Original article

Antiviral activity of 1,4-disubstituted-1,2,3-triazoles against HSV-1 *in vitro*

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Background: Herpes simplex virus 1 (HSV-1) affects a large part of the adult population. Anti-HSV-1 drugs, such as acyclovir, target thymidine kinase and viral DNA polymerase. However, the emerging of resistance of HSV-1 alerts for the urgency in developing new antivirals with other therapeutic targets. Thus, this study evaluated a series of 1,4-disubstituted-1,2,3-triazole derivatives against HSV-1 acute infection and provided deeper insights into the possible mechanisms of action.

Methods: Human fibroblast cells (HFL-1) were infected with HSV-1 17*syn+* and treated with the triazole compounds at 50 μM for 24 h. The 50% effective drug concentration (EC_{50}) was determined for the active compounds. Their cytotoxicity was also evaluated in HFL-1 with the 50% cytotoxic concentration (CC_{50}) determined using CellTiter-Glo® solution. The most promising compounds were evaluated by virucidal activity and influence on virus egress, DNA replication and transcription, and effect on an acyclovir-resistant HSV-1 strain.

Results: Compounds 3 (*(E)-4-methyl-N′-(2-(4-(phenoxymethyl)-1H-1,2,3-triazol1yl)benzylidene)benzenesulfonohydrazide*) and 4 (*2,2′-(4,4′-((1,3-phenylenebis(oxy))bis(methylene))bis(1H-1,2,3-triazole-4,1 diyl))dibenzaldehyde*) were the most promising, with an EC_{50} of 16 and 21 μM and CC_{50} of 285 and 2,593 μM, respectively. Compound 3 was able to inhibit acyclovir-resistant strain replication and to interfere with virus egress. Both compounds did not affect viral DNA replication, but inhibited significantly the expression of ICP0, ICP4 and gC. Compound 4 also affected the transcription of UL30 and ICP34.5.

Conclusions: Our findings demonstrated that these compounds are promising antiviral candidates with different mechanisms of action from acyclovir and further studies are merited.

Introduction

Herpes simplex virus type-1 (HSV-1) infects epithelial cells with formation of water bubbles and wounds mainly in the oral and ocular mucosa [1]. It affects a large part of population, as shown in a recent epidemiological study in Latin America and the Caribbean that estimated about 90% of adults and 60% of children are infected by HSV-1 [2].

HSV-1 can also lead to severe complications such as blindness [3] and encephalitis [4]. In addition, several studies have associated HSV-1 to Alzheimer's disease [3–8]. In 1997, Itzhaki *et al.* [7] suggested the apolipoprotein E ε4 (APOE-ε4) allele, that is a risk factor for Alzheimer's disease, is also a risk of cold sores, and HSV-1 in brain of APOE-ε4 carriers confers higher risk of Alzheimer's disease. More recently, an epidemiological study carried out in Taiwan revealed that the risk of senile dementia is greater in those who are HSV-seropositive than HSV-seronegative, and that antiviral treatment causes a decrease in number of people who later develop senile dementia [8].

Currently, apart from acyclovir (ACV) and other nucleosides analogues, there are few commercially available drugs for the treatment of HSV-1 [9]. ACV is monophosphorylated by viral thymidine kinase, followed by phosphorylation by cellular kinases to become the active form to inhibit DNA polymerase, an enzyme that catalyses the elongation of viral DNA [10]. ACV and related drugs are undoubtedly effective in treating HSV-1 infections [11]. However, due to the widespread use of ACV, cases of ACV-resistant HSV-1 infections

have been increasing, frequently in immunocompromised patients [12–14]. Approximately 95% of resistant cases are due to mutations in the *UL23* gene encoding for thymidine kinase and 5% are due to mutations in the *UL30* gene encoding for viral DNA polymerase [15]. A single mutation in DNA polymerase may confer resistance to many anti-HSV agents [16]. Since most of the anti-HSV drugs have these enzymes as targets, the treatment of resistant HSV-1 infection is problematic and restricted [17]. For this reason, it is necessary to discover new drugs with anti-HSV-1 activity and with a different mechanism of action [18]. Pires de Mello *et al*. [19] have shown several antiviral targets through the infection phases, from the entry of HSV-1 into epithelial cells, the lytic cycle, to latency and reactivation. Thus, essential viral proteins and some cellular targets could be new strategies in antiviral discovery.

Some series of 1,4 disubstituted-1,2,3-triazoles with antiviral activity have been described in literature. Ribavirin, for instance, is a known antiviral against HIV-1, HSV and HCV, and 1,2,3-triazole analogue increased pharmacological activity and reduced ribavirin cytotoxicity [20,21]. Regarding anti-HSV-1 activity, some authors have reported good *in vitro* activity [22–24]. Jordão *et al*. [22], for example, elaborated on a series of arysulfonylhydrazide-1H-1,2,3-triazoles and described 50% effective drug concentration (EC_{50}) values of 1.30 and 1.26 μM against this virus. The effectiveness of 1,2,3-triazole derivatives was also reported in the series of 5-(benzylthio)-4-carbamyl-1,2,3-triazoles where the authors obtained EC_{50} values around 9.9–16.5 μM against HSV-1 [23]. Recently, Cunha *et al*. [24] showed that a series of 1,2,3-triazole linked nitroxyl radical derived from TEMPOL inhibited *in vitro* replication of HSV-1. Four hybrids showed important anti-HSV-1 activity with 50% inhibitory concentration (IC_{50}) values that ranged from 0.80 to 1.32 μM. The potential of the 1,2,3-triazole scaffold present in some compounds with antiviral activity prompted us to investigate the anti-HSV-1 activity of a series of 1,4 disubstituted-1,2,3-triazoles, their cytotoxicity and the effects on viral particle, DNA replication and transcription and viral egress.

Methods

Chemistry

Compounds **1**–**4** (Figure 1) were previously synthesized and reported with potency against highly resistant glioblastoma cells [25]. Compounds **5a**–**c** were recently synthesized as described below.

