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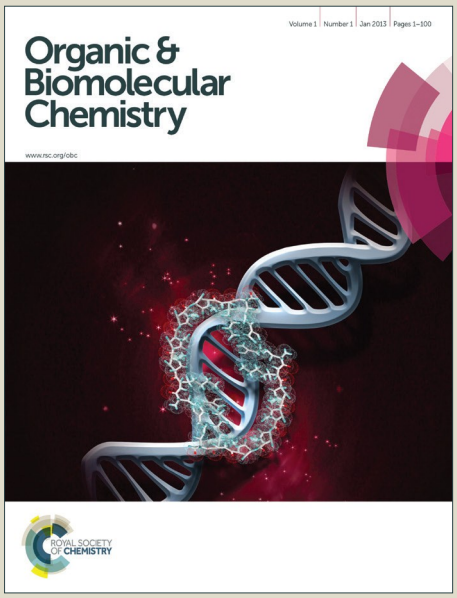

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ARTICLE

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

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We have prepared and studied a series of new brassinosteroid derivatives with *p*-substituted phenyl group in the side chain. To have the best comparison between molecular docking and biological activities both types of brassinosteroid were synthesized; 6-ketones, 10 examples, and B-lactones, 8 examples. The phenyl group was introduced into 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 plant hormone ethylene were also observed in etiolated pea seedlings after treatment with new brassinosteroids. The most active compounds were lactone 8f and 6-oxoderivatives 8c and 9c, their biological activities were comparable or even better than naturally occurring brassinolide. Finally cytotoxicity of new derivatives was studied using human normal and cancer cell lines.

Introduction

Brassinosteroids (BRs, Fig. 1) are a class of plant steroid hormone, that 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 application to medicine due to antiviral,[6,7] immunomodulatory and neuroprotective activity,[8,9] and anti-proliferative effects in animal cells in vitro.[10-15] In contrast to the mammalian nuclear steroid receptor, 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 fit into a highly nonpolar cavity of the receptor cleft and hydroxyl groups of BRs are exposed to the solvent or towards interaction 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 cykloalkyl subtituents at C-24 (replacing the isopropyl group of brassinolide **1**) exhibited significant activity in 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 interaction with the BRI1 receptor. The biological activities of newly prepared derivatives were confirmed using by 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.

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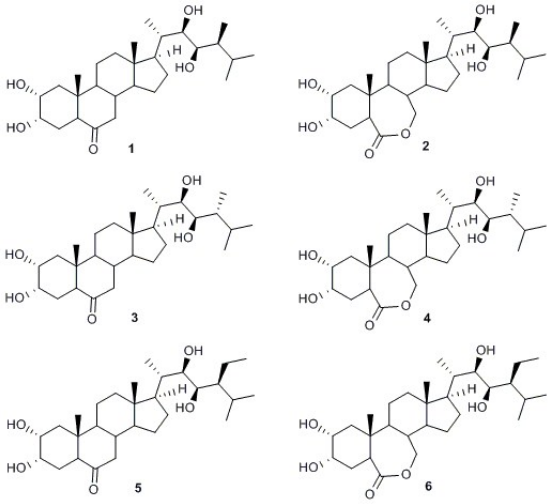


Fig. 1: Structures of most common natural brassinosteroids; castasterone (**1**), brassinolide (**2**), 24-epicastasterone (**3**), 24-epibrassinolide (**4**), 28-homocastasterone (**5**), 28-homobrassinolide (**6**).

Results and discussion

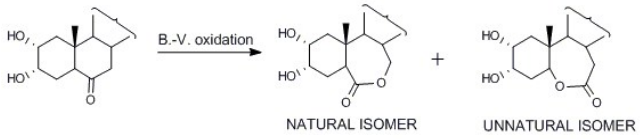
Chemistry

For the preparation of desired phenyl analogues of brassinosteroids, we set out from the known aldehyde **7** ((*20S*)-6,6-ethylenedioxy-5α-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 side chain, we used these substituents in *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 standard use of sodium hydride as a base in the HWE reaction, we observed isomerization of the methyl group on C-20 (see supporting information for detailed reaction conditions and analysis). Using this base along with steric hindrance of the reaction site for bulky aryl group, the reaction time increased and this led to enolization of 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 desired aryl diens **8a**-**17a** with 22*E* configuration were obtained. In almost all 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 ketal group gave aryl dienons **8b**-**17b** which were subjected to dihydroxylation in almost quantitative yield. Simultaneous Sharpless dihydroxylation of both double bonds was used to minimize formation of unnatural configuration of 22 and 23 hydroxy groups. As a chiral ligand, we used hydroquinidine 4-chlorobenzoate. The reaction rate was increased by addition of methansulfonamide.[25] Without methansulfonamide, 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 stereo controlled by A-ring conformation and the presence of a methyl group on C10. The attack of reagent is always from the bottom side of molecule and thus only 2α,3α-diol was detected.

