiment was repeated independently more than three times, with similar results. **i**, Quantification of the cortical meristem cell length (first 20 cells of individual roots) of lines expressing *pSCR-XVE:BAS1-GFP*. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of cells analysed. Numbers of individual roots used in the experiment were 14, 15 and 15 for Col-0, line no. 1 and line no. 4, respectively. Significant differences between transgenic lines and Col-0 were determined by one-way ANOVA and Dunnett's multiple comparison tests. *** P < 0.001. The P value versus Col-0 for line nos. 1 and 4 is <0.0001 and <0.0001, respectively. The experiment was repeated independently more than three times, with similar results. Roots were stained with propidium iodide. NS, not significant. Scale bars, 50 μ m (**a,d,g**) and 1 cm (**b**).

under the transcriptional control of its native promoter, it also showed increased expression in the root elongation zone (Extended Data Fig. 3c). Interestingly, the expression of the BR receptor BRI1 did not increase similarly in the root elongation zone (Extended Data Fig. 3b). These findings are consistent with publicly available expression datasets³⁰ (Extended Data Fig. 3d) and, together, hint at enhanced hormone production in the root elongation zone.

Nuclear accumulation of fluorescently tagged BES1 and BZR1 transcription factors has been used as a readout for active BR signalling²⁹. In accordance with the role of BRs in cell elongation, BR signalling levels show a gradient along the longitudinal root axis with

a maximum in the transition-elongation zone²⁹, but the regulatory mechanisms establishing this gradient are unclear. We hypothesized that longitudinal control of BR biosynthesis is crucial in fuelling the BR signalling maximum. To verify this assumption, we compared BR biosynthetic gene expression patterns with the localization of the BES1-GFP reporter. The nuclear BES1-GFP signal in epidermal and cortical cells started to increase at 200–400 µm from the quiescent centre (QC) and reached a peak in the elongation zone (Fig. 4b,c and Supplementary Video 4), as previously reported²⁹. Similarly, the fluorescence intensity of both CPD-GFP and ROT3-GFP signals increased approximately twofold in the region 200–400 µm from the



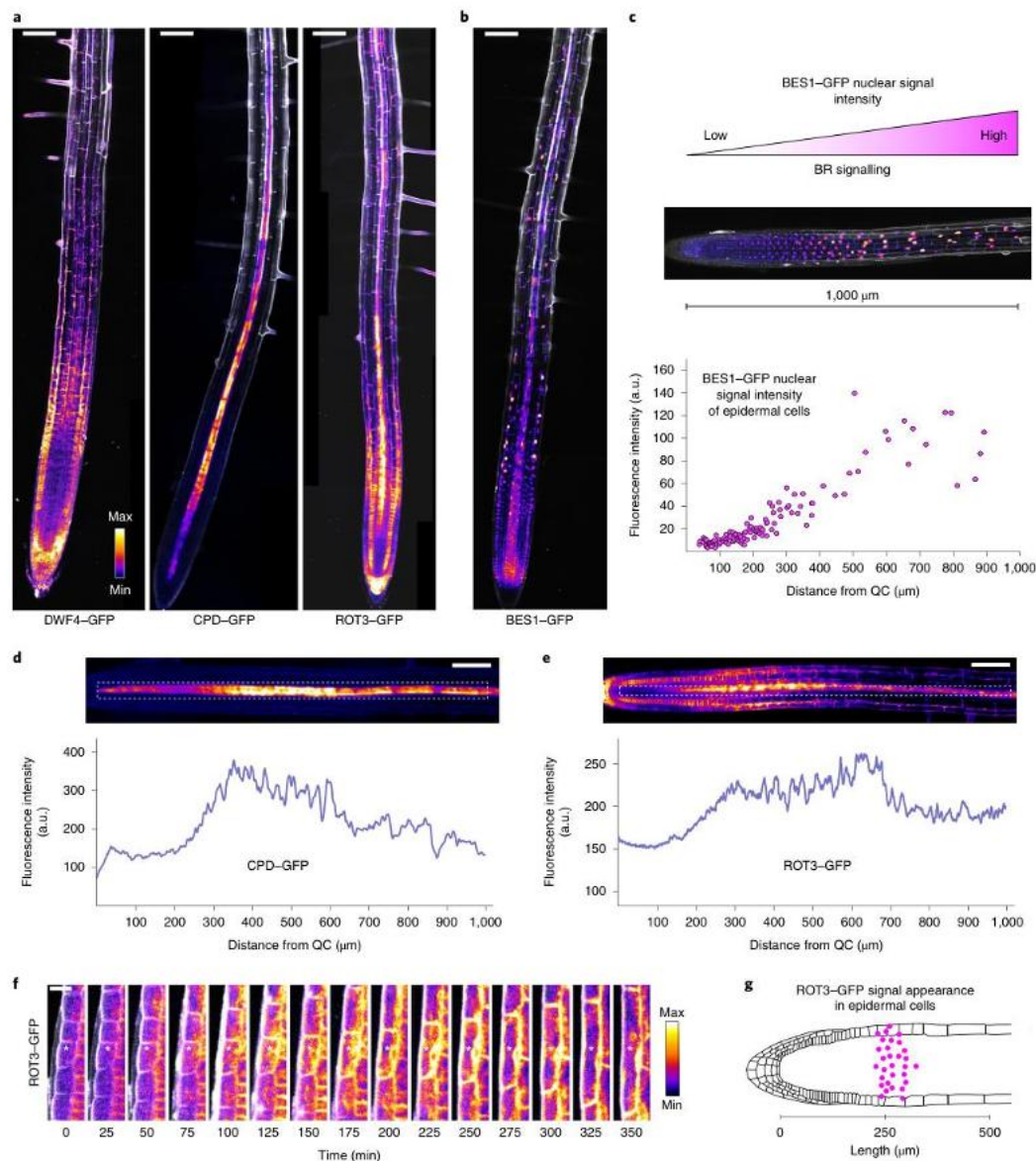


Fig. 4 | BR signalling and synthesis maxima overlap in the *Arabidopsis* root. **a, Six-day-old *Arabidopsis* roots expressing DWF4-GFP/*dwf4*, CPD-GFP/*cpd* and ROT3-GFP/*rot3* under the control of their native promoters. **b**, Six-day-old *Arabidopsis* roots expressing BES1-GFP/*Col-0* under the control of its native promoter. **a, b**, All transgenic lines were imaged in more than three independent experiments. Representative images are shown. **c**, Increase in BES1-GFP nuclear fluorescence intensity used as a readout of active BR signalling. Scatter diagram shows the nuclear fluorescence intensity of BES1-GFP in epidermal cells of a single root in relation to their distance from the QC. **d, e**, Fluorescence intensity profiles of CPD-GFP (**d**) and ROT3-GFP (**e**) along the root elongation zone. GFP signals were measured along the delimited dashed frames and are plotted in the graphs below. Average values of three roots for each line are shown. **f**, Time series analysis of a single epidermal cell expressing ROT3-GFP (asterisk). The ROT3-GFP signal appears in the transition zone and reaches its peak as the cell enters the elongation zone. **g**, Profile of ROT3-GFP signal appearance in root epidermal cells. Ten time points, 25 min apart, of three independent roots are shown. Scale bars, 100 μm (**a, b, d, e**) and 20 μm (**f**). a.u., arbitrary units.**

QC, correlating with the highest BES1–GFP nuclear signal accumulation (Fig. 4d,e). Time-lapse imaging of epidermal cells expressing ROT3–GFP further supported these observations (Fig. 4f). Young epidermal cells had either a weak or no GFP signal while entering the transition zone and, as expansion proceeded, expression started to increase, reaching a peak when cells entered the elongation zone (Fig. 4g). Additionally, the DWF4–GFP expression peak preceded the BR signalling maximum (Extended Data Fig. 4a,b).

Taken together, the expression of BR biosynthetic genes in *Arabidopsis* root peaks in the elongation zone, as a consequence of an increase in expression levels and broadening of the expression domain into additional cell files. Notably, this region of the root largely overlaps with the domain of BR signalling maximum, implying that hormone biosynthesis determines local levels of signalling responses.

Concentration of bioactive BRs is higher in the root elongation zone than in the meristem. Exogenous BL has been shown to restore BR signalling in the *dwf4* mutant root²⁸, hence ruling out the importance of local BR biosynthesis for root growth, but root growth rates and meristem morphology were not fully recovered by the addition of BL²⁹. Therefore, we analysed in detail *dwf4* root growth and morphology under exogenous BL conditions. Because of dose-dependent plant growth responses to BRs⁸, we tested the effect of different increasing concentrations of BL on roots of 6-day-old wild-type and *dwf4* plants. All BL concentrations tested, from the lowest (10 pM) to the highest (250 pM), had a clearly positive effect on root growth after 1 day for both genotypes when compared to mock controls (Fig. 5a and Extended Data Fig. 5a). After 2 days, the effect of exogenous BL became detrimental for root growth in Col-0 at concentrations of 100 pM and higher, whereas growth rates of BL-treated *dwf4* roots were lower than those of the mock-treated wild type at all BL concentrations (Fig. 5a and Extended Data Fig. 5a). Our results support previous observations²⁹ that growth rates of BR-deficient *dwf4* roots cannot be fully recovered to wild-type levels by exogenous BL.

Next, we examined the morphology of root meristems of BL-treated Col-0 and *dwf4* plants and found clear differences between roots grown for 24 h on mock and hormone-supplemented medium (Fig. 5b). Low hormone concentrations had no profound effect on the meristem cell number, but high concentrations (from 100 pM) caused a decrease in cell number in both wild type and mutant (Fig. 5c). In addition, BL treatment reduced the root

meristem diameter of mutant and Col-0 plants (Extended Data Fig. 5b) whereas the root meristem size, as measured from QC to the first elongating cell, was not significantly affected except when *dwf4* roots were treated with 250 pM BL (Extended Data Fig. 5c). Notably, exogenous BL increased the meristem cell length in both genotypes (Fig. 5d). Interestingly, when treated with 10 pM BL, *dwf4* roots displayed meristem architecture parameters almost identical to those of the mock-grown wild type. In contrast, the final size of mature cortical cells of *dwf4* roots, which were fully recovered by treatment with 100 pM BL, was only partially restored by 10 pM BL (Fig. 5e). Finally, we functionally characterized the root meristem after BL treatment by calculating the number of cells produced in the meristem per unit of time²⁷. Root meristem cell production rates of the *dwf4* mutant were rescued to wild-type levels when treated with 10 pM BL, but not with 100 pM (Extended Data Fig. 5d–f).

These data imply that meristem and elongation zones require different concentrations of BR for optimal growth; whereas low hormone concentrations can restore *dwf4* meristem architecture, cell length and cell production rates, they are not sufficient to unlock the full elongation potential of root cells, the reason for partial root growth recovery of the mutant when grown on 10 pM BL. Conversely, high concentrations of exogenous BR enable full elongation of cells in the elongation zone of the mutant root²⁹ but, simultaneously, lead to excessive elongation of meristematic cells, decrease in meristem cell number and cell production rates and, ultimately, reduced root growth. We directly measured the bioactive BR content in different root zones of pea (*Pisum sativum*) radicles. We found that the concentration of BL and CS was roughly fivefold higher in the transition than in the meristematic zone (Fig. 5f,g), which is fully in agreement with the rescue experiments of *Arabidopsis* mutants.

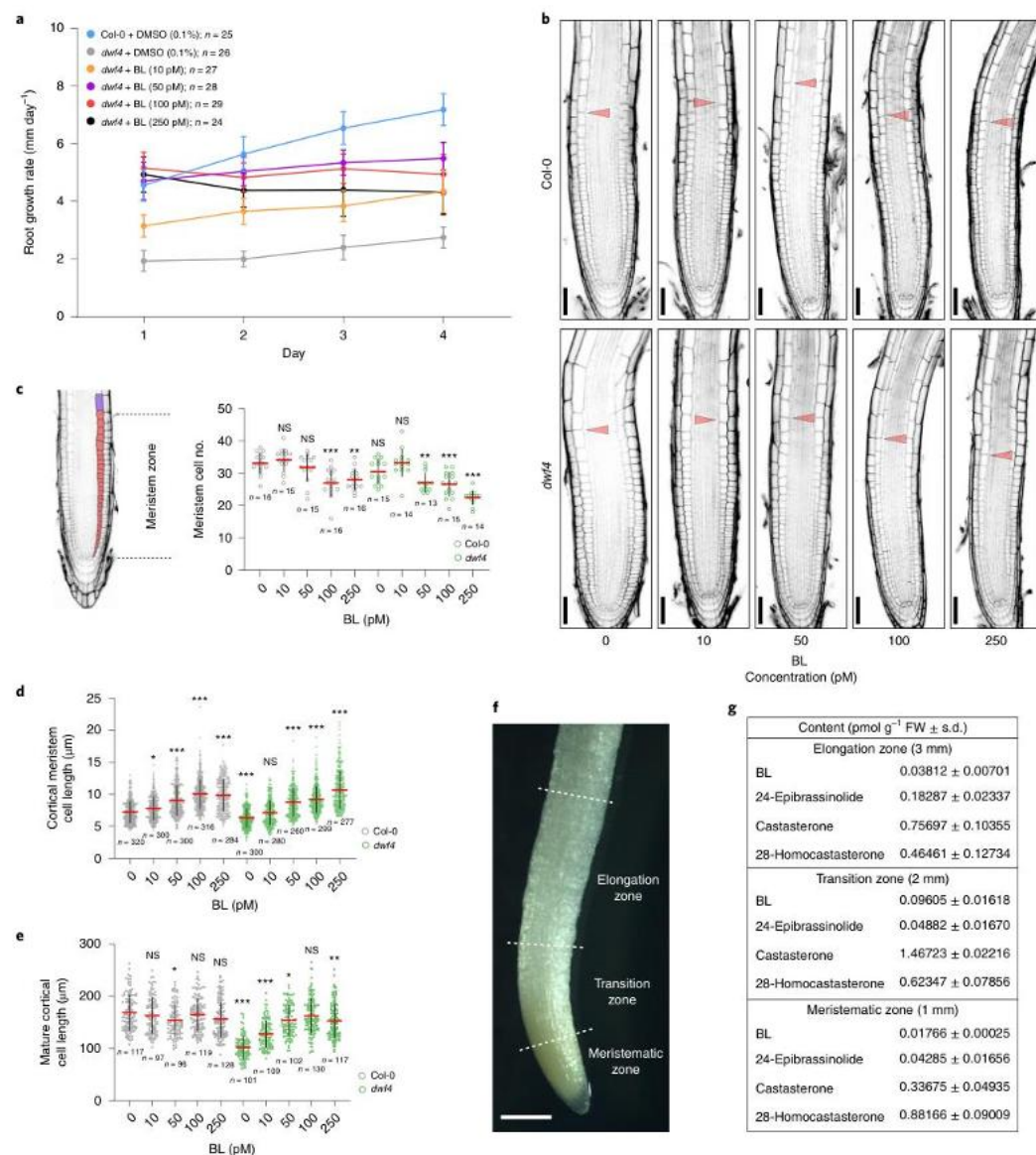
Expression of BR biosynthetic enzymes restricts BR production in the *Arabidopsis* root. If low BR levels were retained in the root meristem by maintaining a reduced expression of BR biosynthetic enzymes, overexpression of these enzymes should mimic growth under exogenous BL and perturb the meristem architecture in a similar fashion. To test this hypothesis, we expressed the rate-limiting enzyme DWF4 fused to mCHERRY under the control of the constitutive 35S promoter. As reported previously³⁰, DWF4–mCHERRY-overexpressing (OE) plants (DWF4–OE) exhibited hypocotyls longer than those of Col-0 and a range of root phenotypes, including roots of length similar to that of the wild type,

Fig. 5 | Root meristem and elongation zones have different demands for BRs. **a**, Root growth rates of *dwf4* plants grown on increasing concentrations of BL compared to Col-0 grown on mock. Six-day-old seedlings were transferred to fresh medium containing BL or mock, and root growth was followed for 4 days. Values represent means $\pm$ s.d. of root growth rates. n = number of roots analysed. **b**, Root meristem architecture of Col-0 and *dwf4* plants treated with different concentrations of BL for 24 h. Roots were stained with propidium iodide. Arrowheads indicate the boundary between meristem and elongation zone. More than three independent experiments were performed. Representative images are shown. Scale bars, 50 μ m. **c–e**, Quantification of cortical meristem cell number (**c**), cortical meristem cell length (that is, the first 20 cells of individual roots) (**d**) and mature cortical cell length (**e**). Roots were treated with BL for 24 h. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots (**c**) and cells analysed (**d,e**). **d**, Numbers of individual roots used in the experiment were 16, 15, 15, 16 and 15 for Col-0 and 15, 14, 13, 15 and 14 for *dwf4* treated with DMSO, 10 pM BL, 50 pM BL, 100 pM BL and 250 pM BL, respectively. **e**, Number of individual roots used in the experiment were 16, 15, 14, 15 and 14 for Col-0 and 14, 17, 13, 15 and 15 for *dwf4* treated with DMSO, 10 pM BL, 50 pM BL, 100 pM BL and 250 pM BL, respectively. Significant differences between Col-0 and *dwf4* seedlings treated with BL and Col-0 grown on mock (DMSO) were determined by two-way ANOVA and Tukey's multiple comparison tests. *** P < 0.001, ** P < 0.01, * P < 0.05. P values versus Col-0 + DMSO control for different treatments are as follows: **c**, Col-0 + 10 pM BL, 0.7073, Col-0 + 50 pM BL, 0.9839, Col-0 + 100 pM BL, 0.0005, Col-0 + 250 pM BL, 0.006, *dwf4* + DMSO, 0.7073, *dwf4* + 10 pM BL, >0.9999, *dwf4* + 50 pM BL, 0.0019, *dwf4* + 100 pM BL, 0.0002, *dwf4* + 250 pM BL, <0.0001; **d**, Col-0 + 10 pM BL, 0.0372, Col-0 + 50 pM BL, <0.0001, Col-0 + 100 pM BL, <0.0001, Col-0 + 250 pM BL, <0.0001, *dwf4* + DMSO, <0.0001, *dwf4* + 10 pM BL, 0.998, *dwf4* + 50 pM BL, <0.0001, *dwf4* + 100 pM BL, <0.0001, *dwf4* + 250 pM BL, <0.0001; **e**, Col-0 + 10 pM BL, 0.9596, Col-0 + 50 pM BL, 0.0235, Col-0 + 100 pM BL, 0.996, Col-0 + 250 pM BL, 0.0765, *dwf4* + DMSO, <0.0001, *dwf4* + 10 pM BL, <0.0001, *dwf4* + 50 pM BL, 0.0186, *dwf4* + 100 pM BL, 0.8985, *dwf4* + 250 pM BL, 0.0031. All experiments were repeated independently more than three times, with similar results. **f**, Image of 2-day-old pea root tip. Material for BR content measurement was collected from three marked zones: meristematic, transition and elongation. Scale bar, 1 mm. **g**, Content quantification of four different BRs in the three root zones shown in **f**. Material was collected from pea radicles (length 2–3 cm). Roots were grown once, and material from different zones was extracted in five separate batches and used for analysis. FW, fresh weight.

slightly shorter and occasionally short, wavy and extremely curled, resembling those treated with high BL concentrations (1 nM) (Fig. 6a). As expected, in the roots of these plants most BR-responsive genes corroborated elevated BR signalling (Fig. 6b). In short DWF4-OE roots, expression of BR-responsive genes indicated higher levels of BR signalling than in long roots, correlating with the increased expression levels of *DWF4* (Fig. 6b and Extended Data Fig. 6a). In addition, the phosphorylation status of BES1 in DWF4-OE roots exhibited a shift towards a dephosphorylated state, indicating activation of BR signalling (Fig. 6c and Extended

Data Fig. 6b). The root meristem architecture of DWF4-OE plants (Fig. 6d) displayed fewer, but excessively elongated, meristematic cells of smaller root diameter (Fig. 6e–g), all indicators of elevated BR signalling.

Next we tested a scenario in which the DWF4 enzyme expression was confined to the root meristem zone, to assess whether BR biosynthetic enzyme expression is linked to hormone production. To this end, we expressed DWF4-GFP under the control of the *WEREWOLF* (*WER*) promoter³⁹ that, like the *DWF4* promoter, is active in epidermal meristematic cells. However, whereas the *DWF4*



expression level increased and its domain expanded in the elongation zone (Fig. 6h), *WER* expression remained limited to epidermal cells and gradually weakened as cells started to elongate (Fig. 6i). Mutant *dwf4* plants carrying the *pWER:DWF4-GFP* construct only partially complemented root length (Fig. 6j and Extended Data Fig. 6c), due to the imperfect rescue of the mature cell length (Fig. 6k). Because meristems of roots expressing *pWER:DWF4-GFP* displayed normal characteristics, or those of roots treated with low amounts of BL (Extended Data Fig. 6d–g), we concluded that their reduced length reflected solely the lack of sufficient DWF4 enzyme expression and possibly of BR biosynthesis in the root elongation zone. Similar results were obtained when expression of another biosynthetic enzyme, CPD, was confined to the meristem using the *WER* promoter. Again, *cpd* mutants had wild-type-like meristems and partially complemented the root length due to the imperfect rescue of the mature cell length (Extended Data Fig. 7a–g). Conversely, when CPD enzyme expression was mostly limited to the root elongation zone, by expression of CPD-mCherry under the control of the *COBRA-LIKE9* (*COBL9*) promoter¹⁰ in the *cpd* mutant, the mature cell length was rescued, but the meristems of these plants displayed characteristics of mutant meristems (Extended Data Fig. 7a–g). Altogether, our findings reveal that spatially limited expression of BR biosynthetic genes can restrict BR production in the *Arabidopsis* root.

Discussion

Root growth is driven by the coordination of cell division and longitudinal expansion, maintaining the optimal size of the meristematic cell pool and simultaneously facilitating cell elongation²⁶. Among other factors, BR signalling regulates both cell division and expansion and shows a gradient along the longitudinal root axis²⁹. A role for BR biosynthesis in establishing this gradient has been ruled out because of the limited mobility of the hormones involved and the general belief that every tissue has the capacity to synthesize BRs³⁰, despite the lack of information on their biosynthesis and accumulation sites.

To explore the importance of BR biosynthesis and distribution for root growth, we examined the localization of all known enzymes

in the pathway. Strikingly, most BR biosynthetic enzymes exhibited restricted expression domains especially in the root meristem, implying that BR intermediates have to move over short distances. The mobile nature of BRs was indicated by the observation that root meristem defects of the *bri1* receptor mutant were largely rescued by the ectopic expression of BRI1 in the epidermis³³, a tissue separated from the CPD expression domain by several cell layers. Our findings, using ectopic expression of CPD and BAS1 in the endodermis, support previous suggestions that BRs could move locally^{11,42}. In this scenario, locally synthesized BR precursors are able to move within certain root domains to facilitate pathway completion and bioactive compound production. In our experimental set-up we could not determine whether precursors or bioactive hormones were able to move and allow compounds to act in a paracrine manner⁴³. Simultaneously, the *dwf4* and *cpd* mutant rescue experiments by spatially limited expression of corresponding enzymes imply that hormones have a limited mobility over long distances. We demonstrate that the feedback inhibition of BR biosynthetic genes via BR signalling is not the regulatory mechanism that determines their expression patterns at the tissue level. Transcriptional modules driving the expression of BR biosynthetic genes remain to be identified.