For the structural elucidation of the synthesized compounds, ^{1}H NMR and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker Avance III spectrometer (operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR; Billerica, MA, USA). The chemical shifts (δ) were given in parts per million (ppm) from internal tetramethylsilane on the δ scale, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet). All coupling constants (*J* values) were given in Hz. Melting points were determined with an electrothermal, analogue model. Infrared spectra were performed using a Varian-3100 spectrometer (Agilent Technologies, Santa Clara, CA, USA). High-resolution mass spectra (HRMS) were obtained by Bruker MicrOTOF II instrument. Reactions were monitored by thin-layer chromatography using Merck TLC Silica gel 60 F254 (Merck, Darmstadt, Germany). Silica gel column chromatography was performed over Merck Silica gel 60 Å (particle size: 0.040–0.063 mm, 230–400 mesh ASTM). All reagents used were commercially obtained.

Synthesis of alkynes

The alkyne (2-(prop-2-yn-1-yloxy)benzaldehyde; **6**) were prepared following the procedure described by Silva *et al*. [25].

2-Hydroxybenzaldehyde (5.3 mmol) and anhydrous K_2CO_3 (10.6 mmol) were added in a 50 ml two-necked flask and dissolved in 15 ml acetonitrile. After 15 min, propargyl bromide (80% in toluene, 6.4 mmol) was slowly added and the reaction mixture was refluxed under N_2 atmosphere for 4.5 h. Then the mixture was diluted with water (30 ml) and extracted with dichloromethane (3×20 ml). The combined organic layers were dried over Na_2SO_4 and the solvent was concentrated under reduced pressure. The desired alkyne (**5**) was obtained without further purification.

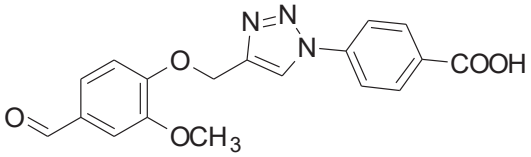
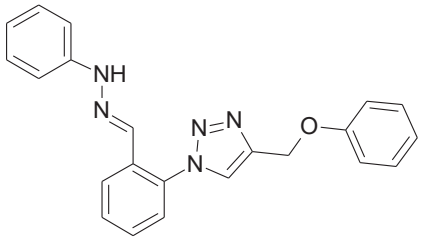
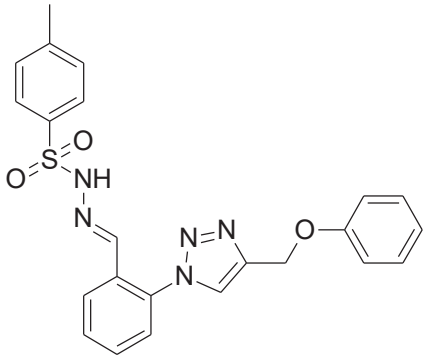
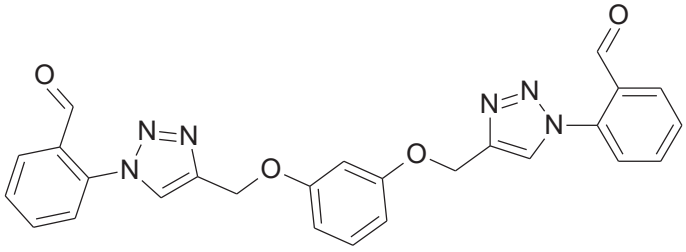
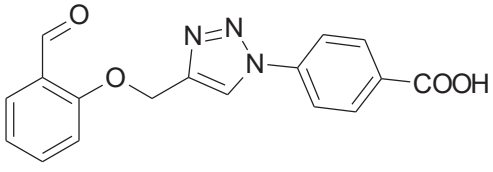
2-(prop-2-yn-1-yloxy)benzaldehyde

Yield 90%; white solid; MP: 67–69°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.49 (s, 1H, C<u>H</u>O), 7.87 (dd, *J*=7.7, 1.8 Hz, 1H, Ar-<u>H</u>), 7.58 (ddd, *J*=8.5, 7.3, 1.8 Hz, 1H, Ar-<u>H</u>), 7.16–6.95 (m, 2H, Ar-<u>H</u>), 4.84 (d, *J*=2.4 Hz, 2H, C<u>H</u>$_2$), 2.57 (t, *J*=2.4 Hz, 1H, C<u>H</u>). IR (KBr, ν_{max}): 3,270 (°CH), 2,118 (C°C), 1,684 (C=O) cm^{-1}.

Synthesis of aryl-azides

The aryl-azides (**7a**–**d**) were prepared following the procedure of Wilkening *et al*. [26]. The aniline derivative (7.5 mmol) was dissolved in 5 ml of water and concentrated sulfuric acid (98%, 1.5 ml) and additional water (1.5 ml) was added. The suspension was cooled to 0°C and a solution of $NaNO_2$ (7.6 mmol) of water (1.5 ml) was slowly added under constant stirring. After 15 min, NaN_3 (9.3 mmol) was added and the mixture was stirred for additional 0.5–1 h. The reaction mixture was extracted with ethyl acetate (3×20 ml) and the combined organic fractions were washed with water (50 ml). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The desired azides (**7a**–**d**) were obtained without further purification.

Figure 1. Cytotoxicity, antiviral activity and selectivity index[a] of the compounds

	Compound	CC_{50}, μM	EC_{50}, μM	SI
1		1,481 ±87.6	76 ±5.5	19.5
2		1,562 ±142	79 ±6.7	19.8
3		285 ±5.1	16 ±8.2	17.8
4		2,593 ±140	21 ±9.8	123.5
5a		997 ±6.8	154 ±11.3	6.5
Acyclovir		1,287 ±237	1.1 ±0.7	1,170

[a]Selectivity index (SI) = CC_{50}/EC_{50}. CC_{50}, concentration that reduces the viability of host cells by 50%; EC_{50}, 50% effective drug concentration.

4-azidobenzoic acid (7a)
Yield 80%; light yellow solid. MP: 185°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.97 (s, 1H, OH), 7.96 (d, *J*=8.7 Hz, 2H, Ar-H), 7.22 (d, *J*=8.7 Hz, 2H, Ar-H). IR (KBr, ν_{max}): 2,105 (N°N), 1,284 (C-O) cm^{-1}.