As brassinosteroids with lactone in the B-ring are known to show higher biological activity than 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 approx. 10-15:1 (with natural isomer favoured). However, these mixtures were inseparable even by HPLC (Scheme 1).



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

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 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 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 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.

<Scheme 2>

Scheme 2: Synthesis of brassinosteroid phenyl analogues: a) benzyltriphenylphosphonium bromide or diethyl arylphosphonates, n-BuLi/THF; b) 5% HCl/THF; c) OsO_4, $CH_3SO_2NH_2$, $K_3[Fe(CN)_6]$, K_3CO_2,hydroquinidine 4-chlorobenzoate/t-BuOH, H_2O; d) Ac_2O/pyridine; e) trifluoroperoxyacetic acid/ CH_2Cl_2; f) NaOH/THF, H_2O.

Molecular docking

Molecular docking is a useful tool for understanding of 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 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 BRs derivatives with a phenyl ring on the tail replacing 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 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 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 sup. inf.). In all docking cases, lactones showed better binding energies than 6-ketones due to better fit to cavity, close to Y599. The best compound to emerge from molecular docking was compound **8f**.

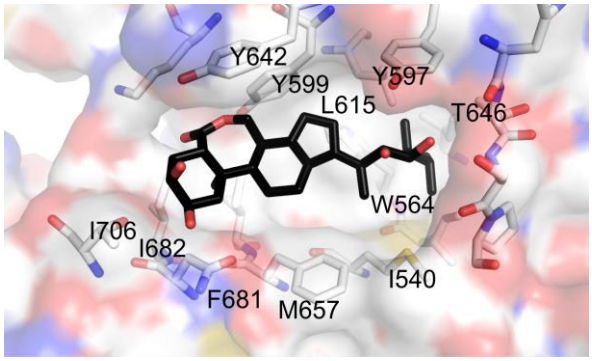


Fig. 2: 3D view of brassinolide with amino acid around active site.

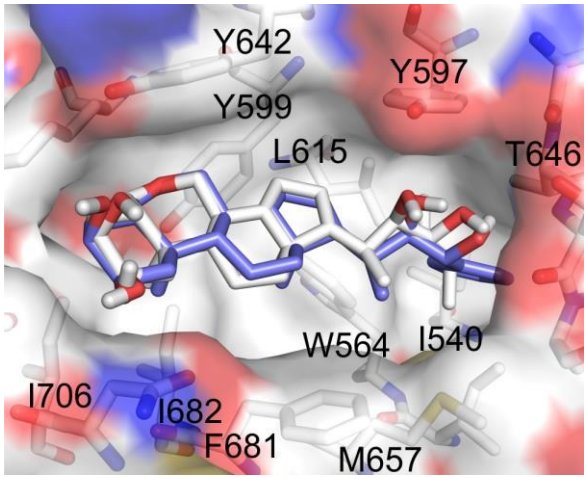


Fig. 3: Pose of **8f** within BRI1 binding site. Structure of brassinolide and BRI1 binding site are shown in white, structure of **8f** is shown in blue.

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 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 it accounts for leaf epinasty.[28] These effects are known as the "triple response" of etiolated seedlings to ethylene.[29]

Tab. 1: IC_{50} (mol/L) values obtained from the pea inhibition biotest.

Compound	IC_{50} (mol.L^{-1})
Brassinolide	$2.2.10^{-5} \pm 2.10^{-6}$
8c	$2.5.10^{-6} \pm 3.10^{-7}$
8f	$1.8.10^{-6} \pm 5.10^{-8}$
9c	$2.0.10^{-6} \pm 3.10^{-7}$
9f	$2.6.10^{-6} \pm 8.10^{-8}$
10c	$1.8.10^{-5} \pm 3.10^{-6}$
10f	$2.3.10^{-5} \pm 2.10^{-6}$
11c	$2.7.10^{-4} \pm 2.10^{-5}$
11f	$1.7.10^{-6} \pm 3.10^{-7}$
12c	$2.1.10^{-2} \pm 4.10^{-3}$
13c	$4.0.10^{-2} \pm 5.10^{-4}$
13f	no inhibition
14c	$1.8.10^{-4} \pm 7.10^{-6}$
14f	$1.4.10^{-6} \pm 2.10^{-7}$
15c	no inhibition
16c	$2.1.10^{-4} \pm 5.10^{-6}$
16f	no inhibition
17c	$2.0.10^{-3} \pm 6.10^{-5}$
17f	$2.1.10^{-4} \pm 5.10^{-6}$

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 BRs used as a positive control, was about ten time 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 BRs derivatives are shown in Fig. 4.

