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New Spermine and Spermidine Derivatives as Potent Inhibitors of *Trypanosoma cruzi* Trypanothione Reductase

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Abstract—Several spermine and spermidine derivatives containing 2-amino diphenylsulfide substituents were prepared and tested for their inhibiting effects on *Trypanosoma cruzi* trypanothione reductase. IC_{50} values were assessed between 0.3 and 3 μM. Compound **32** (K_i = 0.4 μM) is the most potent TR inhibitor described so far. © 1997 Elsevier Science Ltd.

Introduction

Trypanosoma cruzi is the causing agent of the often fatal South American Chagas disease. Today, the drugs used to cure this disease are not specific and therefore fairly toxic to the host.[1,2] Investigation of glutathione regeneration in trypanosomatids has shown that these organisms do not possess the classical glutathione reductase (GR)-based redox system found in mammals. They possess instead an analogous system which relies on a glutathione–spermidine conjugate, N^1,N^8-bis(glutathionyl)spermidine disulfide[3] named trypanothione disulfide ($T(S)_2$). Trypanothione $T(SH)_2$ is regenerated from $T(S)_2$ by an NADPH-dependent flavoprotein, trypanothione reductase (TR).[4,5]

Although TR and human GR share approximately 40% sequence identity and the residues involved in catalysis are quite well preserved, TR and human GR have mutually exclusive substrate specificities.[6,7] This discrepancy in substrate specificity and the observation that many chemicals with trypanocidal properties show activity as regards trypanothione metabolism, suggests it may be possible to develop inhibitors that do not affect the host enzyme.[8–10] We set up a microplate-based assay in order to screen for new TR inhibitors[11] and discovered some structurally related compounds such as the 2-amino-diphenylsulfide **1** and phenothiazine **2** and **3** derivatives (Fig. 1).[12]

This observation was consistent with the fact that several phenothiazines had already been reported both as specific TR inhibitors ($K_i \approx 10$ μM)[13] and for their trypanocidal activity.[13–17] However, the potent neuroleptic activity of these compounds at 40 μM precludes their use in humans as antiparasitic drugs.[18] Compared to phenothiazines, compound **1** presents the advantage of displaying only residual neuroleptic activity, while still being a competitive inhibitor of TR with respect to trypanothione, while not inhibiting human GR. It was found to be active in vitro on the different stages of *T. cruzi* development and was therefore selected as a lead to designing further TR inhibitors.[12] We have previously predicted its position and conformation in the catalytic site of TR by molecular dynamics calculations.[19] Compound **1** appears to interact with two carboxylate groups of glutamic acid residues (466′, 467′) through its *N*-methyl-piperazine extremity and with a hydrophobic pocket of the enzyme (Leu 17, Trp 21, Tyr 110, Met 113, and Phe 114) through the two aromatic rings. The presence of other acidic residues in the vicinity of the

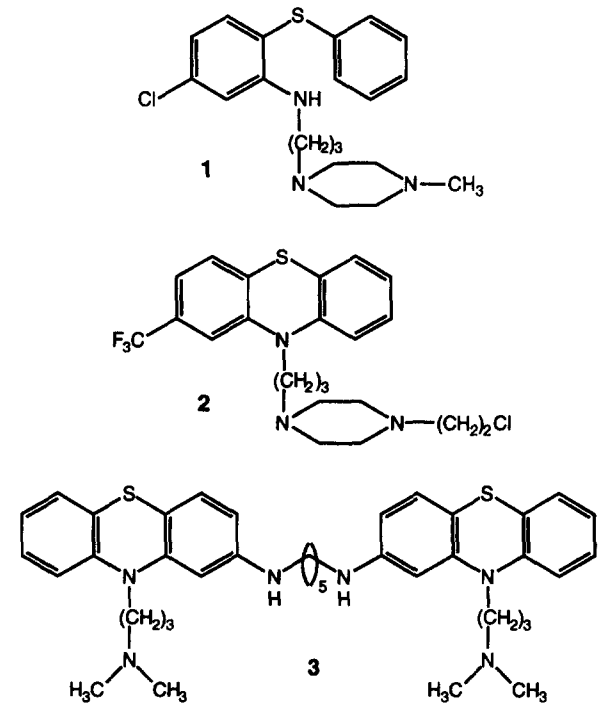

Figure 1. Structures of compounds **1**, **2**, and **3**.

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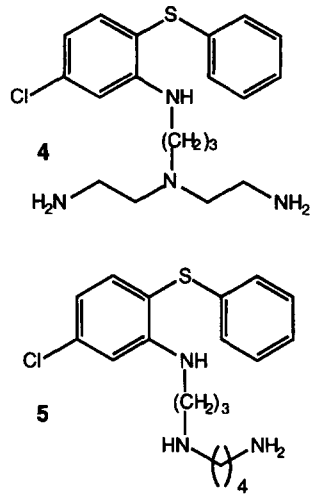


Figure 2. Structures of compounds **4** and **5**.

hydrophobic region in TR while absent in GR, led us to modify the side chain of **1** by replacing the piperazine ring with various linear and branched-amine chains. As expected, this modification enabled us to increase the inhibitory potency towards TR while preserving the inactivity versus GR; the most active compounds were **4** and **5** (Fig. 2).[19]

