Bioorganic & Medicinal Chemistry 16 (2008) 6689–6695

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

The use of natural product scaffolds as leads in the search for trypanothione reductase inhibitors

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ARTICLE INFO

Article history:
Received 11 April 2008
Revised 28 May 2008
Accepted 29 May 2008
Available online 2 June 2008

Keywords:
Trypanosomatid
Trypanothione reductase
Enzymatic inhibition
Linear competitive inhibitors

ABSTRACT

Twenty-three heterocyclic compounds were evaluated for their potential as trypanothione reductase inhibitors. As a result, the harmaline, 10-thiaisoalloxazine, and aspidospermine frameworks were identified as the basis of inhibitors of *Trypanosoma cruzi* trypanothione reductase. Two new compounds showed moderately strong, linear competitive inhibition, namely *N*,*N*-dimethyl-*N*-[3-(7-methoxy-1-methyl-3,4-dihydro-9*H*-β-carbolin-9-yl)propyl]amine (**15**) and 1,3-bis[3-(dimethylamino)propyl]-1,5-dihydro-2*H*-pyrimido[4,5-*b*][1,4]benzothiazine-2,4(3*H*)-dione (**21**), with K_i values of 35.1 ± 3.5 μM and 26.9 ±1.9 μM, respectively. Aspidospermine (**25**) inhibited *T. cruzi* TryR with a K_i of 64.6 ± 6.2 μM. None of the compounds inhibited glutathione reductase. Their toxicity toward promastigotes of *Leishmania amazonensis* was assessed.

1. Introduction

Flagellated protozoa of the genus *Trypanosoma* and *Leishmania* infect humans causing severe illnesses, including Chagas disease (*Trypanosoma cruzi*) in South America, the sleeping sickness (*T. b. gambiense* and *T. b. rhodiensei*) in Sub Saharan Africa and various forms of leishmaniasis (*Leishmania braziliensis* and *Leishmania amazonensis*) in different parts of the world. A common feature of these parasites is their metabolic reliance on trypanothione reductase (TryR) 1, a unique NADPH-dependent flavoenzyme that helps fight oxidative stress by maintaining adequate levels of trypanothione $T[SH]_2$.[1] In humans, this role is assumed by glutathione reductase (GR), a macromolecule closely related to TryR and responsible for catalyzing the glutathione shuttle, GSSG–GSH, as shown in Figure 1.[1] The structural dissimilarities within the active sites of these enzymes accounts for the observed specificity toward their own substrates.[2–4] This supports the strategy of using selective inhibition of TryR as a feasible approach for the control of such infections. A number of compounds competing against $T[S]_2$ have been shown to inhibit TryR and not GR, yet the structural variability of these available leads is still limited. There is therefore a need to identify new, potent and selective TryR inhibitors for evaluation as less toxic trypanocidal and leishmanicidal agents.

Different heterocycles, particularly tricyclics, covalently linked to various alkylamino functionalities, such as clomipramine **1** (K_i = 7 μM),[5] chlorpromazine **2** (K_i = 10 μM),[6] polyamine derivatives

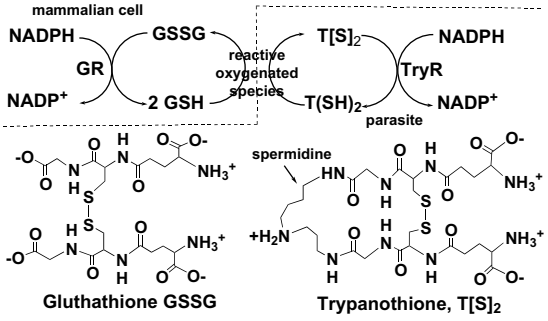

Figure 1. NADPH-dependent reduction of $T[S]_2$ and GSSG by TryR and GR.

of 9,9-dimethylxanthene, **3**,[7] and the anti-malarial pyridoquinoline **4** (K_i = 12 μM),[8] shown in Figure 2, are good examples of competitive inhibitors of TryR. The anti-depressant properties of **2** were diminished in the ring-opened analog **5** (K_i = 1.7 μM).[9] The pendant amino side chain of all of these compounds, that remains protonated at physiological pH, may mimic the spermidine moiety of $T[S]_2$ and can be assumed to be partly responsible for the reported selectivity of these inhibitors.[5] The same could be argued about natural products cepharantine **6** (K_i = 7.6 μM; K_{ii} = 51.6 μM)[10] and N^1,N^2-bis(dihydrocaffeoyl)spermine **7** (K_i = 1.8 μM; K_{ii} = 13 μM),[11] mixed-type inhibitors of TryR, and for lunarine **8**, an irreversible inhibitor (K_i = 144 μM; K_{inact} = 0.116 min^{-1}) discovered with structure-related studies based on the TryR—trypanothione crystal structure.[4]

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doi:10.1016/j.bmc.2008.05.074

