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Polyamine Derivatives as Inhibitors of Trypanothione Reductase and Assessment of their Trypanocidal Activities

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Abstract—Trypanothione reductase (TR) occurs exclusively in trypanosomes and leishmania, which are the etiological agents of many diseases. TR plays a vital role in the antioxidant defenses of these parasites and inhibitors of TR have potential as antitrypanosomal agents. We describe the syntheses of several spermine and spermidine derivatives and the inhibiting effects of these compounds on *T. cruzi* TR. All of the inhibiting compounds displayed competitive inhibition of TR-mediated reduction of trypanothione disulfide. The three most effective compounds studied were N^4,N^8-bis(3-phenylpropyl)spermine (**12**), N^4,N^8-bis(2-naphthylmethyl)spermine (**14**), and N^1,N^8-bis(2-naphthylmethyl)spermidine (**21**), with K_i values of 3.5, 5.5 and 9.5 μM, respectively. Compounds **12**, **14**, and **21** were found to be potent trypanocides in vitro with IC_{50} values ranging from 0.19 to 0.83 μM against four *T. brucei* ssp. strains. However, these compounds did not prolong the lives of mice infected with trypanosomes. This work indicates that certain polyamine derivatives which target a unique pathway in *Trypanosomatidae* have potential as antitrypanosomal agents.

Introduction

An important component of the antioxidative defenses of trypanosomes and leishmania is the glutathione-spermidine conjugate, trypanothione.[1,2] Organisms of the family *Trypanosomatidae* are responsible for many human diseases including African sleeping sickness (*Trypanosoma brucei rhodesiense* and *T. b. gambiense*), Chagas' disease (*T. cruzi*) and kala-azar (*Leishmania donovani*). Other *Trypanosomatidae* species infect livestock and cause diseases such as Nagana cattle disease (*T. b. brucei* and *T. congolense*). Both human and livestock diseases have a dramatic impact on the social and economic conditions in many countries. For example, 16–18 million people are infected with Chagas' disease which occurs predominantly in Central and South America.[3] Current treatment of Chagas' disease and other trypanosome infections is difficult and often ineffectual in controlling the chronic phases of these diseases[4] therefore, more effective antitrypanosomal drugs are urgently needed.

In most organisms, glutathione is an important antioxidant and levels of this thiol are maintained by the action of glutathione reductase (GR).[5] However, *Trypanosomatidae* do not contain GR, instead they have a unique pathway[6,7] in which trypanothione can reduce glutathione disulfide by a nonenzymatic,[8] or possibly enzyme mediated,[9] thiol exchange reaction. Trypanothione disulfide (N^1,N^8-bis(glutathionyl)spermidine) is reduced by the activity of trypanothione reductase (TR) (EC 1.6.4.8.). TR is a NADPH-dependent homodimeric flavoprotein that reduces the disulfide group of trypanothione disulfide.[7,10] TR and GR have structural similarities and operate by an essentially identical mechanism.[11] However, these enzymes have different substrate specificities, TR does not reduce glutathione disulfide and GR does not reduce trypanothione disulfide.[10] Since the antioxidant defenses of trypanosomes are based on the activity of TR, inhibitors of TR are potential antitrypanosomal agents.[1]

Several structurally diverse inhibitors and substrates of TR have been developed,[10,12,13] however, the most effective inhibitors are amines that contain hydrophobic groups. Recently, certain polyamine derivatives that are potent inhibitors of TR have been described.[14–17] We were interested in developing competitive inhibitors of *T. cruzi* TR that are synthetically readily available and decided to focus on derivatives of spermidine and spermine. We have described some of our initial results concerning the inhibiting effects of certain polyamine derivatives on TR.[14] In this paper we give a full account of the syntheses of several spermine derivatives and the evaluation of the effects of these compounds on *T. cruzi* TR. Additionally, we describe the activities of three of our most potent TR inhibitors against trypanosomes in vitro and in vivo.

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Results

The synthetic strategies used to prepare the N^4,N^8- and N^1,N^{12}-bis-substituted spermine derivatives are shown in Figures 1 and 2, respectively. Previously we reported conditions that allow the selective trifluoroacetylation of the primary amino groups of spermine (**1**) to give N^1,N^{12}-bis(trifluoroacetyl)spermine (**2**) isolated as the trifluoroacetate salt. This reaction was an important initial step in the syntheses described in this paper.[18] The secondary amino groups of compound **2** were then reacted, in the presence of base, with one of either di-*t*-butyl dicarbonate, benzyl chloroformate, benzyl bromide, 1-bromo-3-phenylpropane or 2-(bromomethyl)-naphthalene. The incomplete reaction of benzyl bromide with compound **2** enabled the isolation of both the N^4-mono- and N^4,N^8-bis-benzyl derivatives **7** and **9**. The trifluoroacetyl groups of the resulting compounds were then removed by refluxing in a solution of methanol and ammonium hydroxide to give N^4-benzylspermine (**8**) and N^4,N^8-bis-substituted spermine derivatives.

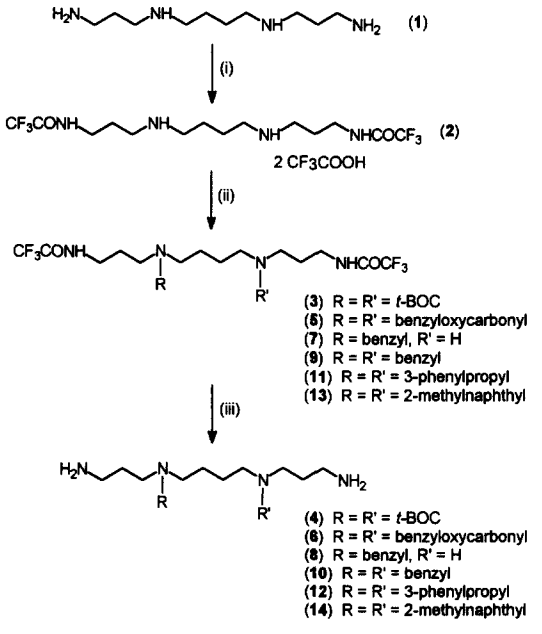
N^1,N^{12}-bis(phenylacetyl)spermine (**16**) was prepared from N^4,N^8-bis(benzyloxycarbonyl)spermine (**6**) by reaction with phenylacetyl chloride in the presence of base to give compound **15**. The benzyloxycarbonyl groups of **15** were then removed by hydrogenolysis in the presence of a catalytic amount of palladium on charcoal to give **16**. N^1,N^{12}-bis(benzyl)spermine (**18**) was also prepared from **6** by reaction with benzaldehyde followed by reduction of the resulting imine groups with sodium borohydride to give compound **17**. The benzyloxycarbonyl groups were then removed to give compound **18** (Fig. 2).