The introduction of a spermine or a spermidine moiety in the side chain of **1** had been considered because of the known antiparasitic properties of substituted polyamines[20,21] but this approach was dismissed, due to the difficulty of selectively protecting primary and secondary amino groups. Recently, two families of polyamines were reported as potent and selective TR inhibitors.[22,23] In one series,[22] synthesis was based on a convenient one-step procedure for the selective trifluoroacetylation of the primary amino groups of polyamines.[24] The most potent inhibitor (K_i = 3.5 μM) was obtained by substituting the secondary amino groups of spermine with 3-phenylpropyl moieties.[22] This result obtained with 'simple' phenylalkyl substituents, led us to reconsider the preparation of spermine and spermidine disubstituted by a 2-amino-diphenylsulfide moiety. Two major reasons justified these syntheses: (i) The bulkiness of the 2-amino-diphenylsulfide moiety; given that the binding site in TR is much larger than in GR, increasing the size of an inhibitor could contribute to increasing its selectivity; (ii) an improved affinity was also expected when using best TR-recognized 2-amino diphenylsulfide moieties instead of 'simple' phenylalkyl fragments.

We evaluated the activities of the inhibitors reported by O'Sullivan et al. using computer methods.[25] The approach includes an automated procedure for the building and conformational sampling of the ligand molecules, leading to representative sets of ligand low-energy conformations. This is then followed by the rigid-body docking of each of the sampled conformers into the TR site model built on the basis of the geometry from X-ray crystallography studies.[26,27] Three-dimensional grids generating the active sites were used to monitor the Van der Waals, Coulomb, hydrophobic, and desolvation potentials created by the enzyme (the latter being based on a continuum solvent model proposed by Gilson and Honig[28]) so that the ligand–enzyme interaction energies are rapidly evaluated as the response of the ligand atoms when placed in the molecular fields produced by the enzyme. An optimal location for each ligand conformation in the site is found by minimizing this intermolecular interaction energy with respect to the translational and rotational degrees of freedom of rigid ligand geometry. Since such optimizations are subject to the risk of being trapped in local or false minima, multiple starting positions of the ligand in the site are tried out to avoid this problem. Eventually, an ensemble of docked conformers originating from one ligand is obtained. Enthalpic and entropic binding 'indices' are then evaluated on the basis of the energies of the conformational ensembles of free and bound ligands. Due to the numerous simplifying assumptions this model is based on, resulting indicies may not accurately reproduce thermodynamic binding enthalpy and entropy, but they nevertheless prove to be valid 'descriptive variables' with respect to the binding affinities of known ligands. Therefore, a linear QSAR relationship between the logarithm of the inhibition constant (log K_i) and the calculated enthalpy and entropy 'indexes' could be established and validated for a control set of TR ligands, with a standard error of predicted vs experimental log K_i values of about one unit.[25] This equation was used to predict the affinity of compounds on the basis of the calculated indexes of O'Sullivan's molecules[22] (which were not included in the control set[25]), as well as for the set of different structures considered for synthesis in our lab.

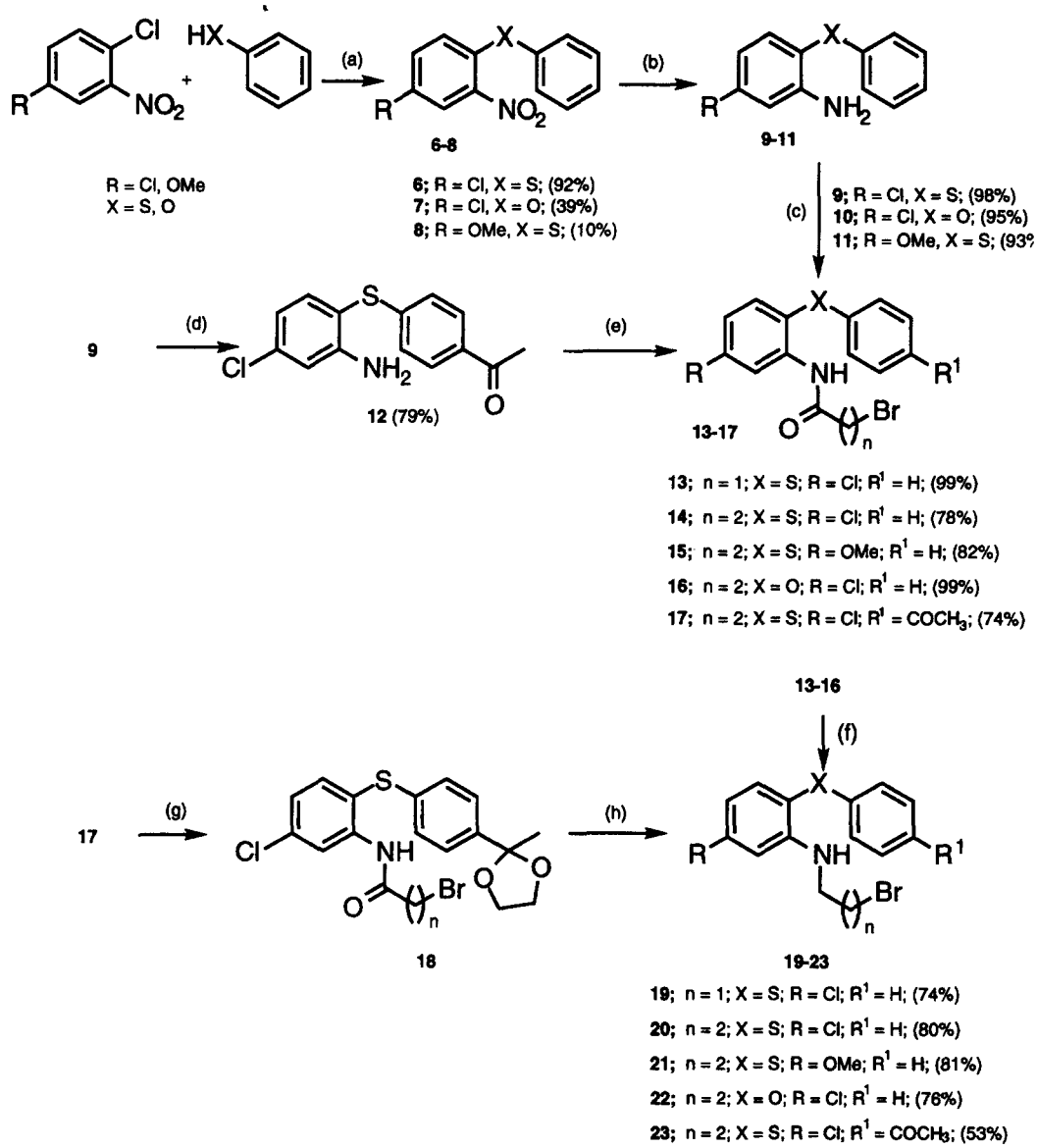
Table 1. Experimental values of the inhibition constants of TR inhibitors reported by O'Sullivan and Zhou[22] vs values obtained from the affinity prediction algorithm

