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Trypanothione Reductase Inhibition/Trypanocidal Activity Relationships in a 1,4-Bis(3-aminopropyl)piperazine Series

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Abstract—A series of symmetrically substituted 1,4-bis(3-aminopropyl)piperazines was synthesized and tested towards trypanothione reductase and for its in vitro trypanocidal potency. The most trypanocidal amongst them was found to be totally inactive towards the enzyme and thus constitutes a lead structure for the identification of new potential *Trypanosoma cruzi* target(s).

Introduction

The antiparasitic activity of piperazine and its derivatives has been known for several decades. In the treatment of Chagas disease, compounds illustrated by Figure 1, in which R_1 is a heterocyclic moiety and R_2 a group consisting of a C_1–C_{12} alkyl chain, have been found to be active against experimental infections of *Trypanosoma cruzi* in mice.[1,2]

The target(s) of these molecules have never been identified but the simultaneous presence of a hydrophobic moiety and of protonated amines, suggests that they could act as inhibitors of trypanothione reductase (TR).[3,4] TR is an NADPH-dependent flavoenzyme which regenerates a glutathione-spermidine conjugate, N^1,N^8-bis(glutathionyl)spermidine, named trypanothione ($T(SH)_2$), from its oxidized form (Fig. 2).[3,5] The enzyme is responsible for the maintenance of the redox balance in trypanosomes, which do not possess the classic redox system based upon the couple glutathione/glutathione reductase (GR). Despite 41% of homology, TR and GR show almost total discrimination toward their respective substrates. Recently, in studies with TR null mutant, the couple trypanothione/trypanothione reductase has been demonstrated to be essential for the survival of the parasites *Leishmania donovani* and *Trypanosoma brucei* within the oxidative environment of the host.[6–8] For these reasons and facing the urgency to find new treatments for trypanosome infections, TR has been described as one of the most promising targets for the design of new trypanocidal drugs.[9]

Numerous molecules corresponding to protonated amines linked to hydrophobic moities: imipramines,[4] phenothiazines,[10] substituted polyamines,[11–13] 2-aminodiphenylsulfides[14–17] have been described as TR inhibitors. This inhibition is thought to occur through the simultaneous interaction with a hydrophobic pocket and a glutamic residue, via one molecule of water, all within the TR active site.[18] From the results of a high-throughput assay set up to screen new TR inhibitors,[19] we selected a series of compounds **1** possessing a piperazine group linked to a 2-aminodiphenylsulfide moiety (Fig. 3).[20] In this series, when the hydrophobic character was enhanced by the association of two aminodiphenylsulfides via an amide flanked spacer linked to the phenyl moiety (compounds **2**, Fig. 3), an increase of in vitro trypanocidal activity and TR inhibition was observed.[16,21] Nevertheless, the association of 2-aminodiphenylsulfides via the 2-amino group by an amino-side chain (compounds **3**, Fig. 3), led to a weak TR inhibition particularly when the spacer was short.[16] In addition, the presence of a piperazine in the linker (compound **4**, Fig. 3) cancelled inhibitory potency

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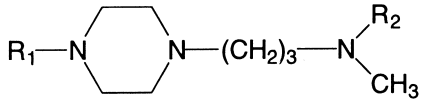


Figure 1. Piperazine derivatives active in vivo against *Trypanosoma cruzi*.

towards TR while displaying potent trypanocidal effects, suggesting that compound **4** could interact with other target(s).[22]

In order to establish structure–activity relationships between TR inhibition and trypanocidal activity from analogues of compound **4** and taking into account that many structurally diverse aldehydes are commercially available, we used a reductive amination reaction to prepare a variety of symmetrical amine derivatives from the 1,4-bis(3-aminopropyl)piperazine. In this paper, we report the synthesis and biological activity of these symmetrically substituted 1,4-bis(3-aminopropyl)piperazines (Fig. 4).

Chemistry

1,4-Bis(3-aminopropyl)piperazine derivatives **6a**–**y** were obtained according to Scheme 1 by condensation of 1,4-bis(3-aminopropyl)piperazine with aldehydes **5a**–**y**. The reaction was carried out in absolute ethanol, in the presence of molecular sieves to remove water and to displace the equilibrium towards diimine intermediates which were directly reduced by sodium borohydride.

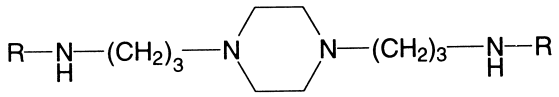


Figure 4. Symmetrical 1,4-bis(3-aminopropyl)piperazine derivatives.

When the required aldehyde **5** was not commercially available for reductive amination, it was synthesized from the corresponding carboxylic acid **7** (Scheme 2). Reduction of carboxylic acid **7** by the borane/tetrahydrofuran complex led to the alcohol intermediate **8**. Oxidation of **8** was carried out in mild conditions using pyridinium chlorochromate (PCC),[23] to avoid the over-oxidation of the sensitive aromatic aldehydes **5**.

