

Diterpenoids from *Azorella compacta* (Umbelliferae) Active on *Trypanosoma cruzi*

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The anti-Trypanosoma cruzi activity of natural products isolated from Azorella compacta was evaluated, with particular emphasis on their effect against intracellular amastigotes. Five diterpenoids from A. compacta derived from mulinane and azorellane were isolated and identified. Only two products, named azorellanol (Y-2) and mulin-11,3-dien-20-oic acid (Y-5), showed trypanocidal activity against all stages of T. cruzi including intracellular amastigotes. At 10 µM, these compounds displayed a strong lytic activity. It ranged from 88.4 ± 0.6 to 99.0 ± 1 % for all strains and stages evaluate, with an IC₅₀/18 h values of 20-84 µM and 41-87 µM, respectively. The development of intracellular amastigotes was also inhibited by nearly 60% at 25 µM. The trypanocidal molecules Y-2 and Y-5 did show different degrees of cytotoxicity depending on the cell line tested, with an IC₅₀/24 h ranging from 33.2 to 161.2 µM.

We evaluated the effect of diterpenoids against intracellular T. cruzi forms by immunofluorescent identification of a specific membrane molecular marker (Ssp-4 antigen) of the T. cruzi amastigote forms. The accuracy and reproducibility of the measurements were found to be outstanding when examined by confocal microscopy.

Key words: *Trypanosoma cruzi* - intracellular amastigotes - lysis - diterpenoids - *Azorella compacta* - chemotherapy - confocal microscopy

Chagas disease caused by *Trypanosoma cruzi* is a major public health problem in South America where nearly 20 million people are infected by *T. cruzi* and 90 million are at risk in endemic areas (WHO 2000).

There is no satisfactory chemotherapy for the disease in any of its phases. The two main drugs used in clinical practice, nifurtimox and benznidazole, produce strong side effects and do not eliminate the parasite during short-term therapies or during the chronic and inapparent phases of the disease (Urbina 1999, Coura & de Castro 2002). Similarly, there are no adequate chemoprophylactic drugs to be used to eliminate the parasite from the blood of serologically positive donors. The prevention of transfusion-associated Chagas disease has been attempted through clinical and serologic screening of blood donors and/or by the addition of trypanomicidal substances such as gentian violet to store blood for 24 h. However the

compounds confers a blue color to the recipients and its use has been restricted in endemic areas. This dye kills trypomastigotes in vitro at 4°C. When used in concentrations of 125 mg/500 ml, and the blood is stored for 24 h before use, transmission is avoided. This strategy has been used extensively in some countries, like Brazil, without apparent side effects, except that patients may become stained for short periods of time (Schmunis 1999).

Due to the high costs involved in the development and registration of new drugs, the pharmaceutical industry has withdrawn funds for research and development of new compounds for the treatment of tropical diseases. Therefore, there is an urgent need to support novel research on natural products with anti-*T. cruzi* activity (WHO 2000).

The high altitude and desert-like environment of the Andean Mountains in Northern Chile allow the growth of a native macroflora under extreme conditions. Two plants, yareta (*Azorella compacta*) and chuquican (*Mulinum crassifolium*), from the Umbelliferae family are rich sources of diterpenoids, compounds with a variety of biological activities (Esquivel et al. 1989, Loyola et al. 1996, 1997a). Both plants are extensively used in folk medicine against diabetes, bronchitis, high-altitude sickness, pneumonia, rheumatism, and have also wound healing effects (Gunckel 1967, Wickens 1995).

In previous studies (Neira et al. 1998), diterpenoids from *A. compacta* showed activity against extracellular amastigotes from highly infected Vero cells. These results do not show what would be the real activity of these compounds against amastigotes since these are intracellular forms of the parasite; therefore, drugs should enter the

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host cell and maintain inside their amastigocidal activity. In the present study, we address this problem by evaluating the anti-*T. cruzi* activity of five diterpenoids isolated from *A. compacta*, against the intracellular amastigote forms, introducing confocal microscopy as an analysis tool.