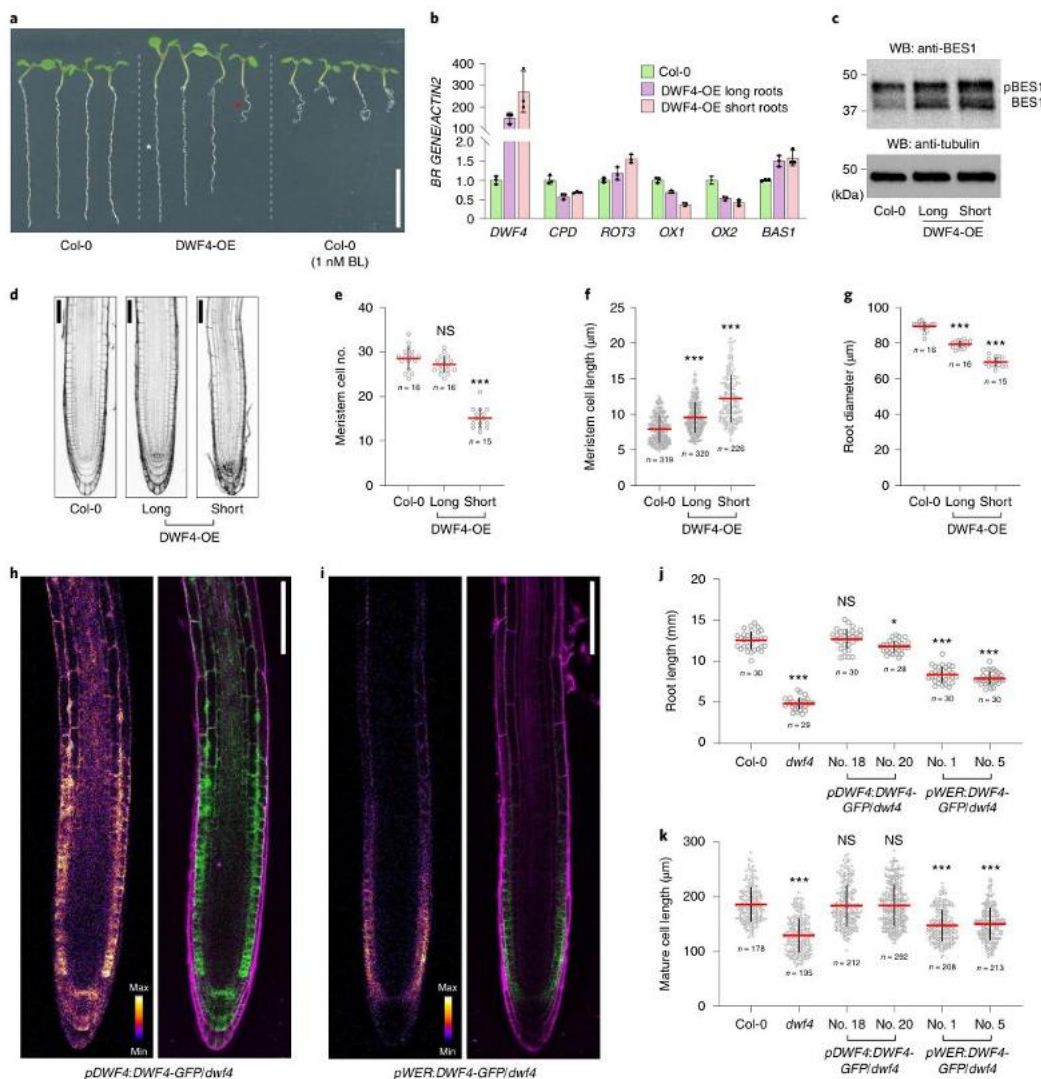
Another striking observation was the elevated expression of BR biosynthetic enzymes in the root elongation zone, correlating with the previously reported BR signalling maximum²⁹. Given the inability of BRs to move over long distances³⁰, a local increase in hormone synthesis in tissues with a high demand for cell elongation, such as the root elongation zone, would be expected. Links between the sites of BR biosynthetic gene expression and those of active hormone biosynthesis have previously been proposed⁴⁴. In addition, the local increase in BR biosynthetic gene expression coincides with the BR signalling maximum at roughly 200–400 µm from the QC, a region where cells cease to divide and enter the elongation zone²⁶. This increase is achieved through either a simple rise in expression levels, such as for CPD, or expansion of the expression domain to additional cell files as observed for the enzymes DWF4, ROT3 and BR6OX1. The question that remains is whether local increases in biosynthetic gene expression and hormone levels occur concomitantly.

Fig. 6 | Local DWF4 enzyme expression determines BR levels. **a**, Comparison of root phenotypes of 6-day-old seedlings carrying the *p35S:DWF4-mCherry* construct (DWF4-OE) with Col-0 seedlings grown on mock or medium supplemented with 1 nM BL. DWF4-OE plants display long roots (white asterisk) with lengths comparable to those of Col-0, as well as short ones (red asterisk) comparable to those of roots treated with high concentrations of BL. **b**, Expression of BR-responsive genes in DWF4-OE roots of 6-day-old seedlings measured by real-time RT-qPCR. Expression of *DWF4*, *CPD*, *ROT3*, *BR6OX1* (*OX1*), *BR6OX2* (*OX2*) and *BAS1* was tested. Transcript levels were normalized to *ACTIN2* gene expression and three biological replicates were quantified. Symbols denote individual samples. Bars represent s.d. **c**, BES1 phosphorylation status tested by immunoblot with α -BES1 antibody in roots of Col-0 and DWF4-OE 6-day-old seedlings. Tubulin was used as loading control. The experiment was performed twice. **d**, Root meristem architecture of 6-day-old *Arabidopsis* wild-type (Col-0) and DWF4-OE seedlings with long or short roots. More than three independent experiments were performed. Representative images are shown. **e–g**, Quantification of different root meristem parameters of 6-day-old Col-0 and DWF4-OE seedlings with long or short roots: cortical meristem cell number (**e**), cortical meristem cell length of the first 20 cells of individual roots (**f**) and root diameter (**g**). All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots (**e, g**) and cells (**f**) analysed. **f**, Numbers of individual roots used were 16, 16 and 15 for Col-0, DWF4-OE long and DWF4-OE short, respectively. Significant differences between transgenic lines and Col-0 control were determined by one-way ANOVA and Dunnett's multiple comparison tests. *** P < 0.001. P values versus Col-0 for different transgenic lines are as follows: **e**, DWF4-OE long, 0.1754 and DWF4-OE short, <0.0001; **f**, DWF4-OE long, <0.0001 and DWF4-OE short, <0.0001; **g**, DWF4-OE long, <0.0001 and DWF4-OE short, <0.0001. All experiments were repeated independently more than three times, with similar results. **h**, Six-day-old *dwf4* mutant roots expressing DWF4-GFP under the control of its native promoter. **i**, Six-day-old *dwf4* mutant roots expressing DWF4-GFP under the control of the *WER* promoter. Note the decrease in GFP signal in the elongation zone. **h, i**, Identical confocal settings were used. More than three independent experiments were performed. Representative images are shown. **j, k**, Quantification of root length (**j**) and mature cortical cell length (**k**) of Col-0, *dwf4* and two independent *dwf4* mutant lines expressing DWF4-GFP under the control of the *DWF4* and *WER* promoters. Six-day-old plants were transferred to fresh medium, lengths were measured after 2 days and plants were imaged by confocal microscopy. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots (**j**) and cells (**k**) analysed. **k**, Number of individual roots used were 25, 23, 23, 24, 17 and 21 for Col-0, *dwf4*, line no. 18, line no. 20, line no. 1 and line no. 5, respectively. Significant differences between transgenic lines and Col-0 control were determined by one-way ANOVA and Dunnett's multiple comparison tests. *** P < 0.001, ** P < 0.01, * P < 0.05. P values versus Col-0 for different transgenic lines are as follows: **j**, *dwf4*, <0.0001, line no. 18, 0.9911, line no. 20, 0.9975, line no. 1, <0.0001, line no. 5, <0.0001; **k**, *dwf4*, <0.0001, line no. 18, 0.9903, line no. 20, 0.0429, line no. 1, <0.0001, line no. 5, <0.0001. All experiments were repeated independently more than three times, with similar results. Roots were stained with propidium iodide. Scale bars, 1 cm (**a**) and 50 µm (**d, h, i**). WB, western blot.

In the case of the rate-limiting enzyme DWF4, maximum expression precedes the signalling peak along the longitudinal root axis, as monitored by the BES1-GFP nuclear accumulation, implying that hormone production is a prerequisite for increased BR signalling in the elongation zone. Overexpression of DWF4 enzyme demonstrates that elevated gene expression leads to increased BR signalling that, in turn, reduces meristem cell numbers and negatively affects root growth, similarly to exogenous BL. Moreover, restricting the expression of DWF4 and CPD enzymes to either the meristem or the elongation zone reveals a clear link between hormone production and local biosynthetic gene expression. Even in the case of auxin, a hormone well known for its directional transport, localized expression of the auxin biosynthetic enzyme TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) plays an

important role in the formation of the local hormone maximum¹⁵. Therefore, we postulate that the expression maximum of BR biosynthetic enzymes also reflects the hormonal maximum, finally determining local BR signalling response levels. This type of localized biosynthesis places BRs in a unique position among the other major groups of plant hormones, which are highly mobile compounds and of which their transport over long distances often plays a crucial role in the establishment of local hormone maxima¹⁶.

Collectively, our data demonstrate that root meristem and elongation zones require different BR concentrations for optimal growth. Such dose-dependent responses of roots to exogenous BRs have been shown previously^{17,29}. Although exogenous BRs, even when applied at extremely high concentrations, have no negative impact on the final size of mature cells in the root elongation zone,



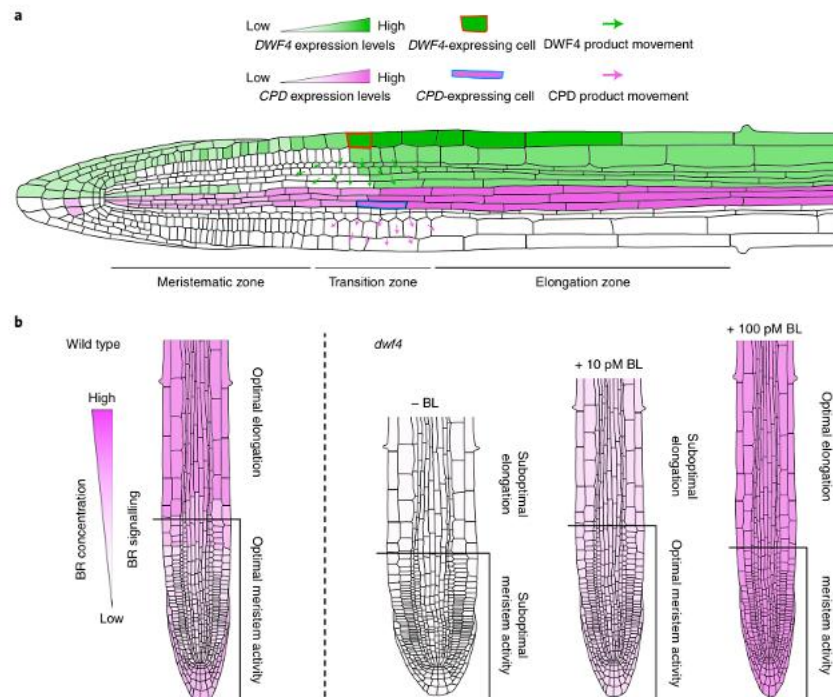


Fig. 7 | Localized BR biosynthesis coordinates root growth. a, Expression domains of BR biosynthetic enzymes that do not always overlap. Expression patterns are shown for DWF4 and CPD, two consecutive enzymes in the biosynthetic pathway. It is likely that BR intermediates need to be exchanged between neighbouring cells for conversion to bioactive molecules. This movement is restricted and creates local BR maxima and a hormone concentration gradient along the longitudinal root axis. **b**, BR signalling shows a gradient along the longitudinal root axis and enables optimal cell division rates in the meristem and cell expansion in the elongation zone. This signalling gradient corresponds to local expression maxima of BR biosynthetic genes and, hence, local BR levels. In *dwf4* biosynthetic mutant roots, meristematic cells divide at a reduced rate and their elongation is impaired, resulting in a short root phenotype. When *dwf4* roots were treated with low concentrations of BL, the meristem activity was recovered but not the cell elongation. Increased concentrations recovered *dwf4* cell elongation defects but simultaneously depleted cells from the meristem. Therefore, both treatments led to *dwf4* roots shorter than those of the wild type, demonstrating that low BR levels in the meristem and high levels in the elongation zone are needed for optimal root growth.

root growth is strongly impaired²⁹. However, increased BR levels negatively affect the meristem activity²⁷. Root growth defects caused by elevated BRs can be explained by a Sizer mechanism for cell elongation and differentiation during root growth⁴⁷, which predicts that cells sense their own length and cease to expand after a threshold value is reached. According to this model, the final length of differentiated cells is independent of changes in meristematic activities and it can therefore explain the retarded root growth phenotypes observed in situations characterized by unaltered mature cell length and decreased meristematic activity. Because minute quantities of exogenous BL can restore the root meristem architecture and function of the *dwf4* mutant but not the elongation zone defects, we postulate that a low level of BR signalling is achieved in the root meristem by the maintenance of low amounts of endogenous BRs. Indeed, direct BR content measurements in different zones of pea roots revealed higher BR concentrations in the elongation and transition zones than in the meristem. Hence, we propose a model (Fig. 7a) in which the BR signalling output reflects the existence of a hormone gradient along the longitudinal root axis. In BR-deficient mutants the elongation of all root cells is impaired, causing two

distinct phenotypic defects, decreased final cell length and decreased meristematic activity. Both phenotypic defects can be fully rescued by exogenous BL, but not simultaneously. Whereas low BL concentrations are able to rescue the root meristem architecture of biosynthetic mutants, only high concentrations enable full cell elongation. Nevertheless, high-concentration treatments result in both untimely elongation of meristematic cells and decrease in meristem cell numbers and mitotic cell production rates (Fig. 7b).

In summary, we postulate that BR signalling levels correspond to a local hormonal accumulation achieved through regulation of the expression of BR biosynthetic enzymes, and radial short-distance transport of active BRs and their precursors. Due to limited mobility, local BR production leads to local hormone accumulation which, in turn, triggers signalling and enables timely transition of meristematic cells to the elongation zone.

Methods

Plant material. *Arabidopsis thaliana* (L.) Heynh. Col-0 plants were used for all experiments. For phenotypic analysis, the following mutant lines were used: *dwf4-102* (At3g50660)⁴⁸, *cpd* (At5g05690)³, *det2-1* (At2g38050)⁴⁹, *rot3* (At4g36380)⁵⁰,

cyp90d1 (At3g13730)³¹, *br6ox1-1* (At5g38970)³², *br6ox2-2* (At3g30180)³² and *br11/br11/br13* (At4g39400/At1g55610/At3g13380)³³. The mutant pairs *rot3/cyp90d1* and *br6ox1/br6ox2* were crossed to obtain double mutants. Genotyping primers are listed in Supplementary Table 1. Transgenic *Arabidopsis* lines expressing *pDET2-DET2-GFP-GUS/det2-9* (ref. ³⁴), *pBES1-BES1-GFP/Col-0* (ref. ³⁵) and *pBR11-BR11-mCitrine/br11* (ref. ³⁵) have been described previously.

Growth conditions. Seeds were surface sterilized with sterilization buffer (80% v/v ethanol, 20% v/v sodium hypochlorite), stratified for 2 days in the dark at 4°C and grown vertically on half-strength Murashige and Skoog (½MS) agar plates supplemented with 1% (w/v) sucrose at 22°C, with a 16/8-h light/dark photoperiod. For the phenotypic analysis, 6-day-old seedlings were transferred to either fresh plates for 3 additional days, for root growth measurement, or to soil and imaged after 3 weeks.

Chemical treatments and root growth assays. For BL (OlChemIm, Ltd.) treatments of *dwf4* mutants, plants were grown on agar plates for 6 days until segregating homozygous mutants were recognizable (wild-type-looking seedlings consisting of Col-0 and *dwf4* heterozygous plants were used as wild-type control), whereafter the seedlings were transferred to fresh medium containing different concentrations of BL. BL was maintained at different stock concentrations in DMSO and diluted 1,000× to reach final concentrations in the medium. For mock treatment, DMSO was used at a final concentration of 0.1% (v/v). Root growth was recorded every 24 h for 4 days. For confocal microscope observations, the procedure was standard and root tips were stained with propidium iodide (Sigma-Aldrich) and imaged 24 h post-treatment. Meristem area was determined as a region extending from the QC to the first elongating cell (cell length is twice its width), and meristem cell numbers as the numbers of cortical cells in a single file spanning from the QC to the first elongating cell. To measure the length of mature cortical cells, cells from the root region where root hairs start to emerge (a sign of cell differentiation after cessation of elongation) were imaged. The root meristem production rate (*P*) was calculated according to ref. ³⁶. Six-day-old Col-0 and *dwf4* seedlings were transferred to fresh agar plates containing different BL concentrations and grown for 3 days. On the third day, root tips were marked and grown for an additional 24 h. Root elongation (*E*) of individual roots was recorded and roots were stained with propidium iodide and imaged with a confocal microscope. For each root, the length of at least 30 mature cortical cells was measured and mean cell length was calculated (*L_{avg}*). *P* was obtained with the equation $P = E/L_{avg}$. For BL and BRZ (Tokyo Chemical Industry) treatment of *pDWF4:DWF4-GFP/dwf4* roots, plants were grown on ½MS agar plates for 5 days before transfer to new plates containing mock, BL or BRZ, grown for 24 h and imaged.

Microscopy and image analysis. Seedlings were imaged on a Leica SP8X confocal microscope. GFP was excited at 488 nm and acquired at 500–530 nm; mCherry was excited at 594 nm and acquired at 600–650 nm. For meristem imaging, roots were stained with propidium iodide (Sigma-Aldrich). Images were taken by a ×20 or ×40 objective. Vertical imaging was done using a Carl Zeiss Axio Observer.7 armed with a VisiScope spinning disk confocal unit based on the Yokogawa CSU-W1-T2 equipped with a VS-HOM1000 excitation light homogenizer and a PRIME-95B Back-Illuminated sCMOS camera, and a Plan-Apochromat ×20/0.8 numerical aperture M27 objective. For time-lapse microscopy experiments with plants expressing *pDWF4:DWF4-GFP/dwf4*, *pCPD:CPD-GFP/cpd*, *pROT3:ROT3-GFP/rot3* and *pBES1-BES1-GFP/Col-0*, 6-day-old seedlings were transferred to a three-dimensional (3D)-printed triple microscopy chamber (five seedlings in each) on ½MS agar blocks, supplemented with propidium iodide and imaged overnight at 25-min intervals. Time series were assembled with stitching of tiled 3D microscopic image acquisitions³⁷ and subsequent analysis with either Fiji (<https://fiji.sc/>) or LASX software. To measure the nuclear intensity signal of BES1-GFP, Z-stack confocal images were acquired for each root. Fluorescence intensity was measured in Fiji software for optical sections that passed through the centre of the nucleus of the cell of interest along the longitudinal root axis. For DWF4-GFP intensity measurements, fluorescence intensity of whole cells was measured. At least five roots were analysed for each line.

Generation of constructs and transgenic lines. To generate transcriptional reporter lines of BR biosynthetic enzymes, promoter regions roughly 2 kbp upstream from the start codons of *DWF4*, *CPD*, *ROT3* and *CYP90D1* were amplified by PCR and cloned into pDONR4-P1R by means of the Gateway BP clonase enzyme mix (Thermo Fisher Scientific). The *BR6OX1* promoter was amplified and cloned with the restriction enzymes BamHI and XhoI into pENTR4-R1 (ref. ³⁸). For *BR6OX2*, a 6.5-kbp promoter region was incorporated into pDONR4-P1R by assembly of four PCR fragments with the Gibson Assembly technology (New England Biolabs). The protocol, including primer design, was according to the instruction manual of the Gibson Assembly Master Mix. All promoters were recombined into pMK7S-NFm14GW.0 (ref. ³⁹) with the Gateway LR clonase enzyme mix (Thermo Fisher Scientific) to generate the expression constructs *PROMOTER:NLS-GFP*, each carrying a kanamycin resistance gene for plant selection. For the *pROT3:NLS-mCherry* construct, the promoter-carrying

entry clone was combined with entry clones carrying the NLS signal peptide and the mCherry gene. These constructs were introduced into the Col-0 wild type by means of the floral-dip method⁴⁰. The lines *pROT3:NLS-mCherry* and *pCPD:NLS-GFP* were crossed to obtain a co-expressing line. To generate translational reporter lines of BR biosynthetic enzymes, coding sequences of the full genomic fragments of *DWF4*, *CPD*, *CYP90D1*, *BR6OX1* and *BR6OX2* were amplified by PCR and cloned by BP clonase enzyme mix into pDONR221 (Thermo Fisher Scientific). The genomic DNA fragment of *ROT3* was isolated by overlap extension PCR and cloned into pDONR221. For *GFP-CYP85A2*, the signal peptide sequence (first 78 bp starting from ATG)⁴¹ was included in the GFP cloning primer, and *SP-GFP* was PCR amplified and introduced into pDONR221. The genomic sequence of *CYP85A2* without the first 78 bp was cloned into pDONR2R-P3. Entry clones were combined with destination vectors in LR reactions to obtain expression clones with GFP-tagged enzymes (pB7m34GW for *DWF4*, *ROT3*, *CYP90D1*, *BR6OX1* and *BR6OX2*, and pK7m34GW for *CPD*). The expression clones were introduced into corresponding mutant backgrounds with the floral-dip method. To generate the *pWER:DWF4-GFP* construct, the complementary DNA of *DWF4* was amplified by PCR, cloned into pDONR221 and subsequently recombined with the *WER* promoter-carrying pDONR4-P1R³⁵ into the pB7m34GW-FAST destination vector. The *DWF4-OE* line was constructed by recombination of pDONR221 with genomic *DWF4* into pDONR4-P1R-35S (cauliflower mosaic virus (*CaMV*) 35S promoter) and of pDONR2R-P3 into the pB7m34GW destination vector. For *CPD* ectopic expression lines, cDNA was cloned into pDONR221 and recombined in an LR reaction with promoter-carrying entry clones into pB7m34GW to obtain *pSCR:CPD-mCherry* and *pCOBL9:CPD-mCherry*, or into pB7m34GW-FAST to obtain *pWER:CPD-GFP* clones. For *BAS1* ectopic expression lines, cDNA was cloned into pDONR221 and recombined in an LR reaction with promoter-carrying entry clones into pB7m34GW to obtain *pSCR:BAS1-mCherry*, or into pB7m34GW-FAST to obtain *pRPS5A:BAS1-GFP* and *pSCR:XVE:BAS1-GFP* (*SCR-XVE* promoter clone was described previously³⁵). Cloning primers are listed in Supplementary Table 1.

Immunoblot analysis. For BES1 immunoblot analysis, experiments were done in duplicate. For protein extraction, approximately 50 roots of 6-day-old *DWF4-OE* seedlings (both long and short roots) were collected. For the line *pSCR:XVE:BAS1-GFP*, transgenic and Col-0 seedlings were germinated on medium containing 10 μM β-oestradiol (Sigma-Aldrich) and grown for 7 days. Approximately 50 roots were collected for each sample. Plant material was frozen in liquid nitrogen, ground by Retsch MM400 and homogenized in 100 μl of ice-cold homogenization buffer (1% v/v SDS, 25 mM Tris/HCl pH 7.5, 150 mM NaCl, 10 mM DTT and Roche Complete protease inhibitor (one tablet 10 ml⁻¹)) and placed on ice for 30 min. The homogenates were centrifuged twice (10 min, 14,000 r.p.m.) at 4°C. After the addition of 4× lithium dodecyl sulfate and sample reducing agent (10×), the samples were heated for 10 min at 70°C, centrifuged again, separated on 4–15% v/v SDS-polyacrylamide gel electrophoresis stain-free protein gel (Bio-Rad Laboratories) and blotted on Trans-Blot Turbo Mini PVDF Transfer Packs. Membranes were blocked at 4°C in 5% v/v Difco Skim Milk. For immunodetection, the anti-BES1 antibody at 1:5,000 was used as primary antibody and donkey anti-rabbit (Merck) at 1:10,000 as the secondary antibody. For tubulin detection, the anti-Tubulin (Abcam) at 1:5,000 was used as primary antibody and sheep anti-Mouse (Merck) at 1:10,000 as secondary antibody. Proteins were detected by the ChemiDoc MP Imaging System (Bio-Rad Laboratories). For the BES1 dephosphorylation assay, the ratio of dephosphorylated BES1 to total BES1 proteins was quantified according to the signal intensity. Loading was adjusted to an equal level based on the amount of tubulin. Signal intensities were determined with Image Lab (Bio-Rad Laboratories).

