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Synthesis of Non Reducible Inhibitors for Trypanothione Reductase from *Trypanosoma cruzi*.

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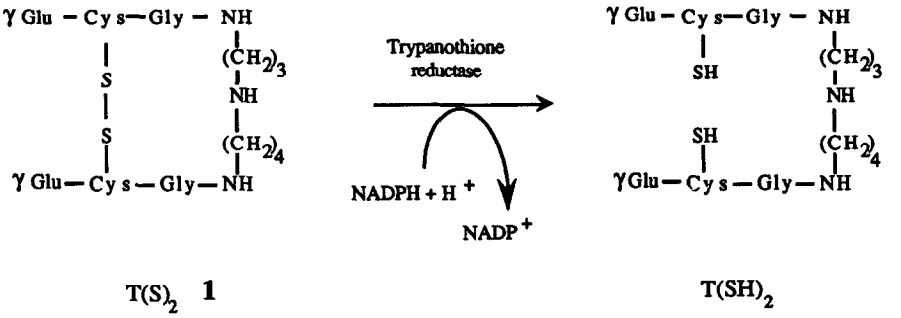
Abstract: A good alternative substrate for the trypanothione reductase from *Trypanosoma cruzi* N,N'-bis(benzyloxycarbonyl)-L-cysteinylglycine 3-dimethylaminopropylamide disulfide, has been modified at the disulfide bridge to obtain non reducible derivatives. Cystine was replaced by three natural diamino diacids (djenkolic acid, lanthionine and cystathionine). The compounds were selective linear competitive inhibitors for trypanothione reductase *versus* trypanothione, its physiological substrate.

In human, *Trypanosoma cruzi* is the agent of the often fatal South American Chagas disease. Today, the drugs used to cure this disease are not specific and therefore fairly toxic to the host himself[1,2]. The need for more rationally designed molecules has led to the evaluation of significant distinctions between the biochemistry of the host and the parasite[3]. Investigation of glutathion (GSSG) metabolism in trypanosomatids has shown that these organisms do not possess a classical GSSG and glutathione reductase (GR) redox based system. They possess instead an analogous system which is based on a glutathione-spermidine conjugate, N^1, N^8-bis(glutathionyl)spermidine called trypanothione ($T(S)_2$) : 1[4]. $T(S)_2$ is maintained in the reduced dithiol form $T(SH)_2$ by a NADPH-dependent, flavoprotein trypanothione reductase (TR) (Fig.1) (for a review, see [5]).



$T(S)_2$ **1** $T(SH)_2$

Figure 1. NADPH-dependent reduction of trypanothione by trypanothione reductase.

As human GR and TR show almost total mutual discrimination toward their respective substrate, it seems possible to design rationally selective TR inhibitors. Strong inhibition of TR which compromises trypanosome's defences against oxydative stress may provide a new drug series[6,7].

Due to the charges at the γ–glutamyl region, trypanothione is an unlikely lead for drug design in terms of bioavailibility ; therefore the polar moiety has been recently substituted for uncharged groups[8,9]. The most efficient replacement was the benzyloxycarbonyl group Z and the corresponding N,N'-bis(benzyloxycarbonyl)-L-cysteinylglycyl-3-dimethylamino-propylamide disulfide **2** was shown to be a good alternative substrate for the TR from *Trypanosoma cruzi* with a better K_m than trypanothione itself (24μM and 55μM respectively).

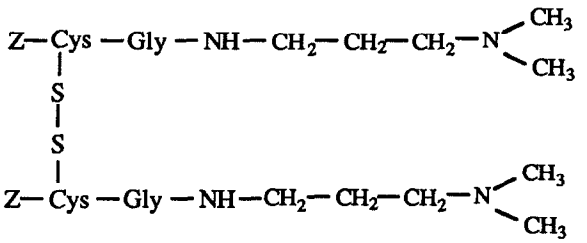


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Figure 2. Structure of N,N'-bis(benzyloxycarbonyl)-L-cysteinylglycyl-3-dimethylamino-propylamide disulfide.

