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Peptoid inhibition of trypanothione reductase as a potential antitrypanosomal and antileishmanial drug lead

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Summary. One route to the design of lead compounds for rational drug design approaches to developing drugs against trypanosomiasis, Chagas' disease and leishmaniasis is to develop novel inhibitors of the parasite-specific enzyme trypanothione reductase. A lead inhibitor based on a peptoid structure was designed in the present study based on the known strong competitive inhibition of trypanothione reductase by *N*-benzoyl-Leu-Arg-Arg-β-naphthylamide and N-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy-β-naphthylamide. In the target peptoid the arginyl residues were replaced by alkylimidazolium units and the benzyloxycarbonyl group by the benzylaminocarbonyl function. The peptoid was synthesised using t-butoxycarbonyl protection chemistry and couplings were activated by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. The resulting peptoid was shown to be a competitive inhibitor of recombinant trypanothione reductase from *Trypanosoma cruzi* with a K_i value of 179μM and with only weak inhibition of human erythrocyte glutathione reductase (the inhibition of glutathione reductase was at least 291-fold weaker than of trypanothione reductase).

Keywords: Drug leads – Glutathione – Antiparasitics – Enzyme inhibitors – Rational drug design

Abbreviations: GR, glutathione reductase; GSSG, glutathione disulphide; GSH, reduced glutathione; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; $T[S]_2$ trypanothione disulphide; $T[SH_2]$, reduced trypanothione as dithiol; TR, trypanothione reductase.

Introduction

Trypanosomiasis and Leishmaniasis are major parasitic diseases of the Third World for which current treatments both few and relatively in effective. There

is a major need for new drugs against parasites of the *Trypanosoma* and *Leishmania* species and for novel drug targets against the diseases caused by other organisms. One characteristic of trypanosomal biochemistry, shared by *Leishmania*, is the use of trypanothione as replacement for the glutathione-based redox defence system of the mammalian host (Fairlamb and Cerami, 1992). The mammalian system uses GSH to protect against oxidative damage and the GSSG produced as a result is recycled to GSH by the action of glutathione reductase. In the parasites GSSG is replaced by trypanothione disulfide ($T[S]_2$) which is recycled analogously to trypanothione dithiol ($T[SH]_2$) by the enzyme trypanothione reductase, Scheme 1. As glutathione and trypanothione reductases show almost total mutual substrate non-recognition (Shames et al., 1986), selective inhibition of TR, soon proposed as a drug design target (Fairlamb et al., 1985; Shames et al., 1986), is now widely recognised as a potential drug target for anti-trypanosomal and anti-leishmanial drug design (Benson et al., 1992; Hunter et al., 1992; Krauth-Siegel and Coombs, 1999; Schirmer et al., 1995). Several classes of compound have now been discovered as TR inhibitors and have been reviewed elsewhere (Austin et al., 1999), amongst which are tricyclics (Benson et al., 1992; Chan et al., 1998; Garforth et al., 1997), nifurtimox (Henderson et al., 1988), quinones (Cenas et al., 1994; Henderson et al., 1988), and 2-aminodiphenylsulfides (Baillet et al., 1996; Girault et al., 1997) as well as combinations of the above with polyamines (Bonnet et al., 1997). In addition, it has now been established that trivalent arsenicals (Cunningham et al., 1994), antimonials (Cunningham and Fairlamb, 1995) and Crystal Violet (Moreno et al., 1994) also inhibit TR.

Peptoids are oligomers of N-substituted glycine and have been used as alternatives to natural peptides in the search for biologically active molecules with acceptable properties to serve as potential drug leads, e.g. they are more stable towards proteolysis than their peptide counterparts (Blommaert et al., 1993; Boyle et al., 1994; Dayey and Horwell, 1993; Heizmann and Felder, 1994; Horwell et al., 1987; Hughes and Woodruff, 1992; Siani et al., 1994; Simon et al., 1992; Zuckermann et al., 1994). Peptoids have been found with similar affinities to their corresponding peptide prototypes for bovine pancreatic β-amylase, hepatitis A viral 3C proteinase and HIV transactivator-responsive element RNA (Simon et al., 1992), and have been especially successful in the field of neuropeptide receptor ligands (Horwell et al., 1999). Oligopeptoids are accessible in a relatively straightforward manner from condensation of the corresponding (protected) monomers, either manually or by automated means (Heizmann and Felder, 1994). Several synthetic routes have now been described to the monomers and oligomers used to construct chemically diverse libraries (Wallace et al., 1999; Zuckermann et al., 1994).