1-azido-4-nitrobenzene (7b)
Yield 60%; dark yellow solid. MP: 65–67°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, *J*=9.1 Hz, 2H, Ar-H), 7.14 (d, *J*=9.1 Hz, 2H, Ar-H). IR (KBr, ν_{max}): 2,110 (N°N) cm^{-1}.

1-azido-4-bromobenzene (7c)
Yield 75%; brown liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J*=8.8 Hz, 2H, Ar-H), 6.93 (d, *J*=8.8 Hz, 2H, Ar-H). IR (KBr, ν_{max}): 2,114 (N°N) cm^{-1}.

General procedure for the synthesis of triazoles
The triazoles (**5a**–**c**) were prepared following the procedure described by Rostovtsev *et al*. [27]. The alkyne (0.6 mmol) and aryl-azide (0.6 mmol) were added to a 1:1 mixture of water and tert-butyl alcohol (6 ml). Sodium ascorbate (0.06 mmol, in 200 µl of water) was added, followed by copper (II) sulphate pentahydrate (0.006 mmol, in 100 µl of water). The reaction mixture was stirred vigorously at room temperature and monitored by thin-layer chromatography until the reagents were completely consumed. At the end of the reaction the mixture was diluted with ice water (50 ml), the precipitate was collected by filtration, washed with cold water (2×25 ml) and dried under vacuum. The procedures for compounds **1**–**4** are described by Silva *et al*. [25].

4-(4-((4-formyl-2-methoxyphenoxy) methyl)-1H-1,2,3-triazol-1-yl)benzoic acid (1)
Yield 80%; white solid; reaction time: 8 h, MP: 238–239°C. ^{1}H NMR (400 MHz, DMSO) δ 13.27 (s, 1H), 9.87 (s, 1H), 9.11 (s, 1H, triazole-H), 8.18–8.05 (m, 4H), 7.59 (dd, *J*=8.2, 1.8 Hz, 1H), 7.46–7.41 (m, 2H), 5.38 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (101 MHz, DMSO-*d6*) δ 191.65, 152.88, 149.32, 143.67, 139.51, 129.93, 125.81, 123.43, 112.72, 109.72, 61.14, 55.14. HRMS(ESI) m/z calculated for $C_{18}H_{15}N_3O_5$+Na [M+Na]$^+$, 376.0903; found 376.0904.

(E)-4-(phenoxymethyl)-1-(2-((2-phenylhydrazono) methyl)phenyl)-1H-1,2,3-triazole (2)
Yield 70%; white solid; MP: 128–129°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (dd, *J*=8.0, 1.1 Hz, 1H), 7.89 (s, 1H), 7.77 (s, 1H), 7.56 (t, *J*=7.1 Hz, 1H), 7.44 (td, *J*=7.6, 1.4 Hz, 1H), 7.39–7.33 (m, 4H), 7.32–7.29 (m, 1H), 7.11–7.01 (m, 5H), 6.92 (t, *J*=7.3 Hz, 1H), 5.37 (s, 2H).^{13}C NMR (101 MHz, $CDCl_3$) δ 158.11, 144.05, 134.23, 131.29, 130.81, 130.14, 129.66, 129.32, 128.51, 126.57, 126.07, 121.45, 120.61, 114.92, 112.87, 61.85. HRMS(ESI) m/z calculated for $C_{22}H_{19}N_5O$+Na [M+Na]$^+$, 392.1481; found 392.1482.

(E)-4-methyl-N'-(2-(4-(phenoxymethyl)-1H-1,2,3-triazol1yl)benzylidene)benzenesulfonohydrazide (3)
Yield 65%; white solid; MP: 167–169°C. ^{1}H NMR (400 MHz, CDCl3) δ 8.57 (s, 1H), 8.09 (dd, *J*=7.3, 2.1 Hz, 1H), 7.82–7.79 (m, 3H), 7.54–7.48 (m, 3H), 7.35–7.27 (m, 4H), 7.05–6.95 (m, 3H), 5.26 (s, 2H), 2.39 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.97, 144.63, 144.14, 141.29, 135.47, 130.66, 130.21, 129.65, 129.12, 127.84, 127.32, 125.74, 125.29, 121.51, 114.82, 61.58, 21.58. HRMS(ESI) m/z calculated for $C_{23}H_{21}N_5O_3S$+Na [M+Na]$^+$, 470.1265; found 470.1261.

2,2'-(4,4'-((1,3-phenylenebis(oxy))bis(methylene)) bis(1H-1,2,3-triazole-4,1 diyl)) dibenzaldehyde (4)
The crude product was purified by flash column chromatography using ethyl acetate/hexane (20:80) as eluent. The product was obtained as a light yellow solid in 50% yield. Reaction time: 16 h, MP: 106–107°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.91 (s, 2H), 8.11 (dd, *J*=7.8, 1.5 Hz, 2H), 8.05 (s, 2H, triazole-H), 7.77 (td, *J*=7.7, 1.6 Hz, 2H), 7.68 (t, *J*=7.5 Hz, 2H), 7.54 (dd, *J*=7.9, 0.9 Hz, 2H), 7.25 (t, *J*=8.2 Hz, 1H), 6.73 (t, *J*=2.3 Hz, 1H), 6.69 (dd, *J*=8.2, 2.4 Hz, 1H), 5.33 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.17, 160.18, 145.69, 138.87, 135.30, 131.14, 131.08, 130.83, 130.34, 126.18, 125.51, 108.51, 102.95, 62.87. HRMS(ESI) m/z calculated for $C_{26}H_{20}N_6O_4$+Na [M+Na]$^+$, 503.1438; found 503.1438.

4-(4-((2-formylphenoxy) methyl)-1H-1,2,3-triazol-1-yl) benzoic acid (5a)
Yield 85%; white solid; reaction time: 8 h; MP: 265–267°C. RMN ^{1}H (400 MHz, DMSO) δ 10.44 (s, 1H), 9.13 (s, 1H, triazole-H) 8.12 (d, *J*=12.3 Hz, 4H), 7.82–7.62 (m, 2H), 7.48 (d, *J*=8.3 Hz, 1H), 7.13 (t, *J*=7.5 Hz, 1H), 5.47 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 189.41, 160.36, 136.39, 127.61, 124.53, 122.82, 121.29, 114.15, 62.17. HRMS(ESI) m/z calculated for $C_{17}H_{13}N_3O_4$+Na [M+Na]$^+$, 346.0798; found 346.0795.