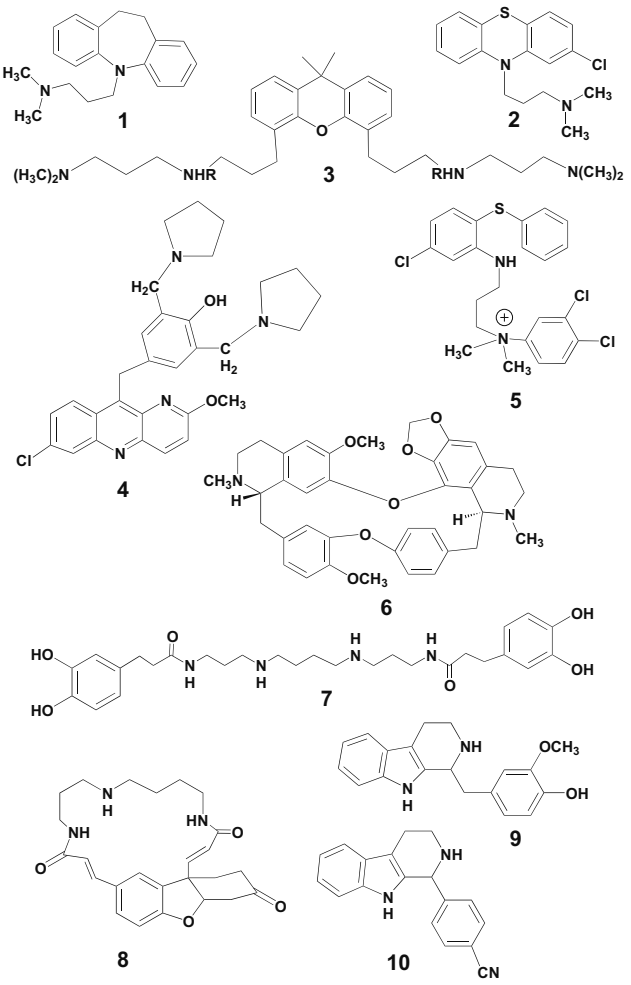


Figure 2. Structures of inhibitors of trypanothione reductase.

Computational models have revealed that the conformation adopted by these tricyclic skeletons at the active site of the enzyme, permits electrostatic interactions between the pendant cationic nitrogen and the negatively charged residues Glu 466′ and Glu467′.[12,13] They also show that the aromatic element of these compounds extends toward a hydrophobic cleft formed by residues Leu 17, Trp 21, Phe 114, Met 113, and Tyr 110. In other studies, it was shown that the introduction of additional aromatic functionality in the pendant side chain, as in compound **5**, improved potency. The rationale behind this strategy was to promote interactions with the hydrophobic residues, Pro 398′, Phe 396′, and Leu 399′, amino acids that collectively constitute an additional hydrophobic surface (Z-site) in the vicinity of the active site.[9,14]

Given the potency and selectivity profile of these inhibitors, a loosely defined putative pharmacophore,[5,12] consisting of a (poly)-cyclic backbone or some ring-opened variant appended to a basic alkylamine moiety, was used in the present studies to prioritize which natural product scaffolds to assess for activity against TryR. This, together with additional criteria described below, was the strategy undertaken to include compounds to identify novel inhibitors of TryR.

Attention was first focused on the tetrahydro-β-carbolines **9** and **10** shown in Figure 2, selected from the 13-member hit list identified by Horvath as virtual inhibitors of TryR.[15] Our interest in this cyclic arrangement was based on the fact that natural products with similar structures, harman, and its analogs, **11**–**14**, Figure 3, have been reported as trypanocidals[16,17] and leismanicidals.[18] Also, the β-carboline skeleton of the harman series was considered to be readily amenable to synthesis and chemical modification. In addition, it was anticipated that, by introducing appropriate basic functionality, the potency of these compounds could be significantly improved. This paper presents the synthesis of new N-alkylated-β-carbolines, **15**–**18**, Figure 3, and compares their inhibition of TryR to that of the related unsubstituted analogs, **11**–**14.**

The pyrimidobenzothiazepine **19** and pyrimidobenzothiazine **20**, in Figure 3, considered as possible isosteres of **1** and **2**, offer the potential to introduce appropriate functionality, such as in

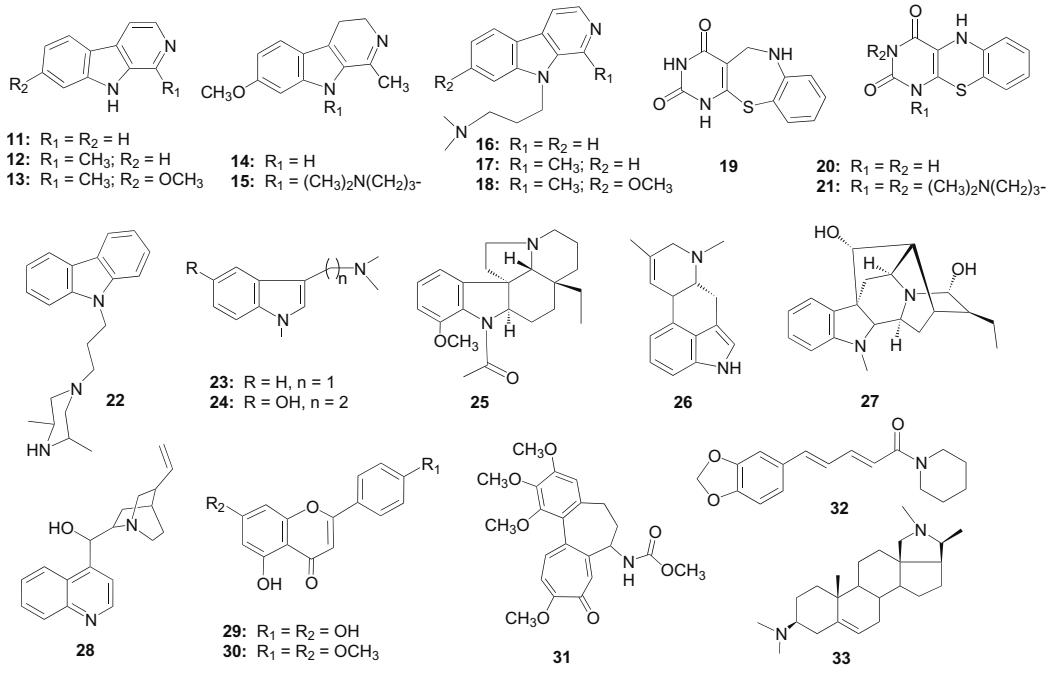
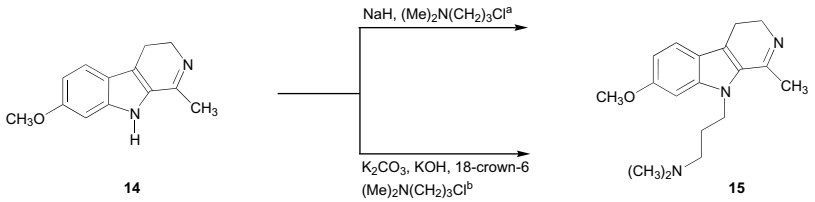


Figure 3. Structures of the compounds tested against recombinant *T. cruzi* TryR.