N^1,N^8-bis(benzyl)spermidine (**20**) was prepared from N^4-(*t*-butoxycarbonyl)spermidine[15] by reaction with benzaldehyde followed by reduction of the imine groups with sodium borohydride and removal of the *t*-butoxycarbonyl group by treatment with trifluoroacetic acid. The synthesis of N^1,N^8-bis(2-naphthylmethyl)-spermidine (**21**) has been previously described.[15]

The inhibiting effects of the prepared polyamine derivatives on *T. cruzi* TR were measured using a standard photometric assay.[19] The TR used in these assays was isolated from *E. coli* SG5, a glutathione reductase deletion mutant, containing the TR expression vector pIBITczTR.[20] 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 the reduction of trypanothione disulfide by TR. For each inhibitor concentration $K_{m(obs)}$ 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:

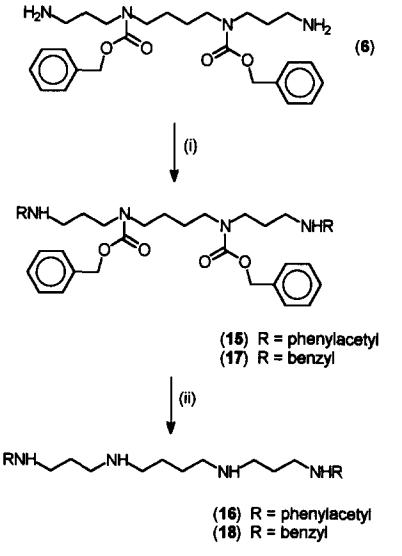
$$K_i = \frac{[I]}{\{(V_{max}K_{m(obs)})/(V_{max(obs)}K_m)\} - 1}$$

The mean K_i value for each compound was calculated



(i) CF_3COOEt, H_2O, CH_3CN, reflux.[18] (ii) for preparation of (**3**) di-*t*-butyl dicarbonate, triethylamine, THF; for preparation of (**5**) benzyl chloroformate, K_2CO_3, THF, H_2O; for preapration of (**7**) and (**9**) benzyl bromide, triethylamine, CH_3CN; for preparation of (**11**) 1-bromo-3-phenylpropane, triethylamine, CH_3CN; for preparation of (**13**) 2-(bromomethyl)naphthalene, triethylamine, CH_3CN. (iii) MeOH, ammonium hydroxide.

Figure 1. Synthesis of N^4,N^8-bis-substituted spermine derivatives.



(i) for preparation of (**15**) phenylacetyl chloride, triethylamine, THF; for preparation of (**17**) benzaldehyde in CH_3CN followed by $NaBH_4$ in EtOH. (ii) H_2, Pd on C, EtOH or MeOH.

Figure 2. Synthesis of N^1,N^{12}-bis-substituted spermine derivatives.

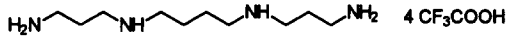
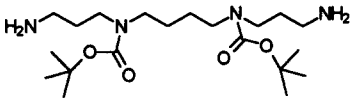
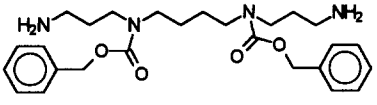
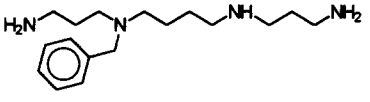
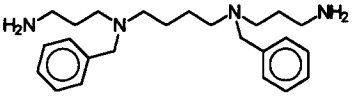
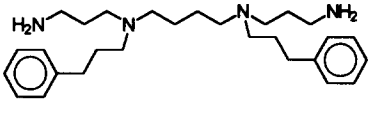
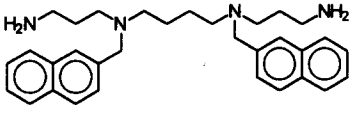
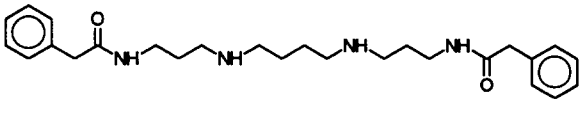
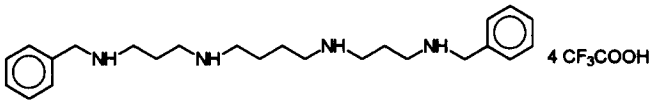
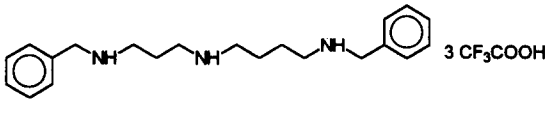
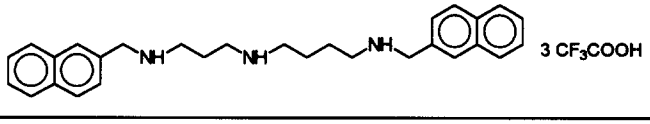
from the K_i values obtained at a minimum of four different inhibitor concentrations. The results of these measurements are presented in Table 1.

None of the compounds tested in this study resulted in the TR mediated oxidation of NADPH in the absence of trypanothione disulfide. Therefore, as expected, compounds were not TR substrates.

The relative specificity of the most potent of these compounds as inhibitors of TR was explored by investigating the effects of compounds **10**, **12**, **14**, and **21** on the reduction of glutathione disulfide by yeast GR (EC 1.6.4.2). The presence of these compounds (at concentrations of 0.255 mM or greater) did not decrease the rate of glutathione disulfide reduction by yeast GR.

The trypanocidal activities of the most effective TR inhibitors in this study, N^4,N^8-bis(3-phenylpropyl)-spermine (**12**), N^4,N^8-bis(2-naphthylmethyl)spermine (**14**), and N^1,N^8-bis(2-naphthylmethyl)spermidine (**21**), were investigated. Initially the effects of the trifluoro-acetate salts of these compounds on bloodstream forms of clinically isolated strains of *T. brucei* ssp. were examined in vitro using a standard growth screen.[21] The

Table 1. Mean K_i values for the competitive inhibition by spermidine and spermine derivatives of trypanothione disulfide reduction by recombinant TR from *T. cruzi*

	Compound	K_i (μM) ± SD
1		>2000
4		>2000
6		81 ± 7.5
8		115 ± 10
10		19 ± 4.8
12		3.5 ± 0.4
14		5.5 ± 0.2
16		114 ± 16
18		153 ± 1.7
20		326 ± 33
21		9.5 ± 2.1

trypanosome strains used were *T. b. brucei* Lab 110 EATRO and three clinical isolates of *T. b. rhodesiense*. In addition, to serve as a control compound for these studies, the effects of the trifluoroacetate salt of spermine (**1**) on these strains was also investigated. The results of these studies are shown in Table 2. Compounds **12**, **14**, and **21** were effective trypanocides with IC_{50} values ranging from 0.19 to 0.83 μM for all of the four strains studied, whereas, the trifluoroacetate salt of spermine (**1**) showed no trypanocidal activity at concentrations below 100 μM.

