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2-Amino Diphenylsulfides as Inhibitors of Trypanothione Reductase: Modification of the Side Chain

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Abstract—A molecular modeling study meant to detect pharmacophore-like patterns in the active site of trypanothione reductase (TR) offered hints about the opportunity of synthesizing and testing diphenylsulfide derivatives with prolonged or branched polyamino side chains as putative TR inhibitors. The inhibition results within the synthesized series confirmed the main working hypothesis inspired by the molecular modeling study. The different compounds were tested in vitro on the enzyme and on *Trypanosoma cruzi* and *Trypanosoma brucei* trypomastigotes as well as in vivo in infected mice. Copyright © 1996 Elsevier Science Ltd

Introduction

The parasitic protozoa *Trypanosoma* and *Leishmania* cause numerous diseases in both man and domestic animals; for example, in humans, *T. cruzi* is the agent of the often fatal Chagas disease for which there is no effective chemotherapy. In these parasites, the mammalian redox defense system based on glutathione and glutathione reductase (GR) is replaced by a spermidine conjugate named trypanothione[1] [N^1, N^8-bis (glutathionyl)spermidine]: $T(S)_2$ (Fig. 1). $T(S)_2$ is maintained in its reduced dithiol form $T(SH)_2$ by trypanothione reductase (TR).[2] Despite ca. 40% homology in their primary sequence, human GR and *T. cruzi* TR show almost total mutual discrimination towards their respective substrates.[3] TR, therefore, appears to be an ideal target for drugs that disrupt the natural defenses of the parasite without interfering with the host enzymes. One possibility consists of designing inhibitors based on the structure of the natural ligand trypanothione, such as non-reducible analogues in which the disulfide bond is converted to a carba analogue.[4] However, such inhibitors have two major disadvantages linked to their peptidic structure: one is the high cost of their synthesis, while the other is their predictable poor intracellular availability against the intracellular form of the parasite. Moreover, using sequence-specific antibodies, we have recently shown that large quantities of TR are present in the kinetoplasma of the parasite,[5] thus defining an additional biological membrane to be crossed between the blood and the target enzyme. Contrary to this structure-based approach, random screening of libraries of organic molecules may yield more promising candidates, both in terms of cost and bioavailability. Therefore, we have developed a high-throughput assay in order to screen for new TR inhibitors.[6] In this work we report the structure of one of the most promising compounds, our attempts to model its binding mode and the synthesis of more potent inhibitors.

We have attempted to model the binding of compound **1** in the catalytic site of TR. Instead of the unknown 3-D structure of TR from *T. cruzi*, we used the one of the highly homologous *Crithidia fasciculata* enzyme (70% of conserved amino acids).[7,8] An exhaustive docking study would require an extensive sampling of the conformational space and the explicit treatment of water molecules and would therefore be extremely time consuming. The binding data available from the X-ray structural study of the complex TR/N^1-bis-glutathionylspermidine[9] suggest that the fixation of the ligand to the TR site does not significantly modify the geometry of the side chains of residues in the catalytic site.

Under these circumstances, a simpler approach consisting of the exploration of a rigid site model by the flexible ligand, by means of a molecular dynamics (MD) simulation should be sufficient to provide the correct overall orientation of the ligand, even if it cannot predict the details of the binding. In other

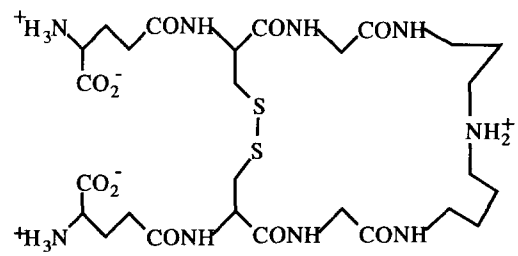


Figure 1. Trypanothione.

words, we wished to use this ligand to detect the preferred binding regions in the TR site and to draw some conclusions concerning this so defined TR 'pharmacophore'. Details concerning this modeling study are described in the Experimental.

Results

By subjecting a series of several ten thousands of compounds (individual molecules or combinatorial libraries) to the above mentioned assay,[6] we discovered two structurally-related inhibitors of TR with K_i in the range of 10 to 25 μM. These inhibitors were the 2-amino-diphenylsulfide (**1**) and the phenothiazine derivative **2** (Fig. 2).[10] Several phenothiazines have been reported previously to be potent and specific inhibitors of TR and active in vitro against *T. cruzi*.[11-15] However, their neuroleptic activity precludes their use in humans as antiparasitic drugs.

Interestingly, compound **1** belonged to a series which was prepared almost 20 years ago, in an attempt to apply the theory of 'open cycles' to phenothiazines but these derivatives displayed a very low neuroleptic activity.[10,16] Although disappointing at that time, the fact that neuroleptic and antiparasitic activities are not correlated and obey different structure–activity rules became in our case an advantage for the search of antiparasitic drugs. Compound **1** was competitive versus trypanothione for TR, while it did not inhibit human GR and was, therefore, selected as a lead to develop TR inhibitors. Previously synthesized analogues of **1** were also tested towards TR, to determine the pharmacophores of the structure.[10,16]

The modeling results showed that the calculated energies for the ligand/enzyme complex were consistently lower when the terminal piperazine nitrogen was protonated. The difference between the calculated energy minima differs by about 20 kcal/mol, out of which 5 kcal/mol represent the difference in intramolecular energies of the two different protonation states. Since the terminally protonated ligand therefore has a net advantage of 15 kcal/mol in the ligand–enzyme interaction energy, we supposed that this species is the one that binds to the TR site.

