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Potent and Specific Inhibitors of Trypanothione Reductase from *Trypanosoma cruzi*: Bis(2-aminodiphenylsulfides) for Fluorescent Labeling Studies

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Abstract—In order to optimise the activity of bis(2-aminodiphenylsulfides) upon trypanothione reductase (TR) from *Trypanosoma cruzi*, a new series of bis(2-aminodiphenylsulfides) possessing three side chains was synthesized. Various moieties were introduced at the end of the third side chain, including acridinyl or biotinyl moieties for fluorescent labeling studies. TR inhibition was improved; the most potent inhibitor ($IC_{50} = 200$ nM) was selective towards TR versus human glutathione reductase and corresponded to a single myristyl group. Compounds were also tested in vitro upon *Trypanosoma cruzi* and *Leishmania infantum* amastigotes, upon-*Trypanosoma brucei* trypomastigotes, and for their cytotoxicity upon human MRC-5 cells. In the presence of serum, acridine derivative was no longer detectable in mass spectrometry and its antitrypanosomal activity no longer observed. This transformation might explain the absence of correlation between the potent TR inhibition and the in vitro and in vivo antiparasitic activity with both of the first generation of 2-aminodiphenylsulfides.

Introduction

Drugs presently being used for trypanosomiasis treatment are non-specific and consequently fairly toxic. Investigation of glutathione metabolism in trypanosomatids has revealed that these organisms possess an original redox system based upon the couple trypanothione/trypanothione reductase (TR). TR, an NADPH-dependent flavoprotein, regenerates trypanothione under its active form $T(SH)_2$ (Fig. 1).[1–3]

Despite 40% identity in their primary sequences, TR and human glutathione reductase (GR) are exclusive towards their respective substrate trypanothione and glutathione.[4] Recently, genetic approaches using TR mutants of *Leishmania donovani* and *L. major* have shown the vital role of TR in the survival of the parasites within activated macrophages.[5,6] These results render TR a potential rational drug design target against trypanosomiasis.

Using a microplate assay to screen TR inhibitors,[7] we discovered, among other potential leads, some 2-aminodiphenylsulfides structurally related to phenothiazines but presenting the advantage of a residual neuroleptic activity.[8] The position and the conformation of the best inhibitor of the series (**A** series, compound **1**, $K_i = 25\ \mu M$, Figure 2) in the catalytic site of TR were studied by molecular dynamics simulation. Molecular modelling clearly indicated interactions of the amino side-chain with acidic residues absent in GR, and of its aromatic rings with a broad hydrophobic pocket.[9] These two features of recognition inspired the synthesis of bis(2-aminodiphenylsulfides) which were found to be more potent and specific TR inhibitors (**B** series, Figure 2).[10] In this series, compound **2** was the most potent inhibitor, with an IC_{50} of 0.55 μM in the presence of 57 μM $T(S)_2$.

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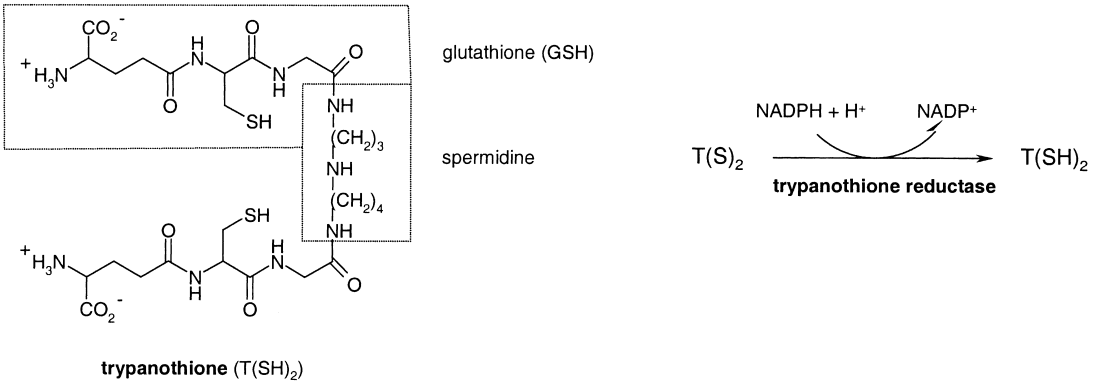


Figure 1. Trypanothione and trypanothione reductase.

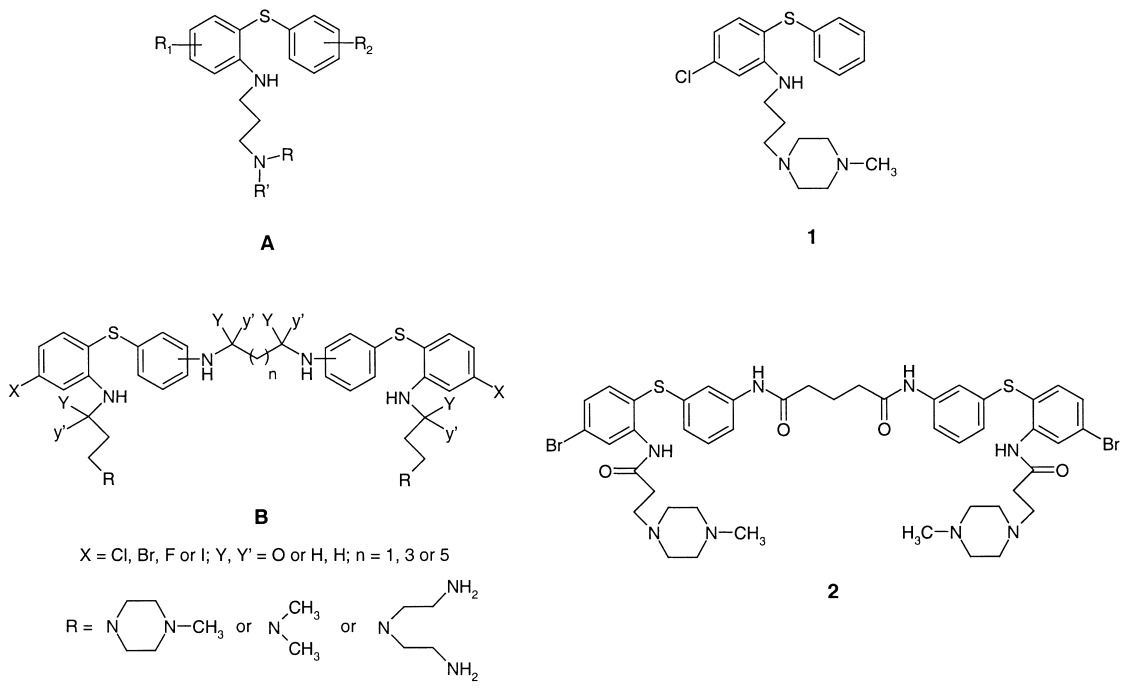


Figure 2. **A** and **B** series of 2-aminodiphenylsulfides and compounds **1** and **2**.

With the aim of further improving TR inhibition, we elected to design and synthesize a new generation of bis(2-aminodiphenylsulfides) (**C** series, Figure 3), corresponding to the attachment of an additional side chain to an analogue of compound **2,** different by the presence of a secondary amino group within the spacer joining the two aromatic moieties. The large volume of the hydrophobic pocket in the TR active site justified the introduction of a hydrophobic moiety to the end of the side chain and, given the narrower catalytic site of GR, the greater bulk of the new molecules should favour the TR/GR specificity. Moreover, this side chain could be functionalized with moieties enabling fluorescent labeling studies to monitor the localisation of the compounds within the parasite. Indeed, the previous generation of bis(2-aminodiphenylsulfides) (**B** series) did not reveal a tangible correlation between TR inhibition and in vitro trypanocidal effect.