We have reported that peptides can provide strong inhibitors of TR, selective over host GR with competitive inhibition kinetics and a K_i value of 13.8μM was determined against *T. cruzi* TR for N-benzoyl-Leu-Arg-Arg-β-naphthylamide, **1** (Garforth et al., 1994). Even stronger inhibition of TR (K_i value 2.4μM) occurs with N-benzyloxycarbonyl-Ala-Arg-Arg-4-methoxy-β-naphthylamide, **2** (McKie et al., 2000). We modelled initial peptoid design on

these leads and used histidine-equivalents at the Arg positions of the original Arg-Arg peptide framework. As the pK_a of N-alkylimidazolium ions is approximately 7.30 (Jencks and Regenstein, 1976), the N-imidazo-peptoid unit has the potential to provide an inhibitor with a cationic side-chain at or near neutrality. This versatility in charge-state of the side-chain was incorporated to allow possible alternative, side chain-neutral, modes of binding to operate and also with the view of bioavailability and membrane

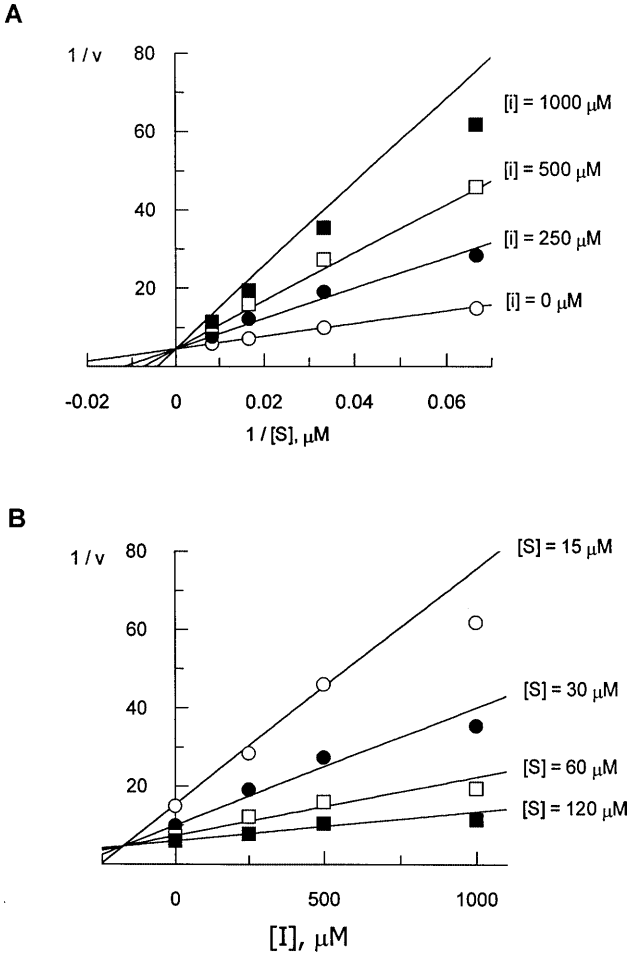


Fig. 1. Diagnostic plots for determination of inhibition type for inhibitor **3**. **A** Lineweaver-Burk plot of 1/V (initial velocity) *versus* 1/[S] (trypanothione disulphide substrate) at pH 7.25, 25°C in 0.02 M Hepes buffer containing 0.15 M KCl, 1 mM EDTA and 0.1 mM NADPH. Points are experimental, lines are theoretical for linear competitive inhibition by **3** with 179 ± 21 μM, V_{max} = 0.220 ΔA/min and K_m = 35.4 ± 5.1 μM. The inhibitor concentrations were 1,000, 500, 250 and 0 μM, respectively. **B** Dixon plot of 1/V *versus* inhibitor (**3**) concentration, [I], at pH 7.25, 25°C in 0.02 M Hepes buffer containing 0.15 M KCl, 1 mM EDTA and 0.1 mM NADPH. Points are experimental, lines are theoretical for linear competitive inhibition with K_i = 179 ± 21 μM, V_{max} = 0.220 ΔA/min and K_m = 35.4 ± 5.1 μM. The trypanothione disulphide concentrations were 120, 60, 30 and 15 μM, respectively

penetration in mind. Consequently, we now report the synthesis of peptoid **3** and studies of its inhibition of recombinant TR from *T. cruzi*.