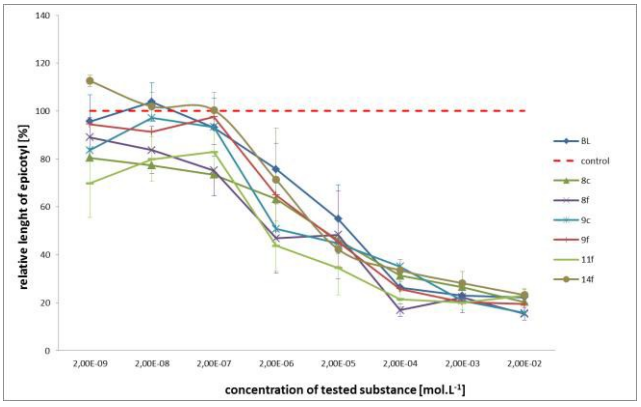


Fig. 4: Effect of selected brassinosteroid derivatives on the inhibition of etiolated pea seedlings. Error bars represent s.d.

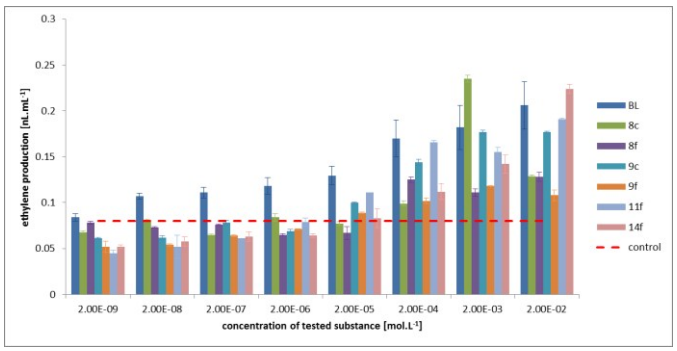


Fig. 5: Effect of selected brassinosteroid derivatives on ethylene production (nL.mL^{-1}) in etiolated pea seedlings determined by GC-FID 24 h after ventilation. Error bars represent s.d.

Levels of ethylene were measured in cultivation vessels during the incubation of etiolated pea plants after treatment with different BR derivatives (Fig. 5). The highest concentration of ethylene (235 and 224 nL. L^{-1}) was determined after 24 h treatment by **8c** and **14f** compared to 206 nL.L^{-1} for BL treatment. While 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*).* Arteca et al[30] also observed stimulated ethylene production in etiolated mung bean segments 4 hours after treatment with 1 µM 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** ≥**8f**>**9c**>**8c** in Arabidopsis sensitivity bioassays. The effects of tested compounds on the Arabidopsis roots and hypocotyls, are shown in supplementary information (Fig. S3 and Fig. S4). Compound **8f** significantly increased dephosphorylation of BES1 (Fig. 6), which is an important transcription factor in the BR signalling pathway. Taken together, these results confirm that the biological activity of compound **8f** is comparable with natural BR-brassinolide.

The antiproliferative activity of prepared brassinosteroid derivatives was tested using several models of normal and 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 Calcein AM cytotoxicity assay are presented in supplementary information (Table S2). Most tested BRs analogues had no detectable cytotoxic activity, even when tested in concentrations of up to 50 µM. Only compounds **10f, 11f** and **13f** showed moderate cytotoxic activity against CEM and Hela cell lines (IC_{50} around 35 µM). No BRs 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 µM).[15]

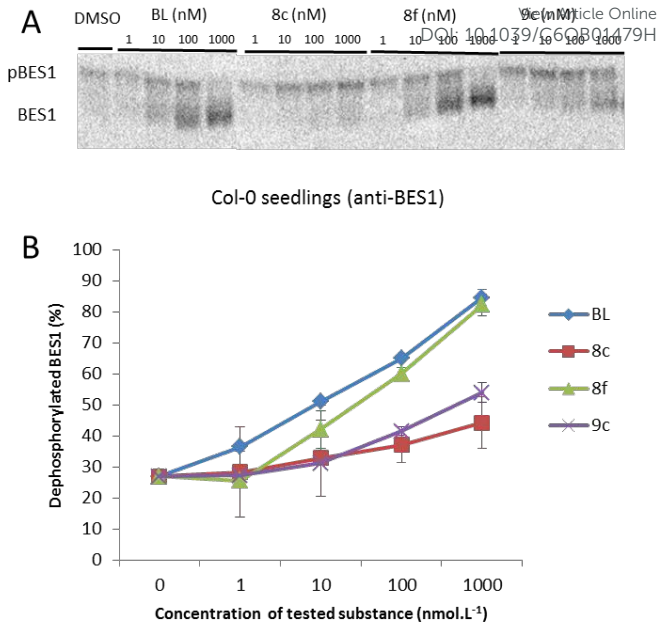


Fig. 6: **A**. Immunoblot analysis of BES1 in *Arabidopsis thaliana* (Col-0) seedlings showing dephosphorylation of BES1 after BRs treatment. **B**. Graph shows percentage of dephosphorylated BES1 relative to the total BES1 detected in Arabidopsis. The data are the average of two biological repeats.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

