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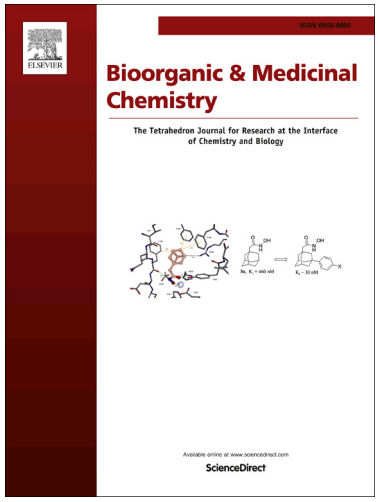

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Title:
Dibenzosuberyl substituted polyamines and analogs of clomipramine as effective inhibitors of trypanothione reductase; molecular docking, and assessment of trypanocidal activities.

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Abstract:

Trypanothione reductase (TR) is an enzyme critical to the maintenance of the thiol redox balance in trypanosomatids, including the genera *Trypanosoma* and *Leishmania* that are parasites responsible for several serious diseases. Analogs of clomipramine were prepared since clomipramine is reported to inhibit TR and cure mice infected with trypanosomes, however its psychotropic activity precludes its use as an anti-trypanosomal therapeutic. The clomipramine analogs contained a tricyclic dibenzosuberyl moiety. Additionally a series of polyamines with *N*-dibenzosuberyl substituents were prepared. All compounds studied were competitive inhibitors of TR and showed trypanocidal activities against *T. brucei in vitro*. The analogs of clomipramine were poor inhibitors of TR, whereas the polyamine derivatives were effective TR

inhibitors with the most potent compound, N^4,N^8-bis(dibenzosuberyl)spermine (**7**), having a K_i value of 0.26 μM. However, compound (**7**) did not prolong the lives of mice infected with trypanosomes. Analysis of docking studies indicated: the tricyclic groups of inhibitors bind at four distinct hydrophobic regions in the active site of TR; the importance of the chlorine substituent of clomipramine in binding to TR; and binding of the dibenzosuberyl groups of (**7**) occur at separate and distinct hydrophobic regions within the active site of TR.

Abbreviations:

TR, trypanothione reductase;
GR, glutathione reductase;
DBS, dibenzosuberyl, 10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptenyl;
DBA, 3-chloro-10,11-dihydro-dibenzo[*b,f*]azepinyl;
$T(SH)_2$, trypanothione dithiol;
$T(S)_2$, trypanothione disulfide;
BR, binding region;
BM, binding mode.

1. Introduction

Several organisms belonging to the family Trypanosomatidae are parasitic protozoa which cause severe diseases including human African trypanosomiasis (*Trypanosoma brucei* sub-species), Chagas disease (*Trypanosoma cruzi*), cutaneous leishmaniasis (including *Leishmaniasis tropica* and *L. major*) and visceral leishmaniasis (including *L. donovani* and *L. infantum*).[1] Diseases caused by trypanosomatids have a serious impact in many countries. For example: in Latin America nearly 8 million people are afflicted with Chagas disease; and 1.3 million new cases of leishmaniasis occur each year, primarily in Asian, African and Latin American countries.[2] Current drugs to treat diseases caused by these protozoa have serious problems including severe toxicity, and there are increasing incidences of drug resistant trypanosomatid strains.[3-5]

One promising target in the development of new chemotherapeutics is the antioxidant defenses of trypanosomatids that rely on an unusual spermidine-glutathione conjugate named trypanothione [N^1,N^8-bis(glutathionyl)spermidine].[6-8] In most organisms, glutathione is an important anti-oxidant and levels of this thiol are maintained by the action of glutathione reductase (GR).[9, 10] However, trypanosomatids have a simpler redox metabolism than mammals and do not contain GR, glutathione peroxidase, or catalase.[8, 11] Trypanosomatids do contain glutathione, however, trypanothione [$T(SH)_2$] is the central thiol in their redox metabolism.

Trypanothione serves as the reducing agent for a myriad of reactions, including the reduction of tryparedoxin (which is the reductant used by trypanosomatids for the ribonucleotide reductase mediated reduction of ribonucleotides).[7, 11, 12] These reactions occur with the concomitant oxidation of $T(SH)_2$ to the trypanothione disulfide [$T(S)_2$]. The dithiol ($T(SH)_2$) is regenerated through the action of trypanothione reductase (TR) which utilizes NADPH to reduce $T(S)_2$.[13-16]

Compounds that disrupt the maintenance of levels of $T(SH)_2$ either through conjugation with $T(SH)_2$, inhibition of the synthesis of polyamines and other components of $T(SH)_2$, or inhibition of TR, have potential to be effective and selective anti-trypanosomal agents.[17-19] Indeed several current anti-trypanosomal drugs impact levels of $T(SH)_2$ in trypanosomatids, although this may not be the primary therapeutic action of these drugs.[3, 4, 20] For example: D,L-α-difluoromethyl ornithine (DFMO, used to treat African trypanosomiasis) inhibits the synthesis of putrescine (1,4-diaminobutane) a component of $T(SH)_2$;[21] pentamidine (used to treat leishmaniasis) inhibits polyamine transport in *L. infantum* and *T. cruzi*[22, 23]; and trivalent melaminophenyl-arsenicals (used to treat African trypanosomiasis) as well as antimonials (used to treat leishmaniasis) impact several sites within the parasites including inhibition of TR and formation of adducts with $T(SH)_2$.[20, 24-26]

Several factors make TR (EC 1.8.1.12) in particular a compelling target for the development of new chemotherapeutics.[14, 15, 18, 27] These factors include that TR is pivotal to the redox metabolism of trypanosomatids, as it appears to be the only route for the transfer of reducing equivalents from NADPH to thiol-containing species.[7] Also, several genetic studies on *Leishmania* and *Trypanosoma* have shown the importance of TR, including studies in which trypanosomatids modified to produce decreased levels of TR were found to be acutely sensitive to oxidative stress with severely compromised abilities to infect cells.[28-31] Additionally, the structure and properties of TR from different genera of trypanosomatids are very similar,[16, 32-34] indicating that an inhibitor of TR may be active against a range of trypanosomatids.

TR is a FAD and NADPH-dependent disulfide oxidoreductase with many similarities to mammalian GR, both being homodimeric flavoproteins of similar size with essentially identical mechanisms and with approximately 40% sequence identity.[13, 35] However, TR and GR have quite different substrate specificities, TR does not reduce glutathione disulfide and GR does not reduce $T(S)_2$.[36, 37]

A range of structurally diverse inhibitors of TR have been developed (for reviews see[14, 15, 17]), with the non-metal inhibitors generally including an aromatic and an amino group (that is protonated at physiological pH). Several of these TR inhibitors are also good trypanocides *in vitro* (i.e., kill trypanosomatids in buffer solutions). However, there are sparse reports of compounds designed to be inhibitors of TR that are also active *in vivo*, i.e., that clear parasitemia in mice[15, 38] (although as mentioned several compounds that cure mammals also inhibit TR).

Thus, we were intrigued by reports that certain tricyclic psychotropics with pendant amino moieties are inhibitors of TR,[39, 40] display trypanocidal activities *in vitro* and that one of these compounds, clomipramine (**1**), was able to cure mice infected by trypanosomes. Specifically, the phenothiazine antipsychotics chlorpromazine, thioridazine and trifluoperazine[16,

[37, 41] and quinacrine (an antiprotozoal)[16, 42, 43] are inhibitors of TR. One of the most potent of these TR inhibitors is clomipramine (**1**), with a K_i value of 6.5 μM against *T. cruzi* TR[41] and an IC_{50} value of 3.4 μM against *T. brucei* TR (at 10.4 μM $T(S)_2$).[16] Also, certain tricyclic drugs including clomipramine and some phenothiazines (including thioridazine and trifluoperazine) are trypanocidal, being effective against species of *Trypanosoma* and *Leishmania in* vitro.[39, 44-48] Additionally, clomipramine is active against trypanosomes *in vivo* and has been shown to cure mice with both acute and chronic infections of *T. cruzi* (with clomipramine administered i.p. at 5mg/kg/day, and in one study co-administered with benznidazole).[49-54] Clomipramine probably impacts several processes in trypanosomatids, and it has been shown to have anti-calmodulin activity[55] in addition to inhibiting TR.

A range of novel derivatives based on the tricyclic drugs imipramine,[40] and promazine[56] have been synthesized and investigated against *T. cruzi* TR. Additionally, other amines with tricyclic groups have been prepared and shown to inhibit TR, including phenothiazines,[37, 56, 57] acridine derivatives[42, 58-61] and methylene blue.[62] Several of these tricyclic derivatives have trypanocidal activities.[56, 57, 59-62] However, clomipramine (**1**) is still one of the more potent inhibitors of TR and to the authors' knowledge it is the only non-metal containing inhibitor of TR that also shows good activity against trypanosomatids *in vivo without* co-administration with other drugs. (The TR inhibitor ebsulfur when administered *with* nifurtimox is active *in vivo*.[38]) For these reasons we sought to investigate the interactions of analogs of clomipramine (**1**) with *T. cruzi* TR. However, recognizing that the psychotropic activity of (**1**) would make it an unlikely candidate for the treatment of trypanosome infections in humans, we chose to prepare analogs in which the tricyclic ring of clomipramine (3-chloro-10,11-dihydro-dibenzo[*b,f*]azepinyl, abbreviated as DBA) was replaced by the dibenzosuberyl group (10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptenyl, abbreviated as DBS) that has a very similar shape (albeit different electronic character) and importantly will have a different pharmacological profile from clomipramine (**1**).

Additionally, we investigated certain polyamine-DBS conjugates, our rationale being primarily based on previous studies of polyamine derivatives with aryl groups as being effective inhibitors of TR several of which also displayed trypanocidal activities.[64-76] Other intriguing factors influenced us including: evidence that conjugation of biologically active moieties to polyamines can potentiate their pharmacological profiles;[77] further reports of polyamine-aryl conjugates with activities against a range of trypanosomatids;[63, 78-80] and the potential of polyamine-DBS conjugates to exploit trypanosomatid polyamine transporters to become accumulated into parasites[81-84] (except for African trypanosomes (*T. brucei*) which do not readily assimilate exogenous polyamines[85]). Consequently we also prepared spermidine and spermine derivatives with *N*-substituted DBS groups.