The two commercially unavailable 2-(phenylsulfanyl)-benzoic acid derivatives **7i** and **7j** were prepared in 60% yield by refluxing the appropriate 2-iodobenzoic acid (X = H or Cl) with thiophenol and aqueous potassium hydroxide (5 equiv), in the presence of a catalytic amount (0.1 equiv) of copper (Scheme 3).[24]

Most of the piperazine derivatives **6a**–**y** were isolated after a simple acid-base extraction with purities >98% (assessed by HPLC). Compounds that needed further

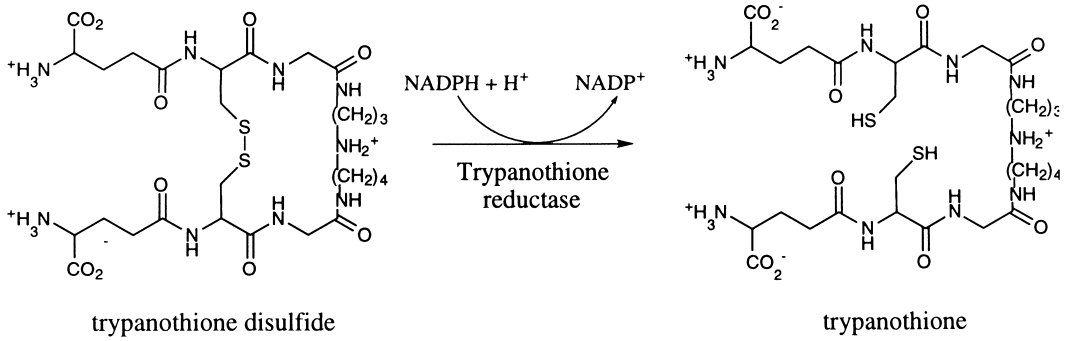


Figure 2. Trypanothione and trypanothione reductase.

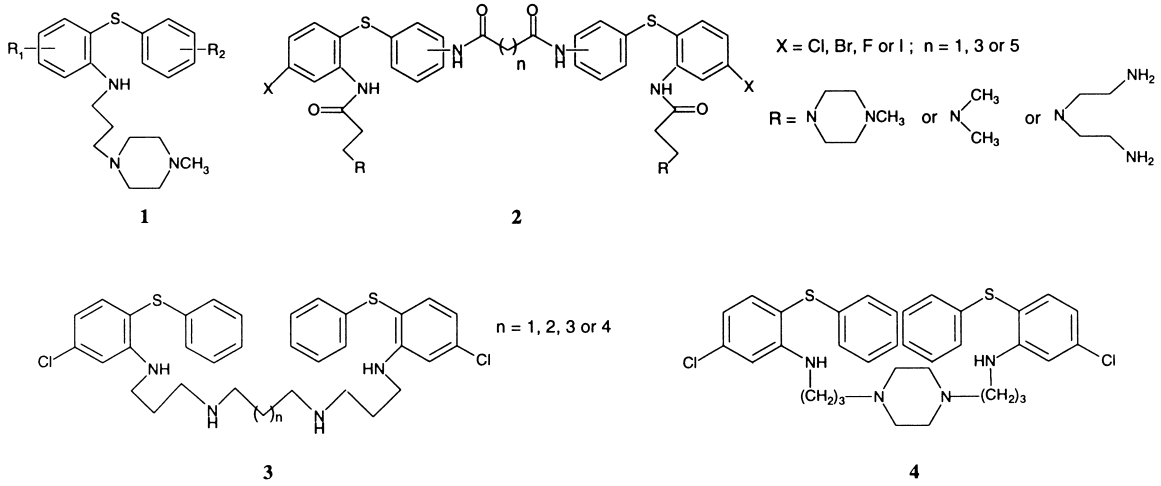
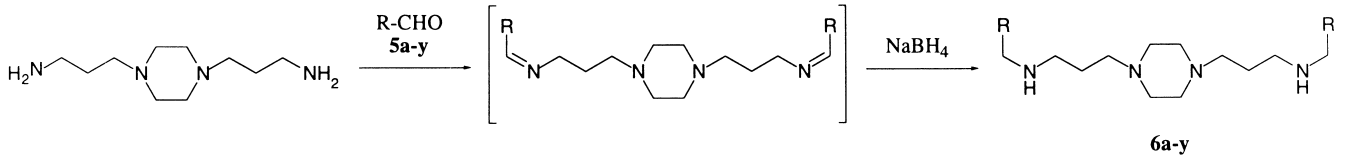
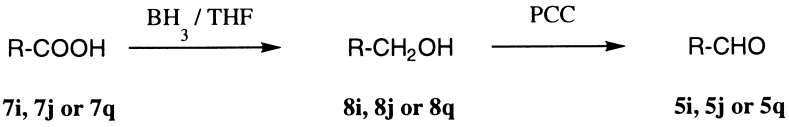


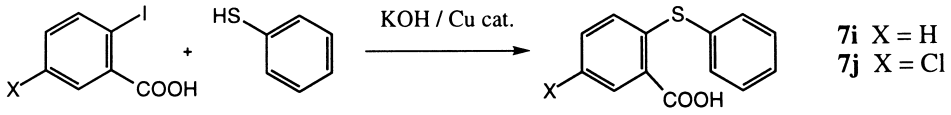
Figure 3. Compounds **1**, **2**, **3** and **4**.



Scheme 1. Synthesis of 1,4-bis(3-aminopropyl)piperazine derivatives **6a**–**y**.



Scheme 2. Synthesis of commercially unavailable aldehydes **5i**, **5j** and **5q**.



Scheme 3. Synthesis of 2-(phenylsulfanyl)benzoic acid derivatives **7i** and **7j**.

purification were purified by flash chromatography on neutral aluminum oxide.