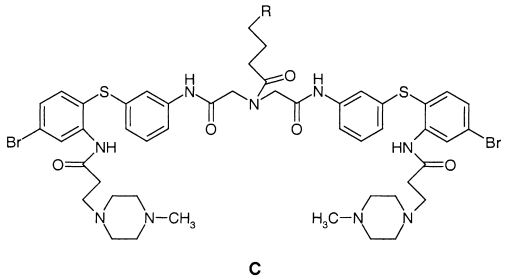


Figure 3. **C** series of bis(2-aminodiphenylsulfides).

In accordance with the distances evaluated by molecular modelling, a constant length of four carbons was selected for the additional side chain. Four terminal groups likely to generate hydrophobic interactions were chosen: fluorenylmethyloxycarbonyl (Fmoc), *tert*-butoxycarbonyl (Boc), naphtyl, myristyl as well as acridinyl and biotinyl moieties, the latter two for fluorescent labeling studies within the parasite. Amine or a carboxylic groups capable of generating polar interactions were also used as controls.

Chemistry

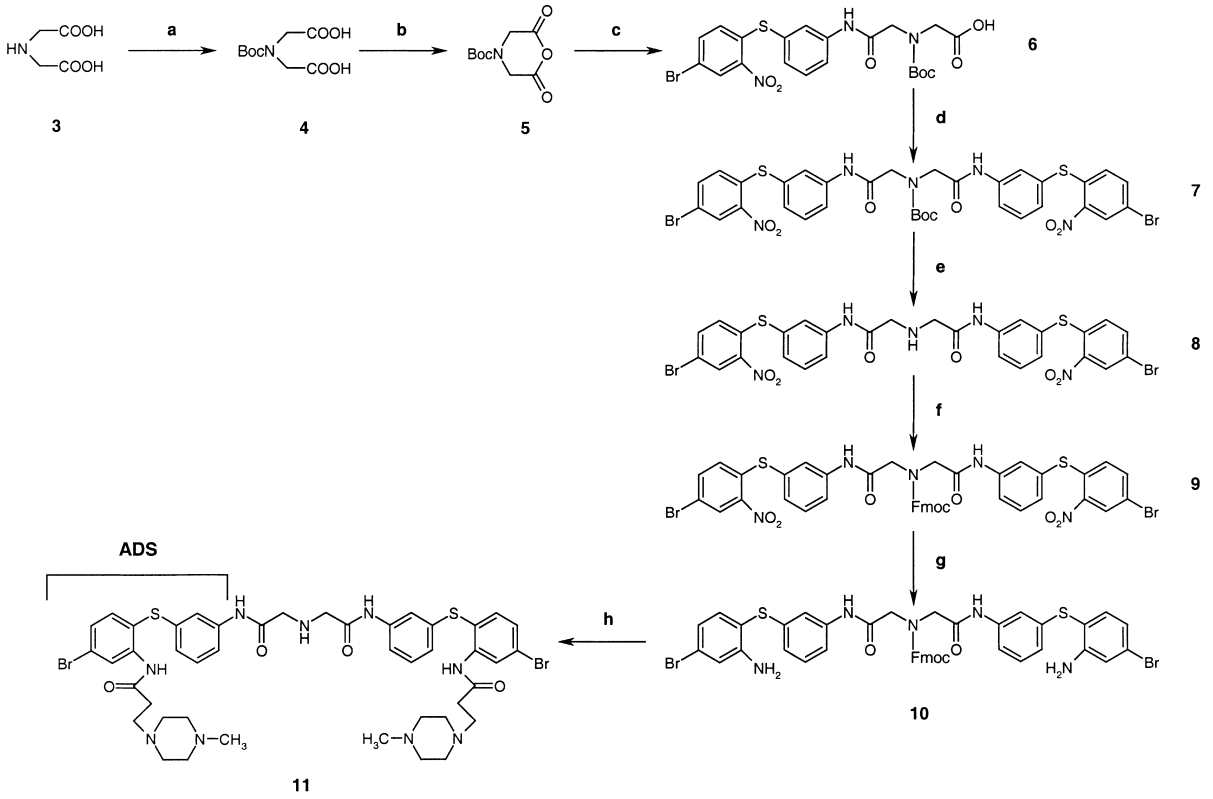
The different compounds of the **C** series were derivatives of the bis(2-aminodiphenylsulfide) **11** which was synthesized as described by Scheme 1. The starting material was iminodiacetic acid **3** whose amino was protected by a Boc group before its transformation into anhydride **5**. This latter was opened by reaction with [3-(4-bromo-2-nitro)phenylsulfanyl]phenylamine to give carboxylic acid **6** which was coupled with the same aromatic amine to produce bis(2-aminodiphenylsulfide) **7**.[11,12] In the first step, the Boc group was preferred to the Fmoc group since the Fmoc analogue of compound **6** did not react with the aromatic amine to form the Fmoc analogue of compound **7**.[13] Since the reduction of nitro groups was carried out under acidic conditions to which the Boc group is sensitive, it should be preceded by a deprotection of the central, secondary amino and its re-protection with a Fmoc group. Nitro groups of compound **9** were then reduced by iron in the presence of concentrated HCl. Attachment of the lateral amino side chains was accomplished in two steps: the reaction of compound **10** with 3-chloropropionyl chloride, using 1-ethylpiperidine as a base, followed by the substitution of the remaining chloro groups by 1-methylpiperazine. 1-ethylpiperidine was preferred as a tertiary amine over pyridine to avoid formation of a pyridinium salt which cannot be converted into the methylpiperazine derivative.[10] The excess of amine permitted the simultaneous elimination of the Fmoc group.

The additional side chain was attached to compound **11** by reaction with the central, secondary amino group. Compounds **12**–**17** were synthesized, as described in Scheme 2, by coupling bis(2-aminodiphenylsulfide) **11** with *N*-*t*-Boc-γ-aminobutyric acid, subsequent deprotection of the terminal amino group, followed by either fixation of a Fmoc group or coupling of the terminal amino group with myristic, 2-naphtoic or 9-acridinecarboxylic acid using PyBroP reagent.[14]