Compounds **12**, **14**, and **21** were then administered to mice infected with *T. b. brucei* LAB 110 EATRO. The compounds were administered using intraperitoneal, subcutaneous and mini-osmotic pump delivery systems. Groups of five mice were infected with 2.5×10^5 trypanosomes and the infection was allowed to progress 24 h before treatment was begun. Animals were initially dosed with 1.0, 5.0, 10, and 25 mg of compound per kg body weight intraperitoneally once daily for three days. Survival times were compared to infected, untreated controls. In a second experiment, compounds were given in Alza® mini-osmotic pumps that release 1.0 μL/h continuously for three days. The doses given in this experiment were 10 and 25 mg/kg/day. In a third experiment, the compounds were administered subcutaneously at 25 mg/kg once daily for three days. In all of the experimental groups treated with compounds **12**, **14**, or **21**, there was no prolongation of life beyond the survival time of the control animals.

The bloodstream parasitemia of animals treated with compounds and untreated animals was also measured using hemocytometer counts of tail vein blood. There was no significant reduction of parasitemia in the animals treated with compounds **12**, **14**, or **21**. However, none of the compounds tested exhibited any overt toxicity.

Discussion

In this study, the inhibiting effects of a series of spermine and some spermidine derivatives on the TR mediated reduction of trypanothione disulfide were investigated. The results obtained indicate some of the specific structural features required for polyamine derivatives to be competitive inhibitors of TR. All of the spermine derivatives described in this study are significantly more effective inhibitors than the corresponding spermidine compounds described in a previous study.[15] For example, the K_i value of N^4,N^8-bis(benzyloxycarbonyl)spermine (**6**) is 81 μM, whereas that of N^4-(benzyloxycarbonyl)spermidine is 280 μM, and the K_i values of compound **14** and N^4-(2-naphthylmethyl)spermidine are 5.5 and 108 μM, respectively.[15]

The difference in inhibitory activities of the spermine and corresponding spermidine analogues may be due to the differences in the number of hydrophobic substituents and/or differences in charge. All compounds that bind to the active site of TR contain hydrophobic groups. Thus, although several spermine and spermidine derivatives containing aromatic substituents are TR inhibitors,[14–17] neither spermine nor spermidine are inhibitors. The hydrophobic moieties of inhibitors presumably interact with hydrophobic regions of the active site of TR.[11] This type of interaction is shown in the crystal structure of the TR-mepacrine complex in which the acridine ring of mepacrine is located close to a hydrophobic wall in the active site.[22] The active site of TR also contains glutamate residues which interact with cationic moieties of bound compounds such as amino groups, that are protonated at physiological pH. Indeed, the carboxylate residue of E18 of *T. congolense* TR is vital for substrate recognition, presumably by interacting with the protonated N^4-amino group of the spermidine moiety of trypanothione.[23] (However, the crystal structure of the TR-N^1-glutathionylspermidine complex shows that E18 interacts with one of the N^1-nitrogen atoms via hydrogen bonding.)[27] Also, modelling studies suggest that the exocyclic nitrogen of clomipramine, a potent competitive inhibitor, interacts with E467′ at the active site of *T. cruzi* TR.[13] The importance of charge in determining which compounds bind to the active site of TR has recently been discussed by Faerman et al.[24] The results presented in our study, indicate that the effectiveness of TR inhibitors is related to the charge of the inhibiting compound, as well as the location, quantity and size of hydrophobic, aromatic substituents.

Table 2. Trypanocidal activities of compounds against four *T. brucei* ssp. strains in vitro

	IC_{50} (μM)			
Compound[a]	**Lab 110**[b]	**K 243**[c]	**K 269**[c]	**K 243-As-10-3**[d]
1	>100	>100	>100	>100
12	0.66	0.79	0.58	0.66
14	0.82	0.83	0.58	0.58
21	0.63	0.23	0.19	0.61

[a]The trifluoroacetate salts of all the compounds were used.
[b]*T. b. brucei* Lab 110 is a drug sensitive strain.
[c]KETRI 243 and KETRI 269 are uncloned clinical isolates of *T. b. rhodesiense*.
[d]KETRI 243-As-10-3 is a pentamidine and melarsoprol resistant clone of a clinical *T. b. rhodesiense* isolate.

Of the spermine derivatives investigated, the most effective inhibitors are N^4,N^8-bis(3-phenylpropyl)-spermine (**12**) and N^4,N^8-bis(2-naphthylmethyl)-spermine (**14**). N^4,N^8-bis(benzyl)spermine (**10**) is a less effective inhibitor than **12** or **14** possibly due to the aromatic moieties in **10** being located closer to the secondary amino groups and/or due to the smaller size of the hydrophobic benzyl compared to the phenylpropyl or methylnaphthyl groups of **12** and **14**. Since the bis-benzyl derivative **10** is a significantly more effective inhibitor than the mono-benzyl derivative **8**, additional hydrophobic interactions may occur between **10** and the active site of TR enabling **10** to bind more effectively to TR than **8**.

Of the N^4,N^8-bis-acylated derivatives investigated in this study, the benzyloxycarbonyl derivative (**6**) was a poor inhibitor and the *t*-BOC (**4**) derivative showed no inhibitory activity. Comparing the activities of the benzyl derivative (**10**) and compound (**6**), the differential inhibitory effects may be due to differences in their protonation states. The bis-acyl derivative (**6**) will have a 2+ charge at physiological pH, whereas the bis-alkyl compound (**10**) will have a 4+ charge. Additionally, the presence of the amide groups in **6** results in a loss of conformational flexibility due to hindered rotation about the carbonyl–N bonds. Thus, **6** may not be able to adopt a conformation that allows for as effective a binding to the active site as **10**.

In this study, N^1,N^{12}-bis(benzyl)spermine (**18**) was shown to be a significantly less active inhibitor than the N^4,N^8-bis-benzyl analogue (**10**). However, N^1,N^{12}-bis(phenylacetyl)spermine (**16**) is a more effective inhibitor than **18**. This suggests that differences in charge are not as important in determining the inhibitory activity of N^1,N^{12}-substituted spermines as they appear to be in N^4,N^8-bis-substituted spermines and N^4-substituted spermidines.[15] Although, the N^1,N^{12}-bis-acylated derivative in this study, compound (**16**), is a relatively poor inhibitor, Ganem has reported N^1,N^{12}-bis-acylated spermines and a N^1,N^8-bis-acylated spermidine that are extremely effective inhibitors of *Crithidia fasciculata* TR.[17] The most effective of these inhibitors was kukoamine A, reported to be a mixed inhibitor with a K_i value of 1.8 and K_{ii} of 13 μM.

Of the polyamines studied containing alkyl substituents at the terminal amino groups, N^1,N^8-bis(2-naphthylmethyl)spermidine (**21**) is a significantly more effective inhibitor than N^1,N^{12}-bis(benzyl)spermine (**18**) and N^1,N^8-bis(benzyl)spermidine (**20**). The methylnaphthyl substituents of **21** presumably interact more effectively with hydrophobic areas of the active site than the smaller benzyl groups of **18** and **20**. This is a further indication of the importance of hydrophobic interactions between compounds and TR in determining inhibitory effectiveness.

The mechanism of TR-mediated reduction of trypanothione disulfide is essentially identical to that of the GR-mediated reduction of glutathione disulfide and these enzymes also have structural similarities.[11] However, the most effective TR inhibitors in this study, compounds **10**, **12**, **14**, and **21**, do not inhibit the reduction of glutathione disulfide by yeast GR, indicating that with respect to GR, these compounds are specific inhibitors of TR.

