

In vitro and in vivo antiherpetic activity of three new synthetic brassinosteroid analogues

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Received 25 November 2003; received in revised form 26 April 2004; accepted 29 April 2004
Available online 1 October 2004

Abstract

Brassinosteroids are a novel group of steroids that appear to be ubiquitous in plants and are essential for normal plant growth and development. It has been previously reported that brassinosteroid analogues exert an antiviral activity against herpes simplex virus type 1 (HSV-1) and arenaviruses. In the present study, we report the chemical synthesis of compounds (2*S*,23*S*)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**2**), (2*S*,23*S*)-5α-fluoro-3β-22,23-trihydroxystigmastan-6-one (**3**), (2*S*,23*S*)-3β,5α,22,23-tetrahydroxy-stigmastan-6-one (**4**) as well as their antiherpetic activity both in a human conjunctive cell line (IOBA-NHC) and in the murine herpetic stromal keratitis (HSK) experimental model. All compounds prevented HSV-1 multiplication in NHC cells in a dose dependent manner when added after infection with no cytotoxicity. Administration of compounds **2**, **3**, and **4** to the eyes of mice at 1, 2, and 3 days post-infection delayed and reduced the incidence of HSK, consisting mainly of inflammation, vascularization, and necrosis, compared to untreated, infected mice. However, viral titers of eye washes showed no differences among samples from treated and untreated mice. Since the decrease in the percentage of mice with ocular lesions occurred 5 days after treatment had ended, we suggest that brassinosteroids **2**, **3**, and **4** did not exert a direct antiviral effect in vivo, but rather may play a role in immune-mediated stromal inflammation, which would explain the improvement of the clinical signs of HSK observed.
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Keywords: Brassinosteroids; HSV-1; Antiviral activity; Human conjunctive cell line; Murine herpetic stromal keratitis; Steroid

1. Introduction

Brassinosteroids (BRs) are recognized as a new class of plant steroidal hormones that control many of the same developmental and physiological processes as their insect and mammalian counterparts, including regulation of gene expression, cell division, differentiation, and homeostasis [1–4].

We recently reported that some natural BRs and synthetic analogues have in vitro antiviral activity against several pathogen viruses [5,6]. Among them, natural compounds (2*R*,23*R*)-2α,3α,22,23-tetrahydroxy-5α-stigmastan-6-one (28-homocastasterone) (**1a**, Fig. 1) and (2*R*,23*R*)-2α,3α,22,23-tetrahydroxy-B-homo-7-oxa-stigmastan-6-one (28-homobrassinolide) (**1b**, Fig. 1) showed selectivity indices (SI) of 2 and 28, respectively, against herpes simplex virus type 1 (HSV-1) multiplication in Vero cells. However, synthetic analogues with 2*S*,23*S* configuration **2**, **3**, and **4** (Fig. 1) showed higher SI (100, 109, and 80, respectively) [7].

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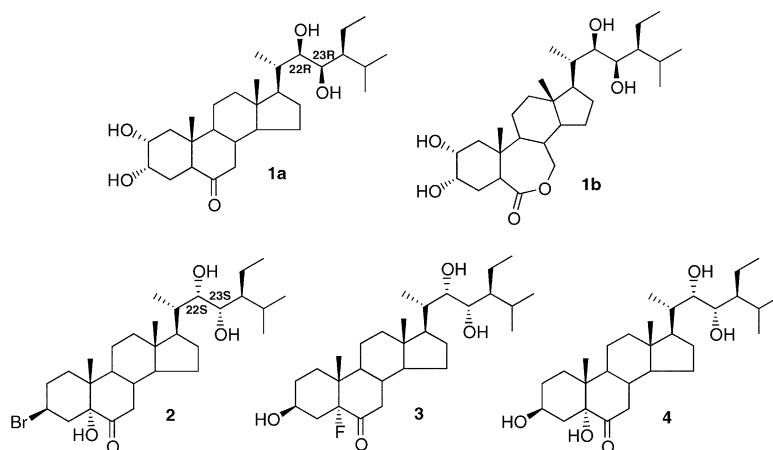


Fig. 1.

HSV-1 induces an ocular disease in humans named herpetic stromal keratitis (HSK), which is a significant cause of ocular morbidity around the world leading to vision impairment and blindness [8,9]. Studies of animal models have been very useful for the characterization of the evolution of HSK. Within one to two weeks, HSV-1 corneal infection in mice initiates a series of events that culminate in a stromal necrotizing keratitis, histologically similar to that of HSK [10]. Furthermore, it resembles many characteristics of the recrudescence disease in humans, both in the duration of virus shedding and with respect to the effect of antiviral drugs [11].

In this paper, we present the chemical synthesis of compounds **2–4** and investigate their antiherpetic activity both in a human conjunctive cell line and in the murine HSK experimental model.

2. Experimental

2.1. Synthesis of compounds

2.1.1. General

All compounds were recrystallized until constant melting point (mp). Melting point were determined on a Fisher Johns apparatus and are uncorrected. ^1H and ^{13}C spectra were recorded on a Bruker AM-500 spectrometer at 500 and 125 MHz, respectively. Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. ^{19}F NMR spectra were recorded on a Bruker AM-500 at 470.4 MHz, and chemical shifts (δ) are given in ppm upfield from CFCl_3 as the internal standard. Coupling constant (J) values are in Hz. Unless otherwise indicated, all solvents and reagents used were of commercial grade. FAB spectra (low and high resolution) were recorded on a VG ZAB-T instrument (VG-Micromass) using 3-NBA as matrix. Low resolution mass spectra (EI) were recorded on a Shimadzu QP-5000 at 70 eV. Reactions were monitored by TLC on plates precoated with silicagel F_{254} 0.2 mm (Merck). Column chromatography was carried out on silica gel 60, 0.04–0.063 mm (Merck).

