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Synthesis and bioactivity evaluation of brassinosteroid analogs

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Abstract

Four new analogs of 28-homocastasterone have been synthesized and completely characterized for the first time from stigmasterol. (22R,23R,24S)-3β-acetoxy-22,23-dihydroxy-5α-stigmastan-6-one (**17**), (22R,23R,24S)-3β-bromo-22,23-dihydroxy-5α-stigmastan-6-one (**18**), (22R,23R,24S)-3β-acetoxy-5,22,23-trihydroxy-5α-stigmastan-6-one (**20**), and (22R,23R,24S)-3β-bromo-5,22,23-trihydroxy-5α-stigmastan-6-one (**21**), were obtained through a synthetic route based on regioselective Δ^5 epoxidation. Compounds **17** and **18**, bearing a 5αH moiety, were prepared through a reductive opening of the 5β,6β epoxy precursor, and compounds **20** and **21**, analogs with a 5αOH moiety were obtained by hydrolytic opening of a mixture of 5α,6α and 5β,6β epoxy precursors. Known compounds **19** and **22** were also obtained following the described synthetic routes, respectively. The new compounds were tested with the traditional auxin-like bioassay for brassinosteroids with **19** and **22** as standards. All compounds were comparatively evaluated for their inhibitory effect on the replication of DNA (HSV-1) virus.

Keywords: Brassinosteroid; Synthesis; Bioactive; Phytohormone; Antiviral

1. Introduction

Brassinosteroids represent a new group of steroidal phytohormones with high growth-promoting activity and antistress effects [1–3]. Since the discovery of the first member named brassinolide in 1979, about 40 other native brassinosteroids have been identified from a broad variety of plants [4]. Research on brassinosteroids has recently been encouraged as studies have suggested that they, like other phytohormones, act in part by regulating gene expression [5,6].

Brassinosteroids synthesis was based on relatively few strategies involving the A-B steroidal ring junction [7,8]. In this paper, we report the synthesis of six analogs (see Fig. 1), four of which are new, through an alternative synthetic strategy that involves regioselective 5,6 epoxidation of the Δ^5 double bond of stigmasterol and subsequent reductive or hydrolytic opening of that epoxide. Scheme 1 depicts the reductive opening of the 5β,6β epoxy precursor to achieve analogs with a 5αH moiety: (22R,23R,24S)-3β-acetoxy-22,23-dihydroxy-5α-stigmastan-6-one (**17**), (22R,23R,24S)-3β-bromo-22,23-dihydroxy-5α-stigmastan-6-one (**18**), and (22R,23R,24S)-2α,3α,22,23-tetrahydroxy-5α-stigmastan-6-one (**19**) (28-homoethylcastasterone).

Scheme 2 describes the hydrolytic opening of a mixture of 5α,6α and 5β,6β epoxy precursors to obtain analogs with a 5αOH moiety: (22R,23R,24S)-3β-acetoxy-5,22,23-trihydroxy-5α-stigmastan-6-one (**20**), (22R,23R,24S)-3β-bromo-5,22,23-trihydroxy-5α-stigmastan-6-one (**21**), and (22R,23R,24S)-2α,3α,5α,22,23-pentahydroxy-5α-stigmastan-6-one (**22**).

Auxin-like bioactivity of the synthetic compounds was evaluated by the rice lamina inclination bioassay. Though larger effects were detected for known compounds **19** and **22** (see Table 1), further studies should be done to determine possible practical applications of the new compounds. Other brassinosteroid analogs have shown bioactivity at field trials notwithstanding they were not active at the bioassay level [3].

Prompted by results indicating antiviral effects for oxysterols [9] and steroidal marine compounds [10], we introduce in this paper some new interesting data concerning inhibition of HSV-1 virus yield in infected Vero cells by synthetic brassinosteroids **17**–**22** (see Table 1).

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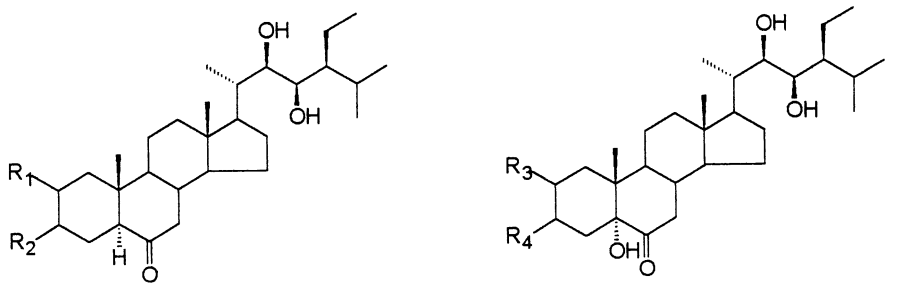


17 R_1 = H R_2= βAcO
18 R_1 = H R_2= βBr
19 R_1 = αOH R_2= αOH

20 R_3 = H R_4= βAcO
21 R_3 = H R_4= βBr
22 R_3 = αOH R_4= αOH

Fig. 1. Target compounds.