BR measurement. Seeds of pea (*P. sativum*), variety Sweet Horizon (Eurotium), were soaked in water for 4 h and placed on water-wetted paper at the bottom of plastic boxes. They were kept in the dark at room temperature for 2 days. Plant material was collected from three successive zones along the longitudinal axis of the radicles: meristem (1 mm), transition (2 mm) and elongation (3 mm). Pea radicles 2–3 cm long were used for material harvesting. Analysis of BRs was performed according to the modified protocol given in ref. ⁴². Briefly, pea root material (20 mg fresh weight of each sample) was powdered in liquid N₂, extracted in 2 ml of ice-cold 60% acetonitrile and centrifuged. The supernatant was supplemented with internal standards of deuterium-labelled BR mix (25 pmol per sample, OlChemIm, Ltd.). Samples were purified on polyamine solid phase extraction columns Discovery DPA-6S (50 mg, Supelco) and evaporated to dryness. Each sample residue was then dissolved in 75 μl of 100% MeOH by vortexing and sonicating for 5 min, made up to 1 ml with PBS buffer (pH 7.2) and passed through immunoaffinity columns (Laboratory of Growth Regulators). BRs were then eluted from the columns using cold 100% MeOH. After evaporation to dryness, samples were reconstituted in 40 μl of MeOH and analysed by liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) on an ACQUITY UPLC I-Class System (Waters) with the use of a triple quadrupole mass spectrometer Xevo TQ-S MS (Waters MS Technologies). Details of the conditions used in UHPLC-MS/MS analysis were described previously⁴³. Each analysis was performed in pentuplicate.

RT-qPCR. Total RNA was extracted by quantitative PCR with reverse transcription (RT-qPCR) from Col-0 and DWF4-OE seedlings (both long and short roots) 6 days after germination with the RNeasy Mini Kit (Qiagen). Genomic DNA was eliminated by on-column digestion with RQ1 RNase-free DNase (Promega) during the isolation procedure. cDNA was generated from 1 µg of total RNA with qScript cDNA SuperMix (Quantabio) and analysed on a LightCycler 480II apparatus (Roche) with the SYBR Green I Master mix (Roche) according to the manufacturer's instructions. Expression levels were normalized to those of *ACTIN2*. Primers are listed in Supplementary Table 1.

Quantification and statistical analysis. All statistical analyses were done in GraphPad Prism v.8 software. Significance of differences was determined with two-tailed Student's unpaired *t*-test analysis for binary comparisons. Comparison of more than two genotypes was done by one-way analysis of variance (ANOVA), with Dunnett's multiple comparisons test subsequently used in the comparison procedure. For *dwf4* mutant rescue treatments with BL, two-way ANOVA was utilized and Tukey's multiple comparison test was subsequently used in the comparison procedure. ****P* < 0.001, ***P* < 0.01, **P* < 0.05.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings in this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions

N.V., Y.W. and E.R. initiated the project and designed experiments. N.V. and Y.W. performed most of the experiments. I.V., M.F. and B.G. performed experiments and analysed data. M.K., P.J. and J.O. contributed materials and hormone measurements. N.V. and E.R. wrote the manuscript. All authors revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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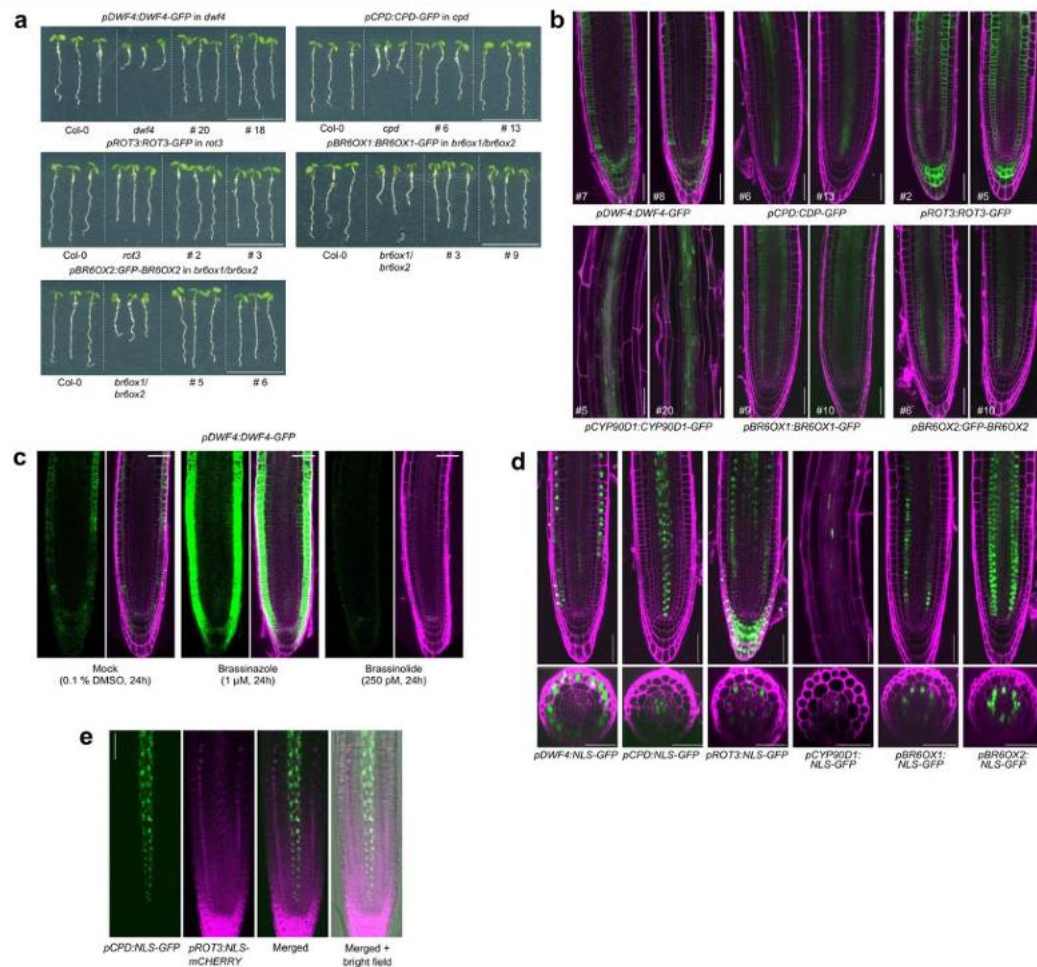
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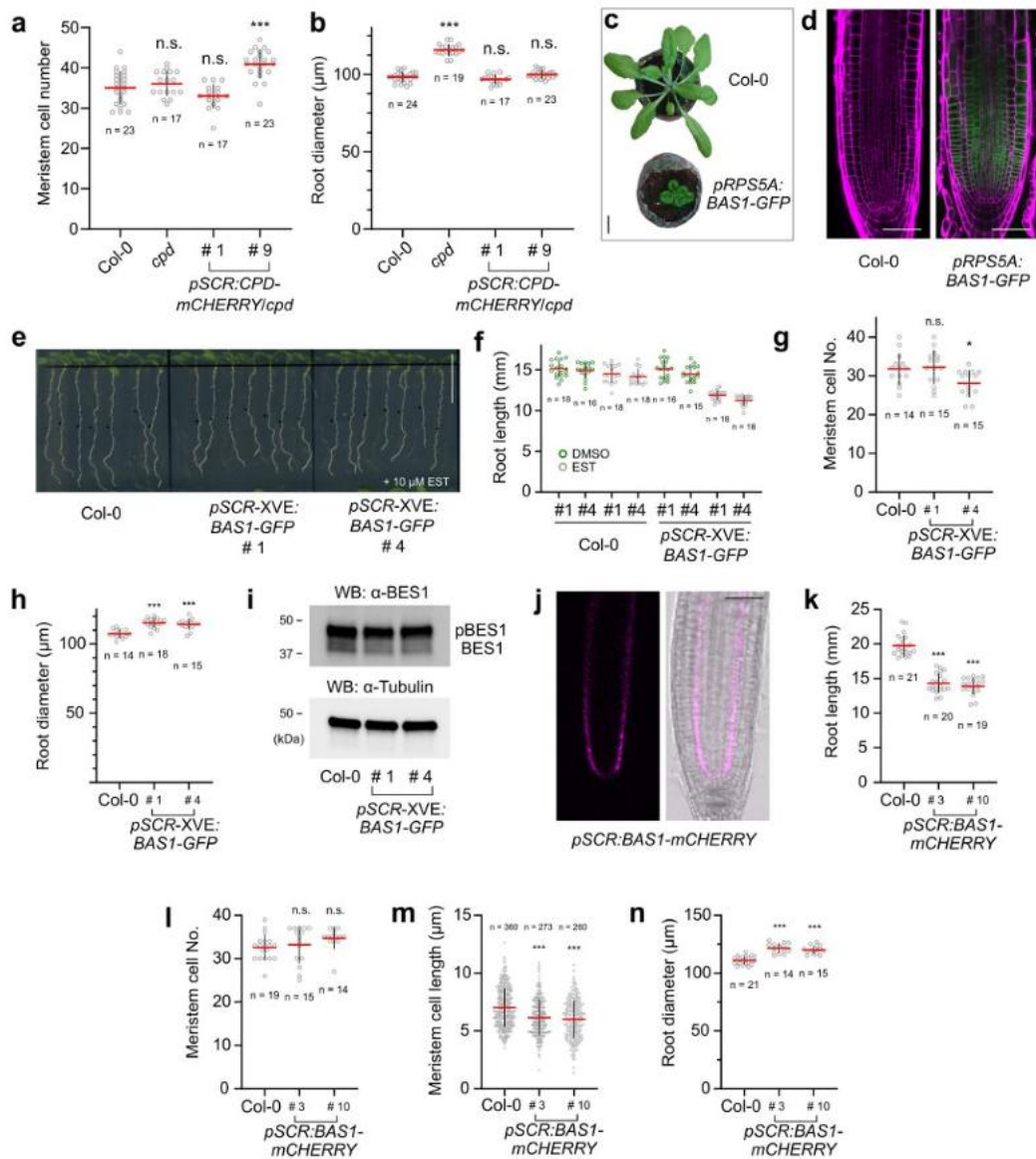
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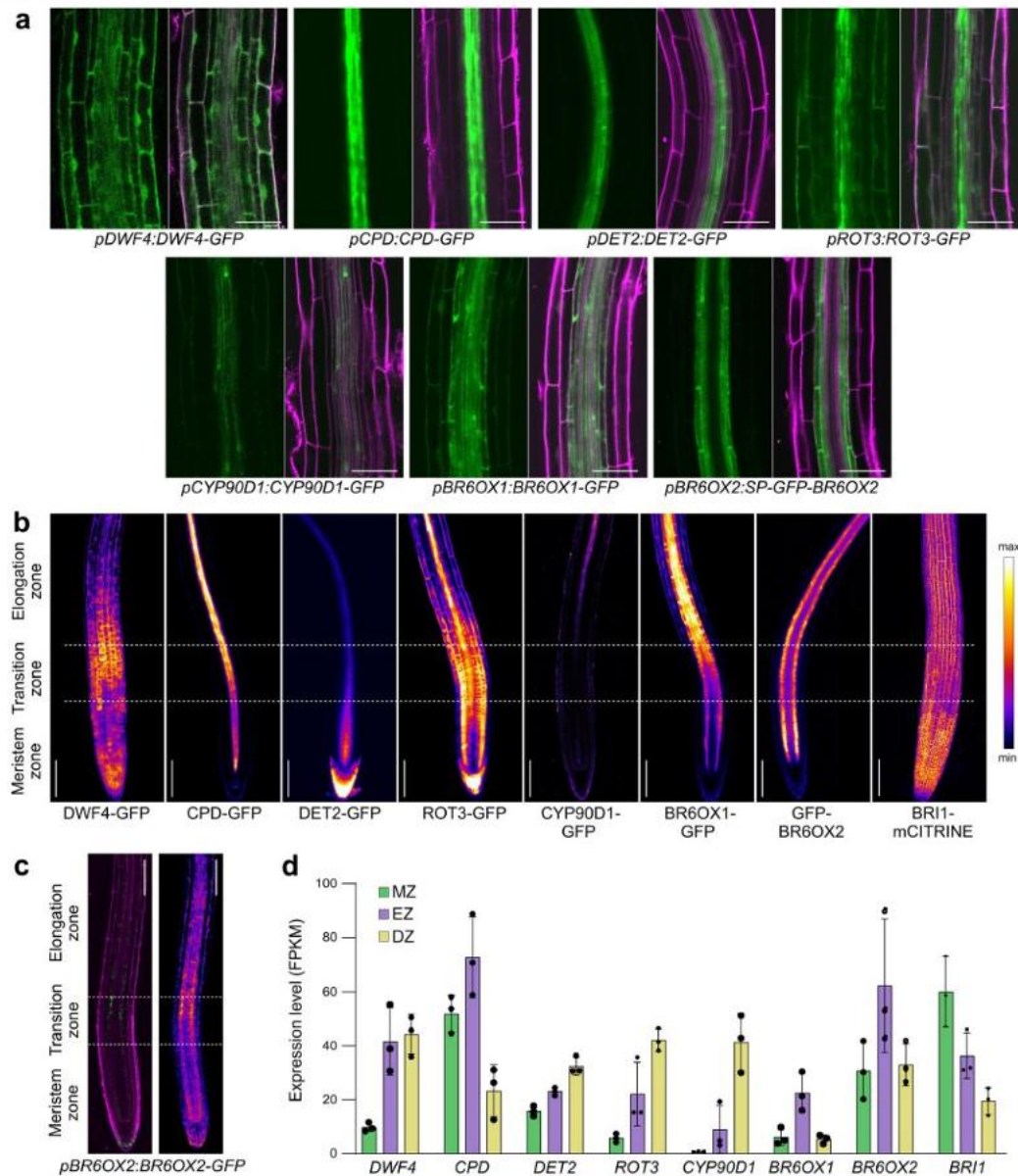


Extended Data Fig. 1 | The GFP-tagged BR biosynthetic enzymes are functional and show similar expression patterns in multiple independent transgenic lines. **a**, Rescue of the BR biosynthetic mutants by expression of the corresponding GFP-tagged enzymes under the control of their endogenous promoters. Two independent transgenic lines for each gene are compared with the wild type (Col-0) and the corresponding mutant. Six-day-old seedlings are shown. Scale bars, 1 cm. **b**, Consistent expression patterns of GFP-tagged BR biosynthetic enzymes under the control of their endogenous promoters in multiple independent transgenic lines. Root meristems of the 6-day-old seedlings from two independent transgenic lines for each GFP-tagged enzyme are shown. For seedlings expressing *pCYP90D1:CYP90D1-GFP* only the root elongation zone is shown, because the signal in the root apical meristem is extremely weak. Scale bars, 50 μ m. **c**, Six-day-old *pDWF4:DWf4-GFP* roots treated with mock (DMSO), brassinazole and brassinolide for 24 h. Scale bars, 50 μ m. **d**, Expression patterns of the *PROMOTER-NLS-GFP* reporters for all BR biosynthetic genes. For each reporter line, 6-day-old root meristems and cross section are shown. Scale bars, 50 μ m. **e**, Co-expression of *NLS-GFP* and *NLS-mCherry* reporters under the control of *CPD* and *ROT3* promoters, respectively. Scale bar, 50 μ m. Roots were stained with propidium iodide.



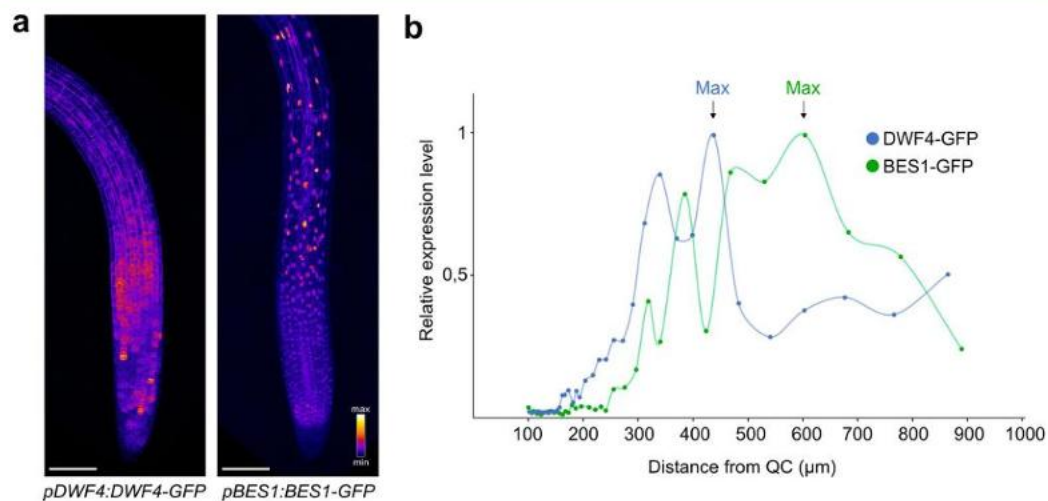
Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Ectopic expression of CPD and BAS1 in the *Arabidopsis* root. **a**, Quantification of the root meristem cell numbers of *cpd* seedlings expressing the *pSCR:CPD-mCherry* construct. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P value versus the Col-0 control for *cpd* is = 0.7077, line # 1 = 0.1849 and line # 9 < 0.0001. The experiment was repeated independently three times with similar results. **b**, Reverted root meristem diameter of the *cpd* mutant to that of the wild type upon ectopic expression of *CPD* in the endodermis. Six-day-old seedlings of the two independent transgenic lines were measured. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P value versus the Col-0 control for *cpd* is < 0.0001, line # 1 = 0.3417 and line # 9 = 0.1597. The experiment was repeated independently three times with similar results. **c**, Active BR depletion by the BAS1-GFP overexpression causing a typical BR-deficient phenotype. Scale bar, 1 cm. **d**, Thickening of the meristem and shortening of the meristem cells caused by the BAS1-GFP overexpression, typical for BR-related mutants. More than three independent experiments were performed. Representative images are shown. Scale bars, 50 μ m. **e**, Decreased root growth by the inducible, tissue-specific expression of BAS1-GFP in the endodermis. Two independent transgenic lines were compared with the wild type (Col-0). Four-day-old seedlings were transferred to fresh medium containing 10 μ M β -estradiol (EST) and imaged after 3 days. Scale bar, 1 cm. **f**, Non-induced *pSCR-XVE:BAS1-GFP* seedlings without impaired root growth. n = number of roots analysed. **g, h**, Quantification of meristem cell number (**g**) and root diameter (**h**) of EST-induced *pSCR-XVE:BAS1-GFP* seedlings. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P values versus the Col-0 control for transgenic lines are as follows: for (**g**) line # 1 = 0.9250 and line # 4 = 0.0294; for (**h**) line # 1 < 0.0001 and line # 4 < 0.0001. More than three independent experiments were performed. Representative images are shown. **i**, BES1 phosphorylation status tested by western blot with the α -BES1 antibody in roots of 7-day-old Col-0 and *pSCR-XVE:BAS1-GFP* seedlings. Tubulin was used as a loading control. Seedlings were germinated and grown on medium containing 10 μ M β -oestradiol. The experiment was performed twice with similar outcome. **j**, Confocal image of a 6-day-old root meristem expressing *pSCR:BAS1-mCherry*. More than three independent experiments were performed. Representative images are shown. Scale bar, 50 μ m. **k-n**, Quantification of root length (**k**) meristem cell number (**l**), meristem cortical cell length – first 20 cells of individual roots (**m**) and root diameter (**n**) of Col-0 and *pSCR:BAS1-mCherry* 6-day-old seedlings. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed for (**k**), (**l**) and (**n**) and n = number of cells analysed for (**m**). For (**m**) number of individual roots used in experiment are 18, 14 and 13 for Col-0, line # 3 and line # 10, respectively. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P values versus the Col-0 control for transgenic lines are as follows: for (**k**) line # 3 < 0.0001 and line # 10 < 0.0001; for (**l**) line # 3 = 0.8157 and line # 10 = 0.1336; for (**m**) line # 3 < 0.0001 and line # 10 < 0.0001; for (**n**) line # 3 < 0.0001 and line # 10 < 0.0001. All experiments were repeated independently more than three times with similar results.



Extended Data Fig. 3 | See next page for caption.

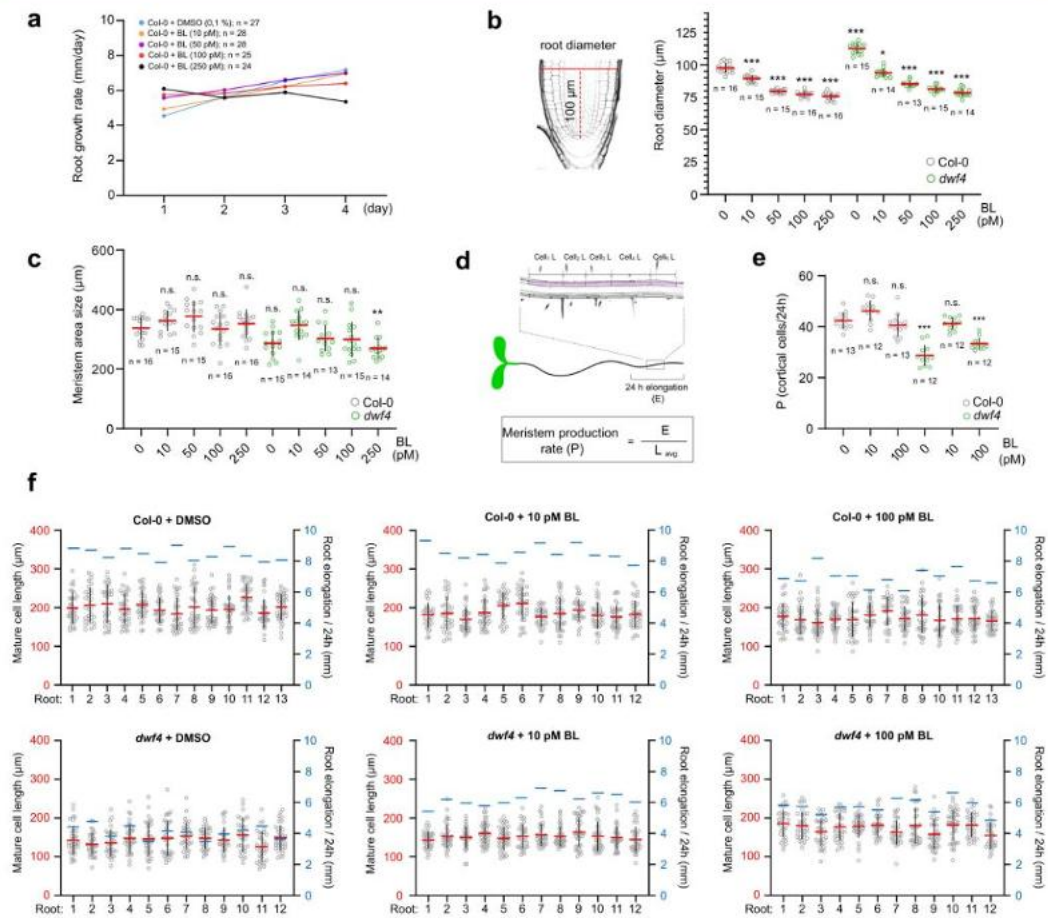
Extended Data Fig. 3 | Expression patterns of BR biosynthetic enzymes in the root elongation zone. **a**, The expression domains of several genes expanding in the root elongation zone. DWF4-GFP expression was very broad and the GFP signal could be observed in all cell types of the elongation zone. The CPD-GFP expression was restricted to the stele of the elongation zone. The DET2-GFP expression was limited to the stele, similarly to the expression of CPD-GFP. The ROT3-GFP expression had maxima in the stele, but a signal also occurred in all other cell types. The expression of the ROT3 homolog, CYP90D1, was limited to the stele. The BR6OX1 and BR6OX2 enzymes both had expression maxima in endodermis and pericycle, but the expression pattern of the BR6OX1 enzyme was expanded in all cell types. Roots were stained with propidium iodide. Scale bars, 50 μ m. **b**, Increase in the expression levels of most of the BR biosynthetic enzymes in the transition and elongation zones as visualized by colour-based fluorescence intensity coding. All reporter lines showed an increase in expression higher in the root except for DET2-GFP and GFP-BR6OX2. The signal intensity of the BR receptor, BRI1-mCITRINE in *pBRI1:BRI1-mCITRINE/bri1* line did not exhibit signal increase in the elongation zone. Maximum Z projections of the same roots from Fig. 2a are shown for BR biosynthetic enzyme lines. Scale bars, 100 μ m. **c**, Roots expressing *pBR6OX2:BR6OX2-GFP* (left panel). Increased GFP signal could be observed in the elongation zone as visualized by colour-based fluorescence intensity coding (right panel). Scale bars, 100 μ m. All transgenic lines in (**a**), (**b**) and (**c**) were imaged in more than three independent experiments. Representative images are shown. **d**, Expression levels of BR biosynthetic genes and BRI1 in the *Arabidopsis* root. All BR biosynthetic genes had an increased expression in the elongation zone, while BRI1-mCITRINE exhibited the highest expression in the meristem. Extracted from Huang and Schiefelbein (2015). MZ, meristematic zone; EZ, elongation zone; DZ, differentiation zone. Symbols depict individual values. Bars represent s.d.