Only the low potential energy conformations in a window of 3 kcal/mol with respect to the minimal energy value were retained. The results of the docking model are presented in Figure 3. Accordingly, the aromatic moiety is preferentially oriented towards a hydrophobic region around Trp 21 and Met 113, while the charged ammonium group is within hydrogen bond distance of Glu 466′ and Glu 467′. The ligand has successfully evidenced a bipolar (hydrophobic pocket/negative charge) pharmacophore in the TR site that is able to accomodate its binding.

Other acidic residues (Glu 18 and Asp 116) can however be found in the vicinity of this hydrophobic pocket and could be taken into account in order to enlarge the considered bipolar pharmacophore. These residues are TR-specific and are not present in human GR. Consequently, the synthesis of polyamino derivatives with a diphenylsulfide aromatic system may lead to inhibitors with improved binding affinity. According to the orientation of the ligand, the most straightforward chemical modifications introducing side chains that should point towards Asp 116 (not seen in Fig. 3), would imply direct aromatic substitutions, which are quite tedious to perform on the available precursors.

The chosen solution was to branch or prolongate the amino chain by means of nucleophilic substitutions on their amino groups, in order to involve both Glu 466′/Glu 467′ and Glu 18 in the binding. The distance

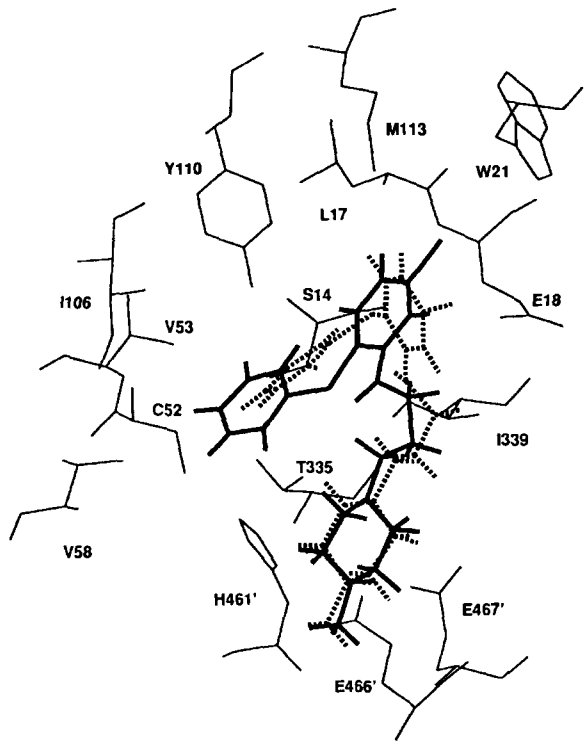
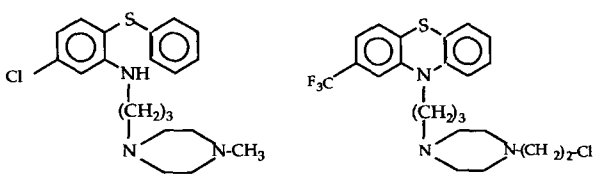


Figure 3. Orientation and binding of compound **1** in the catalytic site of TR as predicted by molecular dynamics calculations (Biosym software, InsightII and Discover modules). In the two conformations, the inhibitor molecule (in sticks) interacts with the glutamate residues Glu 466′ and Glu 467′ and with the hydrophobic region defined around amino acids Trp 21 and Met 113. In addition to Glu 466′ and Glu 467′, only the enzyme residues located at a distance lower than 4 Å from the aromatic diphenylsulfide unit are seen and labelled. The 'character' designs amino acids belonging to the second monomer unit of TR.



1 2

Figure 2. Compounds **1** and **2**.

between the carboxylates of the former and the latter acidic residue is ca. 12–13 Å. Therefore, the length of a linker between two ammonium groups interacting with the two corresponding zones of negative potential should be 6–8 Å.

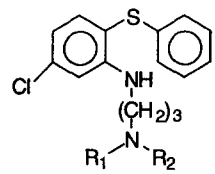
Assuming that the introduced chemical modifications will not prevent the aromatic moiety from binding in the hydrophobic pocket, the considered side chains should be able to accommodate further positively charged groups in the negative potential area due to the considered acidic residues in the site. This conclusion should be, however, considered with caution, since even a very small chemical modification of a ligand may completely change its binding mode. The modeling approach cannot be used to predict the precise chemical modifications within the advanced working hypothesis. On the contrary, testing different modifications of the side chains may show whether a peak in activity can be correlated to the predicted distance of 6 to 8 Å (e.g., 4–5 separating carbon atoms) between the introduced ammonium groups.

The new synthesized compounds have one linear or one ramified side chain (compounds **7–14**, Table 1) or are variants of the piperazine moiety obtained by different substitutions of the terminal nitrogen of **1** (compounds **15–18**, Table 2). These compounds were tested in vitro towards TR from *T. cruzi*, at 10 μM and when they were active, also at 3 and 1 μM. Percentages of inhibition are given in Tables 1 and 2 compared with clomipramine, the most available among the best TR inhibitors.[11,17,18] The compounds were also tested in vitro on *T. cruzi* and *T. brucei* trypomastigotes and, for the most promising ones, tested in vivo as described in the Experimental.

