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SYNTHESIS OF POLYAMINE DERIVATIVES FOR THE PREPARATION OF AFFINITY CHROMATOGRAPHY COLUMNS FOR THE SEARCH OF NEW *Trypanosoma cruzi* TARGETS

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Abstract: The most potent trypanocidal compound of a series of symmetrically substituted 1,4-bis(3-aminopropylpiperazines) which displayed an IC_{50} value of 5 μM on *Trypanosoma cruzi* trypomastigotes, was inactive on trypanothione reductase. Two derivatives **6** and **12** of this compound, one symmetrical and one dissymmetrical, were synthesized *via* a reductive amination reaction, to prepare affinity chromatography columns, which allowed us to isolate three parasitic proteins. Among these, the major ligand **6**- and **12**-binding protein having an apparent molecular weight of 52 kDa has been identified as the thiol-disulfide oxido-reductase Tc52, previously characterized in *Trypanosoma cruzi*.

Introduction

Trypanosoma cruzi is the agent cause of the often fatal South American Chagas disease. Today, the drugs used to cure this disease are non-specific and therefore potentially toxic to the host.[1,2] Faced with an urgent need to find new treatments for trypanosome infections, trypanothione reductase (TR) has been identified as one of the most promising targets for the design of new trypanocidal drugs.[3-5] Nevertheless, the characterization of new targets remains indispensable to the development of new strategies of both rational drug design and lead drug optimization. Affinity chromatography techniques constitute an efficient tool in the purification of new high affinity ligand-binding proteins.[6]

Structure-activity relationships established in our own library of compounds, both for TR inhibition and trypanocidal effect upon the trypomastigote stage of *T. cruzi*, led us recently, to focus our interest upon 1,4-bis(3-aminopropyl)piperazine derivatives. A series of such compounds was therefore synthesized and evaluated for the activities mentioned above. Surprisingly, the most potent trypanocidal compound of the

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series, **1** (Figure 1), which displayed an IC_{50} value of 5 μM and 90% inhibition at 12.5 μM concentration, was inactive on TR.[7]

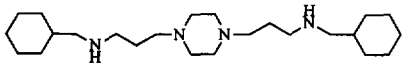


Figure 1. Compound **1**

This absence of correlation between TR inhibition and trypanocidal activity prompted us in the search for possible targets of compound **1** and we report, here, the synthesis of two ligands, one symmetrical and one dissymmetrical, suitable for preparing affinity chromatography columns.

Chemistry

As shown in Figure 2, the anchoring of compound **1** to an *N*-hydroxysuccinimide activated sepharose *via* the primary amino groups of a lysine linker, either at a single terminus or at both extremities to afford a variation in ligand flexibility, was considered. The presence of the lysine moiety provided a basis for load evaluation of the bound ligand by simple amino acid analysis.

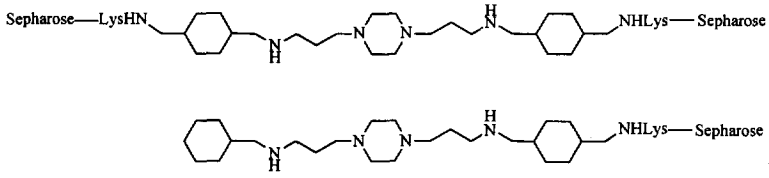
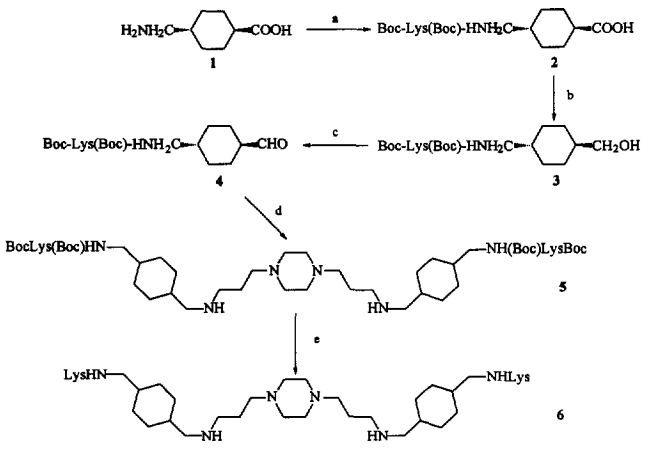


