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INHIBITING EFFECTS OF SPERMIDINE DERIVATIVES ON *TRYPANOSOMA CRUZI* TRYPANOTHIONE REDUCTASE

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Trypanothione reductase is a vital component of the antioxidant defenses of trypanosomes. This enzyme reduces trypanothione, a spermidine-glutathione conjugate. The inhibitory effects of several spermidine derivatives on the reduction of trypanothione by *Trypanosoma cruzi* trypanothione reductase were assessed. Spermidine derivatives containing hydrophobic aromatic substituents were found to be competitive inhibitors of trypanothione reductase. N^4-acylated spermidine derivatives were less effective inhibitors than the corresponding N^4-alkylated derivatives. The most effective compounds studied were N^1,N^8-bis(2-naphthylmethyl)spermidine and N^4-(2-naphthylmethyl)spermidine, with K_i values of 9.5 and 108 μM, respectively.

Keywords: Spermidine; polyamine; trypanothione reductase; *Trypanosoma cruzi*; Chagas disease.

INTRODUCTION

Trypanosomes and leishmanias are protozoans belonging to the family *Trypanosomatidae*. These organisms are the etiological agents of human diseases including Chagas' disease (*Trypanosoma cruzi*), African sleeping sickness (*T. brucei gambiense* and *T. b. rhodesiense*), kala-azar (*Leishmania donovani*) and oriental sore (*L. tropica*).[1] Also, certain diseases of economically important livestock are caused by *Trypanosomatidae*, such as Nagana cattle disease (*T. b. brucei* and *T. b. congolense*). Chagas' disease currently infects 16–18 million people and leads to more than 45,000 deaths each year.[2] Although the disease occurs predominantly in Central and South America, about 100,000 people in the USA are also infected, probably due to transfusion of blood products originating from South America[3] and immigration of infected persons into the USA.[4] Since current treatment of *T. cruzi*

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and other trypanosome infections is difficult and often ineffectual in controlling the chronic phase of these diseases,[5] effective antitrypanosomal drugs are needed.

One strategy for the development of novel trypanocidal agents involves the exploitation of a major difference in the thiol metabolism of trypanosomes and mammals.[6] In most eukaryotes and prokaryotes the predominant low molecular weight thiol is glutathione, which is an important component of cells' antioxidant defenses.[7] Levels of reduced glutathione in these cells are maintained by the action of glutathione reductase. Trypanosoma and leishmania possess a unique mechanism to maintain levels of reduced thiols. These organisms do not contain glutathione reductase, and although they do contain glutathione, the predominant low molecular weight thiols in trypanosoma and leishmania are glutathione-spermidine conjugates including N^1,N^8-bis(glutathionyl)spermidine (trypanothione) and monoglutathionylspermidine.[8,9] Levels of reduced trypanothione are maintained by the action of trypanothione reductase (TR), which can also reduce monoglutathionylspermidine.[9,10] Reduced trypanothione can undergo nonenzymatic,[11] or enzyme-mediated[12] thiol-disulfide exchange reactions with disulfides to produce reduced glutathione and other thiols. Given that the antioxidant defenses of trypanosomes are based on the action of TR, inhibitors of TR are potential antitrypanosomal agents.[6]

TR is a NADPH-dependent, FAD-containing, homodimeric enzyme which reduces the disulfide group of oxidized trypanothione (TS_2) to provide reduced trypanothione ($T(SH)_2$) (for a review see Reference 11).

$$TS_2 + NADPH + H^+ \overset{TR}{\rightleftharpoons} T(SH)_2 + NADP^+$$

Recently, the X-ray crystal structures of *T. cruzi* TR and the NADPH-TR complex were determined to 0.33 nm resolution.[13] Also, the crystal structure of *Crithidia fasciculata* TR complexed with N^1-glutathionylspermidine disulfide has been determined.[14] Additional insights into substrate-TR interactions needed for the rational design of inhibitors have been obtained from mutagenesis and substrate specificity studies. Site-directed mutagenesis studies have shown that the E18 and W21 residues of the active site are crucial for determining the substrate specificity of *T. congolense* TR.[15] The carboxylate residue of E18 is presumed to form an ionic interaction with the protonated N^4-amino group of the spermidine moiety of trypanothione. The tryptophan residue is thought to be involved in hydrophobic interactions with the substrate. Substrate specificity and inhibitor studies have indicated that several structurally diverse compounds bind reversibly to the active site of TR.[10,16–18] These studies have shown that molecules do not require a cyclic structure, a disulfide or glutamyl residue(s) to bind to the active site. However, all compounds that bind to the active site contain amino group(s) and often have

hydrophobic residue(s). The most potent inhibitor described to date is a tricyclic amine, clomipramine (with a K_i, of 6.5 μM against *T. cruzi* TR).[16]

We were interested in developing competitive inhibitors of *T. cruzi* TR that are synthetically readily available. We envisioned that such compounds could be easily modified to contain chemically reactive groups leading to compounds that may act as irreversible inhibitors of TR. Based upon the available information on the structure and substrate/inhibitor specificities of TR, we decided to investigate the inhibiting effects of spermidine and spermine derivatives containing amino and hydrophobic groups. We have recently described some of our preliminary results.[19] In this paper we give a full account of the synthesis of several N^1,N^8-bis- and N^4-mono-substituted spermidine derivatives and evaluation of the inhibiting effects of these compounds on *T. cruzi* TR.