In this paper we report the syntheses of these novel analogs of clomipramine and DBS-polyamine derivatives, their inhibiting activities against *T. cruzi* TR, and a comprehensive molecular docking study of their binding to TR. Three of the compounds studied (compounds (**5**), (**6**) and (**7**)) were very good competitive inhibitors of TR with K_i values of 7.6, 4.0 and 0.26

μM, respectively. Additionally, we describe the activities of these compounds against *T. brucei* subspecies *in vitro* and the activities of compounds (**6**) and (**7**) against *T. b. brucei in vivo*.

2. Results and discussion

2.1. Synthesis

The synthetic strategies used to prepare the analogs of clomipramine (compounds (**2**) and (**3**)) and the DBS-polyamine conjugates (compounds (**4**) to (**7**)) are shown in Scheme 1. Compounds (**2**) and (**3**) were prepared by reaction of *N*,*N*-dimethyl-1,2-diamine or *N*,*N*-dimethyl-1,3-diaminopropane with 5-chlorodibenzosuberane in the presence of triethylamine. Previously we reported the selective trifluoroacetylation of the primary amino groups of polyamines [86] and use of this reaction enabled the syntheses of compounds (**4**) to (**7**) in three steps from their respective polyamines. Thus, the primary amino groups of each polyamine were trifluoroacetylated, followed by *N*-alkylation with 5-chlorodibenzosuberane, and then removal of the trifluoroacetyl groups.

2.2. Enzyme studies

The effects of compounds on the reduction of $T(S)_2$ by *T. cruzi* TR were measured using a standard photometric assay.[13] The TR used was isolated from *E. coli* SG5, a glutathione reductase deletion mutant, containing the TR expression vector pIBITczTR.[87, 88] All of the compounds exhibited linear competitive inhibition of the reduction of $T(S)_2$ by TR. The inhibition constants (K_i values) of compounds are presented in Table 1. Our measured K_i value for (**1**) of 8.40 ± 1.43 μM correlated well with the value of 6.53 μM reported by Benson.[41]

The relative specificity of compounds (**1**) to (**7**) as inhibitors of TR was explored by investigating their effects on the reduction of glutathione disulfide by yeast GR. The presence of these compounds at concentrations of 250 μM did not decrease the rate of this reaction catalyzed by GR. Hence these compounds show specificity for the inhibition of TR with respect to the structurally similar enzyme, GR. This was anticipated since although TR and GR have structural similarities, the active sites of these enzymes have several differences. The active site of TR is large, contains additional glutamate residues (including Glu19) and hydrophobic regions, whereas GR has a smaller active site containing several cationic residues (e.g., Arg37, 38 and 397) that are not present in TR.[89] The active site of TR can therefore accommodate large, cationic compounds and charge is known to be a factor in the selectivity of compounds for TR versus GR.[37] However charge is not the sole factor in the binding of compounds to TR, since the polyamine spermine does not inhibit TR.[65]

There is previous kinetic evidence for the binding of more than one inhibitor molecule to the large active site of TR.[58] Additionally, crystal structures of TR with bound quinacrine

mustard,[43] and TR with a diarylpyrrole[32] show two molecules located in the active site. Our enzyme studies did not show evidence of the binding of more than one inhibitor molecule. However, the maximum inhibitor concentrations used in our TR enzyme assays were approximately twice the K_i, and evidence for the binding of multiple molecules is more pronounced at higher inhibitor concentrations.[58] Thus, our results do not preclude the possibility of more than one inhibitor molecule being bound to the active site of TR at higher inhibitor concentrations.

All the compounds investigated were competitive inhibitors of $T(S)_2$ reduction by TR. Compounds (**2**) and (**3**) which are structurally the most similar to clomipramine (**1**) were significantly less effective inhibitors than (**1**). The size and the concave shape of the DBA ring of compound (**1**), and the DBS tricyclic ring (of compounds (**2**) to (**7**)) are very similar however, the presence of the chlorine atom of DBA is obviously important in the binding of (**1**) to TR. Our docking studies (section 2.4) indicate that the chlorine is involved in binding interactions with residues in the active site, also there are several examples of TR inhibitors containing a halogenated aryl group being more effective than analogs without the halogen.[34, 56, 58, 67] Another possible factor impacting the inhibiting activities is that (**2**) and (**3**) contain an exocyclic nitrogen (versus the endocyclic nitrogen of the DBA group of (**1**)). However, since the shapes of the DBS and DBA rings are similar and docking (section 2.4) showed that both tricycles bind to the same locations in the active site, any effects on binding resulting from differences in ring nitrogen location between compounds (**1**) to (**3**) are likely to be small compared to binding differences due to the presence of chlorine in the DBA ring of (**1**).

The polyamine derivatives, compounds (**4**) to (**7**), were more effective inhibitors than (**2**) and (**3**), thus the presence of an additional amino/aminium group enhanced the inhibiting activities of compounds. The inhibiting activities increased with increased alkyl chain length, i.e., increasing distance between the DBS group and aminium ion. Docking studies (section 2.4) indicate that the longer chain enables the aminium groups to reach H-bond accepting and glutamate residues while still permitting the DBS to be fully sited in one of the tricyclic ring binding regions. The DBS substituted spermine derivative (**7**) was the most effective inhibitor studied with a K_i value of 0.255 μM against $T(S)_2$ reduction by TR. Thus compound (**7**) is one of the most potent competitive inhibitors of TR to date. The presence of two DBS rings and four aminium ions in (**7**) obviously can lead to more favorable hydrophobic and ionic interactions with residues in the active site of TR. However, our docking studies (section 2.4) indicate why the interaction of (**7**) with TR is so much more favorable than the spermidine derivative (**6**) (as reflected by the 20-fold difference in K_i values of these two compounds).

2.3. Trypanosome studies

The *in vitro* trypanocidal activities of compounds (**2**) to (**7**) against bloodstream forms of clinically isolated strains of *T. brucei* ssp. were examined using a standard growth screen.[90] The trypanosome strains used were *T. b. brucei* Lab 110 EATRO and three clinical isolates of *T. b.*

rhodesiense and these results are shown in Table 2. The compounds tested were effective trypanocides with IC_{50} values ≤ 4.5 μM against three of the *T. brucei* ssp. One strain, K 243-As-10-3 (a pentamidine and melarsoprol-resistant clone of *T. b. rhodesiense*), was consistently more resistant to the compounds tested. All the compounds studied had similar, or marginally better, activities against *T. brucei* as clomipramine (**1**).[39] The spermidine and spermine derivatives (**6**) and (**7**) were slightly less effective trypanocides (despite being more potent TR inhibitors) than analogous polyamines with *N*-(3-phenylpropyl) substituents.[64] However, in this earlier work the polyamines with multiple 3-phenylpropyl substituents were less effective trypanocides, despite being more potent TR inhibitors.[64] These polyamines, as well as compound (**7**), are poorly soluble in water and this may affect the accuracy of the trypanosome assays of these compounds.

Our compounds were assessed against *T. cruzi* TR, whereas the trypanosomes studied were *T. brucei* ssp. However differences in the TR enzymes from these species should not impact our studies, since comparison of the crystal structures and kinetic properties of TR from both *T. cruzi* and *T. brucei* have shown no significant differences.[16] Thus TR from both species are considered to be interchangeable in structure-based inhibitor design.[16] Overall we observed no direct correlation between the TR inhibiting and trypanocidal activities of compounds (**2**) to (**7**). An apparent lack of correlation between the TR inhibiting and trypanocidal activities of compounds has been noted in previous studies.[68,75] This suggests that structural features in addition to TR binding, for example, features that facilitate the transport of compounds across the trypanosome cell membrane, are also important to the trypanocidal efficacies of compounds.

The *in vivo* trypanocial activities of the most potent TR inhibitors, compounds (**6**) and (**7**), were investigated by administration of compounds to mice infected with *T. b. brucei* LAB 110 EATRO. After the infection had progressed for 24 h, compound (**6**) was administered ip at 1, 2.5, 5, 10, 25 mg per kg of body weight once per day for three days. For compound (**7**), the drug was administered at several doses ip both twice daily and once a day. Additionally, compound (**7**) was administered at 10, 25 or 50 mg/kg/day using mini-osmotic pumps that release drugs continuously for three days. Compound (**6**) did not prolong the life of animals beyond the survival time of the control animals. Also compound (**6**) showed toxicity at 25 mg/kg with infected animals on this dose dying before untreated, infected animals. Compound (**7**) displayed a slight prolongation of life. Specifically, some infected animals lived a day beyond controls when dosed at 50 mg/kg/day administered in 2 daily doses of 25 mg/kg for three days. However, compound (**7**) showed toxicity against infected animals that were dosed at 50 mg/kg once daily for three days. Thus, with these dosing regimens, the dosage of (**7**) needed to impact the parasite is very close to the dosage resulting in toxicity to the animal.

2.4. Molecular docking studies

2.4.1. Preparation of ligands and enzyme for docking

Compounds (**2**) to (**7**) have multiple amino groups, thus several protonation states of each compound are possible at physiological pH. Anticipating that different protonation states will

have different affinities for TR, we calculated the relative occurrence of protonation states at pH 7.2 (the pH of our TR inhibition assays) leading us to study the docking of one protonated form of clomipramine (**1**) and a total of ten protonated forms of compounds (**2**) to (**7**) (Table 3). An additional factor we considered was the possibility that different conformers of the DBS or DBA tricyclic ring in compounds may impact their affinities for TR. Most docking programs treat rings as rigid with no conformational flexibility, consequently we identified several low energy conformations for the DBS and DBA rings (representative structures of the conformations are shown in Table 4 and all structures investigated are provided in Supplemental Information). Calculations of the energy barriers for interconversion of the conformations indicated that *in vitro* at room temperature the DBS or DBA rings will readily interconvert between the conformations of the ligand structures used in our docking studies (although *in silico* the rings are not able to flex). To the authors' knowledge, no previous docking study of TR using ligands with flexible ring systems has investigated the impact of ring conformation on docking. Consequently, this led us to investigate the docking (using AutoDock Vina[91]) of 33 structures (ligands) of different versions of compounds (**1**) to (**7**) in various protonation states and ring conformations.

There are several crystal structures of TR from various trypanosomatids including *Crithidia fasciculata* (a trypanosomatid that infects insects),[92-94] *T. cruzi* [33, 42, 43, 95, 96], *T. brucei* [16, 34, 96], and *L. infantum*. [20, 32, 97] Several of these structures have ligands located in the active site of TR, including glutathionylspermidine,[94] trypanothione, [32, 33] quinacrine,[42] the covalently bound quinacrine mustard,[43] Sb(III),[20] Ag(0)[97] and more recently, structures with small molecule inhibitors complexed to TR.[32, 34, 96] For our studies we chose to use the crystal structure of *Trypanosoma cruzi* TR in complex with trypanothione (RCSB Protein Data Bank code 1BZL),[33] with removal of the trypanothione ligand from 1BZL before inhibitor docking studies. Using 1BZL allowed us to correlate docking results with experimental results of our inhibitors against *T. cruzi* TR, and enabled the identification of any correspondence between regions of trypanothione binding and regions of ligand (inhibitor) docking (see Fig. 1).