2. Experimental

2.1. General

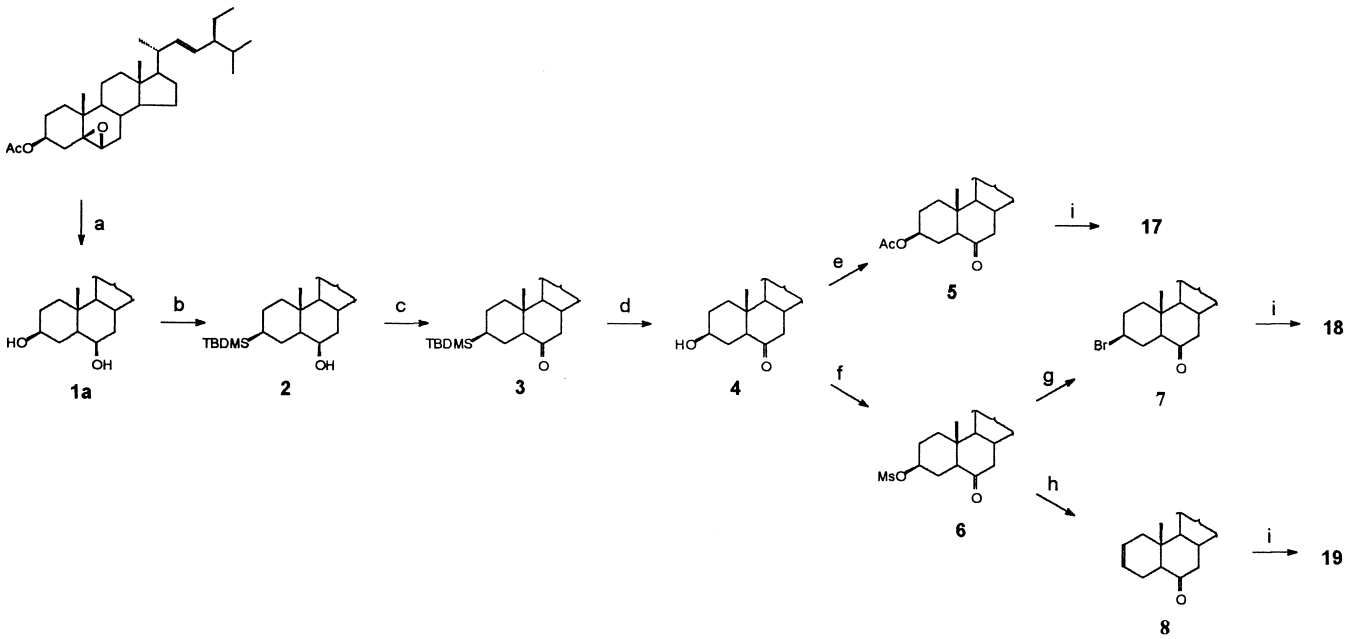
Melting points (m.p.) were determined on a Fisher Johns apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Brucker AC-200 at 200.1 and 50.3 MHz, respectively. Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard, and coupling constants (*J*) values are in Hz. High-resolution mass spectra (EI or FAB) were obtained for all new compounds on a ZAB BEqQ instrument (VG-Micromass). Low resolution mass spectra were recorded on a Varian-MAT CH-7 A, on a VG-TRIO-2, or on a Shimadzu QP-5000 at 70 eV. Unless otherwise indicated, all solvents and reagents used were of commercial grade. Reactions were monitored by TLC on precoated plates with silicagel F_{254} 0.2 mm (Merck, West Point, PA, USA). Column chromatography was carried out on silica gel 60, 0.04–0.063 mm (Merck).

Stereochemistry at C-22 and C-23 was defined by spectroscopic data correspondence with already described compounds.

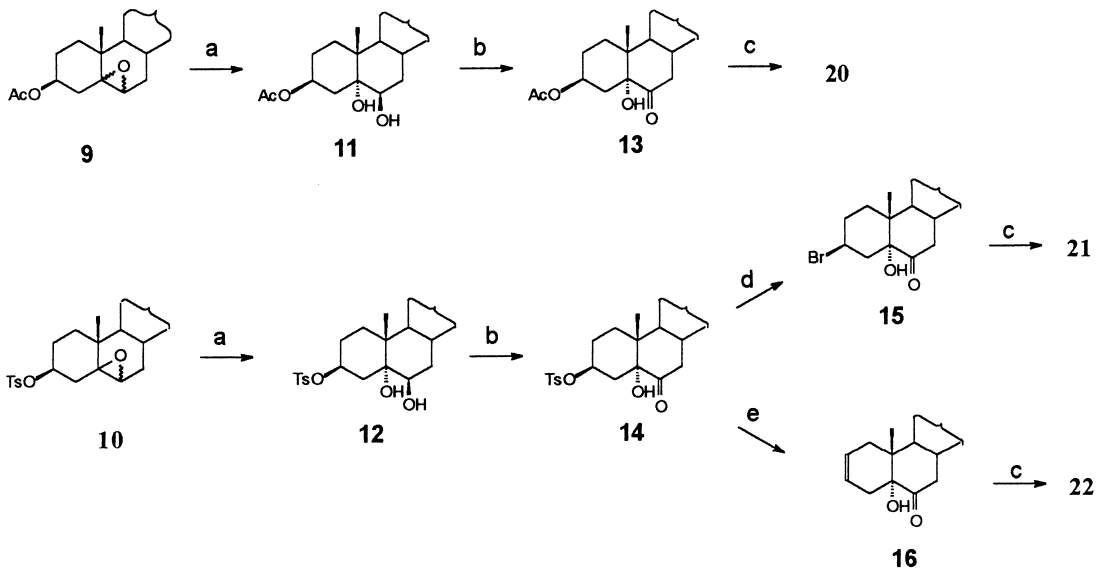
2.2. Syntheses of compounds

*2.2.1. (22E)-5α-Stigmast-22-en-3β,6β-diol (**1a**)*

Lithium aluminum hydride (151 mg, 3.9 mmol) was added to a solution of aluminum chloride (971 mg, 7.3 mmol) in dry THF (10 ml). After 5 min, a solution of (22E)-3β-acetoxy-5β,6β-epoxystigmast-22-ene (300 mg,



Scheme 1. Reagents and conditions: (a) $LiAlH_4/AlCl_3$/THF/r.t. (b) TBDMSCl/imidazole/DMF/r.t. (c) PCC/CH_2Cl_2/0°C. (d) nBu_4NF/THF/r.t. (e) AcCl/Py/r.t. (f) MsCl/Py/r.t. (g) LiBr/MeOH/reflux. (h) LiBr/DMF/reflux. (i) K_2OsO_4. $2H_2O$/$(DHQD)_2$Phal/methanesulfonamide/$K_3Fe(CN)_6$/K_2CO_3/t-BuOH/H_2O.



Scheme 2. Reagents and conditions: (a) $HClO_4$/dioxane/H_2O/r.t. (b) PCC/CH_2Cl_2/0°C. (c) K_2OsO_4. $2H_2O$/$(DHQD)_2Phal$/methanesulfonamide/$K_3Fe(CN)_6$/K_2CO_3/t-BuOH/H_2O. (d) LiBr/acetone/reflux. (e) KI/acetone/reflux.