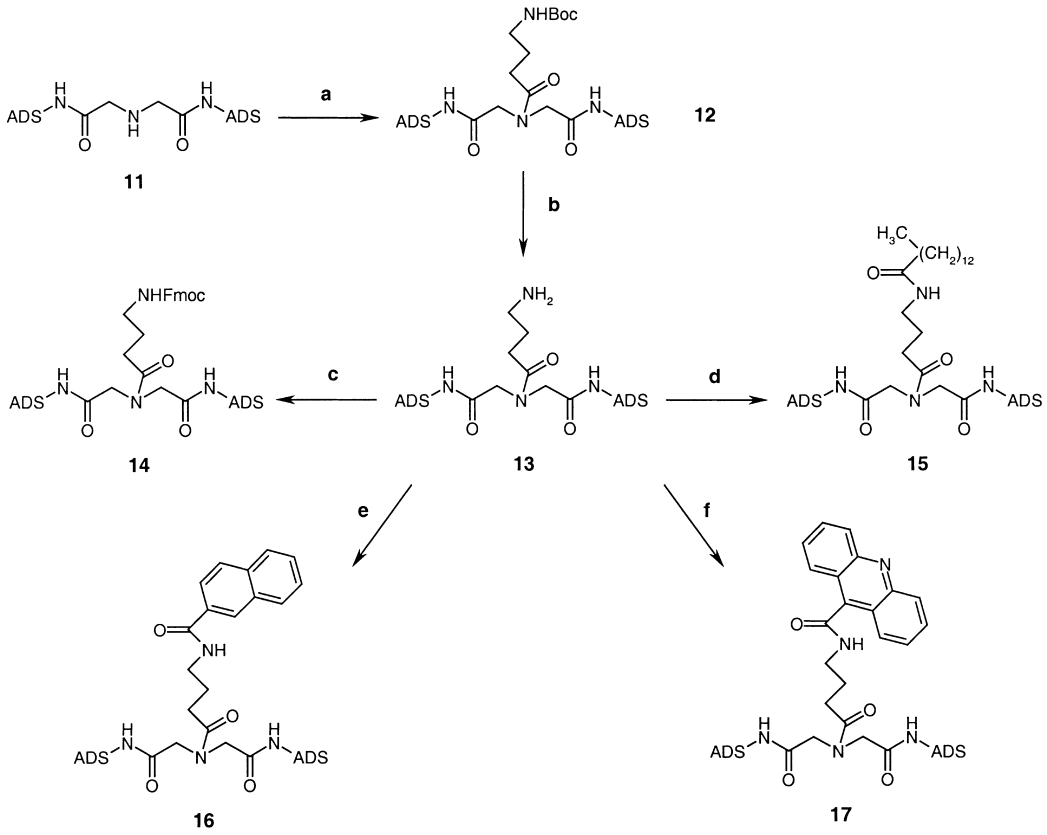
Compound **11** also led to bis(2-aminodiphenylsulfides) **18** and **19** via a coupling of the central, secondary amino group with biotin and glutaric anhydride respectively as shown in Scheme 3.

Results and Discussion

The inhibiting potency of the different compounds of the **C** series was evaluated by measuring the IC_{50} towards TR in the presence of 57 μM of $T(S)_2$ and increasing concentrations of inhibitor (0–60 μM) (Table 1).[10] Compounds were also tested upon human GR, upon *Trypanosoma cruzi* and *Leishmania infantum* amastigotes, upon *Trypanosoma brucei* trypomastigotes,



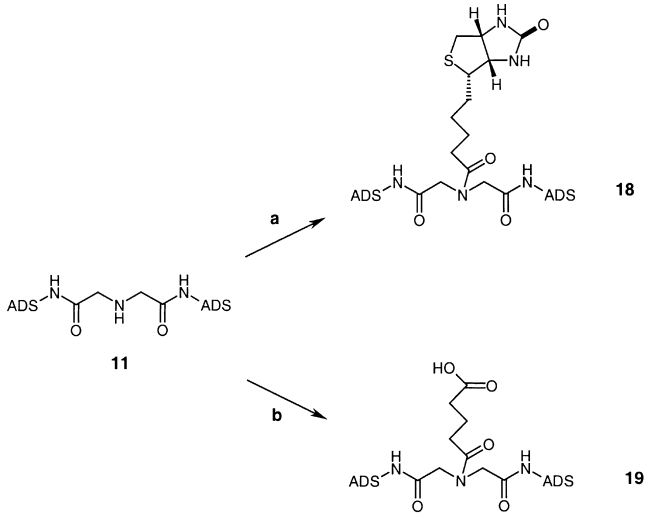
Scheme 1. Synthesis of bis(2-aminodiphenylsulfide) **11**. Reagents and conditions: (a) $(Boc)_2O$, NaOH, dioxane, H_2O, rt, 18 h; (b) DCC, dry THF, rt, 24 h; (c) [3-(4-bromo-2-nitro)phenylsulfanyl]phenylamine, dry THF, reflux, 24 h; (d) cyanuric fluoride, pyridine, dry CH_2Cl_2, rt, 5 h then [3-(4-bromo-2-nitro)phenylsulfanyl]phenylamine, pyridine, dry CH_2Cl_2, rt, 12 h; (e) TFA:CH_2Cl_2 1:1, rt, 4 h; (f) 9-fluorenylmethylchloroformate, Na_2CO_3, dioxane, H_2O, 0 °C, 4 h then rt, 12 h; (g) iron, HCl 12 N, EtOH, reflux, 1 h; (h) 3-chloropropionyl chloride, 1-ethylpiperidine, dry THF, 0 °C, 3 h then 1-methylpiperazine, THF, reflux, 4 h.



Scheme 2. Synthesis of compounds **12**–**17**. Reagents and conditions: (a) *N*-*t*-Boc-γ-aminobutyric acid, PyBroP, DIEA, dry DMF, rt, 2 h; (b) TFA:CH_2Cl_2 1:1, rt, 4 h; (c) 9-fluorenylmethylchloroformate, Na_2CO_3, dioxane, H_2O, 0 °C, 4 h then rt, 12 h; (d) myristic acid, PyBroP, DIEA, dry DMF, rt, 2 h; (e) 2-naphthoic acid, PyBroP, DIEA, dry DMF, rt, 2 h; (f) 9-acridinecarboxylic acid, PyBroP, DIEA, dry DMF, rt, 2 h.

and for their in vitro cytotoxicity upon human MRC-5 cells.

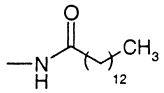
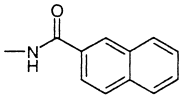
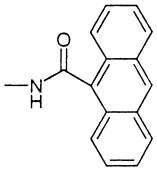
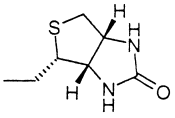
The presence of a protonable site within the spacer of bis(2-aminodiphenylsulfide), caused a decrease of TR inhibition (compound **11**, $IC_{50} = 1.5\,\mu M$, Table 1) when compared with the corresponding methylene group (compound **2**, $IC_{50} = 0.55\,\mu M$).[10] The central side chain possessing a terminal hydrophobic moiety (naphtyl, Boc, myristyl, acridinyl, biotinyl) led in general to an improvement of the activity (IC_{50} values between 0.2 and 0.3 μM, Table 1) while a terminal Fmoc group or a free amino group offered no real influence. In the case of the carboxylic group (**19**), solubility problems prevented IC_{50} measurement. Parallel enzymic studies upon human GR revealed that the different inhibitors were specific towards TR from *T. cruzi* versus human GR.



Scheme 3. Synthesis of compound **18** and **19**. Reagents and conditions: (a) biotin, PyBroP, DIEA, dry DMF, rt, 2 h; (b) glutaric anhydride, dry pyridine, rt, 12 h.

As presented in Table 2, in vitro activities upon parasites were found in the low micromolar range with the exception of *Leishmania*. No correlation between TR inhibition and the in vitro trypanocidal effect was apparent, as had been observed with the previous series of 2-aminodiphenylsulfides (**A** and **B** series). Amongst the most active compounds against *T. cruzi*, **12**–**14**, compounds **12** and **14** were found to be toxic at 12.5 μM upon mouse peritoneal macrophages used in the *Leishmania* test, while compound **13** displayed a similar cytotoxicity upon human MRC-5 cells. Acridinic compound **17** was devoid of cytotoxicity and exhibited the greatest activity against *T. brucei* while those of compounds **12** and **14**, given the sensitivity of this parasite to toxic compounds, suggested a non-specific effect.

Table 1. Inhibitory activities of compounds **2** and **11**–**19** towards TR from *T. cruzi*

Compound	−R	IC_{50} (μM)
2	No central amino group	0.55
11	No central side chain	1.5
12	−NHBoc	0.25
13	$-NH_2$	0.75
14	−NHFmoc	0.6
15		0.2
16		0.3
17		0.25
18		0.3
19	−COOH	*[a]

[a]*: precipitate in the enzymic buffer at 14.25 μM (2% DMSO).

