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Testing the trypanocidal, amoebicidal activity of novel
S-containing sugar derivatives: structure-activity
investigations.

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Introduction

Trypanosoma cruzi is the causative agent of Chagas disease that affects 16-18 million people in Latin America. Further 100 million people are at risk in this area and ca. 50 thousand die annually from this disease. The infection is initiated by metacyclic trypomastigotes present in feces of triatomine bugs. Trypomastigotes invade vertebrate host cells and come in contact with the lysosomal compartment [Burleigh, 1995]. Chagas disease has also been recognized as an opportunistic disease in HIV-infected individuals [Coura and Castro, 2002] in addition to being reported throughout the world due to international immigration [Días, 2007].

Current treatments rely on two old and non-specific chemotherapeutic agents, Nifurtimox and Benznidazole. Despite the major advances that have been made in the identification of specific targets that afford selectivity, the drugs used today have serious side effects. Furthermore, differences in drug susceptibility among different *T. cruzi* isolates have led to varied parasitological cure rates depending on the geographical region. Due to these inadequacies, an intense research program has been directed to find alternative drugs for the treatment of chagasic patients [Urbina, 2010].

A number of drugs were reported to be effective against *T. cruzi* in vitro or in animal models, but no drug proved to be completely satisfactory for clinical use. Similarly, there are no adequate chemoprophylactic drugs to be used to eliminate the parasite from the blood of serologically positive donors in order to prevent transfusion-associated Chagas disease. Therefore, new compounds showing higher potency and selectivity in both the acute and chronic stages of Chagas disease and/or perhaps, even more importantly, better tolerability are urgently needed [Guedes, 2011; Muñoz, 2011].