0.64 mmol) in dry THF (10 ml) was added dropwise, and the reaction mixture was stirred for 3 h. Then acetone (5 ml) and water (5 ml) were added carefully. Extraction with ethyl ether and purification by chromatography (hexane/EtOAc) gave compound **1a** in 44% yield. m.p.: 200–201°C. ^{1}H NMR ($CDCl_3$): 0.68 (3H, s, 18-H_3), 1.00 (3H, s, 19-H_3), 3.60 (1H, m, 3α-H), 3.75 (1H, m, 6α-H), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 12.0, 12.1, 15.5, 21.1, 21.2, 24.1, 18.8, 25.2, 20.9, 31.0, 30.3, 28.8, 35.2, 31.7, 38.4, 34.8, 39.3, 40.3, 39.7, 42.4, 47.4, 51.1, 54.2, 56.0, 56.2, 71.2, 71.6, 129.2, 138.2. MS (EI) *m/z* (%): 430 (M^+, 14), 273 (12), 255 (18), 55 (100). HRMS (EI): Calculated for $C_{29}H_{50}O_2$: 430.3811. Found: 430.3812.

2.2.2. (22E)-3β-(t-Butyldimethylsilyloxy)-5α-stigmast-22-en-6β-ol (2)

(22E)-5α-Stigmast-22-en-3β,6β-diol (**1a**) (522 mg, 1.21 mmol), dissolved in dry dimethylformamide (DMF) (40 ml), was treated with imidazole (210 mg, 3.08 mmol) and *t*-butyldimethylsilyl chloride (220 mg, 1.45 mmol) for 24 h at room temperature. The solvent was evaporated off, and the residue was extracted with methylene chloride/water. The organic layer was dried ($MgSO_4$) and evaporated, yielding 100% of (22E)-3β-(*t*-butyldimethylsilyloxy)-5α-stigmast-22-en-6β-ol (**2**). m.p.: 170–171°C. ^{1}H NMR ($CDCl_3$): 0.70 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 3.60 (1H, m, 3α-H), 3.75 (1H, m, 6α-H), 0.40 (3H, s, CH_3Si-), 0.88 (9H, s, (CH_3)$_3$CSi), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): −4.6, 12.2, 12.3, 15.8, 18.2, 19.0, 21.1, 21.1, 21.2, 24.3, 25.4, 25.9, 28.9, 30.4, 31.9, 32.0, 34.0, 35.8, 38.7, 39.5, 39.9, 40.5, 42.6, 47.6, 51.3, 54.4, 56.1, 56.4, 72.5, 72.3, 129.3, 138.3. MS (EI): *m/z* (%), 487 (M^+-[(CH_3)$_3$C-], 1), 469 (3), 255 (5), 83 (100).

Table 1
Bioactivities of synthetic compounds **17–22**

Compound	Auxin-like effect		Antiviral effect		
	Average angle	%	IC_{50}*	CC_{50}*	IS_{50}
17	41 ± 4	58.6	22.4	238	10.6
18	32 ± 2	45.7	53.3	248	4.7
19	70 ± 3	100.0	41.3	209	5.1
20	20 ± 2	28.6	57.6	230	4
21	34.5 ± 3	49.3	17.7	23	1.3
22	52 ± 4	85.0	40.4	40	1
Stigmasterol	—	—	48.8	479	10

* Concentration expressed in μM.

2.2.3. (22E)-3β-(t-Butyldimethylsilyloxy)-5α-stigmast-22-en-6-one (3)

A solution of **2** (150 mg, 0.27 mmol) in methylene chloride (5 ml) was added to a suspension of pyridinium chlorochromate (260 mg, 1.20 mmol) in methylene chloride (10 ml) at 0°C. The reaction mixture was stirred for 20 min and then allowed to reach room temperature. The suspension was filtered through a pad of silicagel; removal of the solvent from the filtrate gave a crude solid, which was purified by chromatography (hexane/EtOAc) to afford compound **3** (95%). m.p.: 151–152°C. ^{1}H NMR ($CDCl_3$): 0.68 (3H, s, 18-H_3), 0.75 (3H, s, 19-H_3), 3.52 (1H, m, 3α-H), 0.40 (3H, s, CH_3Si), 0.88 (9H, s, (CH_3)$_3$CSi), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): −4.6, 12.2, 13.2, 18.2, 18.2, 19.0, 21.1, 21.2, 21.5, 24.1, 25.4, 25.9, 28.7, 30.3, 31.3, 31.9, 36.9, 37.9, 39.5, 40.4, 41.0, 42.9, 46.8, 51.2, 54.1, 56.0, 57.0, 57.0, 71.5, 129.6, 138.0, 211.1, MS (EI): *m/z* 485 (M^+-(CH_3)$_3$C-, 100), 345 (2), 83 (49), 43 (19). HRMS (EI): Calculated for $C_{31}H_{53}SiO$ [M-[(CH_3)$_3$C-]]$^+$: 485.3815. Found 485.3813.

*2.2.4. (22E)-3β-Hydroxy-5α-stigmast-22-en-6-one (**4**)*

Tetrabutylammonium fluoride (0.85 ml of 1 M in THF) was added to a solution of compound **3** (200 mg, 0.37 mmol) in anhydrous THF (10 ml), and the reaction mixture was stirred for 4 h at room temperature. The solvent was evaporated off, and the crude residue was partitioned between ether/water. The ethereal layer was dried and evaporated to give compound **4** in quantitative yield. m.p.: 145–146°C (lit. 146–147°C [11]). ^{1}H NMR ($CDCl_3$): 0.67 (3H, s, 18-H_3), 0.77 (3H, s, 19-H_3), 4.54 (1H, m, 3α-H), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 12.3, 13.2, 12.3, 19.0, 21.1, 21.2, 24.1, 21.6, 25.4, 30.1, 28.8, 30.8, 38.0, 31.9, 41.0, 43.0, 36.8, 39.5, 40.4, 46.8, 54.0, 51.3, 56.0, 57.0, 56.9, 70.8, 129.7, 138.0, 206.0. MS (EI): *m/z* 428 (M^+, 32), 410 (2), 286 (49), 55 (100).

