Synthesis and Antiviral Evaluation of Some Carbonucleoside Analogues

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1,2-*O*-Isopropylidene-α-L-threofuranosyl heterocyclic derivatives were synthesized from 1,2-*O*-isopropylidene-α-D-xilopentadialdo-1,4-furanose and tested for antiviral activity against herpes simplex virus type 1, dengue virus type 2 and Junín virus. For comparative propose, the antiviral activity of some of their pyranosyl analogues were also tested. The furanosyl derivatives showed to be moderate inhibitors of Junin virus and, in general, proved to be more effective than the pyranosyl analogues.

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The synthesis of heterocycles linked to a sugar residue has received much attention, and this is due to the broad spectrum of biological properties of these compounds [1-4].

Most of the published work is referred to carbonucleosides, in which the heterocycle is linked by a C-C bond to the C-1 of the carbohydrate moiety [1, 5-7].

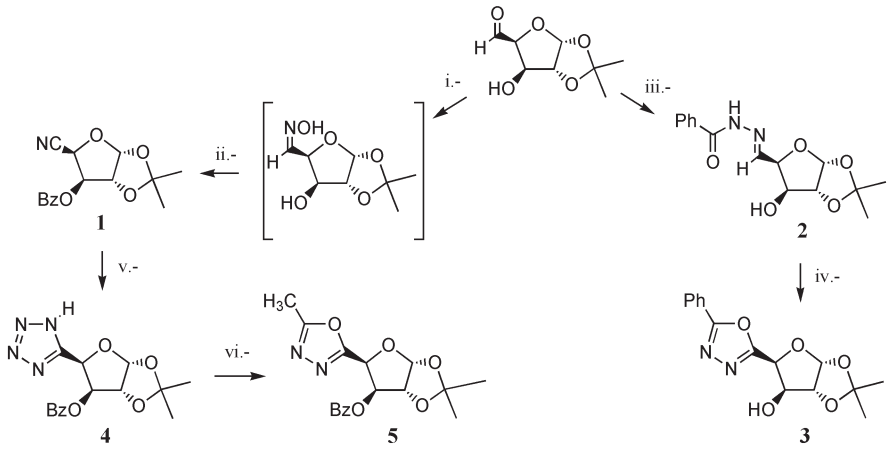
However, chemical and enzymatic stability under physiological conditions could be increased if the heterocycle is linked to a glycosidic carbon other than C-1 [1]. Besides, the biological activity depends on the heterocycle nature as well as the sugar identity.

Looking for new antiviral agents, some L-threofuranosyl and L-arabinopyranosyl residues linked to different heterocycles were tested for antiviral activity against herpes simplex virus type 1, dengue virus type 2 and the etiological agent of Argentine hemorrhagic fever [8], known as Junin virus (JUNV).

For the biological test the following compounds were synthesized: 2-[4-(1,2-*O*-isopropylidene-β-L-threofuranosyl)]-5-phenyl-1,3,4-oxadiazole (**3**), 5-[4-(3-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-threofuranosyl)]tetrazole (**4**) and 2-[4-(3-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-threofuranosyl)]-5-methyl-1,3,4-oxadiazole (**5**).

3-*O*-Benzoyl-1,2-*O*-isopropylidene-α-D-xilofuranurononitrile (**1**) was obtained by dehydration of the corresponding oxime (which was generated *in situ* by reaction of the aldehyde with hydroxylamine hydrochloride in pyridine), using benzoyl chloride. Treatment of 1,2-*O*-isopropylidene-α-D-xilopentadialdo-1,4-furanose with a benzoylhydrazide yielded 1,2-*O*-isopropylidene-β-D-xilopentadialdo-1,4-furanose benzoylhydrazone (**2**). The oxidative cyclization of **2**, using phenyliodide diacetate (PIDA), led to 2-[4-(1,2-*O*-isopropylidene-β-L-threofuranosyl)-5-phenyl-1,3,4-oxadiazole (**3**). 5-[4-(3-*O*-Benzoyl-1,2-*O*-isopropylidene-β-L-threofuranosyl)]tetrazole (**4**) was prepared by treatment of compound **1** with NH_4N_3 in tetrahydrofurane and 2-[4-(3-*O*-benzoyl-1,2-*O*-isopropylidene-β-L-threofuranosyl)]-5-methyl-1,3,4-oxadiazole (**5**) was synthesized by treatment of **4** with acetic anhydride (Scheme 1). We successfully applied this transformation on other carbohydrate derivatives [9], and the mechanism in

