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In Vitro and *In Vivo* Studies of the Trypanocidal Activity of Four Terpenoid Derivatives against *Trypanosoma cruzi*

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Abstract. Four terpenoid derivatives were examined for their activity against *Trypanosoma cruzi.* Our results show that two compounds were very active *in vitro* against both extra- and intracellular forms. These compounds, non-toxic for the host cells, are more effective than the reference drug benznidazole. The capacity to infect cells was negatively affected and the number of amastigotes and trypomastigotes was reduced. A wide range of ultrastructural alterations was found in the epimastigote forms treated with these compounds. Some metabolic changes occurred presumably at the level of succinate and acetate production, perhaps caused by the disturbance of the enzymes involved in sugar metabolism inside the mitochondria. *In vivo* results were consistent with those observed *in vitro*. The parasitic load was significantly lower than in the control assay with benznidazole. The effects of these products showed the reduction of the anti-*T. cruzi* antibodies level during the chronic stage.

INTRODUCTION

Tropical and subtropical diseases caused by protozoal parasites remain a major public health problem in many of the less developed countries of the world, because of the lack of effective drugs or increasing resistance against the few affordable drugs available.[1] American trypanosomiasis, also known as Chagas disease, is one of the most devastating parasitic diseases. It is caused by the kinetoplastid protozoan *Trypanosoma cruzi*, which is vectorially transmitted by a hemiptera depositing feces on the skin surface, containing metacyclic forms, after the blood meal. Other pathways of infection include contaminated blood transfusions, organ transplants, oral contamination caused by food/beverage,[2,3] and even transmission from mother to child during pregnancy or breastfeeding.[4]

Chagas disease manifests itself in the form of an acute infection, during which most patients do not know that they are infected. Actually, the acute phase can be asymptomatic but also the Romana's sign can be present in around 15% of the cases. Only a 30–40% of acute individuals undergo further development and it becomes chronic and systemic, severely affecting the heart, esophagus, and colon.[5] Endemic throughout Latin America, it is the third most widespread tropical disease after malaria and schistosomiasis, according to the World Health Organization (WHO).[5]

An estimated 10 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic. More than 25 million people are at risk of the disease. It is estimated that in 2008 Chagas disease killed more than 10,000 people[6]; unfortunately, current treatment of this disease is very limited, and no successful vaccine has been developed.[7]

Available drugs are mainly nitroheterocyclic compounds such as nitrofuranenifurtimox or the nitroimidazole derivative benznidazole (BZN), but they have proved effective only against the acute phase, exhibiting very limited efficacy in the chronic stage.[8] Furthermore, they are quite toxic, causing severe side effects such as pancreatitis and cardiac toxicity.[2] The search for more effective drugs focuses mainly on their potential action over essential and exclusive components of the trypanosomatids.

The use of natural products as new chemotherapeutic agents has been investigated for some time. Among these, terpenoids, an important group of secondary metabolites, must be emphasized, mainly because of their structural diversity and natural abundance. During the last few years trypanocidal activities for some triterpenes[9] and diterpenes, with *ent*-kaurene,[10] pimarane,[11] and spongiane skeleton,[12] have been reported. Recently, the trypanocidal activity of several oxygenated abietane diterpenoids has been described.[13] Diterpene resin acids are important defense compounds from conifers against potential herbivores and pathogens.[14] The biological activity of natural abietane acids has been reviewed.[15] In recent years, interest in these types of terpenoids has increased as a result of the isolation of compounds, mainly phenols and related derivatives, showing remarkable biological activities.[16–18] Other significant oxidized abietane diterpenes have shown strong inhibition of various human tumors and oncogen-transformed cells.[19] The widespread use of these agents has not yet been established, however, and chemotherapeutic armament against kinetoplastic parasites remains limited. New drug options are clearly needed to fight these pathogens.

Recently, our group synthesized abietane phenols 1–4[20,21] (Figure 1), and their antiproliferative *in vitro* and *in vivo* activities against *T. cruzi* (epimastigote, amastigote, and trypomastigote forms) have been investigated in this work. Unspecific mammal cytotoxicity of the most active compounds was evaluated *in vitro*, and less toxic derivatives have been submitted to *in vivo* experimentation in a more thorough study. Furthermore, we also included a nuclear magnetic resonance (^{1}H NMR) study concerning the nature and percentage of the excretion metabolites to gain information concerning the inhibitory effect of our compounds over the glycolytic pathway, because it represents the prime source of energy for the parasite. Finally, the effect of compounds on the ultrastructure of *T. cruzi* is considered the basis of transmission electronic microscopy (TEM) experiments.