2.1.2. (22E)-3 β -Bromo-5 β ,6 β -epoxystigmast-22-ene (**6**)

A mixture of potassium permanganate (2 g, 12.65 mmol) and ferric nitrate nonahydrate (1 g, 2.47 mmol) was ground to a fine powder, and water (0.1 ml) was added. To a stirred suspension of this mixture in methylene chloride (20 ml), (22E)-3 β -bromostigmasta-5,22-diene [12] (**5**, 1.94 g, 4.08 mmol) and *t*-butyl alcohol (2 ml) were added. After 2 h at room temperature, the reaction was completed, and the product was separated from the inorganic residue by adding ether (50 ml), stirring for 5 min, and filtering through Celite. After evaporation of the solvent, the crude product was purified by column chromatography (CH_2Cl_2 /ethyl acetate 1:1) yielding **6** (73%). mp (acetone): 93 °C.

^1H NMR (CDCl_3): 0.67 (18- H_3 , 3H, s), 0.88 (27- H_3 , 3H, d, J = 6 Hz), 0.95 (26- H_3 , 3H, d, J = 6 Hz), 0.96 (29- H_3 , 3H, d, J = 7 Hz), 1.00 (19- H_3 , 3H, s), 1.02 (21- H_3 , 3H, d, J = 7 Hz), 3.10 (6 α -H, 1H, d, J = 2 Hz), 4.66 (3 α -H, 1H, m), 5.02 (23-H, 1H, dd, J = 15 Hz, 8 Hz), 5.15 (22-H, 1H, dd, J = 15 Hz, 8 Hz).

^{13}C NMR (CDCl_3): 12.1 (C18), 12.1 (C29), 16.9 (C19), 19.1 (C26), 20.9 (C11), 21.2 (C27), 22.0 (C21), 24.3 (C15), 25.3 (C28), 29.7 (C16), 30.0 (C8), 31.9 (C25), 32.8 (C7), 33.6 (C2), 35.4 (C10), 39.9 (C1), 40.1 (C12), 40.3 (C20), 42.5 (C13), 44.3 (C4), 51.3 (C9), 51.8 (C24), 53.4 (C3), 56.4 (C14), 56.5 (C17), 63.4 (C6), 68.2 (C5), 129.7 (C23), 137.9 (C22).

MS (EI) m/z (%): 492 ($[\text{M}]^+$, ^{81}Br , 4); 490 ($[\text{M}]^+$, ^{79}Br , 4); 411 (4); 269 (9); 55 (100).

HRMS (EI): Calculated for $\text{C}_{29}\text{H}_{47}\text{BrO}$: 490.2810; found: 490.2808.

2.1.3. (22E)-3 β -Bromo-5-hydroxy-5 α -stigmast-22-en-6-one (**7**)

Jones reagent (0.5 ml) was added dropwise to a stirred solution of compound **6** (359 mg, 0.73 mmol) in acetone (25 ml). After 1 h, isopropyl alcohol was added to stop the reaction. The mixture was neutralized with solid sodium bicarbonate, filtered, and the solvent was evaporated under reduced pressure. The crude residue was purified by column

chromatography (hexane/ethyl acetate 9:1) to afford compound **7** [13] in 83% yield. mp (methanol): 200 °C.

¹H NMR (CDCl₃): 0.68 (18-H₃, 3H, s), 0.86 (19-H₃, 3H, s), 0.88 (27-H₃, 3H, d, *J* = 6 Hz), 0.95 (26-H₃, 3H, d, *J* = 6 Hz), 0.96 (29-H₃, 3H, d, *J* = 7 Hz), 1.02 (21-H₃, 3H, d, *J* = 7 Hz), 4.32 (3α-H, 1H, m), 5.02 (23-H, 1H, dd, *J* = 15 Hz, 8 Hz), 5.15 (22-H, 1H, dd, *J* = 15 Hz, 8 Hz).

¹³C NMR (CDCl₃): 12.3 and 12.3 (C18 and C29), 14.1 (C19), 19.1 (C26), 21.1 (C11), 21.2 and 21.3 (C21 and C27), 24.0 (C15), 25.4 (C28), 28.7 (C16) 32.0 (C25), 32.2, 32.9 (C1 and C2), 37.4 (C8), 38.8 (C12), 39.5 (C4), 40.4 (C20), 42.0 (C7), 42.3 and 43.1 (C10 and C13), 44.7 (C9), 48.0 (C3), 51.3 (C24), 56.0 and 56.5 (C14 and C17), 81.0 (C5), 129.6 (C23), 138.9 (C22), 211.1 (C6).

MS (EI) *m/z* (%): 508 ([M]⁺, ⁸¹Br, 2); 506 ([M]⁺, ⁷⁹Br, 2); 427 (1); 43 (100).

HRMS (EI): Calculated for C₂₉H₄₇BrO₂: 506.2759; found: 506.2754.

