Brief Articles

Efficient Inhibition of Iron Superoxide Dismutase and of *Trypanosoma cruzi* Growth by Benzo[*g*]phthalazine Derivatives Functionalized with One or Two Imidazole Rings

Ana M. Sanz,[†] Fernando Gómez-Contreras,*[,†] Pilar Navarro,[‡] Manuel Sánchez-Moreno,*[,§] Samira Boutaleb-Charki,[§] Jose Campuzano,[†] Mercedes Pardo,[†] Antonio Osuna,[§] Carmen Cano,[†] María J. R. Yunta,[†] and Lucrecia Campayo[†]

Departamento de Química Orgánica I, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain, Instituto de Química Médica, Centro de Química Orgánica Manuel Lora-Tamayo, CSIC, Juan de la Cierva, 3. 28006 Madrid, Spain, and Instituto de Biotecnología, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain

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The synthesis and trypanosomatic behavior of a new series of 1,4-bis(alkylamino)benzo[*g*]phthalazines **1**−**4** containing the biologically significant imidazole ring are reported. In vitro antiparasitic activity against *Trypanosoma cruzi* epimastigotes is remarkable, especially for compound **2**, whereas toxicity against Vero cells is very low. Conversion of epimastigotes to metacyclic forms in the presence of the tested compounds causes significant decreases in the amastigote and trypomastigote numbers. Fe-SOD inhibition is noteworthy, whereas effect on human Cu/Zn-SOD is negligible.

Introduction

Despite significant advances in the last years, infections and parasitic diseases account for about 25% of the global burden disease.[1] Among them, American trypanosomiasis, also known as Chagas disease, is a potentially fatal, chronic illness that currently affects about 20 million people throughout Mexico and Central and South America and is responsible for about 40000 deaths and 850000 new infections a year.[2,3] It is caused by the hemoflagellate protozoan parasite, *Trypanosoma cruzi*. Additionally, humans can contract the disease by transfusions using contaminated blood of persons who carry the parasite, but do not have active Chagas. The increasing immigration of people from Latin America to other countries is a risk factor for the propagation of Chagas disease in other areas of the world.[4] Because a vast majority of people infected will not develop clinical symptoms for ten to twenty years, blood donors may unknowingly spread the disease.

At present, the chemotherapy developed against trypanosomiasis is unsatisfactory due to the toxicity and limited efficacy of the drugs currently used. Therefore, the development of more effective drugs, particularly against the chronic form, is an urgent priority. Some nitrofurans and nitroimidazoles, like benznidazole, are of variable efficacy in short-term cases, causing anorexia, vomiting, and allergic dermopathy as the major side effects. The chronic infection usually results in cardiac failure and death of the host, and there are no cures for that phase of the disease.[3,5]

The drugs currently used are thought to act by inducing oxidative stress within the parasite through the formation of toxic oxygen metabolites. Benznidazole is capable of generating superoxide radical and hydrogen peroxide, and nifurtimox generates nitro-radicals.[2] However, *Trypanosoma cruzi* possesses a variety of enzymatic antioxidant defenses. Among them, superoxide dismutase (SOD[a]) plays a relevant role.[3] SODs are a group of metal-containing enzymes that have a vital antioxidant role conferred by their scavenging ability for the superoxide anion. Three major classes of SOD have been described on the basis of their prosthetic groups: Fe, Mn, or Cu/Zn. The Fe-SOD, which is found in prokaryotes and some plants, appears to be the enzyme normally associated with trypanosomatides.[6] The chemical reaction of superoxide anion with the prosthetic group of SOD occurs in two steps. The first one begins with the oxidized form of the enzyme (Fe^{3+}) binding $O_2^{\bullet -}$, and follows with protonation and release of molecular oxygen. In the second one, the reduced form (Fe^{2+}) binds a superoxide anion and a proton to liberate hydrogen peroxide, returning to the original oxidized state.[7]

Therefore, compounds with the ability of inhibiting the protective action of SOD are good targets for antiparasitic activity affecting both the growth and survival of parasitic cells. Due to the predominant role of the prosthetic groups, competitive complexation of the metal ion of SOD could be an efficient way of deactivating the antioxidant effect of the enzyme.