MATERIALS AND METHODS

Synthesis

N^1,N^8-Bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) was prepared using the previously described procedure.[20] All other reagents were purchased from commercial sources. THF used was initially dried by distillation over benzophenone and sodium under N_2. The acetonitrile and triethylamine used were initially dried by distillation over calcium hydride under N_2 and were stored over 4 Å molecular sieves. The ammonium hydroxide used contained 29.9% NH_3. Thin layer chromatography was carried out using silica gel (250 μm layer) and compounds were visualized by UV light, ninhydrin in ethanol or phosphomolybdic acid in ethanol. Column chromatography was carried out under pressure (flash chromatography) using silica gel (40 μm). NMR spectra were obtained using a Brucker AC250 NMR spectrometer. ^{1}H NMR spectra were acquired at 250 MHz and ^{13}C NMR spectra were acquired at 62.9 MHz. NMR samples were dissolved in $CDCl_3$ with TMS as an internal reference unless otherwise indicated. CI mass spectra were obtained on a Finnegan 4000 spectrometer, FAB spectra on a Kratos MS50 spectrometer, and EI spectra on an AEI MS9 spectrometer.

N^1,N^8-Bis(trifluoroacetyl)-N^4-(t-butoxycarbonyl)spermidine (2)

To a solution of N^1,N^8-bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) (10.0 g, 22.17 mmol) in THF (50 mL) and triethylamine (8.33 mL, 60.0 mmol) was slowly added di-*t*-butyl dicarbonate (7.26 g, 7.64 mL, 33.26 mmol). The reaction was stirred overnight at room temperature. Saturated aqueous NH_4Cl solution (75 mL) was added to the reaction and then the mixture was extracted with CH_2Cl_2

(4 × 75 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a yellow oil. Compound (**2**) was purified by column chromatography (0.5–4% MeOH in CH_2Cl_2) to give (**2**) as a white solid (9.03 g, 93%). R_f one spot 0.35 (2% MeOH in CH_2Cl_2); mp 66–68°C, ^{1}H NMR δ 3.58 (m, 2 H, CH_2N), 3.30 (m, 4 H, 2 CH_2N), 3.18 (m, 2 H, CH_2N), 1.71 (br s, 2 H, 2 CF_3CONH); 1.57 (m, 6 H, $NCH_2CH_2CH_2N$ and $NCH_2CH_2CH_2CH_2N$) and 1.46 (s, 9 H, $OC(CH_3)_3$) ppm; ^{13}C NMR δ 157.45 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 157.4 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 146.8, 115.95 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 115.9 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 80.5 ($C(CH_3)_3$), 46.5, 43.2, 39.4, 36.0, 28.3 (CH_3) 27.3, 26.3 and 25.6 ppm; MS (FAB) m/z 438.0 ($M + H^+$).

N^4-(t-Butoxycarbonyl)spermidine (3)

To a stirred solution of N^1,N^8-bis(trifluoroacetyl)-N^4-(*t*-butoxycarbonyl)spermidine (**2**) (1.0 g, 2.29 mmol) in MeOH (20 mL) and water (1.2 mL) was added potassium carbonate (3.56 g, 25.7 mmol) and the mixture refluxed overnight. Saturated aqueous NaCl solution (50 mL) was added then the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give (**3**) as a yellow oil (0.414 g, 74%). R_f one spot 0.36 (ethanol:ammonium hydroxide 4:1); ^{1}H NMR δ 3.26 (m, 2 H, CONCH_2), 3.17 (m, 2 H, CONCH_2), 2.94 (s, 4 H, 2 NH_2), 2.68 (m, 4 H, 2 CH_2NH_2), 1.62 (m, 6 H, $NCH_2CH_2CH_2N$ and $NCH_2CH_2CH_2CH_2N$) and 1.40 (s, 9 H, 3 CH_3) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 155.6, 79.2 ($C(CH_3)_3$), 46.4, 44.8 (m), 41.2, 38.4 (m), 31.4 (m), 30.2, 28.0 (CH_3) and 25.6 (m) ppm; MS (CI) m/z 246 ($M + H^+$).

N^1,N^8-Bis(trifluoroacetyl)-N^4-acetylspermidine (4)

To a solution of N^1,N^8-bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) (0.16 g, 0.355 mmol) in pyridine (20 mL) was added acetic anhydride (0.055 g, 0.051 mL, 0.533 mmol) and the mixture was stirred at room temperature for 3 h. The solvent was removed and (**4**) was purified by column chromatography (7% MeOH in CH_2Cl_2) to give (**4**) as a light-yellow colored oil (0.12 g, 89%). R_f one spot 0.66 (15% MeOH in CH_2Cl_2); ^{1}H NMR δ 3.40 (m, 4 H, 2 $CF_3CONHCH_2$), 3.27 (m, 4 H, CH_2NCH_2), 2.13 (s, 3 H, CH_3), 1.75 (m, 2 H, $NCH_2CH_2CH_2N$), 1.64 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 172.3 ($COCH_3$), 157.6 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 157.3 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 115.9 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 115.8 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 48.4, 46.1, 42.3, 39.1, 26.7, 26.1, 25.6, 21.0 ppm; MS (EI) m/z 380 ($M + H^+$).

N^4-Acetylspermidine (5)

To N^1,N^8-bis(trifluoroacetyl)-N^4-acetylspermidine (**4**) (0.16 g, 0.422 mmol) was added a solution of MeOH and ammonium hydroxide (1:1, 15 mL). The mixture was refluxed overnight. The solvents were removed and (**5**) was purified by column chromatography (10% ammonium hydroxide in MeOH) to give (**5**) as a yellow oil (71 mg, 90%). R_f one spot 0.17 (ethanol:ammonium hydroxide 4:1); ^{1}H NMR δ 3.28 (m, 4 H, CH_2NCH_2), 3.12 (br s, 4 H, 2 NH_2), 2.65 (m, 4 H, 2 NH_2CH_2), 1.95 (s, 3 H, $NCOCH_3$), 1.65 (m, 2 H, $NCH_2CH_2CH_2N$) and 1.52 (m. 4 H, $NCH_2CH_2CH_2CH_2N$) ppm, ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 170.8 (d, $COCH_3$), 49.1, 46.9, 41.3 (br), 37.5 (d), 28.6, 26.8, 22.7 (d) and 21.1 (d) ppm; MS (FAB) m/z 188.2 ($M + H^+$).

