Inhibition of Functionalized 1,3-Dienes against *Trypanosoma cruzi*

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Six functionalized 1,3-dienes were synthesized using cross-coupling reactions, catalyzed by palladium complexes, between alkenylboronic acids and α-bromo-α,β-unsaturated carbonylic compounds. Their cytotoxicity against epimastigotes of *Trypanosoma cruzi* and fibroblastic Vero cells was evaluated, using concentrations ranging from 100 μM to 2.5 mM in experiments with three incubation times (4, 8 and 16 h). These tests were performed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric bioassays and its further reduction to formazan, according to the viability of the parasites and cells. With the exception of (5*E*,6*E*)-5-benzylidene-2-methylundec-6-en-4-one, all compounds were cytotoxic to both *Trypanosoma cruzi* and Vero cells, however differential values of IC_{50} were observed for two of these compounds. A possible structure-activity relationship is discussed.

Key words: 1,3-Dienes, *Trypanosoma cruzi*, Cytotoxicity

Introduction

A wide variety of isolated and synthetic compounds have been tested for cytotoxic activity, in a way to find new chemical agents to fight numerous diseases. Many compounds from a broad spectrum of chemical structures and functional groups have been evaluated, comprising isolated natural products from plants or marine organisms and synthetic products derived or not from natural sources (Araya *et al*., 2003; Makarieva *et al*., 2002; Teeyapant *et al*., 1993). Phenothiazines, naphthoquinones, alkaloids, isocoumarins, thiadiazines, and diterpenes are among the wide range of chemical compounds tested in a variety of cell types (*e.g.* Devienne *et al*., 2002; Muelas *et al*., 2002; Scio *et al*., 2003). Some of the evaluated compounds seem to be promising drugs, but their potential is limited by the need of high concentrations (Sepúlveda-Boza and Cassels, 1996). As 1,3-diene moieties are present in the carbon skeleton of many organic molecules, and stereospecific syntheses have been an important tool to obtain conjugated alkadienes (Still and Simpson, 1987), it seems a valid approach to evaluate the cytotoxicity of a group of functionalized 1,3-dienes against fibroblastic Vero cells and epimastigote forms of *T. cruzi*.

In a preliminary communication, Urdaneta *et al*. (1994) reported the first example of cross-coupling reaction between 1-(*E*)-alkenylboronic acids and α-bromo-α,β-unsaturated carbonylic compounds using palladium as catalyst, in a search for an efficient procedure to synthesize functionalized 1,3-conjugated dienes. These reactions have been used successfully in the synthesis of pheromones, retinoids and antibiotics (Kobayashi *et al*., 1987; Roush and Sciotti, 1994).

Trypanosoma cruzi, the causative agent of Chagas disease, constitutes a serious health problem in eighteen countries in Latin America, according to the World Health Organization (WHO, 2003). Although benznidazole is used with a relative success in chagasic patients, there is no effective treatment of this disease (Sepúlveda-Boza and Cassels, 1996), and even though an important number of scientific literature has covered the effects of different chemical agents against *T. cruzi*, the search for new compounds continues.

In this study, six new 1,3-functionalized dienes were synthesized in order to evaluate their *in vitro* cytotoxicity against epimastigote forms of *T. cruzi* as well as in fibroblastic Vero cells during different incubation times. They were synthesized using cross-coupling reactions, catalyzed by palladium complexes, between 1-(*E*)-alkenylboronic acids

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and α-bromo-α,β-unsaturated carbonylic compounds. A family of compounds with cytotoxic properties is reported here, and a possible structure-activity relationship is discussed.

Materials and Methods

Syntheses

The compounds were synthesized using cross-coupling reactions catalyzed by palladium complexes, according to Fig. 1, with the exception of (2*E*,3*E*)-2-benzylidene-4-phenylbut-3-en-1-ol (**4**) obtained by reduction of 1,4-diphenyl-2-(carboxaldehyde)-(1*E*,3*E*)-butadiene (**3a**) with $NaBH_4$ in 95% ethanol at 0 °C.