Based on all the above, we have previously described[8] the synthesis of two series of 1,4-bis(alkylamino)benzo[*g*]phthalazines with structures **I** and **II** (Figure 1) and also their ability for the formation of dinuclear complexes with transition metal ions. The antiparasitic activity of these compounds against *Trypanosoma cruzi* and their Fe-SOD/activity inhibition were studied. The results obtained showed that compounds **I**, with sp^3 and sp^2 nitrogens at the end of the flexible side-chains, have higher activities than the analogues **II**, with terminal hydroxyl groups. This could be related to the remarkable modification in the geometry of the complexation observed when the side-chains

* To whom correspondence should be addressed. Phone: +34-91-394-4229. Fax: +34-91-394-4103. E-mail: fercon@quim.ucm.es.
[†] Departamento de Química Organica, Universidad Complutense.
[‡] Instituto de Química Medica, CSIC.
[§] Instituto de Biotecnologia, Universidad de Granada.

[a] Abbreviations: SOD, superoxide dismutase; TLC, thin-layer chromatography; TMS, tetramethylsilane, MEM, minimal essential medium; MTL, liquid *Trypanosoma* medium; IFCS, inactivated fetal calf serum; EDTA, ethylendiaminetetraacetic acid, STE, Tris NaCl EDTA; NAD(P)H, nicotinamide adenine dinucleotide phosphate.

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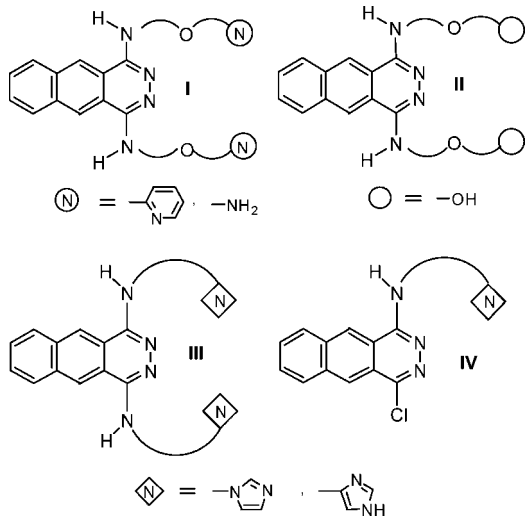


Figure 1. Previous (**I** and **II**) and new models (**III** and **IV**) for the design of benzo[*g*]phthalazine derivatives with potential antiparasitic activity.

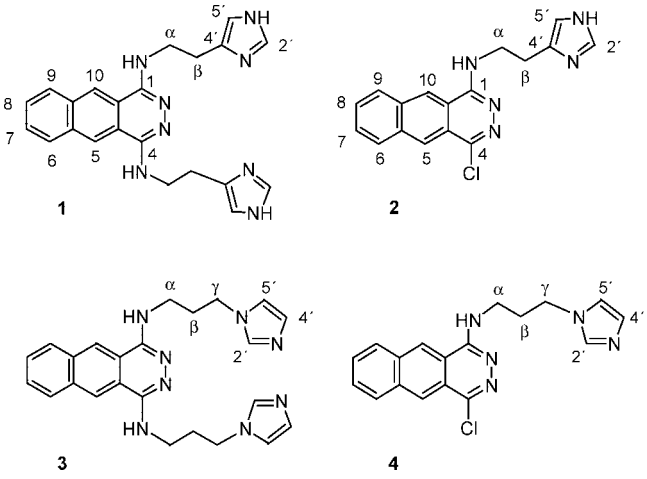


Figure 2. New imidazole containing structures synthesized for testing against SOD and *T. cruzi*.

nitrogens are replaced by oxygens. As a result, dinuclear complexes are not formed.