The trypanocidal activities of the trifluoroactetate salts of the polyamine derivatives **12**, **14**, and **21** and the trifluoroacetate salt of spermine (**1**) were investigated in vitro. The polyamine derivatives **12**, **14**, and **21** all demonstrated significant trypanocidal activity against four different *T. brucei* ssp. strains. The spermine trifluoroacetate salt (**1**) showed no trypanocidal activity at concentrations below 100 μM. Hence, the activities of **12**, **14**, and **21** are not due to the presence of trifluoroacetate. In addition, **1**, **12**, **14**, and **21** are all polycations, thus the observed trypanocidal activities must be related to specific structural features present in **12**, **14**, and **21** and not solely due to the presence of a polycationic species. If the trypanocidal activities of **12**, **14**, and **21** are due to inhibition of TR, these compounds must be able to traverse the cell membrane of trypanosomes. Studies have shown that certain trypanosomes actively concentrate polyamines from their surroundings via transport systems.[25] Possibly, the polycationic nature of compounds **12**, **14**, and **21** enable them cross the trypanosomal cell membrane via a polyamine transporter.

The in vivo trypanocidal properties of compounds **12**, **14**, and **21** were then investigated. Unfortunately, these compounds did not increase the lifetime of mice infected with trypanosomes, or cause a significant decrease in the bloodstream parasitemia of infected mice. However, none of the compounds exhibited any overt toxicity. The lack of in vivo trypanocidal activity for compounds **12**, **14**, and **21** may be due to these compounds being rapidly excreted or metabolized, despite the use of the continuous dosing osmotic pumps. Since these compounds are reversible inhibitors of TR, if concentrations of these compounds are not maintained, TR activity will not be significantly decreased.

Conclusion

Certain polyamine derivatives were shown to be effective competitive inhibitors of *T. cruzi* TR. Some of the structural features necessary for polyamine derivatives to be inhibitors of TR have been indicated in this study. All of the inhibiting polyamines studied contained hydrophobic, aromatic groups and spermine derivatives were more effective inhibitors than the corresponding spermidine derivatives. Other factors that appear to contribute to the effectiveness of inhibitors include the charge of the inhibiting compound, and the size, location and number of hydrophobic, aromatic substituents.

The most effective inhibitors of TR-mediated reduction of trypanothione disulfide were N^4,N^8-bis(3-phenylpropyl)spermine (**12**), N^4,N^8-bis(2-naphthylmethyl)spermine (**14**), and N^1,N^8-bis(2-naphthylmethyl)spermidine (**21**).[15] Compounds **12**, **14**, and **21** were also effective trypanocides in vitro. Although, the polyamine derivatives described in this study did not display trypanocidal properties in vivo, these compounds target a unique pathway in the parasite, and are also synthetically readily available. These compounds, or suitable analogues, could be readily modified to incorporate certain chemically reactive groups resulting in compounds that may be irreversible inhibitors of TR. Such compounds, or other polyamine analogues, may be easily prepared and may lead to derivatives with more promising in vivo trypanocidal activities. Since trypanosome infections occur predominantly in countries with low average household incomes, new chemotherapeutics to combat these infections should be inexpensive. The polyamine derivatives discussed in this paper are nontoxic to the host, easy to prepare and are inexpensive; therefore, these compounds may provide a new direction for the development of affordable antitrypanosomal agents.

Experimental

Synthesis

N^1,N^{12}-bis(trifluoroacetyl)spermine trifluoroacetate salt (**2**), N^1,N^8-bis(2-naphthylmethyl)spermidine (**21**), and N^4-(*t*-butoxycarbonyl)spermidine (**22**) were prepared using previously described procedures.[15,18] All other reagents were purchased from commercial sources. THF was dried by distillation over benzophenone and sodium under N_2. Acetonitrile and triethylamine were 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 and FAB spectra on a Kratos MS50 spectrometer.

N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(*t*-butoxycarbonyl)spermine (3). To a solution of N^1,N^{12}-bis(trifluoroacetyl)spermine trifluoroacetate salt (**2**) (3.0 g, 4.82 mmol) in THF (15 mL) and triethylamine (2.92 g, 4.03 mL, 28.92 mmol) was slowly added di-*t*-butyl dicarbonate (3.16 g, 3.32 mL, 14.47 mmol). The reaction was stirred overnight under N_2. Saturated aqueous $NaHCO_3$ 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 (**3**) was purified by column chromatography (0.5–1.5% MeOH in CH_2Cl_2) to give **3** as a white solid (2.53 g, 88%). R_f one spot 0.33 (1.5% MeOH in CH_2Cl_2); mp 93.5–95.0 °C; ^{1}H NMR ($CDCl_3$ containing 1 drop D_2O) δ 3.31 (m, 8 H, 2 CH_2NCH_2), 3.16 (m, 4 H, 2 $CONHCH_2$), 1.72 (m, 4 H, 2 $NCH_2CH_2CH_2N$), 1.50 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) and 1.46 (s, 18 H, 6 CH_3) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 157.2 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 156.7 (br, OCO), 115.9 (q, $^1J_{CF}$ = 288.0 Hz, CF_3CO), 80.3 ($OC(CH_3)_3$), 46.8, 43.0 (m), 35.9 (m), 28.1 (CH_3), 27.2 (m) and 25.8 (m) ppm.

N^4,N^8-bis(*t*-butoxycarbonyl)spermine (4). To N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(*t*-butoxycarbonyl)spermine (**3**) (2.2 g, 3.7 mmol) was added MeOH and ammonium hydroxide (1:2, 25 mL). The mixture was refluxed overnight. The solvent was removed under vacuum and compound **4** was purified by column chromatography (5% ammonium hydroxide in MeOH) to give **4** as a light-colored oil (1.3 g, 90%). R_f one spot 0.27 (5% ammonium hydroxide in MeOH); ^{1}H NMR ($CDCl_3$ containing 1 drop D_2O) δ 3.25 (m, 4 H, 2 CH_2N), 3.16 (m, 4 H, 2 CH_2N), 2.70 (m, 4 H, 2 $NH_2CH_2CH_2$), 1.68 (m, 4 H, 2 $NCH_2CH_2CH_2N$), 1.49 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) and 1.45 (s, 18 H, 6 CH_3) ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 161.8 (d, CO), 79.9 ($OC(CH_3)_3$), 45.8 (br), 43.5 (br), 37.7 (br), 28.6 (br), 28.0 (CH_3) and 25.6 (br) ppm.

