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Biological activities of new monohydroxylated brassinosteroid analogues with a carboxylic group in the side chain

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ABSTRACT

Thirteen monohydroxylated brassinosteroids analogues were synthesized and tested for their biological activity in plant and animal systems. The cytotoxic activity of the products was studied using human normal and cancer cell lines with 28-homocastasterone as positive control, their brassinolide type activity was established using the bean second-internode test with 24-epibrassinolide as standard.

1. Introduction

Brassinosteroids (BRs) represent a large group of plant steroids which include more than 70 structurally and functionally related compounds [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 pollen, seeds, leaves, stems, roots, flowers and insect galls. They demonstrate various kinds of regulatory action on the growth and development of plants, such as the stimulation of cell enlargement and cell division, improvement of the biomass formation, yield and quality of seeds, and plant adaptability [2]. 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 hormones. Plants perceive steroids at cell membrane, using the membrane-integral receptor kinase brassinosteroid insensitive 1 (BRI1) [3–5]. The encoded protein, BRI1, 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. BRI1 has been established as an authentic brassinosteroid receptor by genetic and biochemical investigations [6]. Crystal structures of BRI1 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 [6,7]. 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* BRI1 enable the rational design of brassinosteroid-like antagonists and agonists. Recent studies have indicated that BRs have antiviral, antiproliferative and antibacterial activity [8]. BRs analogues have been reported to have antiviral activity against herpes simplex virus type 1, arenaviruses as well as against replication of vesicular stomatitis virus in Vero cells [9–11]. Natural types of BRs and their analogues affected the viability, proliferation, apoptosis and expression of some cell cycle related proteins in cancer and normal human cell lines [12–14]. It was shown that natural BRs, 24-epibrassinolide and 28-homocastasterone, inhibited *in vitro* angiogenesis of human endothelial cells [15].

The aim of our study relates to the synthesis of new brassinosteroid monohydroxylated derivatives and to study of their biological and anticancer properties. The plant growth promoting activity of synthetic analogs was assayed using the bean second internode bioassay. The antiproliferative activity of the new BRs analogs was evaluated *in vitro* using cancer cell lines of different histopathological origins and normal human fibroblasts. All derivative

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structures were further subjected to docking studies using AutoDock Vina [16] in order to find structural patterns responsible for the results.

2. Experimental

2.1. General methods

The melting points were determined on a Hund H 600 apparatus (Helmut Hund, Germany). The elemental analyses (C, H, N) were carried out on a Perkin-Elmer 2400 II elemental analyzer. Optical rotations were measured on an Autopol IV polarimeter (Rudolf Research Analytical, Flanders, USA) at 25 °C in chloroform and $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g. The infrared spectra were recorded on a Bruker IFS 55 spectrometer in chloroform. The wave numbers are given in cm^{-1}. The ^{1}H NMR spectra were taken in $CDCl_3$ on a Bruker AVANCE-400 (at 400 MHz) instrument with tetramethylsilane as an internal reference. Chemical shifts are given in ppm (δ-scale), coupling constants (J) in Hz. All of the values were obtained by first-order analysis. The mass spectra (ESI) were obtained with a LTQ Orbitrap XL (Thermo Fisher Scientific). For column chromatography, neutral silica gel (60 μm, Fluka) was used. The HPLC system consisted of a Gilson semi-preparative HPLC system including a quaternary pump, liquid handler, UV–VIS, RI, and ELSD detectors. The semi preparative column was based on silica gel (Labio Biosphere PSI 200).

Reagents and solvents were purchased from Sigma–Aldrich and were not purified.

2.2. Hydroboration of **1**

Solution of borane in THF (1 M, 20 mL) was added dropwise to the solution of methyl ester **1** (1 g, 2.49 mmol) in dry THF (30 mL). The reaction mixture was stirred at room temperature for 6 h. Then, the reaction mixture was quenched by water (10 mL) and cooled to 10 °C. Aqueous solution of NaOH (10%, 20 mL) was added followed by dropwise addition of 30% hydrogen peroxide (30 mL). This reaction mixture was stirred overnight at room temperature. It was then poured into a mixture of sodium sulfite (2 g) in water (50 mL) and acetic acid (1 mL) and stirred for additional 30 min. After then, the reaction mixture was diluted with ethyl acetate, extracted twice with water, the extract was dried over anhydrous sodium sulfate, and solvents evaporated under reduced pressure. A crude mixture of products was dissolved in a mixture of THF and acetone (5:1, 10 mL) and 5% aqueous HCl was added. The reaction mixture was heated under reflux for 3 h. Then it was diluted with ethyl acetate, extracted twice with water, the extract was dried over anhydrous sodium sulfate, and the solvents were evaporated under reduced pressure. Two main products were separated on HPLC in ethyl acetate–hexane (1:1). Two hydroxy compounds **2a** and **2b** were obtained.

2.2.1. Methyl (20S)-3α-hydroxy-6-oxo-5α-pregnane-20-carboxylate (**2a**)

Yield: 380 mg (40%), m.p. 198–200 °C (MeOH), $[\alpha]_D = -6°$ (c 0.27). IR ν (cm^{-1}) 3615, 1727, 1706, 1165. ^{1}H NMR ($CDCl_3$) δ 0.68, 0.73 (both s, 3H, CH_3), 1.19 (d, 3H, J = 6.9 Hz, CH_3), 1.97 (m, 1H), 2.02 (dd, 1H, J = 12.8, J' = 1.0 Hz, H-7β), 2.29 (dd, 1H, J = 12.8, J' = 4.5 Hz, H-7α), 2.43 (dq, 1H, J = 10.3, J' = 6.8 Hz, H-20), 2.73 (dd, 1H, J = 8.9, J' = 6.9 Hz, H-5α), 3.65 (s, 3H, OCH_3), 4.16 (t, 1H, J = 2.1 Hz, H-3β). ^{13}C NMR δ 12.17 (CH_3), 12.30 (CH_3), 17.06 (CH_3), 21.01 (CH_2), 23.96 (CH_2), 26.95 (CH_2), 27.69 (CH_2), 28.16 (CH_2), 31.67 (CH_2), 37.90 (CH), 39.27 (CH_2), 41.49 (C), 42.35 (CH), 43.00 (C), 46.75 (CH_2), 51.34 (CH), 51.66 (CH_3), 52.79 (CH), 53.78 (CH), 56.37 (CH), 65.35 (CH), 177.13 (C), 212.37 (C). HRMS: (ESI+) calculated for $C_{23}H_{36}O_4Na$ (M^++Na) 399.25058. Found 399.25059. Anal. Calcd for $C_{23}H_{36}O_4$: C, 73.37; H, 9.64. Found: C, 73.45; H, 9.60%.