Scheme 1. Reagents and conditions. Method A[20]: NaH in DMF, 3 h at 55 °C; $(Me)_2N(CH_2)_3Cl$, 12 h at 70 °C; 40% yield. Method B[21]: K_2CO_3, KOH, 18-crown-6 in toluene, 2 h; $(Me)_2N(CH_2)_3Cl$, 1 h reflux; 73% yield.

21, which could induce activity. These compounds were also synthesized and tested versus TryR.

Similarly, a list of natural products was generated for evaluation, including **22**–**28**, Figure 3. All of these compounds have an appropriate aromatic scaffold linked to a basic amine side chain and a site amenable for further functionalization. To complement this rational approach, a number of available natural products (that did not conform to the pharmacophore model just described, yet had known anti-parasitic[19] activity) were considered. This compound set included the flavones **29**–**30**, colchinine, **31**, piperine, **32**, and connesine, **33**, none tested before against TryR.

The anti-protozoal activity of several of the compounds shown in Figure 3 was addressed using promastigotes of *L. amazonensis*.

2. Results and discussion

The N-9-alkylation of commercially available β-carbolines **11**–**14**, was achieved by deprotonating the indolic nitrogen with NaH (Method A, Scheme 1) or with K_2CO_3, KOH, and 18-crown-6 in toluene (Method B), followed by the addition of freshly liberated *N*,*N*-dimethylpropyl chloride. Method A has been previously used to alkylate a similar β-carboline,[20] whereas the solid phase transfer catalysis (SPTC) methodology, route B, employed in the alkylation of carbazole.[21] SPTC proved to be more efficient in terms of reaction time and yield (Scheme 1).

The pyrimidobenzothiazepine **19**[22] and 10-thiaisoalloxazine **20**[23] were prepared following available protocols, whereas **21** was obtained in 55% yield by treating **20** with *N*,*N*-dimethylaminopropylchloride in K_2CO_3 and DMF at 60 °C. Under these conditions, the bis-alkylation was unavoidable.

The compounds in Figure 3 were tested for their inhibitory activity toward TryR at 85 μM, following Fairlamb's microplate alternative protocol[24]; the results are summarized in Table 1.

In the β-carboline series, inhibition by the completely planar heterocycles **11**–**13** was not improved by the protonable aminoalkyl chain attached to their indolic nitrogen (compounds **16**–**18**, Table 1). In contrast, harmaline **14**, in its derivatized form **15**, inhibited the activity of TryR at 85 μM by 50%.

Compound **14** was based on virtual inhibitors **9** and **10,** both of which showed predicted K_i values of 7 μM and 2 μM, respectively.[15] Additional interactions between the pending phenyl rings in **9** and **10** and the amino acids at the modeled active site might be responsible for such predicted activity. This modification could improve affinity of **15.**

Molecules **19** and **20** were examined because of their resemblance to dibenzazepine **1** and phenothiazine **2**. Interestingly, the unsubstituted tricycle **20** was more active (36%) than the simple harman-like compounds, **11**–**13** (10%), justifying its transformation into **21**. The inhibitory profile of this template was enhanced to 62%. In compound **19**, the insertion of a methylene unit between the pyrimidine and the aminophenyl rings cause loss of activity. This may be the consequence of a different relative positioning among these two aromatic groups, affecting its overall binding interaction within the active site.

Table 1
Inhibition of recombinant *T. cruzi* TryR by compounds **11**–**33** at 85 μM concentration

Compound	% Inhibition[a]	Compound	% Inhibition[a]
11	10	**23**	8
12	10	**24**	9
13	11	**25**	43
14	19	**26**	28
15	50	**27**	12
16	20	**28**	18
17	22	**29**	13
18	13	**30**	12
19	10	**31**	8
20	36	**32**	11
21	62	**33**	25
22	4	CP[b]	70

Assay conditions: 40 mM Hepes buffer containing 0.15 M KCl, 1 mM EDTA, 150 μM NADPH, 8μM $T[S]_2$ in the presence of 50 μM DTNB at pH 7.4 and 27 °C.[24] With this protocol, a $K_{m\,app}$ of $T[S]_2$ = 8.3 μM ± 0.9 and $K_{m\,app}$ = of NADPH = 3.7 μM ± 0.7 were obtained.

[a] Data shown is the average of three independent experiments.

[b] Chlorpromazine at 12.2 μM was used as positive control. A K_i value of 3.2 μM ± 0.4 and IC_{50} value of 5.8 μM ± 0.09 were determined following the conditions described in Section 3.

The other planar heteroaromatic structures, including carbazole **22** and simple indoles **23** and **24**, were found to be inactive against TryR, Table 1. In the case of tricyclic skeletons (**11**–**13** and **22**), the angle between the two aromatic entities probably is such that the interactions at the active site diminishes. These interactions are partially lost in molecules **23** and **24** since they lack the third ring. This hypothesis is further supported by the fact that piperazine derivatives of non-planar heterocycles are known inhibitors of TryR,[25] whereas carbazole **23**, identified by Horvath[15] as a weak inhibitor, was completely inactive in our assay (Table 1).