Chemistry

A series of 2-amino-4-chloro-diphenylsulfides bearing various amino substituents on the 2-amino group were prepared as outlined in Scheme 1. 2-Amino-4-chloro-diphenylsulfide (**3**) was obtained by reaction of 2,5-dichloro-1-nitrobenzene with thiophenol followed by reduction of the nitro group by catalytic hydrogenation. The reaction of **3** with 3-chloropropionyl chloride led to a mixture of the expected product **4** and of the acrylamide derivative **5** resulting from a β-elimination. This β-elimination occurred only very slowly when **4** was left in the reaction mixture, indicating that it occurred on 3-chloropropionyl chloride before its reaction with **3**. Replacement of triethylamine by pyridine which possesses a lower pK_a (5.2 versus 10.8) avoided the elimination and led quantitatively to **4**. Initial attempts to obtain **7–9** by reaction of **4** with various amines (which allows us to take advantage of the protecting effect provided by the acylation of the nuclear amino group), followed by reduction of the amide were not successful. Thus, **4** was first reduced to **6** by treatment with BH_3 (reaction with $LiAlH_4$ led to an extensive dehalogenation). Compound **6** was then successfully coupled to several diamines leading to **7**, **8**, and **9**. In each case, a large excess of amine (10 equiv) was used to avoid the formation of dimers. Compound **7** was further transformed to **14** by guanidylation with 3,5-dimethylpyrazole-1-carboxamidine nitrate.[19] Triamines in excess also reacted selectively by their primary amino group with **6** leading to **10**, **11**, and **12**. However, protection of the primary amine groups as their phtalimide derivatives[20] and transformation of **6** into its more active iodo analogue were necessary to obtain **13**. In the piperazine series, **1** and **18** were directly obtained by reaction of **6** with an excess of

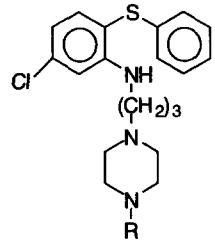
Table 1. Inhibitory activities of compounds **7–14** towards TR from *T. cruzi* and trypomastigote forms of *T. cruzi* and *T. brucei*



Compound concn (μm)	R		% Inhibition TR (*T. cruzi*)			*T. cruzi* % in vitro inhibition			*T. brucei* % in vitro inhibition			
	R_1	R_2	10	3	1	25	12.5	6.25	1.56	0.80	0.40	0.20
7	H	CH_2—CH_2—NH_2	0					0				
8	H	CH_2—CH_2—CH_2—NH_2	33			T[a]	T	T	100	90	0	
9	H	CH_2—CH_2—CH_2—CH_2—NH_2	67	29	8	T	T	T	0			
10	H	CH_2—CH_2—NH—CH_2—CH_2—NH_2	12			T	T	T	100	90	0	0
11	H	CH_2—CH_2—CH_2—NH—CH_2—CH_2—CH_2—NH_2	53	22	11	T	T	T	100	100	100	90
12	H	CH_2—CH_2—N(CH_2—CH_2—NH_2)$_2$	4			T	nd	0	0			
13	CH_2—CH_2—NH_2	CH_2—CH_2—NH_2	75	46	23	T	T	T	0			
14	H	CH_2—CH_2—NH—C(=NH)—NH_2	30			T	nd	0	0			
Clomipramine			45	16	4	40	0		0			

[a]T = cytotoxicity.

Table 2. Inhibitory activities of compound **1** and compounds **15–18** towards TR from *T. cruzi* and trypomastigote forms of *T. cruzi* and *T. brucei*



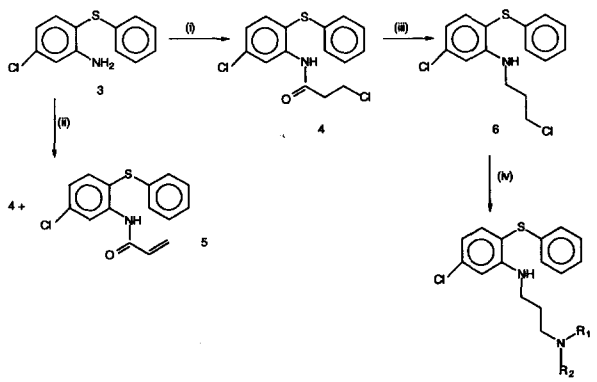
Compound concn (μm)	R	% Inhibition TR (*T. cruzi*)			*T. cruzi* % in vitro inhibition			*T. brucei* % in vitro inhibition			
		10	3	1	25	12.5	6.25	1.56	0.80	0.40	0.20
1	CH_3	22	14	11	T[a]	nd	0	0			
15	$(CH_2)NH_2$	22	5	3	T	nd	T	100	90	60	0
16	$CH_2)_2NH—CO—CH_3$	5			0			0			
17	$CH_2)_2NH—CO—C_6H_5$	5			0			0			
18	H	18			0			0			
Clomipramine		45	16	4	40	0		0			

[a]T = cytotoxicity.

amine, while **15** also required the protection of the primary amine.[20] Acylation of **15** with acetic or benzoyl chloride led, respectively, to **16** or **17**.

Discussion

The substitution of the methyl group in the lead compound **1** with ethylamino (compound **15**) did not improve the inhibitory potency towards TR. However, the acylation of the terminal amine function of **15**, and therefore the corresponding loss of basicity (compounds **16** and **17**) completely abolished its inhibiting potency. The presence of a secondary amino group introduced by the removal of the methyl group of piperazine ring (compound **18**) did not increase the inhibitory activity.



Scheme 1. Synthetic scheme for compounds **1** and **3–18**. Reagents: (i) $Cl(CH_2)_2COCl$, pyridine, $(Et)_2O$, 0 °C; (ii) $Cl(CH_2)_2COCl$, $(Et)_3N$, $(Et)_2O$, 25 °C; (iii) BH_3/THF, 0 °C then 2 h reflux; and (iv) amine, 10 equiv, C_6H_6, 16 h reflux.

On the contrary, the substitution of the piperazine ring with one ethylamino, propylamino or butylamino group could be favourable, depending on the primary amino group–aniline ring distance, as shown with compounds **7–9**. The presence of an additional amino group in the linear side chain (compounds **10–11**) did not improve the best value obtained for **9** while the transformation of the terminal amino group of **7** into a guanidino group, led to an active compound (**14**).

In both compounds possessing a ramified side chain (**12** and **13**), the distance between the two terminal primary amino groups and the aniline ring was still determining since **13** was the best inhibitor synthesized while **12** was practically inactive.