We utilized AutoDock Vina[91] for these docking studies and defined the search space for docking to include the entire active site cleft of TR, but not including the FAD or NADPH binding regions. Since the FAD is tightly bound to TR, this site would not be available to inhibitors *in vitro*, additionally our *in vitro* experiments showed that compounds (**1**) to (**7**) did not compete with NADPH for binding to TR. Tables with the binding energies of the lowest energy poses for each ligand structure along with descriptions of the binding sites of frequently occurring poses for each ligand structure are provided in Supplemental Information.

2.4.2. Docking results

Compound (**7**) was the most potent inhibitor of TR and the binding energies of ligand versions of (**7**) were lower than all other compounds. However, current docking programs are unable to reliably predict inhibition constants of compounds,[98, 99] thus we were unsurprised to observe no direct correlation between the calculated binding energies of ligands and the

inhibition constants of compounds (**1**) to (**6**). Also, there was no apparent correlation between protonation states or ring conformations of ligands with calculated binding energies. However, docking programs (including AutoDock Vina) have more success at predicting the experimentally observed binding modes (poses) of compounds within active sites.[91, 100] Our results showed a notable uniformity in the binding locations of the DBS/DBA groups of the 33 ligand structures docked, with the DBS/DBA rings clustered into four distinct binding regions (BR 1 to 4) in the active site of TR (see Fig. 1). This result is striking since despite the large size of the search space used for docking (that encompassed the entire active site cleft), the DBS/DBA groups of the five lowest energy poses of all modeling runs were located almost entirely to these four binding regions (BRs). Also, the four BRs are clustered in, and overlap with, the space where trypanothione binds (Fig. 1).[33]

Representative poses for each compound are shown in Figures 2 and 3. All 33 ligand structures had poses in BR 1 and 2 and most ligands also had poses in BR 3. However, only ligands for compound (**7**) and occasional poses of compound (**4**) had DBS groups occupying BR 4. (The Supplemental Information provides populations of poses in each BR (Table 5), more detailed descriptions of ligand poses, and information on the binding configurations of low energy poses for each of the 33 ligand structures.) The ring conformation of a ligand clearly affected the number of poses that occupied each BR. However, there was no obvious correlation between ring conformation and relative populations in individual BRs, clearly other aspects of the ligand structures, including protonation state and distance from ring to protonated nitrogen, also impact the docking location of the DBS/DBA rings.

2.4.3. Description of DBS/DBA ring binding regions

The active site of TR is a large cleft (approximately 30 Å deep and 20 Å wide) composed of residues from both subunits of the homodimer (Fig. 1). The active site has several locations where ligands can bind[40, 96] and for some inhibitors is large enough to accommodate two molecules.[32] The residues directly involved in catalysis (including the redox active Cys53 and Cys58, along with His461') are positioned at the deepest part of the cleft, and close to the dimer interface (note, in one subunit the residues are labeled with primed numbers). At the interface of the subunits there is a tunnel that connects both active sites, which has a wider volume at the center of the enzyme, i.e., an interface pocket.[75] The tunnel entrance is close to the catalytic site (entrance is next to His461'), and has a diameter of approximately 8 Å (entrance is lined by Leu399', Lys62, Pro462', Thr463', Glu467', Phe396' and Pro398'). This structural feature (i.e., tunnel with interface pocket) has been reported in GR[101] and occurs in several additional FAD-dependent pyridine nucleotide-disulfide reductases, including glutathione amide reductase (PDB code 2R9Z), thioredoxin glutathione reductase (2V6O) and thioredoxin reductase (1H6V). For GR, crystal structures show that certain tricyclic inhibitors bind in the interface pocket/cavity, including safranin (an acridine derivative),[102] a xanthene[101] and an isoalloxazine[103] derivative. Despite the interface pocket in TR being smaller than that of GR, it has been speculated that this site may be a binding location for inhibitors of TR.[58 75] However, this was not explored in these

docking studies of TR, which included in the search space the start of the tunnel but not the interface pocket.

The locations of the four DBS/DBA binding regions are shown in Figure 1. These sites overlap with regions where $T(S)_2$ binds (from the crystal structure of *T. cruzi* TyrR with bound $T(S)_2$ PDB ID # 1BZL[33]). The DBS/DBA tricyclic rings have a concave shape that enable the ring to maximize interactions with residues that comprise bulges/protuberances into the active site, and with residues lining pockets (or the tunnel entrance) of the active site.

- Binding region 1 (BR 1) is a hydrophobic surface where Ile339 creates a protuberance around which the DBS or DBA tricyclic ring wraps. Depending on the location of the ligand pose in BR1, the DBA/DBS ring may also interact with Pro336 or Asn340. BR 1 corresponds to the region where the γ-Glu-I of $T(S)_2$ binds (the γ-Glu-I interacts with several residues including Ile339 and Pro336[33]).
- Binding region 2 (BR 2) occurs at the entrance of the tunnel that leads to the interface pocket. BR 2 overlaps with the previously identified "Z-site".[56] The DBA/DBS tricyclic ring partially inserts into the tunnel (an aryl group and part of the seven-membered ring is inserted) and the main residues of the tunnel entrance that interact with the DBS/DBA are Leu399', Pro462' and His461'. For compounds (**5**) to (**7**) (i.e., those with longer alkyl chains) often the phenyl group that is not inserted into the tunnel interacts with Lys402' (via a cation-π interaction) which is sited above the tunnel entrance. BR 2 is the location where the γ-Glu-II of $T(S)_2$ binds (the γ-Glu-II group is inserted into the entrance of the tunnel[33]).
- Binding region 3 (BR 3) is a shallow pocket with Tyr111 forming an outward bulge and where the pocket is lined with the hydrophobic residues Leu18, Val54, Ile339 and also Ser15. This site partially overlaps with the previously described "hydrophobic wall".[41, 56] The tricyclic ring of compounds is oriented in the pocket so that it curves around Tyr111 with a polar-π interaction generally occurring between the Tyr111 hydroxyl hydrogen and one of the aryl groups of the tricycle. BR 3 corresponds to the region where the Cys-I and Gly-I groups of $T(S)_2$ bind.[33]
- Binding region (BR 4) is a hydrophobic region closer to the entrance of the active site comprised primarily of Trp22 and Met114. This site also partially overlaps with the previously described "hydrophobic wall".[41, 56] Only compounds (**4**) and (**7**) had poses where the DBS occupied this site (within the five lowest energy poses of eight docking runs). At BR 4, the DBS group is oriented with one aryl group involved in a sulfur-π interaction with Met114.[104-106] The DBS group is also located close to Trp22 with the π-faces of the aryl groups at an approximate 30-40^{o} angle from parallel. This site also has hydrophobic regions from Met114 and Leu18 that interact with the DBS. BR 4 is the region where part of the alkyl chain of the spermidine component of $T(S)_2$ binds.[33]

Several early modeling studies refer to two regions of the TR active site where (tricyclic) inhibitors may bind, described as the "Z-site" (Phe396', Pro398', Leu399') and the "hydrophobic wall/cleft" (Try110, Met113, Phe114, Trp21 and Leu17),[56, 57, 107, 108] and early modeling studies

placed clomipramine at the "hydrophobic wall".[41] (Please note that literature citations of residue numbers for *T. cruzi* TR can differ by one unit depending on source. For results presented in this paper the residue numbering from 1BZL is used.) There is kinetic evidence for multiple binding sites for tricyclic inhibitors of TR,[40] and crystal structures with tricyclic moieties of ligands located at the "Z-site" and "hydrophobic wall".[34, 42, 43] Two of the DBS/DBA binding regions identified in this study occur within the "hydrophobic wall" (BR 3 and 4) and BR 2 overlaps with the "Z-site" (although the tunnel is not mentioned in reports of the "Z-site"). Several crystal structures show aromatic moieties of inhibitors bound at the "hydrophobic wall" of TR, and interacting with Trp22 and Met114 (corresponding to BR 4), and often the inhibitor molecule extends into the region near Tyr111 (BR 3). For example, crystal structures locate in the region of Trp21/22 and Met113/114: the acridine rings of mepacrine (quinacrine)[42] and quinacrine mustard;[43] the dihydroquinazoline ring of *T. brucei* TR inhibitors (with groups also being close to Tyr110 and also in a conformationally induced sub-pocket);[34] also biaryl inhibitors[96] and a diarylpyrrole [32] (both of which also extended into region near Tyr110).

Representative ligand poses compiled from all protonated versions and DBS/DBA configurations of compounds (**1**) to (**7**) are shown in Figures 2 and 3. Tables of frequently occurring ligand poses for each of the 33 ligand structures docked and more detailed descriptions of ligand poses are provided in the Supplemental Information.

2.4.4. Description of ligand amino and aminium ion binding sites

The location of the ligands' amino or aminium moieties depended on the binding location of the DBS/DBA group and to a lesser extent by the ring conformation and protonation state of the nitrogen. However, considering the lowest energy poses for all ligands of (**1**) to (**7**), the most frequent binding site for amino/aminium groups was the region containing Glu466' (ligand interacting with carboxylate), Glu467'(carboxylate) and His461'(imidazole N δ). These three residues are clustered together near the entrance of the tunnel (BR 2). Ligand amino/aminium groups in this location were in general involved in multiple strong ion-pair and/or H-bond interactions to both His461' and Glu466'; or to both Glu466' and Glu467'; or to both Glu467' and Ser470' hydroxyl; or occasionally to both Glu467' and Ser394' hydroxyl.

The next most populated sites were with the carboxylate of Glu19 (sited near Ile339 and BR1); and the hydroxyl or backbone oxygen of Ser470'. Less frequently, amino groups of ligands interacted with the guanidinium of Arg472', and amino/aminiums interacted with Ser110 (hydroxyl or backbone oxygens), or Tyr111 (hydroxyl). Several previous modeling and x-ray studies have noted the importance of electrostatic interactions between ligand amines and Glu19 or Glu466'/Glu467'.[17, 34, 42, 57,70]

2.5. Discussion of interactions of compounds with TR

Figures 2 and 3 show low energy poses that are representative of those that commonly occurred from the collection of all the protonated and ring conformation versions studied for each compound. The common poses that are not shown differ primarily in the locations of the

amino/aminium groups located at the ends of the flexible alkyl chains (further descriptions and tables of the frequent binding locations of poses from each of the 33 docked ligands are provided in the Supplemental Information).