MATERIALS AND METHODS

Extraction and isolation of natural products from *A. compacta* - Plants were collected in the El Tatio area, 4,000 m above sea level. A voucher specimen was deposited at the herbarium of Universidad de Concepción, Concepción, Chile, and identified by Prof. C Marticorena. The dried and finely powdered whole plant was extracted at room temperature with petroleum ether (PE), followed by extraction with dichloromethane and finally with ethanol:water (1:1). The solvent was eliminated using a rotary evaporator at approximately 40°C, and different extracts were obtained. The extracts were submitted to chromatography on silica gel, alumina or Sephadex LH-20 columns and eluted with a solvent mixture of increasing polarity (0-100% EtOAc-PE, or CH₂Cl₂-MeOH). Compound separation was monitored by thin-layer chromatography. The compounds were isolated and purified through column and thin-layer chromatographies, and the derivatives were submitted to crystallization, sublimation and vacuum distillation, as previously described (Loyola et al. 1996). The structure of the natural products was determined by spectroscopic techniques in homo- and heteronuclear 2D-NMR experiments (Loyola et al. 1996).

Natural products - All pure compounds from *A. compacta* were dissolved in water with 1% dimethyl sulfoxide (DMSO) at a final concentration of 10 mM. DMSO was filtered in Millex (0.2 µm) membrane and aseptically added to the tubes.

Cell cultures - Vero cells, HeLa cells, and J-774 murine macrophages were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C, in 75 cm³ growth flasks, using a cell culture incubator under a 5% CO₂ humid atmosphere.

Parasites - Epimastigote, trypomastigote and amastigote forms of the Tulahuen and SPA-14 (González et al. 1995) strains of *T. cruzi*, were used in this study. We also used the CL Brener clone from the CL strain isolated by Brener and Chiari (1963). Parasites were maintained in Swiss mice and axenic growth medium. Epimastigotes were obtained from LIT medium (Camargo 1964) and trypomastigotes from cell cultures (de Diego et al. 1988). Intracellular amastigotes were obtained by infecting Vero cells and/or HeLa cells with cultured trypomastigotes by the method of González (1991). Extracellular amastigotes were obtained as previously described (Tomlinson et al. 1995).

Determination of the trypanocidal activity of the natural products against different stages of *T. cruzi* - Experiments were carried out according to González et al. (1990). Firstly, 45 µl of each compound were added to the first well of a 96-well microtiter plate and two-fold dilutions were performed to reach a final concentration range of 1 to 125 µM. Next, each well received 45 µl of a suspension containing 4x10⁶ epimastigotes and plates were incubated

for 18 h at 28°C. A viability control (1% DMSO) and a mortality control (1 mM crystal violet) were included. No effect on drug toxicity attributable to DMSO was observed at the maximum concentration used of 1%. The trypanocidal activity was measured as percent of mortality after microscopic observation of 50 fields. A viability test was performed using an exclusion dye as described (Jacque et al. 1975). Trypanocidal IC₅₀/18 h against trypomastigotes and extracellular amastigotes was determined as described for epimastigotes, but plates were incubated at 37°C.

Trypanocidal activity against intracellular amastigotes - Five-hundred µl of a suspension containing 7 x 10⁴ Vero cells/ml were seeded on 24-well microplates incubated for 18 h under a 5% CO₂ atmosphere in order to obtain cell adherence. Next, the cells were washed and infected with trypomastigotes of the clone CL Brener at a ratio of 10 parasites per cell, for 3 h. The cells were washed with RPMI to remove extracellular parasites. Finally, the infected culture was treated with each compounds (25 µM) for 24 h. Inhibition of intracellular growth was determined by counting the number of intracellular amastigotes in 100 infected cells. In order to compare the results, two parallel procedures were employed: (a) light microscopy of cells stained using the classical May Grünwald-Giemsa method; (b) confocal immunofluorescence microscopy using Mab 1D9, an anti-Ssp-4 monoclonal antibody (Barros et al. 1997).