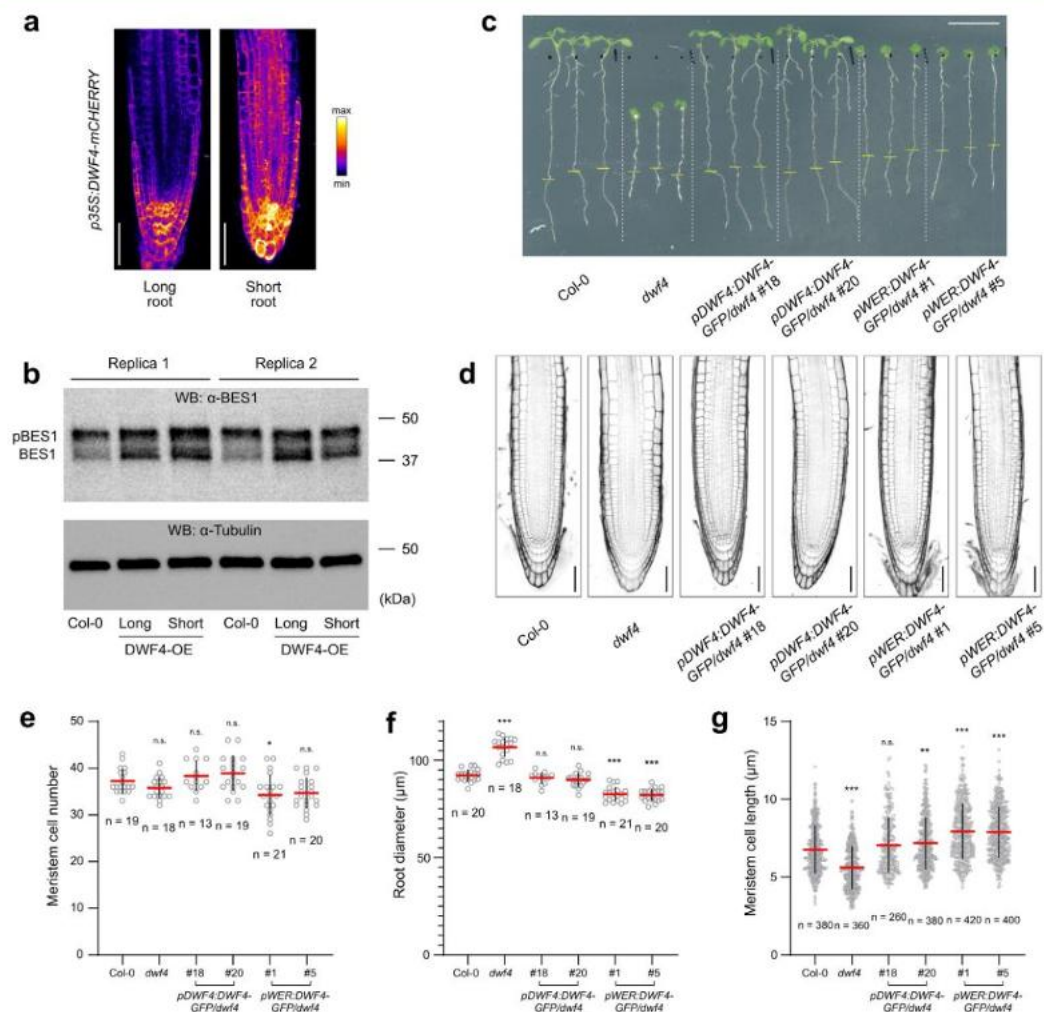
Extended Data Fig. 4 | The *DWF4* expression maximum precedes the BR signalling peak monitored by the nuclear fluorescence intensity of BES1-GFP.

a. Six-day-old *Arabidopsis* roots expressing DWF4-GFP/*dwf4* and BES1-GFP/Col-0 under the control of their native promoters. Scale bars, 100 μm.

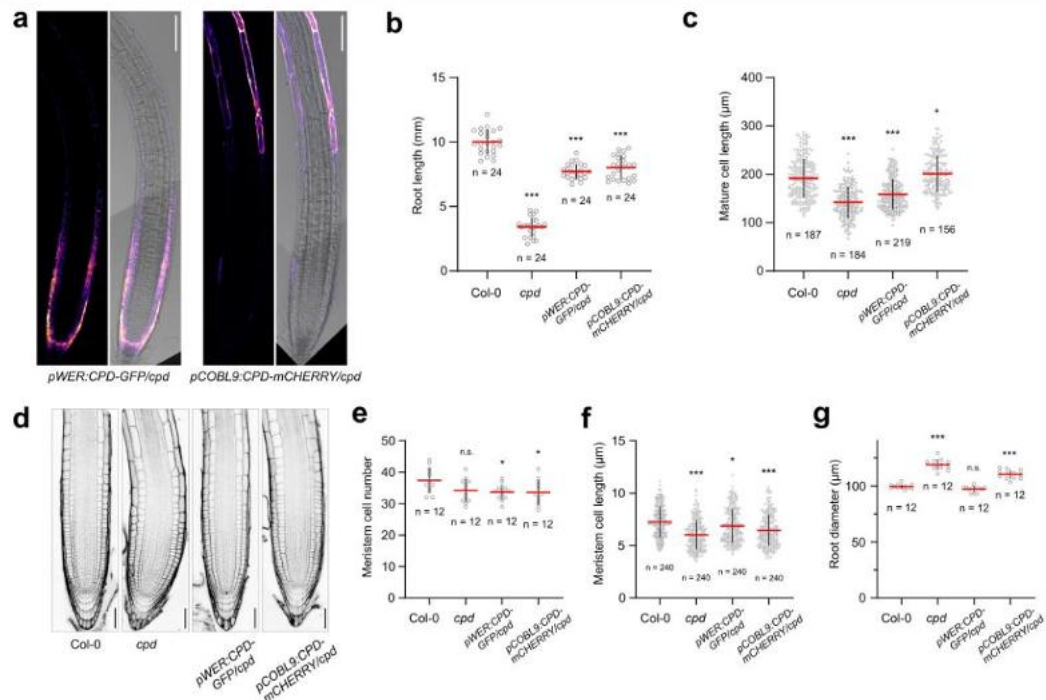
b. Fluorescence intensity of DWF4-GFP and BES1-GFP in single cells within single cell files, along the longitudinal root axis shown in **a**. Note that the DWF4-GFP expression peak precedes the BES1-GFP nuclear accumulation peak in the elongation zone. The DWF4-GFP fluorescence intensity was measured for the whole cells along the trichoblast file whereas the BES1-GFP fluorescence intensity was measured in the nuclei along cortical file.



Extended Data Fig. 5 | Effects of exogenous brassinolide (BL) on root growth. **a**, Root growth rates of Col-0 plants grown on increasing concentrations of BL. Six-day-old seedlings were transferred to fresh media containing BL or mock and root growth was followed for 4 days. Values represent the means of the root growth rates $\pm$ s.d. n = number of roots analysed. **b**, **c**, Quantification of the root meristem diameter (**b**) and the root meristem area size measured from the quiescent center to the first elongated cell (**c**) of Col-0 and *dwf4* treated with increasing concentrations of BL. Roots were treated with BL for 24 h. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. The significant differences between Col-0 and the *dwf4* seedlings treated with BL and Col-0 grown on mock (0) were determined by two-way analysis of variance (ANOVA) and Tukey's multiple comparisons tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P values versus the Col-0 + DMSO control for different treatments are as follows: for (**b**) Col-0 + 10 pM BL < 0.0001 , Col-0 + 50 pM BL < 0.0001 , Col-0 + 100 pM BL < 0.0001 , Col-0 + 250 pM BL < 0.0001 , *dwf4* + DMSO < 0.0001 , *dwf4* + 10 pM BL = 0.0142, *dwf4* + 50 pM BL < 0.0001 , *dwf4* + 100 pM BL < 0.0001 , *dwf4* + 250 pM BL < 0.0001 ; for (**c**) Col-0 + 10 pM BL = 0.9019, Col-0 + 50 pM BL = 0.3871, Col-0 + 100 pM BL = 0.9999, Col-0 + 250 pM BL = 0.9972, *dwf4* + DMSO = 0.0638, *dwf4* + 10 pM BL > 0.9999 , *dwf4* + 50 pM BL = 0.5746, *dwf4* + 100 pM BL = 0.3938, *dwf4* + 250 pM BL = 0.0037. All experiments were repeated independently more than three times with similar results. **d**, Experimental setup for measurements of the root meristem cell production rate (left panel). Six-day-old Col-0 and *dwf4* seedlings were transferred to agar plates supplemented with BL and mock, and after 3 days of growth, elongation of individual roots was recorded for the period of 24 h (E). Subsequently, roots were imaged with confocal microscope, and the average mature cell length was calculated (L_{avg}) for each root and used to calculate the meristem production rate (P). **e**, Quantification of the root meristem production rate (P) for cortical cells. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots analysed. The significant differences between Col-0 and the *dwf4* seedlings treated with BL and Col-0 grown on mock (0) were determined by two-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P value versus the Col-0 + DMSO control for Col-0 + 10 pM BL is = 0.0874, Col-0 + 100 pM BL = 0.7849, *dwf4* + DMSO < 0.0001 , *dwf4* + 10 pM BL = 0.9623 and *dwf4* + 100 pM BL < 0.0001 . The experiment was repeated independently three times with similar results. **f**, Quantification of mature cell length of individual roots used to calculate the meristem production rate in (**e**). All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d.



Extended Data Fig. 6 | Ectopic expression of DWF4 enzyme. **a**, Confocal images of meristems of long and short *p35S:DWF4-mCherry* (DWF4-OE) roots. Six-day-old seedlings were used for imaging. Scale bars, 50 μ m. Confocal imaging was performed in three independent experiments. Representative images are shown. **b**, Two biological replicates of the western blot shown in Fig. 6c. The experiment was repeated independently three times with similar results. **c**, Eight-day-old seedlings expressing DWF4-GFP in *dwf4* mutant background under the control of native or *WEREWOLF* (WER) promoters. Two independent transgenic lines for each construct were compared with wild type (Col-0) and *dwf4*. Plants were grown for 6 days until the point where homozygous *dwf4* plants could be recognized and transferred to fresh agar plates. Root tips were marked (yellow bar), and grown for two additional days. **d**, Confocal images of root meristems of Col-0, *dwf4* and two independent *dwf4* mutant lines expressing DWF4-GFP under the control of the DWF4 and WER promoters. Six-day-old plants were imaged. Roots were stained with propidium iodide. All transgenic lines were imaged in more than three independent experiments. Representative images are shown. Scale bars, 50 μ m. **e–g**, Quantification of cortical meristem cell number (**e**), root meristem diameter (**f**) and cortical meristem cell length of the first 20 cells of individual roots (**g**). All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. n = number of roots (**e**, **f**) and cells (**g**). For (**g**) number of individual roots used in the experiment are 19, 18, 13, 19, 21 and 20 for Col-0, *dwf4*, line # 18, line # 20, line # 1 and line # 5, respectively. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P value versus the Col-0 control for different transgenic lines were as follows: for (**e**) *dwf4* = 0.5724, line # 18 = 0.8377, line # 20 = 0.4041, line # 1 = 0.0308 and line # 5 = 0.0763; for (**f**) *dwf4* < 0.0001, line # 18 = 0.7739, line # 20 = 0.1982, line # 1 < 0.0001 and line # 5 < 0.0001; for (**g**) *dwf4* < 0.0001, line # 18 = 0.0938, line # 20 = 0.0022, line # 1 < 0.0001 and line # 5 < 0.0001. The experiment was repeated independently three times with similar results.



Extended Data Fig. 7 | Confined expression of CPD enzyme can only locally rescue the root phenotype of *cpd* mutant. **a**, Six-day-old *cpd* mutant roots expressing CPD-GFP and CPD-mCHERRY under the control of *WEREWOLF* (*WER*) and *COBRA-LIKE9* (*COBL9*) promoters, respectively. All transgenic lines were imaged in more than three independent experiments. Representative images are shown. Scale bars, 100 μ m. **b**, **c**, Quantification of the root lengths (**b**) and mature cortical cell lengths (**c**) of the Col-0, *cpd*, *pWER:CPD-GFP/cpd* and *pCOBL9:CPD-mCHERRY/cpd* lines. All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. $n =$ number of roots (**b**) and cells (**c**) analysed. For (**c**) the number of individual roots used in the experiment was 12 for all lines. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P value versus the Col-0 control for different transgenic lines are as follows: for (**b**) *cpd* < 0.0001 , *pWER:CPD-GFP* < 0.0001 and *pCOBL9:CPD-mCHERRY* < 0.0001 ; for (**c**) *cpd* < 0.0001 , *pWER:CPD-GFP* < 0.0001 and *pCOBL9:CPD-mCHERRY* = 0.049. The experiment was repeated independently three times with similar results. **d**, Confocal images of root meristems of Col-0, *cpd*, *pWER:CPD-GFP/cpd* and *pCOBL9:CPD-mCHERRY/cpd* lines. All transgenic lines were imaged in more than three independent experiments. Representative images are shown. Scale bars, 50 μ m. **e**–**g**, Quantification of cortical meristem cell number (**e**), cortical meristem cell length of the first 20 cells of individual roots (**f**) and root meristem diameter (**g**). All individual data points are plotted. Red horizontal bars represent the means and error bars represent s.d. $n =$ number of roots (**e**, **g**) and cells (**f**). For (**f**) the number of individual roots used in the experiment was 12 for all lines. The significant differences between transgenic lines and the Col-0 control were determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. The P value versus the Col-0 control for different transgenic lines are as follows: for (**e**) *cpd* = 0.0808, *pWER:CPD-GFP* = 0.0370 and *pCOBL9:CPD-mCHERRY* = 0.0323; for (**f**) *cpd* < 0.0001 , *pWER:CPD-GFP* = 0.011 and *pCOBL9:CPD-mCHERRY* = < 0.0001 ; for (**g**) *cpd* < 0.0001 , *pWER:CPD-GFP* = 0.2712 and *pCOBL9:CPD-mCHERRY* = < 0.0001 . The experiment was repeated independently three times with similar results.

Příloha 10

Plasmodesmata Mediate Cell-to-Cell Transport of Brassinosteroid Hormones

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Plasmodesmata mediate cell-to-cell transport of brassinosteroid hormones

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Brassinosteroids (BRs) are steroidal phytohormones that are essential for plant growth, development and adaptation to environmental stresses. BRs act in a dose-dependent manner and do not travel over long distances; hence, BR homeostasis maintenance is critical for their function. Biosynthesis of bioactive BRs relies on the cell-to-cell movement of hormone precursors. However, the mechanism of the short-distance BR transport is unknown, and its contribution to the control of endogenous BR levels remains unexplored. Here we demonstrate that plasmodesmata (PD) mediate the passage of BRs between neighboring cells. Intracellular BR content, in turn, is capable of modulating PD permeability to optimize its own mobility, thereby manipulating BR biosynthesis and signaling. Our work uncovers a thus far unknown mode of steroid transport in eukaryotes and exposes an additional layer of BR homeostasis regulation in plants.

Brassinosteroids (BRs) are steroidal phytohormones that are crucial for plant growth and regulate diverse developmental processes, such as cell elongation, cell division, photomorphogenesis and xylem differentiation, as well as biotic and abiotic stress responses¹. BRs are perceived by the plasma membrane-localized receptor kinase BR INSENSITIVE 1 (BRI1) and its coreceptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) in the apoplast, triggering sequential phosphorylation between BRI1 and BAK1 (ref. 1). This step is required for BRI1 activation and leads to downstream phosphorylation and dephosphorylation events that activate the transcription factors, such as BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1)/BZR2, either to induce or to repress genes in the nucleus as the final outcome of the BR response^{2,3}.

Normally, the hormone level at any given site within the plant is determined by the relative rates of biosynthesis, catabolism and transport⁴. Until now, a negative transcriptional feedback loop has been considered the main regulatory mechanism of BR biosynthesis and catabolism, in which the transcription of BR biosynthetic and catabolic genes is directly regulated by BES1 and BZR1 (refs. 5,3). Nevertheless, in the *Arabidopsis thaliana* root, the elongation zone is the site of maximum BR biosynthetic expression⁶ and signaling levels, indicating that additional mechanisms control the expression of biosynthetic enzymes. In the root meristem, the expression domains of BR biosynthetic enzymes are separated, implying cell-to-cell movement of hormone precursors⁶ to allow the biosynthetic pathway completion. Hence, the question arises if BR homeostasis in the root meristem is

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also mediated by short-distance BR transport, as well as what is the mode of BR movement between neighboring cells.

BRs are derived from campesterol and converted to brassinolide (BL), the most bioactive BR, after a series of reactions, including reduction, hydroxylation, epimerization and oxidation⁷. All enzymes involved in BR biosynthesis, except the steroid 5- α -reductase, de-etiolated 2 (DET2), are members of the cytochrome P450 (CYP) protein superfamily⁷. The CYPs are well conserved and are predominantly localized in the endoplasmic reticulum (ER)⁸ membrane. For two of the BR CYPs, DWARF4 (DWF4) and CYP85A2, an ER localization has already been demonstrated^{9,10}. Nonetheless, how BRs exit the cell after their synthesis in the ER membranes and how they are transported between neighboring cells is still unknown. The mammalian steroid hormones, after their release from steroidogenic cells, are transported to the target cells through the blood by their carrier proteins¹¹. However, no homologs of the mammalian steroid carrier proteins are found in plants, and BRs do not undergo long-distance movements¹².

Multicellular organisms rely on cell-to-cell communication to coordinate growth and development across tissues and organs¹³. In plants, plasmodesmata (PD) are critical cytoplasmic communication channels¹⁴. PD cross the cell walls of adjacent cells and enable intercellular movement of nutrients, hormones, RNAs, proteins, metabolites and viruses^{15–19}. The typical structure of PD consists of a cytoplasmic sleeve, a plasma membrane leaflet and a desmotubule, a membranous cortical ER-derived rod, connecting adjacent cells²⁰. PD connectivity is exceptionally dynamic, responds to both internal and external stimuli and is regulated by the deposition of callose, a β -(1,3)-glucan polymer, in the PD neck²⁰. Localized production of the polymer by callose synthases can narrow the cytoplasmic sleeve, whereas degradation mediated by β -1,3-glucanases can widen the channel²⁰. Nevertheless, PD conductivity is not directly correlated with the size of the cytoplasmic sleeve²¹, and how exactly PD mediate the exchange of molecules between neighboring cells remains poorly understood. PD number is also controlled because, for instance, secondary PD are often formed during cell growth either to maintain or to increase the PD density in expanding cell walls²². Whether BR precursors are transported via PD to complete the biosynthetic pathway or initiate signaling in adjacent cell files is not known.

Here we corroborate the subcellular localization of BR biosynthetic enzymes in the ER membranes, where hormone biosynthesis likely occurs. By combining genetic and bioorthogonal chemistry approaches, we demonstrate that PD are involved in the short-distance transport of BR precursors, which is required for biosynthetic pathway completion. Finally, we provide evidence that the intracellular BR content negatively regulates PD permeability, possibly through BR signaling inducing callose deposition in the cell walls near the neck zone of the PD and, ultimately, affecting its own mobility and biosynthesis efficiency. Our work reveals an unidentified transport mode of steroidal hormones in eukaryotes and presents an additional layer of BR biosynthesis regulation in plants.

Results

Symplastic communication modulates BR signaling

To determine the subcellular localization of BR biosynthetic enzymes, we co-expressed the GFP-tagged rotundifolia 3 (ROT3) and DWF4 with the mCherry-tagged ER membrane-resident marker cinnamate 4-hydroxylase (C4H)²³ in *Nicotiana benthamiana* (tobacco) leaves (Extended Data Fig. 1a,c) and in roots of transgenic *Arabidopsis* plants (Extended Data Fig. 1b,d). As reported earlier for some *Arabidopsis* CYP proteins^{9,10,24}, both enzymes localized in the ER membrane, marking it as the likely site of BR biosynthesis within cells.