In this paper we report the synthesis and the inhibitory effect of trypanothione analogs for the TR from *Trypanosoma cruzi* which differentiate from **2** at the disulfide bridge moiety. In the new compounds the disulfide bridge ($-CH_2-S-S-CH_2-$) was replaced sucessively by (i) : $-CH_2-S-CH_2-S-CH_2-$ corresponding to the cysteine thioacetal of formaldehyde (djenkolic acid derivative) **3**, (ii) : $-CH_2-S-CH_2-$ (lanthionine derivative) **4**, and (iii) : $-CH_2-S-CH_2-CH_2-$ (cystathionine derivative) **5**. The three compounds were chosen because (a) : they cannot act as substrates, (b) : their affinity compared to **2** can be preserved, (c) : the distance between both peptidic chains is only slightly modified and (d) one or two sulfur atoms can play a recognition role if necessary.

The three analogs **3**, **4**, or **5** were synthesized starting from the protected diamino diacids **8**, **9**[10] or **10** adapted from the procedure previously described by El-Waer *et al.*[8]. Several modifications were made, as depicted in Fig.3.

Z-protected[11] derivatives **8** (from djenkolic acid[12]) and **10** (from cystathionine purchased from Sigma) were obtained according to Zervas[13]. The synthesis of **6** was carried out using BocGlyOH and N,N'-dimethylaminopropylamine according to Hudson's method[14]. The Boc derivative **6** was purified by silica gel chromatography (30g SiO_2 ; eluent : $CH_2Cl_2/CH_3OH/CH_3COOH/H_2O$: 750/270/5/25). Removal of the solvent under reduced pressure yielded a product which was subsequently Boc-deprotected using TFA to afford **7**. This intermediate **7** was then reacted with **8**, **9** or **10**, according to[14]. The silica gel chromatography (30 g SiO_2 ; eluent : $CH_2Cl_2/CH_3OH/CH_3COOH/H_2O$: 70/40/1/9) of the reaction mixture provided a pasty residue which was further purified by RP-HPLC (Preparative Nucleosil C18 column)

using linear gradient A to B over 50 min, then B over 15min (A : H_2O, 0,05% TFA ; B : CH_3CN/ H_2O (50/50), 0,05% TFA), flow rate 1ml/min. The compounds **3**, **4**, or **5**[16] were obtained pure as judged by RP-HPLC (Analytical Nucleosil C18 column, flow rate 0.5 ml/min ; gradient A to B over 30 min, then B over 10 min). Overall yields 20% for **3**, **4** and **5**.