Results and Discussion

All compounds were tested for their inhibitory potency towards trypanothione reductase from *T. cruzi* and their trypanocidal effects upon *T. cruzi* trypomastigote stage (Tables 1 and 2) as well as for their trypanocidal effect upon *T. brucei* trypomastigote stage and their cytotoxicity toward human MRC-5 cells (diploid embryonic lung cell line) (Table 3). The solubility of compounds within the enzymic buffer did not permit the testing of concentrations superior to 60 μM.

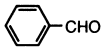
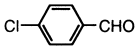
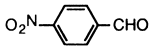
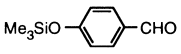
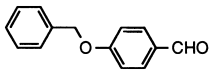
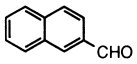
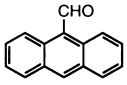
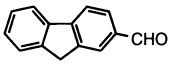
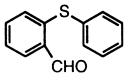
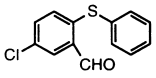
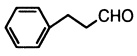
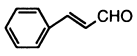
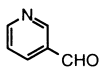
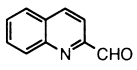
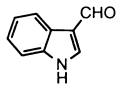
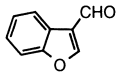
In the aromatic series (Table 1), the most potent TR inhibition was measured with polyphenyl derivatives **6e** and **6g**–**j**. Only one monophenyl derivative **6k** displayed an IC_{50} inferior to 60 μM. The two most potent TR inhibitors **6i** and **6j** (IC_{50} of 0.6 and 4 μM, respectively), are diphenylsulfides, as the lead structure **4**, inactive upon TR, yet they differ from compound **4** structurally by the presence of a benzylamine moiety. The additional methylene group in **6i** and **6j** is likely to be responsible for the TR inhibition since it increases the length of the spacer between the two aromatic moieties and/or introducing an additional site of basicity. The two inhibitors **6g** and **6h** (IC_{50} of 32 and 28 μM, respectively) are tricyclic aromatic compounds, Benson et al. have previously described tricyclic compounds that inhibit TR.[4] While the benzyloxybenzene derivative **6e** constitutes a new lead structure for TR inhibitors, **6f** and **6k** (IC_{50} of 60 and 30 μM, respectively) are structurally close to the N^4,N^8-bis(3-phenylpropyl)spermine, N^4,N^8-bis(2-naphtylmethyl)spermine and N^1,N^8-bis(2-naphtylmethyl) spermidine, already reported as TR inhibitors.[11,12] Heterocyclic compounds were generally less potent than polyphenyl derivatives and benzofuran derivative **6q** alone displayed an IC_{50} inferior to 60 μM. In parallel enzymic studies also revealed that the different inhibitors found with IC_{50} below 30 μM, were specific towards TR versus human glutathion reductase.

In the alicyclic series (Table 2), no TR inhibition was observed below 60 μM proving that an aromatic moiety is really needed for recognition.

A fairly good yet surprising correlation was observed between compounds found to be active upon TR from *T. cruzi* and their cytotoxicity upon mouse peritoneal macrophages used in the test (Table 1). This cytotoxicity was confirmed by studies upon human MRC 5 cells and by activity studies upon *T. brucei*, known for its sensitivity to toxic compounds (Table 3).

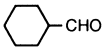
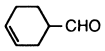
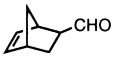
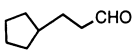
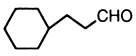
Only two compounds **6a** and **6u**, were found to be non-cytotoxic yet active upon *T. cruzi* trypomastigotes in the low micromolar range. The absence of a potent inhibition by these compounds upon TR from *T. cruzi* suggested that the trypanocidal activities were due to these compounds acting like the lead compound **4**, at an unidentified site or sites. The general absence of a potent TR inhibition has led us to prefer the search for new targets rather than to perform QSAR studies. Since **6u** differs from **6a** and **4** by the absence of aromatic moieties sensitive to stacking interactions, we selected **6u** as a ligand for the preparation of affinity chromatography columns in order to isolate and identify the potential target(s) of these 1,4-bis(3-aminopropyl)piperazine derivatives. Three parasitic proteins were thus isolated and the major of them, possessing an apparent molecular weight of 52 kDa, was identified as a thiol-disulfide oxidoreductase. Work describing the preparation of affinity chromatography columns, isolation and the identification of this protein has very recently been reported.[25]

Table 1. Biological activities of compounds **6a**–**r** prepared from aromatic aldehydes

Compounds	Aldehydes	IC_{50} on TR (μM)[a]	% Inhibition on *Trypanosoma cruzi* (μM)[b]				
			12.5	6.3	3.1	1.6	0.8
6a		>60	80	70	60	20	0
6b		>60	T	T	98	95	20
6c		>60	T	90	70	20	0
6d		>60	0	0	0	0	0
6e		28.5	T	T	T	99	80
6f		60	T	T	T	T	95
6g		32	T	T	T	T	T
6h		28	T	T	T	T	T
6i		0.6	T	T	T	T	T
6j		4	T	T	T	98	90
6k		30	T	T	40	0	0
6l		>60	T	98	80	20	0
6m		>60	0	0	0	0	0
6n		>60	89	40	0	0	0
6o		>60	0	0	0	0	0
6p		>60	0	0	0	0	0
6q		42	T	T	90	60	20
6r		>60	40	20	20	0	0

[a]Clomipramine: $IC_{50} = 12.5$ μM.
[b]T indicates that compound is cytotoxic towards macrophages used in the test.