2.2.2. Methyl (20S)-2α-hydroxy-6-oxo-5α-pregnane-20-carboxylate (**2b**)

Yield: 400 mg (46%), m.p. 153–155 °C (MeOH), $[\alpha]_D = -8°$ (c 0.62). IR ν (cm^{-1}) 3608, 1727, 1708, 1168. ^{1}H NMR ($CDCl_3$) δ 0.68, 0.75 (both s, 3H, CH_3), 1.20 (d, 3H, J = 6.9 Hz, CH_3), 1.95–2.10 (m, 4H), 2.15 (dd, 1H, J = 11.9, J' = 2.6 Hz, H-5α), 2.29 (dd, 1H, J = 13.1, J' = 4.6 Hz, H-7α), 2.43 (dq, 1H, J = 10.3, J' = 6.8 Hz, H-20), 3.65 (s, 3H, OCH_3), 3.77 (m, 1H, $W_{½}$ = 31.5 Hz, H-2β). ^{13}C NMR δ 12.16 (CH_3), 14.14 (CH_3), 17.06 (CH_3), 19.49 (CH_2), 21.20 (CH_2), 23.96 (CH_2), 26.95 (CH_2), 34.57 (C), 37.57 (CH), 39.18 (CH_2), 42.31 (CH), 42.98 (CH_2), 46.56 (CH_2), 47.07 (C), 51.35 (CH_3), 52.76 (CH), 54.12 (CH), 56.26 (CH), 57.95 (CH), 66.93 (CH), 177.13 (C), 211.58 (C). HRMS: (ESI+) calculated for $C_{23}H_{36}O_4Na$ (M^++Na) 399.25058. Found 399.25057. Anal. Calcd for $C_{23}H_{36}O_4$: C, 73.37; H, 9.64. Found: C, 73.42; H, 9.57%.

2.3. 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 water, a saturated solution of potassium hydrogen carbonate, water, and dried over anhydrous sodium sulfate. Solvents were evaporated under reduced pressure and products were separated and purified on HPLC in ethyl acetate–hexane 7:3.

2.4. Baeyer–Villiger oxidation of **2a**

Ketone **2a** (150 mg; 0.40 mmol) was used for Baeyer–Villiger oxidation as described in general procedure. Reaction afforded two lactones **3a** and **3b**.

2.4.1. Methyl (20S)-3α-hydroxy-7-oxa-7a-homo-6-oxo-5α-pregnane-20-carboxylate (**3a**)

Yield: 69 mg (44%), m.p. 185–187 °C (MeOH), $[\alpha]_D = +10°$ (c 0.27). IR ν (cm^{-1}) 3615, 1723, 1182. ^{1}H NMR ($CDCl_3$) δ 0.72, 0.90 (both s, 3H, CH_3), 1.19 (d, 3H, J = 6.9 Hz, CH_3), 1.93 (dt, 1H, J = 6.6, J' = 3.0 Hz), 2.14 (ddd, 1H, J = 15.0, J' = 12.3, J'' = 2.6 Hz, H-4a), 2.43 (dq, 1H, J = 10.3, J' = 6.9 Hz, H-20), 3.19 (dd, 1H, J = 12.2, J' = 4.4 Hz, H-5α), 3.65 (s, 3H, OCH_3), 4.07–4.11 (m, 2H, H-7α, H-7β), 4.17 (m, 1H, $W_{½}$ = 10.9 Hz, H-3β). ^{13}C NMR δ 11.97 (CH_3), 14.54 (CH_3), 17.02 (CH_3), 22.11 (CH_2), 24.88 (CH_2), 26.92 (CH_2), 28.27 (CH_2), 32.53 (CH_2), 32.92 (CH_2), 36.34 (C), 39.47 (CH_2), 39.50 (CH), 41.79 (CH), 42.35 (CH), 42.68 (C), 51.15 (CH), 51.39 (CH_3), 52.80 (CH), 58.36 (CH), 64.87 (CH), 70.32 (CH_2), 176.60 (C), 177.02 (C). HRMS: (ESI+) calculated for $C_{23}H_{36}O_5Na$ (M^++Na) 415.24550. Found 415.24540. Anal. Calcd for $C_{23}H_{36}O_5$: C, 70.38; H, 9.24. Found: C, 70.29; H, 9.30%.

2.4.2. Methyl (20S)-3α-hydroxy-6-oxa-7a-homo-7-oxo-5α-pregnane-20-carboxylate (**3b**)

Yield: 50 mg (32%), m.p. 225–227 °C (MeOH), $[\alpha]_D = +10°$ (c 0.22). IR ν (cm^{-1}) 3614, 1723, 1168. ^{1}H NMR ($CDCl_3$) δ 0.71, 0.90 (both s, 3H, CH_3), 1.18 (d, 3H, J = 6.9 Hz, CH_3), 1.83 (ddd, 1H, J = 14.2, J' = 11.4, J'' = 2.7 Hz, H-4a), 1.92 (d, 1H, J = 12.3, J' = 3.0 Hz), 2.03 (ddd, 1H, J = 14.2, J' = 5.1, J'' = 3.1 Hz, H-4b), 2.43 (dq, 1H, J = 10.3, J' = 6.9 Hz, H-20), 2.46–2.53 (m, 2H, H-7α, H-7β), 3.65 (s, 3H, OCH_3), 4.22 (m, 1H, H-3β), 4.62 (dd, 1H, J = 11.4, J' = 5.1 Hz,

H-5α). ^{13}C NMR δ 11.56 (CH_3), 11.97 (CH_3), 16.99 (CH_3), 22.17 (CH_2), 25.34 (CH_2), 26.57 (CH_2), 27.99 (CH_2), 31.22 (CH_2), 34.87 (CH), 35.69 (CH_2), 38.10 (CH_2), 39.48 (CH_2), 39.94 (C), 42.33 (CH), 42.73 (C), 51.33 (CH_3), 53.13 (CH), 55.18 (CH), 58.02 (CH), 66.29 (CH), 79.62 (CH), 175.07 (C), 177.05 (C). HRMS: (ESI+) calculated for $C_{23}H_{36}O_5Na$ (M^++Na) 415.24550. Found 415.24542. Anal. Calcd for $C_{23}H_{36}O_5$: C, 70.38; H, 9.24. Found: C, 70.31; H, 9.35%.