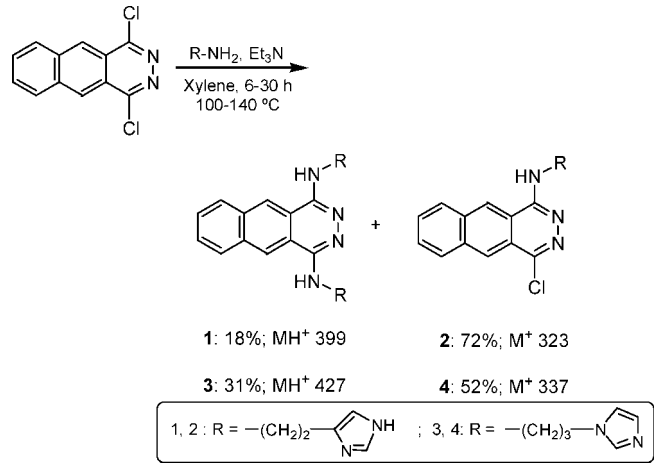
In accordance with those findings and considering both the biological significance of the imidazole ring and the basicity of its nitrogen atoms, we have devised now a new series of benzo[*g*]phthalazine derivatives **III** and **IV** (Figure 1). They have been functionalized at the end of the ethylenic or propylenic side-chains with imidazole rings, linked through carbon or nitrogen. The oxygen atoms present in **I** and **II** have been suppressed in the new design because they are not essential for metal ions complexation.

Results and Discussion

Synthesis. The preparation of compounds **1**–**4** (Figure 2) was performed from 1,4-dichlorobenzo[*g*]phthalazine as shown in Scheme 1, using similar experimental conditions to those reported by our group for the synthesis of **I** and **II** and other analogues.[8,9]

Thus, nucleophilic substitution at the C-1 and C-4 positions of the starting compound with 2-(imidazol-4-yl)ethylamine under reflux of xylene (140 °C) for 10 h afforded a mixture of the bis- and monoalkylamination products **1** and **2**, in 18 and 72% yield, respectively. In a similar way, but reducing the reflux

Scheme 1. Synthesis of the Mono and Bis(alkylamino)benzo[*g*] phthalazine Derivatives



time to 6 h, the reaction with 3-(imidazol-1-yl)propylamine afforded **3** in 31% yield. Unexpectedly, the corresponding monoalkylamino derivative **4** was obtained in very low yield (6%). To improve these results, the reaction was carried out at 100 °C for 30 h. Under these conditions, compound **4** was obtained in 52% yield. Isolation of the pure compounds from the reaction mixtures was performed by flash chromatography on silica gel with a CCl_3H/MeOH mixture of increasing polarity. The monosubstituted derivatives were eluted faster than their disubstituted analogues. All these compounds were obtained as the free polyamines, with the exception of **1**, isolated as the dihydrochloride, that was filtered through a basic alumina column to give the free amine.

All the compounds synthesized were unequivocally identified on the basis of their EI or FAB mass spectra, and IR, ^{1}H NMR, and ^{13}C NMR spectroscopies. The resonance spectra signals were assigned according to heteronuclear multiple quantum coherence experiments. The mono and bisalkylamination products could be easily differentiated in both ^{1}H and ^{13}C spectra by comparing the H_5/H_{10} and C_1/C_4 signals at rings B and A of the benzo[*g*]phthalazine moiety. Disubstituted compounds exhibited an unique signal for C_1 and C_4, and also for H_5 and H_{10} as a singlet. On the contrary, C_1 and C_4 gave clearly different signals in their monosubstituted analogues, being the carbon linked to the chlorine atom shielded about 9–10 ppm. In a similar way, H_5 and H_{10} appeared as two singlets separated by 0.3 ppm, and the proton in the neighborhood of the chlorine was also shielded with respect to that one coplanar with nitrogen. Neat differences were also observed among the hydrogens at ring C in both types of derivatives because H_6 and H_9 exhibited a symmetrical pattern only in the disubstituted compounds.

The mass spectra of the monosubstituted **2** and **4** showed both the loss of chlorine from the molecular ion, and the presence of fragments containing chlorine. Molecular ions corresponding to the formulas of the proposed structures were found for compounds **1**−**4**.