Molecules **25**–**28** contain natural product scaffolds that conform to the pharmacophore model presented. From this group, aspidospermine **25** is the only molecule found moderately active against TryR (43%, Table 1). This skeleton together with condensed heterocycle **26** remain of interest because they are open to chemical manipulation, and many plants containing them are associated with anti-parasitic activity.[26] In the case of inactive molecules **27** and **28**, the hydroxyl groups which are present may affect the lipophilic character required for initial TryR recognition.

Our interest in naringenin **29** and 5-hydroxyflavone **30**, isolated from *Satureja boliviana* and *Lampaya* sp., respectively, was generated by reports that flavonoids are anti-leishmanial and trypanocidal agents.[19,27–29] Similarly, colchinine **31** is a well-known antimalarial compound,[19,30] and piperine **32**, a moderate leishmanicide[31] and strong trypanocidal agent.[32] None of these compounds was active in our TryR assay, Table 1. An unexpected result was obtained with connesine **33**, an anti-amoebic agent,[19] recently reported as an anti-plasmodial compound.[33] The inhibition by this polycyclic, non-aromatic, and lipophilic system was dose dependent: increasing its concentration to 200, 400, and 800 μM resulted in 45%, 73%, and 91% inhibition of TryR, respectively.

The type of inhibition of the herein identified active heterocyclic systems **15**, **21**, and **25** was assessed by plotting (a) $1/v$ versus $1/[S]$ (Lineweaver–Burk plot), (b) $1/v$ versus [I] (Dixon plot), and (c) S/v versus [I] (Cornish–Bowden plot), Figure 4, columns A, B, and C, accordingly. The patterns are indicative of competitive inhibition with K_i values of 35.1 ± 3.5 μM, 26.9 ± 1.9 μM, and 64.6 ± 6.2 μM, respectively.

In addition, the selectivity of these molecules for TryR inhibition was addressed studying their effect over yeast gluthathione reductase[34] at 100 μM. Derivatized harmaline **15** inhibited GR by only 6% whereas no inhibition was detected in the case of **21** or **25**. These are results of three independent experiments.

The parasiticide activity of these compounds was also examined. At this early stage, since TryR occurs in *Trypanosomes* as well as in *Leishmanias*, a simple colorimetric assay based on the reduction of Alamar blue was followed to monitor in vitro growth of promastigotes of *L. amazonensis*,[35] strain readily available to our laboratory. Results are displayed in Table 2.

The growth inhibition effect observed by competitive TryR inhibitor **15** (57% at 100 μM, Table 2) relates well to the IC_{50} reported for unsubstituted harmaline (119 μM in promastigotes of *Leishmania infantum*[36] and 99 μM in promastigotes of *Leishmania mexicana amazonensis*[18]). Therefore, alkylation of the indolic nitrogen of harmaline seems not to diminish its leishmanicidal activity. Compounds **12** and **13** are known to be more potent leishmanicides than harmaline but they are also more toxic to mammalian cells, whereas harmaline has been reported as non-cytotoxic.[36]

The other weak to moderate inhibitors of TryR, **25**, **26**, and **33** were effective over promastigotes of *L. amazonensis*, Table 2, whereas pyrimidinic heterocycles **19**–**21** were inactive. Neither had been assessed previously against *Leishmania*. In the case of lead **25** its IC_{50} value (90.4 ± 0.7 μM) was determined. Since the reported cytotoxicity for this molecule in a human cell line (NIH 3T3 fibroblasts) is 46.2 ± 3.6 μM (IC_{50}),[37] derivatives that would drastically improve the selectivity index are sought for.

The leishmanicide properties of tryptamine analogs related to **23**–**24**,[36] piperine **32**[31] and flavonoids **29**–**30**[28] has been documented elsewhere; hence, they were not studied here.

In conclusion, we have identified three new lead heteroaromatic frameworks (harmaline, pyrimidobenzo thiazine, and aspi-