Cytotoxic activity against mammalian cells - Five-hundred µl of a suspension containing 7 x 10⁴/ml of Vero cells, HeLa cells or J774 macrophages were added to 24-well microplates and the cells were left to adhere and grow for 24 h. Cells were washed with RPMI and incubated with different concentrations of each compound for 24 h. Cytotoxicity was evaluated using exclusion dyes, as indicated previously.

All assays were carried out in triplicate using nifurtimox (35-173 µM) and crystal violet (1 µM) as controls. IC₅₀ values were determined by probit analysis by the method of Finney (1971).

RESULTS

Five diterpenoids derived from mulinane and azorellane were isolated from *A. compacta* extracts (Fig. 1). The chemical structure was elucidated by spectroscopic and chemical methods. The molecules Y-1, Y-4 and Y-5 were chemically identical to mulinolic acid, mulinic acid and mulin-11,3-dien-20-oic acid, respectively. Their carbon skeleton corresponded to mulinane and they have been previously isolated from *Mulinum crassifolium* (Loyola et al. 1996, 1997a). Two other molecules were detected for the first time and were denominated azorellanol (Y-2) and desacetylazorellanol (Y-3) because they have a cyclopropane ring between carbons C-9 and C-12 of the mulinane skeleton. This new carbon skeleton was named azorellane (Loyola et al. 1997b).

The in vitro trypanocidal activity of these diterpenoids against different stages of the Tulahuen and SPA-14 strains and the CL Brener clone of *T. cruzi* is shown in Table I. At 10 µM, after 18 h incubation, only two molecules, the azorellanol (Y-2) and mulin-11,13-dien-20-oic acid (Y-5),

TABLE III

Trypanocidal effect of *Azorella compacta* diterpenoids on intracellular amastigotes of the *Trypanosoma cruzi* clone CL Brener determined by light and confocal immunofluorescence microscopy

Diterpenoids (25 µM)	Number of amastigotes/100 cells	
	Light microscopy (May Grunwald-Giemsa)	Confocal microscopy (Immunofluorescence)
Y-1	101.5 ± 3.5	125.6 ± 4.4
Y-2	42.3 ± 0.6	57.7 ± 0.9
Y-3	104.5 ± 3.6	132.3 ± 4.9
Y-4	111.1 ± 2.5	135.4 ± 3.3
Y-5	39.6 ± 0.8	52.5 ± 1.0
Nifurtimox ^a	37.8 ± 2.4	41.6 ± 3.0

a: control final concentration of nifurtimox = 35 µM

DISCUSSION

In many regions of the world plants have been used in traditional medicine. This fact strongly suggests that they contain products with pharmacological activity. Among parasitic diseases, the examples of quinine and artemisin reinforce the idea that plants could be important sources of new pharmaceuticals.

In Chagas disease, most of the attempts to develop an efficient and safe chemotherapy has been unsuccessful. Currently available drugs (nitrofurans and nitroimidazoles), developed empirically over three decades ago, are unsatisfactory due to frequent toxic side effects and limited efficacy, particularly in the prevalent chronic form of the disease. Furthermore, studies of their mechanism of action have shown that their antiparasitic activity is inextricably linked to mammalian host toxicity (Urbina 2002). The efficacy of these drugs also depends on the susceptibility of *T. cruzi* strains. The drugs are also genotoxic and produce biochemical damage to mammalian tissues. On the other hand, although *T. cruzi*, requires specific endogenous sterols for cell viability and proliferation, the currently available antifungal sterol biosynthesis inhibitors are not powerful enough to induce parasitological cures of human or experimental infections. However, new triazole antifungal compounds, which are potent inhibitors of the sterol C14 alpha demethylase of the parasite and have special pharmacokinetic properties, are capable of inducing parasitological cures in murine models of both acute and chronic Chagas disease. They are currently the most advanced candidates for clinical trials in patients with Chagas disease (Molina et al. 2000, Urbina 2001). Allopurinol and related compounds also reduce the parasitemia and induce a negative xenodiagnosis, but these effects may not be permanent, a fact that invalidates their clinical use (Stoppioni 1999).