2.1.4. (22*S*,23*S*)-3β-Bromo-5,22,23-trihydroxy-5α-stigmastan-6-one (**2**)

A mixture of **7** (1.63 g, 3.20 mmol), THF (20 ml), *t*-butanol/water (1:1) (70 ml), pyridine (25 μl), potassium ferriocyanide (3.2 g, 9.7 mmol), potassium carbonate (1.34 g, 10.5 mmol), and potassium osmate dihydrate (50 mg, 0.13 mmol) was stirred at room temperature for 9 days [14]. An excess of solid sodium bisulfite was added until no evolution of bubbles was observed. Layers were separated, and the aqueous phase was thoroughly extracted with methylene chloride/methanol (5%). The combined organic layers were dried and evaporated, and the crude solid was purified by column chromatography (hexane/ethyl acetate gradient) to give compound **2** (89% yield). mp (methanol/water): 132 °C.

¹H NMR (CDCl₃/CD₃OD 9:1): 0.68 (18-H₃, 3H, s), 0.84 (19-H₃, 3H, s), 0.88 (27-H₃, 3H, d, *J* = 6.7 Hz), 0.95 (26-H₃, 3H, d, *J* = 6.7 Hz), 0.96 (29-H₃, 3H, d, *J* = 6.8 Hz), 1.02 (21-H₃, 3H, d, *J* = 6.8 Hz), 2.71 (7α-H, 1H, t, *J* = 12.6 Hz), 3.60 (22-H and 23-H, 2H, m), 3.34 (3α-H, 1H, m).

¹³C NMR (CDCl₃/CD₃OD 9:1): 11.3 (C18), 13.6 (C19), 13.6 (C21), 14.1 (C29), 17.3 (C26), 18.3 (C28), 21.0 (C27), 21.6 (C11), 23.9 (C15), 26.6 (C25), 27.7 (C16), 31.9 (C2), 32.7 (C1), 37.2 (C8), 38.2 (C4), 39.3 (C12), 41.5 (C7), 42.0 (C10), 42.0 (C20), 43.4 (C13), 44.1 (C9), 48.4 (C24), 49.4 (C3), 52.3 (C17), 55.8 (C14), 70.0 (C23), 71.7 (C22), 80.1 (C5), 213.3 (C6).

MS (FAB) *m/z* (%): 541 ([M + H]⁺, 4); 523 (9); 505 (2); 154 (100).

HRMS (FAB): Calculated for C₂₉H₅₀BrO₄ [M + H]⁺: 541.2892; found: 541.2885.

2.1.5. (22*S*,23*S*)-5-Fluoro-3β,22,23-trihydroxy-5α-stigmastan-6-one (**3**)

Compound **10** [15] (198 mg, 0.44 mmol) was dihydroxylated in a similar way as described for compound **7**. Purification by column chromatography (methylene chlo-

ride/ethyl acetate gradient) afforded **3** in 83% yield. mp (methanol/water): 137 °C.

¹H NMR (CDCl₃/CD₃OD 9:1): 0.68 (18-H₃, 3H, s), 0.88 (27-H₃, 3H, d, *J* = 6.7 Hz), 0.90 (19-H₃, 3H, s), 0.95 (26-H₃, 3H, d, *J* = 6.7 Hz), 0.96 (29-H₃, 3H, d, *J* = 6.8 Hz), 1.02 (21-H₃, 3H, d, *J* = 6.8 Hz), 2.58 (7α-H, 1H, dd, *J* = 12.5 Hz, 12.5 Hz), 3.62 (22-H and 23-H, 2H, m), 3.88 (3α-H, 1H, m).

¹³C NMR (CDCl₃/CD₃OD 9:1): 12.2 (C18), 13.7 (C21), 13.8 (C19, d, *J*_{CF} = 6.2 Hz), 14.1 (C29), 17.3 (C26), 18.3 (C28), 21.1 (C27), 21.2 (C11), 24.0 (C15), 26.6 (C25), 28.6 (C16), 30.0 (C1), 30.8 (C2), 33.9 (C4, d, *J*_{CF} = 22.2 Hz), 37.8 (C8), 39.4 (C12), 42.1 (C20), 42.7 (C7), 42.9 (C13), 43.2 (C10, d, *J*_{CF} = 14.7 Hz), 45.6 (C9), 48.4 (C24), 56.0 (C14), 56.5 (C17), 66.9 (C3), 70.1 (C23), 71.7 (C22), 101.3 (C5, d, *J*_{CF} = 176.9 Hz), 207.2 (C6, d, *J*_{CF} = 27.2 Hz).

¹⁹F NMR (CDCl₃/CD₃OD 9:1): -161.1 (d, *J*_{FH} = 45.0 Hz).

MS (FAB) *m/z* (%): 481 ([M + H]⁺, 19); 463 (20); 305 (21); 154 (100).

HRMS (FAB): Calculated for C₂₉H₅₀FO₄ [M + H]⁺: 481.3694; found: 481.3693.

2.1.6. (22*S*,23*S*)-3β,5,22,23-Tetrahydroxy-5α-stigmastan-6-one (**4**)

Compound **12** [15] (150 mg, 0.34 mmol) was dihydroxylated in a similar way as described for compound **7**. Purification by column chromatography (methylene chloride/ethyl acetate gradient) afforded **4** in 86% yield. mp (methanol/water): 168 °C.

¹H NMR (CDCl₃/CD₃OD 9:1): 0.67 (18-H₃, 3H, s), 0.82 (19-H₃, 3H, s), 0.88 (27-H₃, 3H, d, *J* = 6.7 Hz), 0.95 (26-H₃, 3H, d, *J* = 6.7 Hz), 0.96 (29-H₃, 3H, d, *J* = 6.8 Hz), 1.02 (21-H₃, 3H, d, *J* = 6.8 Hz), 2.58 (7α-H, 1H, dd, *J* = 12.6 Hz, 12.5 Hz), 3.60 (22-H and 23-H, 2H, m), 3.95 (3α-H, 1H, m).

