Antiviral effect of brassinosteroids against herpes virus and arenaviruses

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A natural brassinosteroid and a series of synthetic derivatives were found to be good inhibitors of herpes simplex virus type 1 (HSV-1) and arenavirus replication in cell culture. The synthetic compounds tested were analogues of the 24(S) ethylbrassinone. Compounds (22R,23R,24S)-2α, 3α,5α,22,23-pentahydroxy-stigmastan-6-one and (22R,23R,24S)-3β-bromo-5α,22,23-trihydroxy stigm-astan-6-one were cytotoxic at concentrations of 20–40 μM. (22S,23S,24S)-2α,3α,22,23-tetrahydroxy-5α,stigmastan-6-one, (22R,23R,24S)-3β-acetoxy-22,23-dihydroxy-5α-cholestan-6-one, (22S,23S,24S)-3β-bromo-22,23-dihydroxy-5α-chol-estan-6-one and (22S,23S,24S)-3β-bromo-5α,22,23-trihydroxy-stigmastan-6-one were the most active of the series against HSV-1, with selectivity index (SI) values (CC_{50}/EC_{50}) ranging from 10.6 to 16.5. The majority of the compounds were potent inhibitors of arenaviruses, (22S,23S,24S)-3β-bromo-5α,22,23-trihydroxy-stig-mastan-6-one being the most active, with SI values of 307.8 and 692.5 for Tacaribe and Junin viruses, respectively. The antiviral activity of brassinosteroid derivatives was not because of direct inactivation; time-of-addition experiments suggested that a late step in HSV-1 multiplication was affected, whereas arenaviruses remained susceptible to the compounds throughout the replicative cycle.

Keywords: brassinosteroids; herpes simplex virus type 1; arenaviruses; Junin virus

Introduction

A major goal for new antiviral drugs is agents with different antiviral and toxicity profiles from those drugs currently available. Brassinosteroids are a novel group of steroids that appear to be ubiquitous in plants and essential for normal plant growth and development. They have exciting potential use in agriculture because of their ability to improve crop yield and quality, minimize enviromental stress and herbicidal injury and control pathogenic diseases (Brosa, 1997; Brosa *et al*., 1997).

The natural brassinosteroids that have been identified so far have a common 5-α-cholestane skeleton and their structural variations come from the type and position of functional groups on the skeleton and the stereochemistry present in the A and B rings and the side chain (Fujioka & Sakurai, 1997; McMorris, 1997). We have tested 11 synthetic analogues of the natural bassinosteroid (22R,23R,24S)-2α,3α,22,23-tetrahydroxy-5α-stigmastan-6-one (known as 24(S) ethyl-brassinone) against herpes virus and arenaviruses.

We found that the bassinosteroid and its derivatives inhibited the *in vitro* replication of herpes simplex type 1 (HSV-1) thymidine kinase $(TK)^+$ and TK^- strains, and the arenaviruses Junin (agent of Argentine haemorrhagic fever), Pichinde and Tacaribe viruses.

Materials and Methods

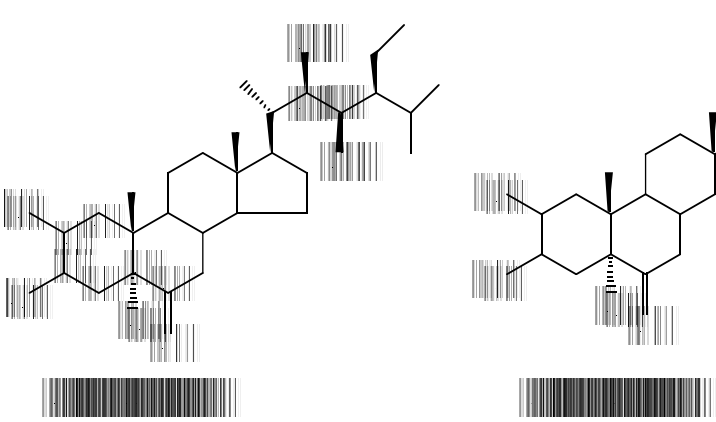
Cells

Vero cells were grown as monolayers in MEM supplemented with 6% inactivated bovine calf serum and 50 μg/ml gentamycin. Maintenance medium consisted of MEM containing 2% inactivated serum.