Compd	Experimental[22] K_i (mM)	Predicted K_i (mM)
1S	280	100
2S	108	4
3S	9.5	6
4S	Inactive	>100
5S	19	7
6S	3.5	8
7S	5.5	7

The numbering of compounds **1S–7S** is identical to that given by the authors in the table on page 1959 of their paper.

Calculated affinity values for O'Sullivan's ligands generally agreed with experimental values, as expected according to the good overall prediction rate reported for previous applications of this algorithm, with exception of compound (**2S**), wrongly predicted by more than an order of magnitude (Table 1).

The affinity prediction algorithm was then applied to one of the structures considered for synthesis (com-



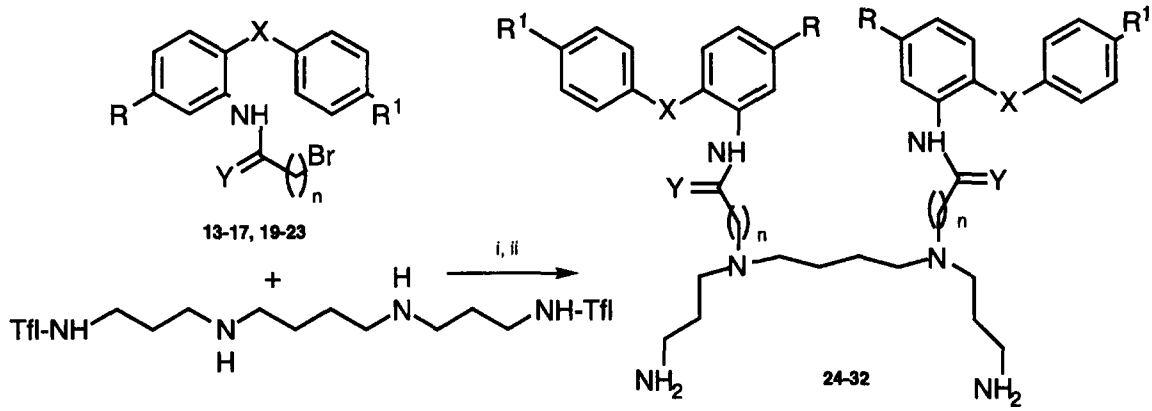
Scheme 1. Synthesis of bromodiphenyl compounds **13–23**. Reagents and conditions: (a) for preparation of **6** and **7**: $CH_3CO_2NH_4$, abs EtOH, reflux, two days; for preparation of **8**: KOH aq, reflux, 12 h; (b) Fe, concd HCl, 95% EtOH, reflux, 1 h; (c) for preparation of **13**: 2-bromoacetyl bromide, ether, pyridine, 0 °C, 2 h; for preparation for **14–16**: 3-bromopropionyl chloride, ether, pyridine, 0 °C, 2 h; (d) acetyl chloride, $AlCl_3$, CS_2, rt, 3 h; (e) 3-bromopropionyl chloride, ether, pyridine, 0 °C, 2 h; (f) BH_3/THF, THF, 0–25 °C, 4 h; (g) ethylene glycol, PTSA catal., toluene, reflux, 12 h; (h) BH_3/THF, THF, 0–25 °C, 4 h then HCl 4 N.

pound **24**, Scheme 2). The estimated inhibition constant of this compound was found to be about 1 μM, which enabled us to predict that the real inhibition constant was lower than 10 μM. Therefore, the new molecules appeared worthy of interest and to be at least as good inhibitors as O'Sullivan's compounds.