Among the various tests used to evaluate cytotoxicity and the viability of cell cultures exposed to drugs or active compounds, cell line systems such as the one used in the present study are the most sensitive and best reproduce the conditions that may occur in mammals.

In vitro cytotoxicity tests against mammalian cells should be followed by more precise methods, such as cytogenetic activity. Nifurtimox has been shown to be at

least four-fold more toxic than benzimidazole in a mutagenesis test against *Salmonella* strain TA100, at a concentration of 35 µg per plaque (Ferreira & Ferreira 1986).

Different publications have described the trypanocidal effect of natural products and extracts from species of *Senecio* (Sarti et al. 1984), some naphthoquinones (Pinto et al. 1987), gossypol (Rovai et al. 1990), boldine (Morello et al. 1994), 1,4 naphthoquinones (Morello et al. 1995) and β-carboline alkaloids (Rivas et al. 1999). In addition, Brengio et al. (2000) have reported that the sesquiterpene lactone dehydroleucodine inhibited the growth of cultured *T. cruzi* epimastigotes.

Our work shows that azorellanol (Y-2) and mulin-11,13-dien-20-oic acid (Y-5) have trypanocidal effects against all developing stages of *T. cruzi*. These appears to be an interesting attribute of these class of compounds, if we consider that several other diterpenoids isolated from plants have shown anti-*T. cruzi* activities. In fact, earlier studies of Alves et al. (1995) showed that diterpene entkaur-16-en-19-oic acid, isolated from *Mikania obtusata* displayed trypanocidal activity against trypomastigotes of *T. cruzi*. On the other hand, we have previously reported the trypanocidal activity of two diterpenoids (Azorellanol and mulin-11,13-dien-20-oic acid) against extracellular amastigotes of the CL Brener clone, SPA-14 and G *T. cruzi* strains transformed in axenic culture at pH 5.0, using the microscopic observation to detect the effect of compounds on amastigote forms (Neira et al. 1998).

Giemsa staining of infected cells has been used for a long time as a routine laboratory procedure for counting intracellular amastigotes. However, this method has been reported to be of low accuracy and with high time consuming (Martinez et al. 2000). This approach is based mainly on the morphological stained parasite identification.

Thus, we propose the use of a complementary method to evaluate the activity of biomolecules against intracellular forms of *T. cruzi* using immunofluorescence to detect a cell membrane marker, the Ssp4 antigen specific for *T. cruzi* amastigotes (Andrews et al. 1987, Burleigh & Andrews 1995). This antigen can be identified with a monoclonal antibody (1D9) that recognizes an Ssp-4 surface glycoprotein which is expressed in all potentially viable intracellular forms of the parasite (Barros et al. 1997).

Confocal scanning fluorescence microscopy has become widely used in cell biology and pathology. Monoclonal antibodies are a powerful tool that enables detailed studies of tissue forms of *T. cruzi* (Mortara et al. 1999, 2000). Confocal microscope systems can store and process digitalized images that can be readily accessible. Although expensive, this procedure has the significant advantage of being a highly sensitive, specific and reproducible method that would improve the evaluation of the activity of different compounds against the intracellular stages of the parasite.

Finally, we may conclude that two diterpenoid compounds isolated from *A. compacta* showed trypanocidal activity as demonstrated by conventional procedures as well as by immunofluorescence confocal microscopy. The next step will be to determine the pharmacokinetic and pharmacodynamic properties of these natural products.