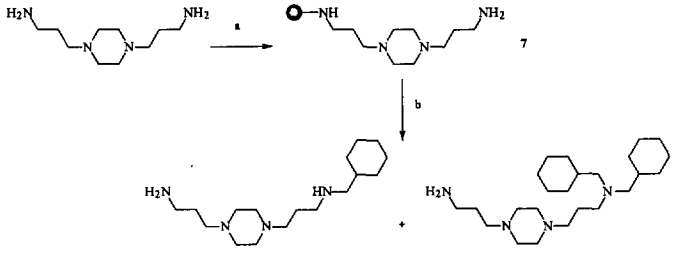
Figure 2. Symmetrical and dissymmetrical anchoring of compound **1** to sepharose

The symmetrical ligand was synthesized as outlined in Scheme 1. Di-*tert*-butyloxycarbonyl lysine was preactivated with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide and then coupled with *trans*-4-(aminomethyl)cyclohexanecarboxylic acid **1** to afford intermediate acid **2**. Then, selective reduction of the carboxylic function into alcohol **3** was achieved with cyanuric fluoride[8] and aldehyde **4** was obtained quantitatively by PCC oxidation on silica gel. Finally, reductive amination of aldehyde **4** carried out with one half-equivalent of 1,4-bis(3-aminopropyl)piperazine, using five equivalents of sodium borohydride, and deprotection of compound **5**[9] with 50% trifluoroacetic acid in dichloromethane gave symmetrical ligand **6**.



Scheme 1. Reagents and conditions: a) 1 eq. Boc-Lys(Boc)-OH, 1 eq. *N*-hydroxysuccinimide, 1 eq. DCC, CH_2Cl_2, 0 to 4°C, 12h, 65%; b) 2 eq. cyanuric fluoride, 1 eq. pyridine, CH_2Cl_2, 0°C, 2h, then 2 eq. $NaBH_4$, MeOH cat., CH_2Cl_2, rt, 5h, 80%; c) 2 eq. PCC/SiO_2, CH_2Cl_2, rt, 5h, 95%; d) 0.5 eq. 1,4-bis(3-aminopropyl)piperazine, molecular sieves, rt, 12h, then 5 eq. $NaBH_4$, CH_2Cl_2, rt, 12h, 40%; e) TFA/CH_2Cl_2-1/1, rt, 2h, 90%.

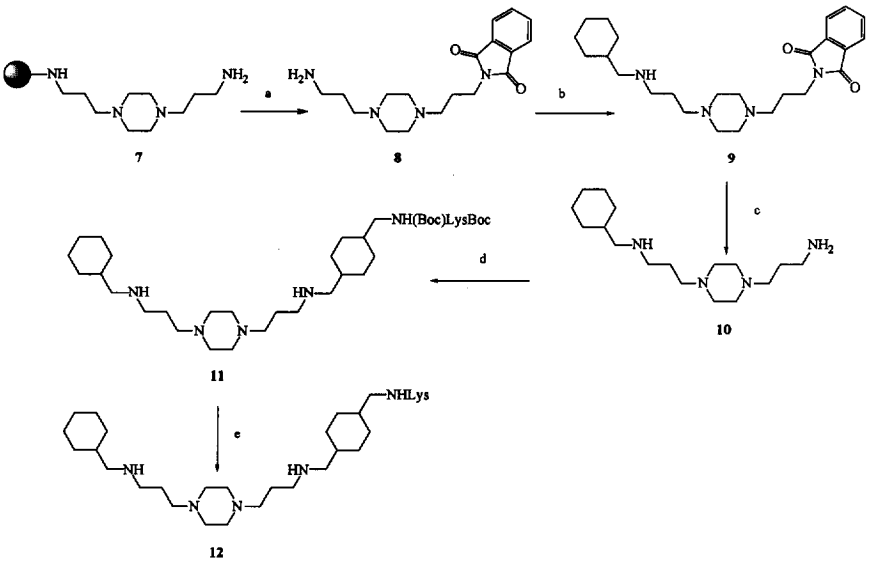
For the preparation of the second ligand, dissymmetry was introduced by the use of 2-chlorotritylchloride resin. Reductive amination of the resulting primary amine **7** with one equivalent of cyclohexanecarboxaldehyde (compared to resin load), and two equivalents of sodium borohydride led, following acidic cleavage from the resin, to the anticipated intermediate but also to the dialkylation product (Scheme 2). Dialkylation which was not observed in the synthesis of the symmetrical ligand, using the more hindered aldehyde **4**, could be due to an over-estimation of the lysine-resin load and therefore to an aldehyde excess.



