Brief Articles

A Trypanocidal Phenazine Derived from β-Lapachone

Cleverson Neves-Pinto,[†] Valéria R. S. Malta,[§] Maria do Carmo F. R. Pinto,[†] Regina H. A. Santos,[§] Solange L. de Castro,[#] and Antônio Ventura Pinto*[,†]

Núcleo de Pesquisas em Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, 21944-970 Rio de Janeiro, RJ, Brazil, Departamento de Química e Física Molecular, Instituto de Química de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil, and Laboratório de Biologia Celular, DUBC, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21045-900 Rio de Janeiro, RJ, Brazil

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An intensive effort has been directed toward finding alternative drugs for treatment of Chagas' disease, caused by *Trypanosoma cruzi*, and prophylaxis of blood in endemic areas. Our research comprises the synthesis and trypanocidal screening of derivatives from naphthoquinones. Herein a new phenazine, obtained from the reaction of β-lapachone with aniline, has its structure established by physical data and X-ray analysis. It was 9 times more active against *T. cruzi* trypomastigotes than crystal violet.

Introduction

Chagas' disease, caused by the parasite *Trypanosoma cruzi*, is endemic in Latin America. It is a very serious public health problem in several countries, with 16−18 million people known to be infected with the parasite, and a further 100 million at risk of infection, either through contact with an insect vector or via blood transfusion.[1] The life cycle of *T. cruzi* involves a vertebrate and an invertebrate host, and the parasite passes through several developmental stages. The trypomastigote form ingested by the insect vector via the blood of an infected individual differentiates into the epimastigote form, which, after proliferation, reaches the posterior intestine and differentiates into metacyclic trypomastigote. This latter infective form, following invasion of vertebrate cells, undergoes differentiation into the amastigote form, which, after several reproductive cycles, transforms into trypomastigote, responsible for the dissemination of the infection. At present, the only available therapeutic agent for Chagas' disease in Latin America is benznidazole. This nitroheterocyclic compound is not always effective, however, and its use is associated with severe side effects. In the absence of specific treatment, the acute phase of Chagas' disease persists for about two months, with an overall mortality of 2 to 8%, especially among children. In the chronic phase, most patients remain asymptomatic (indeterminate phase), with about 20% of the cases developing irreversible cardiac, digestive, or neurologic disturbances.[2] The clinical treatment of chronic patients remains controversial, especially among indeterminate cases.[3] It is worth mentioning that vector control programs in Southern Cone countries in the past decade have markedly reduced the overall incidence of new infections[4] although large numbers of acute cases are still seen in some regions.[5] Furthermore, there is a need to treat about 5 million chronic chagasic patients and to prevent transmission of the disease through blood transfusion. In 1984, the World Health Organization recommended the use of crystal violet in blood banks in endemic areas in order to prevent the transfusional transmission of Chagas' disease.[6] No serious side effects have been reported following use of this dye, although the temporary bluish color conferred to blood and tissues after transfusion of treated blood is not well accepted by the assisted population.

In this context, an intensive research program has been focused on the search for alternative natural and synthetic drugs to both benznidazole and crystal violet.[7−9] In folk medicine, plants containing naphthoquinones have been employed for the treatment of many diseases, especially among Indian populations.[10] The toxicity and therapeutic activities of these compounds involve the formation of reactive oxygen species.[11] Among naturally occurring naphthoquinones, β-lapachone, isolated from the heartwood of trees belonging to the Bignoniaceae and Verbanaceae families, and which are popularly known as "ipes", stands out as having a variety of biological activities, such as antitumor, bactericidal, fungicidal, virucidal, and trypanocidal effects.[12] In recent work involving one of us (Dr. A. V. Pinto), the synergistic cytotoxic effect of β-lapachone and taxol in several human tumor lines has been identified.[13]

As part of a national program on natural therapeutic agents from the Brazilian flora, our group is studying the phytochemical and microbicidal activities of naphthoquinones. Our aim is an understanding of structure−activity relationships among these natural compounds and their semisynthetic derivatives and the possible use

* Address correspondence to Dr. Antônio Ventura Pinto. Tel/Fax: 0055-21-2702683. E-mail: ventura@nppn.ufrj.br.
[†] Universidade Federal do Rio de Janeiro.
[§] Universidade de São Paulo.
[#] Instituto Oswaldo Cruz.