¹³C NMR (CDCl₃/CD₃OD 9:1): 11.8 (C18), 13.6 (C19), 13.6 (C21), 14.0 (C29), 17.3 (C26), 18.3 (C28), 20.7 (C11), 21.0 (C27), 23.6 (C15), 26.6 (C25), 28.3 (C16), 29.5 (C2), 30.1 (C1), 35.7 (C4), 37.0 (C8), 39.1 (C12), 41.6 (C7), 42.0 (C13), 42.0 (C20), 2.6 (C10), 43.9 (C9), 48.4 (C24), 55.5 (C17), 55.8 (C14), 66.1 (C3), 70.0 (C23), 71.6 (C22), 79.7 (C5), 212.8 (C6).

MS (FAB) *m/z* (%): 479 ([M + H]⁺, 40); 461 (100); 447 (17).

HRMS (FAB): Calculated for C₂₉H₅₁O₅ [M + H]⁺: 479.3737; found: 479.3720.

2.2. Antiviral assays

2.2.1. Cells, viruses, and treatment solutions

Vero and IOBA-NHC cells [16] were grown in Eagle's minimal essential medium supplemented with 5% inactivated calf serum (MEM 5%) and 50 μg/ml gentamycin, and maintained in monolayer formation in MEM supplemented with 1.5% inactivated calf serum (MEM 1.5%).

HSV-1 Cgal⁺ (HSV-1 Cgal) KOS strain was propagated at low multiplicity, plaque-assayed on Vero cell monolayers,

and used for in vitro assays. This system contains the *Escherichia coli* lacZ reporter gene encoding β -galactosidase, which is under the control of a strong heterologous promoter from the human cytomegalovirus immediate-early (IE) regulatory region [17], and was kindly provided by Dr. Alberto Epstein (Université Claude Bernard, Lyon, France).

The HSV-1 KOS strain (wt) was also propagated at low multiplicity and was used for in vivo experiments. Compounds **2-4** were dissolved in ethanol and diluted with PBS or culture medium for testing. The maximum concentration of ethanol tested (1%) exhibited no toxicity under the in vitro or in vivo conditions.

2.2.2. Cytotoxicity assay

Cell viability in the presence of the compounds was determined using the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) [18]. The absorbance 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 a percentage of absorbance of treated cell cultures with respect to untreated ones. The CC₅₀ was defined as the concentration of compound that caused a 50% reduction in absorbance.

2.2.3. Virus titration

Vero cells grown in 24-well plates were infected with serial 10-fold dilutions of viral yields and incubated for 1 h at 37 °C. Residual inocula were replaced by maintenance medium with 0.7% of methylcellulose. After 72 h of incubation at 37 °C,

cells were fixed with formaldehyde 10%, stained with Crystal Violet, and plaque forming units were counted.

2.2.4. Measurement of β -gal activity

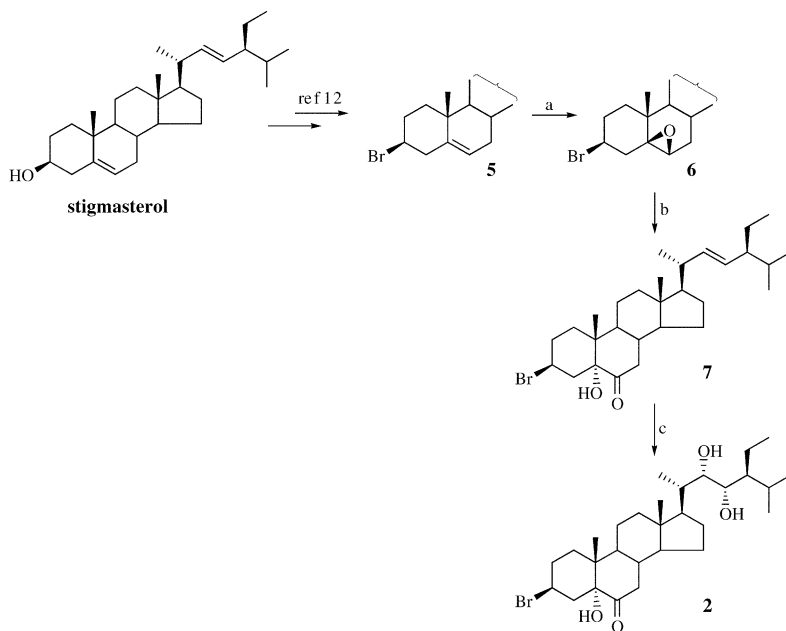
IOBA-NHC cell monolayers grown in coverslips inside 24-well plates were infected with HSV-1 Cgal (m.o.i. = 0.2). After incubation for 1 h at 37 °C, inocula were eliminated, and cells were covered with MEM 1.5%. At 24 h p.i., supernatants were harvested and stored at -70 °C for titration, and cells were stained in situ for β -gal. A solution containing 1% formaldehyde and 0.2% glutaraldehyde in PBS (200 μ l/well) was added for 30 min at 4 °C. Subsequently, supernatants were removed, and cells were washed twice with PBS. A β -gal activity staining solution (50 mM potassium ferrocyanide, 50 mM potassium ferricyanide, 20 mM MgCl₂, and 1 mg/ml X-gal) was added to the cells for 3 h at 37 °C. Coverslips were mounted, and 'blue' cells were observed with an Olympus BX61 microscope, with a slider slot for Normarski DIC, and photographed.