N^1,N^8-Bis(trifluoroacetyl)-N^4-benzoylspermidine (6)

To a solution of N^1,N^8-bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) (1.5 g, 3.33 mmol) in CH_3CN (4.0 mL) and triethylamine (1.0 g, 1.39 mL, 9.98 mmol) was slowly added benzoyl chloride (0.54 mL, 4.66 mmol) and the solution was stirred for 3 h. Saturated aqueous sodium bicarbonate solution (50 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a yellow oil. Compound (**6**) was purified by column chromatography (1–3.5% MeOH in CH_2Cl_2) to give (**6**) as a light-colored oil (1.39 g, 94.6%). R_f one spot 0.55 (3.5% MeOH in CH_2Cl_2); ^{1}H NMR ($CDCl_3$ containing 2 drops of d_4-MeOH) δ 7.32 (m, 5 H, C_6H_5), 3.53 (m, 4 H, CH_2NCH_2), 3.18 (m, 4 H, 2 $CF_3CONHCH_2$), 1.88 (m, 2 H, $NCH_2CH_2CH_2N$) and 1.59 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some of the carbons several peaks were seen) δ 172.5 (d, Ph*C*O), 157.7 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 157.6 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 135.7, 129.7, 128.6, 125.9, 115.8 (q, $^1J_{CF}$ = 287.1 Hz, CF_3), 48.8 (m), 45.0 (d), 41.9, 38.8 (d), 36.6 (d), 27.1 (d) and 25.0 (d) ppm; MS (FAB) m/z 441.8 ($M + H^+$).

N^4-Benzoylspermidine (7)

To a stirred solution of N^1,N^8-bis(trifluoroacetyl)-N^4-benzoylspermidine (**6**) (1.0 g, 2.26 mmol) in MeOH (20 mL) and water (1.2 mL) was added potassium carbonate (3.12 g, 25.45 mmol) and the mixture was refluxed overnight. Saturated aqueous NaCl solution (50 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give (**7**) as a light-colored oil (0.226 g, 40.2%). R_f one spot

0.43 (ethanol:ammonium hydroxide 4:1); ^{1}H NMR δ 7.84 (m, 2 H, aromatic), 7.59 (m, 3 H, aromatic), 3.57 (m, 2 H, NCH_2), 2.80 (m, 2 H, CH_2N), 2.64 (m, 4 H, 2 CH_2NH$_2$), 1.78 (m, 2 H, NCH$_2$CH_2CH$_2$N) and 1.50 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) ppm; ^{13}C NMR δ 167.0 (*C*O), 134.2, 130.7, 127.9, 126.6, 49.1, 47.4, 41.2, 38.5, 30.5, 28.0 and 26.7 ppm; MS (CI) m/z 250 (M + H$^+$).

N^1,N^8-Bis(trifluoroacetyl)-N^4-(benzyloxycarbonyl)spermidine (8)

To a solution of N^1,N^8-bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) (3.0 g, 6.65 mmol) in THF (10 mL) and triethylamine (3.7 mL, 2.69 g, 26.6 mmol) was added benzyl chloroformate (1.70 mL, 2.04 g, 12 mmol) and the solution was stirred for 12 h. The solvent was removed, the residue dissolved in CH_2Cl_2 (50 mL) and saturated aqueous NaCl solution (50 mL) was added. The mixture was extracted with CH_2Cl_2 (3 × 50 mL) and the organic layers collected, dried ($MgSO_4$) and concentrated. Compound (**8**) was purified by column chromatography (1.5–2% MeOH in CH_2Cl_2) to give (**8**) as a colorless oil (2.85 g, 91%). R_f one spot 0.44 (2% MeOH in CH_2Cl_2), ^{1}H NMR δ 8.08 (s, 1 H, N*H*), 7.32 (m, 5 H, C$_6H_5$), 5.13 (s, 2 H, CH_2C$_6$H$_5$), 3.28 (m, 8 H, 4 CH_2N), 1.75 (m, 2 H, NCH$_2$CH_2CH$_2$N), 1.55 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) ppm; ^{13}C NMR δ 157.2 (q, $^2J_{CF}$ = 36.8 Hz. CF$_3$*C*O), 157.3 (q, $^2J_{CF}$ = 36.8 Hz, CF$_3$*C*O), 136.2 (O*C*O), 128.5, 128.4, 128.2 and 127.9 (*C*$_6$H$_5$), 116.0 (q, $^1J_{CF}$ = 287.5 Hz, *C*F$_3$), 67.5, 46.4, 44.0, 39.3, 36.3, 27.1, 25.9, 25.4 ppm; MS (FAB) m/z 472.0 (M + H$^+$).

N^4-(Benzyloxycarbonyl)spermidine (9)

To N^1,N^8-bis(trifluoroacetyl)-N^4-(benzyloxycarbonyl)spermidine (**8**) (0.70 g, 1.49 mmol) was added MeOH and ammonium hydroxide (1:1, 20 mL) and the mixture was refluxed overnight. The solvent was removed and compound (**9**) was purified by column chromatography (10% ammonium hydroxide in MeOH) to give (**9**) as a light-colored oil (0.378 g, 91%). R_f one spot 0.38 (10% ammonium hydroxide in MeOH); ^{1}H NMR δ 7.33 (m, 5 H, C$_6H_5$) 5.11 (s, 2 H, C$_6$H$_5$CH_2), 3.25 (m, 4 H, CH_2NCH_2), 2.66 (m, 4 H, 2 CH_2NH$_2$), 1.66 (m, 2 H, NCH$_2$CH_2CH$_2$N), 1.60 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) and 1.37 (br s, 4 H, 2 NH_2) ppm; ^{13}C NMR δ (rotamers for this compound exist, therefore for some carbons several peaks were observed) 155. 9 (m, *C*OO), 136.6, 128.1, 127.6, 127.5, 67.4 (C$_6$H$_5$*C*H$_2$), 46.3 (br d), 44.2, 41.5, 38.9, 31.8 (br d), 30.5 and 25.3 (br d) ppm; MS (CI) m/z 280 (M + H$^+$).