Viruses

Two strains of HSV-1 were used in the antiviral assays. HSV-1 strain F was obtained from the American Type Culture Collection (Rockville, USA), the TK^- mutants of HSV-1 B2006 were a gift from E De Clercq (Rega Institute, Leuven, Belgium). $XJCl_3$ and IV_{4454} are attenuated strains of Junin virus (Candurra *et al*., 1989), the $TRLV_{11573}$ strain of Tacaribe and AN3739 strain of Pichinde viruses were also used. All virus stocks were propagated and plaque-assayed in Vero cells.

Figure 1. Structural formulae of tested brassinosteroids



Compound	R_1	R_2	R_3
1a–1b	αOH	αOH	H
2a–2b	αOH	αOH	OH
3a–3b	H	βAcO	H
4a–4b	H	βAcO	OH
5a–5b	H	βBr	H
6a–6b	H	βBr	OH

Compounds

Compounds (24S)-2α,3α,22,23-tetrahydroxy-5α-stigmastan-6-one (**1a**–**1b**), (24S)-2α,3α,5α,22,23-pentahydroxystigmastan-6-one (**2a**–**2b**), (24S)-3β- acetoxy -22,23-dihydroxy-5α-stigmastan-6-one (**3a**–**3b**), (24S)-3β-acetoxy-5α,22,23-trihydroxystigmastan-6-one (**4a**–**4b**), (24S)-3β-bromo-22,23-dihydroxy-5α-stigmastan-6-one (**5a**–**5b**) and (24S)- 3β-bromo- 5α,22,23-trihydroxystigmastan-6-one (**6a**–**6b**) were been synthesized from stigmasterol. Compounds **1** and **2** were prepared according to McMorris *et al*. (1994) and Teme Centurión & Galagovsky (1998), respectively. Synthesis and characterization of compounds **3** to **6** have been described elsewhere (Caballero *et al*., 1997).

Stereoisomeric compounds with structure **a** (absolute configuration of 22R,23R) or structure **b** (absolute configuration of 22S,23S) were prepared by catalytic asymmetric dihydroxylation (CAD) of the side chain of each completely functionalized precursor. CAD yielded similar ratio of each pair of stereoisomers, which were easily isolated by silica flash column chromatography (Jacobsen *et al*., 1988).

Stock solutions were prepared in ethanol (1 or 0.1 mg/ml) and further diluted in tissue culture medium shortly before use. Ethanol final concentration in the most concentrated drug solution used was 20 µg/ml. The 50% cytotoxic concentration (CC_{50}) of ethanol, for Vero cells after 72 h of cell contact, was 360 µg/ml.

Acyclovir (9-(2-hydroxyethoxymethyl)guanine) (GlaxoWellcome) was solubilized in DMSO and diluted with maintenance medium to a final concentration of 1 mg/ml. Ribavirin (1-β-D-ribofuranosyl-1,2,4 triazole-3-carboxamide) (Sigma) was dissolved in sterile water to a concentration of 100 mg/ml and conveniently diluted in maintenance medium.

Cytotoxicity assay

Cell viability was determined using the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan) (Denizot & Lang, 1986). 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 the ratio between the absorbance in treated cultures and untreated control cultures. The CC_{50} was defined as the concentration that caused a 50% reduction in absorbance.

Antiviral assay

Antiviral activity was evaluated by two methods; plaque or virus yield reduction. In the plaque reduction test, Vero cell monolayers grown in 24 well plates were infected with 100 p.f.u. of HSV-1/well. After 1 h of adsorption at 37°C, residual inoculum was replaced by maintenance medium containing serial dilutions of the test brassinosteroid (starting at the maximum non-cytotoxic concentration) and 0.7% of methylcellulose. After 48 h of incubation at 37°C, virus plaques were counted. The amount of compound that produced a 50% inhibition (EC_{50}) was obtained by extrapolation from a graph of plaque number versus concentration, in which four different concentrations were plotted.

In the virus yield reduction assay, antiviral activity was evaluated by measuring the reduction of virus yield in the presence of compound. Vero cells grown in 24 well culture plates were infected with HSV-1 or the arenaviruses (Junin, Pichinde or Tacaribe viruses) at a m.o.i. of 1. After 1 h of adsorption at 37°C the cells were covered with maintenance medium containing a non-cytotoxic concentration of the brassinosteroid.

