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Polyamines with *N*-(3-Phenylpropyl) Substituents are Effective Competitive Inhibitors of Trypanothione Reductase and Trypanocidal Agents

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Abstract—Several *N*-(3-phenylpropyl)-substituted spermidine and spermine derivatives were prepared and found to be potent competitive inhibitors of *Trypanosoma cruzi* trypanothione reductase (seven compounds with K_i values $< 5\,\mu M$ are described). The most effective inhibitor studied was compound **12** with a K_i value of $0.151\,\mu M$. Six of the compounds described are also effective trypanocides with IC_{50} values $< 1\,\mu M$.

Several members of the family *Trypanosomatidae* are the causative agents of human and livestock diseases that include African sleeping sickness (*Trypanosoma brucei rhodesiense* and *T. b. gambiense*), Chagas' disease (*Trypanosoma cruzi*) and visceral leishmaniasis or kala-azar (*Leishmania donovani*). Diseases caused by trypanosomes are a serious world problem. For example, Chagas' disease is endemic in 21 Central and South American countries with an estimated 16–18 million people infected.[1] Also, a resurgence of African sleeping sickness is devastating populations in southern Sudan, the Congo, and other sub-Saharan African countries.[2] Current treatments for these diseases and other forms of trypanosomiasis and leishmaniasis are often unsatisfactory. Problems associated with anti-trypanosomal drugs include their limited efficacies, human toxicity, high cost, and the emergence of drug-resistant trypanosome strains.[3] One strategy for the development of novel anti-trypanosomal agents is to target a unique feature of the thiol metabolism of these parasites. In most organisms, glutathione is an important antioxidant and levels of reduced glutathione are maintained by glutathione reductase (GR). However, *Trypanosomatidae* do not contain GR, instead they have a unique enzyme, trypanothione reductase (TR) (EC 1.6.4.8). TR catalyzes the reduction of the disulfide of a glutathione–spermidine conjugate named trypanothione (N^1,N^8-bis(glutathionyl)spermidine). The mechanism of action of TR is essentially identical to that of GR. The two enzymes have structural similarities; however, TR and GR have different substrate specificities.[4] TR is crucial to the antioxidant defenses of *Trypanosomatidae*. Targeted gene replacement studies have indicated that TR is essential for survival of leishmania and trypanosoma.[5] Consequently, inhibitors of TR have potential as effective, anti-trypanosomal drugs.[6]

Inhibitors of TR are structurally diverse, but all reversible inhibitors contain amino and hydrophobic groups.[7,8] Several of the most effective inhibitors are polyamine derivatives with aromatic substituents.[9–11] We have been investigating the inhibition profiles of structurally-simple polyamine derivatives and recently reported that N^4,N^8-bis(3-phenylpropyl)spermine (**1**) is a potent competitive inhibitor of *T. cruzi* TR (K_i 3.48 μM) and an effective trypanocide (IC_{50} values of 0.58–0.79 μM against *T. brucei* strains).[10,11] However, compound **1** did not prolong the lifetimes of mice infected with *T. brucei* and hence does not show anti-trypanosomal activity in vivo.[10] Since, compound **1** is such an effective trypanocide in vitro, and given that no other *N*-(3-phenylpropyl)-polyamine derivatives have been investigated, we

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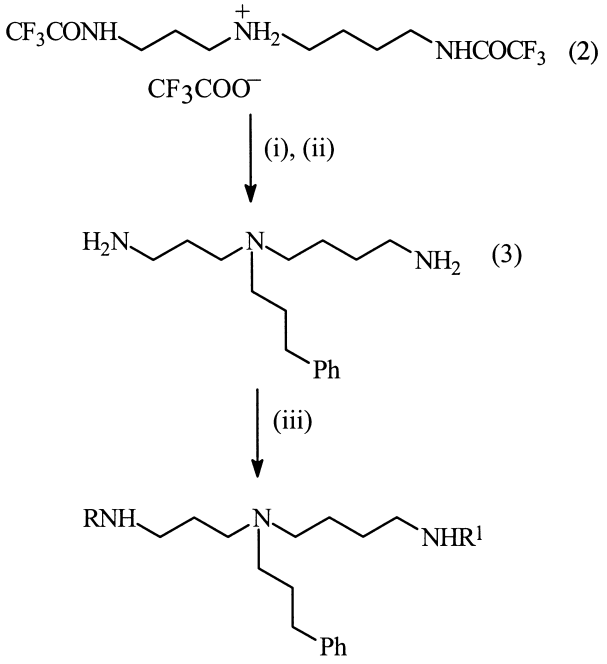
felt compelled to study the inhibiting and trypanocidal activities of this class of compounds since we envisaged that the substitution pattern of the 3-phenylpropyl moiety may profoundly affect the biological activities of these compounds.