*2.2.5. (22E)-3β-Acetoxy-5α-stigmast-22-en-6-one (**5**)*

Compound **4** (500 mg, 1.14 mmol) was dissolved in pyridine (5 ml) and acetic anhydride (2.5 ml) was added. The mixture was allowed to react overnight at room temperature. Then, the solution was poured over 100 ml of ice-cold 5% aq. HCl. After extraction with methylene chloride, the crude product was purified by flash chromatography (hexane/EtOAc), yielding **5** almost quantitatively. m.p.: 146–147°C (lit. 146–147°C [12]). ^{1}H NMR ($CDCl_3$): 0.68 (3H, s, 18-H_3), 0.77 (3H, s, 19-H_3), 4.67 (1H, m, 3α-H), 2.40 (3H, s, CH_3CO), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 11.9, 12.0, 12.7, 18.7, 20.8, 20.9, 21.2, 21.2, 23.7, 25.1, 25.8, 26.6, 28.4, 31.5, 36.1, 37.6, 39.1, 40.1, 40.6, 42.6, 46.3, 50.9, 53.5, 56.1, 56.5, 56.6, 72.5, 129.3, 137.6, 170.5, 209.6. MS (EI): *m/z* 470 (M^+, 20), 427 (3), 410 (M^+-AcOH, 3), 367 (16), 358 (23), 271 (20), 43 (100).

*2.2.6. (22E)-3β-Mesyloxy-5α-stigmast-22-en-6-one (**6**)*

Compound **4** (500 mg, 1.14 mmol) was dissolved in pyridine (5 ml), and methanesulfonyl chloride (0.2 ml) was added. The reaction mixture was stirred at room temperature for 1 h. Then, the solution was poured over 100 ml of ice cold 5% aq. HCl. After extraction with methylene chloride, the organic layer was dried ($MgSO_4$) and evaporated off, yielding **6** quantitatively. m.p.: 148–149°C. ^{1}H NMR ($CDCl_3$): 0.69 (3H, s, 18-H_3), 0.79 (3H, s, 19-H_3), 4.65 (1H, m, 3α-H), 2.98 (3H, s, CH_3SO_2), 5.10 (2H, m, 22-H and 23-H), ^{13}C NMR ($CDCl_3$): 11.9, 12.0, 12.6, 18.7, 20.8, 20.9, 21.1, 23.7, 25.1, 27.0, 27.9, 28.4, 31.5, 36.0, 37.5, 38.5, 39.0, 40.1, 40.2, 42.5, 46.1, 50.9, 53.2, 55.6, 55.9, 56.4, 80.9, 129.3, 137.6, 208.8. MS (EI): *m/z* 506 (M^+, 12), 463 (3), 410 (M^+-MsOH, 21), 394 (34), 367 (28), 271 (39), 55 (100). HRMS (EI): Calculated for $C_{30}H_{50}O_4S$, M^+: 506.3430. Found 506.3424.

*2.2.7. (22E)-3β-Bromo-5α-stigmast-22-en-6-one (**7**)*

Compound **6** (635 mg, 1.25 mmol) dissolved in methanol (50 ml) was treated with lithium bromide (715 mg, 8.31 mmol) under reflux for 6 h. After solvent evaporation, the residue was dissolved in methylene chloride, the organic layer was washed with water, dried ($MgSO_4$), and evaporated. Compounds 3α and 3β-bromo-5α-cholest-22-en-6-one (1:2) were separated and purified by flash chromatography (methylene chloride), yielding 64% of **7**. m.p.: 140–141°C. ^{1}H NMR ($CDCl_3$): 0.67 (3H, s, 18-H_3), 0.69 (3H, s, 19-H_3), 3.92 (1H, m, 3α-H). ^{13}C NMR ($CDCl_3$): 12.1, 12.1, 12.6, 18.9, 21.0, 21.1, 21.2, 23.9, 25.2, 28.6, 31.7, 32.3, 33.3, 37.6, 39.0, 39.2, 40.2, 40.5, 42.7, 46.4, 50.5, 51.1, 53.7, 55.8, 56.7, 58.8, 129.5, 137.8, 209.3. MS (EI): *m/z* 492 (M^+, ^{81}Br, 5), 490 (M^+, ^{79}Br, 5), 410 (M^+-Br, 1), 449 (4), 351 (12), 55 (100).

*2.2.8. (22E,24S)-5α-Stigmasta-2,22-dien-6-one (**8**)*

Compound **6** (421 mg, 0.83 mmol) in dimethylformamide (5 ml) was treated with lithium bromide under reflux for 1 h. The solvent was evaporated and the crude solid was dissolved in methylene chloride. This solution was washed with water, and then the organic layer was dried and evaporated. After purification by column chromatography, compound **8** was obtained (72% yield). m.p.: 133–134°C (lit. 133–135°C [13]). ^{1}H NMR ($CDCl_3$): 0.70 (3H, s, 18-H_3), 0.72 (3H, s, 19-H_3), 5.10 (2H, m, 22-H and 23-H), 5.63 (2H, m, 2-H and 3-H). ^{13}C NMR ($CDCl_3$): 12.1, 12.2, 13.4, 21.1, 21.1, 21.2, 24.0, 18.9, 21.7, 25.3, 28.7, 31.8, 39.4, 40.3, 39.4, 37.7, 47.0, 39.9, 42.7, 51.2, 53.4, 53.8, 55.9, 56.9, 124.4, 124.9, 129.6, 138.0, 211.7. MS: *m/z* 410 (M^+, 5), 395 (2), 271 (10), 55 (100).

*2.2.9. (22R,23R)-3β-Acetoxy-22,23-dihydroxy-5α-stigmastan-6-one (**17**)*

A mixture of **5** (760 mg, 1.61 mmol), THF (8 ml), *t*-butanol/water (1:1) (32 ml), $(DHQD)_2$-PHAL (252 mg, 0.32 mmol), methansulfonamide (308 mg, 2.77 mmol), potassium ferricyanide (1.60 g, 4.80 mmol), potassium carbonate (672 mg, 2.63 mmol), and potassium osmate dihydrate (29 mg, 0.08 mmol) was stirred at room temperature for 9 days. An excess of sodium bisulfite ($NaHSO_3$) was added until no evolution of bubbles was observed. Layers were separated and the aqueous phase was thoroughly extracted with methylene chloride/methanol (5%). Combined organic layers were washed with 0.25 M sulfuric acid and 2% sodium hydroxide. Purification by column chromatography (methylene chloride/acetonitrile gradient) allowed to separate (22R,23R)-3β-acetoxy-22,23-dihydroxy-5α-stigmastan-6-one (**17**) from its 22S, 23S diasteromer, with 22% yield. m.p.: 274–275°C (d). ^{1}H NMR ($CDCl_3/CD_3OD$ 9:1): 0.68 (3H, s, 18-H_3), 0.70 (3H, s, 19-H_3), 4.68 (1H, m, 3α-H), 3.60 (1H, d, J = 8 Hz, 22-H), 3.73 (1H, d, J = 8 Hz, 23-H), 2.40 (3H, s, CH_3CO). ^{13}C NMR ($CDCl_3/CD_3OD$ 9:1): 11.7, 11.7, 12.9, 21.4, 23.7, 25.9, 26.7, 27.4, 36.2, 36.8, 38.0, 39.3, 40.9, 42.7, 46.4, 52.4, 53.6, 56.3, 56.4, 72.9, 210.9. HRMS (FAB): Calculated for $C_{31}H_{53}O_5$ [M + H]$^+$: 505.3893. Found 505.3902.