Table 1. Antiviral EC_{50} and CC_{50} values for brassinosteroid derivatives against HSV-1 strains

		HSV-1 F strain		HSV-1 B2006	
Compound	CC_{50} (μM)*	EC_{50} (μM)†	SI‡	EC_{50} (μM)	SI
(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-5α-stigmastan-6-one (**1a**)	209	41.3	5.1	64.9	3.2
(22S,23S,24S)-2α,3α,22,23-tetrahydroxy-5α-stigmastan-6-one (**1b**)	376	22.8	16.5	41.8	9
(22R,23R,24S)-2α,3α,5α,22,23-pentahydroxy-stigmastan-6-one (**2a**)	40	>40.4	<1	>40.4	<1
(22S,23S,24S)-2α,3α,5α,22,23-pentahydroxy-stigmastan-6-one (**2b**)	364	161.6	2.3	91.5	4
(22R,23R,24S)-3α-acetoxy-22,23-dihydroxy-5β-cholestan-6-one (**3a**)	238	22.4	10.6	14.9	16
(22S,23S,24S)-3β-acetoxy-22,23-dihydroxy-5α-cholestan-6-one (**3b**)	139	>39.6	<3.5	>39.6	<3.5
(22R,23R,24S)-3β-acetoxy-5α,22,23-trihydroxystigmastan-6-one (**4a**)	230	57.6	4	94.2	2.4
(22S,23S,24S)-3β-acetoxy-5α,22,23-trihydroxystigmastan-6-one (**4b**)	461	107.3	4.3	173.5	2.7
(22R,23R,24S)-3β-bromo-22,23-dihydroxy-5α-cholestan-6-one (**5a**)	248	53.3	4.7	47.6	5.2
(22S,23S,24S)-3β-bromo-22,23-dihydroxy-5α-cholestan-6-one (**5b**)	343	30.1	11.4	42.6	8.1
(22R,23R,24S)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**6a**)	23	17.7	1.3	12.9	1.8
(22S,23S,24S)-3β-bromo-5α,22,23-trihydroxystigmastan-6-one (**6b**)	277	18.7	14.8	53.8	5.1
Acyclovir	280	0.3	933.3	5.3	52

*50% Cytotoxic concentration, or compound concentration required to reduce cell viability by 50% of the untreated control after 72 h of incubation at 37°C.
†50% Antiviral effective concentration, or compound concentration require to reduce plaque number by 50%.
‡SI or ratio CC_{50}/EC_{50}.
Data are the average of duplicates.

After 24 h of incubation at 37°C, supernatants were harvested and plaqued in Vero cell monolayers grown in 24 well plates and incubated for 48 h at 37°C for HSV-1 and 7 days for arenaviruses.

The protocol described above was also used to determine the concentrations that produce 50% or 90% inhibition (EC_{50} or EC_{90}) for arenaviruses, the only difference being that after 1 h of adsorption at 37°C the cells were covered with maintenance medium containing fourfold dilutions of the compound. The EC_{50} and EC_{90} values were calculated by plotting percentage inhibition versus four different concentrations of each compound.

Results

Evaluation of the compounds for cytotoxicity and antiviral activity

The brassinosteroid derivatives tested in this study are shown in Figure 1. Compounds **1**, **3** and **5** bear a hydrogen on C5, whereas derivatives **2**, **4** and **6** have a hydroxyl group at that position. In reference to C3 compounds, **1** and **2** have an α-hydroxyl group, **3** and **4** an acetyl group and **5** and **6** a bromide. Compounds **1** and **2** have also a hydroxyl group on the C2 of ring A instead of a hydrogen.

Before the potential antiviral activity of these derivatives was studied, the toxicity of cell cultures was investigated. For that purpose, the CC_{50} after 72 h of incubation at 37°C was determine for each compound using the MTT colourimetric assay. As can be seen in Table 1, CC_{50} values for most compounds ranged between 140–460 μM except for **2a** and **6a**, which were found to be highly cytotoxic (CC_{50} values of 40 and 23 μM, respectively). Interestingly, CC_{50} values of brassinosteroid derivatives were comparable to those obtained with reference drugs such as acyclovir (280 μM) and ribavirin (420 μM). Compounds with **b** structure were less toxic than their respective **a** episomers with one exception, compound **3b**, which was twofold more cytotoxic than compound **3a**.

