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Inhibition of Trypanothione Reductase by Substrate Analogues

Elizabeth A. Garrard, Emily C. Borman, Brian N. Cook, Emily J. Pike, and David G. Alberg*

Department of Chemistry, Carleton College, Northfield, Minnesota 55057

dalberg@carleton.edu

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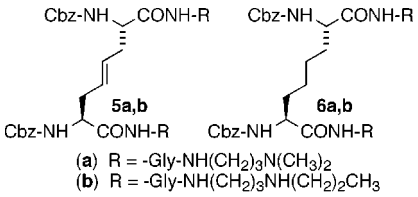
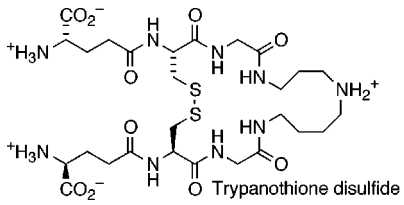
ABSTRACT



Trypanothione reductase (TR) catalyzes the NAPDH-dependent reduction of the spermidine–glutathione conjugate trypanothione, an antioxidant found in *Trypanosomatid* parasites. TR plays an essential role in the parasite's defense against oxidative stress and has emerged as a prime target for drug development. Here we report the synthesis of several trypanothione analogues and their inhibitory effects on *T. cruzi* TR. All are competitive inhibitors with K_i values ranging from 30 to 91 μM.

Trypanothione reductase (TR) is an NADPH-dependent flavoenzyme found in the parasitic protozoa *Trypanosoma* and *Leishmania*.[1] These parasites are responsible for a host of diseases in both humans and domestic animals, including African sleeping sickness (*T. brucei*) and Chagas' disease (*T. cruzi*), among others. To maintain an intracellular reducing environment and to combat oxidative stress, trypanosomatids rely on TR to catalyze the reduction of the antioxidant trypanothione from its disulfide to its dithiol form.[1,2] In the parasites, the trypanothione/TR system appears to serve in place of the related glutathione/glutathione reductase (GR) system found in most other prokaryotes and eukaryotes.[1,3]



Both TR[4] and GR[5] display a high degree of sequence and structural homology, and both catalyze their NADPH-dependent reduction reactions by analogous mechanisms.[3] Despite their similarities, however, the two reductases exhibit almost complete specificity for their respective substrates.[6] This metabolic distinction between the parasites and their hosts, combined with the parasites dependence on TR for growth and virulence,[7] makes TR a promising target for the

(1) Fairlamb, A. H.; Cerami, A. *Annu. Rev. Microbiol.* **1992**, *46*, 695−729.

(2) Fairlamb, A. H.; Blackburn, P.; Ulrich, P.; Chait, B. T.; Cerami, A. *Science* **1985**, *227*, 1485−1487.

(3) (a) Walsh, C.; Bradley, M.; Nadeau, K. *Trends Biochem. Sci.* **1991**, *16*, 305−309. (b) Walsh, C.; Bradely, M.; Nadeau, K. *Curr. Top. Cell. Regul.* **1992**, *33*, 409−417.

(4) For leading references, see: (a) Bond, C. S.; Zhang, Y.; Berriman, M.; Cunningham, M. L.; Fairlamb, A. H.; Hunter, W. N. *Structure (London)* **1999**, *7*, 81−89. (b) Borges, A.; Cummingham, M. L.; Tovar, J.; Fairlamb, A. H. *Eur. J. Biochem.* **1995**, *228*, 745−752. (c) Leichus, B. N.; Bradley, M.; Nadeau, K.; Walsh, C. T.; Blanchard, J. S. *Biochemistry* **1992**, *31*, 6414−6420.

(5) For leading references, see: (a) Schirmer, R. H.; Krauth-Siegel, R. L.; Schulz, G. E. In *Glutathione: Chemical, Biochemical, and Medicinal Aspects. Part A*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; John Wiley and Sons: New York, 1989; pp 553−596. (b) Ghisla, S.; Massey, V. *Eur. J. Biochem.* **1989**, *181*, 1−17. (c) Karplus, P. A.; Schulz, G. E. *J. Mol. Biol.* **1989**, *210*, 163−180. (d) Sweet, W. L.; Blanchard, J. S. *Biochemistry* **1991**, *30*, 8702−8709.

(6) (a) Shames, S. L.; Fairlamb, A. H.; Cerami, A.; Walsh, C. T. *Biochemistry* **1986**, *25*, 3519−3526. (b) Krauth-Siegel, R. L.; Enders, B.; Henderson, G. B.; Fairlamb, A. H.; Schirmer, R. H. *Eur. J. Biochem.* **1987**, *164*, 123−128.