For clomipramine (**1**), the DBA ring was able to dock in BR 1, 2 or 3, and the chlorine atom was often involved in favorable interactions helping to explain the lower K_i value of (**1**) compared to analogs (**2**) and (**3**). Thus, in poses of (**1**) where the DBA group is located in BR 1, the chlorine is able to H-bond to the hydroxyl of Tyr111. When the DBA is in BR 2 with the chlorine inserted into the tunnel, the chlorine is able to accept a H-bond from the amino group of Lys62' (N zeta); and when the chlorine is positioned out of the tunnel, it can H-bond with the hydroxyl of Tyr111. Also, when the DBA is located in BR 3, the chlorine was able to interact with the sulfur of Met114, or the π system of Trp22.[109]

For the polyamine derivatives (**4**) to (**7**) there was a wider range of possible binding interactions for the terminal aminium ions, than occurred with compounds (**2**) and (**3**). This is consistent with compounds (**4**) to (**7**) being more effective inhibitors of TR than (**2**) and (**3**).

Compound (**7**) was a significantly more potent inhibitor than compounds (**1**) to (**6**). Docking studies indicated that both the DBS groups of (**7**) were able to bind to separate binding regions (BR 1 to 4) and the terminal aminiums were able to form H-bond and/or ion-pair interactions with residues (see Fig. 3). There was a striking uniformity in the bound locations for compound (**7**) ligands, with three distinct binding modes into which 90% of the lowest energy poses bound. The most frequent binding mode (BM 1) had one DBS occupying BR 1 (Ile 339) and the other DBS occupying BR 4 (Trp22); in the second mode (BM 2) one DBS occupied BR 2 (tunnel entrance) and the other DBS was in BR 3 (Tyr111); the least frequently occupied mode (BM 3) had one DBS in BR 1 (Ile339) and the other DBS in BR 2 (tunnel entrance). (Data showing the population of poses in each BM and relative energy ranking of poses is provided in the Supplemental Information.) Generally for (**7**) one terminal aminium was associated with the Glu466', 467' and His461' cluster; and the second terminal aminium was H-bonded to the Ser110 hydroxyl, Glu 19 or Ser470' hydroxyl (the aminiums adjacent to DBS had no apparent interaction with active site residues). In addition to interactions with active site residues, several intramolecular interactions occurred in these binding modes of (**7**). In BM 1 and 2 the two DBS groups were positioned so that an aryl group from each DBS had an offset edge to face interaction.[110] Also, in BM 1 one terminal aminium ion is involved in a cation-π interaction with the DBS located in BR 1 (Ile339), in addition to the aminium interacting with both Glu467' and Ser470'.

The modeling results of compound (**7**) provide insights as to why (**7**) is a significantly more potent inhibitor of TR than the other polyamine derivatives in this study. Compound (**7**) can bind in three highly favored configurations (binding modes) in the active site. In all these modes, each DBS group is accommodated into separate hydrophobic areas of the active site, while enabling the terminal aminiums to have strong H-bond and/or ion pair interactions with active site residues. Furthermore, additional intramolecular interactions (cation-π and/or aromatic offset edge to face interactions) further stabilize the bound conformations of (**7**).

Conventional design strategies for improved inhibitors aim to limit the conformational flexibility of molecules (to minimize the entropy decrease on inhibitor-enzyme binding). Although compound (**7**) (and the other polyamine derivatives studied) have a high degree of conformational flexibility, it is likely that intramolecular cation-π interactions (and possibly aryl to aryl interactions) would transiently occur in solution limiting the conformational flexibility of (**7**) resulting in an energetically more favorable binding to TR than anticipated.

3. Conclusions

In the search for new chemotherapeutics to treat diseases caused by infections with *Trypanosoma* and *Leishmania*, the drug target trypanothione reductase (TR) has several desirable qualities, including TR's crucial role in the redox metabolism of a range of parasites from the Trypanosomatidae family. However, TR presents challenges for the development of selective potent inhibitors, partly due to the large size of its active site cleft. Additionally, despite the range of TR inhibitors developed, there are very few TR inhibitors that are active against trypanosome infections *in vivo*.

In this study the inhibiting effects on *T. cruzi* TR of two novel DBS-containing analogs of clomipramine (**1**) were investigated along with four novel polyamine derivatives containing DBS groups. Clomipramine was chosen as an initial design focus since it inhibits TR and additionally cures mice with *T. cruzi* infections, however its psychotropic activity precludes its development as a treatment for trypanosome infections. The rationale for the investigation of polyamine-DBS derivatives was influenced by several factors including previous reports of polyamines with aryl groups being inhibitors of TR and having *in vitro* trypanocidal activities.[64-75] The compounds investigated were competitive inhibitors and selective for TR, in that they did not inhibit GR. The compounds also displayed trypanocidal activities *in vitro*, however the two most effective TR inhibitors (compounds (**6**) and (**7**)) were not active against trypanosomes in *vivo* and were unable to increase the life-span of mice infected with *T. brucei*. The lack of *in vivo* trypanocidal activities for compounds (**6**) and (**7**) may be caused by several factors including rapid mammalian excretion or metabolism of these compounds. For example, the amino groups of (**6**) and (**7**) may be substrates for a variety of mammalian amine oxidases and polyamine acetyltransferases.[111, 112] Also the aminopropyl group of (**7**) may be a substrate for mammalian spermine oxidase (mammalian acetylpolyamine oxidase, formerly known as polyamine oxidase appears to have low activity for non-acetylated polyamines).[111, 113] Since (**6**) and (**7**) are reversible inhibitors of TR, if concentrations of these compounds are not maintained *in vivo*, TR activity will not be significantly decreased.

Also conducted was a comprehensive docking analysis using several protonation states and low energy ring conformations of compounds (**1**) to (**7**), i.e., including the predominant forms of compounds (**1**) to (**7**) that are likely to occur at physiological pH. This approach was necessary to fully explore the likely *in vitro* interactions between these compounds and TR.

Although several docking studies of TR with amines containing ring moieties have been reported, to the authors' knowledge there is no study that accounted for compounds' differing protonation states and ring conformations, even though these factors are likely to impact TR-inhibitor interactions. Our studies indicated that the tricyclic DBS/DBA rings of compounds cluster into four distinct binding regions, which overlap with the binding site of the substrate $T(S)_2$. The concave shapes of the DBS/DBA rings enable them to curve around residues and fit into pockets maximizing hydrophobic interactions. It was evident that similarly-sized planar ring systems would have more limited hydrophobic interactions, and this may explain the 20-fold lower K_i values of (**7**) and (**6**) relative to analogs in which the DBS group is replaced with naphthylmethyl groups.[65, 114] Docking studies also indicated several residues in the active site that participated in H-bond and/or ion pair interactions with the amino/aminium moieties of inhibitors. Commonly, these included multiple interactions with the Glu466'/Glu467'/His461' cluster of residues; or interactions with the Glu19 carboxylate, or Ser470' (with the hydroxyl or backbone oxygen).

The two clomipramine analogs (**2**) and (**3**) were significantly less effective inhibitors of TR than clomipramine (**1**), and docking indicated that although these compounds bind to similar sites in TR, the chlorine atom of (**1**) provides additional binding interactions not possible in the non-halogenated DBS rings of (**2**) and (**3**). This observation is consistent with previous studies in which compounds containing halogenated aryl groups were better TR inhibitors than non-halogenated analogs.[34, 56, 58, 67] The polyamine derivatives (**4**) to (**7**) were more effective competitive inhibitors of TR than compounds (**2**) and (**3**), and the most effective compound was the spermine derivative (**7**) which, with a K_i value of 0.26 μM, is one of the more potent inhibitors of TR known. Other spermine derivatives previously reported to be potent inhibitors of TR include ones substituted with indoles (noncompetitive inhibitors with K_i values of 0.076, to 0.39 μM),[67] 3-phenylpropyl groups (K_i 0.15 μM and greater),[64] or diphenyl groups (K_i 0.4 μM).[69] Since TR has a large active site with multiple hydrophobic binding sites and several H-bond acceptors, a range of compounds in addition to spermine derivatives are also effective inhibitors of TR (with K_i or IC_{50} values of 0.5 μM or less). These include a dichlorobenzyl substituted alkylammonium phenothiazine,[57] a dimethylamine substituted naphthoquinone,[75] certain large bis(2-aminodiphenylsulfide) derivatives,[115] a substituted dihydroquinazoline,[34] and an imidazole and phenylsulfide substituted BTCP derivative.[116] However, all these potent inhibitors of TR including compound (**7**), contain two or more aromatic moieties as well as an amino group that will be protonated at physiological pH. Our docking studies provide insights into how these structural features enhance binding to the active site of TR. Specifically, docking of compound (**7**) showed three low energy binding modes for (**7**) in which the two DBS groups interact with separate, distinct hydrophobic regions of the active site.

Thus, our detailed analysis of the binding of compounds (**1**) to (**7**) provides useful information that can aid the design of more effective and selective inhibitors of TR, and specifically, aid the design of amines with two or more aromatic moieties to exploit binding at two of the hydrophobic regions of the active site. Our docking studies identified four distinct

hydrophobic binding regions in the active site that were accessible to the tricyclic rings of compounds (**1**) to (**7**). Additionally, the enzyme kinetics and *in silico* studies presented indicate that the concave-shaped, aryl-containing ring systems of DBS/DBA are able to bind more effectively than planar naphthyl groups to these hydrophobic regions. The results also provide further evidence that incorporation of a halogen into aryl systems may increase the binding affinity of compounds to TR.

4. Experimental

4.1. Synthesis information

The trifluoroacetate salts of N^1,N^5-bis(trifluoroacetyl)diethylenetriamine (**8**), N^1,N^8-bis(trifluoroacetyl)spermidine (**10**) and N^1,N^{12}-bis(trifluoroacetyl)spermine (**11**) were prepared as previously reported.[86] All other reagents were purchased from commercial sources. Acetonitrile and triethylamine were dried by distillation over calcium hydride under N_2 and were stored over 4 Å molecular sieves. The ammonium hydroxide used contained 29.9% NH_3. Thin-layer chromatography was carried out using silica gel (250 μm layer) and compounds were visualized by UV light, ninhydrin in ethanol, or phosphomolybdic acid in ethanol. Column chromatography was carried out under pressure (flash chromatography) using silica gel (40 μm). Melting points are uncorrected. NMR spectra were obtained at room temperature using either a Bruker DPX 250MHz or Agilent 400MHz MR DD2 spectrometers. ^{1}H NMR spectra were acquired either at 250 or 400 MHz and ^{13}C NMR at 62.9 or 100.5 MHz. NMR samples were dissolved in $CDCl_3$ with TMS as an internal standard unless otherwise indicated. Mass spectra were obtained on a Waters 70-VSE (FAB MS), or a Thermo Scientific LCQ Fleet ion trap LC-MS (ESI MS).