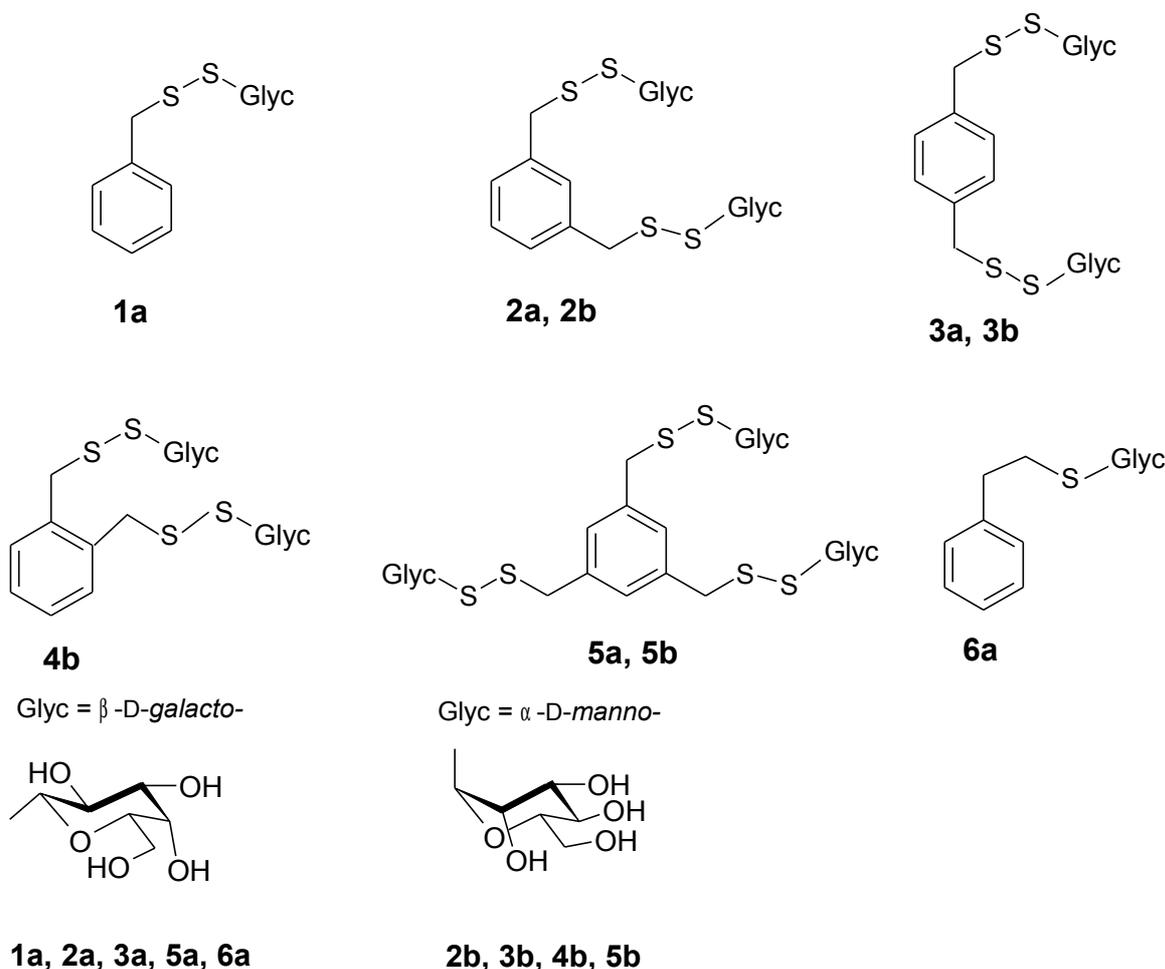
In our grant proposal we suggested to test glycosyl disulfide derivatives against *T. cruzi*. Novel glycomimetics containing disulfide interglycosidic linkages were first synthesized in our laboratory [Szilágyi, 2001]. We have developed further synthetic methods for various structures [Murthy, 2009, Stellenboom, 2010, Illyés, 2011], studied the lectin-binding properties of some derivatives [Murthy, 2009] and determined conformational preferences [Fehér, 2011]. We have reasoned that this type of glycosidic linkage, featuring a three-bond distance between the anomeric carbon and the aglycon as opposed to the two-bond natural glycosidic bonding [Szilágyi & Varela 2006], provides a larger conformational space for these compounds [André, 2006; Fehér, 2011]. Furthermore, differences in the stereoelectronic properties of the bridging S-atoms may promote interactions with proteins which are different from those with O-glycosides. In fact, it was recently shown that appropriately positioned symmetric diglycosyl disulfides are binding to various lectins [Pei, 2005] Subsequently, our own studies disclosed specific lectin-binding affinities of multivalent aromatic glycosyl-disulfide derivatives [Murthy, 2009]. Moreover, increased physiological relevance of these novel carbohydrate structures have been clearly demonstrated in assays with native tumor cells such as human B- or T-lymphoblastoid- and adenocarcinoma cell lines [André, 2006]. A further significant evidence regarding biological activities of disulfide-type sugars was the observation that hemagglutination by *Streptococcus suis* was strongly inhibited by aromatic sugar derivatives [Hansen, 1997] similar to those in the 2nd group of compounds (see below) we proposed to test. The value of such disulfide sugar derivatives in physiologically relevant interactions is, therefore, clearly documented. Based on the foregoing preliminaries we have selected 11 glycosyl disulfide derivatives for exploratory studies of *T. cruzi* inhibition.

Materials and Methods

Chemistry

The chemical structures of the compounds tested in this study are shown in Chart 1. Aromatic glycosyl disulfides **2b** – **5b** have been previously described [Murthy, 2009]. **1a-3a**, **5a** and **6a** were prepared using similar procedures with the difference that the starting product 1-thio-2,3,4,6-tetra-O-acetyl- α -D-mannopyranose was replaced by 1-thio-2,3,4,6-tetra-O-acetyl- β -D-galactopyranose in these reactions. **7** and **8** were synthesized as published [Szilágyi, 2001, Bell & Horton, 1969, resp.].

Structural group 1: multivalent aromatic glycosyl disulfides:



Structural group 2: nonsymmetric diglycosyl disulfides:

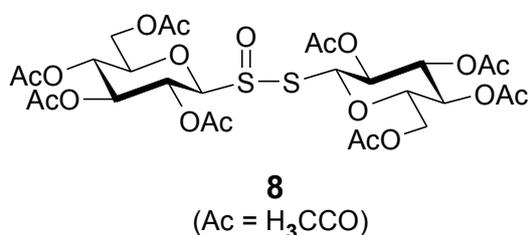
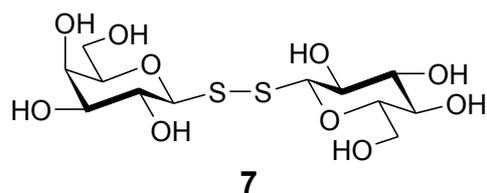
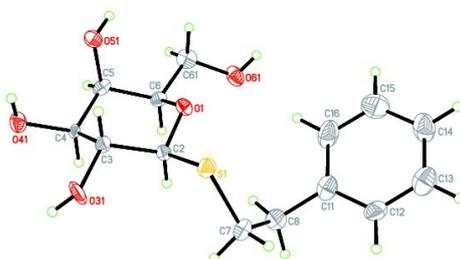


