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Synthesis and plant growth promoting activity of polyhydroxylated ketones bearing the 5α-hydroxy-6-oxo moiety and cholestane side chain

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ABSTRACT

Three polyhydroxylated ketones bearing the 5α-hydroxy-6-oxo moiety were obtained from cholesterol. Two of them show plant growth promoting activity in the bean's second internode bioassay. The obtained results indicate that the presence of the 5α-hydroxy-6-oxo moiety may be capable to induce plant growth promotion even the absence oxygenated functions in the side chain.

1. Introduction

Brassinolide **(1)** and other members of the family of brassinosteroids (Bs) **(2–6)** (Fig. 1) are considered as the sixth class of plant hormones [1–3]. In general, those compounds are characterized by the presence of oxygenated functions in the rings A and B as well as the side chain. The slight structural differences that may be found between the naturally occurring Bs are known to produce moderate to drastic variations in the plant growth stimulating activity. This has led to the establishment of generally accepted structural requirements for a good plant growth stimulating activity [1].

Nevertheless, the fact that a wide variety of synthetic compounds bearing minor, moderate or even drastic structural modifications have shown low to moderate, and in some cases, high plant growth stimulating activity [2–15] suggests that the requirements for a good biological activity are more flexible and variable than those initially established [1]. Several books and reviews have collected and analyzed the profuse available data and account for the former statement [2–5,8] (See for review, [5]).

In particular Brosa and coworkers as well as Galagowsky have described the synthesis of several brassinosteroids analogs (BA) (i.e. **7–9**, Fig. 2) bearing a 5α-hydroxy-6-oxo moiety that have shown low to moderate, or even high plant growth stimulating activity [14]. Interestingly our recently reported BA **10**, which bears the 5α-hydroxy-6-oxo moiety and a furostane side chain also showed moderate biological activity [15] (Fig. 2).

After more than 30 years of brassinosteroids research, two main tasks have become evident:

(a) *The synthesis of naturally occurring brassinosteroids and highly active analogs*. Since this activity is directed to studies on structure–activity, modes of action (interaction substrate–receptor, plant physiology, genetics, etc.) the compounds conceived for this task are expected to bear no – or only minor structural modifications on the known bioactive functions.
(b) *The search for promising candidates for the massive application in agriculture*. In this context and considering that surprises occur, this activity needs a much more open search that should include simple (and even known) compounds with structural modifications that simplify the synthetic procedure and whose plant growth stimulating activity have been not studied. This does not mean a comeback to the trial and error system, but an open search that combines the cumulated experience and some audacity. In addition, the results would feedback task A that may try to explain why some given compounds with drastically modified structures display a significant plant growth stimulating activity.