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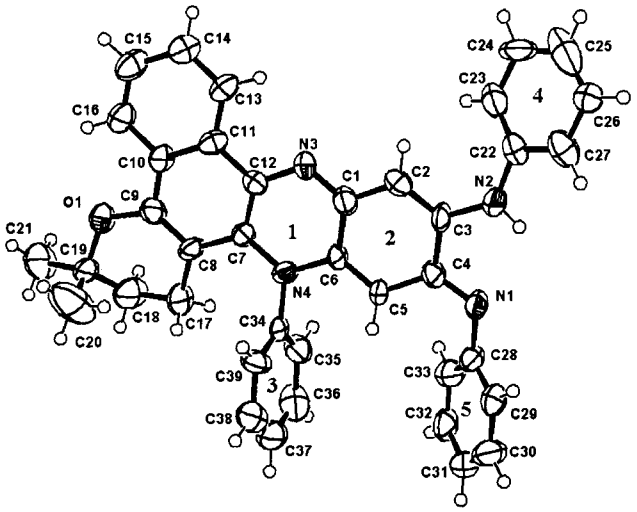
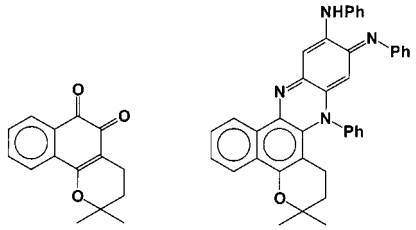


Figure 1. ORTEP projection of compound **2** showing one of the two independent molecules in the crystal asymmetric unit.

Chart 1. Structures of β-Lapachone (**1**) and the Phenazine **2**



of such substances as chemotherapeutic agents against tropical diseases.[14−24] Recently, we synthesized 37 derivatives of naphthoquinones isolated from the Brazilian flora, comprising imidazolic, oxazolic, phenoxazinic, indolic, dipyranic, and cyclopentenic compounds and assayed their activities against *T. cruzi*. The overall analysis showed a tendency of activity among compounds with imidazolic or oxazolic moieties linked to a naphthopyranic structure.[12,25,26]

In the present work, we describe the synthesis of a new semisynthetic phenazine derivative, **2**, and the effect of this substance on trypomastigotes of *T. cruzi*.

Results and Discussion

From the reaction of β-lapachone (**1**) with aniline, compound **2** can be obtained with good reproducibility. This compound is easily crystallized, forming deep violet crystals by slow evaporation of a solution in acetyl acetate. The structure of **2** shown in Chart 1 is in full accordance with physical data and was confirmed by X-ray crystallographic analysis (Figure 1). As expected, **2** shows the characteristics of a typical organic base, being soluble in aqueous acid solution. This compound undergoes a redox reaction, being easily reduced by sodium borohydride to a leuco derivative, which undergoes reoxidation to the original compound in the presence of oxygen.

It is important to point out that although phenazines are usually synthesized through the classical method of reacting *o*-quinones with 1,2-phenylenediamines, as far as we know this is the first report of a phenazine synthesis from a quinone and a mono-amine.[27]

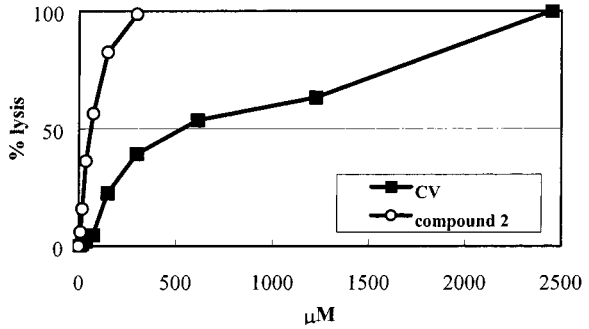


Figure 2. Trypanocidal effect of **2** on bloodstream trypomastigotes of *T. cruzi*: (○) compound **2**; (■) crystal violet.