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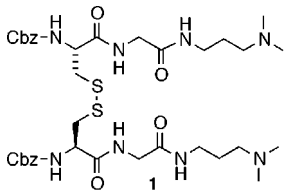
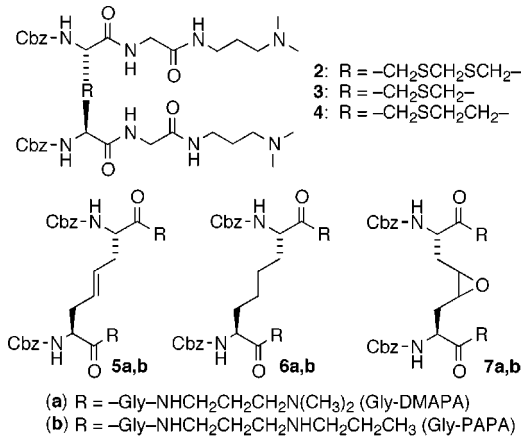
design of antiparasitic drugs.[3] Accordingly, TR has attracted the attention of a number of groups interested in inhibiting this enzyme.[8]

Early substrate specificity studies showed that TR tolerates significant variations in the structure of its substrate. Acyclic substrate analogues incorporating amine-bearing chains in the place of the spermidine group are turned over by the enzyme,[9] as are analogues in which the γ-glutamyl moiety is replaced by various groups, including a simple Cbz moiety.[10] Indeed, we routinely use substrate analogue **1**, reported by El-Waer et al.,[11] as an easily accessible substrate for TR assays.



On the basis of the structure of substrate **1**, Sergheraert and co-workers[12] prepared nonreducible TR inhibitors in which the cystine moiety of **1** is replaced successively by djenkolic acid, lanthionine, and cystathionine (**2**−**4**, respectively). Analogues **2**−**4** all retain sulfur atoms in the bridge connecting the peptidic halves of the molecules. We were interested in exploring similar analogues in which the bridging group is composed exclusively of carbon atoms (**5** and **6)**, with the intent of ultimately using the olefin of **5** as a means of introducing an epoxide moiety, to provide a potential irreversible TR inhibitor (**7**).

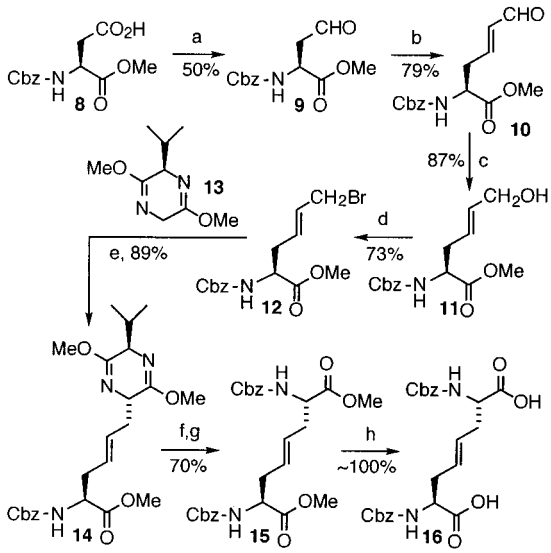


TR is known to be alkylated specifically at Cys-53,[13] an active site nucleophile implicated in an attack on trypanothione's disulfide moiety during the enzymatic reduction, by the thiol-specific reagent iodoacetamide.[6] The enzyme is also covalently inhibited by nitrosourea drugs such as carmustine.[8a] These data suggest that substrate analogues incorporating electrophilic moieties should be potent inactivators of this enzyme.[8,14] We now report the synthesis of reversible TR inhibitors **5** and **6** and the evaluation of the inhibitory activity of these compounds against *T. cruzi* TR. Unfortunately, we have so far been unable to isolate potential epoxide inhibitor **7** because of the instability of its oxirane ring.