2.2.5. Animals

Five to seven-week old male Balb/c mice were purchased from the I.N.T.A. (Castelar, Buenos Aires). All mice were handled according to the Animal Care Guidelines from the National Institute of Health (USA) and the Association for Research in Vision and Ophthalmology (ARVO, USA) resolution on the use of animals in research.

2.2.6. Corneal inoculation of HSV-1

Mice were anaesthetized with 2 mg intraperitoneal ketamine (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (Rompun, Mobay, Shantee, KA). The right cornea of



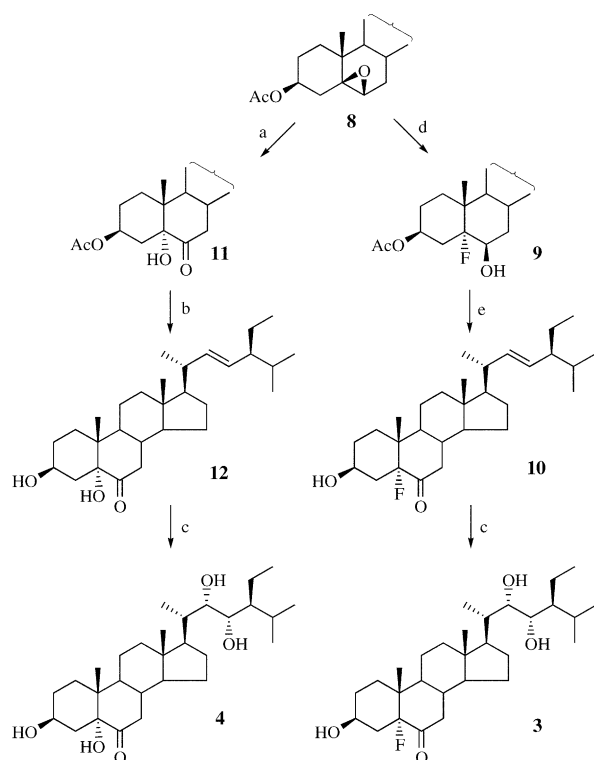
Scheme 1. Synthesis of compound **2**. Reagents and conditions: (a) KMnO₄/Fe(NO₃)₃·9H₂O/t-BuOH/H₂O/CH₂Cl₂, 2 h, r.t. (b) Jones' reagent/acetone, 1 h, r.t. (c) K₂OsO₄/K₄Fe(CN)₆/K₂CO₃/pyridine/t-BuOH/H₂O/THF, 9 days, r.t.

each mouse was scratched eight times in a criss-cross pattern with a 27 gauge needle, and 5 μ l of an HSV-1 suspension containing 2.5×10^4 PFU of virus were instilled in the cul-de-sac. Brassinosteroids were topically administered three times a day at 1, 2, and 3 days post-infection (p.i.) (volume = 5 μ l). The control mice received PBS.

The severity of HSK was determined under a binocular microscope at 8 and 10 days p.i. The diagnosis of disease was based on the density of the inflammatory infiltrates, neovascularization, and the presence of superficial or deep ulcers. Thus, mice exhibiting puffy eyelids with moderate to severe crusting (blepharitis), vessel enlargement with corneal invasion (10% or more) (neovascularization), and slight to severe corneal turbidity/opacity, with or without signs of corneal necrosis or perforation, were considered as ill (stromal keratitis).

3. Results and discussion

Synthesis of compound **2** is summarized in Scheme 1. Compound **5** ((22*E*)-3 β -bromostigmasta-5,22-diene) was obtained in three steps from stigmasterol [12]. Regio and stereoselective epoxidation of compound **5** with $\text{KMnO}_4/\text{Fe}(\text{NO}_3)_3$ [19] yielded 73% of the corresponding 5 β ,6 β epoxide **6**. Hydrolytic opening and further oxidation



Scheme 2. Synthesis of compounds **3** and **4**. Reagents and conditions: (a) Jones' reagent/acetone, 30 min, r.t. (b) $\text{K}_2\text{CO}_3/\text{MeOH}$, 4 h, r.t. (c) $\text{K}_2\text{OsO}_4/\text{K}_4\text{Fe}(\text{CN})_6/\text{K}_2\text{CO}_3/\text{pyridine}/t\text{-BuOH}/\text{H}_2\text{O}/\text{THF}$, 9 days, r.t. (d) $\text{HF}\cdot\text{Py}/\text{CH}_2\text{Cl}_2$, 10 min, 0 $^\circ\text{C}$. (e) $\text{PCC}/\text{HPy}^+\text{CF}_3\text{COO}^-$, 2 h, r.t.

of the oxirane ring of **6** with Jones' reagent gave (22*E*)-3 β -bromo-5-hydroxy-5 α -stigmast-22-en-6-one (**7**) [13]. The side chain double bond of **7** was dihydroxylated with catalytic osmium (VIII) using ferric ferricyanide as cooxidant [14]. No chiral ligand was necessary due to the presence of the C-24 ethyl group, which induced the desired stereoselectivity, yielding 89% of (22*S*,23*S*)-3 β -bromo-5,22,23-trihydroxy-5 α -stigmastan-6-one (**2**).