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; ^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer equipped with a 5 mm JEOL Royal probe. ^{1}H NMR and ^{13}C NMR chemical shifts (δ) were calibrated using tetramethylsilane (TMS, ^{1}H δ = 0 ppm) or solvents: $CDCl_3$ (^{1}H δ = 7.26 ppm, ^{13}C δ = 77.00 ppm) or DMSO-d_6 (^{1}H 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 µg.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 µA and the collision energy at value 4. The probe temperature was ramped up from 50°C to 600°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). 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. For experimental procedures and data for compounds of series b, d, e, and f see supplementary information.

(*22E*)-6,6-Ethylenedioxy-23-phenyl-24-nor-5α-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 °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 °C. The reaction mixture was quenched by 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. ^{1}H 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×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×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×CH), 126.64 (CH), 127.19 (CH), 128.43 (2×CH), 137.30 (CH), 138.08 (C). HRMS: (API+) calculated for $C_{31}H_{43}O_2$ ($[M+H]^+$) 447.3263, Found 447.3266.

General procedure for 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 by 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 gave the desired product.

(*22E*)-6,6-Ethylenedioxy-23-(4-fluorophenyl)-24-nor-5α-chola-2,22-diene (9a)

The general procedure for WHE reaction with diethyl 4-fluorophenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) yielded? 221 mg (89 %) of the title compound **9a** as a colorless oil: IR ν (cm^{-1}) 2933, 1654, 1599. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 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). ^{13}C NMR δ 12.21 (CH_3), 13.57 (CH_3), 20.36 (CH_3), 20.86 (CH_2), 21.42 (CH_2), 24.12 (CH_2), 28.37 (CH_2), 33.34 (CH), 35.90 (C), 39.68 (CH_2), 40.45 (CH), 41.20 (CH_2), 41.24 (CH_2), 42.63 (C), 48.08 (CH), 53.50 (CH), 55.92 (CH), 56.00 (CH), 64.07 (CH_2), 65.55 (CH_2), 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). ^{19}F NMR {^{1}H} δ -116.36 (s, 1F). HRMS: (API+) calculated for $C_{31}H_{42}FO_2$ ($[M+H]^+$) 465.3169, Found 465.3170.

(*22E*)-6,6-Ethylenedioxy-23-(4-chlorophenyl)-24-nor-5α-chola-2,22-diene (10a)

The general procedure for WHE reaction with diethyl 4-chlorophenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) yielded 225 mg (87 %) of the title compound **10a** as a colorless oil: IR ν (cm^{-1}) 2933, 1656, 1593. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 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). ^{13}C NMR δ 12.24 (CH_3), 13.60 (CH_3), 20.30 (CH_3), 20.86 (CH_2), 21.43 (CH_2), 24.14 (CH_2), 28.36 (CH_2), 33.35 (CH), 35.91 (C), 39.68 (CH_2), 40.50 (CH), 41.21 (CH_2), 41.24 (CH_2), 42.67 (C), 48.08 (CH), 53.48 (CH), 55.85 (CH), 55.98 (CH), 64.09 (CH_2), 65.58 (CH_2), 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 $C_{31}H_{42}ClO_2$ ($[M+H]^+$) 481.2873, Found 481.2878.

(*22E*)-6,6-Ethylenedioxy-23-(4-bromophenyl)-24-nor-5α-chola-2,22-diene (11a)

General procedure for WHE reaction with diethyl 4-bromophenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) afforded 231 mg (82 %) of the title compound **11a** as a colorless oil: IR ν (cm^{-1}) 2933, 1656, 1595. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 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). ^{13}C NMR δ 12.23 (CH_3), 13.59 (CH_3), 20.25 (CH_3), 20.85 (CH_2), 21.42 (CH_2), 24.13 (CH_2), 28.35 (CH_2), 33.34 (CH), 35.90 (C), 39.68 (CH_2), 40.51 (CH), 41.20 (CH_2), 41.23 (CH_2), 42.67 (C), 48.07 (CH), 53.47 (CH), 55.82 (CH), 55.97 (CH), 64.08 (CH_2), 65.57 (CH_2), 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 $C_{31}H_{42}{}^{79}BrO_2$ ($[M+H]^+$) 525.2368, Found 525.2370.