N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(benzyloxycarbonyl)spermine (5). To a solution of N^1,N^{12}-bis(trifluoroacetyl)spermine trifluoroacetate salt (**2**) (7.0 g, 11.25 mmol) in THF (25 mL) were added a solution of K_2CO_3 (15.5 g, 113 mmol) in water (15 mL) and benzyl chloroformate (7.67 g, 6.4 mL, 45 mmol). The reaction was stirred for 1 h and saturated aqueous NaCl solution (50 mL) was added. The mixture was extracted with CH_2Cl_2 (4 × 50 mL) and the organic layers were collected, dried ($MgSO_4$), and concentrated to give a light-colored oil. Compound **5** was purified by column chromatography (0.5–1% MeOH in CH_2Cl_2) to give **5** as a light-colored oil (8.92 g, material contains 18% benzyl alcohol (by weight) calculated from ^{1}H NMR integral, calculated yield of compound **5**, 7.31 g, 98%). R_f two spots 0.31 (benzyl alcohol) and 0.25 (compound **5**) (1% MeOH in CH_2Cl_2); ^{1}H NMR δ 8.20 (br s, 2 H, 2 NH), 7.27 (m, 10 H, 2 C_6H_5), 5.09 (s, 4 H, 2 $C_6H_5CH_2$), 3.24 (m, 12 H, 6 CH_2N), 1.67 (br s, 4 H, 2 $NCH_2CH_2CH_2N$), 1.44 (br s, 4 H, $NCH_2CH_2CH_2CH_2N$) and resonances due to benzyl alcohol at 7.27 and 4.61 ppm; ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 157.2 (q, $^2J_{CF}$ = 36.9 Hz, CF_3CO), 157.0 (br), 136.1, 128.4, 127.2, 126.7, 115.8 (q, $^1J_{CF}$ = 281.3 Hz, CF_3CO), 67.3

($C_6H_5CH_2$), 46.3, 43.7, 35.1(br), 26.7 (br) and 25.4 (br) ppm; MS (FAB) *m/z* 663.2 (M + H^+).

N^4,N^8-bis(benzyloxycarbonyl)spermine (6). To N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(benzyloxycarbonyl)spermine (**5**) (2.00 g, 3.02 mmol) was added MeOH and ammonium hydroxide (1:2, 20 mL). The mixture was refluxed overnight. The solvent was removed under vacuum. Compound **6** was purified by column chromatography (5% ammonium hydroxide in MeOH) to give **6** as a light-colored oil (1.29 g, 91%). R_f one spot 0.41 (5% ammonium hydroxide in MeOH); ^{1}H NMR ($CDCl_3$ containing 1 drop D_2O) δ 7.33 (m, 10 H, 2 C_6H_5), 5.11 (s, 4 H, 2 $C_6H_5CH_2$), 3.26 (m, 8 H, 2 CH_2NCH_2), 2.65 (m, 4 H, 2 NH_2CH_2), 1.64 (m, 4 H, 2 $NCH_2CH_2CH_2N$) and 1.50 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ (rotamers for this compound exist, therefore for some carbons several peaks were observed) 162.0 (d, *C*O), 136.7, 128.4, 128.0, 127.8, 67.4 ($C_6H_5CH_2$), 46.7 (br d), 44.3, 38.7 (br d), 31.1 (br d) and 25.4 (br d) ppm.

N^1,N^{12}-bis(trifluoroacetyl)-N^4-benzylspermine (7) and N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(benzyl)spermine (9). To a solution of N^1,N^{12}-bis(trifluoroacetyl)spermine trifluoroacetate salt (**2**) (2.0 g, 3.22 mmol) in CH_3CN (15 mL) and triethylamine (2.24 mL, 16.07 mmol) was added benzyl bromide (0.95 mL, 8.05 mmol). The reaction 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 a light-colored oil. Compounds **7** and **9** were purified by column chromatography (0.5–4% MeOH in CH_2Cl_2 containing 0.5% ammonium hydroxide) to give **7** as a light-colored oil (0.76 g, 49%) and **9** as a light-colored oil (0.55 g, 30%). Analytical data for compound (**7**): R_f one spot 0.23 (5% MeOH and 0.5% ammonium hydroxide in CH_2Cl_2); ^{1}H NMR δ 7.29 (m, 5 H, C_6H_5), 3.52 (s, 2 H, $C_6H_5CH_2$), 3.35 (m, 4 H, 2 $CONHCH_2$), 2.85 (m, 4 H, CH_2NCH_2), 2.50 (m, 4 H, CH_2NHCH_2), 1.90 (p, J = 7.0 Hz, 2 H, $NHCH_2CH_2CH_2N$), 1.73 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) and 1.59 (m, 2 H, $NHCH_2CH_2CH_2NH$) ppm; ^{13}C NMR δ 162.3 (q, $^2J_{CF}$ = 35.1 Hz, CF_3CO), 158.2 (q, $^2J_{CF}$ = 37.4 Hz, CF_3CO), 137.7, 129.1, 128.4, 127.4, 115.9 (q, $^1J_{CF}$ = 287.3 Hz, CF_3CO), 115.8 (q, $^1J_{CF}$ = 287.3 Hz, CF_3CO), 58.4 ($C_6H_5CH_2$), 52.9, 51.3, 48.0, 46.0, 45.3, 36.7, 25.5, 24.9, 24.4 and 23.9 ppm; MS (FAB) *m/z* 484.8 (M + H^+). Analytical data for compound **9**: R_f one spot 0.44 (3% MeOH and 0.5% ammonium hydroxide in CH_2Cl_2); ^{1}H NMR δ 8.45 (br s, 2 H, 2 CO*NH*), 7.26 (m, 10 H, 2 C_6H_5), 3.52 (s, 4 H, 2 $C_6H_5CH_2$), 3.33 (m, 4 H, 2 $CONHCH_2$), 2.55 (t, J = 6.0 Hz, 4 H, 2 CH_2NCH_2), 2.44 (m, 4 H, 2 CH_2NCH_2), 1.66 (p, J = 6.0 Hz, 4 H, 2 $CONHCH_2CH_2CH_2N$) and 1.50 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 156.8 (q, $^2J_{CF}$ = 37.0 Hz, CF_3CO), 138.1, 129.2, 128.4, 127.4, 116.0 (q, $^1J_{CF}$ = 288.1 Hz, CF_3CO), 59.1 ($C_6H_5CH_2$), 53.8, 52.9, 40.3, 24.6 and 24.4 ppm; MS (FAB) *m/z* 574.8 (M + H^+).

N^4-benzylspermine (8). To N^1,N^{12}-bis(trifluoroacetyl)-N^4-benzylspermine (**7**) (180 mg, 0.37 mmol) was added MeOH and ammonium hydroxide (1:1, 25 mL). The mixture was refluxed overnight and the solvent was removed under vacuum. Compound **8** was purified by column chromatography (10–20% ammonium hydroxide in MeOH) to give **8** as a light-colored oil (71 mg, 65%). R_f one spot 0.46 (MeOH:ammonium hydroxide 1:1); ^{1}H NMR δ 7.28 (m, 5 H, C_6H_5), 3.53 (s, 2 H, $C_6H_5CH_2$), 2.70 and 2.45 (m, 12 H, 2 NH_2CH_2, CH_2NCH_2 and CH_2NHCH_2), 1.62 and 1.49 (m, 13 H, $NH_2CH_2CH_2CH_2N$, $NCH_2CH_2CH_2CH_2N$, $NH_2CH_2CH_2CH_2NH$ and 2 NH_2CH_2) ppm; ^{13}C NMR δ 139.9, 128.7, 128.0, 126.6, 58.6 ($C_6H_5CH_2$), 53.6, 51.3, 49.8, 47.8, 40.4, 40.3, 33.5, 30.8, 27.8 and 24.8 ppm; MS (CI) *m/z* 293 (M + H^+).