In this paper, we describe the synthesis and inhibiting effects of different new spermine and spermidine analogues of compounds **1**, **4**, and **5** on *T. cruzi* TR. The compounds differ as to the distance between the aniline ring and the polyamine chain, the nature of the heteroatom and/or the nature of the substituents on the rings (e.g., all parameters known to influence the inhibitory potency of previous analogues of **1**).[12,29]

Chemistry

Different (2-thiophenyl)- or (2-phenoxy)-anilines (compounds **9–12**) were prepared as previously described[19] and outlined in Scheme 1. Compounds **9–11** were obtained by reaction of 1-nitro-2,5-dichloro-benzene or 1-nitro-2-chloro-5-methoxy-benzene with phenol or thiophenol followed by reduction of the nitro group. Compound **12** was prepared from **9** using a standard Friedel–Crafts reaction. The reaction of compounds **9–12** with bromoacetyl bromide or 3-bromopropionyl chloride in the presence of pyridine, led quantitatively to brominated amides **13–17**.[19] Reduction of these amides into amines was carried out with a large excess of borane–tetrahydrofuran complex, either directly (compounds **19–22**), or after temporary protection of the keto group in dioxolan form (compound **23**).



Scheme 2. Synthesis of spermine derivatives **24–32**. Reagents and conditions: (i) 6 equiv DiEA, CH_3CN, reflux, three days; (ii) for preparation of **24–27**: 5 equiv LiOH, H_2O, THF:H_2O (2:1), ultrasonics, 3 h, 25 °C; for preparation of **28–32**: 5 equiv LiOH, H_2O, THF:H_2O (2:1), reflux, 3 h.

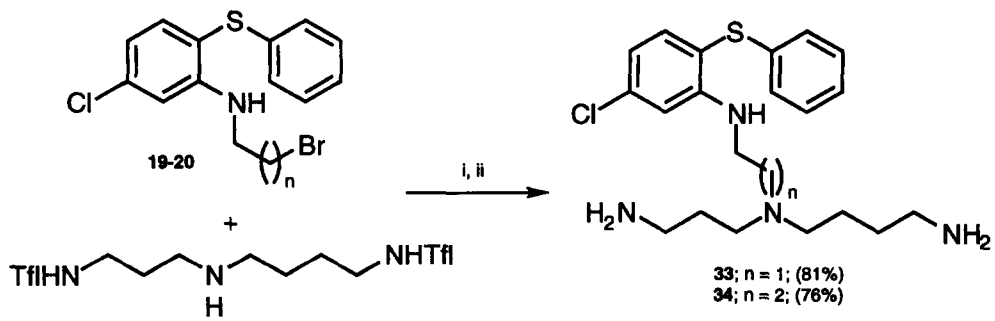
Amides **13–17** or amines **19–23** were reacted by nucleophilic substitution with spermine whose primary amino groups were previously protected (Scheme 2). The one-step di-protection of spermine was carried out with ethyl trifluoroacetate.[24] Final deprotection of the terminal primary amino groups was obtained by action of LiOH in aqueous THF, either by a 3-h reflux treatment (amino compounds **28–32**) or in milder conditions (ultrasonics) for amide compounds **24–27**. These latter proved to be very sensitive and regenerated (2-thiophenyl)- or (2-phenoxy)-anilines in deprotection conditions. Even using ultrasonics, the amide corresponding to keto compound **32** could not be obtained. Bromides similar to **13–17**, **19–23** but with $n = 3$ or 4 did not react with spermine but underwent intramolecular cyclization to yield entropically favoured five- or six-membered rings. Bromides of the same family with $n = 5$ were unreactive towards spermine.

Spermidine derivatives **33** and **34** were synthesized following the procedure described above for the spermine series (Scheme 3).

Results and Discussion

Comparative studies of the catalytic sites of TR and GR showed that, while some acidic residues are shared by both enzymes, others are only present in TR. From compound **1**, selected in a screening assay on TR, we conceived that the binding energy of **1** to TR may be increased by generating additional ionic interactions with additional amine groups, while at the same time preserving the specificity of derivatives of **1** to inhibit TR.[19] Besides, molecular modeling indicated that the hydrophobic pocket in the active site of TR could accommodate a bulky aromatic entity and in fact, we found a higher affinity for TR of **3** compared with its mono derivative.[30] These observations led us to prepare some bis-amino diphenylsulfides, resulting from the alkylation of polyamines by these aromatic moieties.

Inhibiting potency of the different compounds was evaluated by measuring IC_{50} with regard to TR in the presence of 57 μM of TS_2, 0.2 mM NADPH increasing concentrations of inhibitor (0.14–5.7 μM), using clomipramine as a reference inhibitor (the most easily



Scheme 3. Synthesis of spermidine derivatives **33** and **34**. Reagents and conditions: (i) 3 equiv DiEA, CH_3CN, reflux, three days; (ii) 5 equiv LiOH, H_2O, THF:H_2O (2:1), reflux, 3 h.

Table 2. Inhibition of trypanothione reductase by amines **24–34**

Compound	24	25	26	27	28	29	30	31	32	33	34	Clomipramine
IC_{50}(μM)	2.3	1.2	1.3	1.0	0.8	0.4	0.7	0.4	0.3	3.0	1.0	12.5

available of the potent TR inhibitors[13]) (Table 2). While no inhibition was observed with regard to human GR at 10 μM, IC_{50} values were 3 μM and 1 μM, respectively, for spermidine derivatives **33** and **34**, and in the range 0.3–3 μM for spermine derivatives **24–32**. The results show that (i) inhibitory efficiency is higher for amines than for amides: **28** > **24**; **29** > **25**; **30** > **26**; **31** > **27**; (ii) for compounds differing only as to the value of *n*, the presence of an additional methylene group increases the inhibitory potency. Therefore, a four-membered spacer between the aromatic ring and the polyamine chain is the most favourable linker: **25** > **24**; **29** > **28**; **34** > **33**; (iii) the nature of the hetero atom linking the rings has no significant effect on TR inhibition (see amides **25** and **27**; amines **29** and **31**) as is the case for the lead compound **1** (K_i = 25 μM) and its oxygenated analogue (K_i = 50 μM);[30] and (iv) the nature of the substituents on rings can affect the inhibitory potency, especially in the amine series. Thus, the replacement of the methoxy group by a chlorine atom on the anilino ring (compound **30**→**29**) or the introduction of a keto group (compound **29**→**32** on the benzene ring) leads to a decrease in IC_{50} values.