(*22E*)-6,6-Ethylenedioxy-23-(4-iodophenyl)-24-nor-5α-chola-2,22-diene (12a)

General procedure for WHE reaction with diethyl 4-iodophenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) afforded 200 mg (65 %) of the title compound **12a** as a colorless oil: IR ν (cm^{-1}) 2933, 1656, 1595. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 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). ^{13}C NMR δ 12.23 (CH_3), 13.59 (CH_3), 20.24 (CH_3), 20.85 (CH_2), 21.42 (CH_2), 24.13 (CH_2), 28.33 (CH_2), 33.34 (CH), 35.90 (C), 39.67 (CH_2), 40.50 (CH), 41.20 (CH_2), 41.23 (CH_2),

42.67 (C), 48.07 (CH), 53.47 (CH), 55.81 (CH), 55.96 (CH), 64.08 (CH_2), 65.57 (CH_2), 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 $C_{31}H_{42}IO_2$ ([M+H]$^+$) 573.2229, Found 573.2230.

(*22E*)-6,6-Ethylenedioxy-23-(4-nitrophenyl)-24-nor-5α-chola-2,22-diene (13a)

General procedure for WHE reaction with diethyl 4-nitrophenylphosphonate and chromatography on silica (Et_2O/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. ^{1}H NMR ($CDCl_3$) δ 0.76, 0.89 (both s, 3H, CH_3), 1.15 (d, 3H, J = 6.5 Hz, CH_3), 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). ^{13}C NMR δ 12.26 (CH_3), 13.59 (CH_3), 20.02 (CH_3), 20.85 (CH_2), 21.42 (CH_2), 24.14 (CH_2), 28.32 (CH_2), 33.34 (CH), 35.90 (C), 39.68 (CH_2), 40.75 (CH), 41.21 (CH_2), 41.23 (CH_2), 42.79 (C), 48.08 (CH), 53.46 (CH), 55.63 (CH), 55.92 (CH), 64.09 (CH_2), 65.58 (CH_2), 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 $C_{31}H_{42}NO_4$ ([M+H]$^+$) 492.3114, Found 492.3118.

(*22E*)-6,6-Ethylenedioxy-23-(4-methylphenyl)-24-nor-5α-chola-2,22-diene (14a)

General procedure for WHE reaction with diethyl 4-methylphenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) afforded 200 mg (81 %) of the title compound **14a** as a colorless oil: IR ν (cm^{-1}) 2934, 1656, 1595. ^{1}H NMR ($CDCl_3$) δ δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 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, CH_3), 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). ^{13}C NMR δ 12.20 (CH_3), 13.56 (CH_3), 20.42 (CH_3), 20.84 (CH_2), 21.07 (CH_3), 21.40 (CH_2), 24.11 (CH_2), 28.32 (CH_2), 33.32 (CH), 35.87 (C), 39.66 (CH_2), 40.42 (CH), 41.16 (CH_2), 41.21 (CH_2), 42.58 (C), 48.04 (CH), 53.47 (CH), 55.96 (2×CH), 64.03 (CH_2), 65.53 (CH_2), 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 $C_{32}H_{45}O_2$ ([M+H]$^+$) 461.3420, Found 461.3422.

(*22E*)-6,6-Ethylenedioxy-23-(4-methoxyphenyl)-24-nor-5α-chola-2,22-diene (15a)