After cytotoxicity studies were performed, brassinosteroid derivatives were screened against poliovirus (PV), vesicular stomatitis virus (VSV), HSV-1, Junin virus and Tacaribe virus. For that purpose, Vero cell monolayers were infected with each virus at a m.o.i. of 1 and after 1 h adsorption at 37°C the inocula were removed and cultures were incubated with maintenance medium or maintenance medium containing 20 μg/ml of each compound. After 24 h of incubation supernatants were harvested and titrated by plaque assay. The results indicate that the replication of all tested viruses was inhibited by the compounds (data not shown). However, because PV and VSV showed low susceptibility to the majority of brassinosteroid derivatives, we did not consider them for further studies.

Dose–response studies

The response of the brassinsteroid derivatives was concentration-dependent in a plaque reduction assay against HSV-1 TK^+ and TK^- strains. Compounds **1b**, **3a**, **5b** and

Table 2. Inhibitory effect of brassinosteriod derivatives against arenaviruses

		Junin IV_{4454} strain			Junin $XJCl_3$ strain		Tacaribe $TRLV_{11573}$ strain			Pichinde AN_{3739} strain	
Compound	CC_{50} (μM)†	EC_{50} (μM)	SI‡	EC_{90} (μM)	EC_{50} (μM)	SI‡	EC_{50} (μM)	SI‡	EC_{90}* (μM)	EC_{50} (μM)	SI‡
1a	209	11.5	18.2	12.6	1.5	139.3	1.0	209	1.9	3.9	54
1b	376	6.2	60.6	10.0	1.8	206.6	1.7	221.2	5.7	3.5	107.4
2a	40	0.2	200	4.0	ND	ND	0.4	100	0.7	ND	ND
2b	364	1.6	227.5	4.1	ND	ND	1.8	202.2	3.4	ND	ND
3a	238	6.1	39	14.5	ND	ND	2.2	108.2	11.5	ND	ND
3b	139	0.6	231.7	3.6	ND	ND	0.6	231.7	2.0	ND	ND
4a	230	2.5	92	29.6	ND	ND	1.3	177	3.7	ND	ND
4b	461	2.0	230.5	15.9	ND	ND	1.5	307.3	4.8	ND	ND
5a	248	0.8	310	3.6	ND	ND	1.0	248	3.6	ND	ND
5b	343	4.8	71.5	19.1	ND	ND	1.2	285.8	3.7	ND	ND
6a	23	0.7	32.9	6.6	ND	ND	0.1	230	0.9	ND	ND
6b	277	0.4	692.5	12.6	0.5	554	0.9	307.8	4.6	0.6	461.7
Ribavirin	420	11.3	37.2	28	ND	ND	8.5	49.4	19.8	ND	ND

*For formulae description see Table 1. Data are the average of duplicates.
†Compound concentration required to reduce cell viability by 50% of the untreated control after 72 h of incubation at 37°C.
¶Selectivity index or ratio CC_{50} / EC_{50}.
ND, Not done.

6b were the most potent tested, with SI values of 16.5, 10.6, 11.4 and 14.8 for HSV-1 (F strain) (Table 1). Compound **3a** was the most active against HSV-1 B2006 TK^- strain with a SI value of 16 (Table 1).

All compounds were considerably less active than acyclovir, the reference drug. However, acyclovir was almost 20-fold more active against HSV-1 TK^+ than against HSV-1 TK^- (Table 1). Both strains were equally susceptible to brassinosteroid derivatives.

Brassinosteroids were next assessed against arenaviruses by a yield reduction assay in Vero cells. For comparative purposes ribavirin was used as reference drug. The multiplication of Junin virus (strain IV_{4454}) was inhibited by all compounds (Table 2). Compounds **2a**, **2b**, **3b**, **4b**, **5a** and **6b** present SI values 5- to 18.6-fold higher than ribavirin. The susceptibility of Tacaribe virus to brassinosteroid derivatives followed a similar pattern. Pichinde virus and Junin virus (strain $XJCl_3$) were also inhibited by compounds **1a**, **1b** and **6b**, indicating that the arenaviruses are very sensitive to the tested compounds.

Results in Table 2 also show that under more stringent conditions (EC_{90}), the majority of brassinosteroid derivatives are better inhibitors than ribavirin.