Table 2. Biological activities of compounds **6s–y** prepared from nonaromatic aldehydes

Compound	Aldehyde	IC_{50} on TR (μM)[a]	% Inhibition on *Trypanosoma cruzi* (μM)[b]				
			12.5	6.3	3.1	1.6	0.8
6s		>60	40	0	0	0	0
6t		>60	0	0	0	0	0
6u		>60	100	95	80	60	20
6v		>60	40	0	0	0	0
6w		>60	0	0	0	0	0
6x		>60	T	40	0	0	0
6y		>60	T	T	T	70	0

[a]Clomipramine: $IC_{50} = 12.5$ μM.
[b]T indicates that compound is cytotoxic towards macrophages used in the test.

Table 3. Cytotoxicity and activity upon *Trypanosoma brucei* of compounds **6a–y**

Compound	% Cytotoxicity (μM)				% Inhibition on *Trypanosoma brucei* (μM)		
	25	12.5	6.3	3.1	6.3	3.1	1.6
6a	100	17	30	13	0	0	0
6b	100	100	79	0	100	100	0
6c	100	100	0	0	40	0	0
6d	0	0	0	0	0	0	0
6e	100	100	100	0	100	80	0
6f	100	100	100	0	100	100	100
6g	100	100	100	0	100	80	0
6h	100	100	100	0	100	100	100
6i	100	100	100	0	100	100	100
6j	100	100	100	0	100	80	0
6k	100	100	0	0	100	0	0
6l	100	100	0	0	100	0	0
6m	0	0	0	0	0	0	0
6n	100	0	0	0	80	0	0
6o	0	0	0	0	0	0	0
6p	0	0	0	0	0	0	0
6q	100	100	0	0	100	0	0
6r	0	0	0	0	0	0	0
6s	24	0	0	0	0	0	0
6t	0	0	0	0	0	0	0
6u	95	13	25	0	100	0	0
6v	0	0	0	0	0	0	0
6w	0	0	0	0	0	0	0
6x	100	0	0	0	40	0	0
6y	100	0	0	0	100	0	0

Experimental

Chemistry

All melting points were determined on a melting point Büchi apparatus and were uncorrected. All reactions were monitored by thin-layer chromatography (acetone:NH_4OH 28%, 9:1), carried out on 0.2 mm E. Merck silica gel plates (60F-254), using UV light as visualizing agent and 10% ninhydrin in acetone as developing agent; purity of final compounds was checked by HPLC (Nucleosil cyanopropyl), before preparing oxalate or chlorhydrate salts. ^1H and ^{13}C NMR spectra were obtained using a Brucker 300 MHz spectrometer; mass spectra were recorded on a time of flight plasma desorption spectrometer using a Californium source.

Procedure for oxalate salts. To a saturated solution of amine in ethyl acetate (AcOEt), was added dropwise a saturated solution of oxalic acid in AcOEt. The mixture was kept at 4 °C for 3 h; the salt was isolated by filtration, successively washed with ice cold AcOEt and ether, and dried on vacuum.

Procedure for chlorhydrate salts. To a saturated solution of amine in methanol (MeOH), was added an excess of an aqueous HCl 1 M solution (3 equiv). The mixture was stirred at room temperature for 1 h. Then the solvent was evaporated and the resulting salt was washed with ether and dried on vacuum.

General procedure for the preparation of 2-(phenylsulfanyl)-benzoic acid derivatives 7i and 7j. To a solution of KOH (11.2 g, 0.2 mol, 5 equiv), in 75 mL of water were added 2-iodobenzoic acid (9.9 g, 0.04 mol, 1 equiv), or 2-iodo-5-chlorobenzoic acid (11.3 g, 0.04 mol, 1 equiv), copper in powder (254 mg, 4 mol, 0.1 equiv), and thiophenol (4.4 g, 0.04 mol, 1 equiv). After refluxing the reaction mixture for 12 h, the resulting solution was cooled to room temperature, acidified with HCl 4N. The resulting precipitate was isolated by filtration, washed with water and dried on vacuum to yield the desired compound **7**.

2-(Phenylsulfanyl)benzoic acid (7i). White solid (60%); mp 166–168 °C.

5-Chloro-2-(phenylsulfanyl)benzoic acid (7j). White solid (60%); mp 219–221 °C.

General procedure for the preparation of alcohols 8i, 8j and 8q. At 0 °C and under nitrogen atmosphere, a solution of borane/THF complex 1 M in THF (50 mL, 50 mmol, 5 equiv), was added, dropwise, to a solution of acid **7i**, acid **7j** or benzofur-3-ylcarboxilic acid (10 mmol, 1 equiv), in 10 mL of THF. The reaction mixture was stirred for 12 h at room temperature and then hydrolysed with 1 M aq sodium carbonate followed by evaporation under reduced pressure. The residue was taken up in CH_2Cl_2, washed successively with water and NaOH 1 M. The organic layer was dried over $MgSO_4$, filtered and concentrated to yield the crude alcohol **8**, which was used directly in the next step.

2-(Phenylsulfanyl)benzyl alcohol (8i). Yellow oil (59%).

5-Chloro-2-(phenylsulfanyl)benzyl alcohol (8j). Yellow solid (95%); mp 42–44 °C.

Benzofur-3-ylmethyl alcohol (8q). Yellow oil (100%).

General procedure for the preparation of aldehydes 5i, 5j and 5q. To a solution of alcohol **8** (2 mmol, 1 equiv), in 30 mL of CH_2Cl_2 was added pyridium chlorochromate (647 mg, 3 mmol, 1.5 equiv). After stirring for 4 h at room temperature, the reaction mixture was filtered on Celite and concentrated. The residue was purified by thick-layer chromatography (SiO_2, petroleum ether: ether, 90:10), to yield the desired compound **5**.