In this paper, we report the preliminary results of a structure–activity study of *N*-(3-phenylpropyl)-substituted spermine and spermidine derivatives against recombinant *T. cruzi* TR. Additionally, we report the in vitro activities of several of these compounds against four *T. brucei* strains. As expected, the quantity and location of the 3-phenylpropyl moieties dramatically influences the inhibition and trypanocidal profiles of compounds. Several of the compounds described are potent competitive inhibitors of TR, with the most effective compound being N^1,N^1,N^4,N^8,N^{12}-penta(3-phenylpropyl)spermine (**12**) (K_i value of 0.151 μM). Compound **12** is one of the most effective competitive inhibitors of TR described to date. Furthermore, none of the compounds studied inhibited GR, indicating that they show specificity for TR. Compounds that were effective TR inhibitors were also effective trypanocides in vitro.

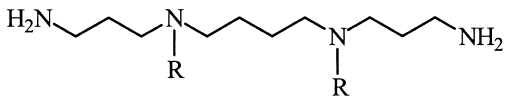
The synthetic strategies used to prepare spermidine and spermine analogues are detailed in Schemes 1 and 2, respectively. The initial steps of both syntheses relied on the one-step selective trifluoroacetylation of the primary amino groups of polyamines as described previously.[12] Compounds **1** and **6** were prepared as described previously.[10] Alkylation of the primary amino groups of compounds **1**, **3**, and **6** was surprisingly problematic. Standard reductive amination reaction conditions (i.e., formation of imine followed by reduction with sodium borohydride, or one-step reductive amination using sodium cyanoborohydride) produced the desired products in negligible yield. However, reaction of **1**, **3**, or **6** with 2 mol equiv of hydrocinnamaldehyde and sodium triacetoxyborohydride[13] produced mixtures of mono- and higher alkylated products that were separable by flash chromatography on silica gel (using a gradient solvent system ranging from 0.3% NH_4OH and 5.0% CH_3OH in CH_2Cl_2 to 1.0% NH_4OH and 20% CH_3OH in CH_2Cl_2). Therefore, this strategy enabled the rapid preparation of a range of *N*-(3-phenylpropyl) substituted spermidine and spermine analogues.[14]

Trypanothione reductase from *T. cruzi* was purified following the procedure of Walsh et al.[15] from an SG5 *Escherichia coli* strain (a glutathione reductase deletion mutant) containing the expression vector pIBITczTR

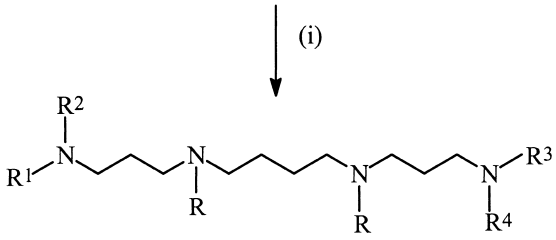


(4) mixture of two isomers:
R = H, $R^1 = PhCH_2CH_2CH_2$;
R = $PhCH_2CH_2CH_2$ and R^1 = H
(5) R = $R^1 = PhCH_2CH_2CH_2$

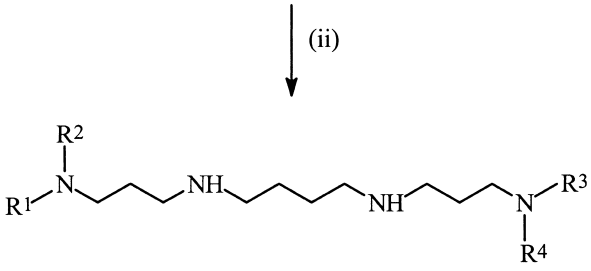
Scheme 1. (i) $PhCH_2CH_2CH_2Br$, Et_3N, CH_3CN, reflux; (ii) NH_4OH, MeOH, reflux; (iii) $PhCH_2CH_2CHO$, $NaBH(OAc)_3$, CH_2ClCH_2Cl.