N^1,N^8-Bis(trifluoroacetyl)-N^4-benzylspermidine (10)

To a solution of N^1,N^8-bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) (1.5 g, 3.33 mmol) and triethylamine (1.4 mL, 9.98 mmol) in CH_3CN (2.5 mL)

was added benzyl bromide (0.595 mL, 5.0 mmol) and the reaction was refluxed overnight. Saturated aqueous NaCl solution (40 mL) was added to the reaction and the mixture was extracted with CH_2Cl_2 (4 × 40 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a light-colored oil. Compound (**10**) was purified by column chromatography (2–3% MeOH in CH_2Cl_2) to give (**10**) as a white solid (0.81 g, 57%). R_f one spot 0.36 (3% MeOH in CH_2Cl_2); mp 63–63.5°C; ^{1}H NMR δ 8.28 (br s, 1 H, N*H*), 7.28 (m, 5 H, C_6H_5), 7.00 (br s, 1 H, N*H*), 3.54 (s, 2 H, $C_6H_5CH_2$), 3.13 (m, 4 H, 2 CONHC*H*$_2$), 2.50 (m, 4 H, C*H*$_2$NC*H*$_2$), 1.67 (m, 2 H, $NCH_2CH_2CH_2CH_2N$) and 1.57 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 157.4 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 157.2 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 138.5, 129.2, 128.4, 127.4, 116.0 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 115.9 (q, $^1J_{CF}$ = 287.6 Hz, CF_3), 59.0 ($C_6H_5CH_2$) 53.2, 52.6, 40.0, 39.6, 26.7, 24.6 and 23.9 ppm; MS (FAB) m/z 428.0 (M + H$^+$).

N^4-Benzylspermidine (11)

To a solution of N^1,N^8-bis(trifluoroacetyl)-N^4-benzylspermidine (**10**) (2.0 g, 4.67 mmol) in MeOH (40 mL) and water (2.5 mL) was added potassium carbonate (7.26 g, 52.54 mmol) and the mixture was refluxed overnight. Saturated aqueous NaCl solution (100 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 100 mL) The organic layers were collected, dried ($MgSO_4$) and concentrated to give (**11**) as a yellow oil (0.825 g, 75%). R_f one spot 0.54 (CH_2Cl_2:MeOH:ammonium hydroxide 20:9:2); ^{1}H NMR δ 7.27 (m, 5 H, C_6H_5), 3.52 (s, 2 H, $C_6H_5CH_2$), 2.67 (m, 4 H, 2 NH_2CH_2), 2.42 (m, 4 H, C*H*$_2$NC*H*$_2$) and 1.50 (m, 10 H, $NCH_2CH_2CH_2N$, $NCH_2CH_2CH_2CH_2N$ and 2 N*H*$_2$) ppm; ^{13}C NMR δ 139.8, 128.5, 127.9, 126.4, 58.5 ($C_6H_5CH_2$), 53.4, 51.1, 41.8, 40.8, 31.3, 30.7 and 24.2 ppm.

N^1,N^8-Bis(trifluoroacetyl)-N^4-(2-naphthylmethyl)spermidine (12)

To a solution of N^1,N^8-bis(trifluoroacetyl)spermidine trifluoroacetate salt (**1**) (0.70 g, 1.56 mmol) and triethylamine (0.65 mL, 4.68 mmol) in CH_3CN (5.0 mL) was added (2-bromomethyl)naphthalene (0.517 g, 2.34 mmol) and the mixture was refluxed overnight. Saturated aqueous sodium bicarbonate solution (50 mL) was added to the reaction and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a light-colored oil. Compound (**12**) was purified by column chromatography (0.5–2.5% MeOH in CH_2Cl_2) to give (**12**) as a light-colored oil (0.330 g, 44%). R_f one spot 0.18 (2.5% MeOH in CH_2Cl_2); ^{1}H NMR δ 8.23 (br s, 1 H, N*H*), 7.80 to 7.40 (m, 7 H, $C_{10}H_7$), 6.73 (br s, 1 H, N*H*), 5.29 (s, 2 H, $C_{10}H_7CH_2$), 3.33 (m, 4 H,

2 CONHCH_2), 2.55 (m, 4 H CH_2NCH_2), 1.70 (m, 2 H, NCH$_2$CH_2CH$_2$N) and 1.59 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) ppm; ^{13}C NMR δ 157.3 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO). 156.9 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 135.6, 133.2, 132.8, 128.3, 127.9, 127.7, 127.6, 127.0, 126.3, 125.9, 116.0 (q, $^1J_{CF}$ = 287.7 Hz, CF_3), 115.8 (q, $^1J_{CF}$ = 287.7 Hz, CF_3), 59.3 (C$_{10}$H$_7$CH$_2$), 53.3, 52.8, 40.0, 39.6, 26.8, 24.8 and 23.9 ppm; MS (FAB) m/z 478.2 (M + H$^+$).

N^4-(2-Naphthylmethyl)spermidine (13)

To N^1,N^8-bis(trifluoroacetyl)-N^4-(2-naphthylmethyl)spermidine (**12**) (0.180 g, 0.31 mmol) was added MeOH and ammonium hydroxide (1:1, 25 mL) and the mixture was refluxed overnight. The solvent was then removed and (**13**) was purified by column chromatography (10% ammonium hydroxide in MeOH) to give (**13**) as a light-colored oil (92 mg, 85.5%). R_f one spot 0.12 (10% ammonium hydroxide in MeOH); ^{1}H NMR δ 7.77 to 7.38 (m, 7 H, C$_{10}H_7$), 3.67 (s, 2 H, C$_{10}$H$_7$CH_2), 2.66 (m, 4 H, 2 NH$_2$CH_2), 2.47 (m, 4 H, CH_2NCH_2), 1.63 (m, 2 H, NH$_2$CH$_2$CH_2CH$_2$N), 1.60 to 1.36 (m, 8 H, NCH$_2$CH_2CH_2CH$_2$N and 2 NH_2) ppm; ^{13}C NMR δ 137.6, 133.2, 132.5, 127.6, 127.5, 127.4, 127.0, 126.9, 125.7, 125.2, 58.8 (C$_{10}$H$_7$CH$_2$), 53.6, 51.3, 42.0, 40.3, 31.5, 30.8 and 24.3 ppm; MS (FAB) m/z 286.2 (M + H$^+$).