2.5. Baeyer–Villiger oxidation of **2b**

Ketone **2b** (150 mg; 0.40 mmol) was used for Baeyer–Villiger oxidation as described in general procedure. The reaction afforded two lactones **5a** and **5b**.

2.5.1. Methyl (20S)-2α-hydroxy-7-oxa-7a-homo-6-oxo-5α-pregnane-20-carboxylate (**5a**)

Yield: 62 mg (40%), m.p. 173–175 °C (MeOH), $[\alpha]_D$ = +20° (c 0.31). IR ν (cm^{-1}) 3610, 1726, 1170. ^{1}H NMR ($CDCl_3$) δ 0.71, 0.92 (both s, 3H, CH_3), 1.19 (d, 3H, *J* = 6.9 Hz, CH_3), 1.58–1.82 (m, 8H), 1.94 (dt, 1H, *J* = 6.6, *J*′ = 4.4 Hz), 1.99–2.07 (m, 2H), 2.23 (m, 1H), 2.43 (dq, 1H, *J* = 10.3, *J*′ = 6.9 Hz, H-20), 2.67 (dd, 1H, *J* = 11.9, *J*′ = 4.6 Hz, H-5α), 3.65 (s, 3H, OCH_3), 3.67 (tt, 1H, *J* = 11.4, *J*′ = 4.0 Hz, H-2β). 4.03 (dd, 1H, *J* = 12.7, *J*′ = 8.9 Hz, H-7a), 4.09 (dd, 1H, *J* = 12.7, *J*′ = 2.1 Hz, H-7b). ^{13}C NMR δ 11.95 (CH_3), 16.04 (CH_3), 17.02 (CH_3), 22.19 (CH_2), 24.86 (CH_2), 24.91 (CH_2), 26.91 (CH_2), 34.09 (CH_2), 38.94 (C), 39.20 (CH), 39.34 (CH_2), 42.31 (CH), 42.64 (C), 46.79 (CH), 49.53 (CH_2), 51.07 (CH), 51.39 (CH_3), 52.77 (CH), 58.89 (CH), 67.02 (CH), 70.33 (CH_2), 176.03 (C), 176.98 (C). HRMS: (ESI+) calculated for $C_{23}H_{36}O_5Na$ (M^++Na) 415.24550. Found 415.24534. Anal. Calcd for $C_{23}H_{36}O_5$: C, 70.38; H, 9.24. Found: C, 70.45; H, 9.31%.

2.5.2. Methyl (20S)-2α-hydroxy-6-oxa-7a-homo-7-oxo-5α-pregnane-20-carboxylate (**5b**)

Yield: 77 mg (49%), m.p. 201–203 °C (MeOH), $[\alpha]_D$ = +6° (c 0.13). IR ν (cm^{-1}) 3611, 1725, 1168, 1041. ^{1}H NMR ($CDCl_3$) δ 0.71, 0.95 (both s, 3H, CH_3), 1.19 (d, 3H, *J* = 6.9 Hz, CH_3), 1.90–2.04 (m, 3H), 2.20 (m, 1H, H-1a), 2.40–2.54 (m, 3H, H-7α, H-7β, H-20), 3.65 (s, 3H, OCH_3), 3.74 (tt, 1H, *J* = 11.3, *J*′ = 4.1 Hz, H-2β). 4.23 (dd, 1H, *J* = 11.1, *J*′ = 5.2 Hz, H-5α). ^{13}C NMR δ 11.96 (CH_3), 13.26 (CH_3), 17.00 (CH_3), 22.26 (CH_2), 25.29 (CH_2), 26.58 (CH_2), 27.84 (CH_2), 33.27 (CH_2), 34.44 (CH), 38.10 (CH_2), 39.37 (CH_2), 41.20 (C), 42.32 (CH), 42.71 (C), 47.14 (CH_2), 51.39 (CH_3), 53.11 (CH), 55.10 (CH), 58.38 (CH), 66.60 (CH), 82.58 (CH), 174.47 (C), 177.02 (C). HRMS: (ESI+) calculated for $C_{23}H_{36}O_5Na$ (M^++Na) 415.24550. Found 415.24536. Anal. Calcd for $C_{23}H_{36}O_5$: C, 70.38; H, 9.24. Found: C, 70.37; H, 9.36%.

2.6. General procedure for ester hydrolysis

Methyl ester (0.15 mmol) in methanol (5 mL) with potassium hydroxide (80 mg, 1.5 mmol) was heated at reflux under nitrogen for 10 h. The solution was neutralized with 5% aqueous HCl (10 mL) and the reaction mixture was left to stand at room temperature for 30 min. The mixture was evaporated to dryness, water (20 mL) was added, and the product was extracted with chloroform (2 × 50 mL). The chloroform solution was dried with anhydrous sodium sulfate and evaporated under reduced pressure. Product crystallized from ethanol (unless otherwise stated).