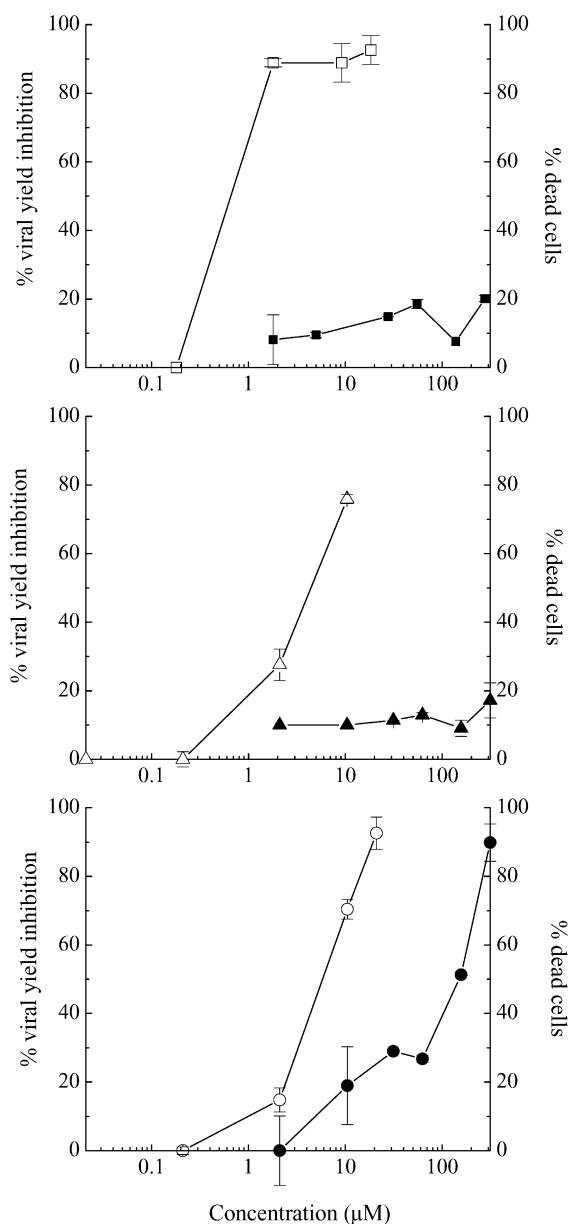


Fig. 2. Cytotoxicity and antiviral activity of compounds **2**, **3**, and **4** in vitro. IOBA-NHC cells grown in 96-well plates were treated with 1–314 μM of **2** (\blacksquare), **3** (\blacktriangle), and **4** (\bullet) for cytotoxicity assays (right axis). Following 24 h of incubation at 37 $^\circ\text{C}$, a MTT colorimetric assay was performed. IOBA-NHC cells grown in 24-well plates were infected with HSV-1 Cgal (m.o.i. = 0.2) and mock-treated or treated with 0.1–20 μM of **2** (\square), **3** (\triangle), and **4** (\circ) (left axis). After 24 h of incubation at 37 $^\circ\text{C}$, free and cell-associated virus was collected and titrated in Vero cells.

The synthesis of compounds **3** and **4** is shown in Scheme 2. Stigmasteryl acetate was converted into its 5 β ,6 β epoxide **8** as previously described [19]. Nucleophilic opening of this epoxide with a hydrofluoric acid–pyridine complex (Olah's reagent) afforded compound **9**; the proper stereochemistry at C-5 was achieved by trans-diaxial acid catalyzed opening of the oxirane ring. Subsequent oxidation at C-6 and hydrolysis of the acetate in C-3 yielded (22*E*)-5-fluoro-3 β -hydroxy-5 α -stigmast-22-en-6-one (**10**) [15]. Dihydroxylation of the Δ^{22} double bond with osmium (VIII) allowed for the synthesis of (22*S*,23*S*)-5-fluoro-3 β ,22,23-trihydroxy-5 α -stigmastan-6-one (**3**) from stigmasterol with a 45% yield. Alternatively, epoxide **8** was treated with Jones' reagent to afford the 5 α -hydroxy-6-keto compound **11** [15]. Hydrolysis of **11** yielded compound **12**. Dihydroxylation of the side chain of **12** gave (22*S*,23*S*)-3 β ,5,22,23-tetrahydroxy-5 α -stigmastan-6-one (**4**).

Anti-HSV-1 activity of brassinosteroid analogues **2**, **3**, and **4** were tested in human conjunctival cells. First, the 50% cytotoxic concentration (CC₅₀) for IOBA-NHC cells was determined. Compounds **2**, **3**, and **4** were added to conflu-

ent non-growing cells in concentrations ranging from 1 to 314 μ M, and after 24 h of incubation at 37 °C, a MTT colorimetric assay was performed. As shown in Fig. 2, brassinosteroids **2**, **3**, and **4** exhibited CC₅₀ values of >277, >314, and 146 μ M, respectively, showing cytotoxicity levels for human conjunctival cells even lower than those corresponding to Vero cells, except for compound **4**, as previously reported [7].

To evaluate the antiviral activity of these compounds, IOBA-NHC monolayers grown in 24-well plates were infected with HSV-1 Cgal (m.o.i. = 0.2) and then, treated with different concentrations of **2**, **3**, and **4** (from 0.1 to 20 μ M) or control media. After 24 h of incubation at 37 °C, free and cell-associated virus were collected and titrated in Vero cells. A dose-dependent inhibition of viral replication was observed in drug-treated cultures, regardless of the compound assayed (Fig. 2). We established that all compounds prevented HSV-1 Cgal multiplication in human conjunctival cells when added after infection, exhibiting 50% inhibition of virus yield at concentrations of 1, 4, and 5 μ M for **2**, **3**, and **4** (Fig. 2), respectively, with SI of >277, >78.5, and 29.2, respectively.