2-((1-(4-nitrophenyl)-1H-1,2,3-triazol-4-yl) methoxy) benzaldehyde (5b)
Yield 60%; yellow solid; reaction time: 24 h; MP: 240–241°C. ^{1}H NMR (400 MHz, DMSO) δ 10.45 (s, 1H), 9.23 (s, 1H, triazole-H), 8.47 (d, *J*=9.1 Hz, 2H), 8.26 (d, *J*=9.1 Hz, 2H), 7.76–7.67 (m, 2H), 7.48 (d, *J*=8.4 Hz, 1H), 7.13 (t, *J*=7.5 Hz, 1H), 5.48 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 189.89, 160.79, 147.29, 144.84, 141.25, 136.87, 128.11, 126.06, 125.06, 123.60, 121.83, 121.22, 114.63, 62.65. HRMS(ESI) m/z calculated for $C_{16}H_{12}N_4O_4$+Na [M+Na]$^+$, 347.0756; found 347.0750.

2-((1-(4-bromophenyl)-1H-1,2,3-triazol-4-yl) methoxy)benzaldehyde (5c)

Yield 65%; light yellow solid; Reaction time: 8 h; ^{1}H NMR (400 MHz, DMSO) δ 10.43 (s, 1H), 9.06 (s, 1H, triazole-H), 7.92 (d, *J*=8.9 Hz, 2H), 7.82 (d, *J*=8.9 Hz, 2H), 7.74–7.67 (m, 2H), 7.48 (d, *J*=8.3 Hz, 1H), 7.14 (dd, *J*=12.4, 4.9 Hz, 1H), 5.46 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 189.37, 160.27, 143.84, 136.38, 132.78, 127.60, 124.51, 122.74, 122.06, 121.26, 114.13, 62.16. HRMS(ESI) m/z calculated for $C_{16}H_{12}BrN_3O_2$+Na $[M+Na]^+$, 380.0011; found 380.0004.

Cell lines and HSV-1 strains

The 1,2,3-triazole derivatives were diluted at the concentration of 50 mM in dimethylsulfoxide (DMSO) and stored at -20°C. We tested ACV as a reference antiviral drug to compare our data.

These compounds were evaluated *in vitro* on human fibroblast (HFL1 ATCC® CCL-153™) cells which were cultivated at 37°C and 5% CO_2, with Dulbecco's Modification of Eagle's medium (DMEM), with 4.5 g/l glucose, L-glutamine and sodium pyruvate (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), and 1% of L-glutamine-penicillin-streptomycin solution (Sigma-Aldrich).

To perform the plaque assay, we used rabbit skin (RS) cells cultivated at 37°C and 5% CO_2 with Minimum Essential Medium Eagle (MEM), with Earle's salts and L-glutamine (Sigma-Aldrich), supplemented with 5% bovine serum (Gibco) and 1% of L-glutamine-penicillin-streptomycin solution (Sigma-Aldrich).

The cells were cultivated until they achieved around 90% of confluence when they were transferred to plates of 24 or 96 wells depending on the assay.

HSV-1 strain 17*syn*+ was used for the experiments. We also tested the compounds on the ACV-resistant clinical strain HO-1 (kindly provided by the D Phelan, College of Medicine, University of Florida, personal communication). Virus stock cultures were prepared from supernatants of infected cells and stored at -80°C until use.

Antiviral activity evaluation

Antiviral activity of the compounds was first assayed by acute HSV-1 infection on HFL-1 cells. HFL cells were seeded in 24-multiwell plates (1×10^5 cell/well) and incubated at 37°C and 5% CO_2 atmosphere until a complete confluence of the cells (24 h approximately). Then, we infected the cells with HSV-1 strain 17*syn*+, in a multiplicity of infection (MOI) of 1.0, for 1 h at 37°C and 5% CO_2. Three wells were not infected to be the control of cell growth. Then, the viral inoculum was removed, the cells were washed with phosphate-buffered saline (PBS), and treated with 50 μM of the compounds in DMEM. DMEM without any compound was used as a control of HSV-1 yield and DMSO was also tested to verify its influence in the HSV-1 yield. At 24 h post-infection (hpi), cells were lysed with 3 cycles of freezing and thawing to collect the extra and intracellular content.

Plaque assay

To determine the viral titre of the experimental groups, the plaque assay was performed according to Lucero *et al.* [28], with few modifications. RS cells were seeded on 24-multiwell plates in a density of 1×10^5 cells/well and incubated at 37°C and 5% CO_2 until the confluence of the cells. Then, the samples were serial diluted (1:10) and RS cells were infected and incubated for 1 h at 37°C and 5% CO_2. The supernatant was aspirated, the cells were washed with PBS and the monolayer was covered with MEM. MEM and 0.3% of immunoglobulin G (IgG) from human serum (Sigma) for 48 h. Then, the monolayer was fixed and stained with 0.5% violet crystal and 20% methanol. The viral titre was determined according to the number of viral plaque-forming units per ml (PFU/ml).

Determination of EC_{50}

In order to determine the EC_{50} of the active compounds, HFL1 cells were seeded in 24-multiwell plates (1×10^5 cells/well) and after cell confluence they were infected with HSV-1 strain 17*syn*+ (MOI of 1.0) for 1 h at 37°C and 5% CO_2. Then, the viral inoculum was removed, the cells were washed and treated with different concentrations (6.25, 12.5, 25 and 50 μM) of the compounds for 24 h. We lysed the cells with 3 cycles of freezing and thawing to collect the samples for the plaque assay that was performed as described under *Plaque assay*. The EC_{50} value was determined by linear regression compared with an infected and untreated control.