(1) R = $PhCH_2CH_2CH_2$
(6) R = $PhCH_2OCO$



For compounds (7), (8) and (9) R = $PhCH_2OCO$; R^1, R^2, R^3 and R^4 = H or $PhCH_2CH_2CH_2$
(10) R = $R^1 = PhCH_2CH_2CH_2$, $R^2 = R^3 = R^4$ = H
(11) R = $R^1 = R^3 = PhCH_2CH_2CH_2$, $R^2 = R^4$ = H
(12) R = $R^1 = R^2 = R^3 = PhCH_2CH_2CH_2$, R^4 = H
(13) R = $R^1 = R^2 = R^3 = R^4 = PhCH_2CH_2CH_2$



(14) $R^1 = PhCH_2CH_2CH_2$, $R^2 = R^3 = R^4$ = H
(15) $R^1 = R^3 = PhCH_2CH_2CH_2$, $R^2 = R^4$ = H
(16) $R^1 = R^2 = R^3 = PhCH_2CH_2CH_2$, R^4 = H

Scheme 2. (i) $PhCH_2CH_2CHO$, $NaBH(OAc)_3$, CH_2ClCH_2Cl; (ii) H_2, Pd/C, EtOH.

described by Sullivan and Walsh.[16] The effects of synthetic compounds on the rate of reduction of trypanothione by *T. cruzi* TR were assayed spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.[17] Enzyme activity was measured at 23 °C in HEPES buffer (100 mM, pH 7.25) containing EDTA (1 mM), NADPH (0.18 mM) and oxidized trypanothione, with a TR concentration of 2.2 μg/mL. For each compound assayed, the velocity of the TR-catalyzed reaction was measured at five trypanothione concentrations (15–75 μM) and five inhibitor concentrations.

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 compounds tested showed linear competitive inhibition against TR reduction of trypanothione. For each inhibitor concentration, $K_{m(obs)}$ (with standard deviation) and V_{max} were determined from a nonlinear regression analysis of the plot of v against $[S_o]$ using the Michaelis–Menten equation. The K_i value was then determined from a weighted regression analysis of the plot of $K_{m(obs)}$ against [I] using the equation below (weighting factors used were 1/(SD of $K_{m(obs)}$)):

$$K_{m(obs)} = (K_m[I]/K_i) + K_m$$

The K_i values for each compound and their standard deviations are given in Table 1. None of the compounds tested caused the TR-mediated oxidation of NADPH in the absence of trypanothione. Thus, as expected, none of the compounds tested were TR substrates. At concentrations of 250 μM, compounds did not inhibit the reduction of glutathione by yeast GR (EC 1.6.4.2), assayed using similar conditions to those described with TR (due to the limited solubilities of **12** and **13** in water, the effects of these compounds against GR were measured at 20 μM). Hence, inhibitors show specificity for TR with respect to GR.

The in vitro trypanocidal activities of compounds on bloodstream forms of clinically isolated strains of *T. b. brucei* ssp. were examined using a standard growth screen.[18] The trypanosome strains used were *T. b. brucei* Lab EATRO and three clinical isolates of *T. b. rhodesiense*. The in vitro trypanocidal activities are given in Table 2.

For each compound studied, inhibition of TR increased with increasing numbers of 3-phenylpropyl substituents, with N^1,N^1,N^4,N^8,N^{12}-penta(3-phenylpropyl)spermine (**12**) being the most effective inhibitor studied. However, the hexa-substituted derivative (**13**) was 1.5 orders of magnitude less effective than **12**, suggesting that the TR active site cannot effectively accommodate the steric bulk of **13**. The location of the substituents also impacted the inhibiting activities of compounds. Compounds with substituents at N^1 and N^{12} were less effective inhibitors than corresponding analogues with N^4 and N^8 substituents, for example, compound **15** was not as effective as **1**. The inhibition profiles of the spermidine analogues studied were similar to those of spermine analogues with the corresponding number of 3-phenylpropyl substituents, that is compounds **4** and **1** have similar K_i values. Therefore, for this class of compounds, the number of hydrophobic substituents has a greater influence on the binding of inhibitors to the active site of TR than the net charge of the inhibitor. Although, TR and GR have similarities, the compounds studied did not inhibit GR. These observations can be rationalized by comparing the electrostatic and steric characteristics of the active sites of TR and GR. The active site of TR is large, contains a glutamate residue (E19) and a

Table 1. K_i values for the competitive inhibition by compounds of trypanothione disulfide reduction by recombinant TR from *T. cruzi*