The type of inhibition of TR displayed by the most potent compound **32** was determined by measuring the rate of TS_2 reduction using several concentrations of TS_2 and inhibitor. The initial velocities of disappearance of NADPH at 340 nm in the presence of different concentrations of inhibitor **32** (0–0.6 μM) were measured and showed that **32** was a competitive-type inhibitor (Fig. 3). The inhibition constant of compound **32** (K_i = 0.4 ± μM) was deduced from a Lineweaver–Burk plot of $1/v$ vs 1/[S] and the corresponding slope report of apparent K_m vs [I]. The type of inhibition was presumed to be identical for the analogues of compound **32** in the series. Work is now in progress to improve TR inhibition by designing asymmetrical analogues in which one of the diphenylsulfide moieties would be replaced by another aromatic entity. Such a new series of compounds should enable us to specify and take advantage of the role played by the second amino diphenylsulfide moiety in TR recognition.

Experimental

Chemistry

All melting points were determined on a melting point Büchi apparatus and were uncorrected. All reactions were monitored by thin-layer chromatography

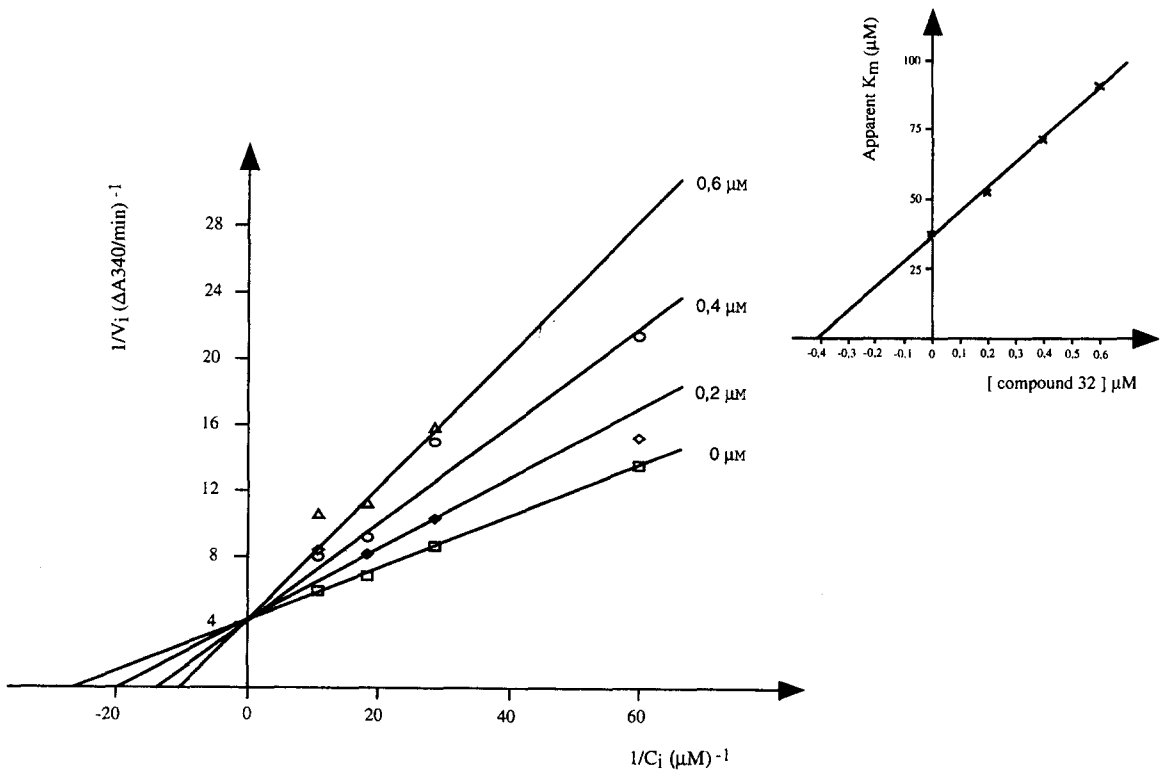


Figure 3. Competitive-type inhibition of TR by compound **32**. Lineweaver–Burk plot of $1/v$ vs 1/[S] at different concentrations of **32** (0–0.6 μM) and the corresponding slope report of apparent K_m vs [I].

(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[31] (R.H.) as developing agents; the purity of final compounds was checked by HPLC (Nucleosil cyanopropyl). ^1H and ^{13}C 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). The chemistry for the analogues of compounds **6–23** has already been described.[12,19]

***N*-(3-bromopropyl)-5-chloro-2-(4-acetoxythiophenyl)-phenylamine (23).** A solution of brominated amide **17** (1 equiv, 2 mmol) in 20 mL of toluene was added to ethylene glycol (1.1 equiv, 2.2 mmol) and para-toluene sulfonic acid (0.03 g, catalytic amount). The mixture was refluxed for 12 h, avoiding water, in a Dean–Stark apparatus. The organic layer was separated and washed with a solution of 1 M NaOH (20 mL) and water (20 mL). The solvent was dried over Na_2SO_4 and evaporated to leave a yellow oil. The crude product **18** was used for the next step.