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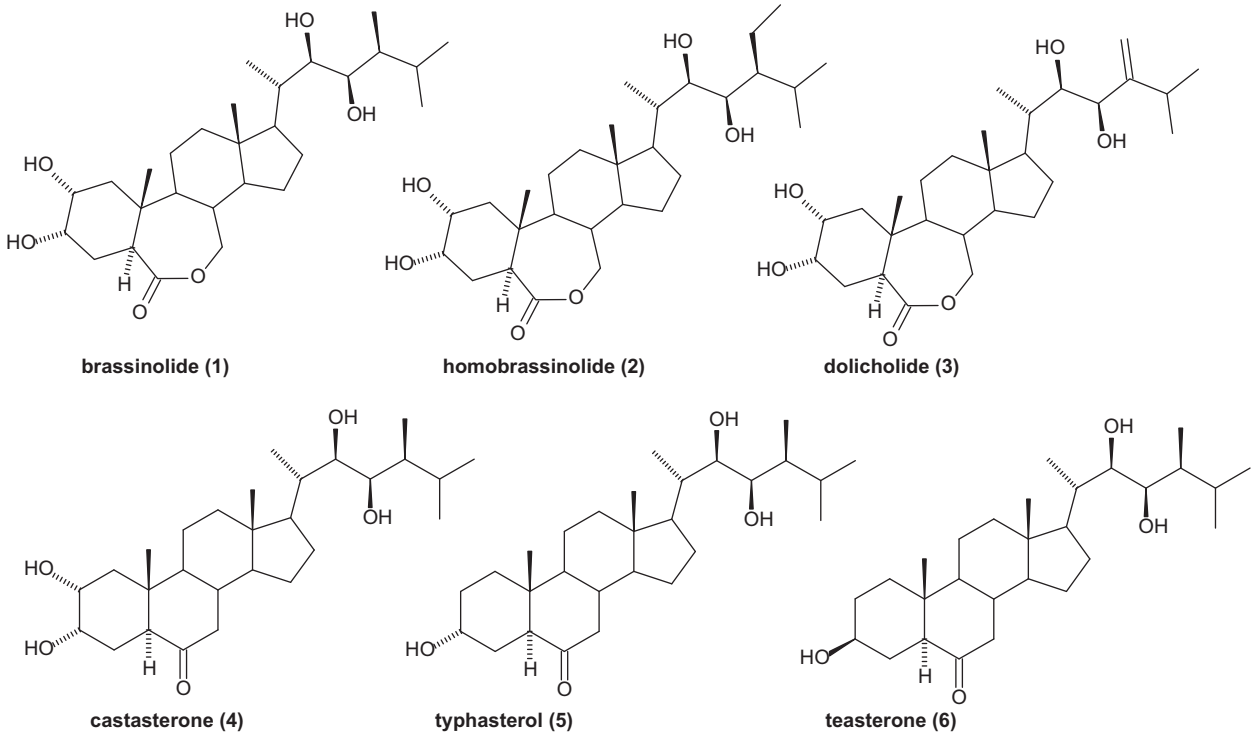


Fig. 1. Some naturally occurring brassinosteroids.

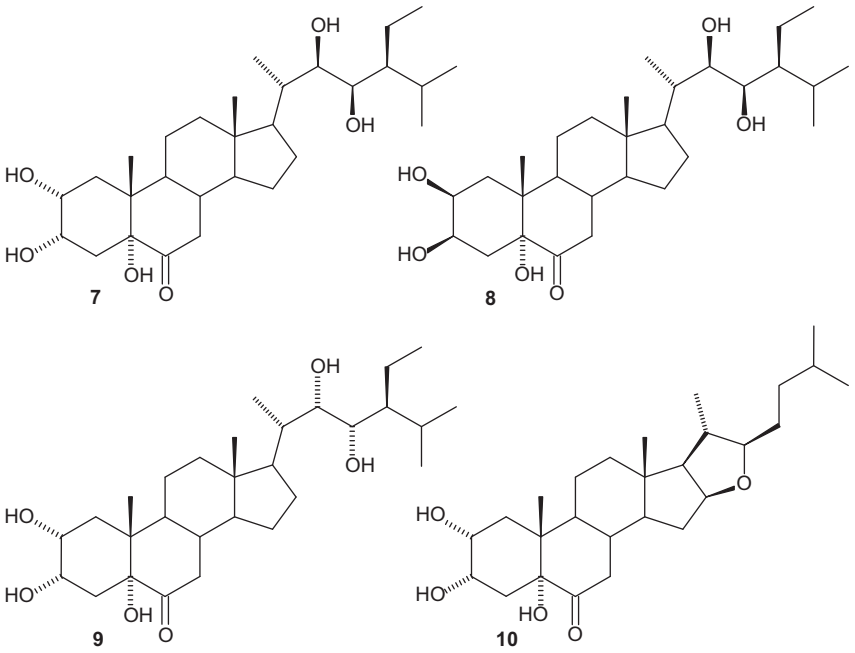


Fig. 2. Some BA bearing the 5α-hydroxy-6-oxo moiety.