4.1.1. General procedures for the *N*-alkylation of amines with dibenzosuberyl groups (10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptene, abbreviated as DBS)

To the trifluoroacetate salt of the amine (1.2 mmol) in acetonitrile (10 mL) at 0 ^{o}C was added triethylamine (2.7 mmol) followed by 5-chlorodibenzosuberane (5-chloro-10,11-dihydro-5*H*-dibenzo[*a,d*]cycloheptene) (1.4 mmol). The resulting solution was stirred overnight at room temperature under N_2. Saturated aqueous NaCl solution (30 mL) was added and the mixture extracted with CH_2Cl_2 (3 x 30 mL). The organic layers were collected, dried (anhydrous Na_2SO_4) and concentrated. Purification by column chromatography (1to 4% ethyl acetate in CH_2Cl_2) afforded the desired compounds (**12**), (**13**) and (**14**).

For the *N*-alkylation of the amines *N*,*N*-dimethyl-1,2-diaminoethane or *N*,*N*-dimethyl-1,3-diaminopropane, the above procedure was followed except 1.2 mol. equiv. of triethylamine was used and purification by column chromatography (4 to 5% CH_3OH in CH_2Cl_2) to afford compounds (**2**) or (**3**), respectively.

4.1.2. *N*-dibenzosuberyl-*N′*,*N′*-dimethyl-1,2-diaminoethane (2)

Light-colored oil; yield, 88%; ^{1}H NMR: δ 7.25 (m, 2 H, aromatic *H*), 7.1 (m, 6 H, aromatic *H*), 4.8 (s, 1 H, cycloheptyl C*H*N), 3.65 (m, 2 H, cycloheptyl CH_2), 2.9 (m, 2 H, cycloheptyl CH_2), 2.6 (t, 2 H, *J* = 5.8 Hz, NCH_2), 2.35 (t, 2 H, *J* = 5.8 Hz, NCH_2) and 2.1 (s, 6 H, 2 CH_3) ppm; ^{13}C NMR: δ 140.2, 139.5, 130.0, 128.8, 127.0 and 125.5 (2 x 6 aromatic *C*), 69.2 (cycloheptyl *C*HN), 58.7, 45.6, 45.0 (2 *C*H_3) and 32.1 (2 cycloheptyl *C*H_2) ppm; MS (ESI) *m/z* 281.02 (MH^+).

4.1.3. *N*-dibenzosuberyl-*N′*,*N′*-dimethyl-1,3-diaminopropane (3)

Light-colored oil; yield, 76%; ^{1}H NMR: δ 7.25 (m, 2 H, aromatic *H*), 7.1 (m, 6 H, aromatic *H*), 4.8 (s, 1 H, cycloheptyl C*H*N), 3.65 (m, 2 H, cycloheptyl CH_2), 2.9 (m, 2 H, cycloheptyl CH_2), 2.55 (m, 2 H, NCH_2), 2.3 (m, 2 H, NCH_2), 2.15 (s, 6 H, 2 CH_3), 1.6 (m, 2 H, CH_2) ppm; ^{13}C NMR: δ 139.9, 139.3, 130.0, 128.5, 127.0 and 125.4 (2 x 6 aromatic *C*), 68.8 (cycloheptyl *C*HN), 57.4, 46.4, 44.7 (2 *C*H_3), 32.0 (2 cycloheptyl *C*H_2) and 27.2 ($CH_2CH_2CH_2$) ppm; MS (FAB) *m/z* 295.2 (MH^+); MS (ESI) *m/z* 295.05 (MH^+).

4.1.4. *N*-[2-(trifluoroacetamido)ethyl]-*N*-dibenzosuberyl-*N′*-trifluoroacetyl-1,2-diaminoethane (12)

Light-colored solid; yield, 76%; mp 114-115 °C; ^{1}H NMR ($CDCl_3$, 250 MHz): δ 7.15 (m, 8 H, aromatic *H*), 6.8 (br. t., 2 H, 2 N*H*), 4.4 (s, 1 H, cycloheptyl C*H*N), 3.8 (m, 2 H, cycloheptyl CH_2), 3.1 (br. q, *J* = 6.0 Hz, 4 H, 2 CONHCH_2), 2.8 (m, 2 H, cycloheptyl CH_2), 2.7 (t, *J* = 6.2 Hz, 4 H, CH_2NCH_2) ppm; ^{13}C NMR: δ 157 (q, $^{2}J_{CF}$ = 37.2 Hz, 2 CF_3CO), 139.5, 138.3, 131.1, 131.0, 128.4 and 126.1 (2 x 6 aromatic *C*), 116 (q, $^{1}J_{CF}$ = 288 Hz, 2 *C*F_3), 77.4 (cycloheptyl *C*HN), 51.5, 38.2 and 31.6 (2 cycloheptyl *C*H_2); MS (ESI) *m/z* 510.31 ($M + Na^+$), 487.95 (MH^+).

4.1.5. *N*-[3-(trifluoroacetamido)propyl]-*N*-dibenzosuberyl-*N′*-trifluoroacetyl-1,3-diaminopropane (13)

Light-colored solid; yield, 69%; mp 124-125 °C; ^{1}H NMR: δ 7.15 (m, 8 H, aromatic *H*), 6.1 (br. s, 2 H, 2 N*H*), 4.3 (s, 1 H, cycloheptyl C*H*N), 3.9 (m, 2 H, cycloheptyl CH_2), 3.2 (br. q, *J* = 6.7 Hz, 4 H, 2 CONHCH_2), 2.8 (m, 2 H, cycloheptyl CH_2), 2.5 (t, *J* = 6.7 Hz, 4 H, CH_2NCH_2), 1.55 (pentaplet, 4 H, *J* = 6.7 Hz, 2 $CH_2CH_2CH_2$) ppm; ^{13}C NMR: δ 157 (q, $^{2}J_{CF}$ = 36.9 Hz, 2 CF_3CO), 139.5, 139.1, 130.9, 130.8, 128.1 and 125.8 (2 x 6 aromatic *C*), 116 (q, $^{1}J_{CF}$ = 288 Hz, 2 *C*F_3), 76.5 (cycloheptyl *C*HN), 47.8, 38.0, 31.6 (2 cycloheptyl *C*H_2) and 25.3 ppm; MS (ESI) *m/z* 538.15 ($M + Na^+$), 515.85 (MH^+).

4.1.6. N^4-dibenzosuberyl-N^1,N^8-bis(trifluoroacetyl)spermidine (14)

Light-colored solid; yield, 85%; mp 87-89 °C; ^{1}H NMR: δ 7.1 (m, 8 H, aromatic *H*), 6.8 (br. t, *J* = 5.5 Hz, 2 H, 2 N*H*), 4.3 (s, 1 H, cycloheptyl C*H*N), 3.9 (m, 2 H, cycloheptyl CH_2), 3.1 (m, 4 H, 2 CONHCH_2), 2.8 (m, 2 H, cycloheptyl CH_2), 2.5 (m, 4 H, CH_2NCH_2), 1.5 (pentaplet, 2 H, *J* = 6.3 Hz, $CH_2CH_2CH_2$) and 1.3 (m, 4 H, 2 NCH_2CH_2) ppm; ^{13}C NMR: δ 157.25 (q, $^{2}J_{CF}$ = 36.7

Hz, CF_3CO), 157.2 (q, $^2J_{CF}$ = 36.7 Hz, CF_3CO), 139.45, 139.43, 130.7, 130.4, 127.7 and 125.5 (2 x 6 aromatic *C*), 115.9 (q, $^1J_{CF}$ = 288 Hz, CF_3), 115.8 (q, $^1J_{CF}$ = 288 Hz, CF_3), 76.1 (cycloheptyl *C*HN), 49.8, 47.7, 39.4, 38.1, 31.4 (2 cycloheptyl CH_2), 26.3, 25.4 and 22.2 ppm; MS (ESI) *m/z* 529.92 (MH^+).

4.1.7. N^4,N^8-bis(dibenzosuberyl)-N^1,N^{12}-bis(trifluoroacetyl)spermine (15)

The general procedure was followed but 2.4 mol. equiv. of 5-chlorodibenzosuberane and 5.0 mol equiv. of triethylamine were used to alkylate the two secondary amino groups. Compound (**15**) was obtained as a light-colored solid; yield, 89%; mp 140-141 °C; ^{1}H NMR: δ 7.1 (m, 16 H, aromatic *H*), 6.2 (br. t, 2 H, 2 CON*H*), 4.3 (s, 2 H, 2 cycloheptyl C*H*N), 3.9 (m, 4 H, 2 cycloheptyl CH_2), 3.1 (q, *J* = 6.2 Hz, 4 H, 2 CONHCH_2), 2.8 (m, 4 H, 2 cycloheptyl CH_2), 2.4 (t, *J* = 6.6 Hz, 4 H, 2 CH_2N), 2.3 (br. m, 4 H, 2 CH_2N), 1.5 (pentaplet, 4 H, *J* = 6.8 Hz, 2 $CH_2CH_2CH_2$) and 1.0 (m, 4 H, 2 NCH_2CH_2) ppm; ^{13}C NMR: δ 157.25 (q, $^2J_{CF}$ = 36.7 Hz, 2 CF_3CO), 139.57, 139.47, 130.8, 130.6, 127.8 and 125.6 (4 x 6 aromatic *C*), 118 (q, $^1J_{CF}$ = 288 Hz, 2 CF_3), 76.1 (cycloheptyl 2 *C*HN), 50.0, 47.5, 38.2, 31.6 (2 cycloheptyl CH_2), 31.5 (2 cycloheptyl CH_2), 25.7 and 22.4 ppm; MS (FAB) *m/z* 779.5 (MH^+); MS (ESI) *m/z* 779.04 (MH^+).

4.1.8. General procedure for the removal of trifluoroacetyl groups of trifluoroacetamides

A solution of the trifluoroacetamide (0.5 mmol) in 1:1 CH_3OH:NH_4OH (20 mL) was refluxed overnight. The solvent was removed under vacuum and purification by column chromatography (1- 10% NH_4OH in CH_3OH) afforded the desired amine, compound (**4**), (**5**), (**6**) or (**7**) respectively.