Table 2. In vitro activities toward amastigote forms of *T. cruzi* and of *L. infantum* and trypomastigote forms of *T. brucei* and in vitro cytotoxicity upon human MRC-5 cells of compounds **11**–**19**

Compound	ED_{50} (μM) upon *T. cruzi*	ED_{50} (μM) upon *T. brucei*	ED_{50} (μM)[a] upon *L. infantum*	CC_{50} (μM) Cytotoxicity
11	12	5.75	> 12.5	21
12	7.5	5	T	10
13	7.5	4.5	> 12.5	10
14	5.2	4	T	9.5
15	> 12.5	> 6.25	> 12.5	> 25
16	10.5	4.75	> 12.5	20.5
17	17	1.8	> 12.5	> 25
18	> 12.5	> 6.25	> 12.5	> 25
19	> 12.5	> 6.25	10	> 25

[a]T: toxic at 12.5 μM upon mouse peritoneal macrophages used in the test.

Acridine compound **17** was found to be highly fluorescent and was therefore used for studying uptake and cellular localization into *T. cruzi* epimastigotes. Maximum excitation occurred at 494 nm and maximum emission at 500 nm (30 μM, 1% DMSO, in buffer pH 7). When epimastigotes from the Y strain of *T. cruzi* were incubated in the presence of 30 μM compound **17**, contrasting effects were observed which were dependent upon the nature of the medium, whether GLSH supplemented with 10% of decomplemented foetal calf serum (SVF), or Hanks-Wallace (HW) buffer (Fig. 4).

In GLSH-SVF, motility of the parasites was not perturbed, no parasitic fluorescence was observed and following 1 h of incubation the mass spectrum of the medium no longer displayed the signal corresponding to compound **17** (Fig. 4A). For the same length of incubation in HW, the mass spectrum expressed the persistent presence of compound **17** (Fig. 4B) and all epimastigotes were dead within 1 h. After a few minutes, three kinds of parasite could be observed: (i) live parasites showing active motility and no fluorescence; (ii) non-motile parasites with a stumpy shape showing several spots of the yellow fluorescence characteristic for compound **17** (Fig. 5, parasite at right side); (iii) remnants of burst parasites with one or two large spot(s) of blue fluorescence characteristic for Hoechst 33258 likely to correspond to remnants of the nucleus and the kinetoplast (Fig. 5, parasite at left side).

The absence of fluorescence inside living cells could be related to changes in fluorescence polarization as the fluorochrome compound interacts with the TR enzyme. Intense fluorescence was only associated with dying parasites in which membrane integrity had been altered and internal structures impaired. Blue and yellow fluorescence were not seen together in the parasites since, as previously reported, Hoechst 33258 staining is associated with affected cells only as an indicator of loss of membrane integrity.[15] No labeling was observed with the biotinyl derivative **18** following streptavidine interaction.

Conclusion

The construction of this new series of potent TR inhibitors assumes the importance of their bioavailability to parasites in order to obtain trypanocidal activity. Rapid transformation of these compounds in the presence of serum rather than absorption to serum proteins might explain the absence of straightforward correlation between the potent TR inhibition and the in vitro and in vivo antitrypanosomal activity with both of the first generations of 2-aminodiphenylsulfides.[8–10,16] The aminodiphenylsulfide moiety is probably responsible for this behaviour since structurally related phenothiazines, used as antipsychotic or antiemetic drugs, display a high bioavailability despite their being highly membrane- or protein-bound. Therefore, structural modifications such as the replacement of the sulfur atom or substitution at the *ortho* position on rings will be introduced to stabilize the aminodiphenylsulfide moiety.

Experimental

Chemistry

All reactions were monitored by thin-layer chromatography performed 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 Hope (R.H.)[17] as developing agents. Chromatography was carried out using silica gel 60 (230–400 mesh ASTM) from Macherey-Nagel. Thick-layer chromatography (TLC) was performed using silica gel from Merck, the compounds were extracted from the silica gel by the following solvent system: acetone:NH_4OH, 80:20. All melting points were determined on a Büchi melting point apparatus and

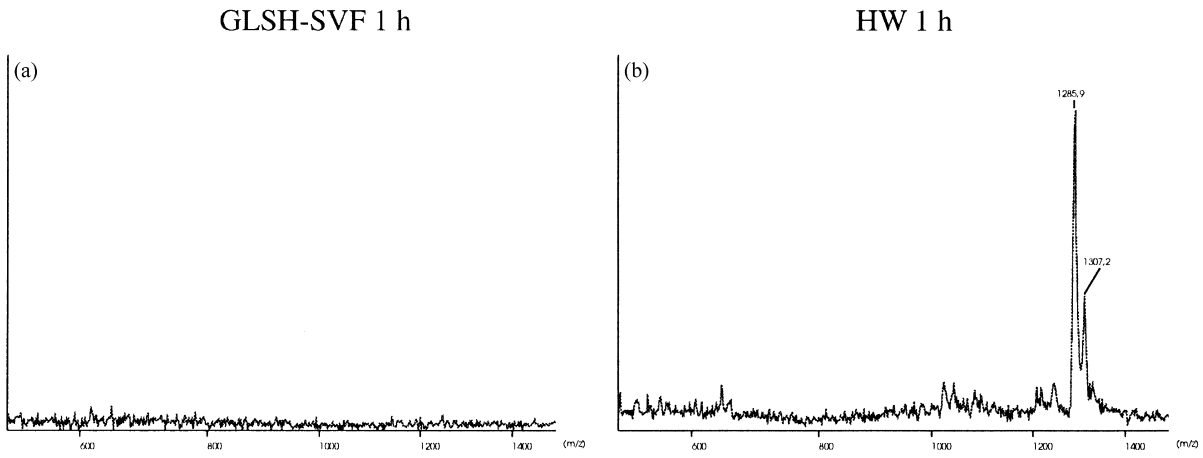


Figure 4. Time of flight mass spectra of compound **17** in the different media (in spectrum b, M^+: 1285.9 and $M^+ + Na^+$: 1307.2).

were uncorrected. ^1H NMR spectra were obtained using a Brücker 300 MHz spectrometer, chemical shifts were expressed in ppm relative to TMS used as an internal standard. Mass spectra were recorded on a time-of-flight (TOF) 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 maintained at 4 °C for 3 h. The salt was isolated by filtration, successively washed with ice cold AcOEt and ether, and dried under vacuum.

Method A: General procedure for preparation of compounds 8 and 13

A solution of *N*-Boc-amino compound (5.9 mmol, 1 equiv), in 87 mL of a 1:1 TFA:CH_2Cl_2 mixture was stirred for 4 h at room temperature. The solvents were evaporated and the oily residue treated with an AcOEt/NaOH 2.5 N mixture. The organic layer was separated, washed with a saturated solution of NaCl, dried over $MgSO_4$ and the solvent evaporated to yield the desired product.