2-(Phenylsulfanyl)phenylcarbaldehyde (5i). Yellow solid (72%); mp 46–48 °C.

5-Chloro-2-(phenylsulfanyl)phenylcarbaldehyde (5j). Yellow oil (57%).

Benzofur-3-ylcarbaldehyde (5q). Yellow oil (49%).

General procedure for the preparation of piperazine derivatives 6a–y. To a solution of 1,4-bis(3-aminopropyl)-piperazine (513 μL, 2.49 mmol, 1 equiv), and adequate aldehyde **5a–y** (5.24 mmol, 2.1 equiv), in 20 mL of absolute ethanol was added 3 Å molecular sieves (5 g). After stirring the mixture at room temperature for 12 h, sodium borohydride (1.9 g, 49.92 mmol, 20 equiv) was added and the mixture was stirred for 12 h at room temperature or under reflux (for compound **6c**). Then the reaction mixture was quenched by dropwise addition of water (20 mL) and ethanol was removed under reduced pressure. The aqueous residue was extracted with $CHCl_3$ (3×30 mL), then the combined organic layers were extracted with HCl 1 N (2×50 mL). The combined aqueous layers were neutralized with NaOH 1 N (50 mL) and extracted with $CHCl_3$ (3×30 mL). The combined organic layers were dried over $MgSO_4$, evaporated to dryness and the oily residue purified by flash chromatography (neutral aluminium oxide, CH_2Cl_2/MeOH), to yield the desired compound **6a–y**.

1,4-Bis{3-[*N*-(benzyl)amino]propyl}piperazine (6a). White solid (87%); mp 80–82 °C; ^{1}H NMR (CD_2Cl_2) δ 7.40–7.34 (m, 8H, aromatic H), 7.31–7.27 (m, 2H, aromatic H), 3.81 (s, 4H, CH_2), 2.73–2.68 (m, 4H, CH_2), 2.45–2.39 (m, 12H, CH_2), 2.29 (s, 2H, NH), 1.75–1.66 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 128.66, 127.12, 57.35, 54.27, 53.79, 48.53, 27.37; *m*/*z* 380; biological assays were performed using the oxalate salt of this compound.

1,4-Bis{3-[*N*-(4-chlorobenzyl)amino]propyl}piperazine (6b). White solid (66%); mp 77–78 °C; ^{1}H NMR (CD_2Cl_2) δ 7.32 (s, 8H, aromatic H), 3.76 (s, 4H, CH_2), 2.68–2.62 (m, 4H, CH_2), 2.40–2.35 (m, 12H, CH_2), 1.69–1.64 (m, 6H, NH and CH_2); ^{13}C NMR (CD_2Cl_2) δ 129.79, 129.28, 128.62, 57.30, 53.83, 48.46, 27.36; *m*/*z* 449; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(4-nitrobenzyl)amino]propyl}piperazine (6c). Yellow solid (24%); mp 58–60 °C; ^{1}H NMR (CD_2Cl_2) δ 8.21–8.17 (m, 4H, aromatic H), 7.58–7.54 (m, 4H, aromatic H), 3.91 (s, 4H, CH_2), 2.70–2.65 (m, 4H, CH_2), 2.42–2.37 (m, 12H, CH_2), 1.74–1.64 (m, 6H, NH and CH_2); ^{13}C NMR (CD_2Cl_2) δ 128.89, 123.75, 57.28, 53.82, 48.61, 27.36; *m*/*z* 470; biological assays were performed using the oxalate salt of this compound.

1,4-Bis(3-{*N*-[4-(trimethylsilyloxy)benzyl]amino}propyl)-piperazine (6d). White solid (27%); mp 92 °C; ^{1}H NMR (CD_3OD) δ 7.21–7.18 (m, 4H, aromatic H), 6.81–6.76 (m, 4H, aromatic H), 3.20 (s, 9H, CH_3), 2.90–2.69 (m, 4H, CH_2), 2.60–2.37 (m, 4H, CH_2), 2.32–2.00 (m, 8H, CH_2), 1.85–1.65 (m, 4H, CH_2), 1.62—1.50 (m, 4H, CH_2); ^{13}C NMR (CD_3OD) δ 162.73, 131.40, 115.41,

53.00, 48.55, 25.13, 21.68; *m*/*z* 556; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis(3-{*N*-[4-(benzyloxy)benzyl]amino}propyl)piperazine (6e). White solid (40%); mp 86–88 °C; ^{1}H NMR (CD_2Cl_2) δ 7.46–7.37 (m, 10H, aromatic H), 7.30–7.26 (m, 4H, aromatic H), 6.97–6.94 (m, 4H, aromatic H), 5.08 (s, 4H, CH_2), 3.73 (s, 4H, CH_2), 2.71–2.66 (m, 4H, CH_2), 2.41–2.35 (m, 12H, CH_2), 1.80 (s, 2H, NH), 1.71–1.68 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 130.89, 129.98, 129.38, 129.01, 70.31, 57.39, 53.70, 53.11, 48.48, 26.99; *m*/*z* 592; biological assays were performed using the oxalate salt of this compound.