Since the introduction of the 5α-hydroxy-6-oxo moiety starting from 3β-hydroxy-Δ^5 steroids is much easier than the preparation of the B-homo-7-oxa-6-oxo moiety that characterizes the more active compounds, BA bearing the 5α-hydroxy-6-oxo moiety may be, in general, more suitable for large scale preparation. Hence, agricultural applications of moderately bioactive BA bearing the 5α-hydroxy-6-oxo moiety may, at the end, prevail over that of the highly active commercially available B-homo-7-oxa-6-oxo analogs whose prohibitive prices preclude the massive and generalized application of brassinosteroids to agriculture.

Those facts have prompted us to initiate a program for the preparation and evaluation of the plant growth promoting activity of a series of simple compounds with the easy to obtain 5α-hydroxy-6-oxo moiety and different side chains, using cheap and readily available steroids as starting materials. Herein we report on an optimized synthesis of three 5α-hydroxy-6-oxo-steroids derived from

cholesterol and the study of their plant growth stimulating activity in the bean second internode bioassay.

2. Experimental

2.1. General conditions

Reactions were monitored by TLC on ALUGRAM® SIL G/UV254 plates from MACHEREY–NAGEL. Chromatographic plates were sprayed with a 1% solution of vanillin in 50% $HClO_4$ and heated until color developed. Melting points were measured on a Melt-Temp II equipment and are uncorrected. Mass spectra were registered in a Thermo-Electron spectrometer model DFS (Double Focus Sector). NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 solution in Varian INOVA 400 or 300 MHz spectrometers using the solvent signals as references. NMR signals assignments were made with the aid of DEPT and a combination of 2D homonuclear (^1H–^1H) and heteronuclear (^1H–^{13}C) correlation techniques, which included ^1H–^1H COSY, ^1H–^1H Nuclear Overhauser Effect Spectroscopy (NOESY), and Heteronuclear Single Quantum Correlation (HSQC). All 2D NMR spectra were recorded using the standard pulse sequences and parameters recommended by the manufacturer.

2.2. Synthetic procedures

2.2.1. 3β-Acetoxy-5-hydroxy-5α-cholestan-6-one (**12**)

m-CPBA (0.968 g, 5.61 mmol) was added to a solution of cholesteryl acetate **(11)** (1.7148 g, 4 mmol) in CH_2Cl_2 (20 ml) and the mixture was stirred until the starting material disappeared (1–1.5 h, TLC). Acetone (50 ml) was added and the mixture was cooled to 0 °C in an ice bath before addition of a solution of CrO_3 (1.4286 g, 14.29 mmol) in water (4.3 ml) The ice bath was removed, the mixture was stirred at room temperature for 20 min and cooled to 0 °C in the ice bath prior to drop wise addition of a solution of CrO_3 (0.7143 g, 7.14 mmol) in water (2.15 ml). The ice bath was removed and the mixture stirred for 50 min, before addition of water (50 ml) and extraction with ethyl acetate (2× 50 ml). The organic layer was washed with water (9× 50 ml), 10% $NaHCO_3$ solution (5× 50 ml), water (2× 50 ml) and saturated NaCl solution (1× 50 ml), dried (Na_2SO_4) and evaporated to afford the desired ketol **12** (1.7597 g, 3.82 mmol, 96%). Mp 230–232 °C (from ethyl acetate); Lit. 232–233 °C [16]. ^1H NMR (300 MHz, $CDCl_3$), δ ppm: 5.05–4.94 (m, 1H, H-3), 2.78 (dd, J = 12.6, 12.6 Hz, 1H, H-7 ax.), 2.01 (s, 3H, CH_3COO-3), 0.91 (d, J = 6.3 Hz, 3H, H-21), 0.87 and 0.85 (d, J = 6.6 Hz, 3H each, H-26 and H-27), 0.81 (s, 3H, H-19), 0.64 (s, 3H, H-18). ^{13}C NMR (75.5 MHz), δ ppm: 29.6 C-1, 26.4 C-2, 71.5 C-3, 32.0 C-4, 79.8 C-5, 213.8 C-6, 41.8 C-7, 37.5 C-8, 44.3 C-9, 42.6 C-10, 21.5 C-11, 39.7 C-12, 43.2 C-13, 56.4 C-14, 24.0 C-15, 28.2 C-16, 56.2 C-17, 12.1 C-18, 13.9 C-19, 35.8 C-20, 18.7 C-21, 36.2 C-22, 24.0 C-23, 39.6 C-24, 28.1 C-25, 22.6 C-26, 22.9 C-27, 171.8 $CH_3\underline{C}OO$-3, 21.5 $\underline{C}H_3COO$-3. MS (EI, 70 eV): 460 M^+, 418, 401, 400 (100%), 318.