Biological Evaluation. In a first step, the inhibitory effect of podands **1**−**4** on the in vitro growth of *T. cruzi* epimastigotes was measured at different times following established procedures (see Supporting Information). Results obtained are displayed in Table 1 using benznidazole as the reference drug and including toxicity values against Vero cells. The four compounds tested resulted to be active against epimastigotes. After 72 h of exposure, **1** and **4** show IC_{50} values of 14.2 and 13.7 μM, respectively, close to those found for benznidazole. However,

Table 1. In Vitro Activity of 1,4-Bis(alkylamino)- (**1** and **3**) and 1-(Alkylamino)-4-chlorobenzo-[*g*]phthalazines (**2** and **4**) on *Trypanosoma cruzi* Epimastigotes

compound	IC_{50} (μM) 24 h	48 h	72 h	toxicity IC_{50}[a] (μM)
benznidazole			15.8	13.6
1	61.1	35.1	14.2	88.7
2	108.5	34.6	<0.3	213.0
3	53.4	34.8	<0.2	69.3
4	51.0	46.9	13.7	145.8

[a] On Vero cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at concentrations employed (2.5, 25, and 125 μM). Note: Average of three separate determinations.

2 and **3** are much more active (IC_{50} <0.3 and 0.2 μM) than **1** and **4**. All the compounds tested are much less toxic against Vero cells than benznidazole. It is worth mentioning that the monosubstitued compound **2** exhibits an inhibitory concentration of 213.0 μM after 72 h of culture. That is 16 times higher than the corresponding value measured for benznidazole (13.6 μM).

Figure 3 illustrates *T. cruzi* propagation in Vero cells (with and without coaddition of the test compounds). When 1×10^5 Vero cells were incubated for 2 days and then infected with 1×10^6 metacyclic forms, the parasites invaded the cells and underwent morphologic conversion to amastigotes within 1 day after infection. During days 1−8, the rate of host-cell infection increased gradually, reaching a 76% of infected cells on day 8. When the four compounds tested were simultaneously added to the infected Vero cells with *T. cruzi* metacyclic forms (10 μM), the infection rate significantly decreased (more than 50%), being especially remarkable the behavior of **2**, **3**, and **4**, with a decrease of the infection of 81, 84, and 74%, respectively, compared with the control substance (Figure 3A).

The average number of amastigotes per infected cell increased on the fourth day of culture, decreasing significantly afterward until day 8 (Figure 3B). This behavior is due to the rupture of the Vero cells with the subsequent release of amastigotes and further transformation into trypomastigotes. The addition of compounds **2** and **3** (10 μM) remarkably diminished the amastigote numbers per infected cell to give reductions of 55 and 44%, respectively, on day 8 with respect to the control culture. The number of trypomastigotes in the medium was 5.3 $\times 10^4$ on day 8, and it was substantially reduced (over 50%) in the presence of **2** and **3** (Figure 3C).

These results prompted us to evaluate the inhibitory effect of **1**−**4** on SOD activity to test their potential as competitors for the metallic ions of the enzyme. We have used epimastigote forms from the Maracay strain of *Trypanosoma cruzi* that excrete Fe-SOD when cultured over calf fetal serum.[10] Data obtained for the inhibition activity of the enzyme are displayed in Table 2.

Significant inhibition values of the enzyme activity are found for all the compounds tested (Table 2). Two of them (**2** and **3**) show a 100% inhibition at 125 μM dose, whereas the monoalkylamino substituted derivative **4** exhibits a 94% inhibition. Compound **4** even gives an 80% inhibition at 2.5 μM concentration, while this value for compounds **1** and **2** fluctuate around 50%. These results could be interpreted considering that the remarkable high activity of compound **2** against epimastigotes of *T. cruzi* obtained in vitro is not only due to the blocking of the metal ion of SOD and that other action mechanisms could be also involved in the trypanosomatic activities observed.

In any case, a good degree of activity against the SOD of the parasite would be of no value if the same pattern was found for human SOD without any discrimination. Therefore, we have also tested the effect of compounds **1**−**4** over CuZn-SOD from human erythrocytes (Table 3).