MATERIALS AND METHODS

Chemical compounds. Compound 1, the methyl ester of 12-hydroxydehydroabietic acid, recently described as a new

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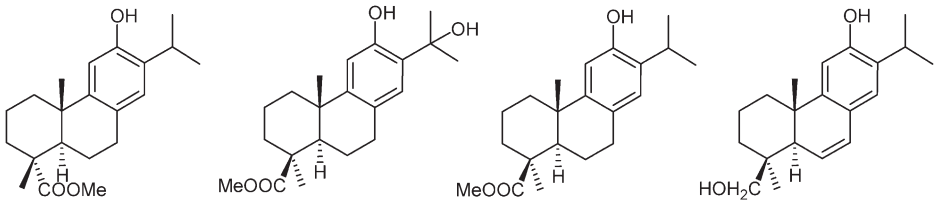

1 2 3 4

FIGURE 1. Terpenoid derivatives structure.

natural product,[22] has been synthesized from commercial abietic acid.[20]

Compounds 2–4 were prepared from *trans*-communic acid,[21] a labdane diterpene very abundant in some species of *Juniperus* and *Cupressus*. Compound 3 is the methyl ester of lambertic acid, isolated from *Podocarpus lambertius* and compound 4, 6,7-dehydroabieta-8,11,13-trien-12,19-diol, named sugikurojin A, is a new diterpene recently isolated from *Cryptomeria japonica*. Compound 2, methyl 12,15-dihydroxyabieta-8,11,13-trien-19-oate, has not yet been found in nature.

Parasite strain, culture. *Trypanosoma cruzi* SN3 strain of (IRHOD/CO/2008/SN3) was isolated from domestic *Rhodnius prolixus* and the biological origin is Guajira (Colombia).[23]

Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy-Nicolle-McNeal) supplemented with minimal essential medium and 20% inactivated fetal bovine serum and afterwards reseeded in a monophasic culture (MTL), following the method of Luque and others.[24]

Cell culture and cytotoxicity tests. Vero cells (Flow) were grown in RPMI (Gibco, Madrid, Spain) supplemented with l0% inactivated fetal bovine serum and adjusted to pH 7.2, in a humidified 95% air-5% CO_2 atmosphere at 37°C for 2 days. For the cytotoxicity test, cells were placed in 30 mL sterile polystyrene container (Deltalab, Barcelona, Spain), and centrifuged at 100 *g* for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1×10^5 cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μL/well and incubated for 2 days at 37°C in humid atmosphere enriched with 5% CO_2.

The medium was removed, and the fresh medium was added together with the product to be studied (at concentrations of 100, 50, 25, l0 and 1 μM). After 72 h of treatment, the cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide (PI) solution (100 μg/mL) was added and incubated for 10 min at 28°C in darkness. Afterward, 100 μL/well of fluorescein diacetate (FDA) (100 ng/mL) was added and incubated under the same conditions as above. Finally, the cells were recovered by centrifugation at 400 *g* for l0 min and the precipitate washed with phosphate buffer solution (PBS). Flow cytometric analysis was performed on a FACS Vantageflow cytometer (Becton Dickinson, Madrid, Spain). The live cells with their plasma membrane intact were associated with the green fluorescence, because of the effect of sterases on FDA. On the other hand, cells that had lost the membrane integrity and were dead allowed the penetration of the IP by passive diffusion and specifically bound to their DNA and then, fluoresce in the range of 580 nm. The percentage of viability was calculated in comparison to that of the control culture (infected but untreated cultures), and the IC_{50} (the concentration required to give 50% of inhibition) was calculated by linear-regression analysis from the Kc values at the concentrations used.

***In vitro* trypanocidal activity assay.** *Epimastigote assay.* The parasite suspension was obtained for the trypanocidal assay by concentrating the epimastigote culture in the exponential growth phase by centrifugation at 1,000 *g* for 10 min, whereupon the number of flagellates were counted in a hemocytometric chamber and distributed into aliquots of 5×10^5 parasites/mL. The compounds were dissolved in dimethyl sulfoxide at a concentration of 0.01%, after being assayed as non-toxic and without inhibitory effects on the parasite growth. The compounds were dissolved in the culture medium, and the dosages used were 100, 50, 25, 10, and 1 μM. After 72 h of incubation, the effect of each compound was evaluated by light microscopy, through the quantification of viable parasite using a Neubauer chamber.