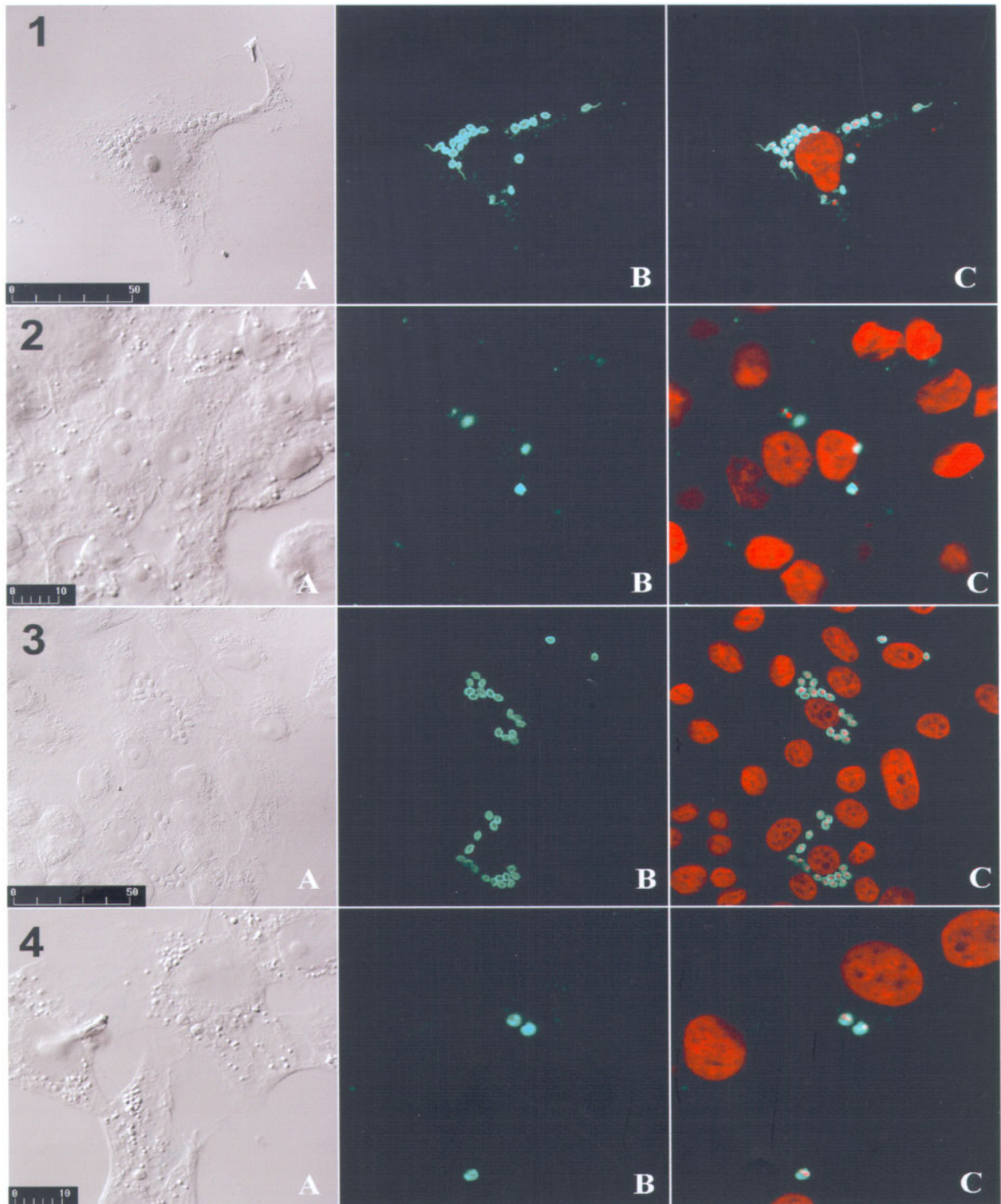


Fig. 2: photomicrographs of intracellular amastigote clone CL Brener after treatment with Y-2, Y-3 and Y-5 diterpenoids (2, 3 and 4). There was a clear decrease in Ssp-4 labeling in amastigotes treated with 25 μ M Y-2 or Y-5 when compared with control (1).

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