The results obtained show that inhibition percentages at the higher dosages are very small in all cases for human SOD, and

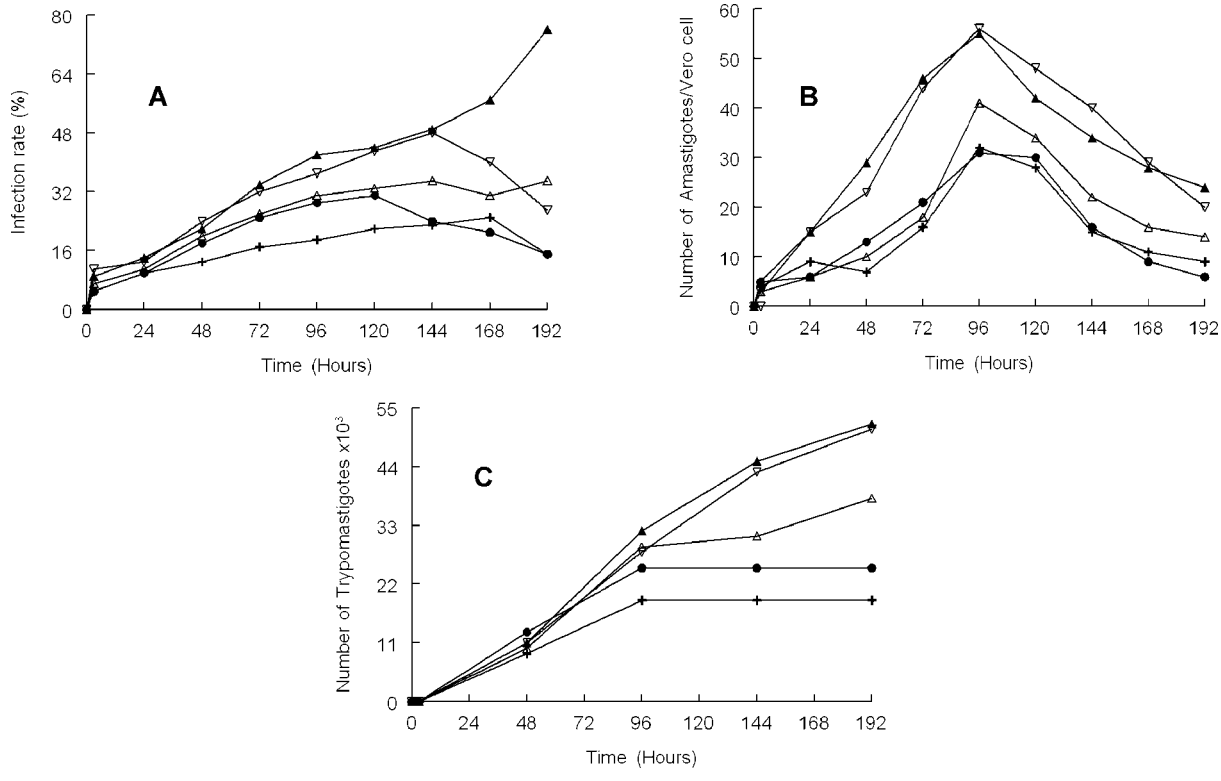


Figure 3. Effect of compounds **1**−**4** on the infection rate and *T. cruzi* growth. (A) Rate of infection. (B) Mean number of amastigotes per infected Vero cell. (C) Number of trypomastigotes in the culture medium. -▲-, control; -Δ-, **1**; -+-, **2**; -●-, **3**; -▽-, **4** (10 μM concentration). The values are means of three separate experiments.

Table 2. In Vitro Inhibition (%) of Fe-SOD in *Trypanosoma cruzi* Epimastigotes: 16.20 ± 1.69 Unit/mg Protein[a]

cmpd	2.5 μM	25 μM	125 μM
1	46	53	86
2	50	91	100
3	32	42	100
4	80	93	94

[a] Values are the average of five separate determinations. Differences between the activities of the control homogenate and the one incubated with the tested compounds were obtained according to the Newman−Keuls test.