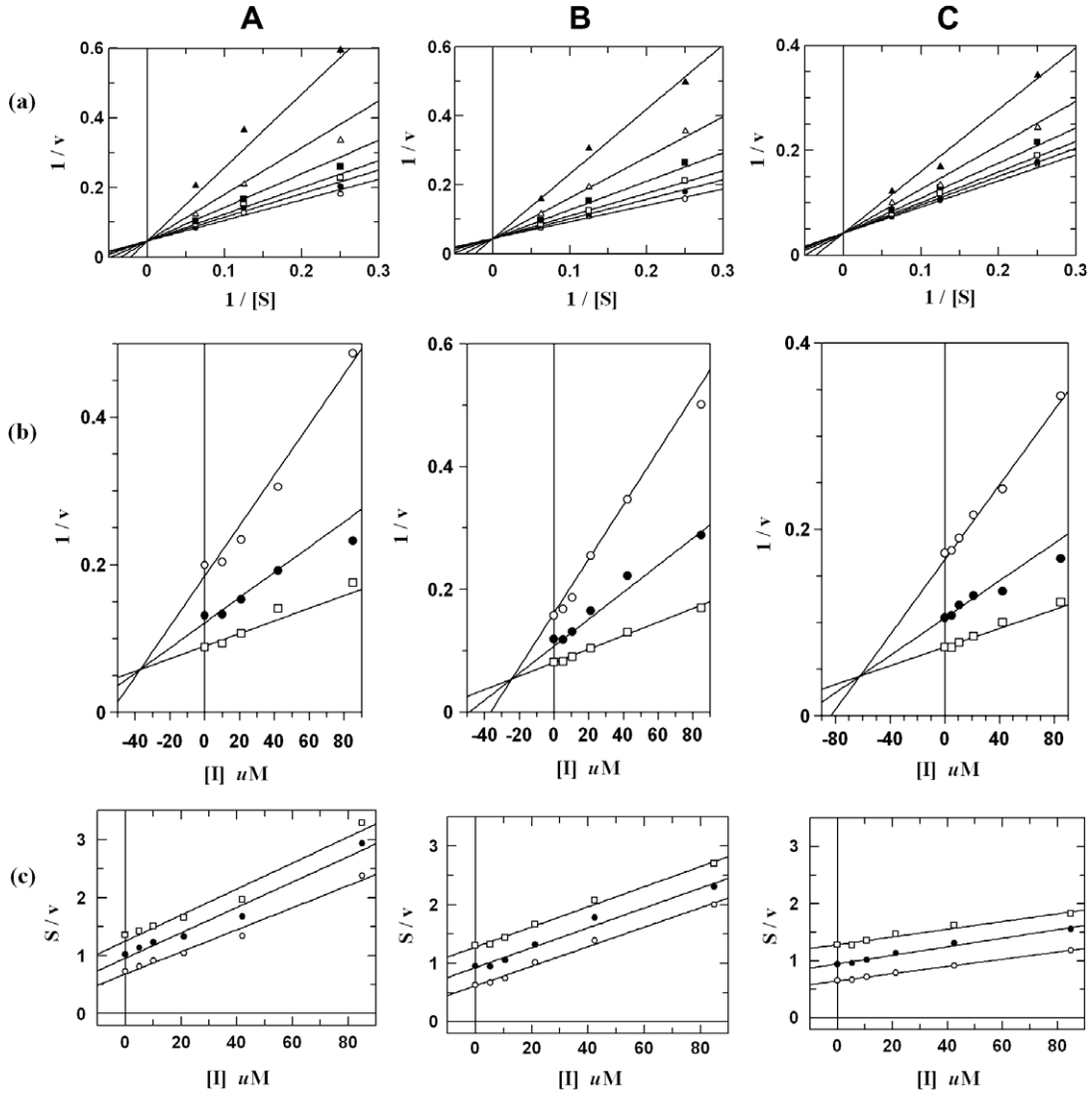


Figure 4. (a) Lineweaver–Burk plot, (b) Dixon plot, and (c) Cornish–Bowden plot showing competitive inhibition of *T. cruzi* TryR by (A) compound **15**, K_i = 35.1 ± 3.5 μM, V_{max} = 19.4 × 10^{-3} ΔA/min and K_m = 10.8 ± 1.3; (B) compound **21**, K_i = 26.9 ± 1.9 μM, V_{max} = 20.6 × 10^{-3} ΔA/min and K_m = 9.4 ± 0.8 and C, compound **25**, K_i = 64.6 ± 6.2 μM, V_{max} = 21.8 × 10^{-3} ΔA/min and K_m = 10.8 ± 0.9. The assays were run in 96-well microplates at pH 7.4, 27 °C, in the presence of TryR (2 mU/mL), DTNB (50 μM), Hepes 40 mM, EDTA 1 mM,[24] using $T[S]_2$ as the variable substrate, 4 μM (○), 8 μM (●), 16 μM (□) and inhibitor at 0 μM (○), 5 μM (●), 10 μM (□), 21 μM (■), 42 μM (Δ), 85 μM (▲). The reaction was initiated with NADPH (150 μM) and monitored at 412 nm. The reported values are the average of three independent experiments.

Table 2
In vitro susceptibility of *L. amazonensis* promastigotes (MHOM/BR/76/LTB-012) to compounds tested (100 μM), evaluated using Alamar blue®

Compound	**15**	**19**	**20**	**21**	**25**	**26**	**27**	**28**	**31**	**33**	Control
% Growth inhibition[a,b]	57	14	15	11	89	70	5	51	6	97	86[c]

[a] Percentage of growth inhibition = $[1 - (\%\text{Red})_{\text{Inh}}/(\%\text{Red})_{\text{control}}] \times 100$ where % Red = $[A_{570\text{nm}} - A_{600\ \text{nm}} \times R_0] \times 100$ with $R_0 = A_{(\text{AB})ox570\text{nm}}/A_{(\text{AB})ox600\text{nm}}$.
[b] The values are average of three independent experiments.
[c] Pentamidine at 1 μM was used as positive control of this in vitro assay. Its IC_{50}, 0.29 ± 0.003 μM, was obtained following the procedure indicated in Section 3.

dospermine) as linear competitive inhibitors of recombinant *T. cruzi* TryR. In doing so, we have corroborated experimentally Horvath's predictions with respect to the β-carboline skeletons. We have also shown that it is worth to pursue further studies due to their selectivity for TryR and for their leishmanicide activities. Work is currently in progress in our laboratory to optimize the potency of the herein identified anti-leishmanial inhibitors of TryR.

3. Experimental

Melting points were determined using a Gallenkamp capillary apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AC-300 MHz instrument in $CHCl_3$-d_1, unless otherwise indicated. Chemical shifts are given in δ (ppm). The assignments of all exchangeable protons were confirmed by the addition of D_2O. Accurate mass spectra were recorded on a Voyager MALDI–TOF (Applied Biosystems) and ABI-Q-star (ESI-Quadrupole-TOF) LC–MS–MS instruments, in the positive mode. Low resolution spectra were performed on a GC–MS Hewlett-Packard 5890 mass spectrometer electron ionization mode (EI(+)). All reactions were monitored by thin-layer chromatography performed on aluminum-backed silica gel 60 F_{254} plates. Silica gel 60, particle size 40–63 μm (mesh 230–400) was used for flash chromatography. Anhydrous DMF and all other reagents were purchased from Sigma–Aldrich Chemicals. The solvents were reagent quality and used without further purification. The pyrimidobenzo thiazepine **19**[22] and pyrimidobenzothiazine **20**[23] were synthesized following available procedures. The data obtained was consistent with the previously reported values. Compounds **23**–**33** were donated by Dr. Manfred Reinecke from Texas Christian University. All of them, except naringenin **29** and 5-hydroxyflavone **30**, are commercial products.