1,4-Bis{3-[*N*-(napht-2-ylmethyl)amino]propyl}piperazine (6f). White solid (97%); mp 50–52 °C; ^{1}H NMR (CD_2Cl_2) δ 7.87–7.80 (m, 8H, aromatic H), 7.54–7.46 (m, 6H, aromatic H), 3.97 (s, 4H, CH_2), 2.76–2.71 (m, 4H, CH_2), 2.42–2.34 (m, 12H, CH_2), 1.79 (s, 2H, NH), 1.75–1.67 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 128.24, 127.99, 127.93, 127.04, 126.68, 126.34, 125.87, 57.39, 54.19, 53.68, 48.62, 27.01; *m*/*z* 480; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(anthr-9-ylmethyl)amino]propyl}piperazine (6g). Yellow solid (25%); mp 65–67 °C; ^{1}H NMR (CD_2Cl_2) δ 8.44–8.38 (m, 6H, aromatic H), 8.06–8.02 (m, 4H, aromatic H), 7.60–7.49 (m, 8H, aromatic H), 4.72 (s, 4H, CH_2), 2.97–2.92 (m, 4H, CH_2), 2.36–2.31 (m, 14H, NH and CH_2), 1.77–1.67 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 131.94, 130.62, 129.37, 127.28, 126.29, 125.32, 124.80, 59.00, 53.84, 49.77, 46.30, 27.29; *m*/*z* 580; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(fluoren-2-ylmethyl)amino]propyl}piperazine (6h). White solid (70%); mp 112–114 °C; ^{1}H NMR (CD_2Cl_2) δ 7.82–7.75 (m, 4H, aromatic H), 7.59–7.55 (m, 4H, aromatic H), 7.40–7.31 (m, 6H, aromatic H), 3.93 (s, 4H, CH_2), 3.85 (s, 4H, CH_2), 2.72–2.67 (m, 4H, CH_2), 2.42–2.36 (m, 12H, CH_2), 1.71–1.64 (m, 6H, NH and CH_2); ^{13}C NMR (CD_2Cl_2) δ 127.08, 127.02, 126.80, 125.39, 125.31, 119.99, 57.35, 54.39, 53.83, 48.57, 37.19, 27.53; *m*/*z* 556; biological assays were performed using the hydrochloride salt of this compound

1,4-Bis(3-{*N*-[2-(phenylsulfanyl)benzyl]amino}propyl)-piperazine (6i). Colourless oil (68%); ^{1}H NMR (CD_2Cl_2) δ 7.40–7.20 (m, 16H, aromatic H), 3.90 (s, 4H, CH_2), 2.71–2.64 (m, 4H, CH_2), 2.45–2.36 (m, 12H, CH_2), 1.82 (s, 2H, NH), 1.69–1.62 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 136.15, 135.13, 134.80, 132.08, 131.11, 130.53, 130.27, 129.78, 127.97, 59.04, 53.86, 51.83, 48.55, 30.50, 28.50; *m*/*z* 596; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis(3-{*N*-[5-chloro-2-(phenylsulfanyl)benzyl]amino}-propyl)piperazine (6j). Colourless oil (33%); ^{1}H NMR (CD_2Cl_2) δ 7.32–7.21 (m, 16H, aromatic H), 3.87 (s, 4H, CH_2), 2.66–2.61 (m, 4H, CH_2), 2.44–2.34 (m, 12H, CH_2), 1.79 (s, 2H, NH), 1.66–1.61 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 134.88, 130.01, 129.85, 129.67, 127.90, 127.22, 58.98, 53.86, 51.83, 48.51, 30.31, 27.30; *m*/*z* 665; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(3-phenylpropyl)amino]propyl}piperazine (6k). Colourless oil (33%); ^{1}H NMR (D_2O) δ 7.39–7.24 (m, 10H, aromatic H), 3.59–3.52 (m, 8H, CH_2), 3.30–3.24 (m, 4H, CH_2), 3.09–2.99 (m, 8H, CH_2), 2.71–2.66 (m, 4H, CH_2), 2.17–2.08 (m, 4H, CH_2), 1.99–1.94 (m, 4H, CH_2); ^{13}C NMR (D_2O) δ 128.89, 126.93, 53.91, 49.48, 47.66, 44.55, 32.17, 27.54, 22.43, 21.23; *m*/*z* 436; biological assays were performed using the hydrochloride salt of this compound.