Like compounds **1**−**4**, inhibitors **5a** and **6a** contain 3-dimethylaminopropylamide (DMAPA, **a**) groups in place of trypanothione's spermidine moiety. Inhibitors **5b** and **6b**, on the other hand, replace the DMAPA groups with two 3-propylaminopropylamide (PAPA, **b**) chains. While the DMAPA group has seen regular use in trypanothione analogues[9−12,15], we wished to examine the PAPA group, since an acyclic trypanothione analogue with two PAPA chains in the place of the spermidine moiety displays both a lower K_m (92 vs 185 μM) and higher k_{cat}/K_m (more than 2-fold) than its analogue with two DMAPA chains.[9]

Inhibitors **5** and **6** were all synthesized from olefin intermediate **16**. We initially prepared **16** from Cbz-aspartic acid α-methyl ester (**8**), as shown in Scheme 1.[16] Notable

Scheme 1[a]



[a] (a) i. BH_3 (76%), ii. NaOCl, TEMPO (65%); (b) $Ph_3P{=}CHCHO$, toluene, Δ; (c) 9-BBN, THF; (d) CBr_4, PPh_3, CH_2Cl_2; (e) *n*-BuLi, THF, −78 °C; (f) 1:1 0.5 M HCl/THF; (g) Cbz-Cl, TEA, THF; (h) i. 0.5 M LiOH, methanol, ii. aqueous HCl.

transformations include the introduction of the *trans* olefin via Wittig addition of (triphenylphosphoranylidene)acetaldehyde to aldehyde **9**, to afford α,β-unsaturated aldehyde

(7) (a) Dumas, C.; Ouellette, M.; Tovar, J.; Cunningham, M. L.; Fairlamb, A. H.; Tamar, S.; Olivier, M.; Papadopoulou, B. *EMBO J.* **1997**, *16*, 2590−2598. (b) Tovar, J.; Cunningham, M. L.; Smith, A. C.; Croft, S. L.; Fairlamb, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5311−5316. (c) Krieger, S.; Schwarz, W.; Ariyanayagam, M. R.; Fairlamb, A. H.; Krauth-Siegel, R. L.; Clayton, C. H. *Mol. Microbiol.* **2000**, *35*, 542−552.

(8) For reviews, see: (a) Schirmer, R. H.; Muller, J. G.; Krauth-Siegel, R. L. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 141−154. (b) Krauth-Siegel, R. L.; Schöneck, R. *FASEB J.* **1995**, *9*, 1138−1146. (c) Krauth-Siegel, R. L.; Coombs, G. H. *Parasitol. Today* **1999**, *15*, 404−409. (d) Austin, S. E.; Khan, M. O. F.; Douglas, K. T. *Drug Des. Discovery* **1999**, *16*, 5−23.

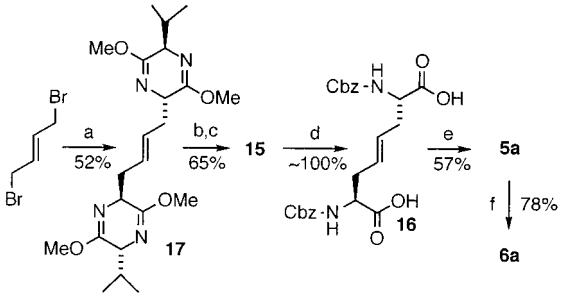
(9) Henderson, G. B.; Fairlamb, A. H.; Ulrich, P.; Cerami, A. *Biochemistry* **1987**, *26*, 3023−3027.

(10) El-Waer, A. F.; Smith, K.; McKie, J. H.; Benson, T.; Fairlamb, A. H.; Douglas, K. T. *Biochim. Biophys. Acta* **1993**, *1203*, 93−98.

10,[17] and the stereospecific alkylation of bromide **12** by the lithiated Schöllkopf dihydydropyrazine **13**, to furnish **14** in 89% yield.[18]

We recently devised a more efficient route to **16**, shown in Scheme 2 along with the elaboration of this olefin to

Scheme 2[a]