N^1,N^8-Bis(acetyl)-N^4-(benzyloxycarbonyl)spermidine (14)

To N^4-(benzyloxycarbonyl)spermidine (**9**) (0.570 g, 2.04 mmol), triethylamine (0.854 mL, 6.13 mmol) and CH_2Cl_2 (10 mL) was added acetyl chloride (0.385 g, 4.9 mmol). After 12 h at room temperature, saturated aqueous NaCl (40 mL) and 1.0 M hydrochloric acid (10 mL) were added. The mixture was extracted with CH_2Cl_2 (3×40 mL) and the organic layers dried ($MgSO_4$). The solvent was removed and (**14**) was purified by column chromatography (3–5% MeOH in CH_2Cl_2) to give (**14**) as a colorless oil (0.628 g, 85%). R_f one spot 0.34 (10% MeOH in CH_2Cl_2); ^{1}H NMR δ 7.35 (s, 5 H, C$_6H_5$), 6.80 (s, 1 H, NH), 6.10 (s, 1 H, NH), 5.12 (s, 2 H, CH_2C$_6$H$_5$), 3.23 (m, 8 H, 4 CH_2N), 1.96 (s, 3 H, CH_3), 1.93 (s, 3 H, CH_3), 1.69 (m, 2 H, NCH$_2$CH_2CH$_2$N), 1.50 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 170.2, 156.8, 156.7, 136.5, 128.4, 128.0, 127.6, 67.1, 46.4 (d), 44.2, 38.7, 35.9 (d), 27.5 (d), 26.7 (d), 25.7 (d) and 23.0 ppm.

N^1,N^8-Bis(acetyl)spermidine (15)

To a solution of N^1,N^8-bis(acetyl)-N^4-(benzyloxycarbonyl)spermidine (**14**) (0.558 g, 1.54 mmol) in ethanol (50 mL) was added 5% palladium on charcoal

(90 mg) and the suspension was shaken under H_2 (60 psi) for 24 h. The suspension was filtered and solvent removed to give (**15**) as a white solid (0.30 g, 85%). R_f one spot 0.49 (4:1 ethanol:ammonium hydroxide); mp 76–77°C; ^{1}H NMR δ 6.83 (br s, 1 H, N*H*), 6.31 (br s, 1 H, N*H*), 3.29 (m, 4 H 2 CH_2NHCO), 2.81 (s, 1 H, N*H*) 2.69 (m, 4 H, CH_2NHCH_2), 1.97 (s, 6 H, 2 CH_3), 1.71 (m, 2 H, NCH$_2$CH_2CH$_2$N), 1.57 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) ppm; ^{13}C NMR δ 170.4, 170.2, 49.0, 47.5, 39.2, 38.2, 28.7, 27.2, 26.8, 23.22 and 23.20 ppm.

N^1,N^8-Bis(benzoyl)-N^4-(t-butoxycarbonyl)spermidine (16)

To a solution of N^4-(*t*-butoxycarbonyl)spermidine (**3**) (0.25 g, 1.02 mmol) in CH_3CN (3 mL) and triethylamine (1.5 mL, 10.8 mmol) was added benzoyl chloride (0.358 g, 2.55 mmol). The reaction was stirred for 45 min then saturated aqueous sodium bicarbonate solution (50 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a yellow oil. Compound (**16**) was purified by column chromatography (1–3% MeOH in CH_2Cl_2) to give (**16**) as a colorless oil (0.396 g, 86%). R_f one spot 0.32 (3% MeOH in CH_2Cl_2); ^{1}H NMR δ 7.80 (m, 4 H, aromatic), 7.43 (m, 6 H, aromatic), 3.46 (m, 4 H, 2 CONHCH_2), 3.33 (m, 2 H, CH_2N), 3.20 (m, 2 H, CH_2N), 1.75 (m, 2 H, NCH$_2$CH_2CH$_2$N), 1.61 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) and 1.45 (s, 9 H, OC(CH_3)$_3$) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons broad signals were observed) δ 167.6, 134.6, 131.2 (d), 128.4 (d), 126.8 (d), 79.7 (O*C*(CH$_3$)$_3$), 46.6, 43.5 (br), 39.5, 36.0 (br), 29.5 (OC(*C*H$_3$)$_3$), 27.6 (br), 26.9 (br) and 25.8 (br); MS (CI) m/z 454 (M + H$^+$).

N^1,N^8-Bis(benzoyl)spermidine (17)

To N^1,N^8-bis(benzoyl)-N^4-(*t*-butoxycarbonyl)spermidine (**16**) (0.80 g, 1.762 mmol) was added trifluoroacetic acid (12 mL). The solution was stirred for 3 h and the solvent was removed. The residue was dissolved in CH_2Cl_2 (10 mL). Saturated aqueous sodium bicarbonate solution (50 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a yellow solid. The solid was recrystallized (ethanol/water) to give (**17**) as a white solid (0.420 g, 67.5%). R_f one spot 0.77 (7.5% ammonium hydroxide in ethanol); mp 124.5–125.5°C (lit.[21] 129.5–130.5°C); ^{1}H NMR δ 7.79 (m, 4 H, aromatic), 7.41 (m, 6 H, aromatic), 3.89 (br s, 3 H, 3 N*H*), 3.41 (m, 4 H, 2 CONHCH_2), 2.65 (m, 4 H, CH_2NHCH_2), 1.78 (m, 2 H, NCH$_2$CH_2CH$_2$N) and 1.61 (m, 4 H, NCH$_2$CH_2CH_2CH$_2$N) ppm; ^{13}C NMR δ 168.26, 168.25, 134.1, 131.2, 131.1, 128.2, 128.15, 126.74, 126.71, 48.8, 46.8, 39.4, 37.8, 28.5, 26.7 and 26.6 ppm; MS (CI) m/z 354 (M + H$^+$).