4.1.9. *N*-(2-aminoethyl)-*N*-dibenzosuberyl-1,2-diaminoethane (4)

Light-colored oil; quantitative yield; ^{1}H NMR: δ 7.1 (m, 8 H, aromatic *H*), 4.39 (s, 1 H, cycloheptyl C*H*N), 4.0 (m, 2 H, cycloheptyl CH_2), 2.82 (m, 2 H, cycloheptyl CH_2), 2.54 (s, 8 H, 4 NCH_2) and 1.42 (br. s, 4 H, 2 NH_2) ppm; ^{13}C NMR: δ 139.8, 139.5, 130.8, 130.7, 127.8 and 125.6 (2 x 6 aromatic *C*), 77.6 (cycloheptyl *C*HN), 55.7, 39.8 and 31.7 (2 cycloheptyl CH_2) ppm; MS (ESI) *m/z* 296.09 (MH^+).

4.1.10. *N*-(3-aminopropyl)-*N*-dibenzosuberyl-1,3-diaminopropane (5)

Light-colored oil; yield, 96%; ^{1}H NMR: δ 7.1 (m, 8 H, aromatic *H*), 4.35 (s, 1 H, cycloheptyl C*H*N), 3.95 (m, 2 H, cycloheptyl CH_2), 2.8 (m, 6 H, cycloheptyl CH_2 and 2 NH_2), 2.5 (m, 8 H, 4 NCH_2) and 1.5 (m, 4 H, 2 CH_2) ppm; ^{13}C NMR (CD_3OD, 62.9 MHz): δ 141.5, 140.9, 131.8, 131.7, 128.8 and 126.6 (2 x 6 aromatic *C*), 77.2 (cycloheptyl *C*HN), 48.5, 40.7, 32.7 (2 cycloheptyl CH_2) and 29.3 ppm; MS (ESI) *m/z* 324.13 (MH^+).

4.1.11. N^4-(dibenzosuberyl)spermidine (6)

Light-colored oil; yield, 64%; ^{1}H NMR: δ 7.1 (m, 8 H, aromatic *H*), 4.35 (s, 1 H, cycloheptyl C*H*N), 3.95 (m, 2 H, cycloheptyl CH_2), 2.8 (m, 2 H, cycloheptyl CH_2), 2.45 (m, 8 H, 4 NCH_2), 2.0 (br. s, 4 H, 2 NH_2), 1.4 (pentaplet, 2 H, *J* = 6.95 Hz, $CH_2CH_2CH_2$), 1.3 (m, 2 H, $CH_2CH_2CH_2$) and 1.2 (pentaplet, 2 H, *J* = 6.95 Hz, $CH_2CH_2CH_2$) ppm; ^{13}C NMR: δ 139.8, 139.2, 130.3, 130.0, 127.2 and 125.1 (2 x 6 aromatic *C*), 75.6 (cycloheptyl *C*HN), 49.4, 46.9, 41.3, 39.7, 31.2 (2 cycloheptyl *C*H_2), 30.8, 29.1 and 21.9 ppm; MS (FAB) *m/z* 338.2 (MH^+); MS (ESI) *m/z* 338.13 (MH^+).

4.1.12. N^4,N^8-bis(dibenzosuberyl)spermine (7)

N^4,N^8-bis(dibenzosuberyl)-N^1,N^{12}-bis(trifluoroacetyl)spermine (**15**) (1.014 g, 1.30 mmol) was dissolved in pyridine (15 mL) and 8 M aqueous NaOH (15 mL) was added. The mixture was refluxed overnight. Saturated aqueous NaCl was added (40 mL) and the mixture extracted with CH_2Cl_2 (4 x 40 mL). The collected organic layers were dried (Na_2SO_4), concentrated and purified by column chromatography (2 to 10% CH_3OH in CH_2Cl_2 containing 1% NH_4OH) to give a light-colored oil (0.745 g, 98%). ^{1}H NMR($CDCl_3$, 300 MHz): δ 7.1 (m, 16 H, aromatic *H*), 4.3 (s, 2 H, 2 cycloheptyl C*H*N), 3.95 (m, 4 H, 2 cycloheptyl CH_2), 2.75 (m, 4 H, 2 cycloheptyl CH_2), 2.45 (t, 4 H, *J* = 7.0 Hz, 2 NCH_2), 2.35 (t, 4H, *J* = 7.0 Hz, 2 NCH_2), 2.3 (br. t, 4 H, 2 NCH_2), 1.4 (pentaplet, 4 H, *J* = 7.0 Hz, 2 $NCH_2CH_2CH_2N$), 1.15 (br. s, 4 H, 2 NH_2) and 1.05 (br. m, 4 H, CH_2CH_2) ppm; ^{13}C NMR: δ 140.1, 139.4, 130.6, 130.3, 127.4 and 125.2 (4 x 6 aromatic *C*), 75.8 (2 cycloheptyl *C*HN), 49.8, 47.2, 40.1, 31.4 (4 cycloheptyl *C*H_2), 29.7 and 22.6 ppm; MS (FAB) *m/z* 587.4 (MH^+); MS (ESI) *m/z* 587.27 (MH^+).

4.1.13. *N*-[3-(trifluoroacetamido)propyl]-*N′*-trifluoroacetyl-1,3-diaminopropane (9)

The general procedure previously described was followed, [86] i.e., to *N*-(3-aminopropyl)-1,3-diaminopropane (2.50 g, 19.1 mmol) in CH_3CN (15 mL) was added ethyl trifluoroacetate (9.77 g, 68.8 mmol) and water (0.41 g, 22.8 mmol). The solution was heated overnight at 80 °C. Solvent was removed under vacuum and CH_2Cl_2 (100 mL) was added. The resulting white solid was filtered and washed with CH_2Cl_2 to give (**9**) as a white solid (6.64 g, 80%); mp 166 °C; ^{1}H NMR (d_6-DMSO, 250 MHz): δ 9.6 (br. s, 2 H, N*H*), 7,7 (br. s, 2 H, N*H*), 3.25 (t, 4 H, *J* = 6.5 Hz, 2 CONHCH_2), 2.9 (br. t, 4 H, *J* = 7.5 Hz, C$H_2NH_2^+CH_2$) and 1.8 (br. pentaplet, *J* = 7.5 Hz, 4 H, 2 $CH_2CH_2CH_2$) ppm; ^{13}C NMR (d_6-DMSO, 62.9 MHz): δ 159.2 (q, $^2J_{CF}$ = 32.0 Hz, CF_3COO^-)156.6 (q, $^2J_{CF}$ = 36.2 Hz, 2 CF_3CO), 117.2 (q, $^1J_{CF}$ = 298 Hz, CF_3COO^-), 116 (q, $^1J_{CF}$ = 288 Hz, 2 CF_3), 44.8, 36.7 and 25.5 ppm; MS (ESI) *m/z* 324.20 (MH^+).

4.2. Enzyme studies

4.2.1. Trypanothione reductase studies

T. cruzi TR was purified following the method of Walsh *et al.*[88] from *E. coli* SG5, a glutathione reductase deletion mutant, containing the TR expression vector pIBITczTR described by Sullivan and Walsh.[87, 88] Prepared compounds were assayed for their effects on the rate of

reduction of trypanothione disulfide by *T. cruzi* TR spectrophotometrically by monitoring the oxidation of NADPH at 340 nm.[13] Stock solutions of compounds (**1**), (**3**) and (**4**) were prepared in HEPES buffer (100 mM, pH 7.25); stock solutions of compounds (**5**) and (**7**) were prepared in 1:1 ethanol:HEPES buffer; and stock solutions of compounds (**2**) and (**6**) were prepared in ethanol . TR activity was measured at 23 ^{o}C in HEPES buffer (100mM, pH 7.25) containing EDTA (1.0 mM), NADPH (0.18 mM), trypanothione disulfide (Bachem Bioscience Inc.) and TR at 1.33 µg/mL. Also, to account for any possible effects of ethanol on TR, for the assays of compounds (**2**), (**5**), (**6**) and (**7**), every 1.0 mL enzyme assay contained a total of 20 µL of ethanol. An estimate of the K_i value for each inhibitor was obtained from initial assays. More accurate K_i values were obtained in subsequent experiments in which a minimum of five inhibitor concentrations (ranging from 0 µM to approximately 2 times the estimated K_i) were assayed for their effects on TR activity in the presence of five trypanothione disulfide concentrations (14.8, 22.2, 29.6, 44.4 and 74.0 µM).

For each compound the inhibition type was assessed by the patterns of three classes of plots: $1/v$ against $1/[S_o]$ for various [I]; $1/v$ against [I] for various $[S_o]$; and $[S_o]/v$ against [I] at various $[S_o]$. For each inhibitor concentration, $K_{m(obs)}$ (with standard deviation) and V_{max} were determined from a non-linear regression analysis of the plot of v against $[S_o]$ using the Michaelis-Menten equation. The compounds all exhibited linear competitive inhibition and the K_i value for each compound was determined from a weighted regression analysis of a plot of $K_{m(obs)}$ against [I] using the following equation (weighting factors used were 1/(SD of $K_{m(obs)}$)):

$$K_{m(obs)} = (K_m[I]/K_i) + K_m$$

4.2.2. Glutathione reductase studies

The effects of compounds (**1**) to (**7**) on the rate of reduction of glutathione disulfide by yeast glutathione reductase (GR) (EC 1.6.4.2) was assayed spectrophotometrically by monitoring the oxidation of NADPH at 340 nm. Stock solutions of compounds (**1**) to (**4**) were prepared in ethanol, and stock solutions of compounds (**5**) to (**7**) were prepared in 1:1 ethanol:HEPES buffer (100 mM, pH 7.25). To account for any possible effects of ethanol on GR, every 1.0 mL enzyme assay contained a total volume of 20 µL of ethanol. GR activity was measured at 23 ^{o}C in HEPES buffer (100 mM, pH 7.25) containing EDTA (1.0 mM), NADPH (0.18 mM), ethanol (20 µL) and five glutathione disulfide concentrations (15.0, 22.5, 30.0, 40.0 and 60.0 µM) with an enzyme concentration of either 0.255 or 0.30 µg/mL. The effects of compounds (**1**) to (**7**) on GR activity were measured at a concentrations of 22.5 and 60 µM glutathione disulfide and at: 0, 250 and 500 µM of compounds (**1**), (**2**), (**3**) or (**4**); 0 and 79.9 µM of (**5**); or 0, 125 and 250 µM of compounds (**6**) or (**7**).