Direct effect of brassinosteroids

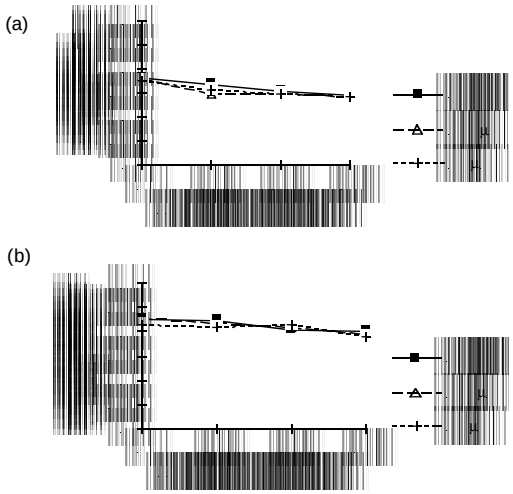
To establish whether brassinosteroids produce a virucidal effect, the following experiments were performed. 10^6 p.f.u of HSV-1 (F strain) or of Junin virus ($XJCl_3$ strain) were diluted in culture medium and incubated for 0, 30, 60 or 90 min at 37°C with two different concentrations of compound **1a** (40 μM and 400 μM) or **6b** (37 μM and 370 μM), respectively. At the indicated times, aliquots were diluted in maintenance medium to a non-cytotoxic drug concentration and titrated by plaque assay. Simultaneously, a virus control without compound was performed. As shown in Figure 2a and 2b, no differences in virus titres were found between treated and untreated viruses, indicating that these compounds are not virucidal.

Time-of-addition studies

The dependence of the inhibitory effects of brassinosteroid derivatives on time-of-addition was next examined. Compound **1a** was added to HSV-1 infected cells at a concentration of 40 μM at different times after infection. At 24 h post-infection, extracellular and cell-associated virus yields were determined. As shown in Figure 3(a), yields of HSV-1 cell-associated or released virus were reduced by more than 99.99% when the compound was added after 1, 3, 5, 7 or 9 h post-infection and remained up to 24 h post-infection. However, when the compound was added at 11 h post infection there was no effect on virus yield. These results indicate that a late event in virus multiplication is inhibited by the drug. On the other hand, time-of-addition studies reported for acyclovir demonstrated that this compound is effective when added up to 6 h after infection (Albin *et al*., 1997).

The effect of compound **6b** at a concentration of 37 μM was assayed in time-of-addition experiments against Junin virus ($XJCl_3$ strain) and Tacaribe virus. This deriv-

Figure 2. Direct inactivation of HSV-1 and Junin virus compounds by compounds **1a** and **6b**



(a) 10^6 p.f.u. HSV-1 F strain diluted in culture medium were incubated at 37°C with either 42 or 420 μM compound **1a** for 30, 60 and 90 min. (b) 10^6 p.f.u. of Junin virus $XJCl_3$ strain diluted in culture medium were incubated at 37°C with either 37 or 370 μM of compound **6b** for 30, 60 and 90 min. At the indicated times, the cultures were chilled and aliquots, conveniently diluted in maintenance medium were taken and tested for virus survival.

ative was selected because it was the most active against both viruses (Table 2). The compound was added at 1, 3, 5, 7, 9, 11, 15 and 17 h post-infection. At 24 h post-infection, supernatants and cell-associated virus were obtained and titrated in Vero cells. Results showed that addition of compound **6b** up to 7 h post-infection inhibited Junin virus replication by approximately 99% (Figure 3b). Afterwards, the levels of both cell associated and free virus increased steadily, however, even at 17 h post-infection an inhibition of 90% was observed.

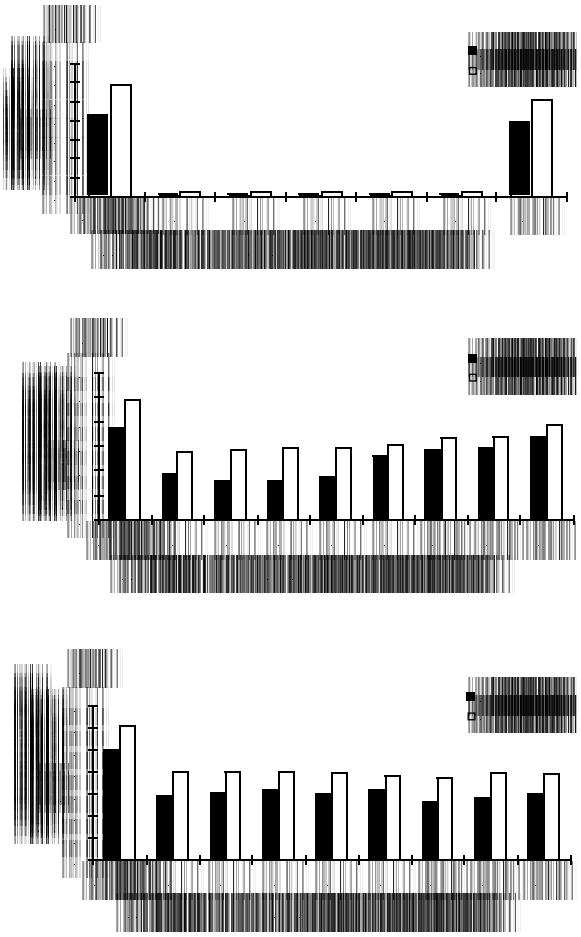
Figure 3(c) shows the results obtained using Tacaribe virus and compound **6b**, added at different times. Virus replication remained sensitive to the compound throughout the viral cycle, showing a similar behaviour to Junin virus.