Cytotoxicity assay

Cytotoxicity values of the compounds were determined using CellTiter-Glo(R) assay (Promega, Madison, WI, USA). This assay determines the number of viable cells based on quantitation of ATP present, which indicates the presence of metabolically active cells, according to the manufacturer [29]. HFL1 cells were cultivated in 96-multiwell plates (5×10^4 cells/well) and incubated at 37°C for 24 h and 5% CO_2. The cells were treated with the compounds in different concentrations (50, 250, 500 and 1,000 μM) diluted in DMEM, and the plates were incubated at 37°C for 24 h and 5% CO_2. Then, 100 μl of the CellTiter-Glo® solution was added to each well. After 12 min stirring at room temperature, the luminescence was recorded in the GloMax® Luminometer (Promega). The concentration that reduces the

viability of host cells by 50% (CC_{50}) was calculated by linear regression analysis of the dose–response curves.

Antiviral activity on resistant HSV-1 strain

The most promising compounds **3** and **4** were tested on the ACV-resistant strain HO-1. HFL1 cells were infected with HO-1 (MOI 0.1) for 1 h. Then, the cells were washed and treated with the compound at 50 μM for 24 h at 37°C and 5% CO_2. After lysing of the cells, the content was harvested and titred as described in *Antiviral activity evaluation*.

Virucidal assay

The virucidal assay was carried out according to Schuhmacher *et al*. [30] with some modifications. HSV-1 strain 17*syn*+ diluted in DMEM (MOI 1.0) was pretreated with the compounds **1** and **4** or remained untreated for 4 h at 4°C. Then, HFL1 cells on the 24-wells plate were infected with the virus for 1 h at 37°C. Viral suspension was removed, the cells were washed with PBS and covered with DMEM. After 24 h at 37°C and 5% CO_2, the cells were lysed and the content was harvested to be titred as described in *Antiviral activity evaluation*. The number of PFU/ml of the treated groups was compared with the untreated group.

Egress assay

In order to evaluate the interference of the compounds on virus egress from the cells, we infected HFL1 cells (1×10^5 cells/well in 24-well plates) for 1 h with a MOI of 1.0. The viral inoculum was removed, the cells were washed and treated with the compounds (50 μM) for 24 h, at 37°C and 5% CO_2. Then, the supernatant correspondent to the virus on the extracellular environment was collected. Cells were washed with PBS, covered with DMEM and lysed by 3 cycles of freezing and thawing to harvest the intracellular viral particles. These samples were diluted and submitted to the plaque assay, as described in *Plaque assay*.

Time-course assay

In order to determine in which phase of infection the compounds would be more effective, we performed time-course assays. HFL1 cells on 24-well plates were infected with HSV-1 strain 17*syn*+ (MOI of 1.0) for 1 h at 37°C and 5% CO_2. Then, the viral inoculum was removed, the cells were washed and treated with the compounds at 50 μM for 2, 4 or 8 hpi. Then, we harvested the content for the plaque assay.

Real-time quantitative PCR

HFL1 cells were infected with HSV-1 17*syn*+ (MOI of 1.0) for 1 h. Then, they were treated with compounds at 50 μM for 8 h. The supernatant was removed, the cells were washed with PBS and the total RNA and DNA were extracted using 500 μl of TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA).

Then, 100 μl of chloroform was added into each tube and after 3 min the samples were centrifuged at 12,000×*g* for 15 min at 4°C. The upper aqueous phase was collected in another tube for RNA extraction and the bottom organic phase was directed for DNA extraction (Zymo Research, Irvine, CA, USA).

RNA extraction was performed using Direct-zol® reagents according to the manufacturer instructions. The RNA samples were treated with DNase (Turbo DNA-free™ Kit; Thermo Fisher Scientific, Carlsbad, CA, USA) and cDNA was obtained by reverse transcriptase reactions using Omniscript® Reverse Transcription Kit (Qiagen, Germantown, MD, USA) according to its protocol.

For DNA extraction the bottom phase was incubated for 3 min with 100% ethanol, centrifuged at 2,000×*g* for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 0.1 M sodium citrate in 10% ethanol (pH 8.5). After 30 min, the samples were centrifuged at 2,000×*g* for 5 min at 4°C. This step was repeated. Then, the pellet was resuspended in 75% ethanol and after 20 min at room temperature, the samples were centrifuged at 2,000×*g* for 5 min at 4°C. The supernatant was discarded and the DNA pellet was dried and resuspended in 8 mM NaOH.

Quantitative PCR (qPCR) was performed using 20 ng input DNA, TaqMan® Fast Universal PCR 2X Master Mix (Applied Biosystems, Austin, TX, USA) with TaqMan® probes (Applied Biosystems) and target-specific primers (Applied Biosystems) and HSV-1 polymerase. Samples were running on a 7900HT Fast Real-Time PCR System (Applied Biosystems).

Results

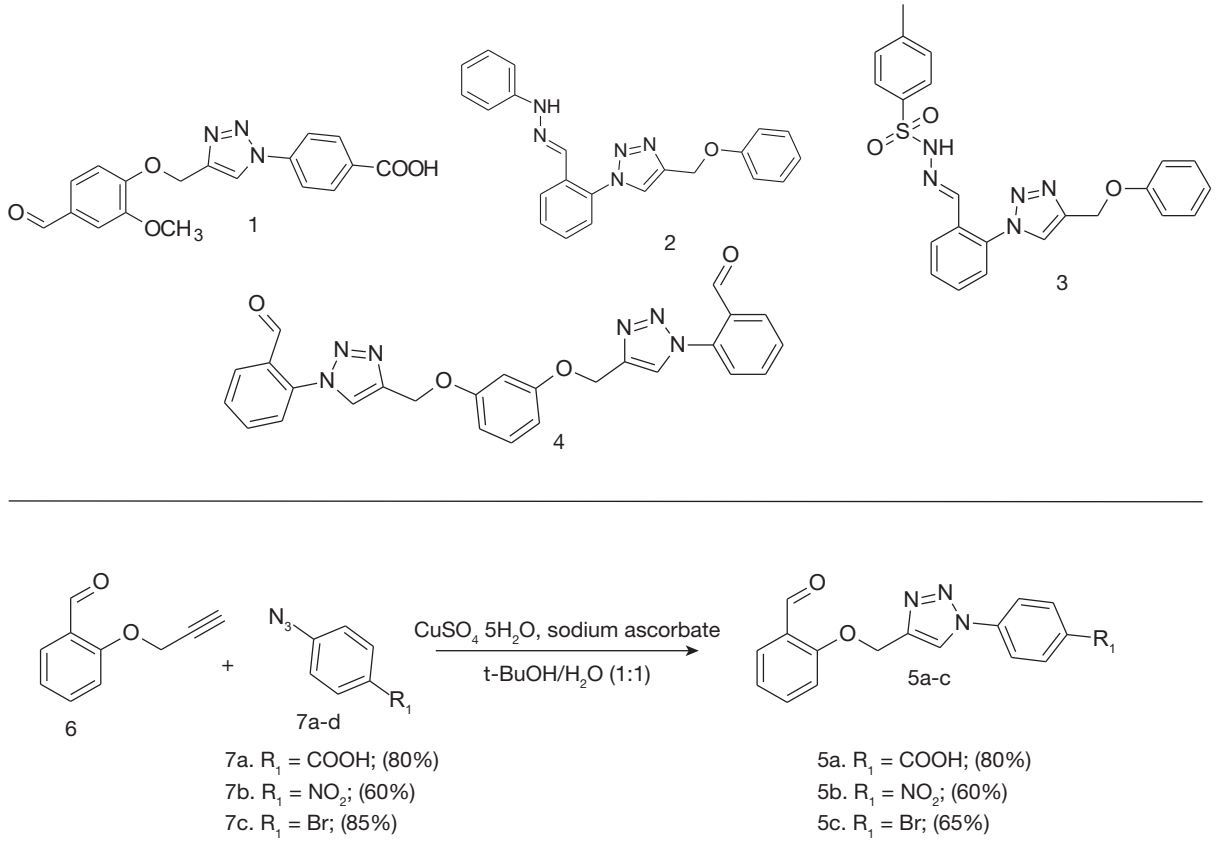
Synthesis of 1,4-disubstituted-1,2,3-triazoles