Method B: General procedure for preparation of compounds 9 and 14

To a cooled solution of Na_2CO_3 (276 mg, 2.6 mmol, 10 equiv), in 1.6 mL of water was added a solution of appropriate compound (0.26 mmol, 1 equiv), in 6 mL of dioxane, then 9-fluorenylmethylchloroformate (68 mg, 0.26 mmol, 1 equiv). After stirring the mixture for 4 h at 0 °C then for 12 h at room temperature, 5.2 mL of HCl 1 N (5.2 mmol, 20 equiv) were introduced. The solvents were evaporated, and the solid residue was treated with a CH_2Cl_2/HCl 1 N mixture. The organic layer was

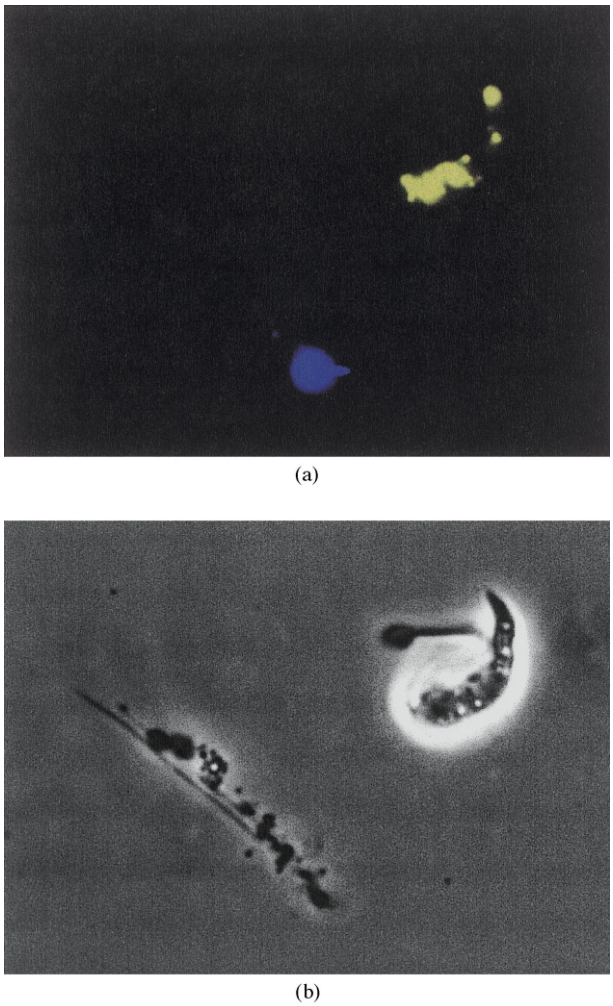


Figure 5. *Trypanosoma cruzi* epimastigotes have been treated with compound **17** for 1 h and observed in the presence of Hoechst 33258 under epifluorescence (a) and phase contrast (b). One stumpy dying parasite (right) shows yellow dots of fluorescence due to compound **17**, whereas a dead burst parasite (left) shows one large spot of blue fluorescence due to Hoechst 33258.

separated, dried over $MgSO_4$ and the solvent evaporated to yield the desired product.

Method C: General procedure for preparation of compounds 12, 15–18

To a cooled solution of the appropriate acid (0.5 mmol, 1 equiv), and DIEA (270 μL, 1.5 mmol, 3 equiv), in 10 mL of dry DMF, PyBroP (234 mg, 0.5 mmol, 1 equiv) was added. After stirring the mixture for 10 min at 0 °C, the appropriate amino derivative (0.6 mmol, 1.2 equiv) was introduced. Following further stirring of the mixture for 10 min at 0 °C then for 2 h at room temperature, the solvent was evaporated and the oily residue treated with a CH_2Cl_2/Na_2CO_3 0.5 M mixture. The organic layer was separated and dried over $MgSO_4$, the solvent evaporated and the oily residue purified by TLC (acetone:NH_4OH, 95:5), to yield the desired product.

***N*-(*tert*-Butoxycarbonyl)iminodiacetic acid (4).** To a cooled solution of iminodiacetic acid (10 g, 75.13 mmol, 1 equiv), in 145 mL of dioxane were added H_2O (50 mL), aqueous NaOH 2 N (50 mL, 100 mmol, 1.3 equiv), and $(Boc)_2O$ (18 g, 82.6 mmol, 1.1 equiv). After stirring the mixture for 30 min at 0 °C then for 18 h at room temperature, the solvents were evaporated and the oily residue was treated with an AcOEt/HCl 1 N mixture. The organic layer was separated, dried over $MgSO_4$ and the solvent evaporated to yield compound **4** as a white solid (11.3 g, 65% yield): R_f 0.3 (CH_2Cl_2); mp 125 °C; ^{1}H NMR (DMSO-d_6) δ 12.50 (bs, 2H, COOH, exchanged in D_2O), 3.93–3.89 (d, J = 9.9 Hz, 4H, CH_2), 1.37 (s, 9H, CH_3); TOFMS m/z 233 (M^+).

***N*-(*tert*-Butoxycarbonyl)-*N*-{*N*′-[3-(4-bromo-2-nitro)phenylsulfanyl]phenylacetamido}aminoacetic acid (6).** To a cooled solution of compound **4** (1.48 g, 6.35 mmol, 1 equiv), in 50 mL of dry THF was added dicyclohexylcarbodiimide (1.3 g, 6.35 mmol, 1 equiv). After stirring the mixture for 24 h at room temperature, [3-(4-bromo-2-nitro)phenylsulfanyl]phenylamine (2.07 g, 6.35 mmol, 1 equiv) was added. Following reflux of the mixture for 24 h, the solvent was evaporated and the oily residue treated with a CH_2Cl_2/citric acid 20% mixture. The organic layer was separated and dried over $MgSO_4$, the solvent evaporated and the oily residue purified by chromatography (CH_2Cl_2:MeOH, 90:10), to yield compound **6** as an orange solid (1.96 g, 57% yield): R_f 0.3 (CH_2Cl_2:MeOH, 9:1); mp 180 °C; ^{1}H NMR (CD_2Cl_2) δ 12.28 (bs, 1H, COOH, exchanged in D_2O), 8.38–8.37 (m, 1H, Ar-H), 7.94–7.93 (m, 1H, Ar-H), 7.89–7.88 (m, 1H, Ar-H), 7.70 (bs, 1H, NHCO, exchanged in D_2O), 7.48–7.41 (m, 2H, Ar-H), 7.29–7.25 (m, 1H, Ar-H), 6.83–6.80 (m, 2H, Ar-H), 3.94 (s, 2H, CH_2), 3.83 (s, 2H, CH_2), 1.38 (s, 9H, CH_3); TOFMS m/z 540 (M^+).

2-(*tert*-Butoxycarbonyl)imino-*N*,*N*′-[3-(4-bromo-2-nitro)-phenylsulfanyl]phenyldiacetylamide (7). At −10 °C, to a solution of compound **6** (5.9 g, 10 mmol, 1 equiv) and pyridine (800 μL, 10 mmol, 1 equiv), in 120 mL of dry CH_2Cl_2 was added, dropwise, cyanuric fluoride (2.7 g, 20 mmol, 2 equiv). After stirring the mixture for 10 min at 0 °C then for 5 h at room temperature, the solvent was evaporated to give crude acyl fluoride. Then to a cooled solution of [3-(4-bromo-2-nitro)phenylsulfanyl]-phenylamine (6.5 g, 20 mmol, 2 equiv), and pyridine (2.4 mL, 30 mmol, 3 equiv), in 120 mL of dry CH_2Cl_2 was added a solution of the latter crude acyl fluoride (10 mmol, 1 equiv), in 35 mL of dry CH_2Cl_2. After stirring the mixture for 10 min at 0 °C then for 12 h at room temperature, the solvent was evaporated and the oily residue treated with a CH_2Cl_2/H_2O mixture. The organic layer was separated and dried over $MgSO_4$, the solvent evaporated and the oily residue purified by chromatography (CH_2Cl_2:MeOH, 95:5), to yield compound **7** as a yellow solid (5 g, 58% yield): R_f 0.5 (CH_2Cl_2:MeOH, 9.5:0.5); mp 115 °C; ^{1}H NMR (CD_2Cl_2) δ 10.04 (bs, 1H, NHCO, exchanged in D_2O), 9.11 (bs, 1H, NHCO, exchanged in D_2O), 8.39–8.38 (m, 2H, Ar-H), 8.04–8.03 (m, 1H, Ar-H), 7.90–7.89 (m, 1 H, Ar-H), 7.81–7.78 (m, 2H, Ar-H), 7.51–7.42 (m, 4H, Ar-H), 7.38–7.33 (m, 2H, Ar-H), 6.88–6.83 (m, 2H, Ar-H), 4.15 (s, 2H, CH_2), 4.06 (s, 2H, CH_2), 1.41 (s, 9H, CH_3); TOFMS m/z 847 (M^+).