2.6.1. (20S)-3α-Hydroxy-7-oxa-7a-homo-6-oxo-5α-pregnane-20-carboxylic acid (**4a**)

Compound **4a** was prepared from methyl ester **3a** (50 mg; 0.13 mmol) according to the above general procedure for ester hydrolysis. Yield: 35 mg (73%), m.p. 129–131 °C (lyophilized from *t*-BuOH), $[\alpha]_D$ = +25° (c 0.14; $CHCl_3$:MeOH–1:1). IR (KBr) ν (cm^{-1}) 3430, 1726, 1711, 1182. ^{1}H NMR ($CDCl_3$) δ 0.74, 0.88 (both s, 3H, CH_3), 1.21 (d, 3H, *J* = 6.8 Hz, CH_3), 1.90–2.13 (m, 7H), 2.43 (dq, 1H, *J* = 10.3, *J*′ = 6.9 Hz, H-20), 3.18 (dd, 1H, *J* = 12.2, *J*′ = 4.3 Hz, H-5α), 3.95 (m, 1H, H-3β), 4.06–4.10 (m, 2H, H-7α, H-7β). ^{13}C NMR δ 11.97 (CH_3), 14.56 (CH_3), 17.00 (CH_3), 22.10 (CH_2), 24.92 (CH_2), 27.08 (CH_2), 28.20 (CH_2), 32.49 (CH_2), 32.91 (CH_2), 36.32 (C), 39.48 (CH_2), 39.53 (CH), 41.76 (CH), 42.23 (CH), 42.76 (C), 51.20 (CH), 52.37 (CH), 58.32 (CH), 64.94 (CH), 70.29 (CH_2), 176.62 (C), 181.14 (C). HRMS: (ESI+) calculated for $C_{22}H_{34}O_5Na$ (M^++Na) 401.22985. Found 401.22976. Anal. Calcd for $C_{22}H_{34}O_5$: C, 69.81; H, 9.05. Found: C, 69.77; H, 9.12%.

2.6.2. (20S)-3α-Hydroxy-6-oxa-7a-homo-7-oxo-5α-pregnane-20-carboxylic acid (**4b**)

Compound **4b** was prepared from methyl ester **3b** (70 mg; 0.18 mmol) according to the above general procedure for ester hydrolysis. Yield: 50 mg (74%), m.p. 272–275 °C (EtOH), $[\alpha]_D$ = +5° (c 0.21; $CHCl_3$:MeOH–1:1). IR (KBr) ν (cm^{-1}) 3428, 1736, 1699, 1151, 1033. ^{1}H NMR ($CDCl_3$ + MeOD) δ 0.71, 0.90 (both s, 3H, CH_3), 1.18 (d, 3H, *J* = 6.9 Hz, CH_3), 1.83 (ddd, 1H, *J* = 14.2, *J*′ = 11.4, *J*″ = 2.7 Hz, H-4a), 1.92 (d, 1H, *J* = 12.3, *J*′ = 3.0 Hz), 2.03 (ddd, 1H, *J* = 14.2, *J*′ = 5.1, *J*″ = 3.1 Hz, H-4b), 2.43 (dq, 1H, *J* = 10.3, *J*′ = 6.9 Hz, H-20), 2.46–2.53 (m, 2H, H-7α, H-7β), 3.65 (s, 3H, OCH_3), 4.22 (m, 1H, H-3β), 4.62 (dd, 1H, *J* = 11.4, *J*′ = 5.1 Hz, H-5α). ^{13}C NMR δ 11.56 (CH_3), 11.97 (CH_3), 16.99 (CH_3), 22.17 (CH_2), 25.34 (CH_2), 26.57 (CH_2), 27.99 (CH_2), 31.22 (CH_2), 34.87 (CH), 35.69 (CH_2), 38.10 (CH_2), 39.48 (CH_2), 39.94 (C), 42.33 (CH), 42.73 (C), 51.33 (CH_3), 53.13 (CH), 55.18 (CH), 58.02 (CH), 66.29 (CH), 79.62 (CH), 175.07 (C), 177.05 (C). HRMS: (ESI+) calculated for $C_{22}H_{34}O_5Na$ (M^++Na) 401.22985. Found 401.22971. Anal. Calcd for $C_{22}H_{34}O_5$: C, 69.81; H, 9.05. Found: C, 69.77; H, 9.09%.

2.6.3. (20S)-2α-Hydroxy-7-oxa-7a-homo-6-oxo-5α-pregnane-20-carboxylic acid (**6a**)

Compound **6a** was prepared from methyl ester **5a** (60 mg; 0.15 mmol) according to the above general procedure for ester hydrolysis. Yield: 42 mg (73%), m.p. 276–278 °C (EtOH), $[\alpha]_D$ = +19° (c 0.34; $CHCl_3$:MeOH–1:1). IR (KBr) ν (cm^{-1}) 3435, 1728, 1684, 1201, 1177. ^{1}H NMR ($CDCl_3$ + MeOD) δ 0.73, 0.91 (both s, 3H, CH_3), 1.21 (d, 3H, *J* = 6.8 Hz, CH_3), 1.91–2.04 (m, 3H), 2.21 (m, 1H), 2.38 (dq, 1H, *J* = 13.5, *J*′ = 6.8 Hz, H-20), 2.71 (dd, 1H, *J* = 11.7, *J*′ = 4.4 Hz, H-5α), 3.67 (tt, 1H, *J* = 11.3, *J*′ = 3.9 Hz, H-2β). 4.05–4.12 (m, 2H, H-7α, H-7β). ^{13}C NMR δ 11.63 (CH_3), 15.64 (CH_3), 16.77 (CH_3), 21.96 (CH_2), 24.64 (2 × CH_2), 26.81 (CH_2), 33.32 (CH_2), 38.62 (C), 38.92 (CH), 39.19 (CH_2), 42.26 (CH), 42.41 (C), 46.60 (CH), 48.90 (CH_2), 50.83 (CH), 52.31 (CH), 58.59 (CH), 66.21 (CH), 70.34 (CH_2), 177.03 (C), 179.05 (C). HRMS: (ESI−) calculated for $C_{22}H_{33}O_5$ (M^+−H) 377.23335. Found 377.23300. Anal. Calcd for $C_{22}H_{34}O_5$: C, 69.81; H, 9.05. Found: C, 69.72; H, 9.16%.