Materials and methods

Benzylamine and 2-napthoxylacetic acid N-hydroxysuccinimide ester were from Aldrich Chemical Co.; HBTU and N-α-t-Boc-sarcosine N-hydroxysuccinimide ester were from Novabiochem. For coupling reactions, anhydrous reactants and dry solvents were used.

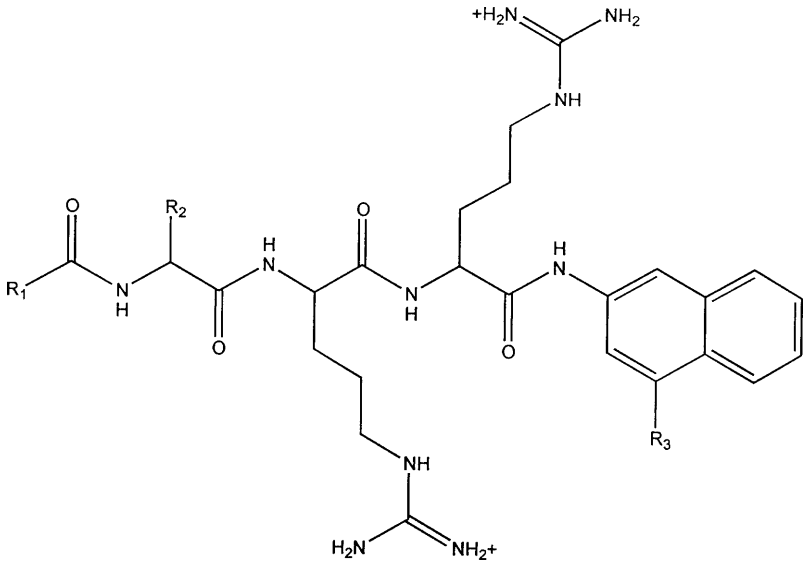


Fig. 2. 1: R_1 = Ph; R_2 = ^{i}Pr; R_3 = H; Benzoyl-Leu.Arg.Arg-β-naphthylamide; **2**: R_1 = $PhCH_2O$; R_2 = Me; R_3 = MeO; Benzyloxycarbonyl-Ala.Arg.Arg-4-methoxy-β-naphthylamide