Previously we showed that BRs move between neighboring cell files in the roots of *Arabidopsis*⁵. Given that BR biosynthetic enzymes are localized in the ER membranes and the hormone is synthesized intracellularly, we hypothesized that the exchange of BRs between adjacent cell files occurs via PD. First, we asked whether the sites of BR biosynthesis might coincide with PD, which could facilitate hormone transport. To this end, we examined the colocalization of the BR biosynthetic enzyme, DWF4–GFP, and the mCherry-tagged PD marker, PD-located protein 1 (PDL1)²⁵, in tobacco leaves (Extended Data Fig. 1e) and estimated the PD enrichment by calculating the PD index²⁵ (Extended Data Fig. 1f). DWF4–GFP exhibited a higher PD index than the ER lumen marker HDEL–BFP, indicating a PD association, but these values were lower than those for MCTP3–GFP, a PD-resident protein²⁶. However, in the roots of *Arabidopsis* plants co-expressing ROT3–GFP and DWF4–GFP with PDL1–mCherry, no clear enrichment of the GFP signal at the PD was found (Extended Data Fig. 1g,h). These findings suggest that the BR biosynthetic machinery might be loosely associated with PD and only in certain cell types. Second, to test if BRs move through the PD, we examined the root growth of transgenic *Arabidopsis* plants expressing the gain-of-function callose synthase 3 (CALS3) from the estradiol (EST)-inducible endodermis 7 (EN7) promoter (*pEN7:icals3m*) that can block PD by callose deposition in the PD neck in the endodermis after induction²⁷ (Fig. 1a). The phenotypic analysis showed that after a 48-h EST treatment, primary root growth was arrested and that the length of the mature cortical cells was substantially shorter than that of the mock-treated plants (Fig. 1b–d). To investigate whether blocking PD in the endodermis affects BR signaling, we analyzed BR-specific phenotypes in the root meristems⁵. To look at relatively early BR signaling responses and minimize the pleiotropic effect of PD closure, we decreased the induction time to 12 h, as this condition was sufficient to observe callose production (Extended Data Fig. 2c). Callose deposition reduced the average length of meristematic cortical cells and slightly increased the root diameter (Extended Data Fig. 2a,b), suggesting decreased BR signaling levels in *pEN7:icals3m* roots after induction. We then analyzed the accumulation of dephosphorylated BES1 (dBES1), which can be used as a readout for BR signaling activation². Compared to the wild-type accession Columbia-0 (Col-0), BES1 was slightly less dephosphorylated in the

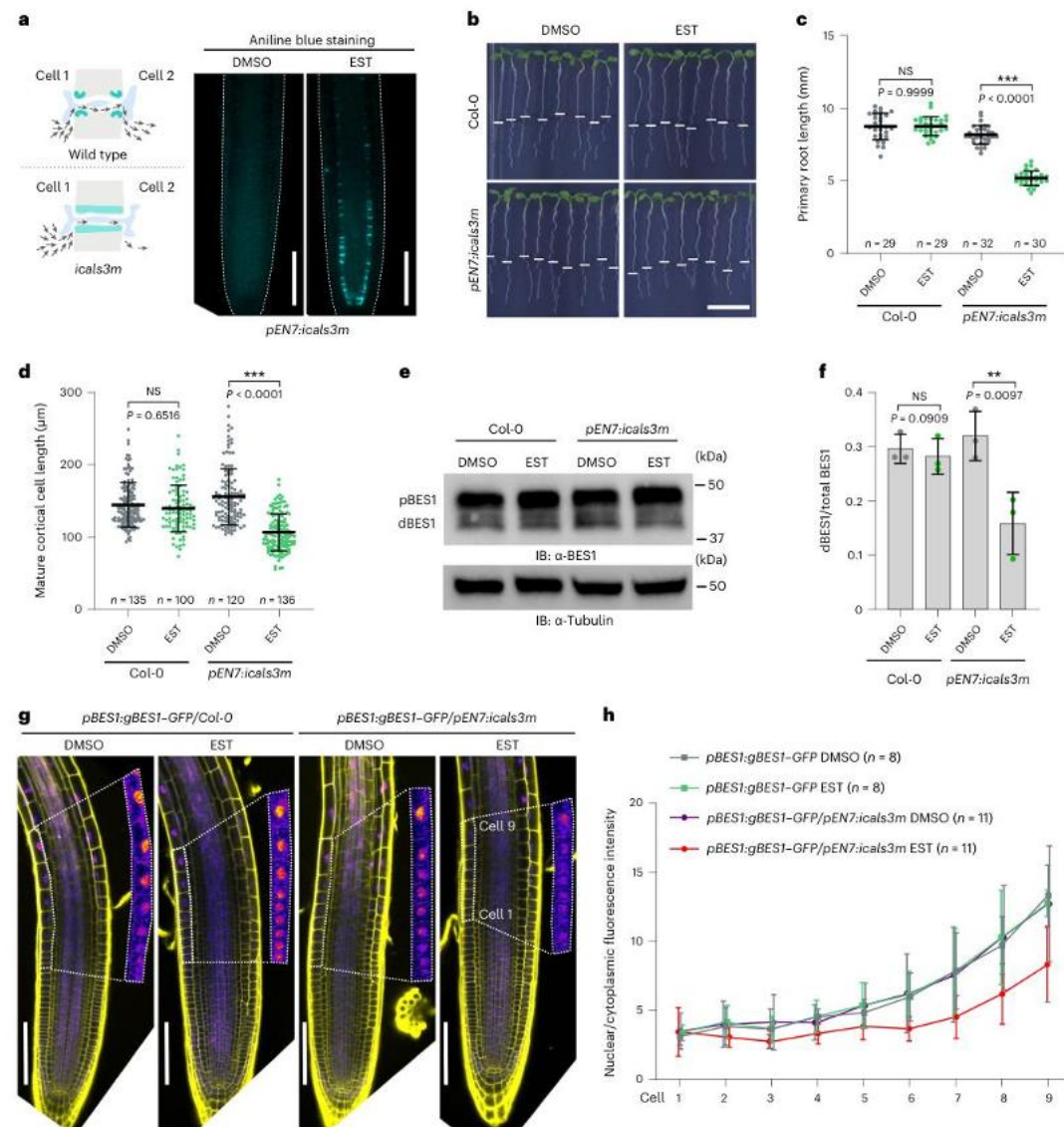
Fig. 1 | PD permeability modulates BR signaling. **a**, Increased callose biosynthesis at plasmodesmata in *icals3m* expressing plants inhibits the exchange of molecules between neighboring cells (left). Aniline blue staining of callose deposition in endodermis of the root tips of *pEN7:icals3m* plants upon EST (5 μ M) or DMSO (mock) treatment for 48 h (right). Scale bars, 100 μ m. **b**, Induction of callose deposition (48-h EST treatment) in the endodermis of *pEN7:icals3m* seedlings causes root growth defects. Root tips were marked immediately after the transfer (white bars). Scale bar, 1 cm. **c,d**, Quantification of the primary root length (c) and mature cortical cell length (d) of seedlings shown in b. All individual data points are plotted. Horizontal and error bars represent the means and s.d., respectively. *n*, number of roots used in c and cells used in d. The significant differences were determined with one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. **e**, Phosphorylation status of BES1 detected by immunoblotting with α -BES1 antibody. Tubulin detected with α -tubulin antibody was used as a loading control. Root tips of 5-day-old *Arabidopsis*

seedlings grown as in a and induced for 12 h were used. **f**, Quantification of BES1 dephosphorylation in e presented as a ratio of dBES1 relative to the total BES1. The significant difference was determined with a two-tailed Student's paired *t*-test analysis. Error bars represent s.d. **g**, Confocal images of root tips of *pBES1:gBES1-GFP/Col-0* and *pBES1:gBES1-GFP/pEN7:icals3m* seedlings. Five-day-old seedlings were transferred to agar media containing EST (5 μ M) or DMSO (mock) and imaged after 12 h. Cell walls were stained with PI. Nine cells used for fluorescence intensity measurements in h are shown in inset panels (1.5 $\times$ enlarged). In the inset panels, only GFP signal is shown. Brightness and contrast were equally adjusted for each treatment. Scale bars, 100 μ m. **h**, Quantification of nuclear/cytoplasmic BES1–GFP fluorescence intensity ratio of epidermal cells in the root transition zone (nine cells shown in the inset panels in g). Values and error bars represent means and s.d., respectively. *n*, number of roots used for each treatment. ****P* < 0.001 and ***P* < 0.01 for c,d and f. For b–h, experiments were performed in three repeats with similar results. IB, immunoblotting.

callose-induced roots (Fig. 1e,f), indicating a decrease in BR signaling. In addition, we introduced the *pBES1:gBES1-GFP* reporter²⁸ into the *pEN7:icals3m* plants²⁷ and examined the nuclear accumulation of BES1, which is also used as an indicator of BR signaling activation². The nuclear/cytoplasmic fluorescence intensity ratio of BES1-GFP was reduced in root epidermal cells after 12 h of EST treatment (Fig. 1g,h), possibly as a result of decreased BR signaling. These findings suggest that the reduced PD permeability might compromise BR signaling, probably due to impaired transport of BRs through the PD.

Limiting the PD transport can be detrimental to root growth because numerous signaling components and nutrients are transported through these intercellular channels^{15–19}. To overcome, or at least

limit the pleiotropic effects of callose-plugged PD on root growth and uncouple BR-specific effects, we ectopically expressed the PD-located protein 5 (PDLPS)-BFP²⁹ fusion to induce overaccumulation of callose at the PD in the native domain of the BR biosynthetic enzyme, constitutive photomorphogenesis and dwarfism (CPD) in the *pCPD:CPD-GFP/cpd* (Extended Data Fig. 2d,e). This fusion allowed us to spatially link CPD enzyme activity with ectopic callose deposition in the central part of the root, the stele, and minimize the interruption of movement of other PD-transported molecules. Roots of this transgenic line grew more slowly than those of *pCPD:CPD-GFP/cpd* (Extended Data Fig. 2f,g) and exhibited BR-deficient phenotypes (Extended Data Fig. 2h,i). Additionally, an increase in phosphorylated BES1 (pBES1)



indicated reduced BR signaling levels in roots expressing PDLP5-BFP (Extended Data Fig. 2j).

To validate our findings that impaired symplastic transport negatively regulates BR signaling, we compared the effect of PDLP5-BFP expression under the *CPD* promoter in *pCPD:CPD-GFP/cpd* and *pSCR:CPD-mCherry/cpd* backgrounds. When the *CPD-mCherry*⁶ enzyme was ectopically expressed in the endodermis, milder growth defects were observed (Extended Data Fig. 2d–i), as well as less affected BR signaling levels (Extended Data Fig. 2j). *CPD* is a BR biosynthetic enzyme with the most restricted expression domain in the stele, whereas all other enzymes are, at least partially, expressed in other tissues⁶. Therefore, displacement of *CPD* enzyme expression to the endodermis allowed possible movement of BR precursors and completion of the pathway by bypassing PDLP5-BFP-induced callose deposition and the reduced PD connectivity in the stele, respectively.

Subsequently, we assessed the BR-related phenotypes and BR signaling in transgenic *Arabidopsis* plants overexpressing the callose-degrading enzyme plasmodesmal-localized β -1,3-glucanase 1 (PdBG1)³⁰ (*p35S:PdBG1-mCitrine/Col-0*, PdBG1-OE), which have increased PD permeability as a result of enhanced callose turnover (Extended Data Fig. 3a,b). The phenotypic analysis revealed that the length of the primary roots of PdBG1-OE seedlings was substantially longer than that of the wild type, with longer meristematic cells and a slightly smaller root radius (Extended Data Fig. 3d–f), thus, resembling, to a certain extent, the phenotype of plants with constitutive BR responses⁶. In agreement, the PdBG1-OE plants accumulated more dBES1 than the control (Extended Data Fig. 3g,h), whereas the expression of *DWF4* was downregulated (Extended Data Fig. 3i), indicating that the increased cell-to-cell connectivity positively affected BR signaling. Taken together, our results show that PD permeability can moderately modulate BR signaling levels.

PD mediate BR movement

We hypothesized that the impaired BR signaling in different PD mutants was due to the altered mobility of BR precursors. To test this assumption, we employed BR precursor feeding experiments with 22-hydroxycampesterol[22-OHCR (compound 1); Supplementary Note 1], which is the product and the substrate of *DWF4* and *CPD* enzymes, respectively (Extended Data Fig. 4a). As expected, exogenous 22-OHCR (500 nM) was able to rescue the root growth of *dwf4* and to dephosphorylate BES1 in this mutant, similarly to BL (500 pM), whereas it was inactive when applied to the *cpd* mutant (Extended Data Fig. 4b–d). Next, the inducible *pEN7:icals3m* construct was introduced into the *dwf4* mutant to conditionally perturb PD permeability in the endodermis. We postulated that after induction of callose deposition at PD in the endodermis, exogenous BL would reach epidermal and cortical cell layers, where BR signaling is sufficient to rescue the root growth of BR-related mutants³¹ (Fig. 2a). Conversely, the inactive BR precursor 22-OHCR would need to move to the stele, where the *CPD* enzyme is expressed, to be converted into the following BR precursor and, finally, to BL to rescue *dwf4* root growth. In a scenario in which BRs move via the PD, callose-induced PD blockage in the endodermis would interrupt the biosynthetic pathway and exogenous 22-OHCR would not rescue the *dwf4* defects (Fig. 2a). Consistent with this hypothesis, we found that without callose induction, both exogenous 22-OHCR (500 nM) and BL (500 pM) rescued the root growth defects of *pEN7:icals3m/dwf4* plants, including primary root and mature cortical cell lengths (Fig. 2b–d). However, after blocking PD in the endodermis, 22-OHCR was less effective in rescuing the *dwf4* root growth defects than BL (Fig. 2b–d and Extended Data Fig. 4e–g).

To further corroborate the different capacities of exogenous BL and 22-OHCR to rescue *dwf4* when PD were closed in the endodermis, we evaluated BR signaling by immunoblot analysis of the phosphorylation status of BES1. Because the *EN7* promoter is only active in the root tip, we collected root tips for immunoblotting. In accordance

with the observed root phenotypes, BR signaling was less efficiently recovered by 22-OHCR than by BL after callose induction (Fig. 2e). Then, we introduced the *pBES1:gBES1-GFP* construct into *pEN7:icals3m/dwf4* plants and quantified the nuclear accumulation of BES1-GFP in the root epidermis as a BR signaling readout. As predicted, BR signaling in the mock-treated *pBES1:gBES1-GFP/pEN7:icals3m/dwf4* seedlings was enhanced by exogenous BL and 22-OHCR with equal efficiency (Fig. 2f,g), but it was only partially complemented by 22-OHCR in the EST-treated seedlings (Fig. 2f,g).

Taken together, these results suggest that PD closure renders BR biosynthesis inefficient, possibly due to the necessity for BR precursors to be exchanged through PD between adjacent root cell files.

Direct visualization of BR movement

To validate the PD-mediated intercellular transport of BRs and directly visualize BRs in *Arabidopsis* roots, we used a bioorthogonal chemistry approach. To this end, castasterone-alkyne (CSA, 2) was synthesized by the introduction of an alkyne group attached to a linker connected to the C-6 position of the castasterone (CS) molecule (Fig. 3a and Supplementary Note 2), which is the direct precursor of BL (Extended Data Fig. 4a). CSA can be visualized after cycloaddition or a click reaction between its alkyne and the azide group of the commercially available green-emitting fluorescent dye azide-BODIPY-fluorescein (azide-BDP-FL)³² to generate CS-BDP-FL (3; Fig. 3a). Although slightly less potent than CS, CSA retained biological activity, because it rescued the short-root phenotype of the *dwf4* mutant and induced BES1 dephosphorylation (Fig. 3b and Extended Data Fig. 5a,b). Subsequently, a click reaction was carried out on transgenic *Arabidopsis* lines with perturbed PD permeability. Initially, the *pEN7:icals3m* line was used, which has increased callose deposition (Fig. 1a) and closed PD in the endodermis after EST induction. In the absence of CSA, no signal was detected in the mock- and EST-treated seedlings (Fig. 3c), indicating that the azide-BDP-FL fluorescent dye did not bind nonspecifically, whereas in the presence of CSA and after a click reaction with azide-BDP-FL, the CS-BDP-FL signal occurred only in epidermal and cortical cells in EST-treated roots, but it was ubiquitous in all cell files in noninduced roots (Fig. 3c,d). These findings suggest that closed PD in the endodermis block the CSA movement from the cortex to the inner tissues.

Consequently, a similar click reaction was carried out with a line expressing *icals3m* from the *WEREWOLF* (*WER*) promoter³³, *pWER:icals3m*. This line specifically accumulated callose in epidermal cells, presumably blocking PD in this tissue (Extended Data Fig. 5c), and caused root growth inhibition (Extended Data Fig. 5d,e). In the root tips of the *pWER:icals3m* seedlings treated with CSA followed by a click reaction, the fluorescent CS-BDP-FL signal accumulated preferentially in epidermal cells when compared to the Col-0 control (Fig. 3e,f), implying that PD closure in the epidermal cells is sufficient to block CSA from moving to the inner tissues. Notably, the uptake of CSA in the epidermal cells was not compromised by extensive callose deposition (Extended Data Fig. 5f), hinting that the lack of fluorescent signal in the inner tissues of the *pWER:icals3m* line is due to the compromised symplastic transport and not to impaired compound uptake. Finally, CSA click chemistry experiments were conducted with the PdBG1-OE plants³⁰, which have open PD. The accumulation of the fluorescent signal in all cell layers was higher than that of the Col-0 control (Fig. 3g,h), suggesting an enhanced accumulation of CSA in the root when the PD aperture is increased. In conclusion, bioorthogonal chemistry allowed the direct visualization of a bioactive BR precursor in plant tissues, confirming the involvement of PD in intercellular BR transport.

BRs modulate PD permeability

As BRs are transported through the PD, we queried whether BRs could, in turn, modulate PD permeability. To investigate this hypothesis, we visualized callose deposition by means of a callose-specific antibody in wild-type Col-0 plants treated with either BL (200 nM) or the

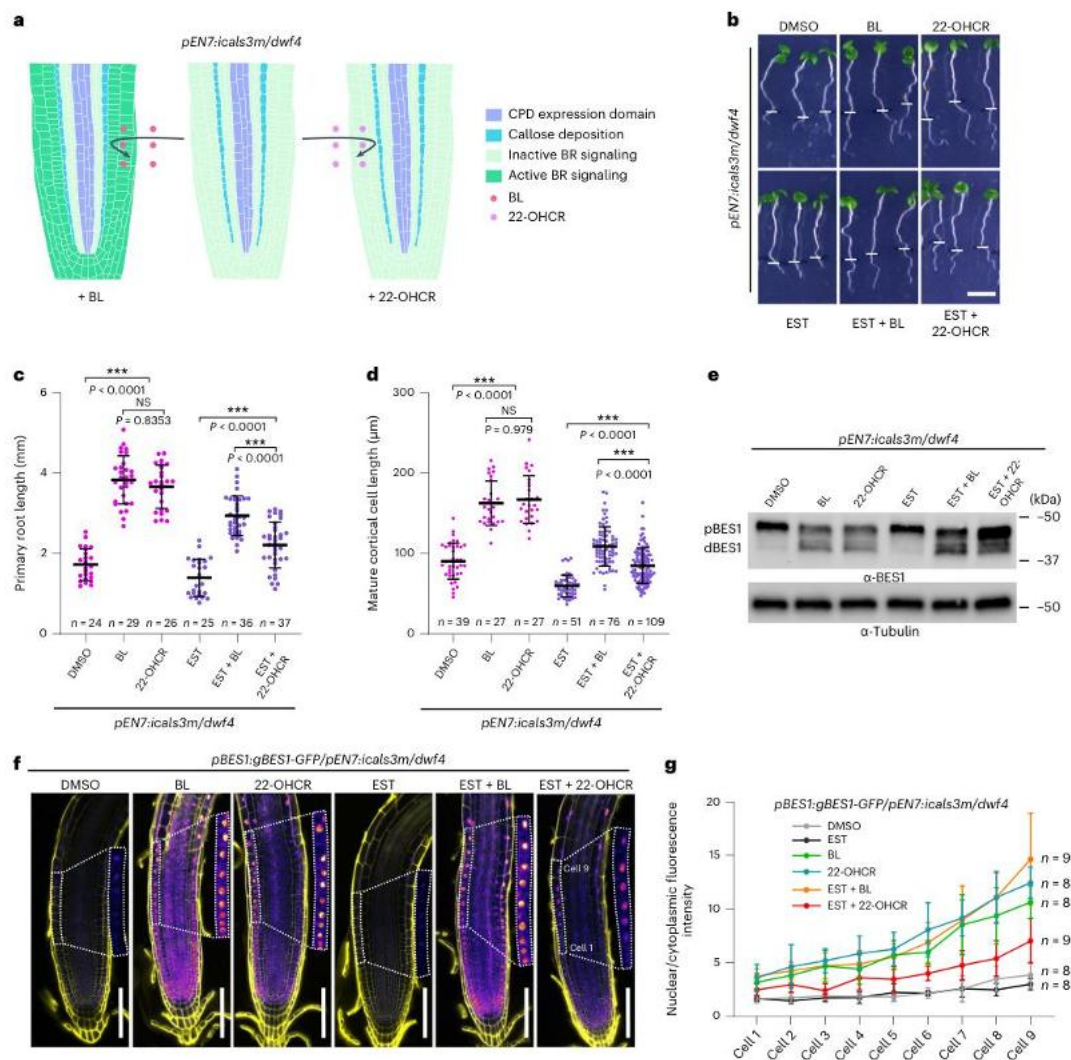
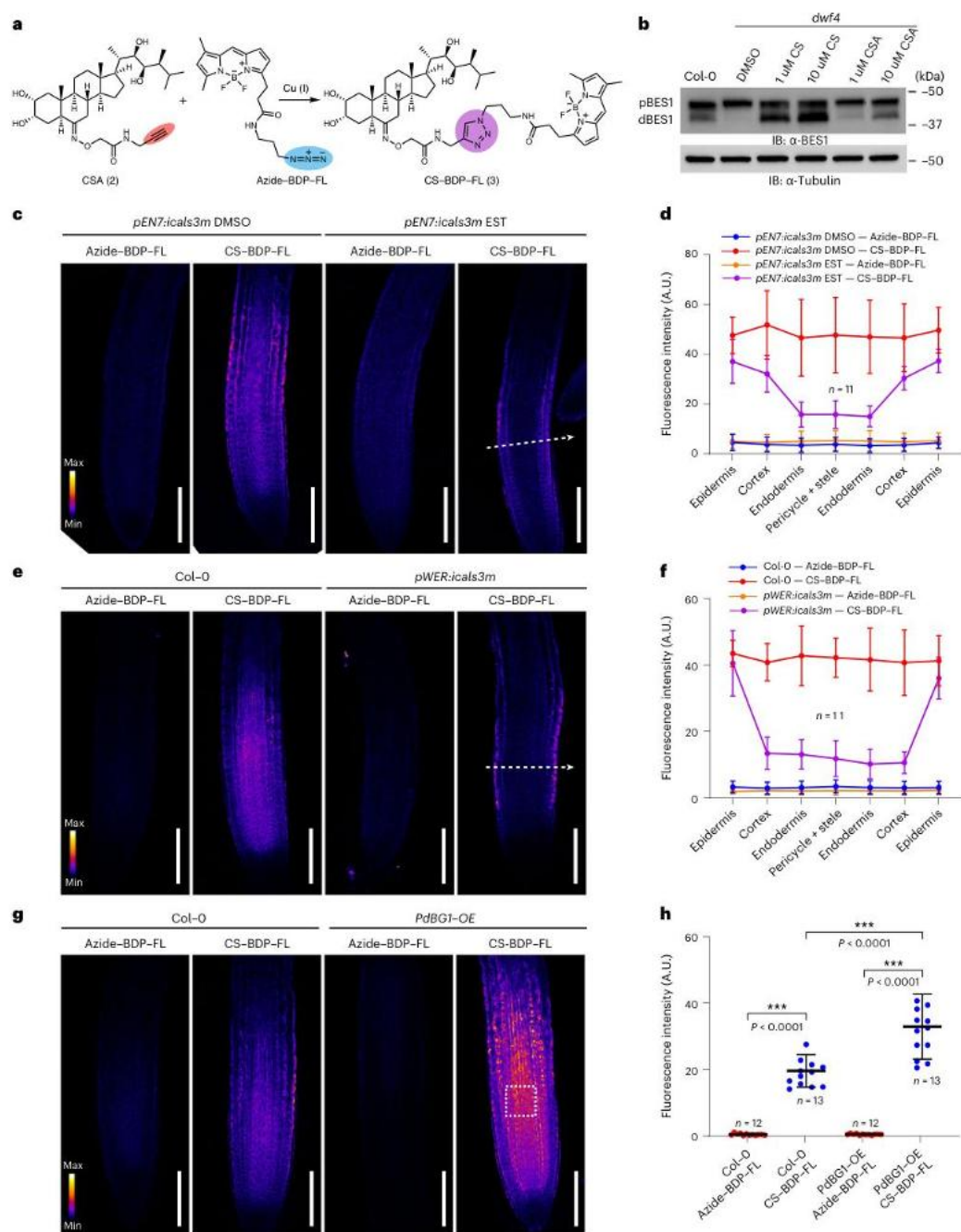


Fig. 2 | BL but not its precursor 22-OHCR rescues *dwf4* mutants after PD closure. **a**, Schematic representation of different complementation principles by BL and 22-OHCR of the *dwf4* root upon callose deposition in the endodermis. **b**, The root growth phenotype of *pEN7:icals3m/dwf4* plants in response to exogenous BL and 22-OHCR upon callose induction. Four-day-old seedlings were transferred to an agar medium containing either DMSO (mock) or EST (5 μ M) for 24 h. Next, seedlings grown on DMSO were transferred to an agar medium containing DMSO, BL (500 pM) or 22-OHCR (500 nM; upper panel). Seedlings grown on EST were transferred to an agar medium containing EST (5 μ M), EST (5 μ M) + BL (500 pM) and EST (5 μ M) + 22-OHCR (500 nM; lower panel) and grown for 24 h. Root tips were marked immediately after the transfer (white bars). Scale bar, 5 mm. **c, d**, Quantification of the primary root (c) and the mature cortical cell (d) lengths from b. Horizontal and error bars represent the means and s.d., respectively. n, number of roots used in c and cells used in d. The

significant differences between the transgenic lines and the wild-type (Col-0) control were determined with ANOVA and Tukey's multiple comparison tests. *** $P < 0.001$. **e**, Phosphorylation status of BES1 detected by immunoblotting with α -BES1 antibody in root tips. Tubulin detected with α -tubulin antibody was used as a loading control. **f**, Confocal images of 6-d-old root tips of *pBES1:gBES1-GFP/pEN7:icals3m/dwf4* Arabidopsis seedlings treated as in b. Cell walls were stained with PI. Nine cells used for fluorescence intensity measurements in g are shown in insets (1.5 $\times$ enlarged). In the inset panels, only the GFP signal is shown. Brightness and contrast were equally adjusted for each treatment. Scale bars, 100 μ m. **g**, Quantification of nuclear/cytoplasmic BES1-GFP fluorescence intensity ratio of epidermal cells in the root transition zone (nine cells from the inset panels in f). Error bars represent s.d. n, number of roots used for each treatment. For b–d and e–g, experiments were performed in three and two repeats, respectively, with similar results.