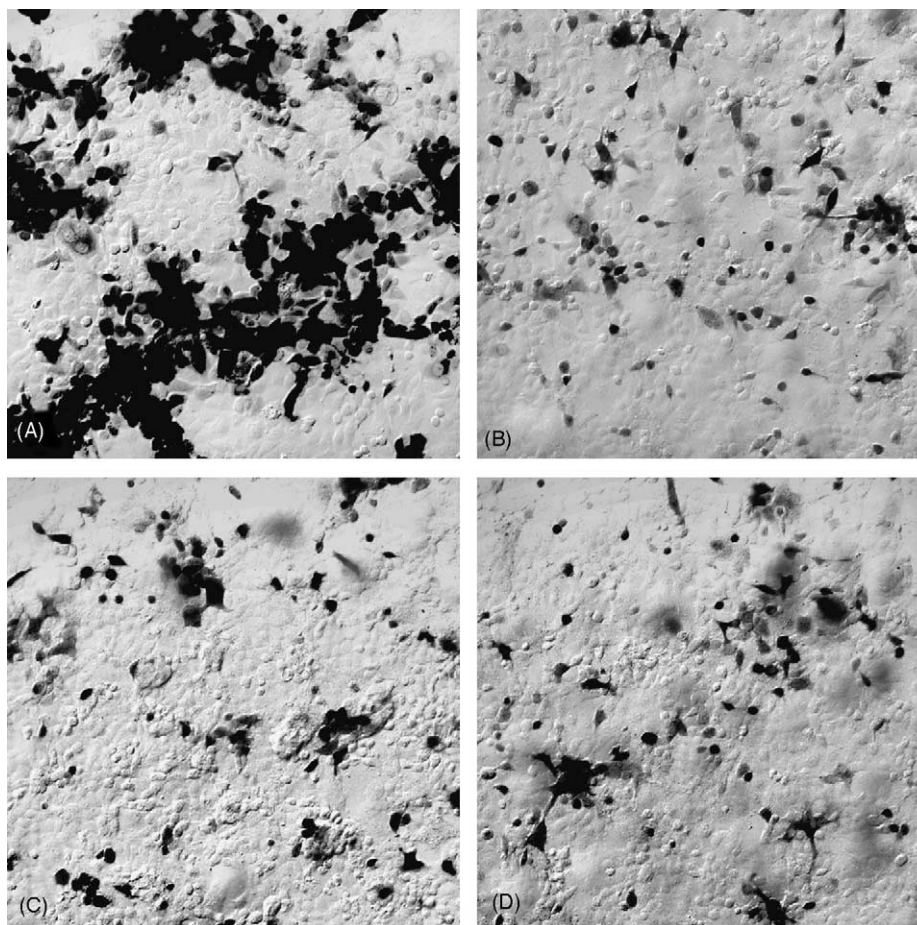


Fig. 3. Effect of brassinosteroids **2**, **3**, and **4** on HSV-1 propagation. IOBA-NHC cell monolayers grown in coverslips inside 24-well plates were infected with HSV-1 Cgal (m.o.i. = 0.2) and incubated for 1 h at 37 °C. Then, cells were covered with MEM 1.5% or media containing 20 μ M of compounds **2**, **3**, and **4** per well. After incubation for 24 h at 37 °C, cells were stained in situ for β -gal. (A) HSV-1 infected -untreated cells; (B) HSV-1 infected **2**-treated cells; (C) HSV-1 infected **3**-treated cells; and (D) HSV-1 infected **4**-treated cells. Magnification: 40 \times .

Only brassinosteroid **2** exhibited a higher SI than that obtained in Vero cells [7].

Considering that brassinosteroids inhibited HSV-1 Cgal multiplication in human conjunctival cells, we investigated whether viral propagation was restrained by these compounds. For this purpose, IOBA-NHC cell monolayers grown in coverslips inside 24-well plates were infected with HSV-1 Cgal (m.o.i. = 0.2) and incubated for 1 h at 37 °C. Then, inocula were eliminated, and cells were covered with MEM alone or MEM 1.5% containing 20 µM of each compound per well. After incubation for 24 h at 37 °C, supernatants were collected and titrated on Vero cells, and human conjunctival cells were stained in situ for β-gal. In the absence of brassinosteroids, ‘blue’ cells clustered in characteristic HSV-1 foci were observed (Fig. 3A), whereas cell cultures treated with any of the three compounds exhibited only scattered ‘blue’ cells (Fig. 3B–D). Therefore, brassinosteroids impeded HSV-1 Cgal propagation in IOBA-NHC cells. Likewise, the inhibition of HSV-1 Cgal multiplication in supernatants from cells treated with a 20 µM concentration of **2**, **3**, and **4** were 92.6, 92.6, and 99.9%, respectively, when titrated in Vero cells, with respect to untreated infected cells.