*2.2.10. (22R,23R)-3β-Bromo-22,23-dihydroxy-5α-stigmastan-6-one (**18**)*

Compound **7** (795 mg, 1.60 mmol) was treated similarly to compound **5**. Purification by silica column chromatography (methylene chloride/acetonitrile gradient) allowed to separate compound **18** from its 22S, 23S diasteromer with 18.3% yield. m.p.: 193–194°C. ^{1}H NMR ($CDCl_3/CD_3OD$ 9:1): 0.70 (3H, s, 18-H_3), 0.82 (3H, s, 19-H_3), 3.93 (1H, m, 3α-H), 3.60 (1H, d, J = 8 Hz, 22-H), 3.73 (1H, d, J = 8 Hz, 23-H). ^{13}C NMR ($CDCl_3/CD_3OD$ 9:1): 11.9, 11.9, 13.1, 21.3, 23.8, 27.6, 32.3, 33.4, 37.0, 37.8, 39.2, 39.4, 40.7, 42.8, 46.5, 50.5, 52.5, 53.8, 56.6, 59.0, 206.6. HRMS (FAB): Calculated for $C_{29}H_{50}O_3Br$ $[M + H]^+$: 525.2943. Found 525.2947.

*2.2.11. (22R,23R)-2α,3α,22,23-Tetrahydroxy-5α-stigmastan-6-one (24-homocastasterone) (**19**)*

Compound **8** (768 mg, 1.87 mmol) was treated as described for compound **5**. After the usual work-up, compound **19** was obtained in 34% yield. All physical and spectral data for **18** were consistent with those previously reported [14].

*2.2.12. (22E)-5,6-Epoxy-3β-tosyloxystigmast-22-ene (**10**)*

(22E)-3β-Tosyloxystigmasta-2,22-diene (800 mg, 1.41 mmol) in methylene chloride (250 ml) was treated with *m*-chloroperbenzoic acid (430 mg, 2.10 mmol) at −78°C. Work-up gave a mixture of 5α,6α and 5β,6β-epoxides (5:1). Purification by chromatography (methylene chloride/hexane) yielded (22E)-5α,6α-epoxy-3β-tosyloxystigmast-22-ene (65%) and (22E)-5β,6β-epoxy-3β-tosyloxystigmast-22-ene (15%).

First eluted compound (5α,6α-epoxide): m.p.: 171–173°C. ^{1}H NMR ($CDCl_3$): 0.63 (3H, s, 18-H_3), 1.00 (3H, s, 19-H_3), 4.60 (1H, m, 3α-H), 2.80 (1H, m, 6β-H), 1.44 (3H, s, CH_3-Ph), 5.10 (2H, m, 22-H and 23-H), 7.26 and 7.72 (4H, d, J = 9 Hz, CH_3-*Ph*). MS (EI): *m*/*z* 410 (M^+-TsOH, 12), 392 (11), 367 (5), 298 (6), 271 (9), 55 (100).

Second eluted compound (5β,6β-epoxide): m.p.: 96°C. ^{1}H NMR ($CDCl_3$): 0.61 (3H, s, 18-H_3), 1.03 (3H, s, 19-H_3), 4.60 (1H, m, 3α-H), 1.44 (3H, s, CH_3-Ph), 2.95 (1H, m, 6α-H), 5.10 (2H, m, 22-H and 23-H) 7.26 and 7.72 (4H, d, J = 9 Hz, CH_3-*Ph*). ^{13}C NMR ($CDCl_3$): 11.8, 12.2, 16.8, 18.9, 21.0, 21.1, 21.5, 21.8, 24.1, 25.3, 27.9, 28.7, 29.5, 31.8, 32.2, 34.7, 36.1, 38.5, 39.5, 40.4, 42.0, 51.0, 51.1, 55.8, 56.1, 62.2, 63.3, 79.3, 127.5, 129.3, 129.6, 134.7, 138.1, 144.2. MS (EI): *m*/*z* (%): 410 (M^+-TsOH, 4), 392 (8), 367 (2), 298 (3), 271 (6), 55 (100).

*2.2.13. (22E)-3β-Acetoxystigmast-22-en-5α,6β-diol (**11**)*

A mixture of 5α,6α and 5β,6β epoxides (**9**) was obtained by epoxidation of (22E)-3β-acetoxystigmasta-5,22-diene with *m*-chloroperbenzoic acid using the same procedure described for compound **10**. The mixture (500 mg, 1.06 mmol) of compound **9** was dissolved in a solution of dioxane (70 ml), water (17 ml), and 70% perchloric acid (6 ml). The reaction mixture was stirred for 2 h at room temperature, and then neutralized with sodium bicarbonate (saturated solution). The solvent was evaporated, and the residue, dissolved in methylene chloride, was thoroughly washed with water. The crude product was purified by chromatography (hexane/EtOAc), yielding 90% of compound **11**. m.p.: 237–238°C. ^{1}H NMR ($CDCl_3$): 0.70 (3H, s, H-18$_3$), 1.19 (3H, s, H-19$_3$), 2.40 (3H, s, CH_3CO), 3.35 (1H, bs, 6-αH), 5.02 (1H, m, 3-αH), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 12.2, 12.3, 16.7, 19.0, 21.1, 21.1, 21.2, 21.2, 24.2, 25.4, 26.7, 28.9, 30.2, 31.9, 32.1, 34.6, 37.0, 38.3, 39.8, 40.5, 42.6, 45.5, 51.2, 55.9, 56.0, 71.2, 75.7, 76.2, 129.3, 138.3, 170.5. MS (EI): *m*/*z* (%), 488 (M^+, 3), 470 (M^+-H_2O, 2), 410 (7), 367 (6), 271 (9), 253 (30), 55 (100).