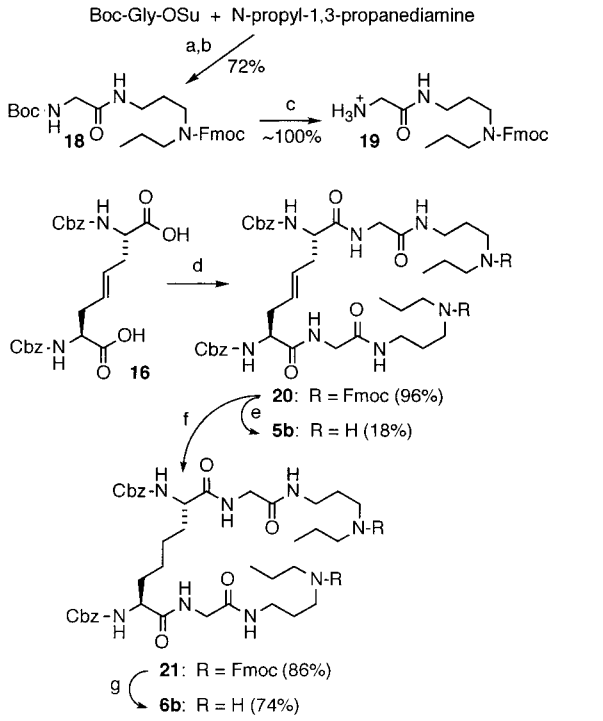


[a] (a) 2.25 equiv **13**, *n*-BuLi, THF, −78 °C; (b) 1:1 0.5 M HCl/THF; (c) Cbz-Cl, TEA, THF; (d) i. 0.5 M LiOH, MeOH, ii. HCl; (e) 2.8 equiv H-Gly-NH$(CH_2)_3$N$(CH_3)_2$, PyBrop, DIEA, CH_2Cl_2; (f) 480 psi H_2, Rh$(PPh_3)_3$Cl, benzene/EtOH.

inhibitors **5a** and **6a**. The double addition of 2 equiv of lithiated Schöllkopf reagent **13** to commercially available *trans*-1,4-dibromobutene gave olefin **17** in 52% yield.[18] Hydrolysis of the bislactim ethers of **17**, followed by treatment of the resulting diamine with 2 equiv of Cbz-Cl, afforded diester **15** in 65% yield for the two steps. Finally, hydrolysis of the methyl esters provided diacid **16** in essentially quantitative yield. PyBrop[19] mediated coupling of 2 equiv of glycine-3-dimethylaminopropylamide[11] to **16** gave inhibitor **5a**. In turn, hydrogenation of a portion of **5a**, in the presence of Wilkinson's catalyst, gave saturated inhibitor **6a** in 78% yield.

The preparation of inhibitors **5b** and **6b** required the synthesis of glycine derivative **19**, shown in Scheme 3. The addition of *N*-propyl-1,3-propanediamine to Boc-glycine *N*-hydroxysuccinimde ester cleanly afforded the corresponding secondary amide, which was treated with Fmoc-Cl to furnish **18** in 72% yield for the two steps. Removal of the Boc moiety under standard conditions provided amine **19**

Scheme 3[a]

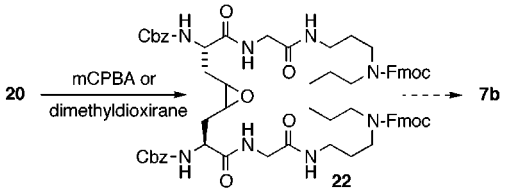


[a] (a) CH_2Cl_2; (b) Fmoc-Cl, DIEA, CH_2Cl_2; (c) TFA, CH_2Cl_2; (d) 2.5 equiv **19**, PyBop, DIEA, CH_2Cl_2; (e) DBU, CH_2Cl_2; (f) 400 psi H_2, Rh$(PPh_3)_3$Cl, benzene/EtOH; (g) diethylamine, CH_3CN.

as its trifluoroacetate salt. PyBop[20] mediated coupling of 2 equiv of amine **19** with diacid **16** afforded **20** in 96% yield. Removal of the Fmoc groups from **20** gave unsaturated inhibitor **5b**,[21] while hydrogenation of **20** in the presence of Wilkinson's catalyst, followed by Fmoc removal, afforded saturated inhibitor **6b**.

We envisioned preparing epoxide **7b** from olefin **20** (Scheme 4). Epoxidation of this olefin with mCPBA ap-

Scheme 4



peared to provide oxirane **22**. However, this compound proved to be very sensitive, and it decomposed during purification attempts. We suspect that decomposition proceeds via intramolecular nucleophilic attack by a glycyl

(11) El-Waer, A.; Douglas, K. T.; Smith, K.; Fairlamb, A. H. *Anal. Biochem.* **1991**, *198*, 212−216.
(12) Tromelin, A.; Moutiez, M.; Meziane-Cherif, D.; Aumercier, M.; Tartar, A.; Sergheraert, C. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1971−1976.
(13) TR amino acid residue numbering is that of the *T. cruzi* enzyme.
(14) One TR-selective covalent inhibitor was recently reported; see ref 4a.
(15) Yuen, C. T.; Garforth, J.; Besheya, T.; Jaouhari, R.; McKie, J. H.; Fairlamb, A. H.; Douglas, K. T. *Amino Acids* **1999**, *17*, 175−183.
(16) This synthesis was inspired by Jurgens' asymmetric synthesis of a diaminopimelic acid derivative, see: Jurgens, A. R. *Tetrahedon Lett.* **1992**, *33*, 4727−4730.
(17) The expected *E*-olefin geometry of **10** was confirmed by the large vinyl coupling constant observed for this material ($J_{CH=CH}$ = 15.6 Hz). No *trans* isomer was observed in the NMR spectrum of this compound.
(18) Schöllkopf, U.; Groth, U.; Deng, C. *Angew. Chem., Int. Ed. Engl.* **1981**, *20*, 798−799.
(19) PyBrop = bromotripyrrolidinophoshonium hexafluorophosphate. Coste, J.; Frérot, E.; Jouin, P. *Tetrahedron Lett.* **1991**, *32*, 1967−1970.
(20) PyBop = benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate. Coste, J.; Le-Nguyen, D.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 205−208.
(21) Our low yield in this normally high-yielding Fmoc deprotection reaction was the result of accidental loss of material during purification.