4.3. Trypanosome studies

4.3.1. Trypanosome isolates

Four trypanosome isolates were used to assess *in vitro* activity: *Trypanosoma brucei brucei* Lab 110 EATRO and three clinical isolates of *T. b. rhodesiense*, KETRI 243, KETRI 269 and KETRI 243-As-10-3. KETRI 243 displays some resistance to melamine-based arsenical drugs (including melarsoprol) and pentamidine. KETRI 243-As-10-3 (a clone of KETRI 243) is completely resistant to melarsoprol and pentamidine.[90]

4.3.2. Determination of *in vitro* antitrypanosomal activities of compounds (2) to (7)

Trypanosome strains were grown as the blood form at 37 ^{o}C in a synthetic medium (HMI-18) with 20% fetal bovine serum. The *in vitro* trypanocidal activities of compounds were assessed by determining IC_{50} values. Measurements were done in duplicate in 24 well plates (1 mL/well). After 48 h, the number of parasites/well was determined using a Z1^{TM} Coulter Counter®. In the absence of inhibiting compounds, controls grew to 5 x 10^6 parasites /mL after 48 h. The approximate range of activity for each compound was initially determined using compound concentrations of 0.1, 1.0, 10, 25 and 100 µM. The 50% inhibitory concentration (IC_{50}) of compounds after incubation with trypanosomes for 48 h, was then determined from additional studies using compound concentrations spaced close to the approximate IC_{50} value. IC_{50} values were determined from semi-log plots. Compounds were dissolved in water then diluted with medium.[90]

4.3.3. *In vivo* studies of trypanocidal activities of compounds (6) and (7)

Female Swiss-Webster mice were infected intraperitoneally (ip) with 2.5 x 10^5 *T. b. brucei* Lab EATRO blood forms of the parasite taken from an infected rat. The infection was allowed to develop for 24 h before treatment was begun. Infected mice were divided into groups of five, including infected, non-treated controls. For compound (**6**), animals were dosed ip with 1.0, 2.5, 5.0, 10 or 25 mg per kg body weight, given once daily for three days. For compound (**7**), animals were dosed ip with a total daily dose of 25 or 50 mg per kg body weight, administered in two doses every day (b.i.d.) for three days. Compound (**7**) was also given at 50 mg per kg body weight, given in a single daily dose for three days. In a second experiment, compound (**7**) was given via Alzet® mini-osmotic pumps (ALZA, Corp., Palo Alto, CA) which dispense 1 µL/h continuously for 3 days. Compound (**7**) was given at 10, 25 or 50 mg/kg/day. Survival times were compared to infected mice that were not treated with compounds.

4.4. Computational methods

4.4.1. Ligand preparation

Compounds (**1**) to (**7**) were built in 2D using AccelrysDraw 4.0[117] and saved as skc files. The predominant protonation states of the compounds at pH 7.2 were estimated using "Calculator Plugins" within MarvinSketch 6.3.0 [118] and compared with literature pK_a values for analogous compounds.[119, 120] This led to the identification of one protonated form of clomipramine (**1**) and ten protonated forms of compounds (**2**) to (**7**) that we investigated.

Using Accelrys Discovery Studio 3.0 Visualizer,[121] the eleven 2D structures in skc format were converted to 3D structures with hydrogen atoms and protons, and the structure files were saved in MDL Molfile format.

Since most docking programs including AutoDock Vina treat rings as rigid with no conformational flexibility during docking calculations (although other bonds in ligands can be designated as rotatable), we investigated the *in vacuo* energies of DBS/DBA conformers. Thus, the *in vacuo* energies of DBS and DBA ring conformations of different rotamers (i.e., while allowing rotation of DBS–N or DBA–C bonds) were explored by employing the MMFF94 force field with the conjugate gradient algorithm available in Avogadro 1.1.0.[122, 123] For these studies the model structures DBS–$NHCH_3$, DBS–$NH_2^+CH_3$ and DBA–CH_3 were used (protonation of the endocyclic N atom in DBA was not studied since this N is not protonated at pH 7.2). Since the DBS and DBA ring conformers are chiral, this led to the identification of *in vacuo* energy minimum conformations for the tricyclic rings (four conformations for compounds with the structure DBS–NHR, two for DBS–NH_2^+R and four for the DBA of clomipramine). Representative structures of the conformations are shown in Table 4 (structures for all the conformations investigated are provided in Supplemental Information).

Since AutoDock programs treat cyclic groups as rigid, the structures in each of the MDL Molfiles were modified using Avogadro 1.1.0[122, 123] to include the appropriate energy-minimized DBS or DBA ring conformations. This led to 33 structure files (of the protonated and uncharged forms of compounds (**1**) to (**7**) along with the identified ring conformations) which were written as SYBYL Mol2 files.

AutoDockTools (v 1.5.4 revision 30)[124, 125] was used to prepare each of the 33 structure files for docking. This involved identifying bonds that will rotate during dockings, and merging carbon atom volumes with attached hydrogens (i.e., non-polar hydrogens) using the unified atom model. The resulting structure/ligand files were written as PDBQT files (which is the AutoDock Vina input file format) and used in subsequent docking runs.

Additional calculations (using the MMFF94 force field in Avogadro) showed that the energy barriers for interconversion of the conformations (i.e., a ring flex involving rotation at C10 and C11) were less than 10 kcal/mol (which is consistent with energy barriers for conformation interconversion of analogous compounds, including cycloheptane and oxacycloheptane, with values of 8.55 and 8.5 kcal/mol[126] and imipramine[127, 128]). This indicates that *in vitro* the ring conformations will interconvert.

4.4.2. Protein preparation

The structure of the biologically active dimer of *T. cruzi* TR containing bound trypanothione was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB Protein Data Bank code ID: 1BZL biological assembly version).[33] AutoDockTools was used to: remove all water molecules, ions and ligands; add polar hydrogens and assign standard protonation states; and to create the unified atom model. The resulting protein/receptor structure was written as a PDBQT file and used for subsequent dockings. A grid

box (the search space) was defined which encompassed the entire active site cleft (the defined grid box was significantly larger than the active site to minimize any "boundary effects" in the docking runs). The grid box did not include the FAD or NADPH binding regions or the interface pocket of TR. The size of the resulting grid box was 30 x 50 x 30 Å (and centered at coordinates x = 66.0, y = 6.0 and z = -6.0 with respect to the original 1BZL file).

4.4.3. Docking method

We utilized AutoDock Vina[91] for docking studies. AutoDock Vina is often better than AutoDock 4, and programs such as Dock and FlexX, for predicting experimentally observed binding modes especially for molecules with 10 or more rotatable bonds.[91, 98, 129] Although several recent TR docking studies utilized AutoDock 4[99, 130] or earlier generations of AutoDock and/or FlexX,[131, 132] given the large conformational flexibility and number of rotatable bonds of our inhibitors, AutoDock Vina was the obvious choice for our studies. Specifically, the Windows version of AutoDock Vina 1.1.2 [91] was used to dock each of the 33 ligand structures to the receptor. The configuration file parameters were changed to include "exhaustiveness = 20" (default is 8) and "num_modes = 10" (this saves the results of the 10 lowest energy poses, the default is 9). This increased level of exhaustiveness was used to ensure comprehensive sampling of the large search space of 30 x 50 x 30 Å (which was required to accommodate the large active site cleft of TR). For each ligand structure 8 docking runs were performed and the coordinates of the 10 lowest energy ligand poses for each run were collected (i.e., a total of 80 ligand poses for each ligand structure). This approach allowed the collection of a large number of low energy poses without the risk of introducing bias, since AutoDock Vina resets starting parameters at the beginning of each new run. The binding energies of the lowest energy poses for all modeling runs for the 33 ligand structures ranged from -5.6 to -8.1 kcal/mol (this range of binding energies is very similar to *in silico* values calculated for tricyclics and quinones docked to TR[99, 131]). Tables of the binding energies of the lowest energy poses for each ligand structure along with descriptions of the binding sites of frequently occurring poses are provided in Supplemental Information.

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Supplementary information

Supplementary information associated with this article is available.

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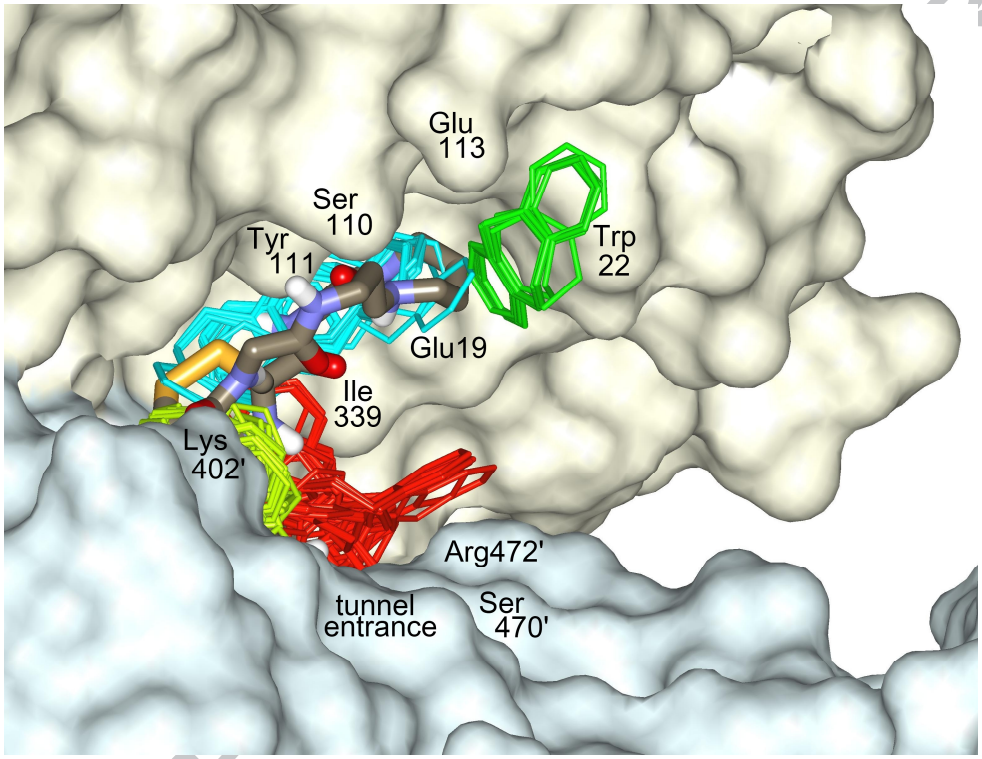
Figure 1. *T. cruzi* TR active site showing the four DBS/DBA binding regions superimposed on the 1BZL structure containing bound trypanothione. Specifically the figure shows superimposed the DBS groups from the five lowest energy poses of docking runs for all conformations of compound (**4**). View is from (A) the side of the active site; and (B) a 90° rotation with a view looking into the active site. Represented is: TR protein subunit A in cream color; protein subunit B in blue/grey (subunit B residues are labeled with a prime (‘)); trypanothione in brown and CPK coloring. DBS groups are shown in wireframe, with DBS in binding region 1(BR 1) as red; DBS in BR 2 as yellow; DBS in BR 3 as blue/aqua; and DBS in BR 3 as green.