Metacyclic trypomastigotes assay. Metacyclogenesis was induced by culturing parasites at 28°C in modified Grace's medium (Gibco) for 12 days as described previously.[25] Twelve days after cultivation at 28°C, metacyclic forms were counted to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

After the metacyclic forms were obtained, Vero cells were cultured in RPMI medium in a humidified 95% air-5% CO_2 atmosphere at 37°C. Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc, Roskilde, Denmark) with rounded coverslips on the bottom and cultivated for 2 days. Afterward, the cells were infected *in vitro* with metacyclic forms of *T. cruzi* at a ratio of 10:1. The compounds (IC_{25} concentrations, concentration required to give 25% of inhibition) were added immediately after infection and were incubated for 12 h at 37°C in a 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing, and the infected cultures were then grown for 10 days in fresh medium without drugs. Fresh culture medium was added every 48 h.

The activity of the compounds was determined from the percentage of infected cells and the number of amastigotes founded per cells infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of three separate determinations. The number of trypomastigotes in the medium was determined as described previously.[24]

Axenic amastigotes assay. Axenic amastigote forms of *T. cruzi* were cultured following the methodology described

previously by Moreno.[26] Thus, epimastigotes transformed into amastigotes after 3 days of culture in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/L β-alanine, 100 mg/L L- asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L MES, 0.4 mg/L hemin, and 10 mg/L gentamicin pH 5.4 at 37°C. The effect of each compound against 1×10^6 axenic amastigote forms/mL, was tested at 48 h using a Neubauer hemocytometric chamber. The inhibitory effect is expressed as IC_{50} values.

***In vivo* trypanocidal activity assay.** This experiment was performed with the permission of the ethical committee of the University of Granada, Spain. Groups of three BALB/c female mice (6 to 8 weeks of age; 25 g) maintained under standard conditions were infected with 1×10^5 *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: i) group 1: uninfected (not infected and not treated); ii) group 2: untreated (infected with *T. cruzi* but not treated); iii) group 3: uninfected (not infected and treated: with 1 mg/kg of body weight/day, for five consecutive days (7–11 post-infection) by the intraperitoneal route[27]; and iv) group 4: treated (infected and treated with 1 mg/kg of body weight/day for five consecutive days (7 to 11 post-infection) by the intraperitoneal route with the compounds and BZN).

Treatments were started 7 days after infection with the parasites. Compounds were administered in a similar way to that explained previously and at the same concentrations.

A blood sample (5 μL) drawn from the mandibular vein of each treated mouse was taken and diluted 1:15 (50 μL of citrate buffer and 20 μL of lysis buffer at pH 7.2). The parasites were counted by fields with the immersion objective. The number of bloodstream *T. cruzi* metacyclic forms were recorded every 2 days from 7 to 40 days post-infection. The number of metacyclic forms was expressed per 100 microscopic fields.

Circulating anti-*T. cruzi* antibodies were quantitatively evaluated at Days 40 and 120 post-infection by the use of an enzyme-linked immunoassay. The blood, diluted to 1:50 in PBS, was reacted with an antigen constituted by a soluble Fe-SODe from *T. cruzi* epimastigotes. The results are expressed as the ratio of the absorbance (*Abs*) of each serum sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.[28]

Metabolite excretion. Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} of the compounds (except for control cultures). After incubation for 96 h at 28°C, the cells were centrifuged at 400 *g* for l0 min. The supernatants were collected to determine excreted metabolites by ^{1}H-NMR spectroscopy as previously described.[29] The chemical displacements were expressed in parts per million (ppm), using sodium 2,2 dimethyl-2-silapentane-5-sulfonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with that described.[29]

Ultrastructural alterations. The parasites, at a density of 5×10^5 cells/mL, were cultured in their corresponding medium, containing the drugs at the IC_{25} concentration. After 96 h, the cultures were centrifuged at 400 *g* for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05M cacodylate buffer (pH 7.4) for 5 h at 4°C. Pellets were prepared for TEM (Zeiss model, Barcelona, Spain) following the technique of Luque.[24]

RESULTS

***In vitro* anti-*T. cruzi* and cytotoxicity evaluation**. The inhibitory effect of the new terpenoid Compounds 1–4 was measured at concentrations ranging from 1 to 100 μM on the *in vitro* growth of. *T. cruzi* epimastigotes.[30] The IC_{50} values registered after 72 h of exposure are shown in Table 1, including BZN as the reference drug. The trypanocidal activity of the derivatives was slightly higher (Compounds 1 and 2, IC_{50} 6.10 and 7.98 μM, respectively) or even slightly less (Compounds 3 and 4, IC_{50} 50.03 and 69.01 μM) with respect to that found for BZN (15.89 μM).