The biological assay against bloodstream forms of *T. cruzi* revealed that **2** (ED_{50}/24 h = 61.3 ± 9.6 μM) is about 9 times more active than the standard compound, crystal violet (ED_{50}/24 h = 536.0 ± 3.0 μM) (Figure 2). In this context, **2** represents a new semisynthetic heterocyclic compound prepared from a quinone isolated from Tabebuia, with a lytic capacity against the infective form of *T. cruzi*.

Phenazines are a class of heterocycles with a wide spectrum of microbicidal activities.[28,29] Such substances are also inhibitors of DNA topoisomerase II, leading to antitumor activity, and are also capable of intercalating DNA.[30] It is interesting to note that clofazimine, an antileprosy drug that is often included in combination chemotherapy for disseminated infections in AIDS patients,[31] bears structural similarities to **2**. However, the only phenazine derivative that has been tested against pathogenic trypanosomatids is 5-methylphenazinium methyl sulfate (PMS, phenazine methosulfate). This compound was active against epimastigotes of *T. cruzi*, with an ED_{50}/4 d of 2.5 μM,[32] and against intracellular amastigotes reducing by 92% macrophage infection after 24 h of treatment at a concentration of 8 μM.[33] It was not assayed against trypomastigote forms. PMS was also active against *Leishmania amazonensis*.[34] PMS can act as an electron acceptor in mitochondria leading to generation of free radicals,[35] and in bacteria the generation of free radicals caused loss of cell viability.[36] The production of superoxide anion radical from PMS in epimastigotes of *T. cruzi* has been described.[32] Due to its redox character, the trypanocidal effect of **2**, like PMS, may be attributable to production of reactive intermediates of oxygen. This possibility needs to be ascertained by experiments with biological reducing agents such as NAD(P)H or reduced glutathione. In relation to other redox type drugs, such as naphthoquinones,[21,37] crystal violet, and nitroaromatic derivatives,[38] the involvement of free radicals in the trypanocidal activity has been already established. The action of such lethal reactive intermediates in the parasite is favored by a partial deficiency in detoxification mechanisms.[39] Furthermore, the known interference of phenazines with electron transport processes raises the possibility that the mode of action of **2** could also involve other cellular processes, such as energy metabolism.

The present work revealed a new trypanocidal compound and stimulates the synthesis of new derivatives of phenazine endowed with redox properties, reinforcing the strategy of a rational approach in of the development

of drugs that may be active against Chagas' disease. The broad spectrum of activity of phenazines also suggests that **2** should be tested against other pathogenic trypanosomatids, such as the agents of leishmaniasis and African trypanosomiasis.

Experimental Section

Chemistry. Melting points were determined in a capillary Thomas-Hoover apparatus (Thomas Co., Philadelphia, PA) and are uncorrected. ^{1}H and ^{13}C NMR were recorded using a Varian Gemini 200 (Varian, Palo Alto, CA) and Bruker 200 MHz spectrophotometer (Bruker-Franzen Analytic, Bremen, Germany), in the solvents indicated, with TMS as internal standard at room temperature. Chemical shifts (δ) are given in ppm and coupling constants (J) in hertz. Infrared spectra were recorded on a Perkin-Elmer 783 spectrophotometer (Perkin-Elmer, Norwalk, CT) and Nicolet FT-IR (Nicolet Instrument Co, Madison, WI) in KBr pellets or liquid films. UV spectra were obtained with a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) as a solution in ethanol. The mass spectra were obtained at 70 eV in a VG Autospec apparatus (Micromass, Manchester, U.K.). The fragments were described as a relation between atomic mass units and the charge (m/z), and the relative abundance was described in percentage of the base peak intensity. The elemental analysis was performed in the Laboratorio Analitico of the Instituto de Quimica da Universidade de Sao Paulo.