As described by Silva *et al*. [25], the key step for the obtention of 1,4-disubstituted-1,2,3-triazoles (**1**–**4**) was the copper-catalysed azide-alkyne cycloaddition, click chemistry reaction. Compounds **5a**–**c** were also obtained in good yields, by click reaction between 2-(prop-2-yn-1-yloxy(benzaldehyde) (**6**) and arylazides (**7a**–**c**), using one equivalent of corresponding azides and alkynes (Figure 2). These kinds of 1,4-disubstituted-1,2,3-triazoles, similar to compounds **5a**–**c**, have been also described and characterized by Gupta *et al*. [31].

Antiviral activity

All 1,4 disubstituted-1,2,3-triazoles were tested at 50 μM on HSV-1 17*syn*+ for 24 hpi and compounds **1**, **2**, **3**, **4** and **5a** showed antiviral activity, while **5b** and

Figure 2. Chemical structure and synthesis of 1,4-disubstituted-1,2,3-triazoles compounds evaluated against HSV-1 [25]



HSV-1, herpes simplex virus type 1.

5c had no effect on viral yield. The active compounds were tested in different concentrations on HFL1 cells infected with HSV-1 17*syn*+ to determine the EC_{50}. The compounds with the higher antiviral activity were **3** and **4**, with EC_{50} of 16 and 21 μM, respectively (Figure 1).

Cytotoxicity assay

To determine the CC_{50} of the compounds with antiviral activity, we evaluated them in different concentrations on HFL1 cells and the viability of the cells was determined indirectly by means of the quantification of ATP, which indicates the presence of metabolically active cells [29]. The CC_{50} of the compounds were between 285 and 2,593 μM (Figure 1).

We also calculated the selectivity index (SI), obtained by the ratio between CC_{50} and EC_{50}. The SI of the compounds were between 6.5 and 123.5.

Antiviral activity on resistant HSV-1 strains

Compounds **3** and **4** were tested on the ACV-resistant HSV-1 strain HO-1. Compound **4** was not able to control the yield of this strain. However, compound **3** showed remarkable antiviral activity against HO-1, with 75% reduction of PFU, whereas ACV reduced only 8.8%.

Effect on the viral particles

In order to evaluate whether the compounds could affect the viral particles, we pretreated HSV-1 17*syn*+ for 4 h with compounds **3** and **4** before infection and estimated the viral titre 24 hpi. Although the group treated with compound **3** showed fewer PFU/ml than the untreated group and compound **4** slightly higher, these differences were not significant. Therefore, the compounds did not affect the viral particles and the viral titre was similar in the untreated and treated groups.

Effects on viral egress from the cells

We performed the egress assay to analyse whether the compounds could disturb the release of virus from the cells. We could verify that the compounds had a lower viral yield in intracellular content than the untreated group. However, compound **4** showed similar viral yield in the extracellular content, while **3** decreased the

viral titre in the extracellular content compared with the untreated group (Figure 3). Additionally, the ratio of the intracellular/extracellular contents was 1.8 for the untreated group, 0.98 for **4** and 9.3 for **3**, which suggests that compound **3** may interfere on the virus egress from the cell, or in some related process.

Time-course assay

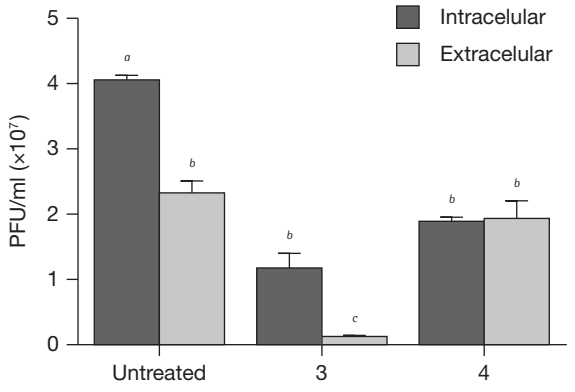
To evaluate which infection phase the compounds could affect, we performed viral plaques at 2, 4 and 8 hpi. The compounds did not affect the virus titre at 2 hpi and they had some effect at 4 hpi, but it was not significant. However, at 8 hpi compound **3** reduced 96% ±0.6% of the virus titre and compound **4** reduced 95% ±2% (Figure 4).

Effects on HSV-1 DNA replication and transcription

In order to investigate whether these compounds may alter HSV-1 DNA replication and transcription, we performed qPCR during acute infection in HFL cells at 8 hpi, when the compounds showed significant antiviral activity. The number of DNA relative quantity was similar in the treated and untreated groups (Figure 5A), which means that the compounds did not affect DNA replication.