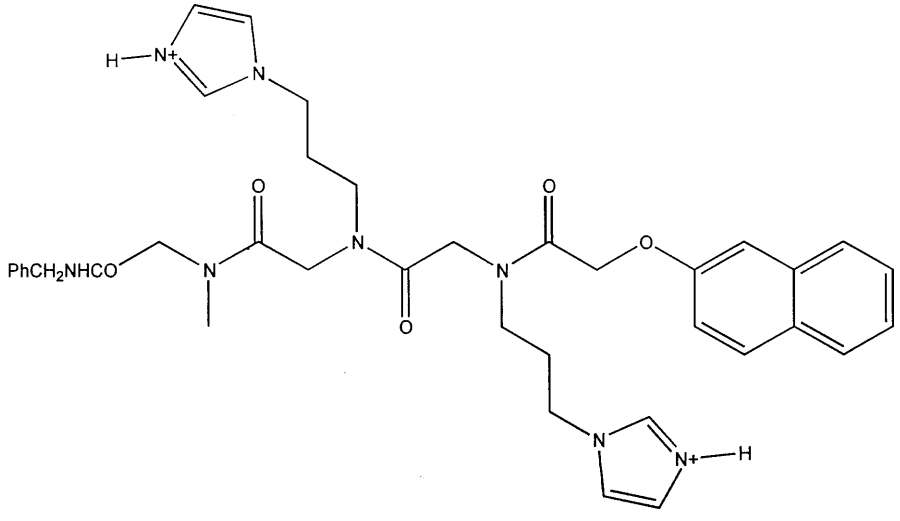
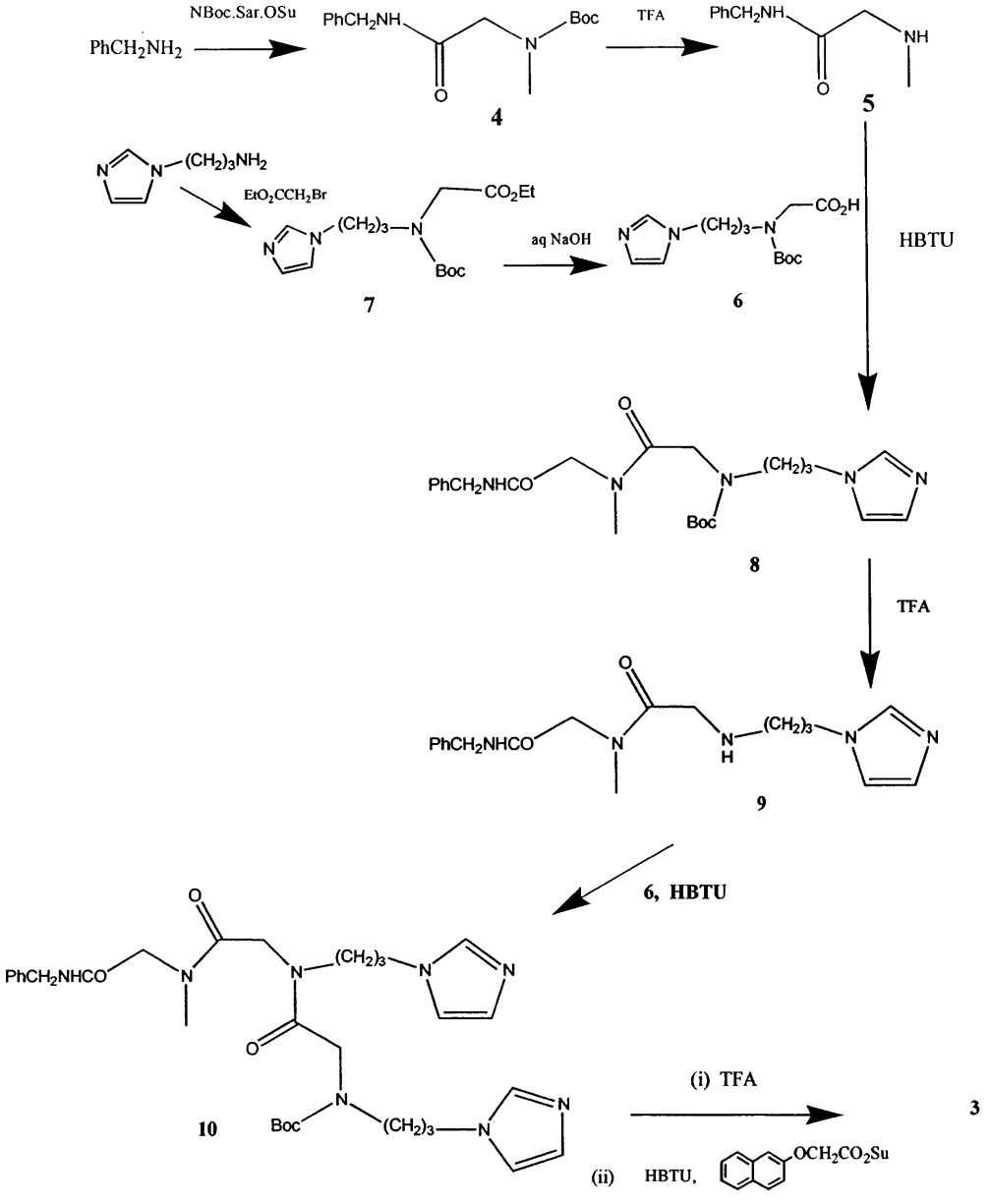


Fig. 3. 3: target peptoid shown as di-imidazolium, protonated form

Tlc was carried out on silica plates. Spots were made visible by UV and light or exposure to iodine vapor. Melting points were taken on a Gallenkamp apparatus with digital thermometer and are uncorrected. NMR spectra were recorded on a Jeol JNM EX 270 spectrometer operating at 270 MHz for ^{1}H. Chemical shifts (δ) were reported in parts per million (ppm) relative to TMS, or the 7.25 ppm residual chloroform peak for ^{1}H nmr spectra. Splitting patterns are abbreviated as: s, singlet; d, doublet; t, triplet; q, quartet; and m, unresolved multiplet. Fast atom bombardment mass spectra (FAB-MS) were taken by means of a Kratos-Concept Instrument operating in the FAB mode (Xe-beam bombardment). Elemental analyses were performed on an EA 1108-Elemental Analyser (Carlo Erba Instruments) in the Department of Chemistry of the University of Manchester.