The best activities were obtained for species with side chains in which two NH^+ groups are separated by four (compound **9**) or five (compound **13**) atoms. In the side chain of **11**, the amino groups are separated by either three or seven atoms, a pattern that may easily adopt a conformation matching the proposed pharmacophore model.

Using Dixon plots, K_i values of 24 and 12 μM were respectively measured for the most potent inhibitors of TR: **9** and **13**, which were linearly competitive with trypanothione. The inhibitory potency of **13** is in the same range as clomipramine. All compounds were tested towards human glutathione reductase at 10 μM and were inactive.

All derivatives were also evaluated in vitro against amastigote and trypomastigote forms of *T. cruzi* in macrophage tissue cultures. Within the drug concentration range of 6.25–25 μM, the derivatives were either cytotoxic or inactive; the presence of the primary

amino groups can explain this cytotoxicity. With some derivatives, a complete inhibition of growth was observed in the in vitro evaluation against *T. brucei* trypomastigotes until 1.56 μM. However, given the greatest sensitivity of *T. brucei* compared with *T. cruzi* in the test, this result can be related to aspecific activity.

The best inhibitors towards TR, compounds **9**, **11**, and **13**, were also tested in vivo on mice. Animals were infected with hemoflagellates and at the same time were orally treated with the test compound at 40 mg/kg, then intraperitoneally at the same dose, on the 4 following days. While untreated control animals usually die between 6 and 7 days after infection, the majority of treated animals died before, suggesting some toxicity for these compounds. After lowering the dose to 10 mg/kg/day, no difference could be observed between treated and untreated animals.

In conclusion, the molecular modeling study of an inhibitor selected by random screening (compound **1**) allowed us to predict its position and its conformation in the catalytic site of trypanothione reductase from *T. cruzi*. Based on the pharmacophore-like binding patterns in the active site of the enzyme provided by molecular dynamics calculations, the modifications achieved on this lead compound in order to increase the overall binding energy led to a derivative as potent as clomipramine, one of the most efficient TR inhibitors known today. Unfortunately, this derivative and its analogues show an in vitro and an in vivo toxicity, frequently observed with polyamines. Moreover, their K_i values are still too high to conclude on the reliability of TR as target in *T. cruzi*. Work is now in progress to cancel or diminish this toxicity, to improve the binding to the hydrophobic pocket and to determine the possible other targets of this series of compounds using one of them as ligand in affinity chromatography.

Experimental

Molecular modeling

According to the p*K*a of piperazines (pK_1 9–10; pK_2 5–6),[21] the free ligand is expected to occur mostly in its monoprotonated form at pH 7 (global charge of +1). In the bound state, these p*K* values may be subjected to important shifts due to the influence of charged groups in the enzymatic site. Both nitrogen atoms of the piperazine ring are almost equiprobable candidates for protonation and therefore, we ran separate simulations with both monoprotonated species.

The CVFF force field (Biosym software, InsightII, and DISCOVER modules)[22] was used to simulate the inhibitor/enzyme complex model, with a cut-off radius of 10 Å and a vacuum dielectric constant $\varepsilon_r = 1$. The optimization of the inhibitor geometry using this force field led to unreasonable values for the torsional angles involving the sulfur atom. On the contrary, MOPAC-AM1[23] was successful in predicting correct ($\sim 90°$) C_{ar}—S—C_{ar}—C_{ar} torsional angles. A constraint term was thus added to the force field calculations in order to preserve the correct geometry of these torsions.

Since the only ligand for which the binding mode is known from X-ray structural studies,[9] N^1-bis-glutathionylspermidine, has no apparent similarity to our compound, we were not able to extrapolate an initial placement for the ligand on the basis of published coordinates. No special initial orientation of the ligand with respect to the site was imposed.

A superposition of the free[8] and complexed[9] TR sites revealed that the root mean square of the deviation of the positions of the residue backbone atoms is as small as 0.3 Å, while for all the heavy atoms, this value is of 0.9 Å. This validates to some extent our working hypothesis considering the site atoms as fixed. The sampling of the conformational space of the inhibitor in the 'rigid' site was performed by molecular dynamics (MD) at constant temperature (300 K), where velocities were rescaled every 10 iterations. The global time of simulation was 500 ps with an integration step of 1 fs.

An *a posteriori* correction for solvent effects was estimated for the sampled geometries, using Gilson and Honigš[24] simple desolvation term. The average atomic volume intervening in this computation was set to 2.2 Å^3 resulting from another docking study of TR inhibitors.[25] This corresponds to an average atomic radius of 0.8 Å, which is as expected lower than typical van der Waals radii since the 'excluded volume' due to atomic overlappings needs to be substracted when estimating this average atomic volume. For each sampled geometry, the so evaluated energetic contribution due to the loss of the solvation shells of both site and ligand upon binding was added to the vacuum potential energy provided by the MD simulation. This contribution does, however, only account for the dielectric effects of the solvent, but not for cavitation or hydrophobic effects.[26]

As evidenced by the binding study of N'-bis-glutathionylspermidine,[9] most of the site–ligand interactions are water-mediated. Performing the sampling of the conformational space in vacuum will enhance stronger site–ligand contacts which are actually unfavourable because they imply an important displacement of solvent. According to the vacuum Hamiltonian, the states that correspond to a minimum of the solvent-corrected energies are 'seen' as high energy levels. If these energy levels are high enough in order to become inaccessible with respect to the available kinetic energy of the ligand in the MD simulation, the sampling in vacuum is inappropriate, for example, in the absence of a solvent contribution the ligand would 'stick' to the site wall, being unable to visit the states it would have explored in the presence of water. Comparing the relative energies of the sampled conformations **i** with respect to the best minimum (**0**) in vacuum and after solvation correction respectively: $\Delta E^{vac}_i = E^{vac}_i - E^{vac}_0$ and $\Delta E^{sol}_i = E^{sol}_i - E^{sol}_0$, we found that within the set of

conformations with $\Delta E_i^{vac} < 20$ kcal/mol, ΔE_i^{vac} and ΔE_i^{sol} differ in average by 2.5 kcal/mol. This difference is small compared with the range of energies of the conformations that were sampled in vacuum, due to the fact that velocity rescaling was employed in order to ensure a constant kinetic energy of the ligand and enhance the crossing of energy barriers.