Chart 1. Compounds used for the antitrypanosoma tests

6a showed moderate trypanocidal activity (Table 1) but, in addition, it is one of the most potent inhibitors of β -galactosidase (EC 3.2.1.23) [De Bruyne, 1977] and a radio labeled derivative has also been used for imaging of *LacZ* gene expression. [Choi, 2003]. Therefore determination of its stereo structure is of multiple interest. We have therefore determined the structure of **6a** by X-ray crystallography [Brito, 2011]



Trypanocidal activities against tissue culture derivated trypomastigotes

T. cruzi (Y strain) trypomastigotes cell culture-derived were used in this study. HeLa cells were infected with trypomastigotes as previously described [González, 1996]. Experiments were carried out according to [González, 1991] with slight modifications. The trypanocidal activity was measured as IC₅₀ using a resazurin method as previously described [Rolon, 2006]. All assays were carried out in triplicate using benznidazole (35-173 μ M) as positive control.

Trypanocidal activity against intracellular amastigotes

100 μ l of a suspension containing 4 x 10⁴ HeLa cells/ml were seeded on 96-well microplates and incubated for 18 h under a 5% CO₂ atmosphere in order to obtain cell adherence. Next, the cells were washed and infected with cell culture derived trypomastigotes (Y strain) at a ratio of 5 parasites per cell, for 3 h. Two days later, the infected culture was treated with each

compound (25 μM) for 24 h and the activities against intracellular growth were determined by counting the number of trypomastigotes released after 5 days of intracellular development.

Cytotoxic activity against mammalian cells

One hundred μl of a suspension containing $4 \times 10^4/\text{ml}$ of HeLa cells, were added to 96-well microplates and the cells were left to adhere and grow for 24 h. Cells were washed with RPMI and incubated with different concentrations of each compound for 24 h. Cytotoxicity was evaluated using a resazurin test.

Results

The direct effect of the glycosyl disulfides on tissue culture derived trypomastigotes was evaluated after 18 h of treatment at 37°C . The most active compounds was **3a** with IC_{50} of $8.7 \pm 1.21 \mu\text{M}$. Other promising compounds were **2a**, **2b** and **5a** that displayed IC_{50} values of 14.8 ± 1.2 , 14.2 ± 1.2 and $14.5 \pm 1.18 \mu\text{M}$, respectively (Table 1). In the same way, the cytotoxic effect of active and inactive compounds were evaluated after 18 h of incubation with confluent HeLa cells cultures. **3a**, the most promising trypanocidal derivative proved to be cytotoxic only at an elevated concentration of $251.3 \pm 1.44 \mu\text{M}$. Cytotoxic concentrations for **2a**, **3b** and **5b** with low IC_{50} against *T.cruzi* were similar or even higher (Table 1). Finally the effects of the glycosyl disulfides on *T. cruzi* infected HeLa cell cultures were evaluated after 18 h of treatment at 37°C (Figure 1). We observed that compound **3a** strongly inhibited the intracellular development of *T.cruzi* amastigotes as well, as evidenced by a low release of trypomastigotes to the supernatant medium. Other compounds like **1a**, **2b**, **3b**, **4b** and **8** also displayed strong inhibition of parasite release suggesting an effect against the intracellular development of *T.cruzi* amastigotes. None of the compounds were cytotoxic at the concentrations assayed (Table 1).