The cytotoxicity evaluation against mammalian cells by using Vero cells as the mammal-cell model (Table 1) showed that the four derivatives were much less toxic than BZN. On the other hand, the selectivity index calculated for the derivatives was about 20-fold (Compound 1) and 24-fold (Compound 2) higher than that of BZN. The SI values found for Compounds 3 and 4 were significantly lower than those found for Analogues 1 and 2.

Axenic amastigotes obtained following the technique described in the Materials and Methods section, were assayed to determine the IC_{50} against the four terpenoids, using BZN as the reference drug (Table 1). Compounds 1 and 2 proved the most effective with IC_{50} of 6.03 and 6.81 μM, respectively. When the SI was determined for Compound 2, it was 32-fold better than the reference drug, whereas Compound 1 was 23-fold better that the BZN again.

TABLE 1

In vitro activity, toxicity, and selectivity index found for the terpenoids derivatives on epimastigote and axenic-amastigote forms of *Trypanosoma cruzi**

Compound	Activity IC_{50} (mM)†		Toxicity on Vero cell IC_{50} (mM)‡	SI§	
	Epimastigote forms	Axenic amastigote forms		Epimastigote forms	Axenic amastigote forms
Benznidazole	15.89 ± 1.1	18.92 ± 1.1	13.60 ± 0.9	0.8	0.7
Compound 1	6.10 ± 0.2	6.03 ± 0.3	98.95 ± 6.2	16.2 (20)	16.4 (23)
Compound 2	7.98 ± 0.8	6.81 ± 0.1	154.66 ± 17.3	19.4 (24)	22.7 (32)
Compound 3	50.03 ± 6.6	30.44 ± 2.3	117.67 ± 21.8	2.4 (3)	3.9 (6)
Compound 4	69.01 ± 7.7	38.72 ± 3.6	163.14 ± 14.5	2.4 (3)	4.2 (6)

* Results are averages of three separate determinations.
† IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations used (1, 10, 25, 50, and 100 μM).
‡ On Vero cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at concentrations used (1, 10, 25, 50 and 100 μM).
§ Selectivity index = IC_{50} Vero cells/IC_{50} epimastigote and axenic amastigote forms of the parasite. In brackets: number of times that compound SI exceeds the reference drug SI.

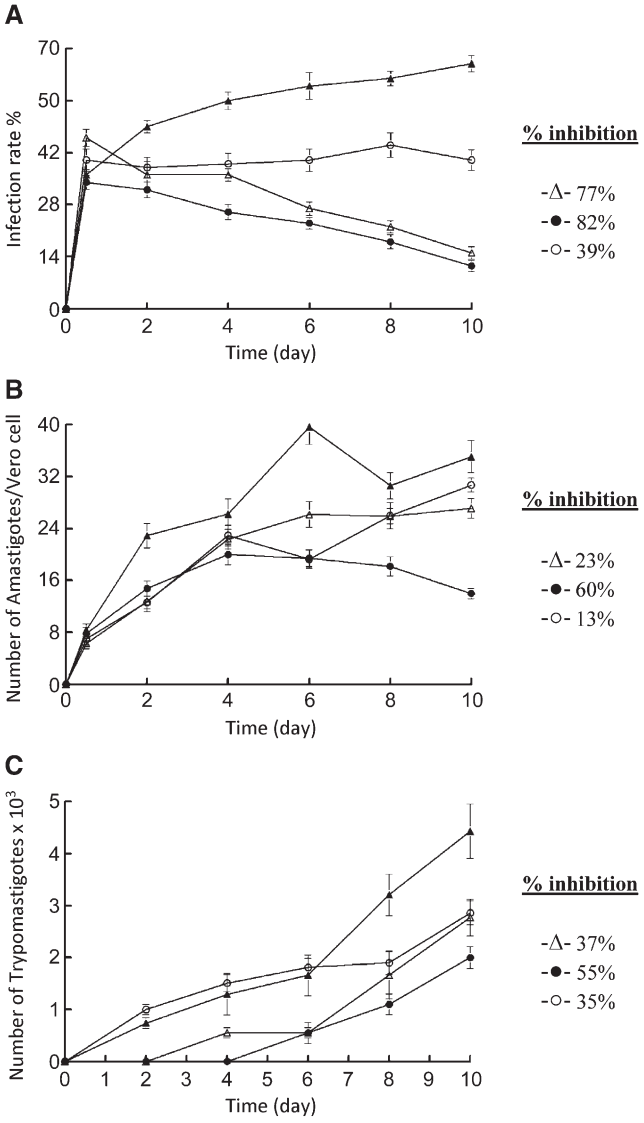

FIGURE 2. Effect of activity of terpenoid compounds on the infection rate and *T. cruzi* growth. (**A**) Rate of infection. (**B**) Mean number of amastigotes per infected Vero cell. (**C**) Number of trypomastigotes in the culture medium. Control (-▲-); benznidazole (BZN) (-○-); Compound **1** (-△-) and Compound **2** (-●-) (at IC_{25} conc.). The values are means of three separate experiments.