Discussion

In this study we found that synthetic analogues of the natural brassinosteroid (22R, 23R, 24S)-2α, 3α, 22, 23-tetrahydroxy-5α-stigmastan-6-one have a significant inhibitory effect on arenavirus replication *in vitro* and a moderate effect on HSV-1 TK^+ and TK^- multiplication.

Except for **2a** and **6a**, all derivatives were not cytotoxic for Vero cells at concentrations ranging from 139 to 461 μM (Table 1). The stereochemistry of side chain functionality appears to play an important role in toxicity because the 22S,23S configuration (**b** structure) was less

Figure 3. Effect of time-of-addition on HSV-1, Junin virus and Tacaribe virus multiplication



Compound **1a** (42 μM) was added to HSV-1 F strain (a) or compound **6b** (37 μM) was added to Junin virus XJ Cl_3 strain (b) and Tacaribe virus (c) at different times after infection. At 24 h post-infection extracellular and cell-associated virus yields in treated or untreated cultures were determined using a plaque assay. Each value is the mean of duplicate determinations.

toxic than the 22R,23R configuration (**a** structure). No relationship between structure and bioactivity was found for the different substituents on C2, C3 or C5 (Figure 1).

Brassinosteroid derivatives exhibited lower SI values for HSV-1 than acyclovir (Table 1). However, similar values were reported for foscarnet (trisodium phosphoformate) used in clinical treatment (Beadle *et al.*, 1998). Interestingly, the derivatives were equally active against TK^+ and TK^- strains. This is an attractive property taking into account the reported emergence of acyclovir resistant mutants *in vivo*. (Chatis & Crumpacker, 1992).

The biological mode of action of brassinosteroid derivatives against HSV-1 was not investigated in this report. However, the results of time-of-addition experiments indicate that a late event in the replication cycle

is affected, suggesting that the mechanism-of-action is different from that of acyclovir (Elion, 1982).

A comparison of the bassinosteroid SI values between HSV-1 and arenaviruses (Tables 1 and 2) indicate that these compounds are potent inhibitors of the latter. The arenavirus Junin virus causes a severe disease in humans known as Argentine haemorrhagic fever (Weissenbacher *et al.*, 1987). Several compounds of different chemical structure have been assayed against Junin virus replication *in vitro* (Rodriguez *et al.*, 1986; Andrei *et al.*,1990; Candurra *et al.*, 1996; Castilla *et al.*, 1998). So far, studies in animal models (Weissenbacher *et al.*, 1986) and patients (Enria & Maiztegui, 1994) have only been reported using ribavirin, a broad spectrum antiviral compound (Sidwell, 1980). However, treatment with ribavirin is not ideal because of lack of efficacy in patients with advanced disease and the development of side effects including thrombocytosis and anaemia (Enria *et al.*, 1987, Kenyon *et al.*, 1986).

Under the conditions used in this experiment, the majority of brassinosteroid derivatives showed SI values five to 18.6-fold higher than ribavirin for all arenaviruses tested (Table 2), indicating that these compounds deserve further studies.

Brassinosteroids are growth promoting compounds that exert anti-stress effects on plants with induced expression of heat shock proteins (Dhaubhadel *et al*., 1999). Like the natural brassinosteroids, the synthetic derivatives tested in the experiments reported here are also plant growth promoters, as shown by the rice lamina inclination test described by Wada *et al.* (1984) (LR Galagovsky, personal communication). To our knowledge, no reports of the antiviral activities of brassinosteroids have been published. Therefore, the data presented here suggest that they are novel compounds that should be considered a new family of antiviral compounds. However, further studies are needed to define the precise *in vitro* antiviral mechanism of these compounds and to correlate molecular structure and bioactivity.

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