2-Imino-*N*,*N*′-[3-(4-bromo-2-nitro)phenylsulfanyl]phenyl-diacetylamide (8). Compound **8** was prepared from compound **7** by method A and was obtained as a yellow solid (4 g, 100% yield): R_f 0.5 (CH_2Cl_2:MeOH, 9:1); mp 75 °C; ^{1}H NMR (CD_2Cl_2) δ 8.71 (bs, 2H, NHCO, exchanged in D_2O), 8.20–8.19 (m, 2H, Ar-H), 7.72–7.70 (m, 2H, Ar-H), 7.65–7.61 (m, 2H, Ar-H), 7.33–7.27 (m, 4H, Ar-H), 7.17–7.13 (m, 2H, Ar-H), 6.68–6.63 (m, 2H, Ar-H), 3.39 (s, 4H, CH_2), 1.75 (bs, 1H, NH, exchanged in D_2O); TOFMS m/z 747 (M^+).

2-(9-Fluorenylmethyloxycarbonyl)imino-*N*,*N*′-[3-(4-bromo-2-nitro)phenylsulfanyl]phenyldiacetylamide (9). Compound **9** was prepared from compound **8** by method B and was obtained as a yellow solid (5 g, 90% yield): R_f 0.55 (CH_2Cl_2:MeOH, 9:1); mp 65 °C; ^{1}H NMR (CD_2Cl_2) δ 10.17 (bs, 1H, NHCO, exchanged in D_2O), 9.67 (bs, 1H, NHCO, exchanged in D_2O), 8.37–8.34 (m, 2H, Ar-H), 8.07–8.03 (m, 1H, Ar-H), 7.92–7.88 (m, 1H, Ar-H), 7.86–7.80 (m, 2H, Ar-H), 7.73–7.70 (m, 2H, Ar-H), 7.67–7.64 (m, 1H, Ar-H), 7.56–7.53 (m, 2H, Ar-H), 7.49–7.30 (m, 7H, Ar-H), 7.18–7.12 (m, 2H, Ar-H), 6.81–6.77 (m, 2H, Ar-H), 4.47–4.42 (m, 2H, CH_2), 4.23–4.07 (m, 5H, 1 CH and 2 CH_2); TOFMS m/z 970 (M^+).

2-(9-Fluorenylmethyloxycarbonyl)imino-*N*,*N*′-[3-(2-amino-4-bromo)phenylsulfanyl]phenyldiacetylamide (10). To a solution of compound **9** (4.3 g, 4.4 mmol, 1 equiv), and iron (1.5 g, 26.4 mmol, 6 equiv), in 15 mL of EtOH was added HCl 12 N (915 μL, 11 mmol, 2.5 equiv). Following reflux for 1 h, the mixture was filtrated on Celite and the solvent evaporated to yield compound **10** as a brown oil (4 g, 100% yield): R_f 0.6 (CH_2Cl_2:MeOH, 9:1); ^{1}H NMR (CD_3COCD_3) δ 7.90–7.50 (m, 8H, Ar-H), 7.55–7.25 (m, 10 H, Ar-H), 7.20–7.10 (m, 2H, Ar-H), 7.03–6.90 (m, 2H, Ar-H), 4.45–4.17 (m, 4H, CH_2), 4.07–3.95 (m, 3H, 1 CH and 1 CH_2); TOFMS m/z 910 (M^+).

2-Imino-*N*,*N*′-(3-{2-[3-(4-methylpiperazin-1-yl)propanoyl-amino-4-bromo]phenylsulfanyl}phenyl)diacetylamide (11). Under N_2, 3-chloropropionyl chloride (904 μL,

9.5 mmol, 5 equiv) was added, dropwise, into a cooled solution of compound **10** (1 g, 1.9 mmol, 1 equiv), in 35 mL of dry THF. After stirring the mixture for 30 min at 0 °C, three additions of 1-ethylpiperidine (781 μL, 5.7 mmol, 3 equiv), were made at intervals of 30 min. Following further stirring of the mixture for 1.5 h at 0 °C, a large excess of 1-methylpiperazine (4.2 mL, 37.9 mmol, 20 equiv), was added at room temperature and the mixture heated and held at reflux for 4 h. The solvent was removed and the oily residue treated with a CH_2Cl_2/water mixture. The organic layer was separated and dried over $MgSO_4$, the solvent evaporated and the oily residue purified by TLC (CH_2Cl_2:MeOH, 80:20), to yield compound **11** as a colourless solid (945 mg, 50% yield): R_f 0.05 (CH_2Cl_2:MeOH, 8:2); mp 60 °C; ^{1}H NMR (CD_2Cl_2) δ 10.66 (bs, 2H, NHCO, exchanged in D_2O), 9.08 (bs, 2H, NHCO, exchanged in D_2O), 8.41–8.40 (m, 2H, Ar-H), 7.58–7.55 (m, 2H, Ar-H), 7.43–7.38 (m, 2H, Ar-H), 7.29–7.20 (m, 6H, Ar-H), 6.91–6.87 (m, 2H, Ar-H), 3.39 (s, 4H, CH_2), 2.59–2.47 (m, 24 H, CH_2), 2.22 (s, 6H, CH_3); TOFMS *m*/*z* 995 (M^+).

2-(*N*-{4-[*N*′-(*tert*-Butoxycarbonyl)amino]butanoyl}imino)-*N*,*N*-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanyl}phenyl)diacetylamide (12). Compound **12** was prepared from *N*-*t*-Boc-γ-aminobutyric acid and compound **11** by method C and was obtained as a colourless solid (472 mg, 80% yield): R_f 0.4 (acetone: NH_4OH, 9.5:0.5); mp 60 °C; ^{1}H NMR (CD_2Cl_2) δ 10.75 (bs, 1H, NHCO, exchanged in D_2O), 10.65 (bs, 1H, NHCO, exchanged in D_2O), 10.57 (bs, 1H, NHCO, exchanged in D_2O), 9.63 (bs, 1H, NHCO, exchanged in D_2O), 8.58–8.49 (m, 2H, Ar-H), 7.68 (bs, 1H, NHC(O)O, exchanged in D_2O), 7.57–7.53 (m, 2H, Ar-H), 7.42–7.35 (m, 2H, Ar-H), 7.30–7.21 (m, 6H, Ar-H), 6.86–6.83 (m, 2H, Ar-H), 4.20 (s, 2H, CH_2), 4.08 (s, 2H, CH_2), 2.54–2.30 (m, 24 H, CH_2), 2.26–2.17 (m, 4H, CH_2), 2.15 (s, 6H, CH_3), 1.88–1.84 (m, 2H, CH_2), 1.24 (s, 9H, CH_3); TOFMS *m*/*z* 1180 (M^+).