Scheme 1. Synthesis of peptoid 3

Enzyme isolation, assay and inhibition studies

Trypanothione reductase from *T. cruzi* was isolated by means of overexpression of the gene in *E. coli* JM109 cells bearing the expression vection PBSTNAV (Meinnel et al., 1988) as previously described (Benson et al., 1992). The enzyme was homogenous by the criterion of SDS PAGE and had a specific activity identical to wild-type TR (Krauth-Siegel et al., 1987). Enzyme activity was assayed as 25°C in 0.02 M HEPES buffer, pH 7.25, containing 0.15 M KCl, 1 mM EDTA, 0.12 mM $T[S]_2$ and 0.1 mM NADPH (Krauth-Siegel et al., 1987) at an enzyme concentration of approximately 0.3 μg ml^{-1}. Recombinant human GR was isolated from pUB 302-59 plasmid-carrying *E. coli* SG5 cells as described (Nordhoff et al., 1993) and assayed following literature conditions (Worthington and Rosemeyer, 1974). The GR isolated had a K_m value for GSSG of 67.9 μM and k_{cat} = 1.50 $\times$ 10^4 min^{-1} (cf. literature values of 65 μM and k_{cat} = 9.5 $\times$ 10^3 min^{-1} for recombinant human GR (Nordhoff et al., 1993)). SDS-PAGE showed the enzyme to be close to homogeneous with a subunit molecular weight of 51,800 daltons.

Inhibitor design used a molecular graphics homology model of *T. congolese* TR (McKie, 1993) constructed in this laboratory by modification of the published coordinates of human erythrocyte GR (Karplus and Schulz, 1987). Potential inhibitors were designed to bind *via* specific interactions, hydrogen-bond charge-charge and hydrophobic. Structures were constructed using the CHEMNOTE function of QUANTA. Energy minimisation was performed using CHARMM. Towards the end of the project, the published *C. fasciculata* (Bailey et al., 1993; Hunter et al., 1992; Kuriyan et al., 1991) and *T. cruzi* (Zhang et al., 1996) X-ray diffraction coordinates were used to replace the modelled coordinates and rationalise the results obtained.

Inhibition type was assessed by analysing the patterns of three diagnostic classes of plot: $1/v_o$ versus $1/[S_o]$ for various [I]; $1/v_o$ versus [I] for various $[S_o]$; and $[S_o]/v_o$ *versus* [I] at various $[S_o]$. Values of K_i for competitive inhibition were determined by direct weighted ($1/v_o^2$ for weighting) least squares nonlinear regression analysis of the raw data using the equation for linear competitive inhibition ($v = V_{max}[S_o]/([S_o] + K_m(1 + [I]/K_i))$) using the Grafit programme, (Erithacus Software Ltd), available from Sigma Chemical Co. Values of I_{50}, the concentration required to give 50% inhibition under the assay conditions described above, were determined by interpolation from a plot of assay velocity *versus* inhibitor concentration using Grafit software to fit the data to the equation for linear competitive inhibition suitably rearranged.

Synthesis

The target peptoid (**3**) was synthesised according to Scheme 1.

N-Boc-sarcosine-benzylamide, **4**

To a solution of N-Boc-sarcosine-O-succinimide (10.42 g, 36.59 mmol) and triethylamine (3.7 g, 1 eq.) in dichloromethane (100 ml) was added benzylamine (3.9 g, 1 eq.) and the mixture stirred overnight. The solvent was then removed by rotary evaporation and the residue partitioned between water and ethyl acetate. The aqueous layer was discarded and the organic phase extracted with water followed by dilute hydrochloric acid. It was further washed with 50% sodium bicarbonate, followed by water. The organic layer was dried over anhydrous magnesium sulphate, filtered and the solvent evaporated to afford the desired product (Dado et al., 1992) as a gum (10.32 g, quantitative), used directly in the next step. ^{1}H NMR ($CDCl_3$) δ_H: 7.36–7.25 (m, 5H Ar-H), 6.30 (br, 1H, NH), 4.47 (d, J = 5.6 Hz, 2H, $ArCH_2$), 3.91 (s, 2H, CH_2), 2.94 (s, 3H, NCH_3), 1.41 (s, 9H, t-Bu).