Synthesis of Compound 2 (7,8-Dihydro-2,2-dimethyl-6-(*N*-phenyl)-3-(*N*-phenylamine)-4-(*N*-phenylimine)-4*H*,6*H*,7*H*-benzo[*a*]pyran[2,3-*c*]phenazine). β-Lapachone (**1**) was obtained from natural lapachol[18] (Chart 1). To a solution containing 0.484 g (2 mmol) of **1** in 4 mL aniline was added two drops of concentrated HCl, and the mixture was incubated at 50 °C for 16 h. The reaction mixture was then poured into 600 mL of 1 N HCl and kept overnight at room temperature. The dark precipitate was filtered off and dried at room temperature. This material was dissolved in 300 mL of ethyl acetate and the solvent allowed to evaporate at room temperature. The dark violet crystals were purified by recrystallization from acetyl acetate, yielding **2** (286 mg, 25%) as deep violet crystals, mp 126 °C: IR (KBr) ν_{max} 2.980, 2.940, 1.600, 1.610, 1.530, 750, 700 cm^{-1}; UV (EtOH) λ_{max} nm (log ϵ) 210 (4.64), 315 (4.56), 570 (4.49); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 9.0 (d, 1H), 8.2 (d, 1H), 6.8−7.7 (m, 19H). 5.8 (s, 1H), 1.8 (t, 2H), 1.5 (t, 2H), 1.3 (s, 6H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 141.6 (s), 141.2 (s), 140.4 (s), 130.4 (s), 129.5 (d), 129.4 (s), 129.3 (s), 129.2 (d), 129.1 (d), 128.6 (d), 127.7 (s), 123.1 (d), 122.9 (d), 121.8 (d), 121.8 (d), 121.7 (d), 121.3 (d), 103.8 (s), 91.5 (d), 74.5 (s), 32.5 (t), 26.0(q), 22.0 (t); MS m/z (abundance) 572 ($M^{+\cdot}$, 100%), 515 (70%), 437 (10%). Anal. Calcd for $C_{39}H_{32}N_4O$: C, H, N.

X-ray Analysis. The analysis was performed using a CAD4 diffractometer with MoKα radiation. The poor quality of the crystal limited the data collection of 2θmax equal to 21.4°, even if the temperature was maintained at 243 K. The structure was solved and analyzed using the WinGX system.[40] The compound crystallizes in the monoclinic $P2_1/a$ space group, with two independent molecules per asymmetric unit ($Z = 8$), probably due to the disorder in the methyl groups of one molecule and to the differences in the dihedral angles involving the phenyl substituted rings for the two molecules. The hydrogen atoms (N1 and N1A) in both molecules were located using a difference Fourier map and agree perfectly with the distances N2−C3 and N2−C22: respectively, 1.37(1) and 1.42(2) Å and 1.36(1) and 1.41(2) Å for molecule A. In the equivalent N1 position, the distances clearly indicate double bond character for N1−C4 (1.31(1) and 1.29(1) Å for molecule 1 and A, respectively). The ORTEP representation of one of the two independent molecules is shown in Figure 1. It is interesting to note that the phenyl ring 3 attached to the N4 (ring 1) makes a dihedral angle of 76.6(5)° in one molecule and 89.8(6)° in the other; and between the ring 2 and the rings 4, 5, the values are 32.2(5)° and 55.1(5)°, 57.6(5) and 45.6(5)°, for the molecule 1 and molecule A, respectively. A complete set of data has been deposited at the Cambridge Crystallographic Data Centre with number CCDC 167872.

Trypanocidal Assay. A stock solution of **2** was prepared in dimethyl sulfoxide (DMSO), with the final concentration of the solvent in the experiments never exceeding 0.1%. The Y strain of *T. cruzi* was used.[41] Trypomastigote forms were obtained at the peak of parasitaemia from infected albino mice, isolated by differential centrifugation and resuspended in Dulbecco's modified Eagle medium (DME) to a parasite concentration of 10^7 cells/mL in the absence or the presence of 10% of blood. This suspension (100 μL) was added to the same volume of a solution of **2**, previously prepared at twice the desired concentration in DME (2−200 μM) in 96-well plates and then incubated at 4 °C. Untreated and crystal violet-treated parasites were used as controls.[23] Cell counts were performed after 24 h of incubation, and the activities of the test compounds were expressed as ED_{50} values, corresponding to the concentration that causes lysis of 50% of the parasites.

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Supporting Information Available: X-ray crystallographic data of compound **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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