Compounds 3 and 4 did not reach values of SI ≥ 20-fold and therefore these compounds were not included in the subsequent studies.[31]

Because the metacyclic trypomastigote forms are responsible for the infection of the mammalian host, Compounds 1 and 2 were assayed against the metacyclic forms to evaluate the infection rate. When 1×10^4 Vero cells were incubated for 2 days and then infected with 1×10^5 metacyclic forms, obtained in the way described in the Materials and Methods section (control experiment; Figure 2A), the parasites invaded the cells and underwent the morphological transformation to amastigotes within 1 day after infection, following the parasite's normal life cycle. On Day 10, the rate of host-cell infection peaked. When Compounds 1 or 2 were added to the infected Vero cells with *T. cruzi* metacyclic forms (IC_{25} concentration), the infection rate significantly decreased with respect to the control, reaching 77% (Compound 1) and 82% (Compound 2) on Day 10 of the experiment. The infection-rate decline found using the compounds tested was substantially more pronounced than that measured for BZN (39%). Furthermore, another indication of the effectiveness of the infection-rate decrease was the average number of amastigotes per infected cell (Figure 2B) increased to 39.6 amastigotes/cell in the control experiment on Day 6, decreased to a value of 30.6 on Day 8, and increased again to 35.0 on Day 10. The break-up of Vero cells implies the transformation of amastigotes into trypomastigotes and release to spread the infection. Therefore, the variation of the trypomastigote number in the culture medium was also measured (Figure 2C) as a third way to test whether the infection rate was initially affected as a consequence of the exposure to the compounds. The control experiment afforded a trypomastigote number of 4.4×10^3 on Day 10, and reductions of 37% and 55% are found for Compounds 1 and 2, respectively. The reduction was higher for Compound 2 than that found for BZN (35%).

***In vivo* anti-*T. cruzi* evaluation.** The good results obtained *in vitro* with Compound 1 and 2 prompted us to study their *in vivo* activity in mice. Their impact on the two significant stages of Chagas disease was evaluated: the acute phase, considered until 60 days post-infection, and the chronic phase, from 60 days post-infection. It has been published that the intravenous doping route results in high mortality rates,[27] and therefore we used the intraperitoneal route, using a concentration of 5 mg/kg,[32] which did not result in any animal mortality. Female Swiss mice were inoculated intraperitoneally with 1×10^5 metacyclic trypomastigotes, and treatment began 7 days post-infection with the intraperitoneally route of 1 mg/kg/day of each compound for 5 days. Administration was performed using a saline solution. A group treated in the same manner with the vehicle (control) was included. During the study of the acute-phase activity, the level of parasitemia was determined every 2 days (Figure 3).

None of the animals treated with Compounds 1 and 2 died during the treatment, whereas the surviving percentage of the mice treated with BZN was 80%.

The data represented in Figure 3 show that the two compounds tested diminished the trypomastigote number on the day of maximum parasitic load, which was Day 25 post-infection, with respect to the control with untreated mice. On Day 40 post-infection a reduction of the parasitemia was found for the two compounds. From these data, the following order for *in vivo* activity could be established: Compound 1 ≈ Compound 2 > BZN.

Concerning the activity in the chronic phase, serological tests were performed 40 and 120 days post-infection (Table 2). Compounds 1 and 2 decreased antibody levels between Days 40 and 120, showing higher performance than did BNZ in this assay.

Studies on the action mechanism. To gain information on the possible mechanism of action of Compounds 1 and 2 on the parasite, we performed the following experiments:

Metabolite-excretion effect. For information concerning the effect of Compounds 1 and 2 on metabolite excretion, the ^{1}H-NMR spectra were registered. As a control, a culture of parasites untreated was used (Figure 4A). In this control experiment, *T. cruzi* excreted acetate and succinate as the major metabolites and, in a lower percentage, L-alanine. These data agree with those reported previously.[33] When the