Sarcosine benzylamide, **5**

To a solution of N-Boc-sarcosine-benzylamide (**4**, 10.32 g, 37.12 mmol) in dichloromethane (100 ml) was added trifluoroacetic acid (100 ml) and the resulting mixture stirred at room temperature for 1 hour. The solvent was then removed *in vacuo*, the residue triturated with ether and the product filtered off as a hygroscopic solid in 75% yield. ^{1}H NMR (D_2O) δ_H: 7.23–7.11 (m, 5H, Ph), 4.22 (s, 2H PhC$\underline{H}_2$), 3.70 (s, 2H, C$\underline{H}_2$N), 2.54 (s, 3H, NC$\underline{H}_3$).

N-Boc-(3-imidazolopropyl)-glycine ethyl ester, **7**

To a solution of ethyl bromoacetate (6.6 ml, 59.87 mmol) in dimethylformamide (100 ml) was added triethylamine (6.06 g, 1 eq.) followed by aminopropylimidazole (7.5 g, 1 eq.) and the resulting solution stirred at room temperature overnight. The solvent was removed by rotary evaporator to afford the desired product in a crude mixture, which was taken up in dichloromethane (100 ml), and triethylamine (6.06 g, 1 eq.) and di-t-butylcarbonate (13.06 g. 1 eq.) added. The resulting solution was stirred at room temperature overnight. The solvent was then removed by rotary evaporation and the residue partitioned between ether and water. The organic phase was collected and dried over anhydrous sodium sulphate. After filtration and solvent evaporation, crude product (6.85 g) was obtained and used directly for the next step without further purification.

N-Boc-(3-Imidazolopropyl)glycine, **6**

To a solution of crude **7** (6.85 g) in tetrahydrofuran (88 ml) was added sodium hydroxide (22 ml of 1 M, 1 eq.) and the mixture stirred at room temperature for 4 hours. Water (22 ml) was added and the organic solvent removed by rotary evaporation. The aqueous solution was then extracted with ethyl acetate. After discarding the organic phase, hydrochloric acid (22 ml of 1 M) was added to the aqueous portion and the whole solution freeze-dried to afford crude **4**. Dichloromethane was added to the crude product to precipitate inorganic material. The organic layer was collected and dried over anhydrous magnesium sulphate. After filtration and evaporation of solvent, the product was obtained as a gum (3.2 g, 19% overall from ethyl bromoacetate). ^{1}H NMR ($CDCl_3$) δ_H: 10.40 (brs, 1H, O$\underline{H}$), 8.45 (s, 1H, imid), 7.18 (s, 1H, imid), 7.08 (s, 1H, imid), 4.39 (t, J = 7.9 Hz, 2H, C$\underline{H}_2$ imid), 3.93 (m, 2H, C$\underline{H}_2$, Glyc), 3.47 (t, J = 5.3 Hz, 2H imid, C$\underline{H}_2$, CH_2, C$\underline{H}_2$N), 2.07 (m, 2H, imid, CH_2, C$\underline{H}_2$, CH_2N), 1.46 (s, 9H, t-Bu).

N-Boc-(3-imidazolopropyl)glycyl-sarcosine benzylamide, **8**

To a solution of N-Boc-(3-imidazolopropyl)glycine (**6**, 1.65 g, 5.83 mmol) in acetonitrile (15 ml) was added triethylamine (1.3 g, 2.2 eq.) followed by sarcosine benzylamide trifluoroacetate (**5**, 1.7 g, 1 eq.) and HBTU (2.2 g, 1 eq.). The resulting solution was stirred at room temperature overnight, the solvent evaporated *in vacuo* and the residue partitioned between ethyl acetate and water. The organic layer was washed with 50% sodium hydrogen carbonate and water before drying over anhydrous magnesium sulphate. Filtration and rotary evaporation to remove solvent afforded 8 as a gum (1.35 g, 53%). ^{1}H NMR ($CDCl_3$) δ_H: 7.74–6.63 (m, 8H, 8x = C$\underline{H}$), 4.44 (m, 2H, Ph, C$\underline{H}_2$), 4.05–3.86 (m, 6H, 3xC$\underline{H}_2$), 3.31 (t, J = 6.6 Hz, 2H, NC$\underline{H}_2$), 3.11 (s, 3H, NC$\underline{H}_3$), 2.05 (m, 2H, C$\underline{H}_2$), 1.36 (s, 9H, t-Bu).