amide nitrogen on the epoxide ring, analogous to the well-known tendency of aspartyl β-ester-containing peptides to cyclize to aminosuccinimides.[22] However, oxidation of **20** with dimethyldioxirane[23] did cleanly provide **22** in essentially quantitative yield, but removal of the Fmoc groups from this compound was not possible without decomposition. We now believe that epoxides **7a,b** are unlikely to be stable to the assay conditions, and we are currently exploring structural modifications that would provide for more robust epoxide compounds. One possibility would be to replace the offending glycyl amide moieties with less nucleophilic esters (i.e. replace the glycyl residues with glycolates).

Compounds **5** and **6** were evaluated as inhibitors of recombinant *T. cruzi* TR.[24] TR activity was assayed using **1** as the disulfide substrate and by following the oxidation of NADPH spectrophotometrically at 340 nm.[25] The K_i values for each inhibitor are given in Table 1.

Table 1. Inhibition of *T. cruzi* TR[a]

entry	inhibitor	K_i (μM)
1	**5a**	74 ± 6
2	**5b**	91 ± 6
3	**6a**	30 ± 3
4	**6b**	48 ± 3

[a] Assays were run at 25 °C in 100 mM HEPES (pH 7.8), 1 mM EDTA, and 150 μM NADPH. K_i values for each inhibitor were determined by measuring the initial rates at three different inhibitor concentrations, ranging from 25 to 105 μM, in the presence of five substrate concentrations, varied from 2.5 to 36 μM. The data were fit to the competitive inhibition model using Cleland's COMP program.[27] The K_m value for assay substrate **1** was 6.7 μM.

As expected, **5** and **6** are all modest competitive inhibitors of TR, with affinities comparable to those found for analogues **2**−**4**.[12] We also assessed the abilities of our compounds to inhibit yeast GR. We observed no inhibition of GR by any of our compounds at up to 250 μM concentrations of each ([glutathione] = 30 μM), indicating a decided specificity of our inhibitors for the parasite enzyme.

It appears that TR has a slight preference for the DMAPA chain (**a**) over the PAPA chain (**b**). This finding is somewhat surprising in light of the substrate specificity results of Henderson, et al.,[9] which would suggest otherwise, although those results were obtained with enzyme from *Crithidia fasciculata*. It is also apparent that the TR active site tolerates the different structural geometries of the saturated versus the unsaturated inhibitors, displaying an approximate 2-fold preference for the saturated inhibitors. Crystallography studies indicate that the TR active site is rigid and undergoes little conformational change upon substrate binding.[4a,26] That TR appears to be reasonably tolerant of the geometry of the tether linking the two peptidic halves of our inhibitors, despite the enzyme's apparent rigidity, is reassuring and bodes well for the eventual success of potential epoxide inhibitors when such compounds become available.

Acknowledgment. We thank Professor Christopher Walsh and Kari Nadeau (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School) for providing the SG5 *E. coli* strain containing TR expression vector pIBITczTR. We are grateful for support from a Camille and Henry Dreyfus Foundation Faculty Start-up Grant and a Bristol-Meyers Squibb Company Award of the Research Corporation. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research. A student stipend for E.A.G. was provided by a grant from the Howard Hughes Medical Institute. We also thank Salwa Salah for optimizing the yields of the first three transformations shown in Scheme 2.

Supporting Information Available: Experimental procedures and characterization data for all compounds and experimental details for the enzyme assays. This material is available free of charge via the Internet at http//pubs.acs.org.

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(26) Bailey, S.; Smith, K.; Fairlamb, A. H.; Hunter, W. H. *Eur. J. Biochem.* **1993**, *213*, 67−75.
(27) We also attempted to fit the data to the uncompetitive and noncompetitive inhibition models using Cleland's UNCOMP and NCOMP programs. As expected, the data for each inhibitor either failed to fit these models or gave a fit significantly worse than that obtained with the competitive model. Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103−138.