General procedure: A 25 ml flask equipped with a reflux condenser and a magnetic stirrer was loaded with 64 mg of $Pd(PPh_3)_4$ (0.06 mmol), then the system was purged with argon, and the catalyst was dissolved in 3.0 ml of THF. 2.0 mmol of α-bromo carbonylic compound dissolved in 3.0 ml of THF were added to the solution, and stirred during 20 min. 2.10 mmol of 1-(*E*)-alkenylboronic acid dissolved in 3.0 ml of THF were added, followed by $NaHCO_3$ (4.0 eq) dissolved in 3.0 ml of water. The mixture was stirred under reflux during 9 h, and then allowed to cool to room temperature. The reaction mixture was diluted with water followed by the addition of 5.0 ml of a saturated solution of NH_4Cl, extraction with three portions of 10 ml of Et_2O and then washed with a saturated solution of NaCl, and finally dried over Na_2SO_4. The organic phase was filtered and concentrated under reduced pressure. The product was purified by column chromatography on silica gel using a mixture of hexane/ethyl acetate (92:8 v/v) as eluent. All coupled compounds were obtained as clear yellow oils.

1,4-Diphenyl-2-(carboxaldehyde)-(1E,3E)-butadiene (**3a**)

68%; 1*E*,3*E* > 98% according to ^{1}H NMR. – ^{1}H NMR ($CDCl_3$, δ in ppm, *J* in Hz): δ = 9.76 (s, 1H, –CHO), 7.64–7.27 [m, 12H, $C_6\mathbf{H}_5C\mathbf{H}$=C(CHO)–, *J* = 17, –CH=C**H**$C_6\mathbf{H}_5$], 7.01–6.96 (d, 1H, *J* = 17, CH=CHC_6H_5). – MS (70 eV): *m*/*z* (%) = 234 (40), 205 (100), 190 (13), 178 (10), 165 (8), 128 (23), 115 (15), 91 (41), 77 (15). – IR: ν = 1696 (C=O), 1637, 1604 cm^{-1} (C=C). – UV: λ_{max} = 207 nm (ε = 18,600).

1,4-Diphenyl-2-(methylcarboxylate)-(1E,3E)-butadiene (**3b**)

69%; mixture of 1*E*,3*E* and 1*Z*,3*E* (68:32) according to ^{1}H NMR. – ^{1}H NMR ($CDCl_3$, δ in ppm): δ = 7.45–7.24 [m, 12H, $C_6\mathbf{H}_5C\mathbf{H}$=C($CO_2CH_3$)–, –CH=C**H**$C_6\mathbf{H}_5$], 7.10–7.09 (d, 1H, –C**H**=CHC_6H_5), 3.89 (s, 3H, –OCH_3). – MS (70 eV): *m*/*z* (%) = 264 (20), 231 (8), 205 (100), 190 (9), 155 (5), 134 (10), 121 (13), 101 (13), 77 (11), 51 (5). – IR: ν = 1721 (C=O), 1643, 1598 (C=C), 1233 cm^{-1} (C–O). – UV: λ_{max} = 323 nm (ε = 17,600).

1-Phenyl-2-(ethylcarboxylate)-(1E,3E)-undecadiene (**3c**)

70%. – ^{1}H NMR ($CDCl_3$, δ in ppm): δ = 7.60–7.36 [m, 6H, $C_6\mathbf{H}_5C\mathbf{H}$=C($CO_2CH_2$ CH_3)–], 6.29–6.27 [m, 2H, –C**H**=C**H**–CH_2–$(CH_2)_5CH_3$], 4.30–4.26 (m, 2H, –O$C\mathbf{H}_2CH_3$), 2.15–2.13 [m, 2H, =CH–C$\mathbf{H}_2$–$(CH_2)_5CH_3$], 1.37–1.27 [m, 13H, –$(CH_2)_5CH_3$], 0.89–0.86 (t, 3H, –CH_3). – MS (70 eV): *m*/*z* (%) = 300 (77), 271 (5), 255 (10), 227 (14), 215 (16), 173 (23), 141 (32), 129 (100), 115 (28), 91 (27), 57 (88).