General procedure for WHE reaction with diethyl 4-methoxyphenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) afforded 203 mg (80 %) of the title compound **15a** as a colorless oil: IR ν (cm^{-1}) 2934, 1656, 1594. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 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, OCH_3), 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). ^{13}C NMR δ 12.21 (CH_3), 13.58 (CH_3), 20.50 (CH_3), 20.85 (CH_2), 21.41 (CH_2), 24.12 (CH_2), 28.38 (CH_2), 33.33 (CH), 35.88 (C), 39.67 (CH_2), 40.43 (CH), 41.18 (CH_2), 41.22 (CH_2), 42.58 (C), 48.06 (CH), 53.48 (CH), 55.24 (CH_3), 55.99 (CH), 56.02 (CH), 64.05 (CH_2), 65.54 (CH_2), 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 $C_{32}H_{45}O_3$ ([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 WHE reaction with diethyl 4-isopropylphenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) afforded 231 mg (88 %) of the title compound **16a** as a colorless oil: IR ν (cm^{-1}) 2935, 1656, 1593. ^{1}H NMR ($CDCl_3$) δ ^{1}H NMR ($CDCl_3$) δ 0.74, 0.89 (both s, 3H, CH_3), 1.11 (d, 3H, J = 6.7 Hz, CH_3), 1.229, 1.231 (both d, 3H, J = 7.0 Hz, CH_3), 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, C*H*(CH_3)$_2$), 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). ^{13}C NMR δ 12.24 (CH_3), 13.60 (CH_3), 20.49 (CH_3), 20.86 (CH_2), 21.43 (CH_2), 23.97 (2×CH_3), 24.14 (CH_2), 28.35 (CH_2), 33.35 (CH), 33.79 (CH), 35.91 (C), 39.68 (CH_2), 40.50 (CH), 41.19 (CH_2), 41.24 (CH_2), 42.61 (C), 48.07 (CH), 53.49 (CH), 55.97 (CH), 56.00 (CH), 64.07 (CH_2), 65.56 (CH_2), 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 $C_{34}H_{49}O_2$ ([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 WHE reaction with diethyl 4-cyanophenylphosphonate and chromatography on silica (Et_2O/cyclohexane - 1/19) afforded 220 mg (87 %) of the title compound **17a** as an amorphous solid: IR ν (cm^{-1}) 2933, 2224, 1646, 1604. ^{1}H NMR ($CDCl_3$) δ 0.75, 0.89 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.7 Hz, CH_3), 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). ^{13}C NMR δ 12.25 (CH_3), 13.59 (CH_3), 20.07 (CH_3), 20.84 (CH_2), 21.42 (CH_2), 24.13 (CH_2), 28.32 (CH_2), 33.34 (CH), 35.90 (C), 39.67 (CH_2), 40.66 (CH), 41.20 (CH_2), 41.22 (CH_2), 42.76 (C), 48.08 (CH), 53.45 (CH), 55.65 (CH), 55.92 (CH), 64.09 (CH_2), 65.58 (CH_2), 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 $C_{32}H_{42}NO_2$ ([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), methansulfonamide (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/20 mL; 0.039 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 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/$CHCl_3$ - 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. ^{1}H NMR (DMSO-d_6) δ 0.30, 0.59 (both s, 3H, CH_3), 0.86 (d, 3H, J = 6.4 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.97 (dd, 1H, J = 13.1, J´ = 4.6 Hz), 2.07 (t, 1H, J = 12.6Hz), 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 (CH_3), 12.47 (CH_3), 13.36 (CH_3), 20.79 (CH_2), 23.28 (CH_2), 26.82 (CH_2), 27.22 (CH_2), 36.29 (CH), 37.02 (CH), 39.14 (C), 41.84 (C), 42.03 (CH_2), 45.95 (CH_2), 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×CH), 127.21 (CH), 128.07 (2×CH), 143.28 (C), 211.57 (C). One CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{43}O_5$ ([M+H]$^+$) 471.3110, Found 471.3108. Anal. Calcd for $C_{29}H_{42}O_5$: C, 74.01; H, 8.99. Found: C, 73.95; H, 9.06 %.

(*22R*, *23R*)-2α,3α,22,23-tetrahydroxy-23-(4-fluorophenyl)-24-nor-5α-cholan-6-one (9c)

The general procedure for dihydroxylation of **9b** and chromatography on silica (MeOH/$CHCl_3$ - 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. ^{1}H NMR (DMSO-d_6) δ 0.33, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.85 (m, 1H), 1.98 (dd, 1H, J = 13.0, J´ = 4.8 Hz), 2.07 (t, 1H, J = 12.6Hz), 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 (CH_3), 12.44 (CH_3), 13.38 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.84 (CH_2), 27.28 (CH_2), 36.35 (CH), 37.04 (CH), 39.15 (C), 41.86 (C), 42.06 (CH_2), 45.97 (CH_2), 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×CH), 128.86 (d, J = 8.4 Hz, 2×CH), 139.58 (d, J = 2.4 Hz, C), 161.26 (d, J = 242.3 Hz, C), 211.62 (C). One CH_2 covered by DMSO multiplet. ^{19}F NMR {^{1}H} δ -115.37 (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, 71.88; H, 8.55 %.

(*22R*, *23R*)-2α,3α,22,23-tetrahydroxy-23-(4-chlorophenyl)-24-nor-5α-cholan-6-one (10c)

The general procedure for dihydroxylation of **10b** and chromatography on silica (MeOH/$CHCl_3$ - 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. ^{1}H NMR (DMSO-d_6) δ 0.34, 0.60 (both s, 3H, CH_3), 0.85 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.85 (m, 1H), 1.98 (dd, 1H, J = 13.0, J´ = 4.8 Hz), 2.07 (t, 1H, J = 12.6Hz), 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 (CH_3), 12.49 (CH_3), 13.38 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.84 (CH_2), 27.29 (CH_2), 36.47 (CH), 37.05 (CH), 39.15 (C), 41.87 (C), 42.07 (CH_2), 45.98 (CH_2), 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×CH), 128.87 (2×CH), 131.52 (C), 142.41 (C), 211.62 (C). One CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{42}ClO_5$ ([M+H]$^+$) 505.2721, Found 505.2723. Anal. Calcd for $C_{29}H_{41}ClO_5$: C, 68.96; H, 8.18. Found: C, 68.90; H, 8.28 %.