N-(3-imidazolopropyl)glycyl sarcosine benzylamide, **9**

This compound was prepared quantitatively by deprotection of **8** by means of trifluoroacetic acid, as described for **5** without trituration with ether. ^{1}H NMR (D_2O) δ_H: 8.62 (s, 1H, imid), 7.38 (s, 1H, imid), 7.32 (s, 1H, imid), 7.24–7.18 (m, 5H, Ph), 4.26–4.19 (m, 4H, 2xC$\underline{H}_2$), 4.04–3.88 (m, 4H, 2xC$\underline{H}_2$), 3.04 (m, 2H, 2xC$\underline{H}_2$), 2.89 (s, 3H, NC$\underline{H}_3$), 2.21 (m, 2H, C$\underline{H}_2$). MS (+ve FAB) m/e: calc. for $C_{18}H_{25}N_5O_2$ 344.2087 (M + 1), found 344.2094 (M + 1).

N-Boc-(3-imidazolopropyl)-glycyl-N-(3-imidazolopropyl)glycyl-sarcosine benzylamide, **10**

This compound was prepared in 27% yield from **9** and **6** using HBTU coupling as described for **8** and using 6 equivalents of triethylamine. ^{1}H NMR ($CDCl_3$) δ_H: 7.51–7.00 (m, 12H, 12xN$\underline{H}$=C$\underline{H}$), 4.42 (m, 2H, PhC$\underline{H}_2$), 4.07–3.74 (m, 10H, 5xC$\underline{H}_2$), 3.41–3.10 (m, 7H, 2xC$\underline{H}_2$, NC$\underline{H}_3$), 2.17–1.91 (m, 4H, 2xC$\underline{H}_2$), 1.42 (s, 9H, t-Bu).

N-(3-imidazolopropyl)glycyl-N-(3-imidazolopropyl)glycyl-sarcosine benzylamide

This compound was prepared quantitatively by deprotection of **10** using trifluoroacetic acid as described for **5**. ^{1}H NMR (D_2O) δ_H: 8.61–8.51 (m, 2H, imid), 7.38–7.28 (m, 4H, imid), 7.24–7.10 (m, 5H, Ph), 4.28–3.72 (m, 12H, 6xC$\underline{H}_2$), 3.28 (m, 2H, C$\underline{H}_2$), 3.06–2.96 (m, 5H, C$\underline{H}_2$NC$\underline{H}_3$), 2.17 (m, 2H, C$\underline{H}_2$), 1.92 (m, 2H, C$\underline{H}_2$). MS (+ve FAB) m/e calc. for $C_{26}H_{36}N_8O_3$ 509.2989 (M + 1), found 509.2992 (M + 1).

N-Naphthoxyacetyl-(3-imidazolopropyl)-glycyl-N-(3-imidazolopropyl)-glycyl-sarcosine benzylamide, **9**

This compound was prepared in 40% yield by coupling N-(3-imidazolopropyl)glycyl-N-(3-imidazolopropyl)glycyl-sarcosine benzylamide from the previous step with naphthoxy-2-acetic acid-N-succinimide as described for **4** using 9 equivalents of triethylamine. ^{1}H NMR ($CDCl_3$) δ_H: 7.75–7.66 (m, 3H, 3xC$\underline{H}$), 7.58–6.84 (m, 16H, NH, 16x = C$\underline{H}$), 4.67 (m, 2H, C$\underline{H}_2$O), 4.40 (m, 2H, Ph, C$\underline{H}_2$), 4.21–3.84 (m, 10H, 5xC$\underline{H}_2$), 3.41–3.20 (m, 4H, 2xC$\underline{H}_2$), 3.02, 2.87 (m, 3H, NC$\underline{H}_3$), 2.16-1.87 (m, 4H, 2xC$\underline{H}_2$). MS (+ve FAB) m/e: calc. for $C_{38}H_{44}N_8O_5$ 693.3513 (M + 1), found 693.3528 (M + 1).