Scheme 1



i.- $NH_2OH.HCl$/Pyridine; ii.-BzCl; iii.- $PhCONHNH_2$/ethanol; iv.-PIDA/$NaCH_3COO.3H_2O$/methanol; v.-NH_4N_3/THF; vi.-Acetic anhydride.

gas phase using the mass spectrometer as reactor was studied [10]. Compounds **6 - 11** were synthesized as we previously described [9, 11] (Figure 1). Compounds **1 - 5** were characterized by ^{1}H nmr and ^{13}C nmr. The chemical shift and coupling constants obtained from the ^{1}H nmr spectra are listed in Table 1. The assignment of the ^{13}C nmr spectra is shown in Table 2.

Besides, compounds were studied by electron ionization mass spectrometry (EI - MS). Even the molecular ion ($M^{+\bullet}$) was only observed in the spectra of compounds **3** and **5**, signals corresponding to ($M^{+\bullet}$- $CH_3^{\bullet}$) together with others expected by fragmentation of this kind of compounds were observed in all spectra (see Experimental).

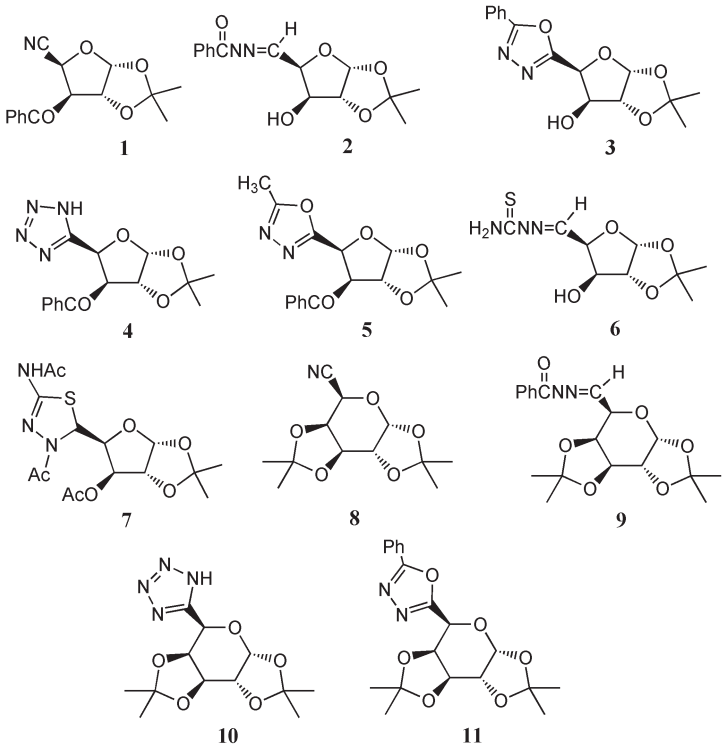


Figure 1

Table 1

^{1}H nmr Chemical Shifts (δ/ppm) and H-H Coupling Constant (*J* / Hz) for Compounds **1** – **5** [a]