2.2.2. 3β,5-Dihydroxy-5α-cholestan-6-one (**13**)

A saturated solution of K_2CO_3 in methanol (100 ml) was added to the solid ketol **12** (2 g, 4.34 mmol) and the mixture was stirred overnight at room temperature. Half the solvent was evaporated, the mixture was poured into ice-water, the produced solid was filtered off, washed with water and dried in the air to afford the dihydroxylated ketone **13** (1.3411 g, 3.21 mmol, 74%). M.p 230–232 °C (from methanol); Lit. 231–232° [16]. ^1H NMR (300 MHz, $CDCl_3$), δ ppm: 4.62 (broad, H-4), 3.94–3.90 (m, H-3), 3.12 (broad, H-7_{eq}), 2.78 (dd, J = 12.6 Hz, 12.6 Hz, H-7_{ax}), 0.91 (d, J = 6.4 Hz, $C\underline{H}_3$-21), 0.86 (d, J = 6.4 Hz, $C\underline{H}_3$-26 and $C\underline{H}_3$-27), 0.77 (s, $C\underline{H}_3$-19), 0.64 (s, $C\underline{H}_3$-18). ^{13}C NMR (75.5 MHz, $CDCl_3$), δ ppm: 29.9 C-1, 28.0 C-2, 66.8 C-3, 35.5 C-4, 80.0 C-5, 214.5 C-6, 41.7 C-7, 37.4 C-8, 44.2 C-9, 42.4 C-10, 21.3 C-11, 39.4 C-12, 43.0 C-13, 56.2 C-14, 23.8 C-15, 29.7 C-16, 56.0 C-17, 11.9 C-18, 13.8 C-19, 35.6 C-20, 18.5 C-21, 36.0 C-22, 23.7 C-23, 39.5 C-24, 27.9 C-25, 22.4 C-26, 22.7 C-27. MS (EI, 70 eV): 420 MH_2^+, 419 MH^+, 418 M^+, 401, 400, 385, 367, 318 (100%), 303.

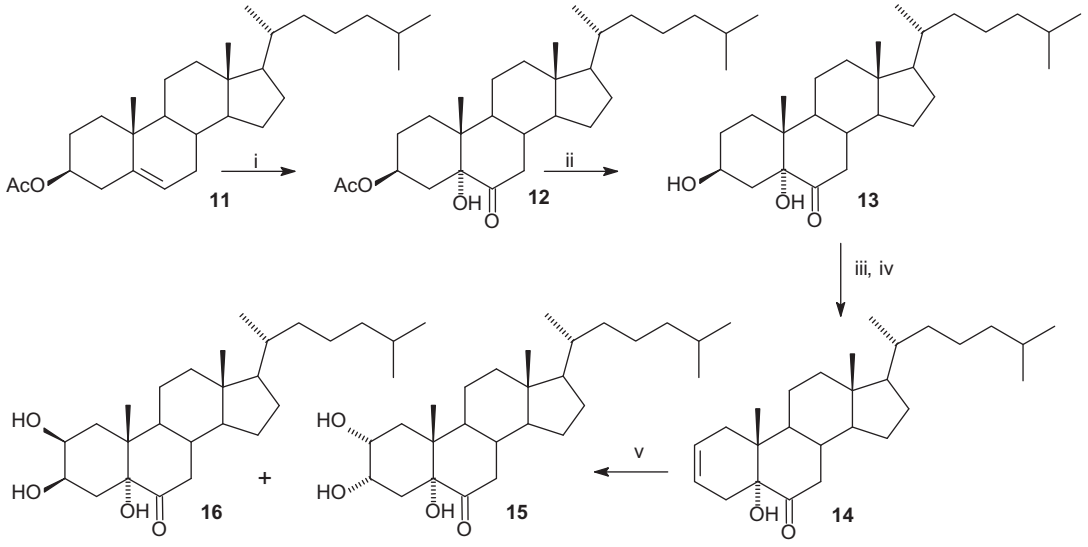
2.2.3. 5-Hydroxy-5α-cholest-2-en-6-one (**14**)