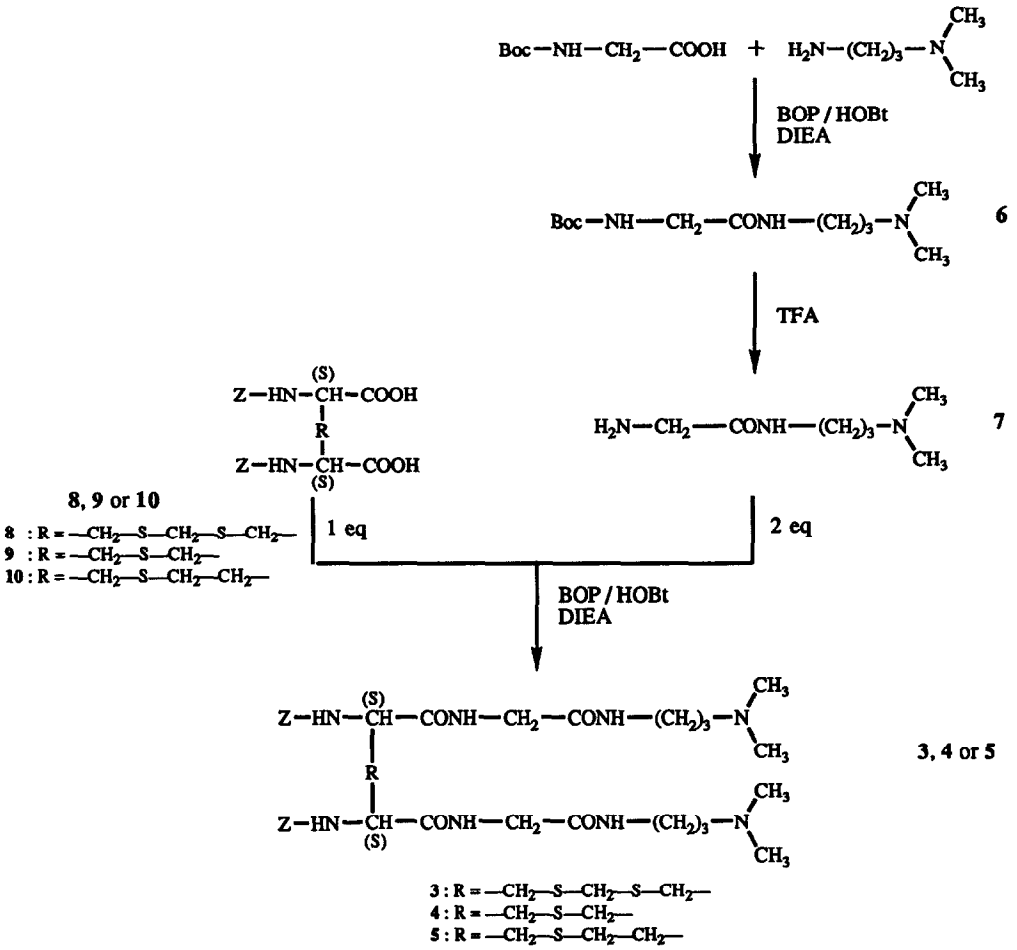

Figure 3. Synthesis of **3**, **4** and **5**.

The inhibitory capacities of these compounds were tested toward recombinant TR from *T. cruzi*. The enzyme was purified as described in[8] and its activity was assayed spectrophotometrically. Trypanothione reductase activity was measured at 25°C in 0.02 M HEPES buffer, pH 7.25, containing 1mM EDTA, 0.15M KCl and 0.15mM NADPH with an enzyme concentration of 0.8µg/ml. The substrate-dependent oxidation of NADPH at 340 nm was monitored as described by Krauth-Siegel *et al.*[15]. The patterns of all

classes of plots indicated that compounds **3, 4, 5** are simple linear competitive inhibitors of TR with its natural substrate trypanothione (Fig. 4 for compound **3**).

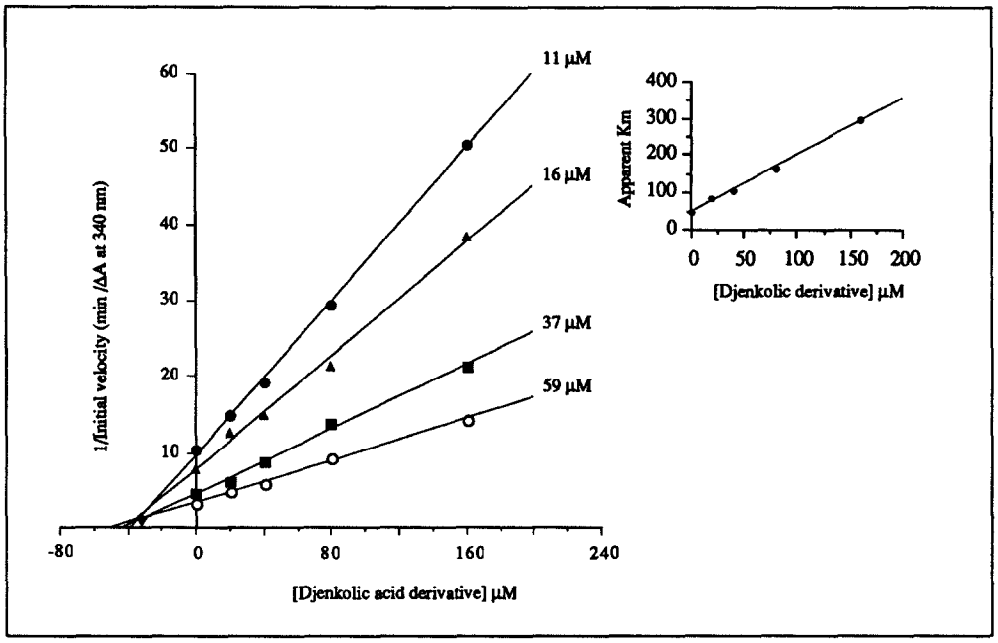

Figure 4. Dixon plot for the inhibition of recombinant TR from *T. cruzi* by compound **3**, using trypanothione as substrate.