Scheme 2. Reagents and conditions: a) 0.1 eq. 2-chlorotritylchloride resin, 0.23 eq. DIEA, CH_2Cl_2, rt, 24h; b) cyclohexanecarboxaldehyde (1 eq./resin load), 2 eq. $NaBH_4$, CH_2Cl_2, rt, 12h, then TFA/CH_2Cl_2-1/10, rt, 5 min.

To evaluate accurately the number of amine equivalents allowed to react with cyclohexanecarboxaldehyde and thus to prevent disubstitution, amine **7** was protected as a phthalimide and cleaved from the resin by treatment with 10% trifluoroacetic acid in dichloromethane (Scheme 3). Reductive

amination could be then carried out upon a known quantity of compound **8** by using exactly one equivalent of cyclohexanecarboxaldehyde in absolute ethanol, followed by two equivalents of sodium borohydride. The phthaloyl protecting group was removed using hydrate hydrazine in refluxing ethanol and a second reductive amination was carried out with aldehyde **4**. Dissymmetric ligand **12** was obtained by deprotecting the two lysine amino groups of compound **11**[9] with 50% trifluoroacetic acid in dichloromethane.



Scheme 3. Reagents and conditions: a) 1.5 eq. *N*-ethoxycarbonylphthalimide, CH_2Cl_2, rt, 24h, then TFA/CH_2Cl_2-1/10, rt, 5 min, 50%; b) 1 eq. cyclohexanecarboxaldehyde, molecular sieves, abs EtOH, rt, 12h, then 2 eq. $NaBH_4$, rt, 12h, 55%; c) 10 eq. hydrazine hydrate, EtOH, reflux, 24h, 80%; d) 1 eq. **4**, molecular sieves, abs EtOH, rt, 12h, then 2 eq. $NaBH_4$, rt, 12h, 50%; e) TFA/CH_2Cl_2-1/1, rt, 2h, 95%.

Biological results

Affinity chromatography columns were prepared from activated CH Sepharose 4B gel (*N*-hydroxysuccinimide ester) and from ligands **6** or **12** in $NaHCO_3$ buffer, pH 8.[10] After recovering ligand excess and washings any remaining activated esters groups were blocked by ethanolamine addition. Gel load was determined after acidic hydrolysis and was evaluated respectively to be 27 μmol/g of gel for **6** and 30 μmol/g of gel for **12**.[11]

Passage of epimastigote lysates from *T. cruzi* on both **6**- and **12**-sepharose gels for affinity chromatography and elution with respective ligand solutions[12] enabled us to isolate one major parasitic protein with an apparent molecular weight of 52 kDa (Figure 3). In the second column (ligand **12**), two minor additive

proteins of 32 and 90 kDa were also detected (Figure 3A). Attention was focused upon the major **12**-binding protein. In both cases, we verified, in the TS_2 reduction-assay and by immunoblotting with anti-TR-antibodies,[13] that the parasitic protein of 52 kDa was not trypanothione reductase, whose apparent molecular weight is 54 kDa.[14] Two peptide sequences of the major ligand **12**-binding protein with an apparent molecular weight of 52 kDa in SDS-PAGE, were obtained following proteolysis with endoproteinase lys-C and sequencing by Edman degradation. Comparison of these sequences with database compilations revealed 98% identity with the thiol-disulfide oxido-reductase Tc52, already characterized in *Trypanosoma cruzi*.[6,15] Both peptides could be located in the amino acid sequence deduced from TcAc2 gene product (peptide 108-117 and peptide 342-350).[16] Assignment of both ligand **6**- and **12**-binding protein to Tc52 was confirmed by immunoblotting with antibodies raised against the peptide L6-A22 from TcAc2 gene product (Figure 3B). Tc52 activity of the **12**-binding protein was confirmed in the hydroxyethyl disulfure reduction assay in the presence of glutathione as hydrogen donor.[15]