*2.2.14. (22E)-3β-Tosyloxystigmast-22-en-5α,6β-diol (**12**)*

(22E)-3β-Tosyloxy-5,6-epoxystigmast-22-ene (**10**) (500 mg, 0.85 mmol) was dissolved in a solution of dioxane (60 ml), water (15 ml), and 70% perchloric acid (10 ml). The mixture was allowed to react for 2 h at room temperature and then neutralized with sodium bicarbonate (s.s.). The solvent was evaporated, and the residue, dissolved in methylene chloride, was thoroughly washed with water. Purification by chromatography (hexane/EtOAc) yielded 94% of **12**. m.p.: 142–143°C (d). ^{1}H NMR ($CDCl_3$): 0.68 (3H, s, H-18$_3$), 1.16 (3H, s, H-19$_3$), 3.52 (1H, bs, 6α-H), 4.90 (1H, m, 3α-H), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 12.3, 12.4, 16.7, 19.1, 21.1, 21.2, 21.2, 21.5, 24.2, 25.4, 27.7, 28.9, 30.2, 31.9, 32.3, 34.6, 37.1, 38.2, 39.8, 40.5, 42.5, 45.7, 51.3, 56.0, 56.1, 75.3, 75.8, 80.5, 127.5, 129.4, 129.6, 134.7, 138.3, 144.2. MS (EI): *m*/*z* 428 (M^+-TsOH, 3), 410 (17), 392 (41), 43 (100).

*2.2.15. (22E)-3β-Acetoxy-5α-hydroxystigmast-22-en-6-one (**13**)*

Compound **11** (300 mg; 0.51 mmol) dissolved in methylene chloride (10 ml) was added to a suspension of pyridinium chlorocromate (170 mg, 0.78 mmol) in methylene chloride (25 ml) at 0°C. After 20 min the ice bath was removed and the reaction was allowed to stand for 4 h at room temperature. The suspension was then filtered off through a pad of silica gel D. The solvent was evaporated, and the crude solid was purified by column chromatography. Elution with hexane/ethyl acetate (8:2) provided **13** [15] in 82% yield. m.p.: 253–254°C. ^{1}H NMR ($CDCl_3$): 0.66 (3H, s, 18-H_3), 0.80 (3H, s, 19-H_3), 2.40 (3H, s, CH_3CO), 5.00 (1H, m, 3α-H), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 12.2, 12.2, 13.9, 19.0, 21.1, 21.2, 21.2, 21.3, 24.0, 25.4, 26.3, 28.8, 29.5, 31.8, 32.3, 37.3, 39.5, 40.4, 41.7, 42.5, 43.0, 44.3, 51.2, 56.0, 56.4, 70.8, 80.2, 129.6, 138.1, 170.5, 212.4. MS (EI): *m*/*z* 486 (M^+, 3), 426 (3), 408 (4), 365 (13), 269 (14), 55 (100).

*2.2.16. (22E)-5α-Hydroxy-3β-tosyloxystigmast-22-en-6-one (**14**)*

Compound **12** (300 mg, 0.50 mmol) was as described for compound **11**. Purification of crude product by flash column chromatography with hexane/ethyl acetate (4:1) as the eluent, yielded 94% of **14**. m.p.: 88–90°C. ^{1}H NMR ($CDCl_3$): 0.67 (3H, s, 18-H_3), 0.73 (3H, s, 19-H_3), 4.42 (1H, m, 3α-H), 1.44 (3H, s, CH_3-Ph), 5.10 (2H, m, 22-H and 23-H), 7.26 and 7.72 (4H, d, J = 9 Hz, CH_3-*Ph*). ^{13}C NMR ($CDCl_3$): 12.3, 12.3, 13.9, 19.1, 21.3, 21.4, 21.3, 21.5, 24.1, 25.5, 27.4, 28.9, 29.8, 32.0, 33.6, 37.4, 39.5, 40.5, 41.7, 42.4, 43.1, 44.2, 51.3, 56.4, 56.0, 79.9, 80.4, 127.5, 129.5, 129.6, 134.7, 138.0, 144.2, 212.2. MS (EI): *m*/*z* 426 (M^+-TsOH, 2), 408 (8), 392 (2), 43 (100).

*2.2.17. (22E)-3β-Acetoxy-5α-hydroxycholest-22-en-6-one (**15**)*

Compound **14** (750 mg, 1.14 mmol), dissolved in acetone (50 ml), was treated with lithium bromide (715 mg, 8.31 mmol) under reflux for 6 h. After solvent evaporation, the residue was dissolved in methylene chloride, the organic layer was washed with water, dried ($MgSO_4$), and evaporated. A mixture of 3β and 3α-bromides (2.3:1) was obtained. Isolation was performed by column chromatography (methylene chloride), yielding 54% of **15**. m.p.: 200–201°C. ^{1}H NMR ($CDCl_3$): 0.68 (3H, s, 18-H_3), 0.86 (3H, s, 19-H_3), 4.32 (1H, m, 3α-H), 5.10 (2H, m, 22-H and 23-H). ^{13}C NMR ($CDCl_3$): 12.3, 12.3, 14.1, 19.1, 21.1, 21.2, 21.3, 24.0, 25.4, 28.7, 32.0, 32.2, 32.9, 37.4, 38.8, 39.5, 40.4, 42.0, 42.3, 43.1, 44.7, 48.0, 51.3, 56.0, 56.5, 81.0, 129.6, 138.9, 211.7. MS (EI): *m*/*z* 508 (M^+, ^{81}Br, 2); 506 (M^+, ^{79}Br, 2), 427 (1), 43 (100). HRMS (EI): Calculated: 506.2759. Found: 506.2754.