Tosyl chloride (953.2 mg, 5 mmol) was added to a solution of the dihydroxylated ketone **13** (1 g, 2.39 mmol) in dry pyridine (12 ml), the mixture was stirred overnight at room temperature and poured into 3% HCl/ice, the solid product was filtered off, washed with water, dissolved in ethyl acetate, dried (Na_2SO_4) and evaporated. The dried solid was refluxed for 30 min. in dry DMF (25 ml) with LiBr (1.3195 g) and Li_2CO_3 (1.0925 g) and the mixture was allowed to cold to room temperature. Ethyl acetate (70 ml) was added, the mixture was filtered, the solid residue was washed with ethyl acetate (4× 5 ml) and the organic layer was washed with water (6× 20 ml), dried (Na_2SO_4) and evaporated. The residue was purified in a chromatographic column (hexane/ethyl acetate 8/1) to afford the unsaturated 5α-hydroxyketone **14** (0.7315 g, 1.83 mmol, 77%). M.p 134–136 °C *(from ethyl acetate);* Lit. 140–141.5 °C [17]. ^1H NMR (300 MHz, $CDCl_3$), δ ppm: 5.69–5.57 (2H, m, H-2, H-3), 2.68 (1H, dd, J = 12.6 Hz, 12.6 Hz, H-7_{ax}), 2.18 (1H, dd, J = 4.1 Hz, 12.7 Hz, H-7_{eq}), 0.90 (3H, d, J = 6.5 Hz, $C\underline{H}_3$-21), 0.85 (6H, dd, J = 1.4 Hz, 6.6 Hz, $C\underline{H}_3$-26 and $C\underline{H}_3$-27), 0.69 (3H, s, $C\underline{H}_3$-19), 0.64 (3H, s, $C\underline{H}_3$-18). ^{13}C NMR (75.5 MHz, $CDCl_3$), δ ppm: 28.0 C-1, 122.4 C-2, 125.5 C-3, 34.5 C-4, 78.0 C-5, 211.5 C-6, 42.6 C-7, 37.5 C-8, 45.2 C-9, 42.3 C-10, 21.0 C-11, 39.5 C-12, 42.9 C-13, 56.4 C-14, 23.8 C-15, 30.1 C-16, 56.1 C-17, 11.9 C-18, 14.5 C-19, 35.7 C-20, 18.6 C-21, 36.1 C-22, 23.9 C-23, 39.6 C-24, 28.0 C-25, 22.5 C-26, 22.8 C-27. MS (EI, 70 eV): 402 MH_2^+, MH^+, 400 M^+, 385, 368, 367 (100%), 355.

2.2.4. 2α,3α,5-Trihydroxy-5α-cholestan-6-one (15) and 2β,3β,5-trihydroxy-5α-cholestan-6-one (**16**)

N-methylmorpholine N-oxide (0.243 g) and a 12.5 mg/ml solution of OsO_4 in *tert*-butyl alcohol (0.39 ml) were added to a solution of the unsaturated ketol **14** (200 mg, 0.5 mmol) in THF (5 ml) and the mixture was stirred overnight under N_2 atmosphere before addition of a solution of Na_2SO_3 (0.4 g) in water (3 ml). The mixture was extracted with ethyl acetate (2× 50 ml) and the organic layer was washed with brine (5× 20 ml), dried and evaporated to afford a mixture of the trihydroxylated compounds **15** and **16** that were separated in a chromatographic column using 1/1 of hexane/ethyl acetate as eluent.