2-[*N*-(4-Aminobutanoyl)imino]-*N*,*N*′-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanyl}-phenyl)diacetylamide (13). Compound **13** was prepared from compound **12** by method A and was obtained as a colourless oil (780 mg, 90% yield): R_f 0.5 (acetone: NH_4OH, 9:1); ^{1}H NMR (CD_2Cl_2) δ 10.65 (bs, 1H, NHCO, exchanged in D_2O), 10.58 (bs, 1H, NHCO, exchanged in D_2O), 10.55 (bs, 1H, NHCO, exchanged in D_2O), 8.90 (bs, 1H, NHCO, exchanged in D_2O), 8.40–8.27 (m, 2H, Ar-H), 7.48–7.37 (m, 2H, Ar-H), 7.33–7.25 (m, 2H, Ar-H), 7.16–7.09 (m, 6H, Ar-H), 6.76–6.73 (m, 2H, Ar-H), 4.12 (s, 2H, CH_2), 3.96 (s, 2H, CH_2), 3.75 (bs, 2H, NH_2, exchanged in D_2O), 2.45–2.25 (m, 24 H, CH_2), 2.11–2.06 (m, 4H, CH_2), 2.03 (s, 6H, CH_3), 1.75–1.72 (m, 2H, CH_2); TOFMS *m*/*z* 1080 (M^+).

2-(*N*-{4-[*N*′-(9-Fluorenylmethyloxycarbonyl)amino]butanoyl}imino)-*N*,*N*-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino - 4 - bromo]phenylsulfanyl}phenyl)diacetylamide (14). Compound **14** was prepared from compound **13** by method B and was obtained as a colourless oil (203 mg, 60% yield): R_f 0.55 (acetone:NH_4OH, 9.5:0.5); ^{1}H NMR (CD_2Cl_2) δ 10.57 (bs, 1H, NHCO, exchanged in D_2O), 10.51 (bs, 1H, NHCO, exchanged in D_2O), 10.45 (bs, 1H, NHCO, exchanged in D_2O), 9.43 (bs, 1H, NHCO, exchanged in D_2O), 8.42–8.33 (m, 3H, Ar-H), 7.67–7.62 (m, 2H, Ar-H), 7.52 (bs, 1H, NHC(O)O, exchanged in D_2O), 7.45–7.42 (m, 2H, Ar-H), 7.31–7.23 (m, 5H, Ar-H), 7.17–7.09 (m, 7H, Ar-H), 6.73–6.69 (m, 3H, Ar-H), 4.33 (s, 2H, CH_2), 4.02 (m, 3H, 1 CH and 1 CH_2), 2.49–2.25 (m, 24 H, CH_2), 2.11–2.05 (m, 6H, CH_2), 2.04 (s, 6H, CH_3), 1.60–1.50 (m, 2H, CH_2); TOFMS *m*/*z* 1302 (M^+).

2-{*N*-[4-(*N*-Tetradecanoyl)aminobutanoyl]imino}-*N*,*N*′-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanyl}phenyl)diacetylamide (15). Compound **15** was prepared from myristic acid and compound **13** by method C and was obtained as a colourless oil (200 mg, 31% yield): R_f 0.3 (acetone:NH_4OH, 9.5:0.5); ^{1}H NMR (CD_2Cl_2) δ 10.93 (bs, 1H, NHCO, exchanged in D_2O), 10.32 (bs, 1H, NHCO, exchanged in D_2O), 10.21 (bs, 1H, NHCO, exchanged in D_2O), 9.99 (bs, 1H, NHCO, exchanged in D_2O), 8.59–8.56 (m, 2H, Ar-H), 7.71–7.67 (m, 2H, Ar-H), 7.54–7.41 (m, 4H, Ar-H), 7.30–7.21 (m, 4H, Ar-H), 6.82–6.72 (m, 2H, Ar-H), 6.56 (bs, 1H, NHCO, exchanged in D_2O), 4.31 (s, 2H, CH_2), 4.17 (s, 2H, CH_2), 3.20–3.17 (m, 2H, CH_2), 2.76–2.45 (m, 32 H, CH_2), 2.44 (s, 3H, CH_3), 2.36–2.32 (m, 7H, 2 CH_2 and 1 CH_3), 1.85–1.70 (m, 4H, CH_2), 1.64–1.57 (m, 2H, CH_2), 1.46–1.41 (m, 2H, CH_2), 1.28 (s, 3H, CH_3), 1.24–1.20 (m, 4H, CH_2), 1.12–1.11 (m, 4H, CH_2); TOFMS *m*/*z* 1290 (M^+).

2-(*N*-{4-[*N*-(Napht-2-oyl)]aminobutanoyl}imino)-*N*,*N*′-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanyl}phenyl)diacetylamide (16). Compound **16** was prepared from 2-naphtoic acid and compound **13** by method C and was obtained as a yellow oil (247 mg, 40% yield): R_f 0.25 (acetone:NH_4OH, 9.5:0.5); ^{1}H NMR (CD_2Cl_2) δ 10.64 (bs, 1H, NHCO, exchanged in D_2O), 10.26 (bs, 1H, NHCO, exchanged in D_2O), 10.04 (bs, 1H, NHCO, exchanged in D_2O), 9.81 (bs, 1H, NHCO, exchanged in D_2O), 8.45–8.41 (m, 2H, Ar-H), 7.79–7.71 (m, 3H, Ar-H), 7.57 (bs, 1H, NHCO, exchanged in D_2O), 7.49–7.25 (m, 10 H, Ar-H), 7.15–7.07 (m, 4H, Ar-H), 6.70–6.60 (m, 2H, Ar-H), 4.23 (s, 2H, CH_2), 4.07 (s, 2H, CH_2), 2.45–2.35 (m, 24 H, CH_2), 2.05–2.03 (m, 10 H, 2 CH_2 and 2 CH_3), 1.74–1.69 (m, 2H, CH_2); TOFMS *m*/*z* 1234 (M^+).

2-(*N*-{4-[*N*-(Acridin-9-oyl)]aminobutanoyl}imino)-*N*,*N*′-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanyl}phenyl)diacetylamide (17). Compound **17** was prepared from 9-acridinecarboxylic acid and compound **13** by method C and was obtained as a yellow oil (257 mg, 40% yield): R_f 0.25 (acetone:NH_4OH, 9.5:0.5); ^{1}H NMR (CD_2Cl_2) δ 10.75 (bs, 1H, NHCO, exchanged in D_2O), 10.28 (bs, 1H, NHCO, exchanged in D_2O), 10.11 (bs, 1H, NHCO, exchanged in D_2O), 9.76 (bs, 1H, NHCO, exchanged in D_2O), 8.39–8.34 (m, 2H, Ar-H), 8.06–8.02 (m, 1H, Ar-H), 7.89–7.86 (m, 2H, Ar-H), 7.80 (s, 1H, NHCO, exchanged in D_2O), 7.67–7.61 (m, 2H, Ar-H), 7.52–7.25 (m, 8H, Ar-H), 7.14–7.06 (m, 4H,

Ar-H), 6.68–6.60 (m, 3H, Ar-H), 4.19 (s, 2H, CH_2), 4.00 (s, 2H, CH_2), 2.52–2.20 (m, 24 H, CH_2), 2.09–2.01 (m, 10 H, 2 CH_2 and 2 CH_3), 1.83–1.74 (m, 2H, CH_2); TOFMS *m*/*z* 1285 (M^+).