3.1. Chemistry

3.1.1. *N,N*-Dimethyl-*N*-[3-(7-methoxy-1-methyl-3,4-dihydro-9*H*-β-carbolin-9-yl)propyl] amine (15)

Method A.[20] To a solution of harmaline **14**, (0.1 g, 0.5 mmol) in anhydrous DMF (1 mL), NaH (60%, 0.07 g, 1.8 mmol) was added under N_2 atmosphere and heated at 55 °C for 3 h. Freshly liberated 3-chloro-1-(*N,N*-dimethylamino)propane (2 mmol in 1.5 mL of toluene) was added dropwise. The reaction was kept at 70 °C for 12 h. The progress of the reaction was followed by TLC. The residual NaH was consumed with drops of EtOH. The mixture was acidified with 1% HCl and washed with toluene (3× 15 mL). The aqueous phase was basified to pH 8.5 and the product extracted with CH_2Cl_2 (3× 30 mL). The organic phase was collected, dried over $MgSO_4$, filtered, and concentrated to dryness. The crude oil was purified by flash chromatography with $CH_3COOC_2H_5/CH_3OH/(C_2H_5)_3N$, 7:3:0.4 as the eluting solvent (R_f 0.32) to yield 0.06 g (40%) of a yellow-green pure oil. ^{1}H NMR δ: 1.79 (m, 2H, CH_2), 2.09 (t, 2H, J = 6.6 Hz, $CH_2N(CH_3)_2$), 2.11 (s, 6H, $N(CH_3)_2$), 2.45 (s, 3H, $ArCH_3$), 2.69 (t, 2H, J = 8.1 Hz, CH_2), 3.65 (t, 2H, J = 7.6 Hz, CH_2), 3.82 (s, 3H, $ArOCH_3$), 4.27 (t, 2H, J = 7.2 Hz, CH_2N_{indol}), 6.71–6.76 (dd overlapped with s, 2H, HAr), 7.39 (d, J = 9.2 Hz, 1H, HAr); ^{13}C NMR (75.47 MHz) δ: 19.97, 24.73, 28.55, 42.81, 45.53, 47.57, 55.81, 56.37, 93.51, 110.93, 119.22, 119.89, 121.14, 130.22, 139.70, 158.20, 158.61. MS (ES) m/z 300.2056 $[M+H]^+$ (calcd for $C_{18}H_{26}N_3O$: 300.207039; error 4.79 ppm). [For an alternative route see below.]

3.1.2. *N,N*-Dimethyl-*N*-[3-(9*H*-β-carbolin-9-yl)propyl]amine (16) following Method A as described for (15)

Norharmane, **11**, (0.3 g, 1.8 mmol), NaH (0.4 g, 10 mmol), DMF (2.6 mL), and 3-chloro-1-(*N,N*-dimethylamino) propane (10 mmol in 1 mL of toluene). Reaction time, 12 h. Flash chromatography ($CH_3COOC_2H_5/CH_3OH/(C_2H_5)_3N$, 7.5:2.5:0.4; R_f 0.35). Yellow oil (0.23 g, 50% yield). ^{1}H NMR δ: 1.99 (m, 2H, CH_2), 2.16 (s, 6H, $N(CH_3)_2$), 2.21 (t, 2H, J = 6.6 Hz, $CH_2N(CH_3)_2$), 4.40 (t, 2H, J = 6.8 Hz, CH_2N_{indol}), 7.21 (t, 1H, J = 6.6 Hz, HAr), 7.49 (m, 2H, HAr), 7.88 (d, 1H, J = 5.2 Hz, HAr), 8.06 (d, 1H, J = 7.7 Hz, HAr), 8.38 (d, 1H, J = 5.5 Hz, HAr), 8.9 (s, 1H, HAr); ^{13}C NMR (75.47 MHz) δ: 26.90, 41.01, 45.30, 56.38, 109.79, 114.72, 119.81, 121.22, 122.04, 128.51, 128.57, 132.33, 136.70, 138.99, 141.38. GC–LRMS (EI) m/z $[M^+]$ 58, 181, 195, 207, 253; MS (MALDI) m/z 254.1640 $[M+H]^+$ (calcd for $C_{16}H_{20}N_3$: 254.1652; error 4.59 ppm).

3.1.3. *N,N*-Dimethyl-N-[3-(1-methyl-9*H*-β-carbolin-9-yl) propyl]amine (17)

Method B.[21] To a suspension of K_2CO_3 (23 mg, 0.16 mmol), KOH (17 mg, 0.3 mmol), and 18-crown-6 (7 mg, 0.03 mmol) in toluene (2.5 mL), harmane, **12** (50 mg, 0.3 mmol) was added under N_2 atmosphere. After 2 h stirring at rt, freshly liberated 3-chloro-*N,N*-dimethylpropylamine (70 mg, 0.44 mmol in 2 mL of toluene) was added dropwise. The suspension was refluxed for 2 h and then filtered. The filtrate was concentrated and washed with saturated KCl (3× 5 mL). The product was extracted with 15% HCl (3× 6 mL). The aqueous layer was basified to pH 8.5 and the product recovered with CH_2Cl_2 (3× 12 mL). The organic layer was concentrated to dryness to obtain an orange-yellow oil, purified by flash chromatography ($CH_3COOC_2H_5/CH_3OH/(C_2H_5)_3N$, 9.4:1.2:0.34; R_f 0.3) to yield 68 mg (85%) of a clear yellow oil. ^{1}H NMR δ: 1.99 (m, 2H, CH_2), 2.24 (s, 6H, $N(CH_3)_2$), 2.34 (t, 2H, J = 7.0 Hz, $CH_2N(CH_3)_2$), 3.07 (s, 3H, $ArCH_3$), 4.63 (t, 2H, J = 7.4 Hz, CH_2N_{indol}), 7.24 (td, 1H, J = 6.63 Hz, J = 1.5 Hz, HAr), 7.52–7.61 (m, 2H, HAr), 7.84 (d, 1H, J = 5.5 Hz, HAr), 8.12 (d, 1H, J = 7.7 Hz, HAr), 8.32 (d, 1H, J = 5.2 Hz, HAr); ^{13}C NMR (75.47 MHz) δ: 23.63, 28.97, 42.92 45.60, 56.66, 110.03, 113.11, 119.79, 121.45, 121.60, 128.29, 129.32, 135.29, 138.12, 141.50, 141.79. GC–LRMS (IE) $[M]^+$ m/z 267, 209, 196, 58; MS (MALDI) m/z 268.1805 $[M+H]^+$ (calcd for $C_{17}H_{22}N_3^+$: 268.1808; error 1.16 ppm).