BR biosynthesis inhibitor BRZ (1 μM) for 24 h. The immunostaining revealed that BL-treated seedlings exhibited increased callose deposition, whereas the callose levels were substantially reduced after

BRZ treatment (Fig. 4a,b and Extended Data Fig. 6). Consistently, the BR-deficient *dwf4* and *cpd* mutants had almost no callose deposition in their root meristems, but the observed callose deficiency was rescued

Fig. 3 | Localization of CSA in *Arabidopsis* root tips. **a**, Chemical compounds used in the bioorthogonal reaction. **b**, CSA is retaining the biological properties of CS. Phosphorylation status of BES1 detected with immunoblotting and α -BES1 antibody. Tubulin detected with the α -tubulin antibody was used as a loading control. Five-day-old wild-type *Arabidopsis* and *duf4* mutant seedlings were transferred to an agar medium containing CS or CSA at the indicated concentrations and DMSO (mock) for 24 h. **c**, Blocking PD in the endodermis arrests CS-BDP-FL signal in epidermal and cortical cells. Five-day-old seedlings were transferred to an agar medium containing either EST (5 μ M) or DMSO (mock) for 24 h. Next, seedlings from each treatment were divided into two and transferred to liquid media containing either CSA (20 μ M) or DMSO (mock) for 4 h, followed by a bioorthogonal reaction. **d**, Fluorescence intensity quantification along the white dashed arrow in **c**, positioned at 200 μ m away

from the root tip shown in **c**. **e**, Blocking the PD in epidermal cells arrests CS-BDP-FL signal accumulation in the epidermis. Six-day-old seedlings were incubated in a liquid medium with either CSA (20 μ M) or DMSO (mock) for 4 h followed by a bioorthogonal reaction. **f**, Quantification of fluorescence intensity in **e**, positioned at 200 μ m away from the root tip shown in **e**. **g**, Highly permeable PD increase the CSA uptake. Six-day-old *PdBG1-OE* plants were treated as in **e**. **h**, Quantification of fluorescence intensity in **g**, in which a 50 $\times$ 50 μ m² area, 200 μ m away from root tip (white dashed box in **g**), was used. The significant differences were determined with ANOVA and Tukey's multiple comparison tests. *** P < 0.001. Scale bars, 100 μ m (**c**, **e** and **g**). Horizontal bars in **h** represent the means, and error bars in **d**, **f** and **h** represent the s.d. A.U., arbitrary units; n , number of roots. For **b**, **e** and **f**, the experiment was repeated two times; for **c** and **d**, four times; and for **g** and **h**, three times with similar results.

by exogenous BL (Fig. 4a,b and Extended Data Fig. 6). These results indicate that BRs can modulate PD permeability and that high and low BR signaling would induce closure and opening of PD, respectively.

To explore whether the symplastic BR transport is regulated by BR signaling, we performed photoactivation of cytosolic DRONPA-s, a reversibly switchable photoactivatable fluorescent protein in single root cells and monitored the spread of the fluorescent signal to the surrounding cells³⁴ (Fig. 4c). We observed substantially faster spread of the signal in mock-treated Col-0 plants in comparison to BL-treated ones (Fig. 4d), demonstrating the ability of BRs to decrease PD permeability. To directly observe the distribution of CSA under conditions of perturbed BR signaling, click chemistry was used. As anticipated, exogenous BL substantially reduced the accumulation of CSA in Col-0 roots when compared with the mock control (Fig. 4e,f). Conversely, CSA accumulation in the root meristems of *duf4* seedlings was stronger than in Col-0 roots (Fig. 4g,h). To examine whether the reduced CSA accumulation is a consequence of increased BR signaling levels and not a competition with BL, we tested CSA accumulation after bikinin (BIK) treatment, which can activate BR signaling downstream of the BRI1 receptor³⁵. Similar to BL, BIK application reduced CSA accumulation in the treated plants (Fig. 4e,f). The BR-induced callose turnover and control of PD permeability are likely under transcriptional control because BR signaling positively regulates expression levels of several callose deposition-related genes in the *Arabidopsis* root³⁶ (Extended Data Fig. 7 and Supplementary Table 1).

In summary, our findings demonstrate the existence of an additional BR signaling regulatory feedback loop that acts at the PD permeability level and ensures optimal BR biosynthesis by modulating BR movement.

Discussion

Polyhydroxylated steroidal molecules are used as signaling molecules in both mammals and plants to control a plethora of developmental processes^{37–39}. Although these molecules share similar structures, they impact gene expression differently. For example, in mammals, steroidal hormones act in an endocrine manner, namely they are synthesized in

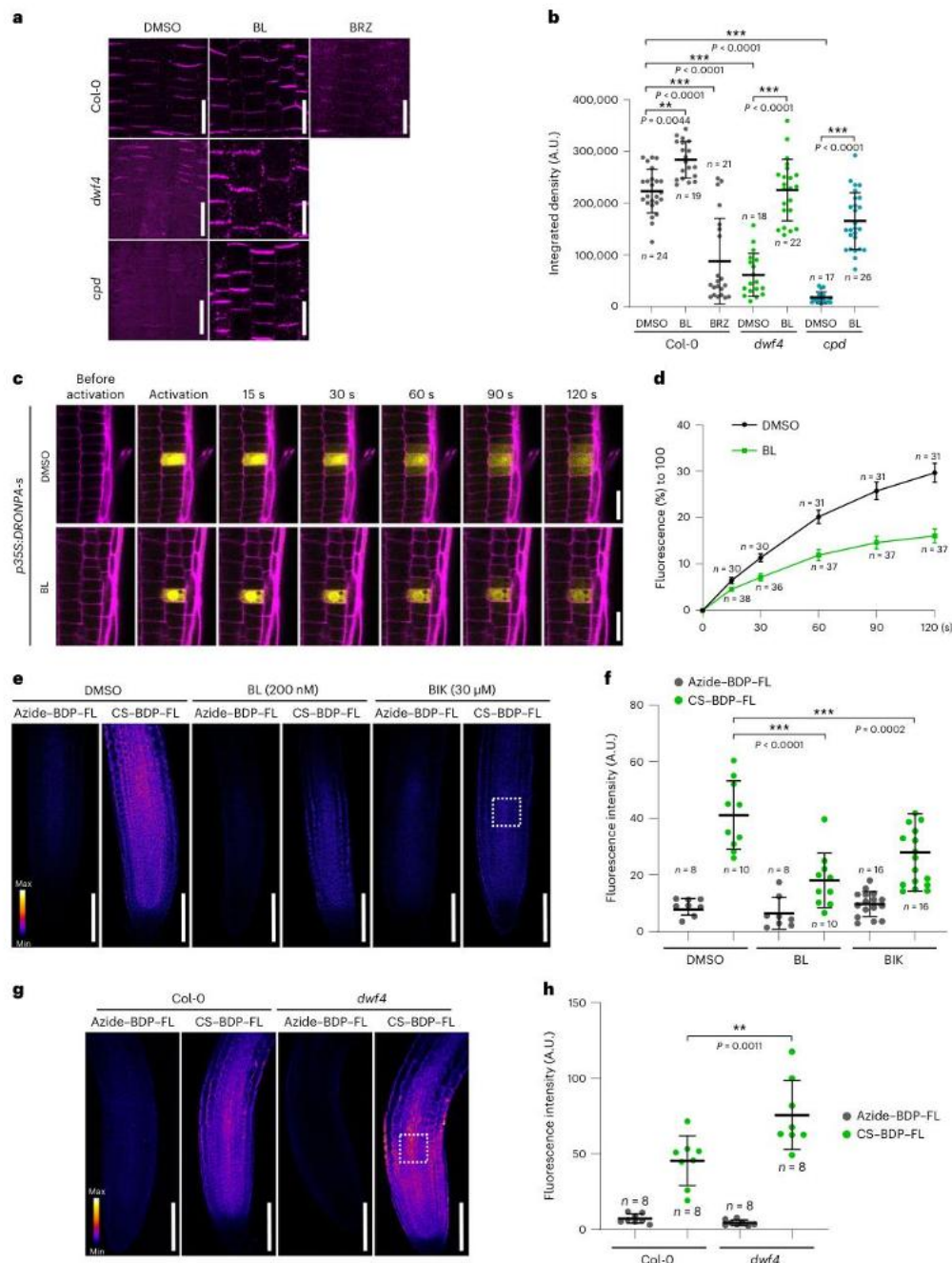
glands, transported by specialized protein carriers and after release into the target cell cytoplasm, they bind to intracellular receptors⁴¹. In plants, a family of plasma membrane-localized receptors, including BRI1 and its homologs BRI1-LIKE1 (BRL1) and BRI1-LIKE3 (BRL3), perceive locally produced BRs in the apoplast⁴⁰ because plant steroidal hormones do not undergo long-distance transport⁴². The textbook knowledge suggests that steroid hormones can freely diffuse across biological membranes due to their hydrophobicity. However, the simple diffusion theory was challenged by finding that the release of the steroid hormone ecdysone in *Drosophila melanogaster* requires ATP-binding cassette transporter-mediated vesicle loading and calcium-mediated vesicle exocytosis⁴³, and the cellular uptake of ecdysone needs a membrane solute carrier transporter. In addition, molecular dynamics simulation experiments demonstrated that the flip-flop transitions of steroids, and hence, their ability to diffuse across lipid membranes, are determined by the number of hydroxyl groups in the molecule⁴⁴. Here we confirmed that the BR biosynthetic enzymes are localized in the ER membrane, which is the most likely place for BR biosynthesis. As BR biosynthetic enzymes are expressed in a nonoverlapping fashion along the radial axis of *Arabidopsis* roots, exchange of precursors between cells of different cell files is necessary for the completion of bioactive BR synthesis. Given that BRs are hydrophobic molecules, it is unlikely that they freely move via an apoplastic route between neighboring cells. Alternatively, PD-mediated symplastic transport can ensure the movement of steroid molecules in plants. This pathway enables the cell-to-cell exchange of nutrients, hormones, RNAs, proteins, metabolites and viruses^{25–29}. Therefore, we speculated that the intermediates or bioactive BRs are transported through the PD after synthesis in the PD vicinity. In the scenario of PD-mediated BR transport, perturbation of PD permeability should affect BR signaling. Examination of *Arabidopsis* plants with altered PD permeability revealed that BR signaling was modulated to a certain extent in plants with perturbed PD permeability, possibly due to the reduced or enhanced ability to transport BRs through the PD. Nevertheless, BR signaling levels were not drastically changed when PD conductivity was altered. The reason for this observation might be the pleiotropic nature of PD mutants and the

Fig. 4 | BRs regulate PD permeability. **a**, Callose immunostaining of *Arabidopsis* wild-type (Col-0), *duf4* and *cpd* roots using an α -callose antibody. Four-day-old seedlings were transferred to an agar medium containing BL (200 nM), BRZ (1 μ M) and DMSO (mock) for 24 h. Scale bars, 20 μ m. **b**, Quantification of callose intensity at the PD in **a**. Callose signal at the cell plate was excluded from measurements. **c**, The reversibly switchable photoactivatable fluorescent protein system DRONPA-s movement after activation in a single meristematic cell of the root. Confocal images of 6-d-old root meristems stained with PI are shown. Roots were treated either with mock or BL (200 nM) for 24 h before imaging. Scale bars, 20 μ m. **d**, Normalized values of the mean fluorescence intensity of the DRONPA-s are extracted from the adjacent cells next to the activated cell. For each time point, n > 15. Error bars represent the standard error of the mean (s.e.m.). **e**, Reduction of the CSA uptake after BL and BIK treatment. Five-day-old seedlings were

transferred to an agar medium containing BL (200 nM), BIK (30 μ M) or DMSO (mock) for 24 h, followed by the bioorthogonal reaction to form CS-BDP-FL. **f**, Quantification of fluorescence intensity of images in **e** in the boxed region of interest positioned 200 μ m away from the root tip. **g**, *duf4* seedlings exhibited an increased CSA uptake capacity. The bioorthogonal reaction was done in 6-d-old *Arabidopsis* seedlings of Col-0 and *duf4*. **h**, Quantification of fluorescence intensity of images in **g** in the boxed region of interest positioned 200 μ m away from the root tip. Horizontal and error bars (**b**, **f** and **h**) represent the means and s.d., respectively. n , number of roots (**b**, **f** and **h**). The significant differences were determined with ANOVA and Tukey's multiple comparison tests. *** P < 0.001 and ** P < 0.01 for **b**, **f** and **h**. Scale bars, 100 μ m (**e** and **g**). For **a** and **b**, experiments were performed in four repeats with similar results; for **c**–**f**, in three repeats; and for **g** and **h**, in two repeats with similar results.

fact that the molecules that negatively regulate BR signaling⁴³ are also transported via PD. Additionally, changes in the cell wall composition of PD mutants might trigger BR signaling without the ligand binding⁴⁴

and mask reduced hormone synthesis. Whether the BR biosynthetic machinery is associated with PD and what physiological relevance this association might have remained to be tested in detail in future studies.



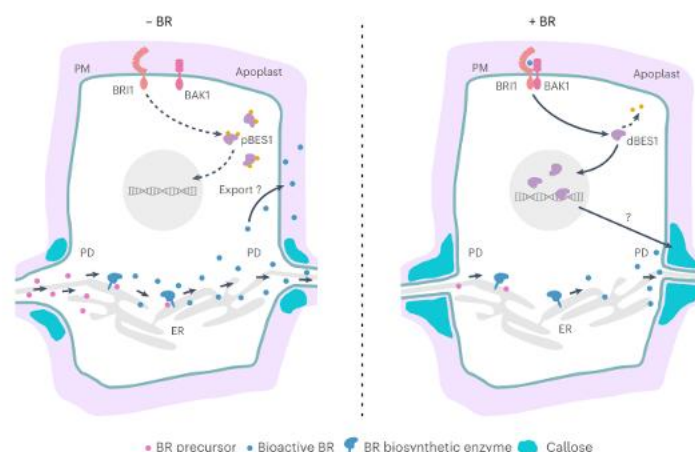


Fig. 5 | PD-mediated transport and homeostasis of BR biosynthesis and signaling. BR biosynthetic enzymes are expressed in neighboring cells, requiring the exchange of BR intermediates through PD to produce bioactive BRs. Once synthesized, BRs exit the cell via an unknown mechanism and reach the apoplast (left panel). Once in the extracellular matrix, bioactive BRs bind to the BR11 receptor and coreceptor BAK1 and initiate a signaling cascade that leads to

dephosphorylation of the BES1 transcription factor. BES1 can then enter the nucleus and initiate transcriptional responses. High levels of BRs increase callose deposition and decrease PD permeability, possibly via BR signaling-initiated transcriptional regulation. Restricted BR precursor movements reduce hormone production and contribute to optimal BR signaling level maintenance (right panel). PM, plasma membrane; PD, plasmodesmata; ER, endoplasmic reticulum.

To further demonstrate the PD-mediated BR movement, we used precursor feeding experiments. Conditionally disrupting cell-to-cell communication, specifically in the endodermis, prevented the rescue of *dwf4* mutants with their exogenous product, 22-OHCR. Hence, we concluded that closed PD in mid-positioned root cell files blocks this BR precursor movement and prevents it from reaching inner tissues where it can be converted to downstream BR precursors. Nevertheless, it seems that the transport of 22-OHCR was not completely blocked by the PD constriction, leaving the possibility for partial apoplastic movement of the hormone. Currently, no tools are available that allow monitoring of the BR distribution or movement in plant tissues, in contrast to other plant hormones⁴⁵. Alexa Fluor 647-CS (AFCS) is the only existing bioactive fluorescently labeled BR that could be used to track BR movements in living cells⁴⁶, but this chemical probe might be problematic because of the bulky fluorophore. Therefore, we took a bioorthogonal chemistry approach and developed the BR probe CSA that can be visualized with confocal microscopy after conjugation with an azide-BDP-FL probe. Click chemistry allowed us to follow the CSA distribution in *Arabidopsis* roots under perturbed PD permeability conditions. When the endodermal PD were closed, CSA accumulated in epidermal and cortical cells and could not reach the stele, but when PD were blocked in the epidermis, the CSA was detected only in epidermal cells. This interesting finding implies that only epidermal cells of the root can directly uptake BRs and possibly other steroid-like compounds frequently used in plant research, such as EST. In addition, plants with an enhanced PD permeability accumulated more CSA in all cell files of the root. Together, these results demonstrate that the PD play a crucial role in short-distance BR transport.

Maintenance and regulation of BR levels in cells are essential for plant growth and development. The feedback transcriptional regulation of key BR biosynthetic genes by direct BES1 and BZR1 binding to their promoters is one of the well-known mechanisms for retaining BR homeostasis⁵. Intriguingly, in *Arabidopsis* roots, the expression domains of BR biosynthetic enzymes are separated. We hypothesize that this spatial organization of the biosynthetic pathway allows more

flexible control of hormone biosynthesis through precursor transport regulation. Indeed, the callose immunostaining together with click chemistry-based visualization of BRs revealed that intracellular BR content, probably through BR signaling, regulates PD permeability. Presumably, control of the callose-regulating turnover, and, hence, BR mobility, can optimize BR biosynthesis and signaling, thus creating a negative feedback regulation loop between PD permeability and intracellular BR content. Nevertheless, how BR signaling impacts callose production and degradation at PD remains to be investigated. Recently, the callose synthase gene, glucan synthase-like 8 with a function in plant defense, has been reported as a direct target of BES1, and its expression to be induced by BRs⁴⁷. Our analysis of existing BR response single-cell RNA-sequencing datasets³⁶ revealed that two other genes implicated in callose turnover regulation in the *Arabidopsis* root, PDL3 (ref. 48) and GSL4 (ref. 49), are upregulated after BR treatment.

Collectively, our results show that BR precursors are transported through PD after their biosynthesis in the PD-neighboring ER membrane to complete BR biosynthesis and to produce bioactive BRs (Fig. 5). In turn, elevated BR levels can alter callose deposition and decrease PD permeability, possibly through transcriptional regulation initiated by BR signaling, thereby reducing hormone production by restricting the movement of BR precursors. In contrast, low intracellular BR levels lead to decreased callose deposition and increased PD permeability, with enhanced hormone production as a consequence. This negative feedback loop ensures the precise amount of bioactive BRs that is beneficial to plant growth and development. Our work reveals a thus far unknown BR transport pathway in plants and expands the regulatory mechanisms of hormone homeostasis.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41589-023-01346-x>.

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Methods

Plant materials and growth conditions

The *A. thaliana* (L.) Heynh., accession Col-0, was used in all experiments. Seeds were surface-sterilized with sterilization buffer (80% (vol/vol) ethanol and 20% (vol/vol) sodium hypochlorite), stratified for 2 d in the dark at 4 °C and grown vertically on half-strength Murashige and Skoog (1/2 MS) medium (1%) agar (wt/vol) plates supplemented with 1% (wt/vol) sucrose at 22 °C, with a 16-h light/8-h dark photoperiod. The following mutant lines were used: *dwf4-102* (ref. 50), *cpd¹*, *pDWF4:DWF4-GFP/dwf4* (ref. 6), *pROT3:ROT3-GFP/rot3* (ref. 6), *pCPD:CPD-GFP/cpd¹*, *pBR6OX2:BR6OX2-GFP/br6ox1;br6ox2* (ref. 6), *pBES1:gBES1-GFP/Col-0* (ref. 6), *pEN7:icals3m/Col-0* (ref. 27) and *p35S:PdBG1-mCitrine/Col-0* (ref. 30). Primers used for genotyping are listed in Supplementary Table 2. The lines *pEN7:icals3m/dwf4* and *pBES1:gBES1-GFP/pEN7:icals3m/dwf4* were obtained by crossing *pEN7:icals3m* with *dwf4-102* and *pEN7:icals3m/dwf4* with *pBES1:gBES1-GFP/Col-0*, respectively.

Plasmid construction and generation of transgenic lines

To generate the *p35S:C4H-mCherry* and *pUBQ10:PDLPI-mCherry* constructs, the genomic fragments of *C4H* (AT2G30490) and *PDLPI* (AT5G43980) were amplified by PCR and cloned into *pDONR221* (Thermo Fisher Scientific). The resulting entry clones were recombined with the cauliflower mosaic virus 35S and the *UBIQUITIN10* promoters in *pDONR4-PIR* with *pDONR2R-P3-mCherry* for *C4H* and *PDLPI*, respectively into the *pH7m34GW²* destination vector by an LR reaction. The resulting constructs were used for transient expression in tobacco and generation of transgenic plants by introducing them into *pDWF4:DWF4-GFP/dwf4* and *pROT3:ROT3-GFP/rot3* plants by *Agrobacterium tumefaciens*-mediated transformation according to the floral dip protocol⁵¹. To generate *pWER:icals3m*, *pDONR4-PIR-WER* (obtained from NASC set 2106366) was recombined with *icals3m* in *pDONRPI-P2* and without in *pDONR2R-P3* into the *pH7m34GW²* destination vector by an LR reaction. The construct was transformed into Col-0. To generate *pCPD:PDLPS-BFP/pCPD:CPD-GFP/cpd* and *pCPD:PDLPS-BFP/pSCR:CPD-mCherry/cpd* lines, the genomic fragment of *PDLPS* was cloned into *pDONR221* and combined in an LR reaction with *pDONR4-PIR-CPD¹* and *pDONR2R-P3-BFP* in the destination vector *PK8m34GW-FAST*. The resulting constructs were introduced into *pCPD:CPD-GFP/cpd¹* and *pSCR:CPD-mCherry/cpd¹* translational lines. To generate *p35S:DWF4-GFP* and *p35S:ROT3-GFP* constructs, the entry clones *pDONR221-DWF4* and *pDONR221-ROT3* (ref. 6) were put into the *pK7FWG2* destination vector by an LR reaction. To generate *p35S:MCTP3-GFP* construct, *MCTP3* (AT3G57880) was amplified by PCR and cloned into *pDONR221* (Thermo Fisher Scientific). The resulting entry clone was put into the *pK7FWG2* destination vector by an LR reaction. The cloning primers are listed in Supplementary Table 2.