	1 [b]	**2** [c]	**3** [b]	**4**[b]	**5** [b]
H-1	6.02 (d, 1H) $J_{1,2}$ = 3.3	5.96 (d, 1H) $J_{1,2}$ = 3.3	6.14 6 (d, 1H) $J_{1,2}$ = 3.7	6.12 (d, 1H) $J_{1,2}$ = 3.6	6.21 (d, 1H) $J_{1,2}$ = 3.3
H-2	4.70 (d, 1H)	4.59 (d, 1H)	4.74 (d, 1H)	4.78 (d, 1H)	4.84 (d, 1H)
H-3	5.58 (d, 1H) $J_{3,4}$ = 3.3	4.70 (d, 1H) $J_{3,4}$ = 4.8	4.72 (d, 1H) $J_{3,4}$ = 2.6	5.72 (d, 1H) $J_{3,4}$ = 3.3	5.69 (s, 1H)
H-4	5.04 (d, 1H)	4.31 (dd, 1H) $J_{4,5}$ = 3.7	5.36 (d, 1H)	5.88 (d, 1H)	5.69 (s, 1H)
H-5	-	7.79 (d, 1H)	-	-	-
N-H	-	10.93 (s, 1H)	-	-	-
-C(CH_3)$_2$	1.54 (s, 3H)	1.47 (s, 3H)	1.52 (s, 3H)	1.55 (s, 3H)	1.61 (s, 3H)
	1.34 (s, 3H)	1.30 (s, 3H)	1.38 (s, 3H)	1.32 (s, 3H)	1.38 (s, 3H)
Aromatics	7.30-7.65 (m, 5H)	7.95-7.40 (m, 5H)	8.15-7.40 (m, 5H)	7.66-7.21 (m, 5H)	7.97-7.39 (m, 5H)
-C-5'(oxadiazol)-CH_3	-	-	-	-	2.38 (s, 3H)
-OH	-	4.67 (s, 1H)	4.01 (s, 1H)	-	-

[a] Chemical shifts are referred to TMS. Multiplicity of coupling and number of protons are given in brackets: s = singlet, d = doublet, dd = double doublet, m = complex multiplet. [b] Chloroform-d_1; [c] dimethylsulfoxide-d_6

Table 2

^{13}C Nmr Chemical Shifts (δ / ppm) for Compounds **1** – **5**

	1 [a]	**2** [b]	**3** [a]	**4** [a]	**5** [a]
C-1	105.6	105.5	105.9	105.4	105.4
C-2	82.6	85.8	84.4	83.6	83.2
C-3	76.5	76.8	75.9	77.6	77.5
C-4	68.6	80.7	73.8	74.0	73.4
C-5	113.8 [c]	147.4	-	-	-
C-2'	-	-	162.3	-	161.4
C-5'	-	-	165.6	153.1	164.9 [c]
-*C*$(CH_3)_2$	113.8 [c]	111.4	112.6	113.3	113.3
-C(*C*$H_3)_2$	27.0 and 26.2	26.4 and 25.7	26.4 and 25.7	26.8 and 25.3	26.8 and 26.3
-C-5'(oxadiazol)-*C*H_3	-	-	-	-	10.8
C=O	165.0	160.3	-	164.9	164.9 [c]
Aromatics	134.1-128.5	131.8-127.6	132.2-123.1	133.7-127.6	133.8-128.6

[a] Chloroform-d_1; [b] Dimethylsulfoxide-d_6; [c] overlapped signals.

The compounds were initially evaluated for their cytotoxicity in Vero cell cultures. None of them were cytotoxic in the range of concentrations tested. The antiviral activity of all the compounds was assayed against three human viruses: herpes simplex virus type 1 (HSV-1), Junín virus (JUNV) and dengue virus type 2 (DEN-2). As shown in Table 3, most of the compounds were totally inactive against HSV-1 and DEN-2, but showed moderate activity against JUNV. The most effective inhibition was observed with the compounds **3**, **5** and **10** with inhibitory concentration 50% (IC_{50}) values in the range 0.23 - 0.26 mmolar.

All the compounds did not show significant cytotoxicity for Vero cells up to 0.5 mmolar, the maximum concentration possible to be evaluated due to their water solubility.

Most of the compounds were inactive against HSV-1 and DEN-2. Only compound **1** was able to inhibit DEN-2, but the value of the antiviral concentration was very near to the cytotoxic dose (Table 3). By contrast, a more potent activity of several products was observed against JUNV, an arenavirus etiological agent of Argentine hemorrhagic fever [8]. In general, the furanosyl derivatives proved to be more effective than the pyranosyl derivatives: compounds **2** - **7** inhibited JUNV multiplication, and the **3**, **5** and **7** products were found the most selective agents with IC_{50} values in the range 0.25 - 0.36 mmoles. By contrast with the furanosic compounds, among the four L-arabinopyranosyl derivatives only compound **10** showed selective activity against JUNV (IC_{50} = 0.23 mmolar); it is surprising that the analog of compound **10** in the L-threofuranosyl group of derivatives, compound **4**, was very weakly active. For comparative purposes, the carboxamide nucleoside ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), the only compound that has shown partial efficacy against JUNV infections [12], was taken as a reference substance. The IC_{50} of ribavirin against JUNV was determined to be 18.3 µmolar and the CC_{50} was >400 µmolar, exhibiting a very significantly higher antiviral potential and selectivity against JUNV than the most active carbonucleoside analogue tested here.