After production of cDNA from the RNA extraction, qPCR was performed for *ICP0* and *ICP4* (immediate early genes), *UL30* and *TK* (early genes) and *ICP34.5* and *gC* (late genes). Compounds **3** and **4** were able to suppress significantly the transcription of the immediate early genes *ICP0* and *ICP4*, and the late gene *gC*. Compound **4** also reduced significantly the transcription of *UL30* and *ICP34.5* (Figure 5B).

Figure 3. Viral titre on intra- and extracellular content to evaluate the effect of the compounds on the virus egress from cells



Different letters are significant (P<0.001). Error bars represent standard deviations. n=3. PFU, plaque-forming units.

Discussion

This study evaluated a series of 1,2,3-triazole derivatives, which showed notable antiviral activity against HSV-1 17*syn*+, and the most promising compounds were **3** and **4**. Compound **4** caused low cytotoxicity and showed the highest SI (SI=CC_{50}/EC_{50}), which is a pharmaceutical parameter to determine the security range for *in vitro* use. Compound **3** had moderate cytotoxicity, although its CC_{50} was more than 17-fold the EC_{50}, offering a security range to manage dosing.

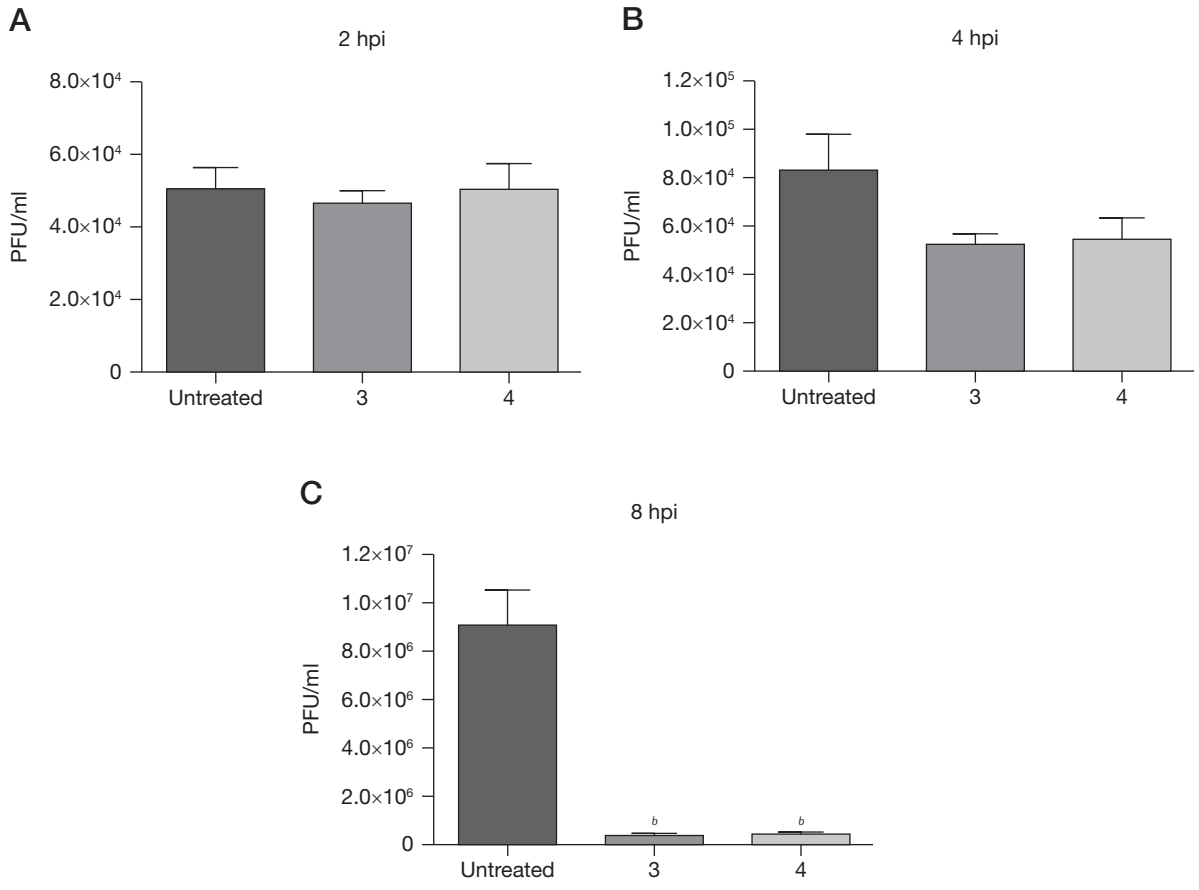
Similarly, other triazole derivatives were evaluated for anti-HSV-1 activity. Arylsulfonylhydrazide-1,2,3-triazoles derivatives presented EC_{50} between 1.3 and 37 μM on viral infection *in vitro* [32]. Pandey *et al.* [33] found 2,3,4-triazole derivatives with EC_{50} between 150 and 250 μg/ml. In another study, among 18 1,2,4-triazole derivatives, only one showed promising antiviral activity against HSV-1, with 88% reduction in plaque formation at 50 μg/ml [34]. However, none of these studies have addressed the possible mechanisms of action of these compounds.

Compounds **3** and **4** were also tested on a clinical ACV-resistant strain, HO-1, and compound **3** had satisfactory antiviral activity against this strain. This fact demonstrates the importance of searching for new compounds with antiviral activity that could serve as alternative treatments of resistant strains, which are prevalent especially in immunocompromised patients [12–14]. Additionally, we suggest that compound **3** may have a different mechanism of action from ACV and other nucleosides analogues.

In order to understand the mode of action of these compounds responsible for the antiviral effect, we performed experiments, such as virucidal assay, time-course, egress assay and qPCR. We verified that the compounds did not have virucidal activity because the pretreatment of viruses did not result in reduction of plaques, suggesting the compounds did not affect the virion envelope structure, and the free viruses were not sensitive to them. Also, we can infer that the molecules did not affect the processes of adsorption and entry into host cells.