2-[*N*-(Biotinoyl)imino]-*N*,*N*'-(3-{2-[3-(4-methylpiperazin-1-yl)propanoylamino-4-bromo]phenylsulfanyl}phenyl)diacetylamide (18). Compound **18** was prepared from biotin and compound **11** by method C and was obtained as a colourless oil (305 mg, 50% yield): R_f 0.15 (acetone: NH_4OH, 9.5:0.5); ^1H NMR (CD_2Cl_2) δ 11.04 (bs, 1H, NHCO, exchanged in D_2O), 10.52 (bs, 1H, NHCO, exchanged in D_2O), 10.46 (bs, 1H, NHCO, exchanged in D_2O), 10.34 (bs, 1H, NHCO, exchanged in D_2O), 8.47–8.40 (m, 2H, Ar-H), 7.74–7.72 (m, 1H, Ar-H), 7.60–7.53 (m, 1H, Ar-H), 7.49 (bs, 1H, NHCO, exchanged in D_2O), 7.46–7.42 (m, 2H, Ar-H), 7.31–7.25 (m, 2H, Ar-H), 7.18–7.10 (m, 4H, Ar-H), 6.72–6.69 (m, 2H, Ar-H), 6.27 (bs, 1H, NHCO, exchanged in D_2O), 4.26 (s, 2H, CH_2), 4.21 (s, 2H, CH_2), 3.50–3.45 (m, 2H, CH_2), 3.38–3.34 (m, 2H, CH_2), 2.60–2.38 (m, 27 H, 3 CH and 12 CH_2), 2.33–2.22 (m, 2H, CH_2), 2.18 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 1.84–1.79 (m, 2H, CH_2), 1.50–1.44 (m, 2H, CH_2); TOFMS *m*/*z* 1221 (M^+).

4-{*N*,*N*-[*N*',*N*''-(3-{2-[4-Bromo-3-(4-methylpiperazin-1-yl)propanoylamino]phenylsulfanyl}phenyl)acetylamido]amido}butanoic acid (19). To a cooled solution of compound **11** (127 mg, 0.13 mmol, 1 equiv), in 1 mL of dry pyridine was added glutaric anhydride (14.6 mg, 0.13 mmol, 1 equiv). After stirring the mixture for 12 h at room temperature, the solvent was evaporated and the oily residue treated with a CH_2Cl_2/cooled water mixture. The organic layer was separated and dried over $MgSO_4$, the solvent evaporated and the oily residue purified by TLC (acetone:NH_4OH, 100:1), to yield compound **19** as a colourless oil (100 mg, 70% yield): R_f 0.25 (acetone: NH_4OH, 10:0.1); ^1H NMR (CD_2Cl_2) δ 11.04 (bs, 1H, NHCO, exchanged in D_2O), 10.27 (bs, 1H, NHCO, exchanged in D_2O), 10.04 (bs, 1H, NHCO, exchanged in D_2O), 10.02 (bs, 1H, NHCO, exchanged in D_2O), 8.60–8.56 (m, 2H, Ar-H), 7.86–7.83 (m, 1H, Ar-H), 7.71–7.68 (m, 1H, Ar-H), 7.52–7.41 (m, 4H, Ar-H), 7.29–7.19 (m, 4H, Ar-H), 6.72–6.68 (m, 2H, Ar-H), 4.34 (s, 2H, CH_2), 4.17 (s, 2H, CH_2), 2.71–2.42 (m, 24 H, CH_2), 2.34 (s, 6H, CH_3), 2.28–2.23 (m, 4H, CH_2), 1.89–1.86 (m, 2H, CH_2); TOFMS *m*/*z* 1109 (M^+).

Biological evaluation

All compounds were evaluated under oxalate form.

Assays for TR inhibition

Recombinant *T. cruzi* trypanothione reductase was produced from the SG5 *Escherichia coli* strain with the overproducing expression vector pIBITczTR.[18] 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 promoted by the addition of the enzyme and the subsequent NADPH oxidation 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.

Assays for GR inhibition

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 presence of 44 μM of GSSG, in 40 mM Hepes, 50 mM KCl and 1 mM EDTA, pH 7.4, and 180 μM of NADPH.

In vitro activity against intracellular *Trypanosoma cruzi* amastigotes

Primary mouse peritoneal macrophages were seeded in 96-well microplates at 30,000 cells per well. After 24 h, about 100,000 trypomastigotes of *T. cruzi* were added per well together with twofold dilutions of the drug. The cultures were incubated at 37 °C in 5% CO_2–95% air for 4 days. Following 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) compared with untreated control cultures. Scoring was performed microscopically and ED_{50}-values were then extrapolated.

In vitro activity against *Trypanosoma brucei* trypomastigotes

Bloodstream forms of *T. brucei* were cultivated in HMI-9 medium.[19] In a 96-well microplate, 10,000 haemoflagellates were incubated at different drug concentrations (12.5, 6.25, 3.13 and 1.56 μM) for 4 days. Parasite multiplication was measured colorimetrically (490 nm) following addition of MTT tetrazolium which converts to an aqueous soluble, formazan product.[20] ED_{50}-values were then extrapolated.

In vitro activity against intracellular *Leishamnia infantum* amastigotes

Primary mouse peritoneal macrophages were seeded in 16-well LABTEK culture slides at 30,000 cells per well. After 24 h, amastigotes of *L. infantum* (derived from the spleen of an infected donor animal) were added at an infection ratio of 10/1 together with a twofold dilution of the drug. The cultures were incubated at 37 °C in 5% CO_2–95% air for 7 days. Treatment of uninfected control cultures was also included to determine a selective index. Drug activity was semi-quantitatively scored as% reduction of the total parasite load or the number of infected macrophages in Wright stained preparations. Scoring was performed microscopically and ED_{50}-values were extrapolated.

Cytotoxicity test upon human MRC-5 cells

A human diploid embryonic lung cell line (MRC-5, Bio-Whittaker 72211D) and primary peritoneal mouse macrophages were used to assess the cytotoxicity for host cells. The peritoneal macrophages were collected

from the peritoneal cavity 48 h after stimulation with potato starch and seeded in 96-well microplates at 30,000 cells per well. MRC-5 cells were seeded at 5000 cells per well. After 24 h, the cells were washed and a twofold dilution of the drug was added in 200 μL standard culture medium (RPMI + 5% FCS). The final DMSO concentration in the culture remained below 0.5%. The cultures were incubated with four concentrations of compounds (25, 12.5, 6.25 and 3.13 μM) at 37 °C in 5% CO_2–95% air for 7 days. Untreated cultures were included as controls. For MRC-5 cells, the cytotoxicity was determined using the colorimetric MTT assay[20] and scored as % reduction of absorption at 540 nm of treated cultures versus untreated control cultures. For macrophages, scoring was performed microscopically. CC_{50}-values were then extrapolated.

Fluorescence microscopy

Hoechst 33258 (stock solution at 5 mg/mL in 100% DMSO) was used at 1 μg/mL final concentration. Compound **17** (stock solution at 3 mM in 100% DMSO) was used at 30 μM final concentration. Fluorescence microscopy was performed using a Zeiss Axiophot equipped for epifluorescence; Hoechst 33258 and compound **17** fluorescence were visualized simultaneously using the following filter combination (excitation G365, beam splitter FT395, emission LP 420) and pictures taken with a Princeton Micromax CCD camera driven by the Metaview software. Blue and yellow fluorescence were recorded simultaneously as black and white signals and were artificially reconstituted with metaview and merged using Adobe Photoshop.

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