N^1,N^8-Bis(benzyloxycarbonyl)-N^4-(t-butoxycarbonyl)spermidine (18)

To a solution of N^4-(*t*-butoxycarbonyl)spermidine (**3**) (1.50 g, 6.1 mmol) in THF (25 mL) were added a solution of potassium carbonate (8.42 g, 61 mmol) dissolved in water (15 mL) and benzyl chloroformate (2.60 g, 15.25 mmol). The reaction was stirred under N_2 for 1 h. Saturated aqueous NaCl solution (50 mL) was added to the reaction and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and (**18**) was purified by column chromatography (0.5–2% MeOH in CH_2Cl_2) to give (**18**) as a white solid (2.96 g, 94.6%). R_f one spot 0.45 (2% MeOH in CH_2Cl_2); mp 101–102°C; ^{1}H NMR δ 7.32 (s, 10 H, 2 C_6H_5), 5.08 (s, 4 H, 2 $C_6H_5CH_2$), 3.16 (m, 8 H, 4 NCH_2) and 1.64 (m, 2 H, $NCH_2CH_2CH_2N$), 1.51 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) and 1.45 (s, 9 H, $OC(CH_3)_3$) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons broad peaks were observed) δ 156.4, 136.6, 136.5, 128.3, 127.8, 79.6 ($OC(CH_3)_3$), 66.5 ($C_6H_5CH_2$), 66.4 ($C_6H_5CH_2$), 46.4, 43.5 (br), 40.5, 39.2, 37.6 (br), 28.3 ($C(CH_3)_3$), 27.2 and 25.5 (br) ppm; MS (FAB) m/z 514.0 ($M + H^+$).

N^1,N^8-Bis(benzyloxycarbonyl)spermidine (19)

To N^1,N^8-bis(benzyloxycarbonyl)-N^4-(*t*-butoxycarbonyl)spermidine (**18**)(0.500g, 0.975 mmol) was added trifluoroacetic acid (7 mL). The mixture was stirred at 0°C for 30 min and then the solvent was removed, and the residue dissolved in CH_2Cl_2 (10 mL). Potassium hydroxide solution (0.01 M, 50 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a solid. The solid was recrystallized (ethanol/water) to give (**19**) as a white solid (0.210 g, 52%). R_f one spot 0.51 (10% MeOH and 2% ammonium hydroxide in CH_2Cl_2); mp 100–101°C (lit.[22] 104.5–105°C); ^{1}H NMR δ 7.33 (m, 10 H, 2 C_6H_5), 5.07 (s, 4 H, 2 $C_6H_5CH_2$), 3.20 (m, 4 H, 2 $CONHCH_2$), 2.61 (m, 4 H, 2 CH_2NH), 2.38 (br s, 2 H, 2 NH), 1.67 (m, 2 H, $NCH_2CH_2CH_2N$) and 1.50 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 156.6, 136.6, 128.3, 127.9, 127.8, 66.55, 66.49, 49.1, 47.1, 40.6, 39.2, 29.4, 27.5 and 26.9 ppm.

N^1,N^8-Bis(2-naphthylmethyl)-N^4-(t-butoxycarbonyl)spermidine (20)

A solution of N^4-(*t*-butoxycarbonyl)spermidine (**3**) (0.60 g, 2.45 mmol in CH_3CN (15 mL) was dried over 3 Å molecular sieves. The resulting solution was added to 2-naphthaldehyde (1.53 g, 9.8 mmol) and stirred under N_2 for 3 h. The solvent was removed and ethanol (5 mL) was added followed by sodium borohydride (0.930 g, 24.6 mmol). The suspension was stirred for 0.5 h, then water (1 mL) was slowly

added. Saturated aqueous sodium bicarbonate solution (50 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a colorless oil. Compound (**20**) was purified by column chromatography (3–7% MeOH in CH_2Cl_2 containing 0.5% ammonium hydroxide) to give (**20**) as a light colored oil (0.686 g, 53%). R_f one spot 0.12 (5% MeOH and 0.5% ammonium hydroxide in CH_2Cl_2); ^{1}H NMR δ 7.72 (m, 8 H, aromatic), 7.43 (m, 6 H, aromatic), 3.82 (s, 4 H, 2 $C_{10}H_7CH_2$), 3.14 (m, 4 H, CH_2NCH_2), 2.55 (m, 4 H, 2 $NHCH_2$), 1.69 (m, 2 H, $NCH_2CH_2CH_2N$), 1.50 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) and 1.39 (s, 9 H, $C(CH_3)_3$) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons broad peaks were observed) δ 155.2 (*C*O), 137.46, 137.41, 133.0, 132.2, 127.6, 127.3, 127.2, 126.2, 126.0, 126.09, 125.5, 125.1, 78.6 ($C(CH_3)_3$), 53.6, 53.5, 53.1, 48.6, 46.4, 46.0 (br d), 44.5 (br d), 28.0 (CH_3), 26.9, 25.9 (br d) ppm.