*2.2.18. (22E)-5α-Hydroxystigmasta-2,22-dien-6-one (**16**)*

Compound **14** (500 mg, 0.83 mmol), dissolved in acetone (100 ml) was treated with potassium iodide (350 mg, 2.10 mmol) under reflux for 2 h. Then, the solvent was evaporated off, and the crude residue was dissolved in dimethylsulfoxide (aprox. 20 ml). Lithium carbonate (700 mg, 3.81 mmol) was added, and this new solution was stirred for 2 h at 120°C. The solvent was evaporated again, and the residue was extracted with ethyl ether/water. The organic phase was dried ($MgSO_4$) and purified by chromatography (hexane/ethyl acetate) to afford **16** in 74% yield. m.p.: 154–155°C. ^{1}H NMR ($CDCl_3$): 0.67 (3H, s, 18-H_3), 0.71 (3H, s, 19-H_3), 5.10 (2H, m, 22-H and 23-H), 5.65 (2H, m, 2-H and 3-H). ^{13}C NMR ($CDCl_3$): 12.4, 12.4, 14.6, 19.1, 21.1, 21.3, 21.3, 24.1, 25.5, 28.8, 32.0, 34.6, 34.6, 37.5, 39.5, 40.5, 42.4, 42.7, 42.9, 45.3, 51.3, 56.6, 56.9, 78.1, 122.4, 125.4, 129.5, 137.9, 211.2. MS (EI): *m*/*z* 426 (M^+, 10), 408 (3), 83 (23), 55 (100).

*2.2.19. (22R,23R)-3β-Acetoxy-5α,22,23-trihydroxy-stigmastan-6-one (**20**)*

Compound **13** (779 mg, 1.60 mmol) was treated in a similar way as described for compound **5**. Crude solid was purified from starting material and 22S, 23S diasteromer by column chromatography (methylene chloride/acetonitrile) to give compound **20** (12% yield). m.p.: 197–199°C. ^{1}H NMR ($CDCl_3$/CD_3OD 9:1): 0.71 (3H, s, 18-H_3), 0.77 (3H, s, 19-H_3), 2.40 (3H, s, CH_3CO), 4.68 (1H, m, 3α-H), 3.60 (1H, d, J = 8.1 Hz, 22-H), 3.75 (1H, d, J = 8.1 Hz, 23-H). ^{13}C NMR ($CDCl_3$/CD_3OD 9:1): 11.7, 11.7, 12.9, 21.2, 21.4, 23.7, 25.9, 26.7, 27.4, 36.2, 36.8, 38.0, 39.3, 40.9, 42.7, 46.4, 52.4, 53.6, 56.3, 56.4, 72.9, 170.5, 210.9. HRMS (FAB): Calculated for $C_{31}H_{53}O_6$ $[M + H]^+$: 521.3842. Found 521.3849.

*2.2.20. (22R,23R)-3β-Bromo-5α,22,23-trihydroxy-stigmastan-6-one (**21**)*

Compound **15** (816 mg, 1.60 mmol) (816 mg, 1.60 mmol) was treated in a similar way as described for compound **5**. Crude solid was purified from starting material and 22S, 23S diasteromer by column chromatography (methylene chloride/acetonitrile gradient) to give compound **21** (18.7% yield). m.p.: 205°C (d). ^{1}H NMR ($CDCl_3$/CD_3OD 9:1): 0.66 (3H, s, 18-H_3), 0.85 (3H, s, 19-H_3), 4.35 (1H, m, 3α-H), 3.57 (1H, d, J = 8.1 Hz, 22-H), 3.73 (1H, d, J = 8.1 Hz, 23-H). ^{13}C NMR ($CDCl_3$/CD_3OD 9:1): 11.6, 11.6, 13.4, 13.6, 21.0, 19.2, 18.7, 23.6, 20.9, 27.3, 28.8, 32.7, 31.9, 38.2, 37.3, 42.0, 39.4, 36.8, 44.1, 41.5, 42.7, 46.3, 48.4, 52.3, 56.0, 72.2, 74.2, 80.2, 213.4. HRMS (FAB): Calculated for $C_{31}H_{53}O_3Br$ $[M + H]^+$: 541.2892. Found 541.2891.

*2.2.21. (22R,23R)-2α,3α,5α,22,23-Pentahydroxy-stigmastan-6-one (**22**)*

Compound **16** (735 mg, 1.7 mmol) was treated in a similar way as described for **5**. After the usual work-up, compound **22** was obtained in 12% yield. All physical and spectral data for **22** were consistent with those previously reported [16].

2.3. Rice lamina inclination test

The activities of the compounds as plant growth promoters were evaluated by means of a highly sensitive, modified [16] rice lamina inclination test based on the procedure described by Wada et al. [17]. Seedlings of rice (Oryza sativa, Chuy variety) were washed with ethanol and water and then were left in water at 30°C for 2 days (with a 16 h photoperiod). Germinated seeds were cultivated on agar under the same growing conditions for 4 days. Intact seedlings (4–5 cm long) were inoculated with 0.5 μl of the test compound solution (1 mg/ml in ethanol) just under the 2nd leaf joint. After 48 h of growing in the dark (at 30°C), the magnitude of the induced angle between the leaf and the sheath was measured.

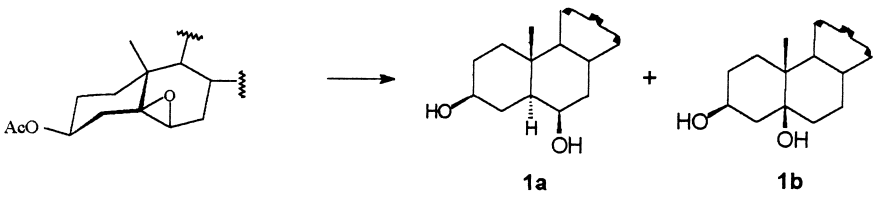


Fig. 2. Reductive opening of 5β,6β epoxide.

2.4. Antiviral assays

2.4.1. Cells and virus

Monkey kidney Vero cells were grown as monolayers in minimum essential medium (MEM) supplemented with 6% inactivated calf serum and 50 μg/ml gentamycin. Maintenance medium (MM) consisted of MEM containing 2% inactivated serum. The virus used was herpes simplex virus type 1 (HSV-1) F strain (American Type Culture Collection; Rockville, MD, USA).

2.4.2. Cytotoxicity assay

Cytotoxicity was determinated by the MTT colorimetric assay [18]: cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) was used to assay growth and cell survival.

Vero cells grown in 96-well culture plates for 24 h were treated with serial dilutions of the test brassinosteroid for 24, 48, or 72 h. After this time, cell monolayers were washed with Hanks solution, and 50 μl of MTT (0.1 μg/ml) were added to each well. After 2 h of incubation at 37°C, 4% CO_2 and 100% relative humidity, supernatants were removed and 200 μl of 96% ethanol were added to solubilize the formazan. Optical density of each well was measured on an Eurogenetics MPR-A 4i microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Results were expressed as the ratio between the optical density in treated cultures and the optical density in the untreated control cultures. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that caused a 50% reduction in optical density.