(2E,3E)-2-Benzylidene-4-phenylbut-3-en-1-ol (**4**)

80%. – ^{1}H NMR ($CDCl_3$, δ in ppm, *J* in Hz): δ = 7.32–7.40 (m, 6H, arom.), 7.11–7.31 (m, 5H, $C_6\mathbf{H}_5$), 6.81–6.88 (d, 2H, =CH, *J* = 16), 4.57 (s, 2H, –CH_2–), 2.34 (1H, –OH). – MS (70 eV): *m*/*z* (%) = 236 (20), 218, (5), 205 (100), 127 (9), 115 (10), 91 (4), 77 (11).

1-Phenyl-2-(methylcarboxylate)-(1E,3E)-octadiene (**3d**)

78%; 1*E*,3*E* > 98% according to ^{1}H NMR. – ^{1}H NMR ($CDCl_3$, δ in ppm, *J* in Hz): δ = 7.40–7.33 [m, 6H, $C_6\mathbf{H}_5C\mathbf{H}$=C($CO_2CH_3$)], 6.51–6.26 (m, 2H, –CH=CHCH_2–), 3.82 (s, 3H, –CO_2CH_3), 2.16–2.11 (m, 2H, –CH=CHC$\mathbf{H}_2$–), 1.39–1.34 [m, 4H, =$CHCH_2(C\mathbf{H}_2)_2CH_3$], 0.92–0.87 (t, 3H, *J* = 7, –CH_3). – MS (70 eV): *m*/*z* (%) = 244 (17), 213 (6), 201 (13), 187 (38), 169 (23), 155 (47), 141 (77), 129 (100), 121 (34), 115 (92), 102 (13), 91 (81), 72 (43), 65 (30), 59 (81), 41 (58). – IR: ν = 1723 (C=O), 1643, 1595 (C=C), 1235 cm^{-1} (C–O). – UV: λ_{max} = 220 nm (ε = 12,600).

(5E,6E)-5-Benzylidene-2-methylundec-6-en-4-one (**3e**)

50%. – ^{1}H NMR ($CDCl_3$, δ in ppm): δ = 5.96–5.98 (m, 1H), 2.62–2.63 (d, 2H, $-CH_2-$), 2.12–2.28 (m, 4H, $-CH_2-$), 1.25–1.42 (m, 6H, $-CH_2-$), 0.95–0.97 (d, 6H, $2CH_3$), 0.88–0.92 (t, 3H, $-CH_3$). – MS (70 eV): *m/z* (%) = 270 (28), 255 (4), 227 (26), 213 (20), 143 (10), 128 (11), 115 (12), 91 (18), 85 (17), 57 (88).

Parasites

Epimastigote forms of *T. cruzi* (EP strain) were grown at 27 °C in liver infusion tryptose (LIT) medium, supplemented with 10% fetal bovine serum and 2% w/v of antibiotics (gentamicins and penicillins). Once in the logarithmic phase, they were collected and seeded into 96-well plates (Costar) (1×10^6 parasites/well) in minimum essential medium (MEM), with and without the test compounds during different incubation times (4 h, 8 h and 16 h). All assays were carried out twice, three replicates per assay.

Cells

Fibroblastic Vero cells were grown in Petri dishes in MEM, in a humidified 5% CO_2/95% air atmosphere at 37 °C. After washing twice with phosphate buffered saline (PBS) solution, the cells were dissociated by trypsinization, and aliquots with 2×10^4 cells/ml were seeded into 96-well tissue culture plates (Nunclon). After a 24 h period, the medium was removed, and the cells were incubated with and without the compounds as described below.