Table 3

Antiviral Activity and Cytotoxicity of Compounds **1-11**

Compound	CC_{50} (mmolar) [a]	IC_{50} (mmolar) [b]		
		HSV-1	DEN-2	JUNV
1	>0.50	>0.50	0.50	>0.50
2	>0.50	>0.50	>0.50	0.50
3	>0.50	>0.50	>0.50	0.25
4	>0.50	>0.50	>0.50	0.43
5	>0.50	>0.50	>0.50	0.26
6	>0.50	>0.50	>0.50	0.46
7	>0.50	>0.50	>0.50	0.36
8	>0.50	>0.50	>0.50	>0.50
9	>0.50	>0.50	>0.50	>0.50
10	>0.50	>0.50	>0.50	0.23
11	>0.50	>0.50	>0.50	>0.50

[a] Cytotoxic concentration 50%: Compound concentration required to reduce cell viability by 50 %. Cytotoxicity was determined by the MTT assay; [b] Inhibitory concentration 50%: Compound concentration required to inhibit virus yield (JUNV and DEN-2) or virus plaques (HSV-1) by 50%. Results are presented as the mean from duplicate independent tests.

In conclusion, seven of the derivatives tested showed a moderate degree of antiviral activity, meanwhile three of them (**3, 5**, and **7**) proved to be moderate inhibitors of the arenavirus JUNV at non cytotoxic concentrations while no significant activity was observed against HSV-1 and DEN-2. Particularly, when the heterocycle was an oxadiazole ring (**3** and **5)** the activity of the compounds was slightly higher than when a thiadiazole was present (**7**).

These results showed that future syntheses, in this particular field, must be oriented to the generation of L-threofuranosyl oxadiazole derivatives, with improved capabilities against JUNV. Actually, we are already working on the synthesis of new compounds including these particular groups.

EXPERIMENTAL

General Methods.

Melting points were determined on a Thomas Hoover capillary apparatus and are uncorrected. Precoated Merck silica gel 60F-254 plates were used for thin layer chromatography (tlc). Flash chromatography was performed using silica gel 60G (mean particle size 15 μm) purchased from Merck, Inc. ^{1}H nmr (200 MHz) and ^{13}C nmr (50 MHz) spectra were measured with a 200 MHz Bruker spectrometer in deuteriochloroform or hexadeuterated dimethylsulfoxide, as is indicated in each case. Heteronuclear correlated spectra were obtained using the standard Bruker program (HETCOR; program name: XHCORRDC.AUR) at 500 MHz, with a spectral width of 2551.02 Hz in the ^{1}H direction (resolution 128 Hz per point) and 151151.52 Hz in the ^{13}C direction (resolution 2048 Hz per point).

Mass spectra were recorded at 70 eV on a Shimadzu QP-5000 spectrometer. Elemental analyses were within ±0.4% of the theoretical values.

Synthesis of Carbonucleosides and Related Compounds.

3-*O*-Benzoyl-1,2-*O*-isopropylidene-α-D-xilofuranurononitrile (**1**).

To a stirred solution of 1,2-*O*-Isopropylidene-α-D-xilopentadialdo-1,4-furanose [13] (0.19 g; 1 mmol) in pyridine, hydroxylamine hydrochloride (0.076 g, 1.1 mmoles) was added. The mixture was heated in a boiling water bath for 0.5 hour and the reaction was followed by tlc (cyclohexane/ethyl acetate 4:1 v/v), then cooled using an ice bath and, after that, 0.7 mL of benzoyl chloride were slowly added. After heating the mixture for 0.5 hour in a water bath at 60°, the reaction was completed; then the reaction medium was dissolved in dichloromethane and successively extracted with water, hydrochloride acid (1 *N*), water, sodium hydroxide (10%) and water. The organic layer was treated with charcoal, filtered through celite and concentrated. The residue was separated by flash column chromatography using mixtures of cyclohexane/ethyl acetate as eluent. Compound **1** was isolated as a solid (0.22 g, 76%) of mp 115-117° (ethanol); $[\alpha]_D$ -44.3 (*c* 1, chloroform); ms: *m/z* 274 ($M^{+\bullet}$-$CH_3^\bullet$); 231 ($M^{+\bullet}$-$CO(CH_3)_2$), 214 ($M^{+\bullet}$-$CH_3^\bullet$-CH_3COOH), 152 ($M^{+\bullet}$-$CH_3^\bullet$-C_6H_5COOH).