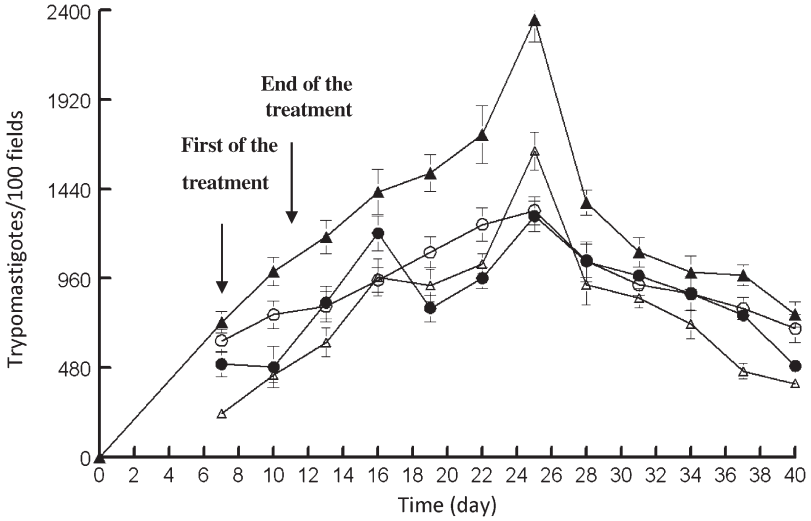

FIGURE 3. Parasitemia in the murine model of acute Chagas disease. Control (-▲-) and dose receiving 5 mg/kg of: Benznidazole (BZN) (-○-); Compound 1 (-△-) and Compound 2 (-●-). The values are means of three separate experiments.

trypanosomatids were treated with Compound 2, the excretion of some of these catabolites (mainly acetate) was clearly disturbed (Figure 4B) at the dosages assayed (IC_{25}). Similarly, succinate, L-alanine and ethanol levels rose in comparison with the control experiment.

Compound 1 provoked similar effects, although at a lesser magnitude (spectra not shown). The BZN did not trigger any alteration in the energy metabolism of the parasites (spectra not shown).

Ultrastructural alterations. The study of the ultrastructural alterations caused by Compounds 1 and 2 in the *T. cruzi* epimastigotes reflected notable changes in parasites, as reflected in (Figure 5B, C, and D), with respect to the control cultures (Figure 5A). Both compounds induced ultrastructural alterations. The most evident changes were the destruction of the treated parasites. Furthermore, there was evident intense vacuolization in a high number of parasites. Compound 2 triggered the dilatation of the kinetoplast of some parasites (Figure 5D).

DISCUSSION

In most studies on activity assays of new compounds against parasites, forms that develop in invertebrate host are used because they are easier to handle *in vitro*, but a preliminary test using extracellular epimastigote forms should always be complemented by a subsequent evaluation using

TABLE 2

Differences in the level of anti-*Trypanosoma cruzi* antibodies between Days 40 and 120 post-infection for Compounds **1** and **2** and benznidazole (BZN), expressed in absorbance units (abs)

Compounds*	A†
Control (untreated)	0,206 ± 0.07
BZN	0,116 ± 0.03
Compound 1	−0.237 ± 0.01
Compound 2	−0.005 ± 0.00

*1 mg/kg/day, intraperitoneal route administered during 5 days (see Material and Methods).
† ΔA = absorbance at 490 nm, Day 120 p.i. absorbance at 490 nm, day 40 p.i.