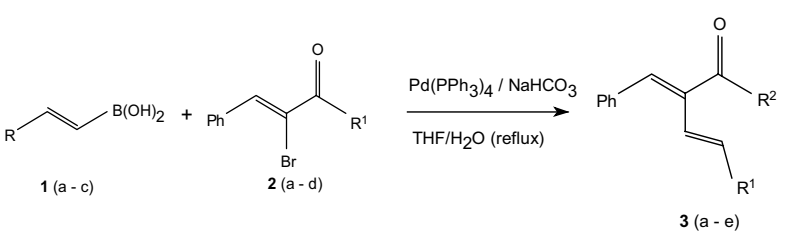
MTT assays

The chemical compounds were dissolved in DMSO, and dilutions with PBS solution were done to obtain different test concentrations ranging from 100 μM to 2.5 mM. Benznidazole (Rochagan®, Roche, Brazil) was used as a control with the same concentrations as the test compounds. The final concentration of DMSO (less than 1% for the highest concentration of 2.5 mM) did not affect parasite or cell viability. 50 μl of each dilution were added to the wells and after an incubation period (4 h, 8 h, or 16 h) with the compounds, 35 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phenazine methosulphate (PMS) solution (Muelas-Serrano *et al.*, 2000) (2.5 mg/ml of MTT with 0.22 mg of PMS/ml) were added to each well, followed by an additional incubation time of 75 min. Then 50 μl of sodium dodecyl sulphate (SDS) solution (20% in 0.01 M HCl) were added to dissolve the blue formazan crystals, followed by another incubation period of 75 min. The sample absorption was read on a scanning multiwell spectrophotometer (Biorad model 450) at 595 nm.

Results and Discussion

Syntheses

An efficient method for a stereo-controlled synthesis of these novel 1,3-functionalized dienes was developed using a palladium complex as catalyst in coupling reactions between 1-(*E*)-alkenylboronic acids and α-bromo-α,β-unsaturated carbonylic compounds (Fig. 1). The α-bromo-α,β-unsaturated carbonylic compounds were prepared according to procedures found in the literature (Kowalsky *et al.*, 1982). Bromination/dehydrobromination of



1 (a - c) **2** (a - d) **3** (a - e)

R = *n*-Bu: **1a**
R = *n*-Hept: **1b**
R = Ph: **1c**

R^1 = H: **2a**
R^1 = OMe: **2b**
R^1 = OEt: **2c**
R^1 = *i*-Bu: **2d**

R^1 = Ph; R^2 = H: **3a**
R^1 = Ph; R^2 =OMe: **3b**
R^1 = *n*-Hept; R^2 =OEt: **3c**
R^1 = *n*-Bu; R^2 = OMe: **3d**
R^1 = *n*-Hept; R^2 = *i*-Bu: **3e**

Fig. 1. Scheme of synthesis of functionalized 1,3-dienes **3a**–**3e** (see text for synthesis of compound **4**).

trans-cinnamaldehyde and *trans*-methylcinnamate produced (*Z*)-α-bromo cinnamaldehyde (79%) and (*Z*)-α-bromo methylcinnamate (70%), respectively. Due to low selectivity in producing the 1*E*,3*E* isomer, an additional way to obtain (*Z*)-α-bromo methylcinnamate was carried out, oxydizing (*Z*)-α-bromo cinnamaldehyde with SeO_2 and H_2O_2 in *t*-BuOH with higher yields and a purity >99%. For the final reaction, 1-(*E*)-alkenylboronic acids were synthesized by hydroboration with catecholborane of the correspondent alkynes: 1-hexyne, 1-nonyne and phenylacetylene (Brown and Gupta, 1975), producing 1-(*E*)-alkenylboronic esters, which then were hydrolyzed to obtain 1-(*E*)-hexenylboronic acid (80%), 1-(*E*)-nonenylboronic acid (80%) and 1-(*E*)-styrylboronic acid (78%), respectively.

Styrylboronic acid was treated with α-bromo methylcinnamate in the presence of tetrakis(triphenylphosphine)Pd(0) and potassium bicarbonate using as solvent a mixture of THF/H_2O, producing compound **3b**, 1,4-diphenyl-2-(methylcarboxylate)-(1*E*,3*E*)-butadiene, along with its isomer 1*Z*,3*E* in the proportion 68:32.