2.6.4. (20S)-2α-Hydroxy-6-oxa-7a-homo-7-oxo-5α-pregnane-20-carboxylic acid (**6b**)

Compound **6b** was prepared from methyl ester **5b** (40 mg; 0.10 mmol) according to the above general procedure for ester hydrolysis. Yield: 33 mg (85%), m.p. 284–285 °C (EtOH), $[\alpha]_D$ = +12° (c 0.15; $CHCl_3$:MeOH–1:1). IR (KBr) ν (cm^{-1}) 3425, 1720, 1697, 1188, 1038. ^{1}H NMR ($CDCl_3$ + MeOD) δ 0.73, 0.94 (both s, 3H, CH_3), 1.21 (d, 3H, *J* = 6.9 Hz, CH_3), 1.89–2.02 (m, 3H), 2.17 (m, 1H, H-1a), 2.39 (dq, 1H, *J* = 10.3, *J*′ = 6.9 Hz, H-20), 2.47–2.52 (m, 2H, H-7α, H-7β), 3.68 (dd, 1H, *J* = 14.9, *J*′ = 7.6, *J*″ = 3.7 Hz, H-2β). 4.28 (dd, 1H, *J* = 10.7, *J*′ = 4.7 Hz, H-5α). ^{13}C NMR δ 11.64 (CH_3), 12.85 (CH_3), 16.78 (CH_3), 22.01 (CH_2), 25.09 (CH_2), 26.47 (CH_2), 27.60 (CH_2), 32.55 (CH_2), 34.26 (CH), 37.79 (CH_2), 39.20 (CH_2), 40.82 (C), 42.26 (CH), 42.47 (C), 46.49 (CH_2), 52.65 (CH), 54.85 (CH), 58.04 (CH), 65.73 (CH), 82.87 (CH), 175.44 (C), 179.08 (C). HRMS: (ESI+) calculated for $C_{22}H_{34}O_5Na$ (M^++Na) 401.22985.

Found 401.22968. Anal. Calcd for $C_{22}H_{34}O_5$: C, 69.81; H, 9.05. Found: C, 69.71; H, 9.20%.

2.6.5. Methyl (20S)-3α-(4-nitrobenzoyloxy)-6-oxo-5α-pregnane-20-carboxylate (**8**)

Diethyl azodicarboxylate (40% in toluene; 0.6 mL; 1.53 mmol) was added to a solution of hydroxyketone **7** (200 mg; 0.53 mmol), triphenylphosphine (400 mg; 1.53 mmol), and 4-nitrobenzoic acid (260 mg; 1.56 mmol) in dry THF. Reaction was stirred at 80 °C for 4 h. THF was evaporated under reduced pressure and the mixture was subjected to column chromatography in ethyl acetate-hexane 1:3 to afford 4-nitrobenzoate **8** (245 mg; 88%), m.p. 193–195 °C (MeOH), $[\alpha]_D = -23°$ (c 0.26). IR ν (cm^{-1}) 1720, 1609, 1600, 1530, 1277. ^1H NMR ($CDCl_3$) δ 0.63, 0.74 (both s, 3H, CH_3), 1.13 (d, 3H, J = 6.9 Hz, CH_3), 1.85–2.01 (m, 4H), 2.27 (dd, 1H, J = 13.2, J' = 4.5 Hz, H-7α), 2.37 (dq, 1H, J = 10.3, J' = 6.8 Hz, H-20), 2.56 (dd, 1H, J = 12.4, J' = 3.1 Hz, H-5α), 3.58 (s, 3H, OCH_3), 5.35 (m, 1H, $W_{½}$ = 5.3 Hz, H-3β), 8.12 (m, 2H, 2 × H_{Ar}), 8.24 (m, 2H, 2 × H_{Ar}). ^{13}C NMR δ 12.20 (CH_3), 12.43 (CH_3), 17.09 (CH_3), 21.09 (CH_2), 23.97 (CH_2), 25.10 (CH_2), 25.40 (CH_2), 26.98 (CH_2), 32.69 (CH_2), 37.88 (CH), 39.19 (CH_2), 41.30 (C), 42.32 (CH), 43.02 (C), 46.61 (CH_2), 51.39 (CH), 52.83 (CH_3), 52.87 (CH), 53.94 (CH), 56.32 (CH), 70.93 (CH), 123.58 (2 × CH), 130.59 (2 × CH), 136.14 (C), 150.51 (C), 163.75 (C), 177.12 (C), 210.99 (C). HRMS: (ESI+) calculated for $C_{30}H_{39}NO_7$Na (M^++Na) 548.26187. Found 548.26169. Anal. Calcd for $C_{30}H_{39}NO_7$: C, 68.55; H, 7.48; N, 2.66. Found: C, 68.50; H, 7.56; N, 2.54%.

2.7. Baeyer–Villiger oxidation of **8**

Ketone **8** (200 mg; 0.38 mmol) was used for Baeyer–Villiger oxidation as described in general procedure. The reaction afforded two lactones **9a** and **9b**.

2.7.1. Methyl (20S)-3α-(4-nitrobenzoyloxy)-7-oxa-7a-homo-6-oxo-5α-pregnane-20-carboxylate (**9a**)

Yield: 109 mg (53%), m.p. 227–230 °C (MeOH), $[\alpha]_D$ = +25° (c 1.27). IR ν (cm^{-1}) 1724, 1609, 1600, 1531, 1278. ^1H NMR ($CDCl_3$) δ 0.73, 0.97 (both s, 3H, CH_3), 1.19 (d, 3H, J = 6.9 Hz, CH_3), 2.33 (ddd, 1H, J = 15.1, J' = 12.3, J'' = 2.6 Hz, H-4a), 2.44 (dq, 1H, J = 10.2, J' = 6.8 Hz, H-20), 3.10 (dd, 1H, J = 12.2, J' = 4.4 Hz, H-5α), 3.65 (s, 3H, OCH_3), 4.06–4.17 (m, 2H, H-7α, H-7β), 5.39 (m, 1H, $W_{½}$ = 5.2 Hz, H-3β), 8.20 (m, 2H, 2 × H_{Ar}), 8.32 (m, 2H, 2 × H_{Ar}). ^{13}C NMR δ 11.95 (CH_3), 14.57 (CH_3), 17.01 (CH_3), 22.16 (CH_2), 24.85 (CH_2), 25.30 (CH_2), 26.88 (CH_2), 29.77 (CH_2), 33.99 (CH_2), 36.33 (C), 39.32 (CH_2), 39.46 (CH), 42.27 (CH), 42.63 (C), 42.89 (CH), 51.07 (CH), 51.38 (CH_3), 52.79 (CH), 58.78 (CH), 70.46 (CH_2), 70.52 (CH), 123.61 (2 × CH), 130.54 (2 × CH), 135.99 (C), 150.52 (C), 163.78 (C), 175.55 (C), 176.91 (C). HRMS: (ESI+) calculated for $C_{30}H_{39}NO_8$Na (M^++Na) 564.25679. Found 564.25603. Anal. Calcd for $C_{30}H_{39}NO_8$: C, 66.52; H, 7.26; N, 2.59. Found: C, 66.46; H, 7.36; N, 2.55%.