Results and discussion

The target peptoid (**9**) was found to inhibit TR from *T. cruzi* with linear competitive kinetics, the inhibition model being diagnosed by a combination of three plotting methods, viz., $1/v_0$ versus $1/[S_o]$ at various values of [I]; $1/v_o$ *versus* [I] at various $[S_o]$; $[S_o]/v_o$ *versus* [I] at various $[S_o]$. The plots are shown in Fig. 1. The value of K_i was determined as 179 ± 21 μM at 25°C in pH 7.25 0.02 M HEPES buffer containing 0.15 mM KCl and 1 mM EDTA.

Peptoid **3** inhibited human glutathione reductase weakly, but the weakness of the inhibition only allowed estimation of the I_{50} value because of solubility requirements. The percent inhibition detected for 0.5 mM and 1 mM

concentrations of **3** in 0.1 M potassium phosphate buffer at pH 7.0, containing 0.2 M KCl and 1 mM EDTA, 0.1 mM NADPH and 1 mM GSSG were 7.8 ± 0.4 and 15.3 ± 1.9, respectively, from which the I_{50} value was estimated as ≥3.3 ± 0.4 mM by linear extrapolation (as the correct relationship is curved this is very much lower limit). The value of K_i for **3** with human GR can be estimated using the relationship $K_i = I_{50}/(1 + [S_o]/K_m)$ assuming competitive inhibition. The GSSG concentration used in the assay was 1 mM and the K_m was determined as 67.9 μM, giving an estimated minimal value of ≥52 mM for the K_i of **3** with human GR.

On the basis of the measured K_i for **3** with TR and the extrapolated estimate of K_i for human GR, a selectivity ratio of $K_i(GR)/K_i(TR) = 291$ can be calculated.

This is the first example of a peptoid inhibitor for this class of enzyme and the structure of this initial peptoid structure is completely distinct from the peptide backbone of the trypanothione substrate. The structural differences between template peptide structure (**2**) and the peptoid (**3**) cause a change in K_i value by 75-fold. We have carried out superposition comparisons of the low energy conformations adopted by **2** and **3** using molecular modelling methods, but this is not reported in detail here because of the lack of availability of an X-ray structure for a docked peptide structure. The following comparative analysis is based on measured effects on binding strength of structural changes of fragments of the inhibitor framework. The effect of replacement of the N-terminal $PhCH_2OCO$ group of **2** by $PhCH_2NHCO$ in **3** can be estimated approximately from data available from studies with alternative substrates. The effect of this change for substrate action of (N-substituted)-cysteinylglycyl-3,3-dimethylaminopropylamides (Yuen et al., 1999) is to decrease k_{cat}/K_m by 5-fold, with most of this arising from the k_{cat} term rather than K_m which only increases by some 2-fold. There is evidence that the effect of change in K_m values for these substituents reflects binding energy differences (Yuen et al., 1999). Apart from the side-chain alterations discussed below, the other structural alteration is the length of linker attaching the naphthalene site. The major structural compromise made in the design was to incorporate histidine-like side-chains in the peptoid for ease of synthesis and potential for reasonable absorption. The pK_a of the N-alkylimidazolium group is usually approximately 7 (Jencks and Regenstein, 1976), but this is subject to major changes within enzyme active-sites. In comparison, arginine-containing peptides have been found to provide reported inhibitors specific for TR over GR and with K_i values as low as 13.8 μM (Garforth et al., 1994) and 2.4 μM (Austin et al., 1999; McKie et al., 2000). Thus, an arginine-equivalent in peptoid analogues of **3** may improve inhibition substantially. While there are synthetic routes to arginine surrogates for peptoids (Heizmann and Felder, 1994; Murphy et al., 1999), the possession of a permanent dicationic charge may prove problematic in terms of delivery, especially in situations wherein the parasite is in the central nervous system. However, in preliminary tests compound **3** gave 43% inhibition of the growth of the amastigote stage of *T. cruzi*, although it was inactive against *T. brucei* and *Leishmania donovani* (S.L. Croft, personal

communication). Thus, compound **3** provides a specifically inhibitory, lead peptoid amenable to knowledge-guided optimisation, using combinatorial or related approaches.

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