Prompted by the anti-HSV-1 activity of compounds **2**, **3**, and **4** in cultured cells from conjunctival tissue, we decided to evaluate their ability to heal HSK in a murine experimental model. First, a toxicity assay was performed. Daily observation of groups of five mice each that received compounds **2**, **3**, and **4** three times a day during 3 consecutive days confirmed no toxic effect at a concentration of 40 µM. To determine if treatment with compounds **2**, **3**, and **4** after viral inoculation affected the clinical signs of murine HSK, four groups

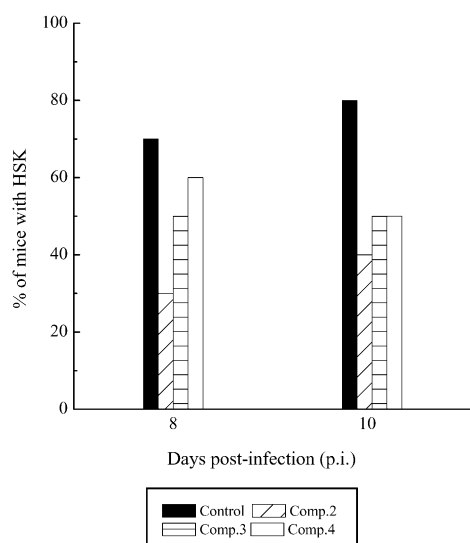


Fig. 4. Effect of brassinosteroids **2**, **3**, and **4** on the incidence of HSV-1 induced-ocular disease in mice. Forty adult Balb/c mice were inoculated with 10^5 UFP/5 µl of HSV-1 KOS strain in their corneas. Three groups of 10 mice each received 40 µM of **2**, **3**, or **4** three times a day for 3 consecutive days, beginning at 1 day p.i. The remaining animals (10) were used as a control for HSV-1 infection. Signs of HSK (blepharitis, neovascularization, corneal clouding, edema, irritation and necrosis) were assessed for 10 days.

of 10 mice each were infected with HSV-1 KOS strain in the right eye. One group was mock-treated with PBS (control), whereas the others were treated topically with a concentration of 40 µM of compounds **2**, **3**, and **4** three times a day at 1, 2, and 3 days p.i.

Signs of ocular disease that gradually developed from 6 to 7 days of infection onwards were observed until day 14 p.i. in all groups (data not shown). The percentage of animals with HSK at 8 and 10 days p.i. was registered (Fig. 4), and the percentages were compared using the χ^2 -test, with 90% confidence intervals. At the onset of disease (day 8 p.i.), 70% of untreated, infected mice exhibited lesions of keratitis, which increased to 80% after 10 days of infection. By day 8 p.i., compound **2** significantly restrained the evolution of HSK, since only 30% of animals had signs of keratitis. A healing effect of compounds **3** and **4** was less evident. Although the percentage of sick animals decreased to 50% at 10 days p.i., no significant differences with respect to untreated infected animals were observed (Fig. 4). Among mice with keratitis, those evolving to necrosis showed no significant differences in the incidence of this lesion between untreated and treated infected animals, regardless of the compound administered (data not shown).

To determine whether compounds **2**, **3**, and **4** inhibited HSV-1 multiplication in vivo, the eyes of untreated and treated infected mice were gently washed with a 10 µl drop of MEM 1.5%, at 1, 3, 6, and 9 days after infection. Each sample was diluted 1:10 and titrated in Vero cells. No significant differences in viral titers among samples from untreated and treated animals were detected, indicating that none of the compounds exerted an antiviral effect in-vivo (Fig. 5).

It is remarkable that the decrease in the percentage of mice with ocular lesions occurred 5 days after the treatment with

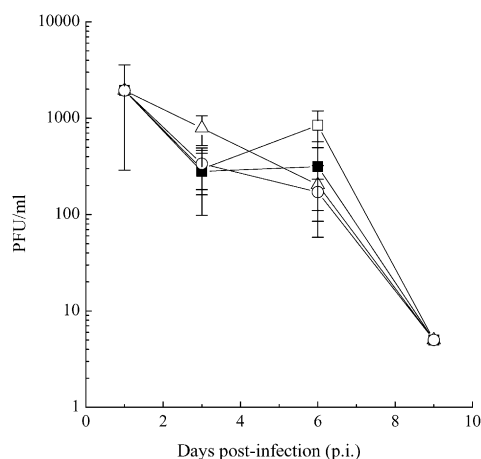


Fig. 5. Effect of brassinosteroids **2**, **3**, and **4** on HSV-1 multiplication in the eye. The eyes of infected adult Balb/c mice untreated and treated with 40 µM of **2** (■), **3** (△), and **4** (○) were gently washed with a 10 µl drop of MEM 1.5% at 1, 3, 6, and 9 days after infection. To quantify viral infectivity, each sample diluted 1:10 was titrated in Vero cells. HSV-1 (□).

2, **3**, and **4** had ended, suggesting that brassinosteroids did not exert a direct antiviral effect, as shown in Fig. 5. It is suspected that multiple immunopathological mechanisms are at play during the development of HSK and that early lesions are principally the consequence of CD4+ T cells recognizing virally derived peptides [20]. Since intact HSV is rarely found in the cornea beyond day 6 p.i., it seems likely that the persistence of incomplete viral antigens and the viral immune alteration of cell membranes in the corneal stroma incite inflammatory reactions [21]. In fact, keratocytes in the corneal stroma of the mouse can function as antigen-presenting cells [22]. Despite the lack of anti-HSV activity in vivo, the fact that compounds **2**, **3**, and **4** improved the clinical signs of HSK led us to consider that these compounds may play a role in immune-mediated stromal inflammation. Further studies to elucidate an eventual antiinflammatory effect of compounds **2**, **3**, and **4** are in progress.

Acknowledgements

This work was supported by grants from the Universidad de Buenos Aires, UBA X-051 and Agencia Nacional de Promoción Científica y Técnica (ANPCYT) PICT 6594/99. Mass spectrometry was provided by the Washington University Mass Spectrometry Resource, an NIH Research Resource (Grant No. P41RR0954).

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