3.1.4. *N,N*-Dimethyl-*N*-[3-(7-methoxy-1-methyl-9*H*-β-carbolin-9-yl)-propyl]amine (18) following Method A

Harmine, **13** (0.3 g, 1.4 mmol), NaH (0.6 g, 14 mmol), DMF (2 mL), and 3-chloro-1-(*N,N*-dimethylamino)propane (14 mmol in 10 mL of toluene). Reaction time, 28 h. Flash chromatography ($CH_3COOC_2H_5/CH_3OH/(C_2H_5)_3N$, 9.3/1.0/0.3; R_f 0.35). Orange oil (0.35 g, 84 % yield); ^{1}H NMR δ: 1.92 (m, 2H, CH_2), 2.18 (s, 6H, $N(CH_3)_2$), 2.26 (t, 2H, J = 6.8 Hz, $CH_2N(CH_3)_2$), 2.97 (s, 3H, $ArCH_3$), 3.88 (s, 3H, $ArOCH_3$), 4.49 (t, 2H, J = 7.4 Hz, CH_2N_{indol}), 6.81 (dd, J = 8.6, 1.8 Hz, HAr), 6.91 (d, 1H, J = 2.2 Hz, HAr), 7.67 (d, 1H, J = 5.5 Hz, HAr), 7.89 (d, 1H, J = 8.8 Hz, HAr), 8.20 (d, 1H,

J = 5.2 Hz, HAr); ^{13}C NMR (75.47 MHz) δ: 23.40, 28.82, 42.79, 45.65 55.80, 56.56, 93.49, 109.15, 112.41, 115.21, 122.44, 129.65, 135.40, 138.26, 140.74, 143.40, 161.05. MS (MALDI) m/z 298.1935 $[M+H]^+$ (calcd for $C_{18}H_{24}N_3O$: 298.1914, error 6.96 ppm).

3.1.5. 1,3-Bis[3-(dimethylamino)propyl]-1,5-dihydro-2*H*-pyrimido[4,5-*b*][1,4]benzothiazine-2,4(3*H*)-dione (21)

Compound **20**[23] (100 mg, 0.44 mmol) and K_2CO_3 (146 mg, 1 mmol) in anhydrous DMF (2 mL) were stirred at 60 °C for 30 min under a N_2 atmosphere. The reaction was allowed to cool, followed by the addition of K_2CO_3 (160 mg, 1.2 mmol) and 3-chloro-*N*,*N*-dimethylpropylamine hydrochloride salt (153 mg, 1 mmol). The reaction was stirred at 60 °C for 6 h. The DMF was removed under high vacuum and the orange residue was purified by flash chromatography ($CHCl_3$/MeOH/Et_3N, 9.6:0.1:0.3; R_f 0.25). The compound was obtained as an orange solid (96 mg, 54% yield): mp 81 °C; ^{1}H NMR (CD_2Cl_2) δ: 1.78 (m, 2H, CH_2), 1.87 (m, 2H, CH_2), 2.22 (s, 6H, $N(CH_3)_2$), 2.24 (s, 6H, $N(CH_3)_2$), 2.36 (m, 4H, $CH_2N(CH_3)_2$), 3.99 (m, 4H, $CH_2N_{pyrimidine}$), 6.28 (s, 1H, NH) 6.76 (dd, 1H, J = 6.6, 1.2 Hz, HAr), 6.89 (t, 1H, J = 6.6 Hz, HAr), 7.04 (dd, 1H, J = 6.3, 1.5 Hz, HAr), 7.12 (t, 1H, J = 6.3 Hz, HAr) ^{13}C NMR (75.47 MHz) δ: 26.04, 27.24, 40.77, 45.26, 45.56, 45.69, 57.03, 57.45, 114.16, 115.56, 116.90, 123.36, 127.68, 127.96, 129.45, 143.39, 150.47, 156.22. MS (ES) m/z 404.2127 $[M+H]^+$ (calcd for $C_{20}H_{30}N_5O_2S^+$: 404.211474, error 3.033 ppm).

Alternative route for *N*,*N*-dimethyl-*N*-[3-(7-methoxy-1-methyl-3,4-dihydro-9*H*-β-carbolin-9-yl)propyl]amine (**15**), following Method B. Harmaline (50 mg, 0.23 mmol), K_2CO_3 (20 mg, 0.14 mmol), KOH (15 mg, 0.26 mmol), 18-crown-6 (6 mg, 0.02 mmol), toluene (2.5 mL), and 3-chloro-*N*,*N*-dimethylpropylamine (0.5 mmol in 2 mL of toluene). Reaction time 1 h. Flash chromatography ($CH_3COOC_2H_5$/CH_3OH/$(C_2H_5)_3N$, 8.6:1.5:0.4; R_f 0.30). 50 mg (73% yield) of a clear yellow oil. The NMR data was identical to **15** obtained by Method A.