Table 3. CuZn-SOD Activity Inhibition (%) in Human Erythrocytes: 23.36 ± 4.21 Unit/mg Protein

cmpd	2.5 μM	25 μM	125 μM
1	0	5	6
2	0	0	11
3	2	12	18
4	7	22	27

all the compounds are nearly inactive at the lower dose employed (2.5 μM) in the test. These results enhance the potential antiparasitical interest of the alkylaminobenzo[*g*]phthalazine derivatives studied in this work.

In conclusion, we have prepared a new series of benzo[*g*]-phthalazine derivatives **1**−**4** containing one or two imidazole rings as the key structural feature. These compounds exhibit excellent antiparasitic in vitro properties against *T. cruzi* epimastigotes and remarkably low toxicity against Vero cells. Furthermore, they are nearly inactive against human SOD, but active against Fe-SOD, although inhibition of this latter enzyme may not be the main factor for the activities observed. Although the results obtained here are very promising, further studies are necessary to determine the mechanism involved in the activity pattern observed for compounds **1**−**4**.

Experimental Section

The starting amines: 3-(imidazol-1-yl)propylamine and 2-(imidazol-4-yl)ethylamine (histamine) were purchased from Aldrich and used without further purification. 1,4-Dichlorobenzo[*g*]phthalazine was obtained from 2,3-naphthalenedicarboxylic acid following a method previously described.[11]

Synthesis of 1 and 2. A solution of 1,4-dichlorobenzo[*g*]phthalazine (386 mg, 1.56 mmol), 2-(imidazol-4-yl)ethylamine (692 mg, 6.23 mmol), and 473 mg (4.68 mmol) of triethylamine in xylene (60 mL) was heated at 120–130 °C for 10 h. The reaction mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The solid residue was purified by column chromatography with a polarity increasing chloroform/methanol mixture as eluent to obtain both products.

1,4-Bis[2-(imidazol-4-yl)ethylamino]benzo[*g*]phthalazine (1). The last eluted fraction afforded 239 mg of the dihydrochloride of **1** as a yellow solid, mp 218−220 °C (d). ^{1}H NMR (CD_3OD): δ 8.98 (s, 2H, H-5, H-10), 8.20 (m, 2H, H-6, H-9), 7.83 (m, 2H, H-7, H-8), 7.81 (s, 2H, H-2′), 7.02 (s, 2H, H-5′), 3.79 (t, 4H, Hα), 3.11 (t, 4H, Hβ). MS (FAB) *m*/*z* (%): 471 (M^+, 3), 436 (4), 399 (M^+ + 1 − 2HCl, 25), 398 (19), 317 (7), 304 (14), 223 (13), 210 (8), 179 (13). Anal. ($C_{22}H_{22}N_8 \cdot 2HCl$) C, H, N, Cl.

Flash column chromatography treatment of the hydrochloride of **1** on basic alumina using a polarity increasing mixture of chloroform/methanol as eluent gave 108 mg (18%) of free **1** as a yellow solid (R_f = 0.28, $CHCl_3$/MeOH/NH_4OH, v/v, 7:3:1), mp 123–125 °C. ^{1}H NMR (DMSO-d_6): δ 11.82 (br s, 2H, NH), 8.78 (s, 2H, H-5, H-10), 8.11 (m, 2H, H-6, H-9), 7.69 (m, 2H, H-7, H-8), 7.54 (s, 2H, H-2′), 6.88 (1 s, 2H, H-5′), 3.67 (t, 4H, Hα), 2.94 (t, 4H, Hβ). MS (FAB) *m*/*z* (%): 399 (M^+ + 1, 100), 398 (M^+, 38), 331 (10), 317 (21), 304 (5), 223 (9), 210 (12), 179 (11). Anal. ($C_{22}H_{22}N_8 \cdot 3H_2O$) C, H, N.