Chemical treatments

BL (OChemIm), BRZ (Tokyo Chemical Industry), β -EST (EST; Sigma-Aldrich), BIK (Sigma-Aldrich), 22-OHCR and CSA were kept at different stock concentrations in dimethyl sulfoxide (DMSO) and were diluted 1,000 $\times$ to reach the final concentrations in the media. For the mock treatment, DMSO was at a final concentration of 0.1% (vol/vol). Specific treatments are described in the main text and/or figure legends.

Immunoblot analysis

For the BES1 immunoblot analysis of *pEN7:icals3m* seedlings, the experiments were done in triplicate. Five-day-old Col-0 and *pEN7:icals3m* seedlings were transferred to fresh 1/2 MS medium agar plates containing DMSO or 5 μ M EST. The root tips were collected for immunoblotting after 12 h of treatment. For the BES1 immunoblot analysis of *pCPD:PDLPS-BFP/pCPD:CPD-GFP/cpd* and *pCPD:PDLPS-BFP/pSCR:CPD-mCherry/cpd* lines, the experiments were done in

triplicate. Six-day-old seedlings (root part) were collected for the BES1 assay. For the *p35S:PdBG1-mCitrine* line, experiments were done in duplicate. Six-day-old seedlings of *p35S:PdBG1-mCitrine* and Col-0 were collected for the BES1 assay. For the 22-OHCR activity tests, experiments were done in duplicate. Five-day-old Col-0, *dwf4* and *cpd* seedlings were transferred to 1/2 MS medium agar plates containing DMSO, BL (500 pM) or 22-OHCR (500 nM) for 24 h and collected for the BES1 assay. For the CSA activity test, experiments were done in duplicate. Five-day-old *dwf4* seedlings were transferred to fresh 1/2 MS medium agar plates containing DMSO, CS (1 μ M and 10 μ M) or CSA (1 μ M and 10 μ M) and collected after 24 h for the BES1 assay. All plant material was frozen in liquid nitrogen, ground by Retsch MM400 and homogenized in 100 μ l of ice-cold homogenization buffer (1% (vol/vol) SDS, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM dithiothreitol and Roche Complete protease inhibitor (one tablet/10 ml)) and placed on ice for 30 min. The homogenates were centrifuged twice (10 min, 20,000g) at 4 °C. After the addition of 4 $\times$ lithium dodecyl sulfate and sample-reducing agent (10 $\times$), the samples were heated for 10 min at 70 °C, centrifuged again, separated on 4–15% (vol/vol) SDS-polyacrylamide gel electrophoresis stain-free protein gel (Bio-Rad Laboratories) and blotted on Trans-Blot Turbo Mini PVDF Transfer Packs. Membranes were blocked at 4 °C in 5% (vol/vol) skimmed milk (Difco). For immunodetection, the anti-BES1 antibody at 1:5,000 was used as the primary antibody and donkey anti-rabbit (Merck) at 1:10,000 as the secondary antibody. For tubulin detection, the anti-tubulin (Abcam) at 1:5,000 was used as the primary antibody and the sheep anti-mouse (Merck) at 1:10,000 as the secondary antibody. Proteins were detected by the ChemiDoc MP Imaging System (Bio-Rad Laboratories). For the BES1 dephosphorylation assay, the ratio of dBES1 to total BES1 proteins was quantified according to the signal intensity. Loading was adjusted to an equal level based on the amount of tubulin. Signal intensities were determined with Image Lab (Bio-Rad Laboratories).

Synthesis of chemical compounds and bioorthogonal chemistry

CSA and 22-OHCR were synthesized as described (Supplementary Notes 1 and 2). Azide-BDP-FL (Jena Bioscience, CLK-044-1) and Click-&-Go Cell Reaction Buffer Kit (Click Chemistry Tools, 1263) were used for click chemistry labeling experiments. The plant material was incubated without or with CSA (20 μ M) in liquid 1/2 MS medium for 4 h, washed twice with PBS, fixed with 3.7% (vol/vol) formaldehyde in PBS for 15 min and washed twice with 3% (wt/vol) BSA in PBS. The samples were permeabilized by 0.5% (vol/vol) Triton X-100 in PBS for 20 min at room temperature and washed three times with 3% (wt/vol) BSA in PBS. The click chemistry was done according to the manufacturer's instructions (Click Chemistry Tools), using Azide-BDP-FL at a 4 μ M concentration in the reaction mixture for 30 min. Samples were washed with 3% (wt/vol) BSA in PBS, counterstained by propidium iodide (PI) at 1:1,000 dilution in H₂O and imaged.

Aniline blue staining

Seedlings were fixed and destained in 1:3 acetic acid/ethanol until the material was transparent (usually 2 h), then washed in 150 mM K₂HPO₄ for 30 min. Next, seedlings were incubated for at least 4 h in 150 mM K₂HPO₄ and 0.01% (wt/vol) aniline blue (staining solution) in tubes wrapped in aluminum foil for light protection, washed in 150 mM K₂HPO₄ for 5 min and imaged.

Callose immunostaining

Arabidopsis seedlings were vertically grown on 1/2 MS medium agar plates for 4 d, then transferred to fresh media with the following treatments for another 24 h: 200 nM BR, 1 μ M BRZ and DMSO mock. The immunolocalization procedure was done according to the published protocol⁵⁴. In summary, seedlings were fixed in 4% (vol/vol) paraformaldehyde in microtubule stabilization buffer [MTSB; 50 mM piperazine-

N,N'-bis(2-ethanesulfonic acid), 5 mM (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 5 mM MgSO₄, pH 7 with KOH]. Root tips were cut and mounted on poly-lysine-coated microscopy slides. Unspecific binding was prevented by blocking in neutral donkey serum before incubation with the antibody. The callose antibody (Australia Biosupplies) was diluted to 1:500 in MTSB containing 5% (vol/vol) neutral donkey serum and incubated with the samples for 4 h at room temperature. The secondary goat anti-mouse IgG (H&L)—Alexa Fluor 594 was diluted to 1:500 in MTSB buffer containing 5% (vol/vol) neutral donkey serum and incubated with the samples for 1 h.

Reverse transcription–quantitative PCR

Total RNA was extracted by reverse transcription–quantitative PCR from 6-d-old seedlings with the RNeasy Mini Kit (Qiagen). Genomic DNA was eliminated by on-column digestion with RQ1 RNase-free DNase (Promega) during the isolation procedure. cDNA was generated from 1 μg of total RNA with qScript cDNA SuperMix (Quantabio) and analyzed on a LightCycler 48 II apparatus (Roche) with the SYBR Green I Master mix (Roche) according to the manufacturer's instructions. Expression levels were normalized to those of ACTIN2. Primers are listed in Supplementary Table 2.

Analysis of BL scRNA-seq

To examine the transcriptional regulation of PD-related genes by BRs, we reanalyzed a previously described scRNA-seq dataset (GEO: GSE212230) in which wild-type *Arabidopsis* plants were grown on 1 μM BRZ for 7 d and transferred to 1 μM BRZ versus 100 nM BL for 2 h (ref. 36). First, we constructed a heatmap by calculating the log₂ fold-change of BL 2 h/BRZ using aggregated counts across all cell types and developmental stages. The heatmap was visualized with ComplexHeatmap (v2.10.0)³⁵. Differentially expressed genes from each combination of cell type and developmental stage of the root³⁶ were used to identify BL-regulated PD-related genes. We then plotted the log-normalized, 'corrected' counts produced by the SCTransform function³⁶ for *PDL1* and *CalS8/GSL4* on the two-dimensional uniform manifold approximation and projection embedding.

Microscopy and image analysis

Most of the images were captured by a Leica SP8X confocal microscope. Images were collected using Las-X software (v 3.5.0.18371). GFP and mCherry were excited at 488 nm and 594 nm and acquired at 500–530 nm and at 600–650 nm, respectively. For the study of subcellular localization of BR biosynthetic enzymes, images were taken by a ×40/1.10 WATER objective and signal accumulation was used during confocal imaging for *Arabidopsis* lines related to ROT3 and DWF4. For the experiments related to the transgenic line *pBES1:GFP*, *Arabidopsis* roots were mounted in a PI (Sigma-Aldrich) (10 ng ml⁻¹) solution between slides and coverslips and images were taken by a ×25/0.95 WATER objective. Nine cells from the root transition zone were used for BES1–GFP signal quantification. For the click chemistry experiments, the BDP–FL signal was detected with a 503-nm laser excitation and a 505- to 519-nm emission filter, and images were taken by a ×25/0.95 WATER objective. For *pDWF4:DWF4–GFP/pUBI10:PDL1–mCherry* and *pROT3:ROT3–GFP/pUBI10:PDL1–mCherry* in Extended Data Fig. 1g, roots were imaged under a vertical Zeiss LSM900 microscope equipped with a Plan-Apochromat M27 20×/0.8 n.a. objective. GFP and mCherry were excited at 488 and 587 nm and acquired at 410–546 nm and at 595–700 nm, respectively. For immunostaining, samples were imaged with a Zeiss LSM880 microscope with an ×40/NA 1.3 oil lens. Atto550 excitation was done with 0.3% of 561-nm power, and fluorescence was collected between 566 and 700 nm. Zeiss ZEN 3.3 (blue edition) software was used for image collection with Zeiss LSM880 and Zeiss LSM900 microscopes. Callose deposition at PD was quantified for whole root meristems with the Fiji software (<https://fiji.sc/>) macroinstruction program (Supplementary Note 3). The callose signal at the

cell plate was excluded from the measurements. The experiment was repeated two times with similar results. For aniline blue staining, a 405-nm laser excitation and a 505-nm long-pass emission filter were used for imaging. Image analysis was done with the Fiji software. To measure the length of mature cortical cells, seedlings were stained with PI (Sigma-Aldrich) and images were taken with ×25/0.95 WATER objective. The cells from the root region, in which root hairs start to emerge (a sign of cell differentiation after cessation of elongation), were imaged and measured.

Two-photon microscopy and cell diffusion assay with DRONPA-s

Five-day-old 35S-DRONPA-s seedlings were grown on 1/2 MS agar plates under long-day conditions at 21 °C and 70% humidity. Then, they were transferred to plates containing either DMSO or 200 nM BL for an additional 24 h. Before imaging, plants were incubated for 2 min in 1/500 PI in a water solution. In total, 15–20 roots were analyzed with two to three regions of interest (ROIs) per root. For the DRONPA-s assay, root tips were analyzed with a Leica TCS SP5–multiphoton confocal microscope set up. PI was excited at 488 nm and detected at 620–700 nm. Fluorescence intensities were quantified with the Leica LAS AF software. For DRONPA-s deactivation, root tips were illuminated with 488-nm light for 45 s at 70% of the total laser intensity (argon laser, 20 mW; Leica Microsystems). For DRONPA-s activation, three ROIs (corresponding to three cells per root) were set to the center of the cell to avoid activation of the adjacent cells. ROIs were illuminated with 800-nm light for 5 s using a two-photon infrared (IR) (Titane-Saphir pulsating laser). Laser power is circa 2.6 W with a 20% gain. For DRONPA-s acquisition, the detection was made 2 s after single-cell activation up to 120 s, with 20% (488 nm) argon laser at emission 500–575 nm. For DRONPA-s movement analysis, mean fluorescence intensities were measured from the activated cell and the two adjacent cells in ImageJ. DRONPA-s movement was calculated as a fluorescent signal moving from the activated cell into the adjacent cells. X–Y drifts were corrected with the StackReg plugin. After normalization and background subtraction, values were transformed into percentages of the average fluorescence from the two adjacent cells. The activated cell is considered to be at 100% (for example, 100% of the DRONPA-s molecules were activated in the ROI). A total of 15–20 roots were analyzed with two to three ROIs per root.

PD index

PD enrichment was assessed by calculating the fluorescence intensity of FP-tagged DWF4, PD localized MCTP3 and the ER protein HDEL³⁷, at (1) PD (indicated by PDL1–mCherry or PDCB1–mCherry) and (2) the cell periphery. Constructs of interest were transiently co-expressed in *N. benthamiana* leaves with PDL1–mCherry or PDCB1–mCherry (PD markers). Confocal images of leaf epidermal cells were acquired by sequential scanning of PDL1–mCherry or PDCB1–mCherry in channel 1 and GFP/BFP-tagged proteins in channel 2. Approximately 20 images of leaf epidermal cells were acquired for each combination. The quantification was carried out according to the standard protocol²⁵. Individual images were processed using ImageJ by defining six ROIs at PD (using PD marker to define the ROI in channel 1) and 12 ROIs at the cell periphery outside PD. The GFP/BFP-tagged protein mean intensity (channel 2) was measured for each ROI and then averaged for a single image. The PD index corresponds to the intensity ratio between the fluorescence intensity of the protein of interest at PD versus the cell periphery outside of PD. Roots expressing DWF4–GFP/ROT3–GFP and PDL1–mCherry were imaged in a similar fashion to tobacco leaves, and the PD index for more than 20 cells per transgenic line (four roots per line) was calculated as explained above.

Statistical analysis

All statistical analyses were carried out with GraphPad Prism v.8.0 and v.9.0 software. Two-tailed Student's paired *t*-test was used for

BES1 immunoblot analysis. Comparison of more than two genotypes or treatments was done with a one-way analysis of variance. Tukey's multiple comparison tests were used in the comparison procedure (** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Numerical source data files and uncropped scans of blots are provided for figures and extended data figures. Primer lists, Fiji macro for callose deposition quantification and notes on synthesis of chemical compound used in this study can be found in Supplementary Information. Source data are provided with this paper.

Code availability

Single-cell RNA-sequencing data analysis code used in this study is deposited at GitHub at <https://github.com/tmnolan/Plasmodesmata-mediate-cell-to-cell-transport-of-brassinosteroid-hormones>.

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Author contributions

Y.W., N.V., E.M.B. and E.R. initiated the project and designed experiments. Y.W. performed most of the experiments. N.V. prepared constructs and performed imaging. Y.L. performed imaging and calculated the PD index. J.P.S. and M.S. performed callose immunostaining and DRONPA-s imaging and analyzed the data. T.M.N. analyzed scRNA-seq data. M.P.P. generated transgenic lines and contributed materials. B.C. and J.M.W. synthesized CSA, and M.K. and K.F. synthesized 22-OHCR. Y.W., N.V., T.S., E.M.B., W.B., P.N.B. and E.R. analyzed the data and wrote the article. All authors revised the manuscript.

Competing interests

P.N.B. is the cofounder and Chair of the Scientific Advisory Board of Hi Fidelity Technologies, a company that works on crop root growth. The remaining authors declare no competing interests.

Additional information

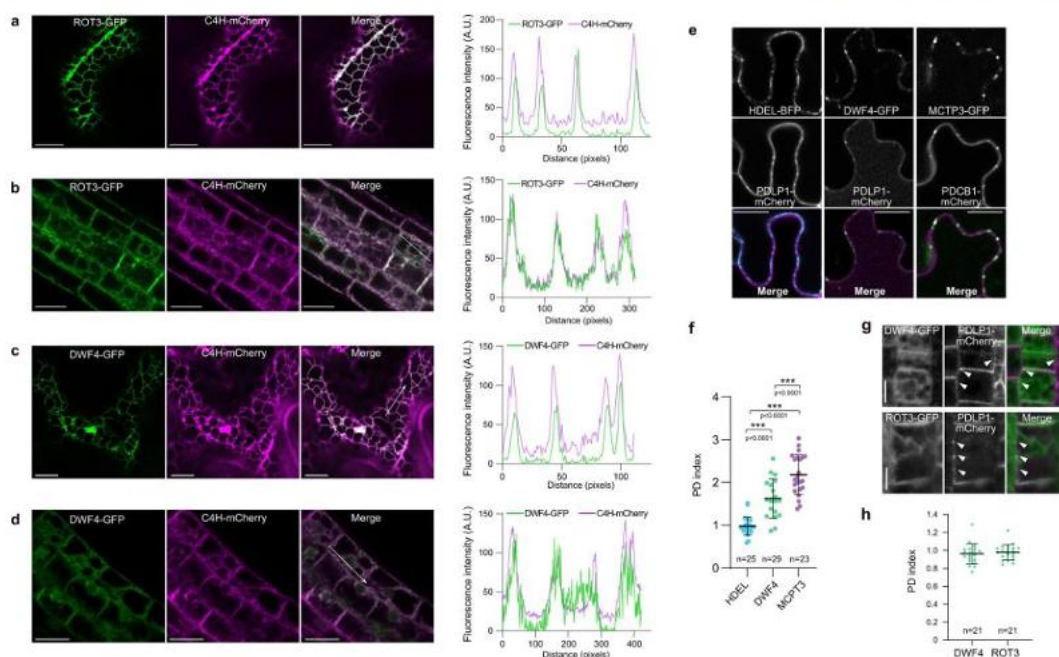
Extended data is available for this paper at <https://doi.org/10.1038/s41589-023-01346-x>.

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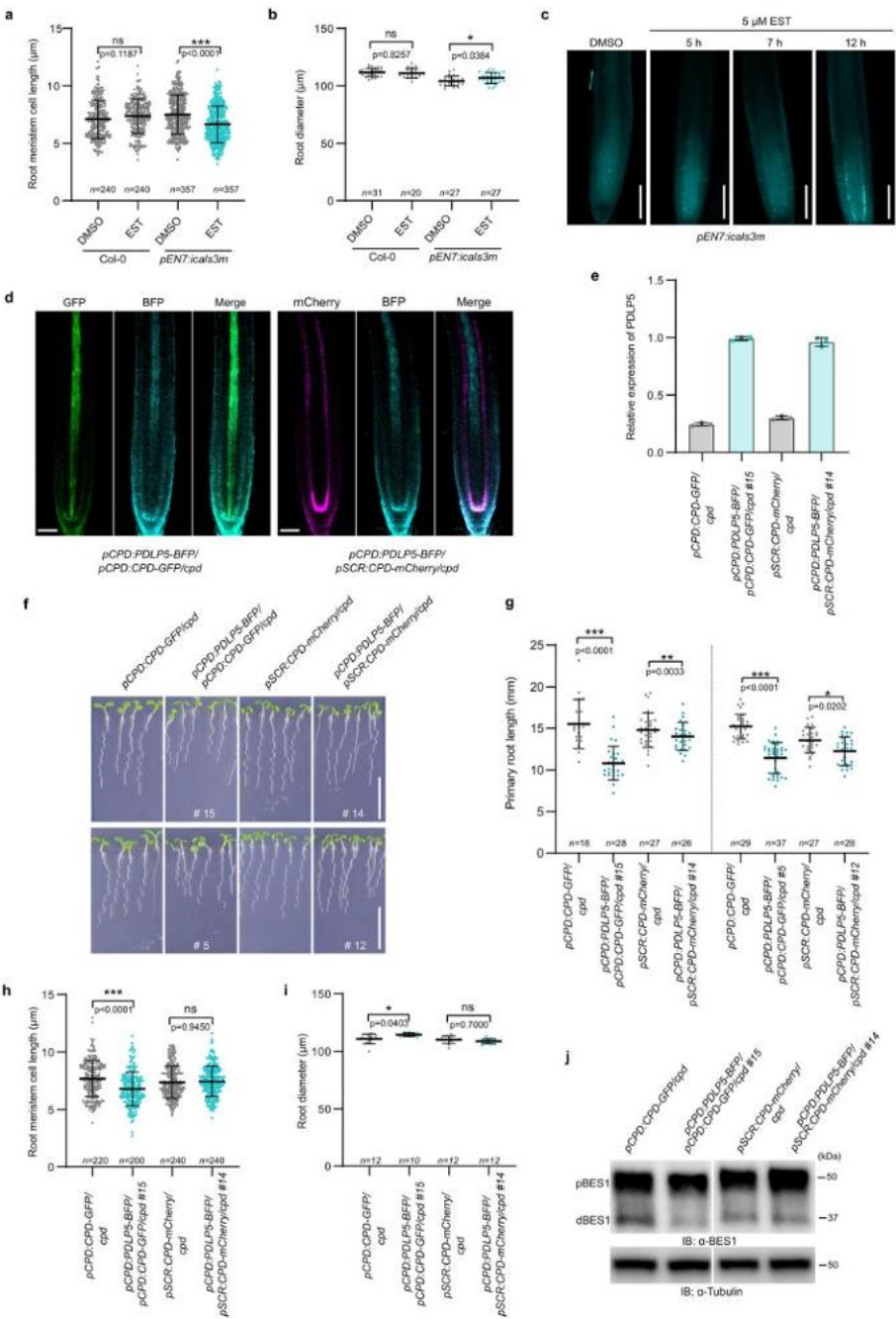
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Extended Data Fig. 1 | Subcellular localization of BR biosynthetic enzymes. **a,b,c,d.** The BR biosynthetic enzyme ROT3-GFP and DWF4-GFP co-localize with the ER marker C4H-mCherry when transiently co-expressed in tobacco leaves (**a,c**) and in *Arabidopsis* root epidermal cells expressing ROT3-GFP and DWF4-GFP under the control of their native promoters (**b,d**). The fluorescence intensity profiles along the white arrows are shown on the right. A.U., arbitrary units. **e.** Co-localization of HDEL-BFP and DWF4-GFP with plasmodesmata (PD) marker PDL1-mCherry and MCTP3-GFP with PD marker PDCB1-mCherry when transiently co-expressed in tobacco leaves. These co-expression combinations were used to calculate PD indexes in (**f**). **f.** The PD index of DWF4-GFP biosynthetic enzyme compared to the indexes of ER marker HDEL-BFP and PD-resident protein MCTP3-GFP. DWF4-GFP index above 1 indicates partial enrichment at PD. All

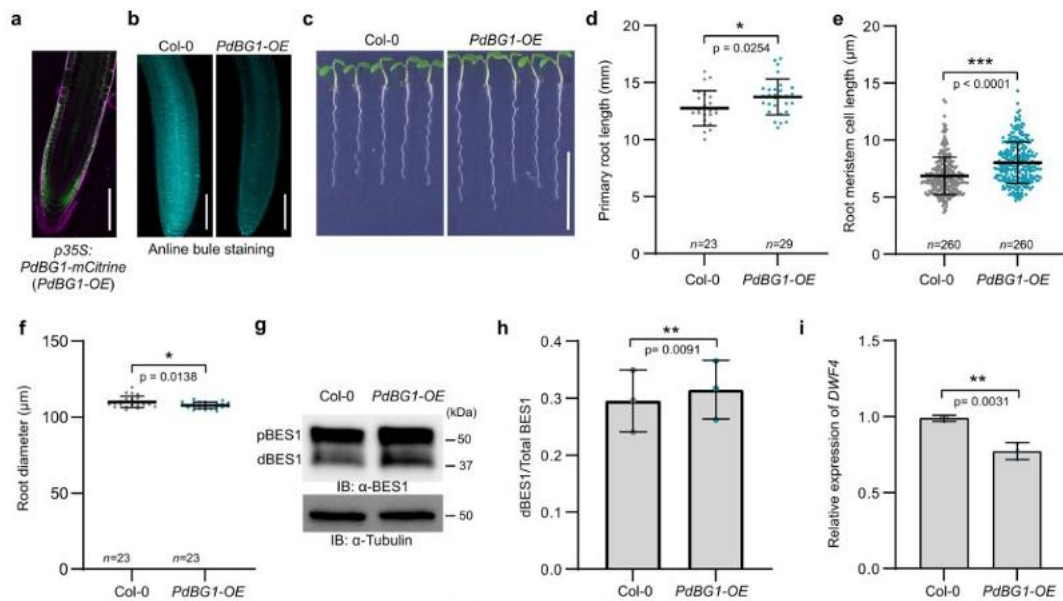
individual data points are plotted. Horizontal and error bars represent the means and s.d., respectively. n , number of ROIs used to calculate average PD index. The significant differences were determined with one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. *** $P < 0.001$. **g.** Co-localization of DWF4-GFP and ROT3-GFP biosynthetic enzymes expressed under their native promoters with the PD marker PDL1-mCherry in *Arabidopsis* roots. Epidermal and cortical cells of the root transition zone were imaged for DWF4-GFP and ROT3-GFP, respectively. White arrowheads mark PD, labeled by PDL1-mCherry. No clear co-localization of BR biosynthetic enzymes and PDL1-mCherry were observed. Scale bars, 10 μ m (**a,c,g**), 20 μ m (**e**) and 25 μ m (**b,d**). For **a,b,c,d,e,f**, the experiment was repeated three times and for **g,h**, twice with similar results.