*2.2.4.1. 2α,3α,5-Trihydroxy-5α-cholestan-6-one (**15**).* Yield (134.4 mg, 0.31 mmol, 62%). M.p 190–192 °C (from ethyl acetate) [18]. ^1H NMR (400 MHz, DMSO-d_6), δ ppm: 5.61 (1H, s, OH-5), 5.44 (1H, d, J = 3.0 Hz, OH-3), 4.54 (1H, d, J = 5.8 Hz, OH-2), 3.92 (1H, m, H-3), 3.63–3.59 (1H, m, H-2), 2.56 (1H, dd, J = 12.4 Hz, 12.4 Hz, H-7_{ax}), 1.84 (1H, dd, J = 4.1 Hz, 12.4 Hz, H-7_{eq}), 0.89 (3H, d, J = 6.4 Hz, $C\underline{H}_3$-21), 0.84 (6H, dd, J = 1.9 Hz, 6.6 Hz, $C\underline{H}_3$-26 and $C\underline{H}_3$-27), 0.67 (3H, s, $C\underline{H}_3$-19), 0.61 (3H, s, $C\underline{H}_3$-18). ^{13}C NMR (100 MHz, DMSO-d_6), δ ppm: 27.6 C-1, 66.4 C-2, 69.2 C-3, 34.3 C-4, 79.1 C-5, 210.4 C-6, 40.9 C-7, 36.4 C-8, 44.1 C-9, 42.6 C-10, 20.6 C-11, 39.2 C-12, 44.6 C-13, 55.8 C-14, 23.4 C-15, 30.5 C-16, 55.4 C-17, 11.7 C-18, 14.3 C-19, 35.1 C-20, 18.4 C-21, 35.5 C-22, 23.2 C-23, 38.8 C-24, 27.3 C-25, 22.3 C-26, 22.6 C-27. MS (IE, 70 eV): 436 MH_2^+, 435 MH^+, 434 M^+, 416, 398, 383, 367, 355, 331, 318, 317, 285, 247, 243, 231, 211, 189, 175, 161, 149, 137, 135, 109, 95, 81,69 (100%), 55. HRMS-EI: observed 434.3385; required for $C_{27}H_{46}O_4$ 434.3391.



Scheme 1. Synthetic procedure.

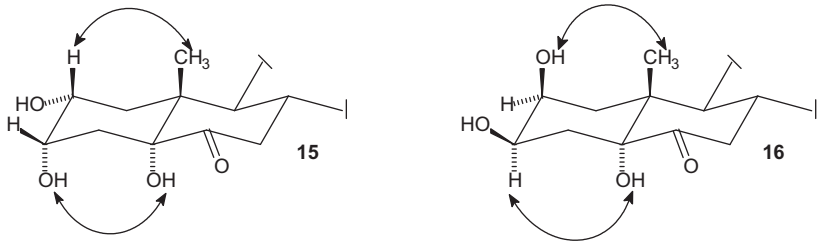


Fig. 3. Selected NOE correlations in triols **15** and **16**.