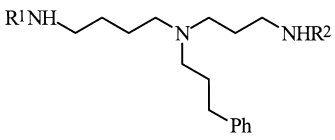
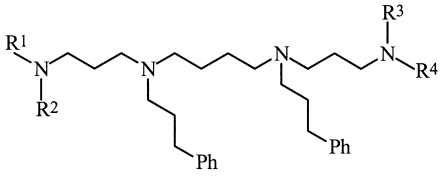
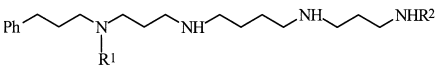
Compound	K_i (μM)±SD
	
3 $R^1 = R^2 = H$	14.1±0.9
4 Mixture of two isomers: $R^1 = H$, $R^2 = PhCH_2CH_2CH_2$ and $R^1 = PhCH_2CH_2CH_2$, $R^2 = H$	4.67±1.10
5 $R^1 = R^2 = PhCH_2CH_2CH_2$	3.50±0.34
	
1 $R^1 = R^2 = R^3 = R^4 = H$	3.48±0.38
10 $R^1 = PhCH_2CH_2CH_2$, $R^2 = R^3 = R^4 = H$	1.38±0.30
11 $R^1 = R^3 = PhCH_2CH_2CH_2$, $R^2 = R^4 = H$	0.614±0.147
12 $R^1 = R^2 = R^3 = PhCH_2CH_2CH_2$, $R^4 = H$	0.151±0.032
13 $R^1 = R^2 = R^3 = R^4 = PhCH_2CH_2CH_2$	10.3±4.7
	
14 $R^1 = R^2 = H$	37.6±6.8
15 $R^1 = H$, $R^2 = PhCH_2CH_2CH_2$	12.3±1.7
16 $R^1 = R^2 = PhCH_2CH_2CH_2$	3.62±1.10

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

Compound[a]	IC_{50} (μM)			
	Lab 110[b]	K 243[c]	K 269[c]	K 243-As-10-3[d]
1	0.66	0.79	0.58	0.66
4	0.31	0.53	0.38	0.57
5	0.22	0.36	0.54	0.46
10	0.27	0.16	0.16	0.16
11	0.12	0.14	0.16	0.15
12	3.05	3.35	2.2	5.5
13	22	35	30.5	24
15	0.81	1.5	1.8	2.2
16	0.40	0.16	0.27	0.53

[a]The hydrochloride salts of compounds were used (the trifluoroacetate salt of **1** was used as previously reported[10]).
[b]*T. b. brucei* Lab 110 EATRO 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 *T. b. rhodesiense* KETRI 243.

hydrophobic area, whereas GR has a smaller active site containing several cationic residues (R37, R38, and R347) that are not present in TR.[6,19] The active site of TR can therefore accommodate large, positively charged compounds. The charge of inhibitors is known to be a major factor in their selectivity for TR versus GR.[8] However, charge is not the sole factor involved in the binding of compounds to TR, since spermine does not inhibit TR.[10] The crystal structures of the trypanothione–TR complex and that of the mepacrine–TR complex indicate that hydrophobic interactions between ligands and a hydrophobic wall in the active site (L18, W22, Y111, and M114) play an important role in ligand binding.[19,20] Hence, the hydrophobicity of the 3-phenylpropyl moiety and possibly its ability to participate in cation–π interactions[21] are important contributing factors to the binding of *N*-(3-phenylpropyl)-substituted polyamines to the active site of TR.

Most of the compounds studied also displayed in vitro trypanocidal activities. These activities correlated approximately with each compound's ability to inhibit TR. One factor that possibly may have contributed to discrepancies in the correlation between K_i and IC_{50} values is the presence of polyamine oxidase which occurs in fetal bovine serum (this is required in the culture medium for the blood form of *T. brucei*). However, since polyamine oxidase catalyzes the oxidation of primary amines, it is extremely unlikely that polyamine oxidase would degrade compound **12**. Compound **12** was the major exception to the observed overall correlation, being the most effective inhibitor of TR but one of the less effective trypanocides studied. Another factor that could contribute to disparities in the observed correlation is the possibility that the compounds studied may cross the trypanosomal cell membrane at differing rates.

In conclusion, this paper describes a class of novel, effective TR inhibitors that also display significant trypanocidal activity. Efficient, selective syntheses of the compounds in this study are being developed in order to produce the quantities of compounds needed for in vivo trypanocidal studies.

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