2.7.2. Methyl (20S)-3α-(4-nitrobenzoyloxy)-7-oxo-7a-homo-6-oxa-5α-pregnane-20-carboxylate (**9b**)

Yield: 70 mg (34%), m.p. 205–208 °C (MeOH), $[\alpha]_D = -10°$ (c 1.64). IR ν (cm^{-1}) 1725, 1609, 1600, 1531, 1272. ^1H NMR ($CDCl_3$) δ 0.73, 0.98 (both s, 3H, CH_3), 1.18 (d, 3H, J = 6.9 Hz, CH_3), 2.19–2.31 (m, 2H), 2.43 (dq, 1H, J = 10.3, J' = 6.9 Hz, H-20), 2.46–2.58 (m, 2H, H-7α, H-7β), 3.65 (s, 3H, OCH_3), 4.55 (dd, 1H, J = 11.3, J' = 5.3 Hz, H-5α), 5.44 (m, 1H, $W_{½}$ = 5.1 Hz, H-3β), 8.19 (m, 2H, 2 × H_{Ar}), 8.33 (m, 2H, 2 × H_{Ar}). ^{13}C NMR δ 11.60 (CH_3), 11.92 (CH_3), 16.96 (CH_3), 22.20 (CH_2), 24.95 (CH_2), 25.29 (CH_2), 26.53 (CH_2), 32.07 (CH_2), 32.93 (CH_2), 34.76 (CH), 38.15 (CH_2), 39.31 (CH_2), 39.86 (C), 42.24 (CH), 42.67 (C), 51.35 (CH_3), 53.11 (CH), 55.04 (CH), 58.30 (CH), 71.61 (CH), 79.60 (CH), 123.62 (2 × CH), 130.58 (2 × CH), 135.72 (C), 150.56 (C), 163.71 (C), 174.31 (C), 176.95 (C). HRMS: (ESI+) calculated for $C_{30}H_{39}NO_8$Na (M^++Na) 564.25679. Found 564.25612. Anal. Calcd for $C_{30}H_{39}NO_8$: C, 66.52; H, 7.26; N, 2.59. Found: C, 66.43; H, 7.35; N, 2.50%.

2.8. Hydrolysis of 4-nitrobenzoates **4a** and **4b**

Compound **4a** and **4b** were prepared from methyl esters **9a** and **9b** (each 50 mg; 0.09 mmol) according to general procedure for hydrolysis of ester. Spectral characteristics for both acids were in agreement with the data reported in experiments above.

2.9. 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 [16]. All 3D structures of BRI1 ligands were obtained with Marvin 5.10.3 [7], 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) [17] 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 the docked ligand with brassinolide crystal-like poses and the best crystal-like poses of each ligand were analyzed.

2.10. 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.

2.11. 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 4-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.

Table 1
Activity in the bean second-internode bioassay.

Compound	Maximal prolongation of the second internode (mm)	SD (mm)	Amount applied for maximal prolongation (mol)
24-Epibrassinolide	54.8	±10.8	10^{-10}
2a	13.1	±6.6	10^{-9}
2b	17.1	±7.7	10^{-10}
3a	14.5	±4.6	10^{-11}
3b	9.7	±3.6	10^{-11}
4a	10.1	±6.1	10^{-10}
4b	3.9	±1.1	10^{-9}
5a	8.1	±5.3	10^{-8}
5b	6.6	±3.2	10^{-8}
6a	11.6	±2.2	10^{-9}
6b	8.3	±4.8	10^{-9}
8	3.4	±0.9	10^{-12}
9a	7.3	±6.3	10^{-10}
9b	4.4	±0.8	10^{-9}

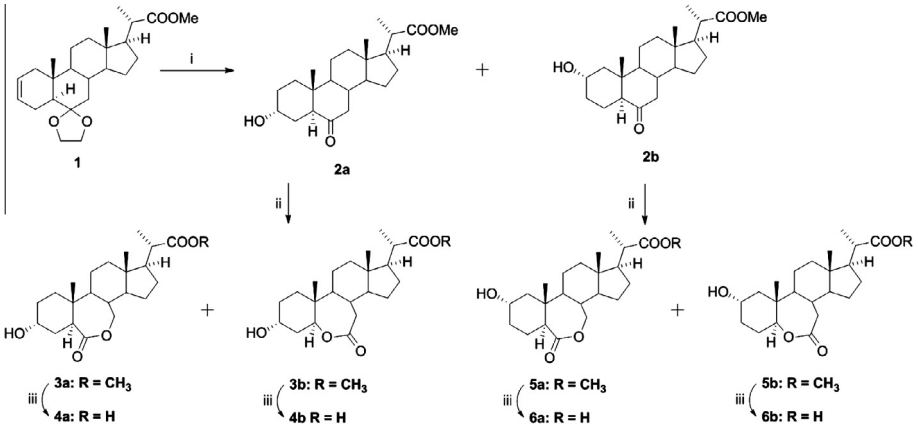
2.12. The bean second-internode bioassay

Brassinolide-type activity was measured by the bean second-internode bioassay modified by us [18]. Seeds of bean (*Phaseolus vulgaris* L., cv. Pinto) germinated for 2 days were selected for uniformity from a large population of seedlings and then transferred into pots containing perlite and 1/10 diluted Hoagland solution (half concentration, pH 5.7). Seedlings were grown in a light-controlled cultivation room (25–27 °C, light 48 W m^{-2}, light/dark period 16/8 h). Seven days old plants with second internodes 2 mm long were treated with different amounts of tested compounds in 5 μL fractionated lanolin. The substances were applied in microdrops to the scar left after the removal of bract from the base of the second internode. 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 length of the second internodes was measured after 5 days and the difference in length between treated and control plants provided a measure of activity (Table 1).