1-[2-(Imidazol-4-yl)ethylamino]-4-chlorobenzo[*g*]phthalazine (2). The less-retained fraction (R_f = 0.62) afforded 381 mg (72%) of **2** as a yellow solid, mp 240–245 °C (d). ^{1}H NMR (DMSO-d_6): δ 9.05 (s, 1H, H-10), 8.74 (s, 1H, H-5), 8.35 (m, 1H, H-9), 8.16 (m, 1H, H-6), 7.78 (m, 2H, H-7, H-8), 7.68 (s, 1H, H-2′), 6.92 (s, 1H, H-5′), 3.79 (t, 2H, Hα), 2.98 (t, 2H, Hβ). MS (FAB) *m*/*z* (%) 323 (M^+, 15), 256 (29), 242 (74), 229 (92), 213 (4), 179 (100), 151 (28). Anal. ($C_{17}H_{14}N_5Cl \cdot 4H_2O$) C, H, N.

Synthesis of 3 and 4. A solution of 1,4-dichlorobenzo[*g*]phthalazine (400 mg, 1.61 mmol), 3-(imidazol-1-yl)propylamine (0.77 mL, 6.45 mmol), and 489 mg (4.84 mmol) of triethylamine in xylene (25 mL) was refluxed for 6 h. Work-up of the reaction mixture by the same procedure used for compounds **1** and **2** allowed the isolation of the two substitution products.

1,4-Bis[3-(imidazol-1-yl)propylamino]benzo[*g*]phthalazine (3). The last eluted fraction (R_f = 0.23, Cl_3CH/MeOH/NH_4OH, v/v, 7:3:0.1) afforded 215 mg (31%) of a yellow solid, which was identified as **3**, mp 122–124 °C. ^{1}H NMR (DMSO-d_6): δ 8.81 (s, 2H, H-5, H-10), 8.14 (m, 2H, H-6, H-9), 7.71 (m, 2H, H-7, H-8), 7.70 (s, 2H, H-2′), 7.20 (s, 2H, H-5′), 6.89 (s, 2H, H-4′), 4.11 (t, 4H, Hγ), 3.39 (t, 4H, Hα), 2.13 (q, 4H, Hβ). MS (FAB) *m*/*z* (%) 427 (M^+ + 1, 100), 426 (M^+, 19), 359 (47), 263 (22), 250 (11), 179 (13). Anal. ($C_{24}H_{26}N_8 \cdot 3H_2O$) C, H, N.

1-[3-(Imidazol-1-yl)propylamino]-4-chlorobenzo[*g*]phthalazine (4). The less-retained fraction (R_f = 0.44, Cl_3CH/MeOH, v/v, 4:1) afforded 31 mg (6% yield) of **4** as a yellow solid: mp 217–218 °C. ^{1}H NMR (DMSO-d_6): δ 9.03 (s, 1H, H-10), 8.74 (s, 1H, H-5), 8.36 (m, 1H, H-9), 8.17 (m, 1H, H-6), 7.97 (m, 1H, H-8), 7.80 (m, 1H, H-7), 7.71 (s, 1H, H-2′), 7.25 (s, 1H, H-5′), 6.91 (s, 1H, H-4′), 4.13 (t, 2H, Hγ), 3.55 (t, 2H, Hα), 2.18 (q, 2H, Hβ). MS (EI) *m*/*z* (%) 337 (M^+, 12), 302 (21), 221 (2), 234 (70), 194 (22), 179 (100), 151 (18). Anal. ($C_{18}H_{16}N_5Cl \cdot 2H_2O$) C, H, N, Cl. The yield of compound **4** was substantially improved by increasing the reaction time to 30 h and diminishing the reaction temperature to 100 °C. Under these conditions, **4** was obtained in 52% yield, while compound **3** was not detected.

Biological Tests. The biological evaluation tests have been performed according to procedures previously described in the literature or developed by the authors following standard methods. Experimental conditions used for trypanocidal in vitro studies, cell culture and cytotoxicity tests, transformation of epimastigotes to metacyclic forms,[12] amastigote Vero cells assays,[13] and also Fe-SOD and human SOD enzymatic ihhibition studies[14,15] are explained in detail in the Supporting Information.

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Supporting Information Available: Details on chemical procedures and instruments used for isolation and identification of the newly synthesized compounds, combustion analysis, and also on IR and ^{13}C NMR data. Methodology followed in the biological evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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