2.4.3. Antiviral assay: virus yield inhibition method

The virus used was herpes simplex virus type 1 (HSV-1) F strain. Vero cells were grown at 37°C with Eagle Minimum Essential Medium (MEM) containing 5% of bovine serum. Maintenance medium (MM) consisted of MEM with 1.5% of bovine serum. Compound solutions were prepared in ethanol at a concentration of 1 mg/ml and diluted with MM to a final concentration of 20 μg/ml.

Antiviral activity was evaluated by plaque reduction method: Vero cell monolayers grown in 24-well plates were infected with about 100 PFU of virus/well. After 1 h of adsorption at 4°C residual inoculum was replaced by MM containing 0.7% of methylcellulose and the different concentrations of the brassinosteroid. After 48 h of incubation at 37°C, virus plaques were counted. The 50% antiviral effective concentration (IC_{50}) was calculated as the concentration required to reduce virus plaques by 50%. All determinations were performed in duplicate.

Selectivity indexes were calculated as the rate between CC_{50} and IC_{50}.

3. Results and discussion

Synthesis of compounds **17**–**19** with a 5αH moiety required (22E)-3β-acetoxy-5β,6β-epoxystigmast-22-ene as the starting compound (see Scheme 1), that was obtained by regio and stereoselective epoxidation of stigmasteryl acetate [19]. The reductive opening of the 5β,6β-epoxide to achieve a 6β-hydroxy derivative required the use of alane [20] because other regular reductive reagents produced large amounts of the 5β-hydroxy derivative. This alane reagent ensured a predominant *trans* diaxial attack of the hydride on the more hindered C-5 position [21] of the epoxide (see Fig. 2), leading to (22E)-3β,6β-dihydroxy-5α-stigmastan-22-ene (**1a**) and to (22E)-3β,5β-dihydroxy-5β-stigmastan-22-ene (**1b**) in a 3:1 ratio [22].

Lack of selectivity on direct oxidation [23] of compound **1a** required silyl ether protection for the hydroxyl group at C-3. Compound **2** was then oxidized with pyridine chlorochromate (PCC) to give compound **3**. Deprotection of **3** with *t*-butylammonium fluoride led to (22E)-3β-hydroxy-5α-stigmastan-6-one (**4**), which was acetylated to yield compound **5**. Sharpless' catalytic, asymmetric dihydroxylation (CAD) [24] of compound **5** furnished target compound **17** (22R,23R)-3β-acetoxy-22,23-dihydroxy-5α-stigmastan-6-one. CAD also yielded not natural occurring diastereomeric 22S,23S compounds, in minor proportion (rate 3:1).

Target compounds **18** and **19** were obtained through a bifurcation of this synthetic route. Mesylation of **4** led to compound **6**, which depending on the temperature of reflux conditions, yielded either **7** or **8** when treated with lithium bromide in methanol or dimethylformamide, respectively. CAD performed on compound **7** yielded (22R,23R)-3β-bromo-22,23-dihydroxy-5α-stigmastan-6-one (**18**), and CAD performed on compound **8** yielded (24S)-homoethylcastasterone (**19**).

Brassinosteroid analogs bearing a 5α-hydroxyl group (**10**-

22) were obtained by the synthetic pathway diagrammed in Scheme 2. Compounds **11** and **12** were obtained as single products by hydrolytic opening of the mixture of the 5α,6α and 5β,6β epoxides of (22E)-5,6-epoxystigmast-22-en-3β-yl acetate (**9**) or tosylate (**10**), respectively. Oxidation of **11** and **12** with PCC yielded **13** and **14**, respectively. Compound **13** was dihydroxylated (CAD) to yield (22R,23R)-3β-acetoxy-5α,22,23-trihydroxystigmastan-6-one (**20**).

Compound **14** was treated with lithium bromide in acetone to give the 3β-bromo derivative (**15**), which was dihydroxylated to yield (22R,23R)-3β-bromo-5α,22,23-trihydroxy-stigmastan-6-one (**21**). Compound **14** was also subjected to elimination to give (22E)-5α-hydroxystigmasta-2,22-dien-6-one (**16**) which, in turn, was tetrahydroxylated to obtain (22R,23R)-2α,3α,5α,22,23-pentahydroxystigmastan-6-one (**22**).

Bioactivities of compounds **17–22** were evaluated by the modified rice lamina inclination bioassay [16,17] for testing auxin-like properties. Chuy, a local variety of rice, was used. Average angles and percentage of bioactivity are shown in the Table. Compound **19** showed a larger angle value and was used as the standard. Compound **22**, in agreement with previous reports [16], elicited high activity. However, the introduction of a 5α hydroxy group did not always affect the bioactivity in the same way and depended on the C-3 functionality (see auxin-like bioactivity in the Table for the pairs **17** and **20**, **18** and **21**, and **19** and **22**). Further studies should be done to investigate possible practical agricultural applications of these synthetic compounds. Other brassinosteroid analogs have proved to be quite suitable at field trials although exhibited extremely low activity at the bioassay level [3].

It is known that oxysterols can interact with cell membranes modifying their fluidity and consequently modulating cellular function [25–27]. Some oxygenated derivatives of cholesterol have been tested in vitro on the replication of HIV virus [9] yielding inhibition with modest but reproducible selectivity indexes.

It has been also reported that sulfated polyhydroxysterols isolated from marine organisms have shown antiviral effects on herpes simplex virus [10,28].

These results prompted us to test antiviral activity of compounds **17–22**. Tests were performed in vitro in Vero cells infected with herpes simplex virus (HSV-1, F strain). In the table are shown the effective concentration required to reduce by 50% virus plaques (IC_{50}), the cytotoxic concentration (CC_{50}), that was determined as the concentration of each compound required to reduce cell viability by 50% of the standard, and the selectivity index, calculated as the ratio between CC_{50} and IC_{50}. As shown in Table 1, all compounds proved to be active against herpes simplex virus. Values indicate that compounds **21** and **22** were cytotoxic at the concentrations that elicited antiviral effects and that compounds **17–20** showed antiviral bioactivity with modest selectivity indexes, similar to that of stigmasterol. Although further studies should be done to evaluate mechanisms of action, recent studies have demonstrated that these compounds are not virucides, but interesting antivirals [29].

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