Chemistry

All melting points were determined on a melting point Büchi apparatus and were uncorrected. All reactions were monitored by TLC (acetone:NH_4OH 28%, 9:1) carried out on 0.2 mm E. Merck silica gel plates (60F-254) using UV light as a visualizing agent and 10% ninhydrin in acetone or Reindel Hoppe[27] (R.H.) as developing agents; the purity of the final compounds was checked by HPLC (Nucleosil cyanopropyl) before preparing the oxalate salts. ^1H spectra were obtained using a Bruker 300 MHz spectrometer; mass spectra were recorded on a time of flight plasma desorption spectrometer using a Californium source. Microanalyses were obtained from CNRS (France) and were calculated for oxalate salts.

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 and successively washed with ice cold, AcOEt, and ether.

***N*-(3-Chloropropyl)-5-chloro-2-phenylthiophenylamine (4).** To a cooled solution of 2-amino-4-chloro diphenylsulfide (2.5 g, 10.6 mmol) in 44 mL of ether and 1.7 mL of pyridine was added dropwise 3-chloropropanoyl chloride (1.2 mL, 12.7 mmol). The mixture was stirred for 20 min, then allowed to reach room temperature. It was extracted successively with a saturated solution of NaCl (30 mL), 1 M HCl (30 mL) and 2.5 M NaOH (30 mL). The organic layer was separated, dried over Na_2SO_4, evaporated to yield quantitatively **4** as a colourless solid (3.5 g) which was used without further purification: R_f (pentane:ether, 10:2): 0.3; mp 77 °C; ^1H NMR (CD_2Cl_2): δ 8.33 (broad, 1H, NH), 7.56–7.59 (m, 1H, Ph), 7.13–7.33 (m, 7H, Ph), 3.77 (t, $J = 7$ Hz, 2H, CH_2), 2.73 (t, $J = 7$ Hz, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 8.33 exchanged; *m/z* 325,9; anal. calcd for $C_{15}H_{13}NOCl_2S$: C, 55.22; H, 4.01; N, 4.29. Found: C, 55.53; H, 4.21; N, 4.25.

4-Chloro-2-(3-chloropropylamino) diphenylsulfide (6). Under N_2, to a cooled solution of **4** (1.0 g, 3.1 mmol) in 3 mL of anhydrous THF was added dropwise for 1 h 25 mL (25 mmol) of commercial 1 M BH_3/THF solution. The mixture was stirred for 30 min, allowed to react for 2 h at room temperature, and refluxed for 2 h. Excess BH_3 was neutralized by methanol at 0 °C and the solution was evaporated to dryness. The oily residue was treated with a mixture of CH_2Cl_2 and brine. The organic layer was separated and evaporated to dryness to yield quantitatively **6** (940 mg) as a colourless solid, which was used without further purification: R_f (pentane:ether, 10:2): 0.5; mp 48 °C; ^1H NMR (CD_2Cl_2): δ 7.08–7.46 (m, 6H, Ph), 6.69–6.73 (m, 2H, Ph), 5.05 (broad, 1H, NH), 3.43 (t, $J = 7$ Hz, 2H, CH_2), 3.30–3.36 (m, 2H, CH_2), 1.98 (qt, $J = 7$ Hz, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.05 exchanged, 3.32 (t, $J = 7$ Hz, 2H); *m/z* 311.5; anal. calcd for $C_{15}H_{15}NCl_2S$: C, 57.69; H, 4.84; N, 4.48. Found: C, 57.50; H, 4.79; N, 4.46.

4-Chloro-2-(3-iodopropylamino) diphenylsulfide. This analogue of **6** was synthesized as follows. To a solution of 0.1 g (0.32 mmol) of 4-chloro-2-(3-chloropropylamino) diphenyl sulfide **6** in 1 mL of acetone, were added 0.24 g (5 equiv) of NaI. After refluxing for 3 h the mixture was evaporated to dryness. The residue was treated with a mixture of CH_2Cl_2 and H_2O; the organic phase was separated, dried, and evaporated to give the iodo analogue of **6** directly used for preparing **13**.

General procedure for the substitution of 6 by polyamines

To a solution of 0.2 g (0.65 mmol) of 4-chloro-2-(3-chloropropylamino) diphenyl sulfide **6** in 1.3 mL of benzene were added 10 equiv of polyamine. After refluxing the mixture for 15 h, the solvent was removed and the oily residue was treated with a mixture of CH_2Cl_2 and saturated aqueous NaCl. Products of the nucleophilic substitution were obtained as colourless oils by thick-layer chromatography (SiO_2, acetone: NH_4OH 28%, 9:1).