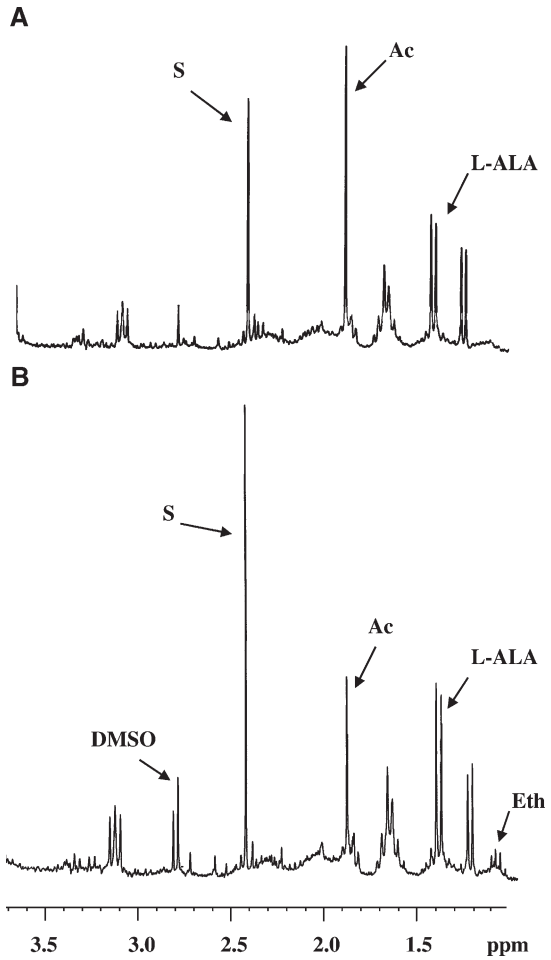

FIGURE 4. Nuclear magnetic resonance (^{1}H-NMR) spectra epimastigote forms of *Trypanosoma cruzi* treated against terpenoids compounds (at a concentration of IC_{25}): (**A**) Control (untreated) and (**B**) Compound 2. Ala = L-alanine; Ac = acetate; S = succinate; Eth = ethanol, and DMSO = Dimethyl sulfoxide.