N^4,N^8-bis(benzyl)spermine (10). To N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(benzyl)spermine (**9**) (0.20 g, 0.35 mmol) was added MeOH and ammonium hydroxide (1:1, 25 mL). The mixture was refluxed overnight and the solvent was removed under vacuum. Compound **10** was purified by column chromatography (10% ammonium hydroxide in MeOH) to give **10** as a light-colored oil (0.10 g, 75%). R_f one spot 0.16 (10% ammonium hydroxide in MeOH); ^{1}H NMR δ 7.29 (m, 10 H, 2 C_6H_5), 3.50 (s, 4 H, 2 $C_6H_5CH_2$), 2.60 (t, J = 7.0 Hz, 4 H, 2 NH_2CH_2), 2.42 (m, 8 H, 2 CH_2NCH_2), 1.56 (p, J = 7.0 Hz, 4 H, 2 $NCH_2CH_2CH_2N$), 1.45 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) and 1.39 (br s, 4 H, 2 NH_2) ppm; ^{13}C NMR δ 140.0, 128.6, 128.0, 126.6, 58.6 ($C_6H_5CH_2$), 53.6, 51.2, 40.3, 30.8 and 24.8 ppm; MS (CI) *m/z* 383 (M + H^+).

N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(3-phenylpropyl)spermine (11). To a solution of N^1,N^{12}-bis(trifluoroacetyl)spermine trifluoroacetate salt (**2**) (1.0 g, 1.61 mmol) in CH_3CN (5 mL) and triethylamine (1.35 mL, 9.65 mmol) was added 1-bromo-3-phenylpropane (0.98 mL, 6.44 mmol). The solution 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 a light-colored oil. Compound **11** was purified by column chromatography (0.5–5% MeOH in CH_2Cl_2 containing 0.5% ammonium hydroxide) to give **11** as a light-colored oil (0.410 g, 40.4%). R_f one spot 0.33 (3% MeOH and 0.5% ammonium hydroxide in CH_2Cl_2); ^{1}H NMR δ 9.38 (br s, 2 H, 2 N*H*), 7.28–7.21 (m, 10 H, 2 C_6H_5), 3.44 (m, 4 H, 2 $CONHCH_2$), 2.59 (m, 8 H, 2 $C_6H_5CH_2$ and 2 $C_6H_5CH_2CH_2CH_2N$), 2.45 (m, 8 H, 2 CH_2NCH_2), 1.78 (m, 4 H, 2 $C_6H_5CH_2CH_2CH_2N$), 1.68 (m, 4 H, 2 $NHCH_2CH_2CH_2N$) and 1.36 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 156.8 (q, $^2J_{CF}$ = 36.7 Hz, CF_3CO), 141.6, 128.4, 128.2, 125.9, 116.1 (q, $^1J_{CF}$ = 287.9 Hz, CF_3CO), 54.4, 53.9, 53.4, 41.1, 33.6, 28.1, 24.6 and 23.9 ppm.

N^4,N^8-bis(3-phenylpropyl)spermine (12). To N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(3-phenylpropyl)spermine (**11**) (0.20 g, 0.317 mmol) was added MeOH and

ammonium hydroxide (1:1, 15 mL). The mixture was refluxed overnight. The solvent was removed under vacuum. Compound **12** was purified by column chromatography (10% ammonium hydroxide in MeOH) to give **12** as a light-colored oil (110 mg, 79%). R_f one spot 0.18 (10% ammonium hydroxide in MeOH); ^{1}H NMR δ 7.19 (m, 10 H, 2 C_6H_5), 2.70 (t, J = 7.0 Hz, 4 H, 2 NH_2CH_2), 2.60 (t, J = 7.6 Hz, 4 H, 2 $C_6H_5CH_2$), 2.43 (m, 12 H, 6 CH_2N), 1.76 (m, 4 H, 2 $C_6H_5CH_2CH_2$), 1.54 (p, J = 7.0 Hz, 4 H, 2 $NH_2CH_2CH_2CH_2N$) and 1.39 (m, 8 H, $NCH_2CH_2CH_2CH_2N$ and 2 NH_2) ppm; ^{13}C NMR δ 142.3, 128.2, 128.1, 125.5, 54.0, 53.5, 51.8, 40.7, 33.7, 31.0, 28.8 and 25.0 ppm; MS (FAB) m/z 439.2 (M + H^+).

N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(2-naphthylmethyl)spermine (13). To a solution of N^1,N^{12}-bis(trifluoroacetyl)spermine trifluoroacetate salt (**2**) (1.0 g, 1.61 mmol) in CH_3CN (5 mL) and triethylamine (6 mol equiv, 1.35 mL, 9.65 mmol) was added 2-(bromomethyl)naphthalene (1.07 g, 4.83 mmol). The reaction 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 a light-colored oil. Compound **13** was purified by column chromatography (0.5–2.5% MeOH in CH_2Cl_2) to give **13** as a light-colored oil (380 mg, 35.5%). R_f one spot 0.25 (2.5% MeOH in CH_2Cl_2); ^{1}H NMR δ 8.51 (br s, 2 H, 2 NH), 7.83–7.34 (m, 14 H, 2 $C_{10}H_7$), 3.30 (s, 4 H, 2 $C_{10}H_7CH_2$), 3.30 (m, 4 H, 2 $CONHCH_2$), 2.55 (t, J = 5.8 Hz, 4 H, 2 CH_2NCH_2), 2.47 (m, 4 H, 2 CH_2NCH_2), 1.67 (p, J = 5.8 Hz, 4 H, 2 $NHCH_2CH_2CH_2N$) and 1.51 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 156.8 (q, $^2J_{CF}$ = 36.2 Hz, CF_3CO), 135.7, 133.2, 132.8, 128.9, 127.9, 127.7, 127.6, 127.0, 126.2, 125.9, 116.0 (q, $^1J_{CF}$ = 287.7 Hz, CF_3CO), 59.4 ($C_6H_5CH_2$), 53.8, 53.1, 40.4, 24.5 and 24.4 ppm; MS (FAB) m/z 676.5 (M + H^+).

N^4,N^8-bis(2-naphthylmethyl)spermine (14). To N^1,N^{12}-bis(trifluoroacetyl)-N^4,N^8-bis(2-naphthylmethyl)spermine (**13**) (190 mg, 0.29 mmol) was added MeOH and ammonium hydroxide (1:1, 15 mL). The mixture was refluxed overnight. The solvent was removed under vacuum. Compound **14** was purified by column chromatography (10% ammonium hydroxide in MeOH) to give **14** as a light-colored oil (109 mg, 81%). R_f one spot 0.25 (10% ammonium hydroxide in MeOH); ^{1}H NMR δ 7.73 and 7.47 (m, 14 H, 2 $C_{10}H_7$), 3.63 (s, 4 H, 2 $C_{10}H_7CH_2$), 3.23 (br s, 4 H, 2 NH_2CH_2), 2.65 (t, J = 6.8 Hz, 4 H, 2 NH_2CH_2), 2.44 (m, 8 H, 2 CH_2NCH_2), 1.56 (p, J = 6.8 Hz, 4 H, 2 $NH_2CH_2CH_2CH_2N$) and 1.48 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR δ 137.7, 133.2, 132.6, 127.6, 127.52, 127.49, 127.1, 127.0, 125.7, 125.3, 58.8 ($C_6H_5CH_2$), 53.7, 51.4, 40.4, 30.9 and 24.8 ppm; MS (FAB) m/z 483.2 (M + H^+).