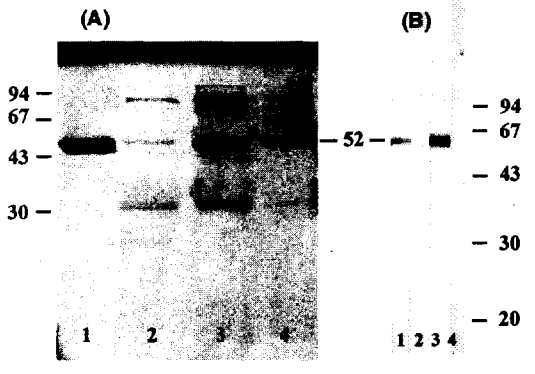


Figure 3. 15% SDS-PAGE analysis of ligand **12**-binding proteins after elution with ligand **12** and Western blots.
(A) Line 1: Tc52 purified on S-hexylglutathione-agarose column. Lines 2-4: elution profile with a linear gradient 0 to 100% 5 mM ligand **12** in the column buffer, over 2 h.
(B) Lines 1 and 3: respectively, ligand **12**-binding proteins and Tc52, purified as previously described, immunoblotted with antibodies raised against peptide L6-A22 from TcAc2 gene product. Lines 2 and 4: no reactivity against parasitic proteins was observed when using a pre-immune serum.

Acknowledgments

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9. All new compounds gave ^{1}H NMR, ^{13}C NMR and MS consistent with their structure. Selected data for protected ligands **5** and **11**: compound **5**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.42 (br s, 2H, NH), 5.25 (br s, 2H, NH), 4.71 (br s, 2H, NH), 4.04-3.99 (m, 2H, CH), 3.14-3.11 (m, 8H, CH_2), 2.08-1.90 (m, 4H, CH_2), 1.86-1.79 (m, 10H, CH_2), 1.70-1.28 (m, 60H, 12 CH_3, 10 CH_2 and 4 CH), 1.00-0.90 (m, 12H, CH_2); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 172.57, 156.66, 80.36, 79.63, 71.13, 66.82, 57.98, 54.98, 45.66, 40.25, 37.51, 35.35, 32.14, 30.13, 28.75, 27.70, 23.11; m/z 1107; compound **11**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.34 (br s, 1H, NH), 5.21 (br s, 1H, NH), 4.70 (br s, 1H, NH), 4.00-3.85 (m, 1H, CH), 3.05-3.02 (m, 4H, CH_2), 2.60-2.55 (m, 6H, CH_2), 2.38-2.25 (m, 12H, CH_2), 1.78-1.53 (m, 19H, 8 CH_2 and 3 CH), 1.45-1.24 (m, 24H, 3 CH_2 and 6 CH_3), 0.93-0.75 (m, 8H, CH_2); ^{13}C NMR ($CDCl_3$, 300 MHz) δ 173.01, 156.67, 80.53, 79.60, 63.98, 57.20, 56.30, 56.01, 54.95, 53.87, 53.17, 52.39, 49.41, 49.11, 45.73, 43.50, 40.82, 40.30, 39.74, 36.79, 32.17, 31.12, 30.10, 28.84, 26.28, 25.74, 25.07, 23.60, 23.13; m/z 750.
10. Preparation of affinity columns with ligands **6** and **12**: activated CH Sepharose 4B gel (Pharmacia) (2 g) was suspended in 1 mM HCl, and washed with 400 mL 1 mM HCl for 15 min on a sintered glass filter. Compound **6** or **12** (0.45 mmol) was dissolved in 12 mL coupling buffer: 0.1 M $NaHCO_3$ pH 8, containing 0.5 M NaCl. The solution was mixed with the 6 mL gel and the mixture was rotated 3.5 h at room temperature. The excess of ligand was recovered and the gel washed with 30 mL of coupling buffer pH 8. Remaining active groups were blocked with 20 mL 1M ethanolamine at pH 8 for 1 h. The product was washed with three cycles of alternating pH, starting with 0.1 M acetate buffer pH 4 containing 0.5 M NaCl, then with 0.1 M Tris-HCl buffer pH 8 containing 0.5 M NaCl. The column was kept at 4 °C, in 0.1 M Tris-HCl buffer pH 8 containing 0.5 M NaCl.
11. Acidic hydrolysis conditions: 6 N HCl, 110°C, 24 h.
12. Parasite culture, passage of epimastigote lysates on columns and elution by ligand: epimastigotes culture of *Trypanosoma cruzi* Y was performed according to the previously described procedure.[6] All the early steps were carried out as described for supernatant preparation from epimastigotes culture.[6] The supernatant was loaded onto a 5 mL affinity column previously equilibrated with the column buffer for one night. Following extensive washing with 300 mL of the same buffer, elution was performed using a linear gradient 0 to 100% 5 mM ligand in the column buffer, over 2 h, 0.5 $ml.min^{-1}$, detection at 280 nm. The eluate was concentrated by centrifugation through an Amicon Centriprep 10 concentrator. 15% SDS-PAGE was carried out and protein bands were stained with Coomassie blue.
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