Anal. Calcd. for $C_{15}H_{15}NO_5$: C, 62.28; H, 5.19; N, 4.84. Found: C, 62.09; H, 5.32; N, 4.80.

1,2-*O*-Isopropylidene-α-D-xilopentadialdo-1,4-furanose Benzoylhydrazone (**2**).

To stirred solution of 1,2-*O*-isopropylidene-α-D-xilopentadialdo-1,4-furanose (1.75 g; 9.3 mmoles) in ethanol, benzoyl hydrazide (1.29 g, 9.5 mmoles) was added. The reaction mixture was heated at reflux with continuous stirring for 0.5 hour. After that, the mixture was concentrated and from this solution crystallized compound **2** (1.14 g, 40%); mp 153° (ethanol); $[\alpha]_D$ -67.3 (*c* 1, ethanol); ms: *m/z*: 291 ($M^{+\bullet}$-$CH_3^\bullet$); 218 ($M^{+\bullet}$-$CO(CH_3)_2$-CO-H_2), 176 ($C_9H_8N_2O_2^{+\bullet}$), 147 ($C_8H_7N_2O^+$).

Anal. Calcd. for $C_{15}H_{18}N_2O_5$: C, 58.82; H, 5.88; N, 9.15. Found: C, 58.91; H, 5.67; N, 8.93.

2-[4-(1,2-*O*-Isopropylidene-β-L-threofuranosyl)]-5-phenyl-1,3,4-oxadiazole (**3**).

To a stirred solution of compound **2** (0.31 g; 1 mmol) in 8 mL of methanol, sodium acetate trihydrate (0.27 g, 2 mmoles) and PIDA (0.32 g, 1 mmol) were added. The mixture was stirred at room temperature and the reaction was followed by tlc (cyclohexane/ethyl acetate 4:1 v/v) until no changes was observed. The reaction medium was evaporated *in vacuo* and the residue extracted with dichloromethane-water. The combined organic layers were dried over sodium sulfate, and evaporated *in vacuo*. The residue was purified by flash chromatography, using mixtures of cyclohexane/ethyl acetate as eluent. Compound **3** was isolated as syrup (0.27 g, 88%); $[\alpha]_D$ +1.96 (*c* 0.8, chloroform); ms: *m/z* 304 ($M^{+\bullet}$), 289 ($M^{+\bullet}$-$CH_3^\bullet$), 245 ($M^{+\bullet}$-$CH_3^\bullet$-CH_2CO-H_2), 229 ($M^{+\bullet}$- $CH_3^\bullet$-CH_3COOH), 187 ($M^{+\bullet}$- $CH_3^\bullet$-CH_3COOH-C_2H_2O).

Anal. Calcd. for 2($C_{15}H_{16}N_2O_5$)•$C_4H_8O_2$: C, 58.62; H, 5.75; N, 8.05. Found: C, 58.27; H, 5.48; N, 8.33.

5-[4-(3-*O*-Benzoyl-1,2-*O*-isopropylidene-β-L-threofuranosyl)]-tetrazole (**4**).

Compound **1** (2.02 g; 7 mmoles) and sublimated ammonium azide (0.60 g, 10 mmoles) were suspended in tetrahydrofurane (25 mL), and then allowed react at room temperature. The reaction was followed by tlc (cyclohexane/ethyl acetate 4:1 v/v) and after 21 days the conversion was not complete. The mixture was evaporated *in vacuo* to give a syrup, which was purified by flash chromatography, using mixtures of cyclohexane/ethyl acetate as eluent. Compound **4** was isolated as syrup (1.49 g, 64%); $[\alpha]_D$ -59.4 (*c* 1, chloroform); ms: *m/z* 317 ($M^{+\bullet}$-$CH_3^\bullet$), 274 ($M^{+\bullet}$-acetone or $M^{+\bullet}$-$CH_3^\bullet$-N_3H), 214 ($M^{+\bullet}$-$CH_3^\bullet$-CH_3COOH), 195 ($M^{+\bullet}$-$CH_3^\bullet$-C_6H_5COOH), 153 ($M^{+\bullet}$ -$CH_3^\bullet$-C_6H_5COOH-CH_2CO), 125 ($M^{+\bullet}$-$CH_3^\bullet$-C_6H_5COOH-CH_2CO-CO).