N^1,N^{12}-bis(phenylacetyl)-N^4,N^8-bis(benzyloxycarbonyl)spermine (15). To a solution of N^4,N^8-bis(benzyloxycarbonyl)spermine (**6**) (0.60 g, 1.28 mmol) in THF (5 mL) and triethylamine (0.71 mL, 5.10 mmol) was added phenylacetyl chloride (0.79 g, 0.68 mL, 5.10 mmol). The reaction was stirred under N_2 for 5 h. 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 a light-colored oil. Compound **15** was purified by column chromatography (0.5–3% MeOH in CH_2Cl_2) to give **15** as a white solid (0.36 g, 40%). R_f one spot 0.13 (2% MeOH in CH_2Cl_2); mp 108–109 °C; ^{1}H NMR ($CDCl_3$ containing 1 drop D_2O) δ 7.25 (m, 20 H, 4 C_6H_5), 5.06 (s, 4 H, 2 $C_6H_5CH_2OCO$), 3.52 (s, 4 H, 2 $C_6H_5CH_2CO$), 3.13 (m, 12 H, 6 NCH_2), 1.60 (m, 4 H, 2 $NCH_2CH_2CH_2N$) and 1.42 (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 171.0 (CO), 156.6 (d, OCO), 136.6, 135.2 (br), 129.2, 128.8, 128.5, 127.9, 127.0, 67.1 ($C_6H_5CH_2OCO$), 53.4 ($C_6H_5CH_2CO$), 46.5 (br d), 44.0 (br d), 36.3 (br d), 27.9 (br d) and 25.3 (br d) ppm.

N^1,N^{12}-bis(phenylacetyl)spermine (16). To a solution of N^1,N^{12}-bis(phenylacetyl)-N^4,N^8-bis(benzyloxycarbonyl)spermine (**15**) (0.20 g, 0.28 mmol) in MeOH (15 mL) was added palladium on activated carbon (5% Pd on C, 0.24 g). The suspension was shaken vigorously under H_2 (45 psi) overnight. The mixture was then filtered through Celite with MeOH to give **16** as a white solid (83 mg, 67.7%). R_f one spot 0.66 (25% ammonium hydroxide in MeOH); mp 245–247 °C (dec); ^{1}H NMR (MeOH-d_4) δ 7.31 (m, 10 H, 2 C_6H_5), 3.55 (s, 4 H, 2 $C_6H_5CH_2CO$), 3.32 (m, 4 H, 2 $CONHCH_2$), 2.90 (m, 8 H, 2 CH_2NCH_2), 1.91 (p, J = 6.8 Hz, 4 H, 2 $NHCH_2CH_2CH_2N$) and 1.75 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR (MeOH-d_4) δ 174.8 (CO), 136.2, 129.7, 129.3, 127.7, 47.6 ($C_6H_5CH_2$), 45.8, 43.5, 36.6, 27.1 and 23.7 ppm.

N^1,N^{12}-bis(benzyl)-N^4,N^8-bis(benzyloxycarbonyl)spermine (17). To a solution of N^4,N^8-bis(benzyloxycarbonyl)spermine (**6**) (0.10 g, 0.212 mmol) in CH_3CN (6 mL) was added benzaldehyde (0.065 mL, 0.64 mmol). The mixture was stirred for 5 h at room temperature and the solvent was removed. The residue was dissolved in EtOH (10 mL). Sodium borohydride (48 mg, 1.28 mmol) was added at 0 °C and the mixture was stirred for 1 h. Water (1 mL) then saturated aqueous $NaHCO_3$ solution (50 mL) was added. 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 **17** was purified by column chromatography (5–6% MeOH in CH_2Cl_2 containing 0.5% ammonium hydroxide) to give **17** as a yellow-colored oil (44 mg, 32%). R_f one spot 0.40 (7% MeOH and 0.5% ammonium hydroxide in CH_2Cl_2); ^{1}H NMR δ 7.32 (m, 20 H, 4 C_6H_5), 5.10 (s, 4 H, 2 $C_6H_5CH_2OC$), 3.72 (d, J = 12.8 Hz, 4 H, 2 $C_6H_5CH_2N$), 3.25 (m, 8 H, 4 CH_2N), 2.57 (m, 4 H, 2 $NHCH_2$), 1.70 (m, 4 H,

2 N*H* and 2 $NCH_2CH_2CH_2N$), 1.48 (m, 4 H, $NCH_2CH_2CH_2CH_2NH_2$); ^{13}C NMR (rotamers for this compound exist, therefore for some carbons several peaks were observed) δ 156.1 (*C*O), 140.2, 136.8, 128.4, 128.3, 128.1, 128.0, 127.9, 127.7, 126.8, 66.8 ($C_6H_5CH_2CO$), 53.9 ($C_6H_5CH_2NH$), 47.0 (br), 46.3 (d), 44.9 (d), 28.6 (d) and 25.5 (d) ppm.

N^1,N^{12}-bis(benzyl)spermine (18). To a solution of N^1,N^{12}-bis(benzyl)-N^4,N^8-bis(benzyloxycarbonyl)spermine (**17**) (0.220 g, 0.34 mmol) in ethanol (10 mL) was added palladium on activated carbon (5% Pd on C, 0.10 g, 0.06 mmol). The mixture was shaken under H_2 (50 psi) overnight. The catalyst was removed by filtration through Celite. Compound **18** was purified by column chromatography (7% MeOH in CH_2Cl_2 containing 0.5% ammonium hydroxide–5% ammonium hydroxide in MeOH) to give **18** as a light-colored oil (60 mg, 46%). R_f one spot 0.18 (5% ammonium hydroxide in MeOH); ^1H NMR δ 7.30 (m, 10 H, 2 C_6H_5), 3.78 (4 H, 2 $C_6H_5CH_2$), 2.65 (m, 12 H, 6 NCH_2), 1.69 (m, 4 H, 2 $NCH_2CH_2CH_2N$), 1.49 (m, 4 H, $NCH_2CH_2CH_2CH_2N$); ^{13}C NMR δ 140.4, 128.3, 128.0, 126.8, 54.0 ($C_6H_5CH_2$), 49.9, 48.4, 47.8, 30.3 and 27.9 ppm.