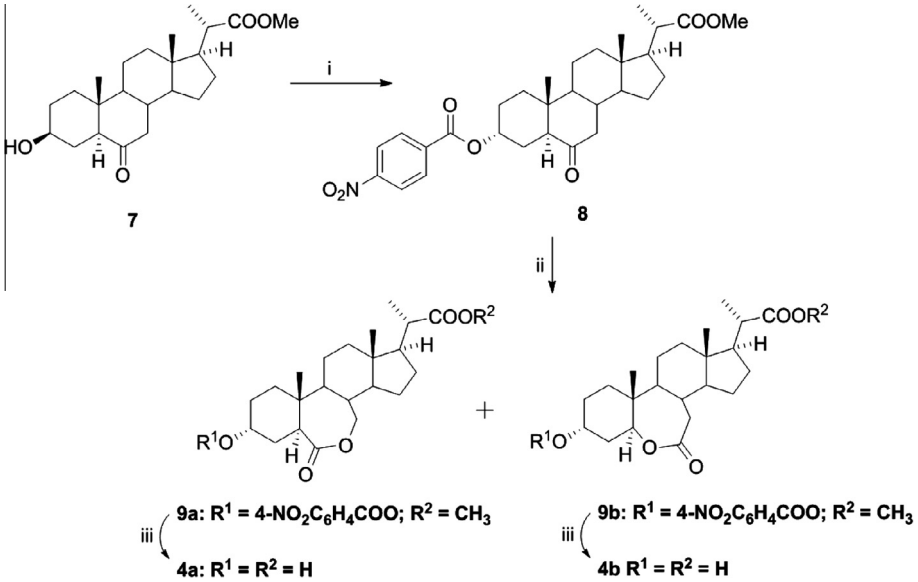
3. Results and discussion

3.1. Chemistry

For the preparation of above mentioned compounds we set out from the known [19] methyl (20*S*)-6,6-ethylenedioxy-5α-pregn-2-en-20-carboxylate (**1**). This olefin was used for hydroboration reaction [20] was followed by acidic hydrolysis of the ketal group. Two



Scheme 1. (i) (a) B_2H_6, THF, r.t.; (b) 30% H_2O_2, NaOH, H_2O, r.t.; Na_2SO_3, AcOH, H_2O, r.t.; (ii) TFAA, 30% H_2O_2, CH_2Cl_2, r.t.; (iii) (a) KOH, MeOH, refl.; (b) 5% HCl, H_2O, r.t.



Scheme 2. (i) DEAD, Ph_3P, 4-nitrobenzoic acid, THF, 80 °C; (ii) TFAA, 30% H_2O_2, CH_2Cl_2, r.t.; (iii) (a) KOH, MeOH, refl.; (b) 5% HCl, H_2O, r.t.

main products were isolated and characterized as 3α-hydroxyester **2a** and 2α-hydroxyester **2b**. Their structure was determined by 1D and 2D NMR spectra. Both hydroxyesters were then used for Baeyer–Villiger oxidation. In both cases, two isomers of lactone were obtained, **3a** and **3b** from hydroxyester **2a**, and **5a** and **5b** from hydroxyester **2b**. Unlike 2α,3α-dihydroxy derivatives when the mostly desired 7-oxa-6-oxo isomer is obtained in very high yield, monohydroxy derivatives afforded both isomers in ratio almost 1:1, even a higher yield of 6-oxa-7-oxo isomer **5b** in case of 2α-hydroxyester **2b**. This fact proved that presence of hydroxy group on A ring is essential for oxidation to a less sterically hindered position. [21] All four lactones were then hydrolyzed with potassium hydroxide to prepare four acids **4a**, **4b**, **6a**, and **6b**.

Acids **4a** and **4b** were prepared also by alternative way using Mitsunobu acylation. As a starting material we used known [22] methyl (20*S*)-3β-hydroxy-6-oxo-5α-pregnan-20-carboxylate (**7**). Standard Mitsunobu procedure [23] used also for steroidal alcohols afforded 4-nitrobenzoate **8** in a very high yield. The following reactions were the same as in previous experiments. Baeyer–Villiger oxidation was followed with alkaline hydrolysis of lactones **9a** and **9b** prepared. Both products were identical with acids **4a** and **4b**. All chemical reactions and conditions can be seen in Scheme 1 and 2. All compounds were characterized by NMR, IR and MS techniques, together with elemental analysis and optical rotation.

3.2. Biological activity and docking

Antiproliferative activity was determined by comparing human normal fibroblasts (BJ) and cancer cell lines (T-lymphoblastic leukemia CEM, breast carcinoma MCF7, cervical carcinoma cell line HeLa). 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 IC_{50} (concentration leading to 50% inhibition of viability) values obtained from Calcein AM cytotoxicity assay were calculated. 28-homocastasterone was used as a positive control, which is most potent natural brassinosteroid towards CEM cells (IC_{50} 13 μM, [12]). All tested monohydroxylated BRs had no detectable activity, even when tested in concentrations of up to 50 μM. No BRs derivative mediated loss of viability was observed in the BJ fibroblasts.

Brassinolide-type activity was measured by the second internode bean bioassay [24]. 24-Epibrassinolide showed very good biological activity and therefore was used as a positive control. This compound elicited nearly 200% increase in elongation of the

Table 2
Resulting binding free energies for best poses and for crystal-like pose and its ranking between all poses. Star (*) denotes structures with common first pose binding more deeply in the receptor groove similarly as shown on Fig. 2.

Compound	$\Delta G_{bind\ best}$ (kcal/mol)	$\Delta G_{bind\ X\text{-}tal}$ (kcal/mol)
Brassinolide	−10.6	−10.6 (1.)
24-Epibrassinolide	−9.8	−9.8 (1.)
28-Homocastasterone	−9.6	−9.3 (2.)
2a	−10.1*	−9.1 (2.)
2b	−10.1*	−9.0 (2.)
3a	−9.7*	−8.9 (4.)
3b	−10.5*	−9.0 (4.)
4a	−9.8*	−9.0 (4.)
4b	−10.4*	−8.2 (11.)
5a	−9.8*	−8.9 (2.)
5b	−10.2*	−8.0 (10.)
6a	−9.9*	−9.0 (3.)
6b	−10.1*	−9.0 (3.)
8	−8.9	−8.6 (2.)
9a	−8.9	−8.3 (3.)
9b	−8.9	−7.7 (14.)