The results from the time-course assay showed the both compounds had effective antiviral activity at 8 hpi. The cellular replication cycle of HSV takes 3–12 h [35], thus after 8 h most of the viral particles might be in the late phase of the lytic infection. In this phase, there is, predominately, the production of the proteins from tegument and envelope, and proteins for the assembly of the viral particle [36].

Figure 4. Time-course for viral titre[a]



[a]Multiplicity of infection 1.0 on HFL cells. [b]Statistical significance (Tukey's Test): $P<0.001$. **(A)** 2 h post-infection (hpi). **(B)** 3 hpi. **(C)** 8 hpi. Error bars represent standard deviations. $n=3$.

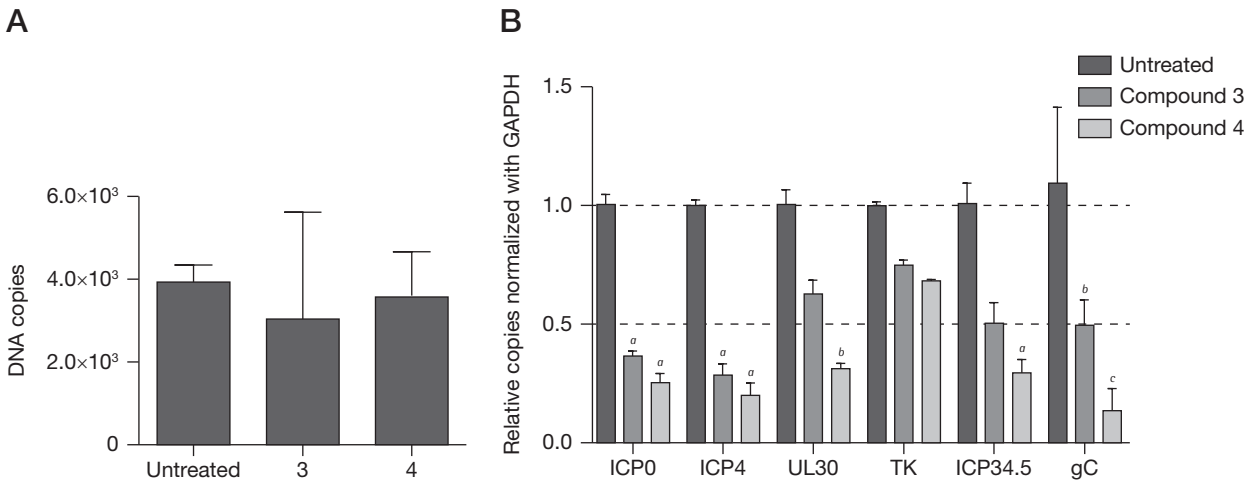
Our compounds did not affect DNA replication. This is the main mechanism of action of ACV and its analogues, which inhibit the DNA polymerase and interrupt viral DNA elongation [10]. Thus, this further supports that the 1,2,3-triazole derivatives presented in this study have a different mechanism of action from the current anti-HSV-1 drugs.

1,2,3-Triazole derivatives showed efficacy on gene transcription suppression. Compounds **3** and **4** had significant activity on the immediate early genes *ICP0* and *ICP4*, which are upstream genes of the expression cascade of viral genes and their proteins regulate the transcription of genes from the following phases [37]. Both compounds also reduced significantly the expression of *gC* gene. gC is one of the glycoproteins of the viral envelope that is produced during the late phase and it is involved in the initial attachment phase through the binding to glycosaminoglycan of the plasma membrane [38].

Compound **4** also had significant activity on the expression of *UL30* and *ICP34.5*. *UL30* is a gene of the early phase of the lytic infection and with *UL42* encode the viral DNA polymerase [39], an enzyme that catalyses the elongation of viral DNA and it is the main target for anti-HSV drugs [10]. The suppression of UL30 gene is relevant for the development of a new antiviral agent that will be able to have a possible synergic effect with ACV. Inhibiting the *ICP34.5* gene is also interesting as an antiviral candidate because this is a major viral neurovirulence factor and is essential for efficient viral replication in neurons [40]. Furthermore, previous studies reported the importance of ICP34.5 in the viral spread and in the progress from lytic infection to latency and reactivation [41,42].

Additionally, in the egress assay, we noted that compound **3** induced the highest reduction of extracellular virus titre and the intra- and extracellular ratio was 9.3, whereas it was 1.8 in the untreated group,

Figure 5. Quantitative PCR assays for HSV-1 cDNA copy number normalized with GAPDH



Normalized copies amount relative to untreated group 2 -ΔΔCt [46]. Error bars represent standard deviations. *n*=3. Statistical significance (Tukey's Test): [a]*P*<0.01; [b]*P*<0.05; [c]*P*<0.001. HSV-1, herpes simplex virus type 1.

suggesting that this compound interfered with virus release from the cells or in some maturation process. It is plausible that this compound has extensive activity in the late phase during which structural proteins that compose the virion are produced [43]. Consequently, this compound may be interfering in the previous step of egress, the envelopment of capsids by Golgi vesicles, which is mediated by interactions between tegument proteins and cytoplasmatic portions of viral glycoproteins binding to the Golgi membrane [44]. Although the mechanism of action of drugs that disturb viral egress is unclear, brefeldin A, a drug that inhibits transport from the endoplasmic reticulum to the Golgi could block HSV-1 nuclear egress [45]. Combined, these results suggest that compounds **3** and **4** have promising profiles as antiviral agents with possible targets related to transcription and the viral egress for compound **3**.

In conclusion, we presented a series of 1,2,3-triazole derivatives with antiviral activity and compounds **3** and **4** were the most promising. Our findings revealed that antiviral activity of these compounds is closely related to the inhibition of the expression of some important genes such as *ICP0*, *ICP4* and *gC*. Compound **3** also affected the release of the viral particle from the host cell and showed activity against the ACV-resistant HSV-1 strain. These results suggest that the mechanism of action of 1,2,3-triazole derivatives is distinct from those of ACV and its analogues, which makes them even more interesting for antiviral development because they might be an alternative for HSV-1 treatment, particularly for resistant strains.

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Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: Further information on ^{1}H NMR and ^{13}C NMR spectra of all the final compounds can be found at https://www.intmedpress.com/uploads/documents/4830_Viegas_Addfile_1.pdf

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