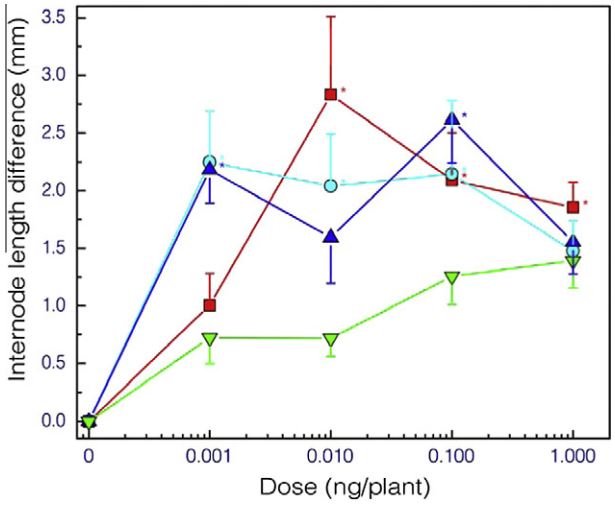


Fig. 4. Biological activity of the studied compounds. Homobrassinolide **2** (■), or compounds **13** (●), **15** (▲), or **16** (▼). The abscissa scale is logarithmic, but zero dosage was arbitrarily placed in the beginning of the graph. Length values are plotted as differences from control plants treated with vehicle (lanolin), therefore at zero dose, the length becomes zero.

*2.2.4.2. 2β,3β,5-Trihydroxy-5α-cholestan-6-one (**16**).* Yield (62.3 mg, 0.14 mmol, 28%). M.p. 262–264 °C (from diethyl ether) [18]. ^{1}H NMR (400 MHz, DMSO-d_6), δ ppm: 5.33 (1H, s, OH-5), 4.29 (1H, d, J = 6.2 Hz, OH-3), 4.08 (1H, d, J = 5.8 Hz, OH-2), 3.75 (2H, m, H-2 and H-3), 2.66 (1H, dd, J = 12.5 Hz, 12.5 Hz, H-7_{ax}), 1.82 (1H, dd, J = 5.0 Hz, 12.7 Hz, H-7_{eq}), 0.88 (3H, d, J = 6.5 Hz, C$\underline{H}_3$-21), 0.87 (3H, s, C$\underline{H}_3$-19), 0.84 (6H, dd, J = 2.0 Hz, 6.6 Hz, C$\underline{H}_3$-26 and C$\underline{H}_3$-27), 0.60 (3H, s, C$\underline{H}_3$-18). ^{13}C NMR (100 MHz, DMSO-d_6), δ ppm: 27.7 C-1, 68.3 C-2, 67.1 C-3, 36.6 C-4, 79.4 C-5, 212.3 C-6, 41.2 C-7, 36.0 C-8, 44.1 C-9, 42.7 C-10, 21.0 C-11, 39.4 C-12, 41.5 C-13, 55.9 C-14, 23.5 C-15, 30.5 C-16, 55.6 C-17, 11.8 C-18, 15.7 C-19, 35.2 C-20, 18.5 C-21, 35.6 C-22, 23.2 C-23, 38.9 C-24, 27.4 C-25, 22.4 C-26, 22.7 C-27. MS (EI, 70 eV): 436 MH_2^+, 435 MH^+, 434 M^+, 416, 390, 373, 355, 331, 318, 316, 303, 255, 247, 221, 175, 161, 147, 121, 137, 107, 95, 81, 69, 57 (100%). HRMS (EI, 70 eV): observed 434.3368, required for $C_{27}H_{46}O_4$ 434.3391.

2.3. Biological assay

2.3.1. Bean's second internode elongation assay

The details of this test have been described elsewhere [15]. Briefly, bean seed were germinated in sterile wet paper towels. Bean seedling were planted in small pots with agrolite and watered with Hoghland solution for 2 weeks. Compounds were dissolved in a mixture of lanolin and ethanol, and 2 μl of the solution containing the dose indicated was applied to the base of the second internode. After 48 h, the length of the second internode was measured. Groups of 10–15 plants were used for each treatment. The data are from three independent experiments. Data is presented as length differences, i.e. the second internode length was recorded and the median value

of the internode length for control plants was subtracted to all data. For each compound tested, the overall statistical significance of the differences between the treatments (different dosages) was tested using the distribution-independent test of Kruskal–Wallis one way analysis of variance (ANOVA on ranks), individual differences against control were isolated with Dunn's method.