3.2. Enzyme assay and inhibition studies

Recombinant *T. cruzi* TryR was kindly donated by Dr. Alan Fairlamb from the University of Dundee, UK. Trypanothione was purchased from Bachem-California, DTNB and *Saccharomyces cerevisiae* GR, from Sigma–Aldrich. Microtitre plate assays were carried out in 96-well plates on the Versamax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). GR inhibition was evaluated with a Perkin-Elmer Lambda 2 UV-Win Lab. Kinetic data analysis was processed using Grafit version 5 (Erithacus Software Ltd, distributed by Sigma–Aldrich, Poole Dorset, UK).

TryR activity was monitored pre-incubating 5 min at 27 °C a mixture of *T. cruzi* TryR (2 mU/mL), $T[S]_2$ (8 μM), DTNB (50 μM) in Hepes 40 mM, EDTA 1 mM, pH 7.4. Reaction was initiated with NADPH (150 μM), and the increase in absorbance at 412 nm was recorded for 5 min. The total volume was 200 μL.[24] Determination of the $K_{m\,app}$ of $T[S]_2$ and NADPH were performed incubating various concentrations of $T[S]_2$ (2–200 μM) and NADPH (16–268 μM), respectively, at the conditions above.

TryR inhibition was measured at 85 μM concentration of the inhibitor, dissolved in DMSO or ethanol. The final DMSO or EtOH content never exceeded 1% in the 200 μL assay. Values of IC_{50} (concentration required to give 50% inhibition) were determined using the four-parameter fitting equation (Grafit). Chlorpromazine was used as a positive control. Its IC_{50} was obtained from 12 different inhibitor concentrations in the range of 0–1000 μM. The inhibition constants (K_i) of **15**, **21**, and **25** were determined at six inhibitor concentrations (0, 5, 10, 21, 42, and 85 μM) with three different concentrations of trypanothione (4, 8, and 16 μM), whereas for chlorpromazine the concentrations used were 0, 3, 6, 12, and 24 μM with 5, 10, and 20 μM of $T[S]_2$. All assays were repeated thrice. The K_i value was calculated through least-squares non-linear regression analysis of the data fitted to the competitive inhibition equation, $v = V_{max}.[S]/K_m\,[1 + [I]/K_i] + [S]$. Values of V_{max} and K_m were obtained from least-squares non-linear regression analysis.

Glutathione reductase activity was assayed at 20 °C with *S. cerevisiae* GR (10 mU/mL), GSSG (1 mM), NADPH (89 μM) in 40 mM potassium phosphate buffer, 200 mM KCl, 1 mM EDTA, pH 6.9, with and without inhibitor (100 μM) in a total volume of 1 mL, according to published procedure.[34] Methylene blue (10 μM, 30% inhibition) was used as a positive control.[34]

3.3. Biological testing

3.3.1. Growth of *L. amazonensis* (strain MHOM/BR/76/LTB-012)

A cloned line of *L. amazonensis* promastigotes cultured at 24.7 °C in M199 (Sigma) with 25 mM Hepes, 2 mM $NaHCO_3$ (pH 7.2) without phenol red and supplemented with 10% heat-inactivated fetal bovine serum (HIFBS, BioWhittaker) were maintained in logarithmic phase by two weekly seeding. This protocol was adapted from a previous report.[38] The doubling time during logarithmic phase was 8–10 h.

3.3.2. In vitro assay for *L. amazonensis* promastigotes

Parasites (1×10^6/mL) were incubated in M199 in the absence or presence of inhibitor (100 μM, dissolved in ethanol or DMSO) at 24.7 ° C for 72 h in 96-well microtiter plates. The final concentration of ethanol or DMSO never exceeded 1% and at this concentration it had no effect on parasite growth. Alamar blue (AB, 20 μL, Bio-Source) was added during the last 6 h[39] of incubation time and viability measured spectrometrically using the Versamax microplate reader at λ_{570nm} and λ_{600nm}. The total final assay volume was 200 μL. The percentage of growth inhibition (GI) was calculated from the decrease (which indicated inhibition) of absorbance (Alamar blue reduction) with respect to the control: $GI = [1 - \%(Red)_{Inh}/(\%Red)_{control}] \times 100$, with $\%\ Red = [A_{570nm} - A_{600nm} \times R_0] \times 100$ and correction factor $R_0 = A_{(AB)ox570nm}/A_{(AB)ox600nm}$.[40] The 50% inhibitory concentration (IC_{50}) values were calculated plotting percentage of reduction against drug concentration using the four-parameter logistic method (Grafit). Pentamidine at 1 μM was used as positive control. The IC_{50} of pentamidine was obtained from 10 different inhibitor concentrations in the range 0–2.1 μM, whereas for **25**, the concentration range was 0–150 μM.

Acknowledgments

We are extremely grateful to K. Douglas (University of Manchester) for his kind support throughout this work. Our thanks goes to A. H. Fairlamb (University of Dundee) for providing the recombinant *T. cruzi* Trypanothione reductase, to M. Reinecke (Texas Christian University) for donating some of the molecules studied and to R. Rieger (Proteomics SUNY-Stony Brook) for the mass spectra analysis. Our appreciation to S. Croft and V. Yardley from the London School of Hygiene and Tropical Medicine for the advice provided at the early stages of our parasite work. Thanks to J.-L. Lemesre (IRD-Montpellier) for the *L. amazonensis* strain and to J. Arevalo and R. Loayza (Instituto de Medicina Tropical *AvH*) for training us in parasite culture maintenance. Financial support was received from The Wellcome Trust Travel Awards 2000, 2002; TWAS 02-051; CONCYTEC: 2002-2003, 441-2004 and PUCP-DAI: 3054, 0198, 3414, and E-032.

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