Extended Data Fig. 2 | See next page for caption.

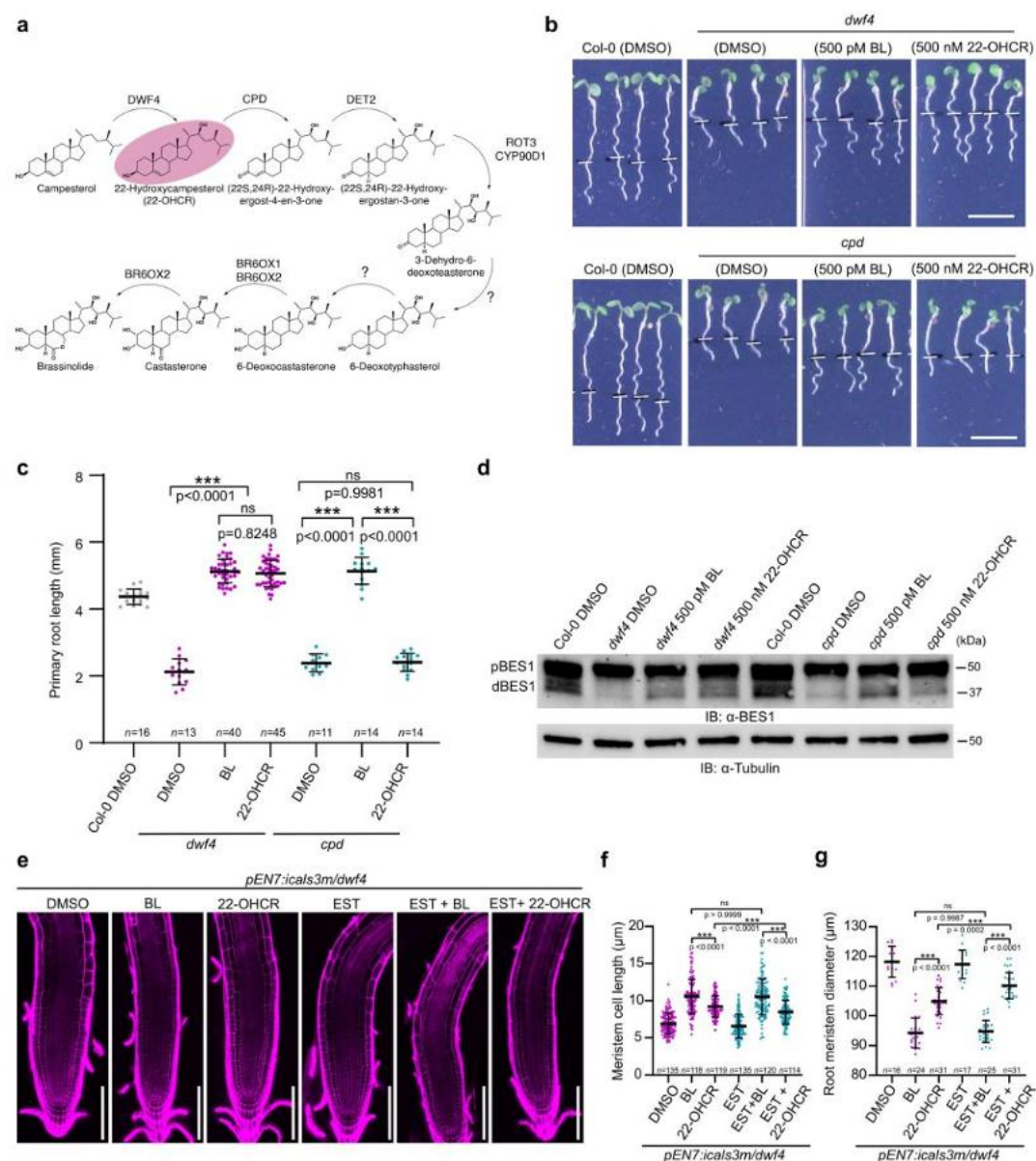
Extended Data Fig. 2 | Reduced cell-to-cell connectivity negatively affects BR signaling. **a,b.** Quantification of root meristem cell length in **(a)** and root diameter in **(b)** of 5-d-old Col-0 and *pEN7:icals3m* seedlings which were grown for 12 h on agar medium containing either estradiol (EST) (5 μ M) or DMSO (mock). For **(a,b)** the significant differences were determined with two-way analysis of variance (ANOVA) and Šidák's multiple comparisons test. *** $P < 0.001$ and * $P < 0.05$. **c.** Aniline blue staining of callose deposition in the root tips of *pEN7:icals3m* plants. Five-day-old seedlings were transferred to agar medium containing estradiol (EST) (5 μ M) for the indicated times and DMSO (mock), followed by aniline blue staining. Scale bars, 100 μ m. **d.** Confocal images of *pCPD:PDLPS-BFP/pCPD:CPD-GFP/cpd* and *pCPD:PDLPS-BFP/pSCR:CPD-mCherry/cpd* lines. Scale bars, 50 μ m. **e.** The relative gene expression of *PDLPS* in *pCPD:PDLPS-BFP/pCPD:CPD-GFP/cpd* (line #15) and *pCPD:PDLPS-BFP/pSCR:CPD-mCherry/cpd* (line #14) compared to their segregating siblings that do not express *pCPD:PDLPS-BFP*. Error bars represent s.d. **f.** Phenotypes of 6-d-old *pCPD:CPD-GFP/cpd*, *pCPD:PDLPS-BFP/pCPD:CPD-GFP/cpd*, *pSCR:*

CPD-mCherry/cpd, and *pCPD:PDLPS-BFP/pSCR:CPD-mCherry/cpd* seedlings from two independent transgenic lines. For each line, segregating siblings that do not express *pCPD:PDLPS-BFP* are shown. Scale bar, 1 cm. **g.** The quantification of primary root length of transgenic lines shown in **(f)**. **h,i.** Quantification of root meristem cell length in **(h)** and root diameter in **(i)** of seedlings shown in **(f)**. **j.** Phosphorylation status of BES1 detected by immunoblotting (IB) with α -BES1 antibody in roots. Tubulin detected with α -tubulin antibody was used as loading control. pBES1, phosphorylated BES1, dBES1, dephosphorylated BES1. Two panels from each row are from the same blots and were cropped and arranged for clarity. For **a,b,g,h,i**, all individual data points are plotted. Horizontal and error bars represent the means and s.d., respectively. *n*, number of roots used in **b,g,i**, and cells used in **a,h**. The significant differences for **g,h,i** were determined with one-way ANOVA and Tukey's multiple comparison tests. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. For **a,b,h,i,j**, the experiment was repeated twice and for **f,g**, three times with similar results.



Extended Data Fig. 3 | Increased cell-to-cell connectivity positively affects BR signaling. **a**, A confocal image of 6-d-old *p35S::PdBG1-mCitrine* (PdBG1-OE) root meristem. Cell walls were stained with propidium iodide. **b**, Aniline blue staining of callose deposition in the root tips of PdBG1-OE plants. Scale bars, 100 μm (**a**, **b**). **c**, Phenotype of 6-d-old PdBG1-OE seedlings. Scale bar, 1 cm. **d**, **e**, **f**, Quantification of the primary root length (**d**), root meristem cell length (**e**) and root meristem diameter (**f**) shown in (**c**). All individual data points are plotted. Horizontal and error bars represent the means and s.d., respectively. n , number of roots (**d**, **f**) and cells (**e**). The significant difference was determined with two-tailed Student's unpaired *t*-test analysis. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. **g**, Phosphorylation

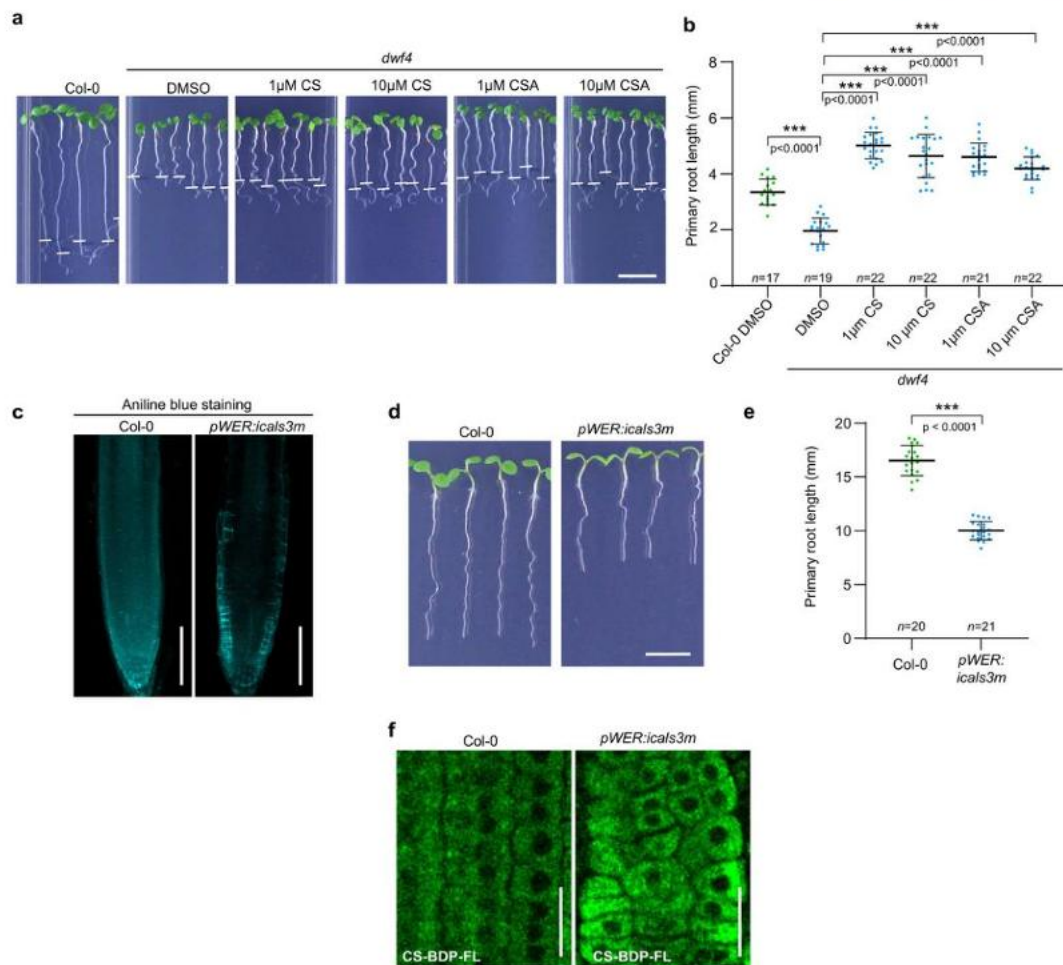
status of BES1 detected by immunoblotting with α -BES1 antibody in whole seedlings. Tubulin detected with α -tubulin antibody was used as loading control. pBES1, phosphorylated BES1; dBES1, dephosphorylated BES1. **h**, Quantification of BES1 dephosphorylation in (**g**) represented as a ratio of dephosphorylated BES1 (dBES1) relative to the total BES1. pBES1, phosphorylated BES1. **i**, The relative gene expression of *DWF4* in Col-0 and PdBG1-OE line. Error bars represent the s.d. For **h**, **i**, the significant difference was determined with two-tailed Student's unpaired *t*-test analysis. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. For **c**, **d**, **e**, **f**, the experiment was done in two and for **g**, **h**, **i**, in three independent biological repeats with similar results.



Extended Data Fig. 4 | See next page for caption.

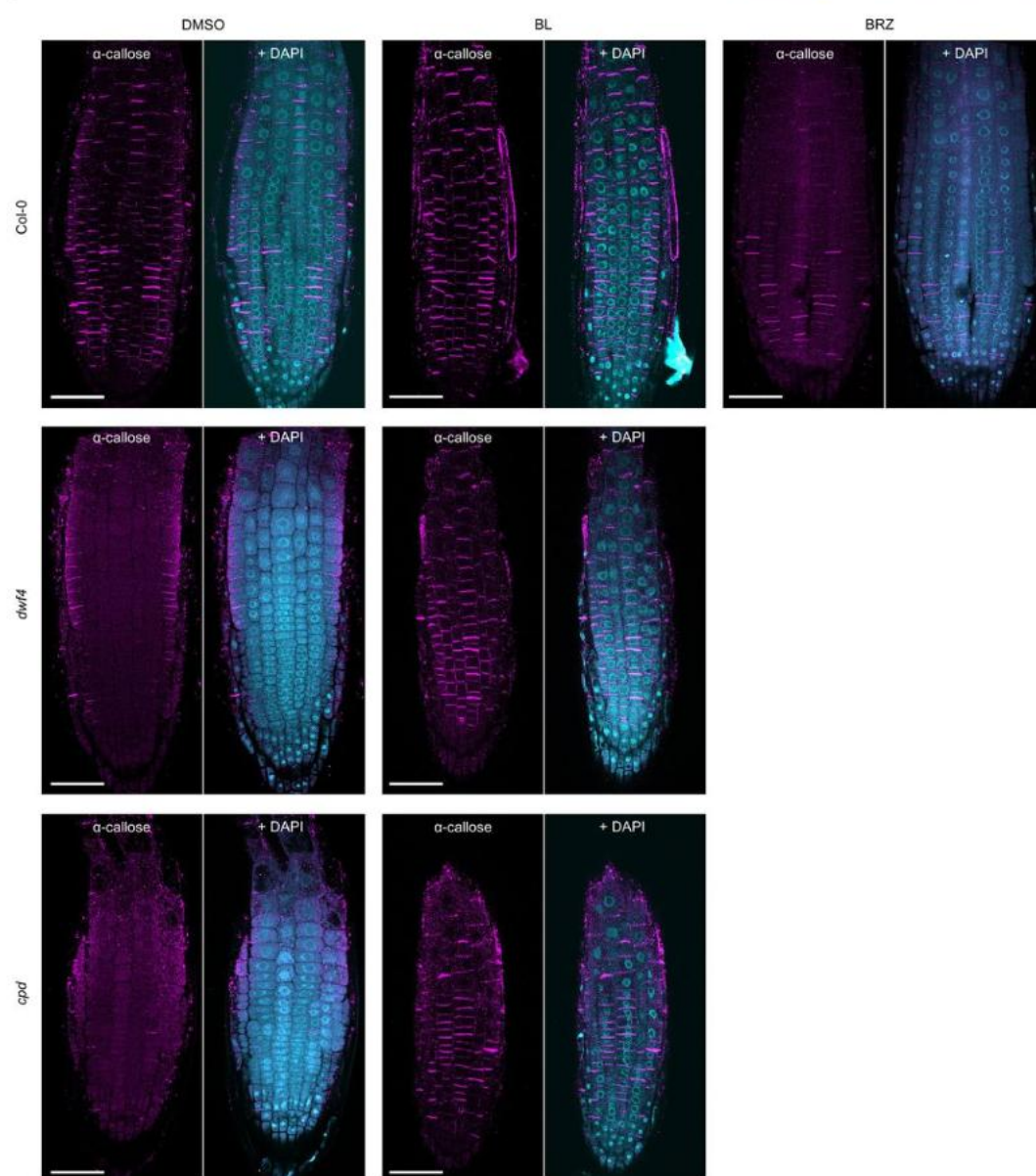
Extended Data Fig. 4 | 22-Hydroxycampesterol (22-OHCR) is an inactive BR precursor. **a**, BR biosynthetic pathway with all known enzymes and their presumed position within the pathway. The position of 22-OHCR is highlighted. **b**, 22-OHCR rescued the root phenotype of *duf4*, but not of *cpd*. Five-day-old *Arabidopsis* wild type (Col-0), *duf4*, and *cpd* seedlings were transferred to agar medium containing BL (500 pM), 22-OHCR (500 nM), and DMSO (mock), and imaged after 24 h. Root tips were marked immediately after the transfer (white bars). Scale bars, 5 mm. **c**, Quantification of the primary root length of Col-0, *duf4* and *cpd* in **(b)**. Horizontal and error bars represent the means and the s.d., respectively. *n*, number of roots analyzed. The significant differences between the wild type (Col-0) and the mutants were determined with one-way analysis

of variance (ANOVA) and Tukey's multiple comparison tests. *** $P < 0.001$. **d**, Phosphorylation status of BES1 detected by immunoblotting (IB) with the α -BES1 antibody in seedlings in **(b)**. Tubulin detected with the α -tubulin antibody was used as loading control. pBES1, phosphorylated BES1, dBES1, dephosphorylated BES1. **e**, Confocal images of 6-day-old root tips of *pBES1::gBES1-GFP/pEN7:icals3m/duf4* *Arabidopsis* seedlings stained with PI. Scale bars, 100 μ m. **f, g**, Quantification of the meristem cell length (**f**) and root meristem diameter (**g**) of roots shown in **(e)**. Horizontal and error bars represent the means and s.d., respectively. *n*, number cells. The significant differences were determined with one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. *** $P < 0.001$. For **b, c, d, e, f, g**, the experiment was repeated twice with similar results.



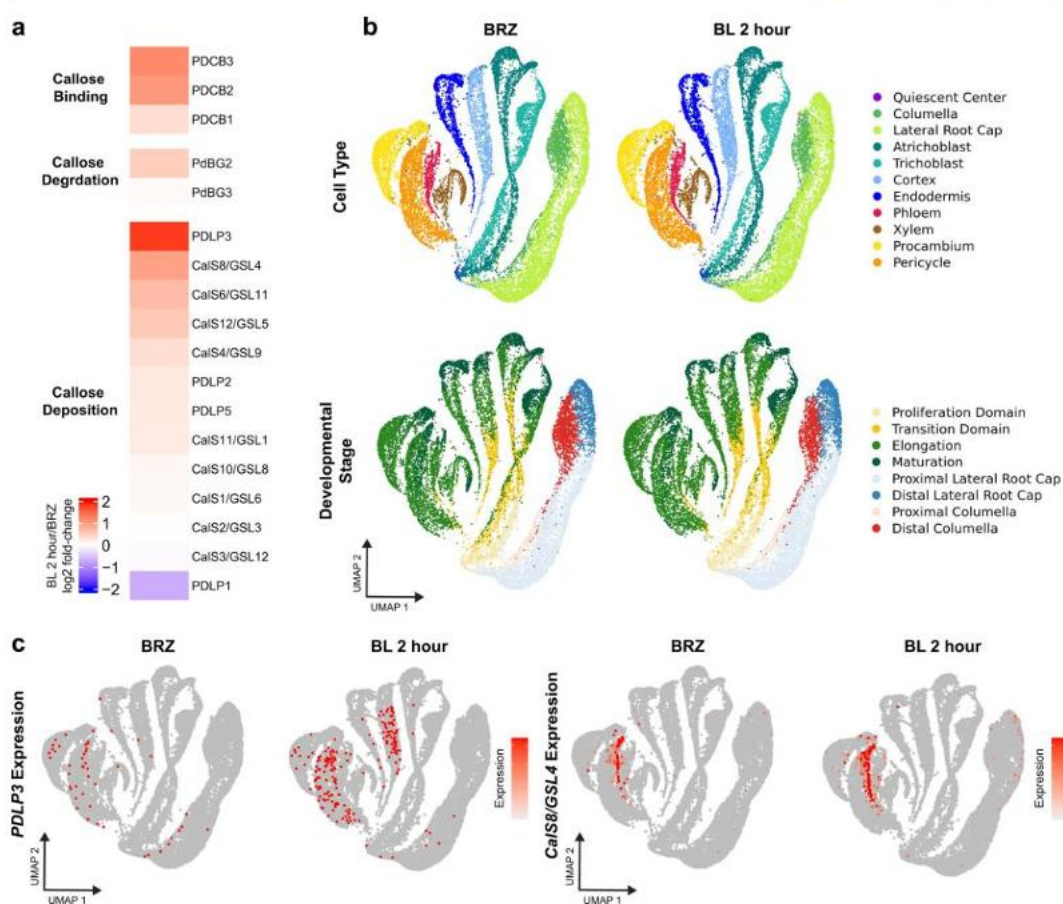
Extended Data Fig. 5 | Biological activity and uptake of castasterone-alkyne (CSA). **a**, CSA retains the biological properties of castasterone (CS). Five-day-old seedlings were transferred to agar media containing different concentrations of CS or CSA as indicated and DMSO (mock) for 24 h. Root tips were marked immediately after the transfer (white bars). Scale bar, 5 mm. **b**, Quantification of primary root length in **(a)**. Horizontal and error bars represent the means and the s.d., respectively. *n*, number of roots analyzed. The significant differences were determined with one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests. *** $P < 0.001$. **c**, Aniline blue staining of callose deposition in

6-day-old *pWER:icals3m* plants. Wild type (Col-0) was used as control (left panel). Scale bars, 100 μ m. **d**, Phenotypes of 6-d-old wild type (Col-0) and *pWER:icals3m* seedlings. Scale bar, 5 mm. **e**, Quantification of primary root length in **(c)**. Horizontal and error bars represent the means and the s.d., respectively. *n*, number of roots analyzed. The significant differences were determined with two-tailed Student's unpaired *t*-test analysis. *** $P < 0.001$. **f**, Accumulation of CS-BDP-FL signal in the epidermal cells of Col-0 and *pWER:icals3m* 6-d-old seedlings. Scale bars, 25 μ m. For **a**, **b**, **d**, **e**, the experiment was repeated twice with similar results.



Extended Data Fig. 6 | BRs positively regulate callose deposition in roots. Callose immunostaining of *Arabidopsis* wild type (Col-0), *dwf4* and *cpd* roots. Four-day-old seedlings were transferred to agar medium containing brassinolide (BL) (200 nM), brassinazole (BRZ) (1 μ M) and DMSO (mock) for 24 h. Cell nuclei

were stained by 4',6-diamidino-2-phenylindole (DAPI). Epidermal cell layers are shown. Scale bars, 50 μ m. The same experimental material was used to quantify callose deposition at PD in Fig. 4b.



Extended Data Fig. 7 | Expression levels of callose deposition-related genes are positively regulated by BRs. **a**, Heatmap showing relative expression of PD-related genes across all cells from publicly available brassinolide (BL) 2-hour scRNA-seq compared to brassinazole (BRZ) scRNA-seq. Color represent log2 fold-change of BL 2-hour versus BRZ. **b**, Two-dimensional uniform manifold

approximation and projection (UMAP) embedding of BRZ and BL 2-hour treated cells from scRNA-seq dataset³⁶. Colors indicate cell type (top) or developmental stage (bottom). **c**, *PDL3* and *CalS8/GSL4* expression in BRZ and BL 2-hour scRNA-seq. The color scale on the UMAP projection represents log-normalized, corrected UMI counts.