N^1,N^8-bis(benzyl)-N^4-(*t*-butoxycarbonyl)spermidine (19). To N^4-(*t*-butoxycarbonyl)spermidine (**22**) (2.43 g, 9.9 mmol) was added $MgSO_4$ (2.34 g, 19.5 mmol), CH_3CN (36 mL) and benzaldehyde (2.23 g, 2.14 mL, 21.04 mmol). The mixture was stirred overnight and the solvent removed. The residue was dissolved in dry ethanol (30 mL) and sodium borohydride (3.0 g, 80.8 mmol) was added at 0 °C. The mixture was stirred for 30 min. Water (3 mL) then saturated aqueous $NaHCO_3$ solution (40 mL) was added. The mixture was extract with CH_2Cl_2 (4 × 50 mL). The organic layers were collected, dried ($MgSO_4$) and concentrated to give a yellow oil. Compound **19** was purified by column chromatography (5% MeOH in CH_2Cl_2 to 7% MeOH in CH_2Cl_2 containing 0.5% ammonium hydroxide) to give **19** as a light-yellow colored oil (1.05 g, 25%). R_f one spot 0.50 (7% MeOH and 0.5% ammonium hydroxide in CH_2Cl_2); ^1H NMR δ 7.25 (m, 10 H, 2 C_6H_5), 3.78 (m, 4 H, 2 $C_2H_5CH_2$), 3.23 (m, 2 H, CH_2NCO), 3.15 (m, 2 H, CH_2NCO), 2.65 (m, 4 H, 2 CH_2NH), 2.26 (m, 2 H, 2 N*H*), 1.71 (p, *J* = 6.95 Hz, 2 H, $NCH_2CH_2CH_2N$), 1.51 (m, 4 H, $NCH_2CH_2CH_2CH_2N$), 1.43 (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 (*C*O), 139.9, 128.3, 128.11, 128.05, 126.91, 126.88, 79.1 ($OC(CH_3)_3$), 53.82 ($C_6H_5CH_2$), 53.77 ($C_6H_5CH_2$), 48.9, 46.8, 46.2 (br), 45.0 (br), 44.4 (br), 28.3 (CH_3), 27.1 and 26.2 ppm.

N^1,N^8-bis(benzyl)spermidine (20). To N^1,N^8-bis(benzyl)-N^4-(*t*-butoxycarbonyl)spermidine (**19**) (0.70 g, 1.65 mmol) was added trifluoroacetic acid (2 mL, 25.9 mmol). The mixture was stirred overnight and the solvent removed. The residue was washed with CH_2Cl_2 to give the trifluoroacetic salt of **20** as a white solid (1.11 g, 100%). R_f one spot 0.29 (5% ammonium hydroxide in MeOH); mp 220–222 °C; ^1H NMR (MeOH-d_4) δ 7.47 (m, 10 H, 2 C_6H_5), 4.22 (s, 2 H, $C_6H_5CH_2$), 4.20 (s, 2 H, $C_6H_5CH_2$), 3.0–3.2 (m, 8 H, 4 $CH_2NH_2^+$), 2.16 (m, 2 H, $NCH_2CH_2CH_2N$) and 1.80 (m, 4 H, $NCH_2CH_2CH_2CH_2N$) ppm; ^{13}C NMR (MeOH-d_4) δ 132.4, 132.2, 130.97, 130.91, 130.73, 130.68, 130.28, 130.26, 52.5, 52.3, 48.2, 47.7, 45.8, 45.4, 24.2, 24.1 and 24.0 ppm.

Enzyme studies

T. cruzi TR was purified following the method of Walsh et al.[26] from *E. coli* SG5, a glutathione reductase deletion mutant, containing the TR expression vector pIBITczTR described by Sullivan and Walsh.[20] Prepared compounds were assayed for their effects on the rate of reduction of trypanothione disulfide by *T. cruzi* TR spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.[19] 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 15 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), trypanothione disulfide (Bachem Bioscience Inc.) 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 a minimum of four inhibitor concentrations (ranging from 0.3–3.7 times the estimated K_i) were assayed for their effects on TR activity in the presence of varying concentrations of trypanothione disulfide (a minimum of four concentrations ranging from 14.8 to 44.4 μM, or 14.8–74 μM). Compounds **1** and **4** were either extremely weak inhibitors, or had no inhibitory effects on TR activity. For these compounds, assays were conducted using two concentrations of trypanothione disulfide (22.2 and 44.4 μM) and three concentrations of either **1** or **4** (ranging from 470 to 2000 μM).

The effect of compounds **10**, **12**, **14**, and **21** on the rate of reduction of glutathione disulfide 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 glutathione disulfide (14.3, 28.6, 42.9, or 57.2 μM) with an enzyme concentration of 0.27 μg/mL. The effects of compounds **10**, **12**, **14**, and **21** on GR activity were measured at a concentration of 14.3 μM glutathione disulfide and either 10.3 mM of **10**, 0.255 mM of **12**, 0.84 mM of **14**, or 0.36 mM of **21**.

Trypanosome studies

Trypanosome isolates. Four trypanosome isolates were used to assess in vitro activity: *T. b. brucei* Lab

110 EATRO and three clinical isolates of *T. b. rhodesiense*, KETRI 243, KETRI 269, and KETRI 243-As-10-3, a clone of KETRI 243. KETRI 243 displays some resistance to melamine-based arsenical drugs (e.g., melarsoprol) and pentamidine, while KETRI 243-As-10-3 is completely resistant to melarsoprol and pentamidine.[21]

Determination of in vitro antitrypanosomal activities of compounds 1, 12, 14, and 21. Trypanosome strains were grown routinely as the blood form at 37 °C in a synthetic medium (IMDM) with 20% horse serum. The in vitro trypanocidal activities of compounds were assessed by determining IC_{50} values. Measurements were done in duplicate in 24 well plates (1 mL/well) with final compound concentrations of 0.1, 1.0, 10, 25, and 100 μM. After 48 h, the number of parasites/well was determined in a model Z1 Coulter Counter, and the approximate range of activity for each compound was determined. Controls grew to 5×10^6 parasites/mL after 48 h. The 50% inhibitory concentration (IC_{50}) of compounds, after incubation with trypanosomes for 48 h, was then determined from additional studies with closely spaced concentration points. Compounds that displayed <50% inhibition at 100 μM were not studied further. Compounds were dissolved in water, then diluted further with medium.[21] IC_{50} values were determined from semi-log plots.

In vivo studies of trypanocidal activities of compounds 12, 14, and 21. Initial in vivo testing was done with the *T. b. brucei* Lab 110 EATRO mouse model infection. Female Swiss–Webster mice were infected intraperitoneally (ip) with 2.5×10^5 *T. b. brucei* Lab 110 EATRO blood forms taken from an infected rat. The infection was allowed to develop for 24 h before treatment was begun. Infected mice were divided into groups of five, including infected, non-treated controls. Initially, animals were dosed at 1.0, 5.0, 10, and 25 mg/kg intraperitoneally once daily for three days. In a second experiment, compounds were given via Alza® mini-osmotic pumps (Alza, Palo Alto, CA) which dispense 1 μL/h continuously for 3 days. Each compound was given at 10 and 25 mg/kg/day. In a third experiment, animals were given 25 mg/kg subcutaneously once daily for three days. Survival times were compared to infected untreated controls. The bloodstream parasitemia of animals was also monitored in thin blood films and by hemocytometer counts.

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