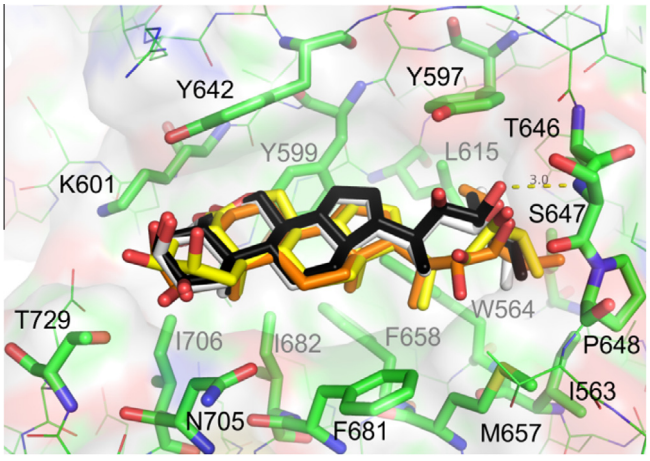


Fig. 1. Comparison of brassinolide analogues with brassinolide crystal pose (black; PDB ID: 3RGZ). Brassinolide interacts with mostly nonpolar groove in a receptor. Only one hydrogen bond is present between the 23-hydroxyl group and main chain nitrogen in S647 residue. Brassinolide docked into the same pose as in crystal (cf. white and black). Both 24-epibrassinolide (yellow) and 28-homocastasterone (orange) showed similar pose with slightly less binding affinity accountable to the small variation in the tail region, while still retaining the hydrogen bond found in crystal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

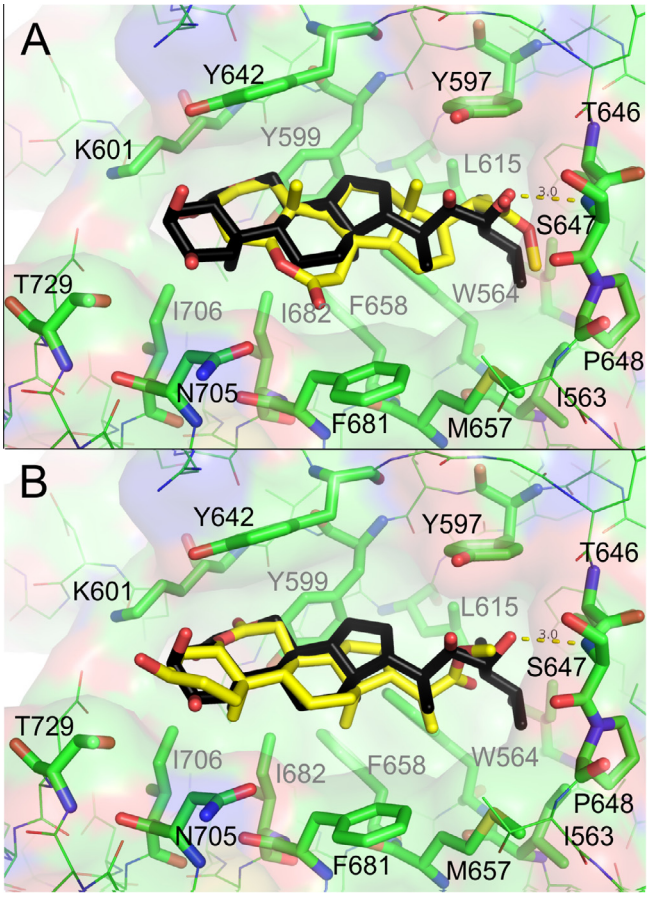


Fig. 2. Comparison of monosubstituted brassinolide analogue **3b** (yellow) with brassinolide crystal pose (black; PDB ID: 3RGZ). Two major poses were detected – (A) more deeply immersed pose with best binding free energy and with hydrogen bonding pattern to S647, (B) brassinolide-like binding pose with lower affinity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

second internode, causing its elongation, swelling, twisting and splitting [25]. The newly prepared monohydroxylated BRs analogs were very weakly active inducing elongation of the second bean internode only (Table 1); the best activity was found in **2b** (+17.1 mm) and in **3a** (+14.5 mm). In the case of **2b**, applied amount was 10^{-10} mol and in the case of **3a** 10^{-11} mol per plant.

The weak activity of derivatives was further analyzed with use of molecular docking of their structures into the receptor domain of kinase brassinosteroid insensitive 1 (BRI1). Firstly, we docked into the structure of brassinolide, 24-epibrassinolide and 28-homocastasterone in order to analyze their best fir. All these compounds docked into the receptor groove in a similar fashion to brassinolide crystal pose (Fig. 1) with only a slightly less affinity to the receptor than brassinolide itself (Table 2). Binding pose is mainly dictated by the number of nonpolar residues in a receptor groove forming a hydrophobic pocket interacting with nonpolar parts of rings and tail of brassinolide and its derivatives. Hydroxyl groups are mostly pointing outward from the receptor groove to the solvent, whereas 23-hydroxyl group interacts with the main chain nitrogen in S647 residue. Brassinolide, 24-epibrassinolide and 28-homocastasterone all have the nonpolar tail pointing deeper into the hydrophobic pocket close to W564 and L615 residues. In such a pose, 2,3-dihydroxy group, thought to be important for biological response [7], positioned in the same area pointing outward from the binding pocket. It was thus shown, that Autodock Vina is able to dock ligands into the binding pocket in crystal-like pose within energetically best poses (Table 2).

However in the case of the monohydroxylated derivatives synthetized in this work, we have found that these compounds showed two distinct binding poses: (i) one with the best binding free energy characterized with a more deeply immersed pose and retained hydrogen bonding pattern to S647, and (ii) a brassinolide-like binding pose with a lower affinity lacking interactions with hydrophobic pocket due to the shorter and more polar tail (Fig. 2). Moreover, the introduction of 4-nitrobenzoic acid on 3-hydroxyl group further lowered the affinity towards the BRI receptor. As most of the interactions between the receptor binding groove and the ligand are mediated through nonpolar interactions, the larger the area of contact between ligand and pocket would govern the affinity. In such case, the shorter aliphatic tail is moving prepared the monohydroxylated brassinosteroid derivatives out of the productive pose more deeply into the receptor groove, thus effectively blocking 2- or 3-hydroxyl groups, which are thought to be important for the biological response of the receptor [7] and diminishing their proposed function in brassinolide-type activity. In future, we will focus on the aliphatic tail elongation in the brassinosteroid derivatives synthesis, as this seems to be the key feature of the brassinolide-like substrates interacting with BRI receptor.

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