N^1,N^8-Bis(2-naphthylmethyl)spermidine Trifluoroacetate Salt (21)

To N^1,N^8-bis(2-naphthylmethyl)-N^4-(*t*-butoxycarbonyl)spermidine (**20**) (0.30 g, 0.571 mmol) was added trifluoroacetic acid (2 mL). The mixture was stirred for 3 h and the solvent was removed to give (**21**) as a white solid (0.388 g, 88%). R_f one spot 0.19 (5% ammonium hydroxide in MeOH); mp 221–222°C (dec); ^{1}H NMR (d_6-DMSO) δ 9.28 and 9.23 (br s, 4 H, 2 NH_2^+), 8.9 (br s, 2 H, NH_2^+), 8.01 (m, 8 H, aromatic H), 7.58 (m, 6 H, aromatic H), 4.34 (s, 4 H, 2 $C_{10}H_7CH_2$), 3.01 (m, 8 H, 4 $CH_2NH_2^+$), 2.04 (m, 2 H, $NCH_2CH_2CH_2N$) and 1.69 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR (d_6-DMSO) δ 158.7 (q, $^2J_{CF}$ = 34.0 Hz, CF_3CO), 158.6 (q, $^2J_{CF}$ = 34.0 Hz, CF_3CO), 132.90, 132.87, 132.6, 129.5, 129.4, 129.3, 128.4, 127.8, 127.7, 127.1, 126.9, 126.7, 117.1 (q, $^1J_{CF}$ = 297.3 Hz, CF_3CO), 50.3, 50.2, 46.2, 46.0, 43.9, 43.8, 22.7, 22.6 and 22.4 ppm.

Enzyme Studies

T. cruzi TR was purified following the method of Walsh *et al.*[23] from *E. coli* SG5, a glutathione reductase deletion mutant, containing the TR expression vector pIBITczTR described by Sullivan and Walsh.[24] Prepared compounds were assayed for their effects on the rate of reduction of trypanothione by *T. cruzi* TR spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.[25] Stock solutions of the compounds were prepared in either HEPES buffer (100 mM, pH 7.25) or ethanol. The maximum amount of ethanol added to the 1.0 mL enzyme assays did not exceed 40 μL, and this quantity of ethanol did not inhibit TR activity

in control assays. TR activity was measured at 23°C in HEPES buffer (100 mM, pH 7.25) containing EDTA (1 mM), NADPH (0.18 mM), oxidized trypanothione and TR at 1.22 μg/mL. An estimate of the K_i value for each inhibitor was obtained from initial assays. More accurate K_i values were obtained in subsequent experiments in which four inhibitor concentrations (ranging from 0.5 to 3.0 times the estimated K_i) were assayed for their effects on TR activity in the presence of varying concentrations of trypanothione (four concentrations ranging from 14.8 to 74 μM, or 22.2 to 128 μM). Compounds (**1**), (**3**), (**5**), (**7**), (**15**) and (**17**) were either extremely weak inhibitors, or had no inhibitory effects on TR activity. For these compounds, control assays were conducted using two concentrations of trypanothione (ranging from 22.2 to 128 μM) and three concentrations of either (**1**), (**3**), (**5**), (**7**), (**15**) or (**17**) (ranging from 700 to 3000 μM).

The effect of compound (**21**) on the rate of reduction of glutathione by yeast glutathione reductase (GR) (EC 1.6.4.2) was assayed spectrophotometrically by monitoring the oxidation of NADPH at 340 nm. GR activity was measured at 23°C in HEPES buffer (100 mM, pH 7.25) containing EDTA (1 mM), NADPH (0.18 mM) and oxidized glutathione (14.3, 28.6, 42.9 or 57.2 μM) with an enzyme concentration of 0.27 μg/mL. The effects of (**21**) on GR activity were measured at two oxidized glutathione concentrations (14.3 and 57.2 μM) using two concentrations of (**21**) (360 and 520 μM).

For each inhibitor, the inhibition type was assessed by the patterns of three classes of plots: $1/v$ against $1/[S_o]$ for various $[I]$; $1/v$ against $[I]$ for various $[S_o]$; and $[S_o]/v$ against $[I]$ at various $[S_o]$. All of the inhibitors exhibited linear competitive inhibition of TR reduction of trypanothione. For each inhibitor concentration $K_{m(\mathrm{obs})}$ and $V_{\max}$ were determined from a least-squares linear regression analysis of the plot of $1/v$ against $1/[S_o]$. (The correlation confidence value, R, of all lines was greater than 0.93). K_i values were determined for each inhibitor concentration using the equation:

$$K_i = \frac{[I]}{\{(V_{\max} K_{m(\mathrm{obs})})/(V_{\max(\mathrm{obs})} K_m)\} - 1}$$

The mean K_i value for each compound was calculated from the K_i values obtained at four different inhibitor concentrations.

RESULTS AND DISCUSSION

Synthetic strategies leading to N^4- and N^1,N^8-bis-substituted spermidine derivatives are outlined in Figures 1 and 2, respectively. Previously, we have reported

TABLE I Mean K_i values ($n = 4$) for the competitive inhibition by N^4-substituted spermidine derivatives of trypanothione reduction by recombinant *T. cruzi* TR.

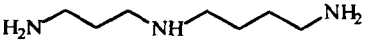
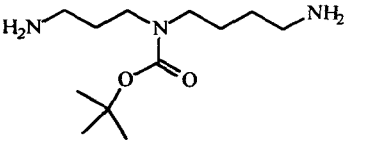
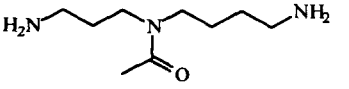
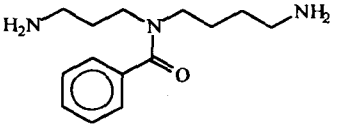
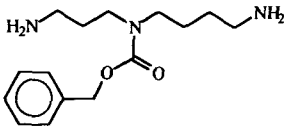
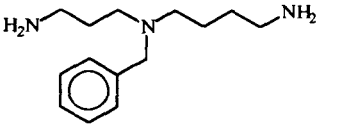
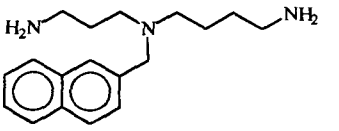
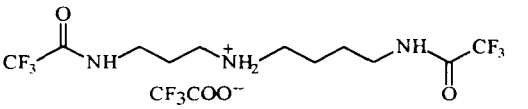
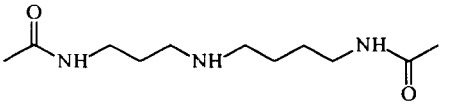
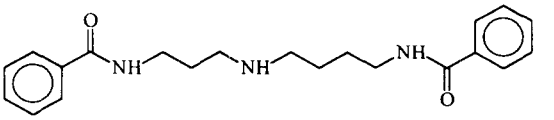
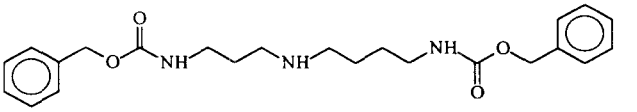
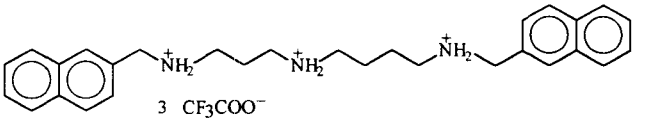
Compound		K_i (μM)±*SD*
		> 2,000
(3)		> 2,000
(5)		> 2,000
(7)		> 2,000
(9)		280 ± 17
(11)		185 ± 32
(13)		108 ± 7