1,4–Bis{3-[*N*-(cinnamyl)amino]propyl}piperazine (6l). Yellow oil (96%); ^{1}H NMR (CD_2Cl_2) δ 7.43–7.23 (m, 10H, aromatic H), 6.58–6.53 (m, 1H, CH), 6.38–6.30 (m, 1H, CH), 3.42–3.39 (m, 4H, CH_2), 2.71–2.66 (m, 4H, CH_2), 2.45–2.36 (m, 12H, CH_2), 1.72–1.62 (m, 6H, NH and CH_2); ^{13}C NMR (CD_2Cl_2) δ 130.81, 129.62, 128.87, 127.56, 126.53, 57.32, 53.82, 52.17, 48.48, 27.54; *m*/*z* 432; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(pyrid-3-ylmethyl)amino]propyl}piperazine (6m). Yellow oil (91%); ^{1}H NMR (CD_2Cl_2) δ 8.56–8.54 (m, 2H, aromatic H), 8.49–8.46 (m, 2H, aromatic H), 7.77–7.68 (m, 2H, aromatic H), 7.29–7.24 (m, 2H, aromatic H), 3.79 (s, 4H, CH_2), 2.69–2.64 (m, 4H, CH_2), 2.40–2.35 (m, 12H, CH_2), 1.71–1.62 (m, 6H, NH and CH_2); ^{13}C NMR (CD_2Cl_2) δ 150.06, 148.58, 135.85, 123.57, 57.26, 54.20, 53.78, 51.64, 48.52, 27.36; *m*/*z* 382; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(quinol-2-ylmethyl)amino]propyl}piperazine (6n). Orange oil (99%); ^{1}H NMR (CD_2Cl_2) δ 8.87–8.85 (m, 2H, aromatic H), 8.16–8.09 (m, 4H, aromatic H), 7.74–7.70 (m, 2H, aromatic H), 7.63–7.57 (m, 2H, aromatic H), 7.51–7.49 (m, 2H, aromatic H), 4.28 (s, 4H, CH_2), 2.83–2.78 (m, 4H, CH_2), 2.44–2.39 (m, 12H, CH_2), 1.87 (s, 2H, NH), 1.78–1.69 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 150.74, 130.43, 129.24, 126.64, 123.87, 120.11, 57.29, 53.83, 50.52, 49.12, 27.28; *m*/*z* 482; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(indol-3-ylmethyl)amino]propyl}piperazine (6o). White solid (31%); mp 130–132 °C; ^{1}H NMR (CD_3OD) δ 7.74–7.70 (m, 2H, aromatic H), 7.49 (s, 2H, aromatic H), 7.45–7.41 (m, 2H, aromatic H), 7.21–7.11 (m, 4H, aromatic H), 4.36 (m, 4H, CH_2), 3.18–3.13 (m, 4H, CH_2), 2.34–2.29 (m, 12H, CH_2), 1.83–1.73 (m, 6H, NH and CH_2); ^{13}C NMR (CD_3OD) δ 126.47, 122.37, 120.03, 118.01, 111.88, 56.23, 52.25, 47.99, 42.53, 22.29; *m*/*z* 458; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(fur-2-ylmethyl)amino]propyl}piperazine (6p). Yellow oil (78%); ^{1}H NMR (CD_2Cl_2) δ 7.38 (s, 2H, aromatic H), 6.35–6.34 (m, 2H, aromatic H), 6.20–6.19 (m, 2H, aromatic H), 3.76 (s, 4H, CH_2), 2.67–2.62

(m, 4H, CH_2), 2.46–2.34 (m, 14H, NH and CH_2), 1.69–1.62 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 143.27, 141.87, 110.98, 106.76, 59.32, 53.84, 48.26, 46.63, 27.40; *m*/*z* 360; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(benzofur-3-ylmethyl)amino]propyl}piperazine (6q). Colourless oil (32%); ^1H NMR (CD_2Cl_2) δ 7.57–7.54 (m, 2H, aromatic H), 7.47–7.44 (m, 2H, aromatic H), 7.27–7.21 (m, 4H, aromatic H), 6.61–6.60 (m, 2H, aromatic H), 3.92 (s, 4H, CH_2), 2.74–2.69 (m, 4H, CH_2), 2.43–2.36 (m, 12H, CH_2), 1.78 (s, 2H, NH), 1.73–1.65 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 128.97, 123.98, 122.93, 120.99, 111.19, 103.68, 27.24, 53.83, 48.38, 47.01, 27.21; *m*/*z* 460; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(thien-2-ylmethyl)amino]propyl}piperazine (6r). Yellow oil (90%); ^1H NMR (CD_2Cl_2) δ 7.24–7.21 (m, 2H, aromatic H), 6.95–6.93 (m, 4H, aromatic H), 3.98 (s, 4H, CH_2), 2.72–2.67 (m, 4H, CH_2), 2.42–2.35 (m, 12H, CH_2), 1.72 (s, 2H, NH), 1.70–1.62 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 126.84, 124.78, 124.37, 57.26, 53.79, 48.85, 48.36, 27.38; *m*/*z* 388; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(cyclopropylmethyl)amino]propyl}piperazine (6s). Yellow oil (44%); ^1H NMR (CD_2Cl_2) δ 2.72–2.68 (m, 4H, CH_2), 2.49–2.39 (m, 18H, NH and CH_2), 1.74–1.57 (m, 4H, CH_2), 1.03–0.94 (m, 2H, CH), 0.54–0.47 (m, 4H, CH_2), 0.18–0.13 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 59.56, 54.95, 54.56, 49.03, 26.61, 11.09, 3.66; *m*/*z* 308; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(cyclobutylmethyl)amino]propyl}piperazine (6t). Colourless oil (50%); ^1H NMR (CD_2Cl_2) δ 2.69–2.64 (m, 8H, CH_2), 2.54–2.38 (m, 16H, NH, CH_2 and CH), 2.10–2.07 (m, 4H, CH_2), 1.95–1.88 (m, 4H, CH_2), 1.72–1.67 (m, 8H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 57.61, 56.12, 53.70, 49.33, 41.11, 35.68, 26.81, 26.53, 18.89; *m*/*z* 336; biological assays were performed using the hydrochloride salt of this compound