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*-[2-amino-ethyl]-1,3-propylenediamine (7).** 215 mg, 93%; R_f 0.7; ^1H NMR (CD_2Cl_2): δ 7.07–7.42 (m, 6H, Ph), 6.63–6.67 (m, 2H, Ph), 5.50 (br, NH), 3.19–3.25 (m, 2H, CH_2), 3.02 (br, NH), 2.69–2.75 (m, 2H, CH_2), 2.54–2.63 (m, 4H, CH_2), 1.74 (qt, $J = 6$ Hz, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.50 exchanged, 3.02 exchanged, 3.21 (t, $J = 6$ Hz, 2H, CH_2), 2.73 (t, $J = 6$ Hz, 2H, CH_2); *m/z* 334.8; anal. calcd for $C_{17}H_{22}N_3ClS$: C, 48.88; H, 5.08; N, 8.14. Found: C, 48.72; H, 5.17; N, 7.97.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*-[2-aminopropyl]-1,3-propylenediamine (8).** 190 mg, 85%; R_f 0.8; ^1H NMR (CD_2Cl_2): δ 7.07–7.41 (m, 6H, Ph), 6.63–6.68 (m, 2H, Ph), 5.48 (br, NH), 3.18–3.24 (m, 2H, CH_2), 3.04 (br, NH), 2.73–2.78 (m, 2H, CH_2), 2.52–2.64 (m, 4H, CH_2), 1.60–1.72 (m, 4H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$) 5.48 exchanged, 3.20 (t, $J = 6$ Hz, 2H, CH_2); 3.04 exchanged, 2.74 (t, $J = 6$ Hz, 2H, CH_2); *m/z* 349.2; anal. calcd for $C_{22}H_{28}N_3O_8ClS \cdot H_2O$: C, 48.22; H, 5.51; N, 7.66. Found: C, 48.34; H, 5.47; N, 7.68.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*-[2-aminobutyl]-1,3-propylenediamine (9).** 172 mg, 74%; R_f 0.6; ^1H NMR (CD_2Cl_2): δ 7.07–7.42 (m, 6H, Ph), 6.64–6.69 (m, 2H, Ph), 5.55 (br, NH), 3.14–3.35 (m, CH_2 and

NH), 2.67–2.75 (m, 2H, CH_2), 2.53–2.59 (m, 4H, CH_2), 1.74 (qt, $J = 6$ Hz, 2H, CH_2), 1.49–1.53 (m, 4H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.55 exchanged, 3.19 (t, $J = 7$ Hz, 2H, CH_2), 2.73 (t, $J = 7$ Hz, 2H, CH_2); *m/z* 364.3; anal. calcd for $C_{23}H_{30}N_3O_8ClS$: C, 50.78; H, 5.55; N, 7.72. Found: C, 50.69; H, 5.63; N, 7.37.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*-[2-(2-amino-ethyl)amino-ethyl]-1,3-propylenediamine (10).** 180mg, 74%; R_f 0.3; ^1H NMR (CD_2Cl_2): δ 7.07–7.41 (m, 6H, Ph), 6.63–6.68 (m, 2H, Ph), 5.48 (br, NH), 2.42–3.29 (br, CH_2 and NH), 1.76 (m, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$) 5.48 exchanged, 3.23 (t, $J = 6$ Hz, 2H, CH_2), 2.84–2.88 (m, 4H, CH_2), 2.57–2.70 (m, 4H, CH_2); 2.76 (t, $J = 6$ Hz, 2H, CH_2); *m/z* 406.2; anal. calcd for $C_{27}H_{37}N_4O_{12}ClS \cdot H_2O$: C, 45.01; H, 5.28; N, 8.39. Found: C, 44.93; H, 5.22; N, 8.39.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*-[3-(3-amino-propyl)amino-propyl]-1,3-propylenediamine (11).** 200 mg, 77%; R_f 0.2; ^1H NMR (CD_2Cl_2): δ 7.07–7.41 (m, 6H, Ph), 6.63–6.687 (m, 2H, Ph), 5.54 (br, 1H, NH), 3.11–3.19 (m, 2H, CH_2), 2.25–2.80 (br, CH_2 and NH), 1.54–1.77 (m, 6H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.54 exchanged, 2.25–2.70 (m, 4H, CH_2), 2.76 (t, $J = 6$ Hz, 2H, CH_2), *m/z* 406.2; anal. calcd for $C_{27}H_{37}N_4O_{12}ClS$: C, 47.89; H, 5.50; N, 8.27. Found: C, 47.99; H, 5.63; N, 8.36.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'* [*N*,*N*-(bis(2-aminoethyl)-2-aminoethyl]-1,3-propylenediamine (12).** 183 mg, 71%; R_f 0.2; ^1H NMR (CD_2Cl_2): δ 7.08–7.41 (m, 6H, Ph), 6.64–6.68 (m, 2H, Ph), 5.48 (br, NH), 2.42–3.29 (br, CH_2, and NH), 1.76 (m, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.48 exchanged, 3.23 (t, $J = 6$ Hz, 2H, CH_2), 2.84–2.88 (m, 4H, CH_2), 2.57–2.70 (m, 10H, CH_2); *m/z* 421.6; anal. calcd for $C_{29}H_{40}N_5O_{16}ClS$: C, 44.53; H, 5.15; N, 8.95. Found: C, 44.45; H, 5.32; N, 9.04.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*,*N'*-[2-aminoethyl]-1,3-propylenediamine (13).** Bis-(3-phthaloyl-aminopropylamine)[20] (530 mg, 1.5 mmol) was added to a solution of the iodo analogue of **6** (prepared as mentioned above, 150 mg, 0.4 mmol) in 1.2 mL of benzene. The mixture was refluxed for 15 h and the solvent was removed. The crude product was treated with a mixture of CH_2Cl_2 and 0.5 M sodium thiosulfate and the organic layer was washed with brine. After purification by thick-layer chromatography (SiO_2, CH_2Cl_2), the bis-phthaloyl derivative of **13** was obtained as a colourless solid (110 mg, 46%); R_f (CH_2Cl_2) 0.2; ^1H NMR (CD_2Cl_2): δ 7.66–7.68 (m, 8H, Ph), 7.0–7.33 (m, 6H, Ph), 6.59–6.63 (m, 1H, Ph), 6.46 (s, 1H, Ph), 5.24 (NH, br), 3.60 (t, $J = 6$ Hz, 4H, CH_2), 2.86–2.92 (m, 2H, CH_2), 2.70 (t, $J = 6$ Hz, 4H, CH_2), 2.48 (t, $J = 6$ Hz, 2H, CH_2), 1.57 (qt, $J = 6$ Hz, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.24 exchanged, 2.89 (t, $J = 6$ Hz, 2H, CH_2); *m/z* 639.2.