(*22R*, *23R*)-2α,3α,22,23-tetrahydroxy-23-(4-bromophenyl)-24-nor-5α-cholan-6-one (11c)

The general procedure for dihydroxylation of **11b** and chromatography on silica (MeOH/$CHCl_3$ - 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. ^{1}H NMR (DMSO-d_6) δ 0.34, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.79 (m, 1H), 1.87 (m, 1H), 1.98 (dd, 1H, J = 13.0, J´ = 4.6 Hz), 2.07 (t, 1H, J = 12.5Hz), 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×Ar-H), 7.51 (d, 2H, J = 8.6 Hz, 2×Ar-H). ^{13}C NMR δ 11.49 (CH_3), 12.50 (CH_3), 13.38 (CH_3), 20.81 (CH_2), 23.31 (CH_2), 26.84 (CH_2), 27.30 (CH_2), 36.50 (CH), 37.06 (CH), 39.15 (C), 41.87 (C), 42.08 (CH_2), 45.99 (CH_2), 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×CH), 131.01 (2×CH), 142.83 (C), 211.62 (C). One CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{42}{}^{79}BrO_5$ ([M+H]$^+$) 549.2216, Found 549.2216. Anal. Calcd for $C_{29}H_{41}BrO_5$: C, 63.38; H, 7.52. Found: C, 63.29; H, 7.55 %.

(*22R*, *23R*)-2α,3α,22,23-tetrahydroxy-23-(4-iodophenyl)-24-nor-5α-cholan-6-one (12c)

The general procedure for dihydroxylation of **12b** and chromatography on silica (MeOH/$CHCl_3$ - 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. ^{1}H NMR (DMSO-d_6) δ 0.35, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.79 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, J = 13.2, J´ = 4.9 Hz), 2.07 (t, 1H, J = 12.5Hz), 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×Ar-H), 7.67 (m, 2H, 2×Ar-H). ^{13}C NMR δ 11.49 (CH_3), 12.48 (CH_3), 13.36 (CH_3), 20.80 (CH_2), 23.29 (CH_2), 26.83 (CH_2), 27.28 (CH_2), 36.51 (CH), 37.03 (CH), 39.14 (C), 41.85 (C), 42.05 (CH_2), 45.96 (CH_2), 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×CH), 136.83 (2×CH), 143.19 (C), 211.57 (C). One CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{29}H_{42}IO_5$ ([M+H]$^+$) 597.2077, Found 597.2076. Anal. Calcd for $C_{29}H_{41}IO_5$: C, 58.39; H, 6.93. Found: C, 58.32; H, 7.01 %.

(*22R*, *23R*)-2α,3α,22,23-tetrahydroxy-23-(4-nitrophenyl)-24-nor-5α-cholan-6-one (13c)