Table 1. Trypanocidal activities and cytotoxic concentrations

| Compounds | Trypanocidal activity IC_{50} (μM) | Cytotoxicity CC_{50} (μM) |
|------------|---|--|
| 1a | $52,2 \pm 1.23$ | >2000 |
| 2a | $14,8 \pm 1.2$ | $247,5 \pm 1.42$ |
| 2b | $14,2 \pm 1.2$ | >2000 |
| 3a | $8,7 \pm 1.21$ | $251,3 \pm 1,44$ |
| 3b | $42,8 \pm 1.34$ | $500,8 \pm 1.47$ |
| 4b | $98,4 \pm 1.24$ | >2000 |
| 5a | $14,5 \pm 1.18$ | >2000 |
| 5b | $233,3 \pm 1.44$ | >2000 |
| 6a | $238,2 \pm 1.38$ | >2000 |
| 7 | $241,4 \pm 1.42$ | >2000 |
| 8 | $111,8 \pm 1.31$ | 256 ± 1.38 |
| Beznidazol | $99,7 \pm 1,5$ | ND |

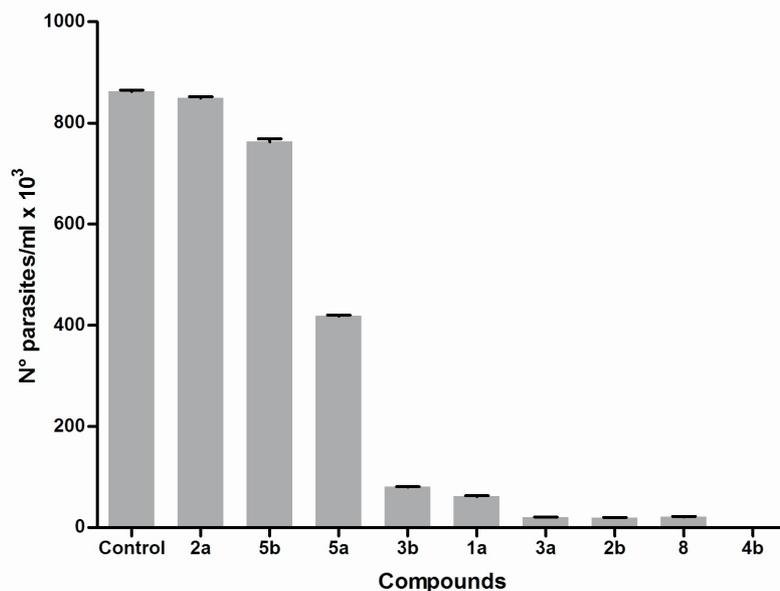


Fig.1 Infected HeLa cell cultures were incubated during 18 h with the compounds listed on the x-axis. The activity against intracellular development was evaluated by counting the number of released trypomastigotes per mL.

Discussion

Among possible drug targets in trypanosome organisms the enzyme trypanothione reductase (TR) emerges as the most thoroughly studied system. This enzyme is essential in protecting parasitic protozoa against oxidative stress by catalyzing the reduction of trypanothione disulfide (TS₂) to trypanothione (T(SH)₂) [Krauth-Siegel, 2005]. This supports the strategy of using selective inhibition of TR as a feasible approach for the control of trypanosomal infections. A recent high-throughput screening campaign covering 100 000 compounds [Holloway, 2009], mostly synthetic, identified five classes of chemical structures as potential candidates to be developed into antiparasitic drugs. Interestingly, just a few of the inhibitors tested contained disulfide bond [El-Waer, 1991; Gallwitz., 1999]. Aromatic sulfides, on the other hand, were found to exert antitrypanosomal effects on several occasions [Stump, 2007; Girault, 2001].

In the present study we have reasoned that compounds incorporating disulfide bonds might be effective against *T. cruzi* via interfering with the TR-mediated redox mechanism of the parasite. The compounds selected for the tests are characterized by the attachment of one or more monosaccharide sugars to an aromatic core (**1a**, **2a,b**, **3a,b**, **4b**, **5a,b**) or to another monosaccharide (**7** & **8**) by disulfide linkages. Compound **6a**, of similar structure but containing a simple sulfide bond, rather than disulfide, was added for comparison, see Chart 1. Then, inspecting the results of our inhibition tests listed in Table 1 some conclusions can be drawn regarding possible structure-activity relationships.