Compound **3d**, 1-phenyl-2-(methylcarboxylate)-(1*E*,3*E*)-octadiene, was obtained from the reaction of α-bromo methylcinnamate and 1-(*E*)-hexenylboronic acid with $Pd(PPh_3)_4$ as catalyst and sodium bicarbonate, using a mixture of THF/H_2O as solvent. Additionally to compound **3d**, its isomer 1*Z*,3*E* was also obtained (87:13).

In order to obtain compound **3a** with a higher yield, and knowing that the base could play an important role in the coupling reaction of these substrates, $NaHCO_3$ was used in the reaction of α-bromo cinnamaldehyde and 1-(*E*)-styrylboronic acid, in addition to $Pd(PPh_3)_4$ (3% mol) and a mixture of THF/H_2O as solvent. The yield was 68% and the isomer 1*E*,3*E* was the main product (>98%).

Cytotoxicity assays

The Vero cells and *T. cruzi* epimastigotes were seeded and incubated with the different compounds under study for a 16 h period, in a concentration range from 100 μM to 2.5 mM. After this period of incubation, the cytotoxic effect of the different compounds was determined by the MTT assay (Muelas-Serrano *et al.*, 2000). As can be observed in Fig. 2, with the exception of compound **3e**, all the other compounds increased the cytotoxicity in a dose-dependent manner as compared with the control signal, indicating that the mitochondrial function and growth of parasites and cells were depressed by these compounds.

In both cases, a steep inhibition was observed between 100 μM and 500 μM concentration of the different active compounds. Therefore, further experiments were conducted with Vero cells and parasites using this range of concentration (Fig. 3), and the inhibitory concentration (50%; IC_{50}) was calculated for all active compounds (Table I). The IC_{50} values obtained were different for Vero cells

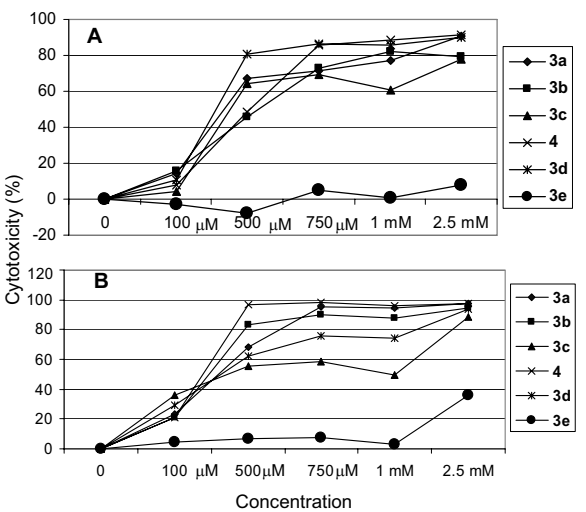


Fig. 2. Percentage of mean cytotoxicity of compounds in the concentration range from 100 μM to 2.5 mM (A) against *T. cruzi* epimastigote forms and (B) in fibroblastic Vero cells (16 h incubation time).

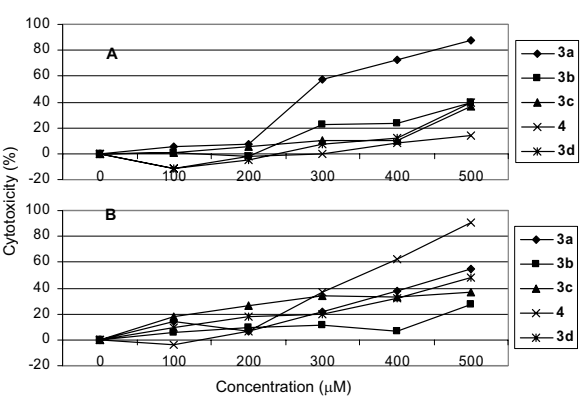


Fig. 3. Percentage of mean cytotoxicity of compounds in the concentration range from 100 μM to 500 μM (A) against *T. cruzi* epimastigote forms and (B) in fibroblastic Vero cells (16 h incubation time).