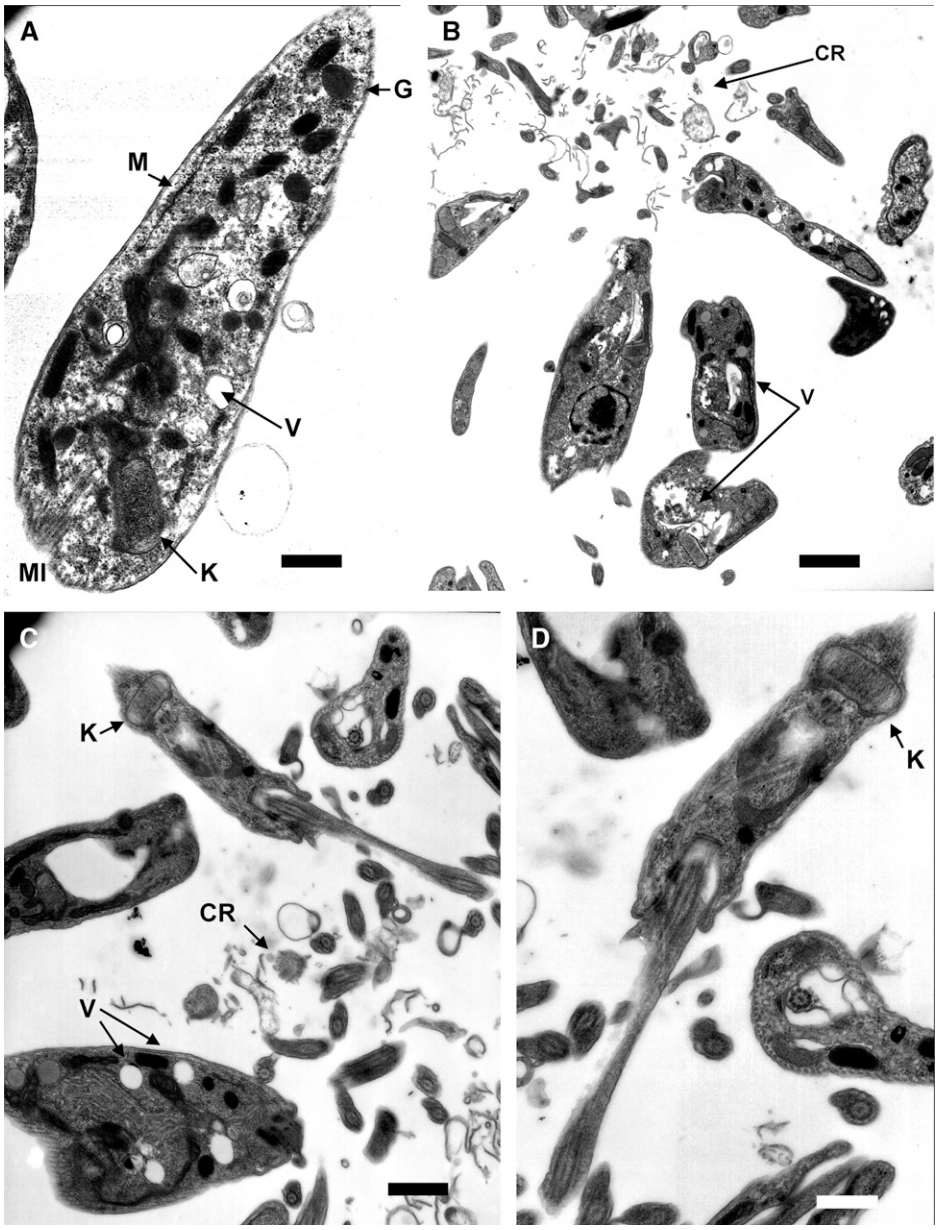

FIGURE 5. Ultrastructural alterations by transmission electron microscopy (TEM) in *Trypanosoma cruzi* treated with terpenoids compounds. (**A**) Control parasite of *T. cruzi* with structures as vacuoles (V) and mitochondrion (M), kinetoplast (K), glycosomes (G), and microtubules (MI) (Bar: 583 μm). (**B**) Epimastigotes of *T. cruzi* treated with Compound **1** with cellular debris from dead parasites (CR) and vacuoles (V) (Bar: 1.59 μm). (**C** and **D**) Epimastigotes of *T. cruzi* treated with Compound 2 with cellular rest (CR), vacuoles (V), and swelling kinetoplast (K) (Bar: 1.00 μm and Bar: 583 μm, respectively).

intracellular forms (amastigotes in vertebrate host cells) for a better understanding of the activity results found. For this reason, we studied the activity of axenic amastigote forms.

The data on the axenic amastigotes match those found for the extracellular forms, where Compounds 1 and 2 were the most effective.

The trypanocidal effect of terpenoid compounds against epimastigote and amastigote forms, has been previously shown. Cassane diterpenes isolated from leaves of *Myrospermum frutescens*, have anti-*T. cruzi* activity against these forms.[34]

According to the activity criteria for the development of new drugs,[31] only the compounds that SI ≥ 20-fold were included in the following studies.

The assays showed that Compounds 1 and 2 were effective enough to undergo the next stage. To study the drug's effect on the infective parasite forms, we performed the metacyclic assay. The IC_{25} of each product was used as the test dosage. This concentration was chosen for being harmful but not totally lethal,[35] revealing that Compound 2 was the most effective.

Diterpenes isolated from *Aristolochia cymbifera* have been shown to be selective against trypomastigotes.[36]

The *in vivo* assays showed that the differences in the level of anti-*T. cruzi* antibodies are consistent with the parasitemia findings.

As far as is known, no trypanosomatid studied to date is capable of completely degrading glucose to CO_2 under

aerobic conditions, and thus these parasites excrete into the medium a great part of their carbon skeleton as fermented metabolites, which differ depending on the species considered.[37,38] *Trypanosoma cruzi* consumes glucose at a high rate, thereby acidifying the culture medium caused by incomplete oxidation to acids. The ^{1}H-NMR spectra enables us to determine the fermented metabolites excreted by the trypanosomatid during *in vitro* culture. One of the major metabolites excreted by *T. cruzi* is succinate, the main role of which is probably to maintain the glycosomal redox balance, by providing two glycosomal oxidoreductase enzymes that allow reoxidation of NADH, produced by glyceraldehydes-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers the significant advantage of requiring only half of the phosphoenolpyruvate produced to maintain the NAD+/NADH balance. The remaining phosphoenolpyruvate is converted into acetate, L-lactate, L-alanine, and/or ethanol, depending on the species considered. The role of the acetate is probably to maintain the glycosomal redox balance. In the case of Compound 2 the inhibition of acetate excretion explains the observed increase in succinate, L-alanine, and ethanol production, probably because these compounds act over any level in the energy metabolism, still unknown.

In conclusion, our results show that four new terpenoid derivative compounds were active *in vitro* against both extra- and intracellular forms of *T. cruzi* (in the order 2 > 1 > 3 > 4 > BZN). These compounds are not toxic for the host cells and are effective at concentrations lower than the reference drug used in this study. The *in vitro* growth rate of *T. cruzi* was reduced, its capacity to infect cells was negatively affected, and the multiplication of the amastigotes and subsequent transformation into trypomastigotes was greatly lowered. Moreover, a wide range of ultrastructural alterations in epimastigote forms of *T. cruzi* treated with these new terpenoid compounds were found. These alterations mainly at the mitochondria level could explain the metabolic changes in the productions of succinate and acetate, which may be caused by the disturbance of the enzymes involved in sugar metabolism within the mitochondria. The *in vivo* studies revealed results that were consistent with those observed *in vitro*. On the one hand, during the treatment of mice with the compounds (Compounds 1 and 2), no signs of toxicity were observed. On the other hand, the parasitic load was significantly decreased in comparison with the reference drug. The effects of these products were also demonstrated with the anti-*T. cruzi* antibody level modification during the chronic stage. So far nothing is known about the structure-activity relationship for these substances, and the results described here do not allow it. Additional research is needed for the activity of other structurally related molecules. These results support further research of terpenoid compounds as potential agents against Chagas disease. The synthesis of new derivatives and preclinical studies, such as doses, schedule, strains, and toxicological studies, is currently in progress.

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