The general procedure for dihydroxylation of **13b** and chromatography on silica (MeOH/$CHCl_3$ - 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. ^{1}H NMR (DMSO-d_6) δ 0.34, 0.59 (both s, 3H, CH_3), 0.87 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.97 (dd, 1H, J = 13.2, J´ = 4.6 Hz), 2.06 (t, 1H, J = 12.5Hz), 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 (CH_3), 12.64 (CH_3), 13.37 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.83 (CH_2), 27.32 (CH_2), 36.77 (CH), 37.03 (CH), 39.14 (C), 41.86 (C), 42.09 (CH_2), 45.97 (CH_2), 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 CH_2 covered by DMSO multiplet. HRMS: (ESI-) calculated for $C_{29}H_{41}NO_7$ ($[M^{\bullet}]^-$) 515.2883, Found 515.2888. Anal. Calcd for $C_{29}H_{41}NO_7$: 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 ($MeOH/CHCl_3$ - 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-d_6) δ 0.32, 0.59 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, J = 13.2, J´ = 4.6 Hz), 2.07 (t, 1H, J = 12.5Hz), 2.27 (s, 3H, CH_3), 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). ^{13}C NMR δ 11.50 (CH_3), 12.44 (CH_3), 13.40 (CH_3), 20.83 (CH_2, CH_3), 23.32 (CH_2), 26.85 (CH_2), 27.28 (CH_2), 36.38 (CH), 37.07 (CH), 39.15 (C), 41.89 (C), 42.07 (CH_2), 46.00 (CH_2), 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 CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{30}H_{45}O_5$ ($[M+H]^+$) 485.3267, Found 485.3270. Anal. Calcd for $C_{30}H_{44}O_5$: 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 ($MeOH/CHCl_3$ - 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-d_6) δ 0.33, 0.60 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.80 (m, 1H), 1.86 (m, 1H), 1.98 (dd, 1H, J = 13.2, J´ = 4.6 Hz), 2.07 (t, 1H, J = 12.7Hz), 2.58 (dd, 1H, J = 12.2, J´ = 3.4 Hz), 3.42-3.49 (m, 2H), 3.72 (s, 3H, CH_3), 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). ^{13}C NMR δ 11.48 (CH_3), 12.38 (CH_3), 13.37 (CH_3), 20.80 (CH_2), 23.30 (CH_2), 26.83 (CH_2), 27.26 (CH_2), 36.31 (CH), 37.04 (CH), 39.14 (C), 41.86 (C), 42.04 (CH_2), 45.97 (CH_2), 50.30 (CH), 51.89 (CH), 52.88 (CH), 54.97 (CH_3), 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 CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{30}H_{45}O_6$ ($[M+H]^+$) 501.3218, Found 501.3216. Anal. Calcd for $C_{30}H_{44}O_6$: 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 ($MeOH/CHCl_3$ - 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-d_6) δ 0.31, 0.59 (both s, 3H, CH_3), 0.84 (d, 3H, J = 6.7 Hz, CH_3), 1.17 (d, 6H, J = 6.9 Hz, 2×CH_3), 1.77-1.86 (m, 2H), 1.98 (dd, 1H, J = 13.2, J´ = 4.6 Hz), 2.07 (t, 1H, J = 12.5Hz), 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). ^{13}C NMR δ 11.46 (CH_3), 12.50 (CH_3), 13.37 (CH_3), 20.80 (CH_2), 23.32 (CH_2), 23.92 (CH_3), 24.02 (CH_3), 26.85 (CH_2), 27.20 (CH_2), 33.11 (CH), 36.33 (CH), 37.05 (CH), 39.14 (C), 41.87 (C), 42.07 (CH_2), 45.98 (CH_2), 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 CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{32}H_{49}O_5$ ($[M+H]^+$) 513.3580, Found 513.3585. Anal. Calcd for $C_{32}H_{48}O_5$: 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 ($MeOH/CHCl_3$ - 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-d_6) δ 0.33, 0.59 (both s, 3H, CH_3), 0.85 (d, 3H, J = 6.7 Hz, CH_3), 1.74-1.88 (m, 2H), 1.98 (dd, 1H, J = 13.2, J´ = 4.8 Hz), 2.06 (t, 1H, J = 12.5Hz), 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). ^{13}C NMR δ 11.47 (CH_3), 12.64 (CH_3), 13.41 (CH_3), 20.83 (CH_2), 23.33 (CH_2), 26.87 (CH_2), 27.33 (CH_2), 36.71 (CH), 37.08 (CH), 39.14 (C), 41.89 (C), 42.11 (CH_2), 46.00 (CH_2), 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 CH_2 covered by DMSO multiplet. HRMS: (API+) calculated for $C_{30}H_{42}NO_5$ ($[M+H]^+$) 496.3063, Found 496.3064. Anal. Calcd for $C_{30}H_{41}NO_5$: C, 72.70; H, 8.34. Found: C, 72.69; H, 8.39 %.

Molecular docking

Docking was performed to obtain prediction of conformation and energy ranking between BRI1 receptor (PDB ID: 3RGZ) and the steroid molecule. The docking studies were carried out using AutoDock Vina 1.05.[31] All 3D structures of BRI1 ligands were obtained with Marvin 5.10.3[32], 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)[16] program prior to docking with Autodock Vina program. Grid box with size of 40 Å were centered on active site of 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 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.

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) equipped with a flame 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.

Arabidopsis brassinosteroid sensitivity assays

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 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, plants were exposed to light for 6 h and grown in 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 (1:10000; 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). Cells were cultured in DMEM (Dulbecco's Modified Eagle 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% CO_2 in a humid environment. Cells were subcultured twice or three times a week using the standard trypsinization procedure.

Calcein AM cytotoxicity assay

Suspensions with approximately 1.0×10^5 cells/mL were distributed in 96-well microtiter plates and after 24 h of stabilization the BRs 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 a further hour. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of 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 control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} 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 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. 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 resistance of plants against various biotic and abiotic stresses. Because recent progress in the chemical synthesis also leads to overcome economically restriction, which are currently the major constraints to use BRs at large scale in the fields.

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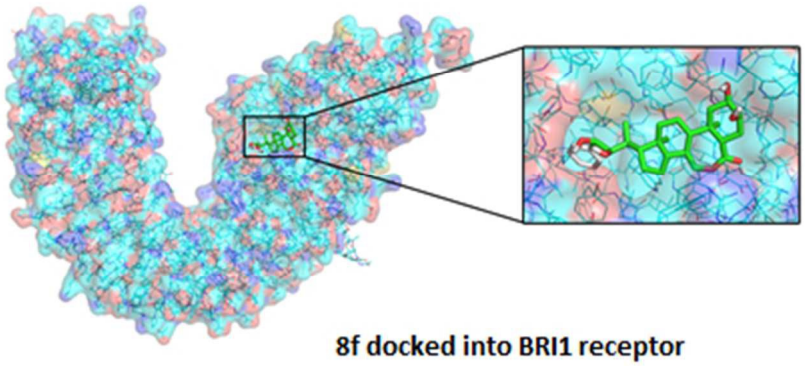

8f docked into BRI1 receptor

110x53mm (96 x 96 DPI)