Anal. Calcd. for $C_{15}H_{16}N_4O_5$•$C_4H_8O_2$: C, 54.28; H, 5.71; N, 13.33. Found: C, 54.47; H, 5.65; N, 13.01.

2-[4-(3-*O*-Benzoyl-1,2-*O*-isopropylidene-β-L-threofuranosyl)]-5-methyl-1,3,4-oxadiazole (**5**).

Compound **4**, (0.83 g; 2.5 mmoles) was suspended in 25 mL of acetic anhydride, and then heated at reflux for 4.5 hours, following the reaction by tlc (cyclohexane/ethyl acetate 9:1 v/v). The reaction was stopped by addition of ethanol and the medium evaporated. The residue was purified by flash chromatography, using mixtures of cyclohexane/ethyl acetate as eluent. Compound **5** was isolated as syrup in (0.53 g, 61%); $[\alpha]_D$ -58.4 (*c* 1, chloroform); ms: *m/z* 346 ($M^{+\bullet}$), 331 ($M^{+\bullet}$-$CH_3^\bullet$), 209 ($M^{+\bullet}$-$CH_3^\bullet$-C_6H_5COOH), 167 ($M^{+\bullet}$-$CH_3^\bullet$-C_6H_5COOH-CH_2CO), 139 ($M^{+\bullet}$-$CH_3^\bullet$- C_6H_5COOH-CH_2CO-CO).

Anal. Calcd. for 2($C_{17}H_{18}N_2O_6$)•C_6H_{12}: C, 61.18; H, 6.19; N, 7.22. Found: C, 61.17; H, 6.42; N, 7.18.

Biological Tests.

Cells and Viruses.

Vero (African green monkey kidney) cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% inactivated calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 mosquito cell line from *Aedes albopictus* was cultured at 28° in L-15 Medium (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% fetal calf serum.

The virus strains used were as follows: JUNV strain IV4454, DEN-2 strain NGC, and HSV-1 strain F. DEN-2 stocks were prepared in C6/36 cells and titrated by plaque formation in Vero cells. JUNV and HSV-1 stocks were prepared and titrated in Vero cells.

Cytotoxicity Assay.

Stock solutions of the compounds for the biological assays were prepared at a concentration of 50 mmolar in dimethylsulfoxide. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT, supplied by Sigma-Aldrich, USA) method in Vero cells. Confluent cultures in 96-well plates were exposed to two-fold dilutions of the compounds, with three wells for each dilution, during 48 hours of incubation at 37°. Then, 10 µl of MM containing MTT (final concentration 0.5 mg/ml) was added to each well. After 2 hours of incubation, the supernatant was decanted and 200 µl ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader and cytotoxicity was calculated as the cytotoxic concentration 50% (CC_{50}), compound concentration required to reduce the MTT signal by 50% compared to controls.

Antiviral Assays.

For JUNV and DEN-2, the antiviral activity was determined by a virus yield inhibition assay. Vero cells grown in 24-well plates were infected at a multiplicity of infection of 0.1 PFU/cell. After 1 hour adsorption at 37º, cells were washed and re-fed with MM containing different concentrations of the compounds (2 wells per concentration). After 48 hours of incubation at 37º, supernatant cultures were harvested and extracellular virus yields were determined by a plaque assay. The IC_{50} was calculated as the concentration required to reduce virus yield by 50% in the compound-treated cultures compared with untreated ones.

The anti-HSV-1 activity was determined by a plaque reduction assay. Vero cells grown in 24-well microplates were infected with about 50 PFU/well. After 1 hour adsorption at 37º, residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. Plaques were counted after 2 days of incubation at 37º and IC_{50} was calculated as the compound concentration required to reduce virus plaques by 50%.

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