The bis-phthaloyl derivative of **13** was added to a mixture hydrazine hydrate (0.5 mL) and ethanol (1 mL). After 10 min at 50 °C, the solvent was removed and the oily residue was treated with a mixture of CH_2Cl_2 and brine. Purification by thick-layer chromatography (SiO_2, acetone:NH_4OH 28%, 9:1) yielded quantitatively **13** (27 mg) as a colourless oil; R_f (acetone: NH_4OH 28%, 9/1): 0.3; ^1H NMR (CD_2Cl_2): δ 7.07–7.42 (m, 6H, Ph), 6.65–6.70 (m, 2H, Ph), 5.25 (br, NH), 3.16–3.28 (m, 2H, CH_2), 2.38–2.69 (m, CH_2, and NH), 1.66–1.71 (m, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.25 exchanged, 2.64–2.68 (m, 4H, CH_2), 2.38–2.45 (m, 4H, CH_2), 2.13 (t, $J = 6$ Hz, 2H, CH_2); *m/z* 377.5; anal. calcd for $C_{25}H_{33}N_4O_{12}ClS \cdot H_2O$: C, 45.01; H, 5.28; N, 8.39. Found: C, 45.07; H, 5.34; N, 8.34.

***N*-[5-Chloro-2-phenylthio)phenyl]-*N'*-[2-guanidino-ethyl]-1,3-propylenediamine (14).** According to the method of Bernatowicz et al.,[19] 1*H*-pyrazole-1-carboxamidine nitrate (80 mg, 0.4 mmol) was added to a solution of **7** (90 mg, 0.26 mmol) and sodium bicarbonate (22 mg, 0.26 mmole) in 4.3 mL of ethanol. After refluxing the mixture for 24 h, the solvent was removed; the oily residue was suspended in MeOH, filtered and the filtrate was purified by HPLC (Nucleosil cyanopropyl) to obtain the TFA salt of **14** (49 mg, 48%, hygroscopic); ^1H NMR (CF_3COOD): δ 7.45–7.60 and 7.07–7.36 (m, 13H, Ph, and NH), 3.73–3.81 (m, 2H, CH_2), 3.56–3.59 (m, 2H, CH_2), 3.41–3.50 (m, 2H, CH_2), 3.27–3.32 (m, 2H, CH_2), 2.31–2.42 (m, 2H, CH_2); *m/z* 379.7.

1-{*N*-[(4-Chloro-2-phenylthio)phenyl]-3-aminopropyl}-4-(2-aminoethyl) hexahydropyrazine (15). To a solution of 4-chloro-2-(3-chloropropylamino) diphenylsulfide (500 mg, 1.6 mmol) in 3.2 mL of benzene was added *N*-(2-(*N'*-piperazinyl)ethyl phtalimide[20] (2.0 g, 8 mmol) and the mixture was refluxed for 24 h. The solvent was removed and the crude solid was treated with a mixture of CH_2Cl_2 and brine. The organic layer was separated, evaporated to dryness, and purified on thick-layer chromatography (SiO_2, CH_2Cl_2:MeOH, 9:1) to obtain the phthaloyl derivative of **15** (535 mg, 63%) as a yellow oil; R_f (CH_2Cl_2:MeOH, 9:1) 0.7, R.H. positive; ^1H NMR (CD_2Cl_2): δ 8.44 (s, 1H, Ph), 7.45–7.86 (m, 4H, Ph), 7.08–7.44 (m, 8H, Ph), 5.08 (br, NH), 3.75 (t, $J = 6$ Hz, 2H, CH_2), 3.17–3.22 (m, 2H, CH_2), 2.49–2.54 (m, 12H, CH_2), 1.73 (qt, $J = 6$ Hz, 2H, CH_2); ∂ ($CD_2Cl_2 + CD_3OD$); 5.08 exchanged, 3.19 (t, $J = 6$ Hz, 2H, CH_2); *m/z* 534.5.

Hydrazine hydrate (85 μL) was added to a refluxing solution of the phthaloyl derivative of **15** (278 mg, 0.52 mmol) in 2.5 mL of ethanol. After 1 h, the solvent and the excess of hydrazine were removed; the oily residue was suspended in ether and filtered. The filtrate was concentrated and treated with a mixture of CH_2Cl_2 and water. The organic layer was separated, dried, and evaporated to dryness. Compound **15** was obtained as a yellow oil (177 mg, 84%); R_f (acetone:NH_4OH 28%, 9:1) 0.7, ninhydrin positive; ^1H NMR (CD_2Cl_2): δ 7.06–7.41 (m, 6H, Ph), 6.63–6.72 (m, 2H, Ph), 5.05 (br, NH), 3.76 (br, NH), 3.17–3.21 (m, 2H, CH_2), 2.80–2.89 (m, 2H, CH_2), 2.23–2.54 (m, 12H, CH_2), 1.71 (qt, $J = 7$

Hz, 2H, CH_2); ∂ ($CD_2Cl_2+CD_3OD$); 5.05 and 3.76 exchanged, 3.18 (t, $J = 7$ Hz, 2H, CH_2); *m/z* 404.4; anal. calcd for $C_{27}H_{35}N_4O_{12}ClS \cdot 2H_2O$: C, 45.60; H, 5.52; N, 7.87. Found: C, 45.53; H, 5.75; N, 8.12.