Under N_2, 20 mL (20 mmol, 10 equiv) of 1 M BH_3/THF solution were added dropwise, for 1 h, to a cooled solution of **18** (2 mmol, 1 equiv) in 3 mL of anhydrous THF. The mixture was stirred for 30 min and allowed to react for 2 h at room temperature. Excess BH_3 was hydrolysed by a solution of 1 M $NaHCO_3$ at 0 °C and the solvent was evaporated. The residue was treated with a mixture of CH_2Cl_2 and brine. The organic layer was separated and evaporated to dryness. The yellow oil was dissolved in 40 mL THF and treated for 2 h with 4 N HCl. After usual treatment, the crude product was purified by flash chromatography (SiO_2, diethyl ether: petroleum ether: 40:60) to produce a white solid (53% yield); mp 49 °C; ^1H NMR (CD_2Cl_2) δ 2.06 (m, 2H, CH_2 $\underline{CH_2}$ CH_2Br), 2.52 (s, 3H, CH_3CO), 3.25 (2t, 4H, $\underline{CH_2}$ $\underline{CH_2}$ $\underline{CH_2}Br$), 5.00 (bs, 1H, 2 NH), 6.80 (m, 2H, aromatic H), 7.05 (d, 2H, aromatic H, J = 8 Hz), 7.46 (d, 2H, aromatic H, J = 8 Hz), 7.85 (d, 2H, aromatic H, J = 8 Hz); ^{13}C NMR (CD_2Cl_2) δ 26.69, 31.10, 31.87, 41.73, 110.91, 111.24, 117.40, 125.81, 127.54, 127.68, 129.24, 134.89, 138.44, 139.18, 143.55, 150.34; 197.12. *m/z* 399.5.

General procedure for the synthesis of spermine derivatives 24–32

One equivalent of protected spermine (1 mmol) and 6 equiv of DIEA (6 mmol) were added to a solution of brominated amides **13–17** or brominated amines **19–23** (2.5 mmol, 2.5 equiv) in 5 mL of acetonitrile. After refluxing the mixture for three days, the solvent was removed and the oily residue was treated with a mixture of CH_2Cl_2 and water. Protected spermine derivatives were obtained as yellow oils for amine derivatives or yellow solids for amide derivatives after thick-layer chromatography (SiO_2, CH_2Cl_2:MeOH, 92:8).

The deprotection reaction required lithium hydroxide (5 equiv, 2.5 mmol), which was added to the solution of protected spermine derivatives (1 equiv, 0.5 mmol) in 20 mL of a mixture of THF:H_2O, 2:1. For the amine derivatives, the mixture was refluxed for 3 h whereas for the amide derivatives it was stirred at room temperature under ultrasonics. Ether (50 mL) was then added, the organic layer was separated, washed twice with water and evaporated to dryness.

Compounds **24–32** were obtained as yellow oils (overall yield: 35–81%) and they were tested for TR inhibition in the hydrochloride form.

Compound **24**: ^1H NMR (CD_2Cl_2) δ 1.30 (m, 4H, 2 CH_2 of spermine group), 1.57 (m, 4H, 2 CH_2 of spermine group), 2.31 (m, 4H, 2 CH_2 of spermine group), 2.42 (4H, 2 CH_2 of spermine group), 3.08 (s, 4H, 2 CH_2 of ethyl group), 3.19 (6H, 2 CH_2 of spermine group + 2 NH_2), 7.05–7.58 (m, 14H, aromatic H), 8.74 (m, 2H, aromatic H), 10.23 (s, 2H, 2 Ph-NH-CO-); ^{13}C NMR (CD_2Cl_2) δ 25.25, 29.52, 30.21, 42.12, 54.38, 60.37, 110.74, 115.21, 118.45, 119.76, 122.39, 130.14, 131.28, 138.55, 141.32, 152.79, 172.45. *m/z* 756.4.

Compound **25**: ^1H NMR (CD_2Cl_2) δ 1.25 (m, 4H, 2 CH_2 of spermine group), 1.60 (m, 8H, 4 CH_2 of spermine group), 2.41 (m, 4H, 2 CH_2 of spermine group), 2.52 (m, 4H, 2 CH_2 of propyl group), 2.65 (m, 4H, 2 CH_2 of spermine group), 3.20 (m, 6H, 2 CH_2 of propyl group + 2 NH_2), 6.5–7.30 (m, 16H, aromatic H), 10.41 (s, 2H, 2 Ph-NH-CO-); ^{13}C NMR (CD_2Cl_2) δ 26.25, 30.62, 30.99, 31.87, 41.25, 55.62, 61.10, 110.85, 115.23, 118.53, 119.52, 122.41, 130.10, 131.32, 138.93, 141.25, 152.83, 171.72. *m/z* 784.2.

Compound **26**: ^1H NMR (CD_2Cl_2) δ 1.26 (m, 4H, 2 CH_2 of spermine group), 1.46 (m, 4H, 2 CH_2 of spermine group), 2.34 (m, 4H, 2 CH_2 of spermine group), 2.40 (m, 8H, 2 CH_2 of propyl group and 2 CH_2 of spermine group), 2.55 (m, 4H, 2 CH_2 of spermine group), 3.58 (m, 6H, 2 CH_2 of propyl group + 2 NH_2), 3.87 (s, 6H, 2 CH_3O), 6.68–7.51 (m, 14H, aromatic H), 8.19 (m, 2H, aromatic H), 10.28 (s, 2H, 2 Ph-NH-CO-); ^{13}C NMR (CD_2Cl_2) δ 24.00, 29.95, 31.92, 35.20, 40.89, 49.71, 51.38, 55.84, 107.43, 110.81, 125.65, 129.40, 138.52, 143.24, 162.03, 171.70. *m/z* 775.2.