The K_i values of the different compounds are reported in Table 1. None of the derivatives inhibited GR activity from bovine intestinal mucosa, assayed in the same conditions as described with TR.

Compound	K_i (µM) (mean ± S.E.M.#)
Djenkolic acid derivative (**3**)	31 ± 4
Lanthionine derivative (**4**)	92 ± 9
Cystathionine derivative (**5**)	43 ± 6

Standard error of the estimate of K_i.

Table 1. Linear competitive K_i values for the inhibition of recombinant TR from *T. cruzi* by compounds **3, 4** and **5**.

Our results confirm that the synthesized compounds are non reducible and therefore they are not substrates for the reaction catalysed by TR.

The observed inhibition *versus* trypanothione indicates that the modifications made do not prevent a good and specific recognition at the active site of TR. No competition at all was observed for GR *versus* GSSG (same buffer as TR).

The djenkolic acid derivative (compound **3**) was the best inhibitor (K_i : 31 μM). The compounds were also tested as competitors against the alternative substrate **2**[8] and showed a similar inhibition potentiality as *versus* trypanothione. These results could be explained by a satisfying conformational freedom in the rigid active site[17]. The inhibitors are likely to undergo the essential induced fit particularly the djenkolic acid derivative **3** which introduces the most important entropic component.

These derivatives are the first non reducible inhibitors (except the melarsen-trypanothione adduct[18]) close to the trypanothione family ; our work is now in progress to modify the disulfide region (sulfur atoms replacement) and analyze the importance of sulfur in the recognition and the inhibition mechanisms.

Acknowledgements

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11. Abbreviations : Boc, *tert*-butyloxycarbonyl ; BOP, benzotriazoloxy-tri-(dimethylamino) phosphonium ; DIEA, diisopropylethylamine ; HOBt, 1-hydroxybenzotriazole ; RP-HPLC, reverse phase high pressure liquid chromatography ; TFA, trifluoroacetic acid ; Z, benzyloxycarbonyl.
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16. ^{1}H NMR (D_2O, 300 MHz)
3 δ 7.45 (10H, s, 2xAr, 2Z), 5.2 (4H, q, $2xCH_2$, 2Z), 4.4 [2H, m, $2xC_\alpha H$, (djenkolic)], 3.9 (4H, br s, $2xC_\alpha H2$, 2xGly), 3.8 [2H, br s , CH_2 (djenkolic)], 3.27 (4H, m, $2x^1CH_2$), 3.1 (4H, m, $2x^3CH_2$), 3.01 to 2.9 [4H, m, $2xC_\beta H_2$, (djenkolic)], 2.85 (12H, s, $4xCH_3$), 1.95 (4H, m, $2x^2CH_2$)
4 δ 7.45 (10H, s, 2xAr, 2Z), 5.3 (4H, q, $2xCH_2$, 2Z), 4.4 to 4.35 [2H, m, $2xC_\alpha H$, (lanthionine)], 3.9 (4H, br. s, $2xC_\alpha H_2$, 2xGly), 3.35 to 3.2 (4H, m, $2x^1CH_2$), 3.2 to 3.1 (4H, m, $2x^3CH_2$), 3 to 2.9 [4H, m, $2xC_\beta H_2$, (lanthionine)], 2.9 (12H, s, $4xCH_3$), 1.9 (4H, m, $2x^2CH_2$)
5 δ 7.45 (10H, s, 2xAr, 2Z), 5.2 (4H, q, $2xCH_2$, 2Z), 4.4 to 4.25 [2H, $2xC_\alpha H$, (cystathionine)], 3.9 (4H, m, $2xC_\alpha H_2$, 2xGly), 3.35 to 3.22 (4H, m, $2x^1CH_2$), 3.15 to 3.05 (4H, m, $2x^3CH_2$), 2.9 (12H, s, $4xCH_3$), 2.75 to 2.6 [4H, m, $2xC_\beta H_2$, (cystathionine)], 2.15 to 2 [2H, m, $C_\varepsilon H_2$, (cystathionine)],1.9 (4H, m, $2x^2CH_2$).
Plasma Desorption Mass Spectrometry (Time of Flight): **3** : 805 (M_{th} = 805) ; **4** : 759 (M_{th} = 759) ; **5** : 773 (M_{th} = 773).
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