3. Results and discussion

3.1. Chemistry

Cholesteryl acetate **(11)** was converted into the acetylated ketol **(12)** following our recently published one-pot epoxidation-oxidative cleavage procedure [19]. Saponification of **12** afforded the dihydroxylated ketone **13** that on consecutive tosylation and elimination afforded the unsaturated ketol **14**. Dihydroxylation of **14** employing OsO_4 and NMMO as co-oxidant produced a mixture of the 2α,3α and 2α,3β diols **15** and **16** (Scheme 1).

Although an analogous preparation of compounds **15** and **16** has been previously reported by Rodriguez and coworkers in the synthesis of cytotoxic 6*E*-hydroximino-4-ene steroids [18], we decided to carry out a complete high field NMR characterization and provide unambiguous evidences on the orientation of the diol moiety introduced at positions C-2 and C-3, not provided in the above cited report. In particular, the orientation of the introduced cis-diol moiety can be easily established in NOESY experiments. While in the 2α,3α,5α-triol **15** the observed **H**-2β↔**H**-19 and 3α-O**H**↔5α-O**H** NOE effects indicate the α-orientation of the 2,3-diol, the 2β−oH ↔**H**-19 and **H**-3α↔5α-o**H** NOE effects observed in the 2β,3β,5α-triol **16** evidence the β-orientation of the diol moiety at positions C-2 and C-3 (Fig. 3).

3.2. Biological activity

A number of recent findings on the Bs signal transduction pathways [20] have paved the way for more direct molecular tests of the Bs effects. However, it has also been shown that these molecular responses are not always proportional to the applied dose [21]. Therefore, until a molecular test is properly validated, the classic physiological assays continue to be more reliable. Hence, the biological activities of compounds **13**, **15** and **16** were determined employing the bean's second internode elongation assay using homobrassinolide (**2**) as active control, and the vehicle (lanolin) as a control lacking activity.

The compounds were tested at concentrations from 1 pg to 1 ng per plant, using the bean second internode assay. From the non-parametric statistical test of the data, homobrassinolide (**2**) and compounds **13** and **15** were found to stimulate plant growth in the employed assay. Compound **16** appeared to give some effect, but the data lacked statistical significance, therefore, we consider this compound as inactive.

Table 1
Bean's second internode elongation differences (mm).

Dose (ng/plant)	**2** homobrassinolide	**13**	**15**	**16**
0.001	1.01 ± 0.28	2.25 ± 0.44 (∗)	2.18 ± 0.29 (∗)	0.72 ± 0.22
0.010	2.83 ± 0.68 (∗)	2.04 ± 0.45 (∗)	1.60 ± 0.40	0.72 ± 0.15
0.100	2.09 ± 0.41 (∗)	2.15 ± 0.64	2.61 ± 0.37 (∗)	1.26 ± 0.25
1.000	1.86 ± 0.22 (∗)	1.48 ± 0.26	1.56 ± 0.28	1.39 ± 0.24

(∗) Statistically significant difference from control in a Kruskal–Wallis ANOVA, followed by the Dunn's test ($p < 0.05$). The length of the control plants internode was subtracted from that of the plants treated with the studied compounds.

Compounds **13** and **15** gave the maximal response (i.e. the highest internode length difference recorded for each compound) nearly as good as the natural phytohormone (**2**), and their effect at the lower dose tested (1 pg/plant) was at least as large as the effect observed with **2** (there was no statistically significant difference between the data for **2**, and the data for the compounds **13** and **15** at this dose). Therefore, we can safely conclude that **13** and **15** were as effective as the natural phytohormone (**2**) in the dosage range tested (Fig. 4 and Table 1).

4. Conclusions

Three brassinosteroid analogs bearing the 5α-hydroxy-6-oxo moiety and different hydroxylated A-rings were obtained from cholesterol. Compounds **13** and **15** showed interesting properties as plant growth stimulators in the bean's second internode elongation assay. The obtained results indicate that the presence of the 5α-hydroxy-6-oxo moiety, even in the absence of oxygenated functions in the side chain, is capable to induce plant growth stimulation. The active compounds **13** and **15** have the advantage that can be easily obtained from the inexpensive and ready available cholesterol acetate **(11)**.

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