1-{*N*-[(4-Chloro-2-phenylthio)phenyl]-3-aminopropyl}-4-{2-(acetyl)aminoethyl}hexahydropyrazine (16) and 1-{*N*-[(4-chloro-2-phenylthio)phenyl]-3-aminopropyl}-4-{2-(benzoyl)aminoethyl}hexahydropyrazine (17). Under N_2, to an ice-cooled solution of amine **15** (173 mg, 0.43 mmol) in pyridine (3.5 mL) was added dropwise 1.2 equiv of acetyl chloride (0.51 mmol, 37 mL) or benzoyl chloride (0.51 mmol, 60 mL). After 1 h, evaporation of pyridine under reduced pressure gave a yellow oil which was chromatographed on a silica gel column using the mixture CH_2Cl_2:MeOH (98/:2) as eluent to yield the amide derivatives **16** and **17**.

16. 176 mg, 92%; R_f (CH_2Cl_2:MeOH: 9:1): 0.6; R.H. positive; ^1H NMR (CD_2Cl_2): δ 7.07–7.41 (m, 6H, Ph), 6.64–6.73 (m, 2H, Ph), 6.05 (br, NH), 5.56 (br, NH), 3.17–3.30 (m, 4H, CH_2), 2.28–2.48 (m, 12H, CH_2), 1.29–1.69 (m, 2H, CH_2); δ (CD_2+CD_3OD); 6.05 and 5.56 exchanged, *m/z* 447.2; anal. calcd for $C_{23}H_{31}ClN_4OS$: C, 61.67; H, 6.92; N, 12.51. Found: C, 61.59; H, 7.09; N, 12.60.

17. 160 mg, 74%; R_f (CH_2Cl_2/:MeOH: 9:1): 0.40; R.H. positive; ^1H NMR (CD_2Cl_2): δ 7.76–7.80 (m, 2H, Ph), 7.39–7.57 (m, 4H, Ph), 7.07–7.28 (m, 5H, Ph), 6.82 (br, NH), 6.64–6.73 (m, 2H, Ph), 5.55 (br, NH), 3.48–3.53 (m, CH_2), 3.18–3.24 (m, CH_2), 2.29–2.61 (m, 12H, CH_2), 1.67–1.76 (m, CH_2), δ ($CD_2Cl_2+CD_3OD$): 6.82 and 5.55 exchanged; *m/z* 508.1; anal. calcd for $C_{28}H_{33}ClN_4OS$: C, 66.08; H, 6.49; N, 11.01. Found: C, 65.94; H, 6.58; N, 10.84.

1-{*N*-[(4-chloro-2-phenylthio)phenyl]-3-aminopropyl}-hexahydropyrazine (18). To a solution of 500 mg (1.6 mmol) of 4-chloro-2-(3-chloro) propylamino diphenyl sulfide in 3.2 mL of benzene was added piperazine (690 mg, 8 mmol). After refluxing for 20 h, the solvent was removed and the oily residue was treated with a mixture of CH_2Cl_2 and brine. The organic layer was separated, evaporated, and purified by thick-layer chromatography (SiO_2, acetone:NH_4OH 28%, 9:1) to give (580 mg, 83%) as a colourless oil; R_f: 0.6; ^1H NMR (CD_2Cl_2): δ 7.07–7.41 (m, 6H, Ph), 6.64–6.72 (m, 2H, Ph), 5.55 (br, NH), 3.17–3.43 (m, 2H, CH_2), 2.83–2.86 (m, 4H, CH_2), 2.25–2.32 (m, 6H, CH_2), 1.88 (NH); 1.71 (qt, $J=7$ Hz, 2H, CH_2); ∂ ($CD_2Cl_2+CD_3OD$); 5.55 exchanged, 3.19 (t, $J = 7$ Hz, 2H, CH_2), 1.88 exchanged; anal. calcd for $C_{23}H_{28}N_3O_8ClS$: C, 50.97; H, 5.20; N, 7.75. Found: C, 50.85; H, 5.39; N, 7.59.

Assay for TR inhibition

TR activity was measured at 25 °C in a 0.04 M Hepes buffer, pH 7.4, containing 0.05 M KCl, 1 mM EDTA, 0.1 mM NADPH, 0.02 mM trypanothione, with an enzyme concentration of 0.0125 U mL^{-1}. The reaction was started by the addition of the enzyme and the absorbance decrease was followed at 340 nm.

In vitro activity on trypomastigotes (*T. cruzi*)

The method, as described by Neal and Croft[28] for anti-leishmanial drugs, was adapted for *T. cruzi*. Briefly, cultured mouse peritoneal macrophages were infected with trypomastigotes derived from a continuous in vitro culture and incubated at different drug concentrations (between 6.25 and 25 mM) for 4 days. After fixation in methanol and Giemsa staining, the number of infected macrophages was determined microscopically and compared to untreated control cultures.

In vitro activity on trypomastigotes (*T. brucei*)

Blood stream forms of *T. brucei* were cultivated in HMI-9 medium as described by Hirumi et al.[29] In a 96-well microplate, 10,000 haemoflagellates were incubated at different drug concentrations (between 1.56 and 6.25 μM) for 4 days. Parasite multiplication was measured colorimetrically (490 nm) after addition of MTS tetrazolium which converts in an aqueous soluble formazan product.[30]

In vivo activity on *T. brucei* and *T. cruzi*

Female Swiss mice (20–25 g) were intraperitoneally infected with 10,000 haemoflagellates in 0.2 mL inoculum. At the same time, the animals were orally treated with the test compound at 40 mg/kg (drug formulation in 100% DMSO), then the following 4 days by the intraperitoneal route. Untreated control animals usually died between 6 and 7 days after infection. Drug activity was evaluated as a prolongation of the mean survival time (MST) compared with untreated controls.

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