Compound **27**: ^1H NMR (CD_2Cl_2) δ 1.16 (m, 4H, 2 CH_2 of spermine group), 1.71 (m, 8H, 4 CH_2 of spermine group), 2.25 (m, 4H, 2 CH_2 of spermine group), 2.47 (m, 4H, 2 CH_2 of propyl group), 2.66 (m, 4H, 2 CH_2 of spermine group), 3.08 (m, 6H, 2 CH_2 of propyl group + 2 NH_2), 6.72–7.36 (m, 14H, aromatic H), 8.62 (m, 2H, aromatic H), 9.37 (s, 2H, 2 Ph-NH-CO-); ^{13}C NMR (CD_2Cl_2) δ 24.21, 27.29, 30.71, 32.08, 34.43, 40.75, 49.48, 118.64, 118.72, 123.14, 123.22, 123.24, 124.29, 124.35, 130.29, 145.29, 156.86, 171.86. *m/z* 752.0.

N^4,N^8-bis(2-*N*-((5-chloro-2-phenylthio)-phenyl)-aminoethyl)-spermine (28). ^1H NMR (CD_2Cl_2) δ 1.34 (m, 4H, 2 CH_2 of spermine group), 1.55 (m, 4H, 2 CH_2 of

spermine group), 2.28 (m, 4H, 2 CH_2 of spermine group), 2.45 (4H, 2 CH_2 of spermine group), 3.08 (m, 8H, 2 CH_2 of ethyl group + 2 NH_2), 3.19 (8H, 2 CH_2 of spermine group and 2 CH_2 of ethyl group), 5.72 (s, 2H, 2 NH-Ph), 6.63–7.41 (m, 16H, aromatic H); ^{13}C NMR (CD_2Cl_2) δ 25.09, 31.77, 40.67, 41.02, 52.41, 53.79, 55.17, 110.87, 116.48, 125.81, 126.78, 129.29, 138.86, 151.05. *m/z* 726.2.

N^4,N^8-bis(3-*N*-((5-chloro-2-phenylthio)-phenyl)-aminopropyl)-spermine (29). ^1H NMR (CD_2Cl_2) δ 1.31 (m, 4H, 2 CH_2 of spermine group), 1.47 (m, 4H, 2 CH_2 of spermine group), 1.66 (m, 4H, 2 CH_2 of propyl group), 2.30 (m, 4H, 2 CH_2 of propyl group), 2.39 (m, 8H, 2 CH_2 of spermine group and 2 CH_2 of propyl group), 2.68 (m, 4H, 2 CH_2 of spermine group), 3.15 (m, 6H, 2 CH_2 of propyl group + NH_2), 5.55 (s, 2H, 2 NH-Ph), 6.6–7.40 (m, 16H, aromatic H); ^{13}C NMR (CD_2Cl_2) δ 25.26, 26.82, 30.12, 41.04, 42.55, 53.12, 56.45, 110.52, 116.47, 125.87, 126.69, 129.36, 137.65, 138.87, 151.09, 156.52. *m/z* 754.2.

N^4,N^8-bis(3-*N*-((5-methoxy-2-phenylthio)-phenyl)-aminopropyl)-spermine (30). ^1H NMR (CD_2Cl_2) δ 1.31 (m, 4H, 2 CH_2 of spermine group), 1.49 (m, 4H, 2 CH_2 of spermine group), 1.69 (m, 4H, 2 CH_2 of propyl group), 2.31 (m, 4H, 2 CH_2 of spermine group), 2.38 (m, 8H, 2 CH_2 of spermine group and 2 CH_2 of propyl group), 2.65 (m, 4H, 2 CH_2 of spermine group), 3.16 (m, 8H, 2 CH_2 of propyl group + 2 NH_2), 3.83 (s, 6H, 3 CH_3O), 5.35 (s, 2H, 2 NH-Ph), 6.24–6.29 (m, 4H, aromatic H), 7.00 (m, 12H, aromatic H); ^{13}C NMR (CD_2Cl_2) δ 25.23, 26.96, 32.07, 41.05, 42.51, 53.12, 56.45, 58.26, 96.84, 102.09, 105.35, 125.32, 125.02, 129.18, 138.71, 139.35, 151.63, 163.29. *m/z* 745.4.

N^4,N^8-bis(3-*N*-((5-chloro-2-phenyloxy)-phenyl)-aminopropyl)-spermine (31). ^1H NMR (CD_2Cl_2) δ 1.32 (m, 4H, 2 CH_2 of spermine group), 1.46 (m, 4H, 2 CH_2 of spermine group), 1.71 (m, 4H, 2 CH_2 of propyl group), 2.29 (m, 4H, 2 CH_2 of spermine group), 2.39 (m, 4H, 2 CH_2 of spermine group), 2.44 (m, 4H, 2 CH_2 of propyl group), 2.62 (m, 4H, 2 CH_2 of spermine group), 3.18 (m, 6H, 2 CH_2 of propyl group + 2 NH_2), 3.36 (m, 2H, 2NH-Ph), 6.5–7.30 (m, 16H, aromatic H); ^{13}C NMR (CD_2Cl_2) δ 25.26, 26.44, 30.99, 40.99, 43.13, 52.39, 52.54, 54.47, 110.97, 115.28, 117.67, 120.20, 123.19, 130.06, 130.33, 141.92, 142.56, 157.84. *m/z* 722.5.