1,4-Bis{3-[*N*-(cyclohexylmethyl)amino]propyl}piperazine (6u). Yellow oil (68%); ^1H NMR (CD_3OD) δ 2.80–2.70 (m, 4H, CH_2), 2.68–2.39 (m, 16H, CH_2), 1.86–1.71 (m, 12H, CH_2), 1.63–1.53 (m, 2H, CH), 1.40–1.20 (m, 8H, CH_2), 1.04–0.89 (m, 4H, CH_2); ^{13}C NMR (CD_3OD) δ 56.99, 56.26, 48.00, 37.51, 31.46, 26.63, 26.07, 25.50; *m*/*z* 392; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(cyclohexen-4-ylmethyl)amino]propyl}piperazine (6v). Colourless oil (84%); ^1H NMR (CD_2Cl_2) δ 5.73–5.65 (m, 4H, CH of double bond), 2.66–2.61 (m, 4H, CH_2), 2.52–2.35 (m, 12H, CH_2), 2.20–2.00 (m, 6H, CH_2 and CH), 1.90–1.60 (m, 14H, NH and CH_2), 1.30–1.22 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 127.41, 126.64, 57.50, 56.36, 53.83, 49.34, 34.32, 30.47, 27.41, 25.32; *m*/*z* 388; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis{3-[*N*-(5-norbornen-2-ylmethyl)amino]propyl}piperazine (6w). Colourless oil (49%); ^1H NMR (CD_2Cl_2) δ 6.16–6.11 (m, 2H, CH of double bond), 5.96–5.93 (m, 2H, CH of double bond), 2.90–2.75 (m, 4H, CH_2), 2.66–2.57 (m, 4H, CH_2), 2.50–2.24 (m, 18H, NH and CH_2), 1.95–1.80 (m, 2H, CH), 1.75–1.60 (m, 6H, CH_2 and CH), 1.47–1.20 (m, 6H, CH_2 and CH); ^{13}C NMR (CD_2Cl_2) δ 137.42, 132.52, 57.48, 54.80, 53.48, 49.81, 49.34, 45.50, 42.80, 39.69, 31.78, 30.99, 27.53; *m*/*z* 412; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis(3-{*N*-[3-(cyclopentyl)propyl]amino}propyl)piperazine (6x). Colourless oil (60%); ^1H NMR (CD_2Cl_2) δ 2.74–2.53 (m, 8H, CH_2), 2.50–2.34 (m, 12H, CH_2), 1.85–1.40 (m, 22H, CH_2 and CH), 1.38–1.32 (m, 4H, CH_2), 1.25–1.02 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 57.45, 56.76, 53.81, 50.59, 49.08, 41.13, 40.54, 34.27, 33.07, 29.63, 27.38, 25.54; *m*/*z* 420; biological assays were performed using the hydrochloride salt of this compound.

1,4-Bis(3-{*N*-[3-(cyclohexyl)propyl]amino}propyl)piperazine (6y). Colourless oil (62%); ^1H NMR (CD_2Cl_2) δ 2.74–2.64 (m, 4H, CH_2), 2.61–2.55 (m, 4H, CH_2), 2.52–2.14 (m, 18H, NH and CH_2), 1.76–1.59 (m, 14H, CH_2 and CH), 1.57–1.48 (m, 4H, CH_2), 1.29–1.17 (m, 8H, CH_2), 0.96–0.85 (m, 4H, CH_2); ^{13}C NMR (CD_2Cl_2) δ 59.08, 58.29, 55.37, 52.04, 50.71, 39.56, 37.06, 35.34, 29.01, 28.66, 28.36; *m*/*z* 448; biological assays were performed using the hydrochloride salt of this compound.

Biological evaluation

Assays for TR inhibition. Recombinant *T. cruzi* trypanothione reductase was produced from the SG5 *Escherichia coli* strain with the overproducing expression vector pIBITczTR.[26] TR activity was measured at 21 °C in a 0.02 M Hepes buffer, pH 7.25 containing 0.15 M KCl, 1 mM EDTA and 0.2 mM NADPH with an enzyme concentration of 0.02 U mL^{-1}. The reaction began with the addition of the enzyme and the subsequent decrease in absorbance was followed at 340 nm. IC_{50} of the different compounds was evaluated in the presence of 57 μM of $T(S)_2$, and 2% DMSO.

In vitro activity upon trypomastigotes (*T. cruzi*). Primary mouse peritoneal macrophages were seeded in 96-well microplates at 30,000 cells/well. After 24 h, about 100,000 trypomastigotes of *T. cruzi* were added per well together with 2-fold dilutions of the drug. The cultures were incubated at 37 °C in 5% CO_2 for 4 days. After fixation in methanol and Giemsa staining, the drug activity was semi-quantitatively scored as % reduction of the total parasite load (free trypomastigotes and intracellular amastigotes) and compared with untreated control cultures. Scoring was performed microscopically.

Cytotoxicity test upon MRC-5 cells. A human diploid embryonic lung cell line (MRC-5, Bio-Whittaker 72211D) was used to assess the cytotoxicity for host cells. MRC-5 cells were seeded in 96-well microplates at 5,000 cells per well. After 24 h, the cells were washed

and 2-fold dilutions of the drug were added to 200 mL standard culture medium (RPMI +5% FCS). The final DMSO concentration in the culture remained below 0.5%. The cultures were incubated at 37 °C in 5% CO_2 for 7 days. Untreated cultures were employed as controls. The cytotoxicity was determined using the colorimetric MTT assay[27] and scored as % reduction of absorption at 540 nm of treated cultures versus untreated control cultures.

In vitro activity upon trypomastigotes (*T. brucei*). Blood stream forms of *T. brucei* were cultivated in HMI-9 medium as described by Hirumi et al.[28] In a 96-well microplate, 10,000 haemoflagellates were incubated at different drug concentrations for 4 days. Parasite multiplication was measured colorimetrically (490 nm) following addition of MTS tetrazolium which converts to an aqueous soluble formazam product.[29]

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