First, sizeable inhibitory activities were observed only for aromatic disulfide derivatives (**1a**, **2a**, **2b**, **3a**, **3b**, **4b**, **5a**, with the exception of **5b**). Second, the presence of both the aromatic ring and the disulfide linkage appear to be essential. This is clearly demonstrated by the much lower activity of derivatives **7** and **8** (no aromatic rings) on the one hand, and by the comparison of **1a** to **6a**, on the other. While the disulfide **1a** is a moderate inhibitor of *T. cruzi* growth, **6a**, lacking just one S-atom in comparison with **1a**, is ca. four times less active. Another remarkable correlation between activity and structure concerns the

substitution pattern on the aromatic ring: di- and trisubstituted derivatives (**2a**, **3a**, **5a**) are more efficient than the monosubstituted **1a**. Of the former group **3a**, bearing the sugar disulfide substituents in *para* position, stands out with an IC₅₀ of 8.7 ± 1.21 μM, being ca. two times more active than **2a** or **5a** which have the same substituents in *meta* positions. And finally, the striking difference of the inhibition efficiencies between **5a** and **5b** and, to a lesser extent, between **3a** and **3b** bears evidence for the importance of the sugar configurations attached to the aromatic ring: the β-*galactosyl* derivative **5a** is ca sixteen times more active than α-*mannosyl*-substituted **5b** and, similarly, **3a** exceeds **3b** five times in inhibition efficiency against *T. cruzi* epimastigotes. Molecule **3a** clearly stands out as the most potent inhibitor of the tested panel with an efficiency comparable to or even exceeding that of the known drugs. In fact, the antitrypanosoma activity of compound **3a** was found more than eleven times higher than that of benznidazol (Table 1), a drug currently used in the treatment of Chagas disease. However in strong opposition to benznidazole, **3a** was also capable of inhibiting the intracellular development of *T. cruzi* amastigotes.

Although it may seem premature to speculate about possible molecular mechanism of inhibition from tests conducted on the whole parasites, some preliminary considerations may still be advanced at this stage. First, it seems reasonable to hypothesize that our disulfides may bind to the TR enzyme, the parasite-specific disulfide reductase. The binding may be facilitated / enhanced by the presence of the aromatic rings in our molecules which displayed inhibitory activities. Note that most of the known TR inhibitors contain aromatic moieties [Jacoby, 1996; Krauth-Siegel, 2005; Holloway, 2009]. The significant specificity of the inhibition potency on the chemical structure, as discussed above, is a further indication for the enzyme binding. TR is, however, not a carbohydrate-binding enzyme. The fact that the inhibition potency of **3a**, a β-*galactosyl* derivative is much higher (Table 1) than that of the α-*mannosyl*-bearing **3b** may indicate that another Trypanosome-specific enzyme may be implicated to interact with **3a**. We hypothesize that this enzyme might be the *T. cruzi* trans-sialidase (TcTS). This transglycosidase is essential for the parasite to survive in the host's bloodstream by transferring sialic acid from glycoconjugates on the surface of the host cells to terminal *galactose* units on the surface of the parasite as the latter is incapable to synthesize sialic acid [Damager, 2008]. The negatively charged sialic acid coat protects the parasite's antigenic surface. Although validation of TcTS as a target for anti-*T. cruzi* chemotherapies is not without problems [Buscaglia, 2006] it is possible that **3a** may interfere with this process via competition as an alternate acceptor for TcTS. Significantly, **3a** was found to be the most efficient agent against intracellular amastigotes as well. In fact, its potency exceeded that of the benznidazol used as control by one order of magnitude (Fig. 1). Remarkably enough, **2a**, a positional isomer of **3a**, on the other hand, was very poor in preventing the release of trypomastigotes from the infected cells (Fig. 1). This may be a consequence of the strict steric requirements for the putative binding of these inhibitors at the binding site of TcTS. Evidently, further studies with recombinant or purified enzymes would have to be conducted to elucidate these points. Finally, it is important to note that none of the compounds in this study were cytotoxic for the host cell (see Table 1). In fact, several compounds reported in the literature have been shown to kill *T. cruzi*. However, of outstanding interest is the observation that several of the compounds we tested were also able to inhibit intracellular proliferation or parasite differentiation when the number of parasites released by *T. cruzi*-infected Vero cells was evaluated. These observations show a remarkable effect of these compounds against the intracellular stage of *T. cruzi*, an activity that is missing in the currently available drugs like benznidazol and nifurtimox [Gutiérrez, 2011].

Work aimed at optimizing the chemical structures of glycosyl disulfides and other closely related analogues is currently being pursued in our laboratory together with experimental approaches focused to elucidate the inhibitory effect on TR and TcTS.

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