N^4,N^8-bis(3-*N*-((5-chloro-2-(4′-acetyl-phenylthio))-phenyl)-aminopropyl)-spermine (32). ^1H NMR (CD_2Cl_2) δ 1.35 (m, 4H, 2 CH_2 of spermine group), 1.49 (m, 4H, 2 CH_2 of spermine group), 1.59 (m, 4H, 2 CH_2 of propyl group), 2.25 (m, 12H, 2 CH_2 of propyl group + 4 CH_2 of spermine group), 2.50 (s, 3H, CO-CH_3), 2.55 (m, 4H, 2 CH_2 of spermine group), 2.68 (m, 4H, 2 CH_2 of spermine group), 3.15 (m, 8H, 2 CH_2 of propyl group + 2NH_2), 5.75 (s, 2H, 2NH-Ph), 6.6–7.80 (m, 14H, aromatic H); ^{13}C NMR (75 MHz, CD_2Cl_2) *d* 25.13, 26.60, 31.12, 41.05, 42.88, 52.20, 53.11, 54.56, 110.49, 116.64, 127.48, 129.16, 134.57, 138.37, 139.18, 144.41, 151.36, 197.03. *m/z* 878.6.

General procedure for the synthesis of spermidine derivatives 33–34

The procedure was similar to the preparation of the spermine derivatives **24–32**. In this case, it was necessary to use 1.5 equiv of brominated compounds **19–20**, 1 equiv of protected spermidine and 3 equiv of DIEA in acetonitrile. Compounds **33** and **34** were obtained as yellow oils (overall yield: 76 and 81%, respectively).

N^4-(2-*N*-((5-chloro-2-phenylthio)-phenyl)-aminoethyl)-spermidine (33). ^1H NMR (CD_2Cl_2) δ 1.40 (m, 4H, 2 CH_2 of spermidine group), 1.53 (m, 2H, CH_2 of spermidine group), 2.39 (m, 6H, 4 CH_2 of spermidine group and CH_2 of ethyl group), 2.66 (m, 2H, CH_2 of spermidine group), 3.28 (m, 8H, CH_2 of spermidine group + CH_2 of ethyl group + 2NH_2), 5.75 (s, 1H, NH-Ph), 6.65–7.49 (m, 8H, aromatic H); ^{13}C NMR (75 MHz, CD_2Cl_2) *d* 27.80, 41.05, 42.59, 53.68, 56.22, 110.88, 116.44, 125.80, 125.88, 126.73, 129.29, 138.75, 138.89, 151.09. *m/z* 407.5

N^4-(3-*N*-((5-chloro-2-phenylthio)-phenyl)-aminopropyl)-spermidine (34). ^1H NMR (CD_2Cl_2) δ 1.43 (m, 4H, 2 CH_2 of spermidine group), 1.53 (m, 2H, CH_2 of spermidine group), 1.67 (m, 2H, CH_2 of propyl group), 2.39 (m, 6H, 4 CH_2 of spermidine group and CH_2 of propyl group), 2.66 (m, 2H, CH_2 of spermidine group), 3.18 (m, 8H, CH_2 of spermidine group + CH_2 of propyl group + 2NH_2), 5.55 (s, 1H, NH-Ph), 6.63–7.41 (m, 8H, aromatic H); ^{13}C NMR (CD_2Cl_2) δ 24.63, 26.67, 40.92, 42.56, 53.17, 58.22, 110.52, 116.42, 125.87, 126.67, 129.37, 137.65, 138.88, 151.07. *m/z* 421.8

Assays for TR and GR activity

Recombinant trypanothione reductase was produced from the SG5 *Escherichia coli* strain with the overproducing expression vector pIBITczTR.[32] 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 was started by adding the enzyme, and the absorbance decrease was followed at 340 nm. Inhibiting potency of the different compounds was evaluated by measuring IC_{50} in the presence of 57 μM of $T(S)_2$ and increasing inhibitor concentrations (0.14–5.7 μM). DMSO was included at 2% final concentration as maximum for the highest concentration of inhibitor (5.7 μM). Because **32** was difficult to solubilize and showed a tendency to precipitate when adding H_2O or phosphate buffer to DMDO mother solutions, we checked the entire solubilization of the compound by measuring the TR activity of the inhibitor solutions before and after filtration on a 0.22 μM filter (Millipore). Free inhibitor concentration was checked by a standard calibration of known inhibitor concentration by HPLC. Compound **32** was first dissolved by adding 1 M HCl (20 μL) before the preparation of mother solutions in 100% DMSO (200 μM). Kinetic constants were determined in duplicate for compound **32** accord-

ing to Lineweaver–Burk with four different inhibitor concentrations (0–0.6 μM) and four different substrate concentrations (20–100 μM) in the presence of an enzyme concentration of 0.012 U mL^{-1}.

Human GR[33] inhibition was evaluated according to conditions described above for TR assay. Inhibitory potencies of the compounds at four concentrations (from 0.3 μM to 10 μM) were also determined with regard to human glutathione reductase, in the presence of 44 μM of GSSG, in 40 mM Hepes, 50 mM KCl, 1 mM EDTA, pH 7.4, and 180 μM of NADPH.

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