Table I. Cytotoxicity of functionalized 1,3-dienes expressed in inhibitory concentration (IC_{50}) to epimastigote forms of *T. cruzi* and to fibroblastic Vero cells (in parenthesis).

Compound	IC_{50} [μM] (mean ± sd)
1,4-Diphenyl-2-(carboxaldehyde)- (1*E*,3*E*)-butadiene (**3a**)	315 ± 22 (489 ± 95)
1,4-Diphenyl-2-(methylcarboxylate)- (1*E*,3*E*)-butadiene (**3b**)	684 ± 59 (ns)
1-Phenyl-2-(ethylcarboxylate)- (1*E*,3*E*)-undecadiene (**3c**)	840 ± 150 (ns)
(2*E*,3*E*)-2-Benzylidene-4-phenylbut-3-en-1-ol (**4**)	1112 ± 250 (367 ± 80)
1-Phenyl-2-(methylcarboxylate)- (1*E*,3*E*)-octadiene (**3d**)	797 ± 180 (529 ± 120)
(5*E*,6*E*)-5-Benzylidene-2-methylundec-6-en-4-one (**3e**)	* (**)

* Did not cause an effect at any tested concentration.
** Only caused an effect at the higher concentration (2.5 mM), but more than 50% cytotoxicity.
ns: not significant.

and parasites. As can be observed in Table I, for *T. cruzi* epimastigotes the lowest IC_{50} was observed for compound **3a**, 1,4-diphenyl-2-(carboxaldehyde)-(1*E*,3*E*)-butadiene (315 μM). In accordance with this result, compound **3a** was the only to show cytotoxic effects at lower incubation periods (32% at 4 h; 57% at 8 h), contrary to compounds **3b**–**3d**, **4** which were cytotoxic only after a 16 h incubation of these compounds with *T. cruzi* epimastigotes. The IC_{50} value of compound **3a** for Vero cells (489 μM) was higher than the value obtained for epimastigotes. The lowest IC_{50} value for these cells was obtained for compound **4,** (2*E*,3*E*)-2-benzylidene-4-phenylbut-3-en-1-ol (367 μM), observing an opposite effect in *T. cruzi* epimastigotes (1112 μM).

Previous studies showed that the cytotoxic effect of synthetic compounds is directly related with their lipophilicity (*e.g.* Kajiya *et al.*, 2001; Devienne *et al.*, 2002). The 1,3-dienes evaluated in this study can be divided in two groups according to their structural characteristics: compounds **3a**, **3b** and **4** have a carbon skeleton that is based on a C_4 chain, in contrast, compounds **3c** and **3d** are structured in base on C_8 and **3e** on C_{11}. For compounds **3a**, **3b** and **4**, the order of lipophilicity is **4** < **3a** < **3b**, due to the presence, in compounds **4** and **3a**, of a hydroxy group and an aldehyde group, respectively. It is interesting to note that in accordance with this, the degree of lipophilicity of these three compounds correlates with their cytotoxic activity. In *T. cruzi* epimastigotes, as can be concluded from the IC_{50} values, the order is similar, that is **4** < **3b** < **3a**. The different IC_{50} values obtained for compounds **3b** and **3a** could be due to the smaller steric effect of the aldehyde group in compound **3a** compared to the carboxylate group in compound **3b**. However, in the case of Vero cells compound **4**, although less lipophilic than compounds **3b** and **3a**, is the more active. This could be explained by different interactions of these chemically related compounds with both types of cells.

Another factor to be considered is that biological activity is not only related to hydrophobicity, but is the sum of electrostatic and steric effects of the molecule. This could explain the lack of cytotoxic effects observed for compound **3e**. This compound is the only functionalized 1,3-diene that has an additional larger and branched lateral carbon chain. In conclusion, we have synthesized six functionalized 1,3-dienes with cytotoxic activity to *T. cruzi* epimastigotes and fibroblastic Vero cells, observing differential values of IC_{50} for two of these compounds.

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