TABLE II Mean K_i values ($n = 4$) for the competitive inhibition by N^1,N^8-bis-substituted spermidine derivatives of trypanothione reduction by recombinant *T. cruzi* TR.

Compound	*K_i (μM)±SD*
(1) 	> 2,000
(15) 	> 2,000
(17) 	> 2,000
(19) 	311 ± 31
(21) 	9.5 ± 2.1

conditions that allow the selective trifluoroacetylation of the primary amino groups of spermidine.[20] This is a critical step in the syntheses of N^4-substituted spermidines.

Several synthetically readily available spermidine derivatives have been prepared. Five of these compounds are effective competitive inhibitors of TR activity (see Tables I and II). None of the tested compounds resulted in the TR mediated oxidation of NADPH in the absence of trypanothione. Thus, as expected, compounds tested were not TR substrates.

The results of these studies indicate the relative importance of specific structural features required for spermidine derivatives to be inhibitors of TR. A feature

common to all the inhibiting compounds studied is the presence of a hydrophobic aromatic moiety. The most potent inhibitors in this study contained naphthyl substituents. Compounds with benzyl substituents were discovered to be less effective inhibitors.

N^4-(2-naphthylmethyl)spermidine (**13**) is the most effective TR inhibitor of all the N^4-substituted spermidines investigated. The relative importance of the size of the hydrophobic aromatic substituent for the binding of spermidine derivatives to TR is indicated by comparing the K_i values of (**13**) (108 μM) with N^4-benzylspermidine (**11**) (185 μM). Spermidine is not an inhibitor of TR, therefore the aromatic substituents of (**11**) and (**13**) are crucial to their inhibitory effects. Crystal structures of TR indicate that regions of the active site are lined with hydrophobic amino acid residues[13,14,26] which may interact with the aromatic moieties of (**11**) and (**13**).

Although N^4-benzylspermidine (**11**) is an inhibitor of TR, N^4-benzoylspermidine (**7**) does not inhibit TR. The differential inhibitory effects of (**11**) and (**7**) may be due to differences in their protonation states. At physiological pH amino groups are protonated; consequently, compound (**11**) will have a 3+ charge, whereas (**7**) will have a 2+ charge. Additionally, the presence of an amide group in (**7**) results in a loss of conformational flexibility due to hindered rotation about the carbonyl-N bond, a feature not present in (**11**). Thus (**7**) may not be able to adopt a conformation which allows for effective binding to the active site. A more conformationally flexible analog of (**7**), N^4-(benzyloxycarbonyl)spermidine (**9**), does show inhibitory activity, consistent with the idea that the presence of the amide group in (**7**) negatively impacts binding of this spermidine derivative in the active site.

The most potent TR inhibitor developed in this study was N^1,N^8-bis(2-naphthylmethyl)spermidine (**21**). Compound (**21**) inhibits TR with a potency of similar magnitude to the most effective competitive inhibitor described previously (clomipramine with a K_i of 6.5 μM).[16] Since the bis-naphthyl spermidine derivative (**21**) is a significantly more effective inhibitor than the mono-naphthyl derivative (**13**), additional hydrophobic interactions may occur between (**21**) and the active site of TR enabling (**21**) to bind more effectively to TR than (**13**).

Other bis-substituted spermidines investigated in this study were N^1,N^8-acylated compounds, one of which (**19**) was a poor inhibitor while the others showed no inhibitory effects. N^1,N^8-bis-acylated spermidines have a 1+ charge at physiological pH whereas bis-alkylated spermidines like (**21**) have a 3+ charge. This charge difference may account for the difference in the K_i values observed for these compounds.

In conclusion, certain spermidine derivatives with hydrophobic aromatic substituents are effective competitive inhibitors of *T. cruzi* TR. The most potent inhibitor developed in this study was N^1,N^8-bis(2-naphthylmethyl)spermidine

(**21**). The mechanism of TR reduction of trypanothione is essentially identical to the mechanism for reduction of glutathione by glutathione reductase[27] and these enzymes also have structural similarities.[11,13–15] However, (**21**) did not inhibit the reduction of glutathione by yeast glutathione reductase, indicating that with respect to glutathione reductase, (**21**) is a specific inhibitor of TR. Previous work has indicated that certain amines containing hydrophobic groups are inhibitors of TR,[16,17] however, these compounds are either not as effective as (**21**) and/or are more complex to synthesize. The results presented in this paper indicate that certain readily available spermidine derivatives are inhibitors of TR. Such compounds could be modified to contain chemically reactive groups leading to compounds that may be irreversible inhibitors of TR. Since trypanosome infections occur predominantly in lower socio-economic populations, new chemotherapeutics to combat these infections should be inexpensive. Spermidine derivatives such as (**21**) are easy to prepare and are inexpensive; therefore, they may provide a new direction for the development of affordable antitrypanosomal agents.

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