(A)



(B)

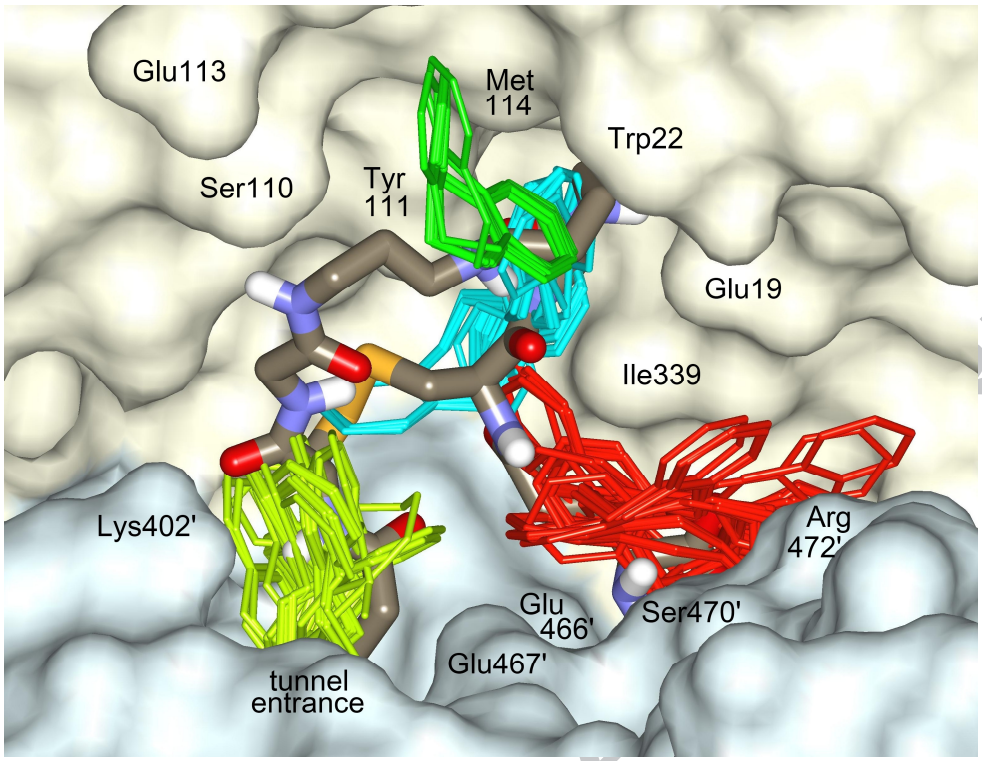
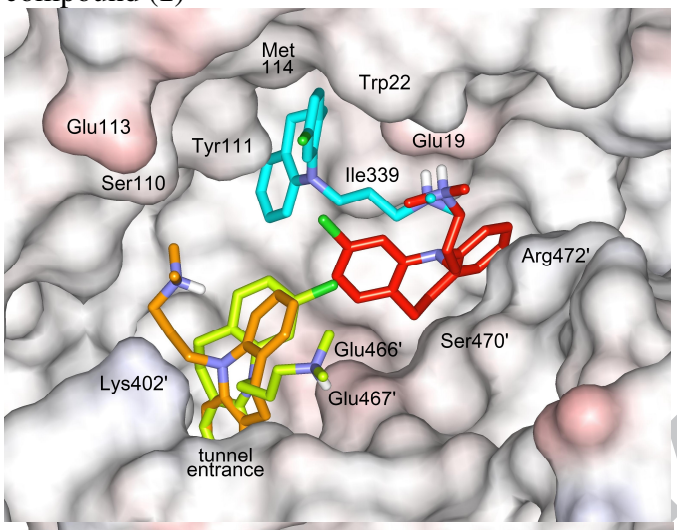
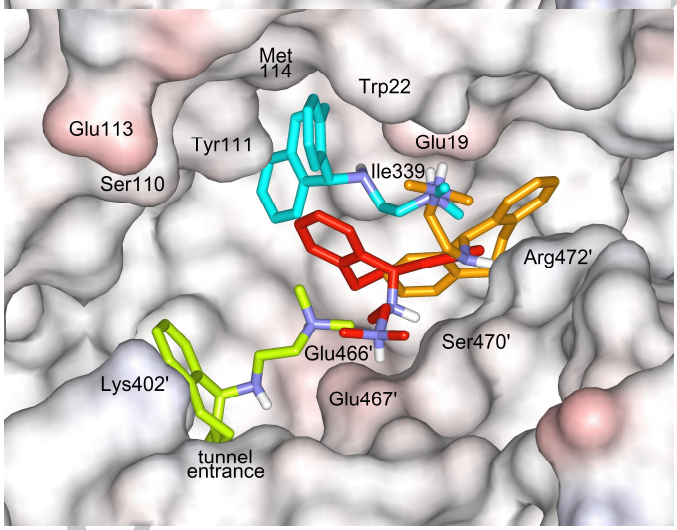


Figure 2. Representative low energy poses of compounds (**1**) to (**6**) from the docking of all ligand structures to the *T. cruzi* TR active site using AutoDock Vina. Poses with the DBS/DBA group located in: BR 1 (Ile339) are in red; BR 2 (tunnel) yellow; BR 3 (Tyr111) blue-aqua; BR 4 (Trp22) green; and a second pose in any BR is shown in brown. Ligand nitrogen atoms are blue-violet and hydrogen atoms on protonated nitrogen atoms are white. Residues from protein subunit B are labeled with a prime (').

compound (**1**)

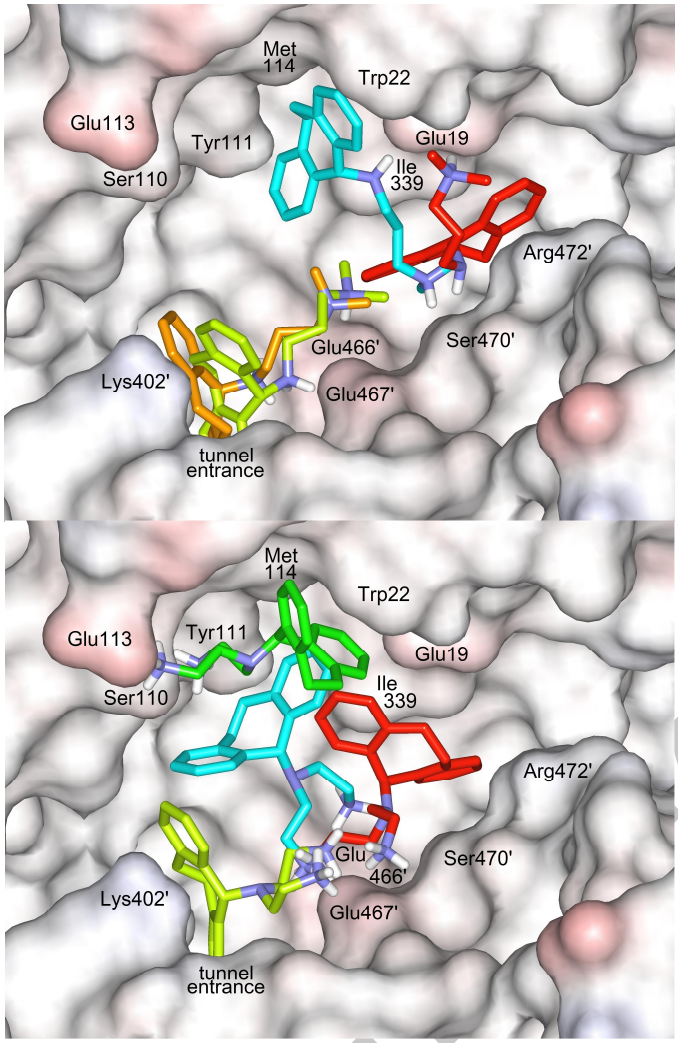


compound (**2**)



compound (**3**)

compound (**4**)



compound (**5**) compound (**6**)

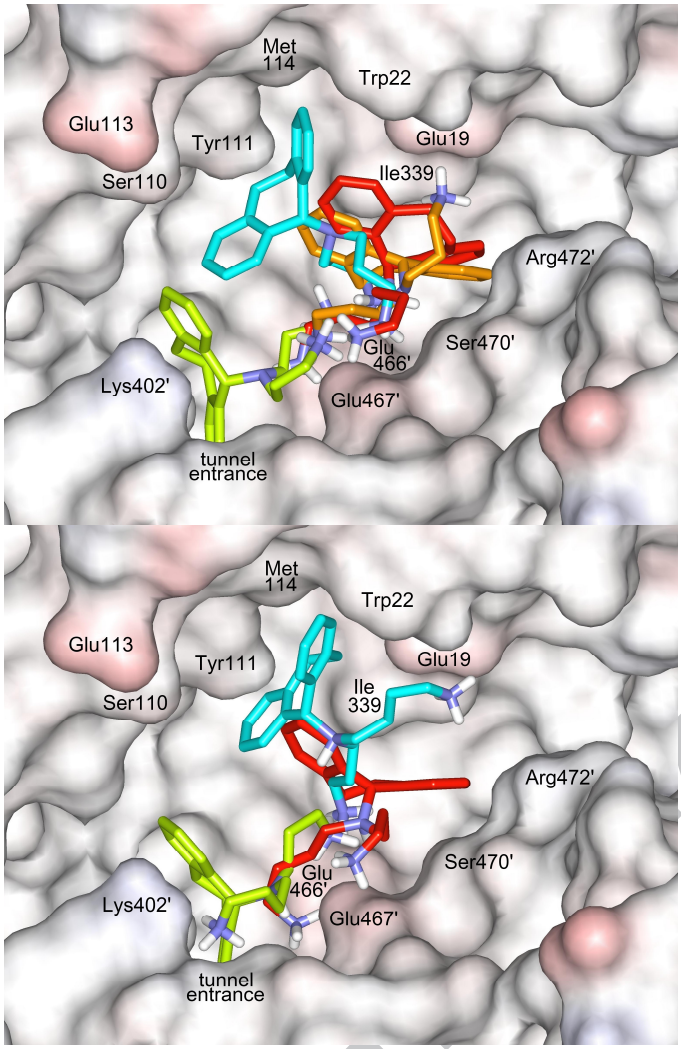
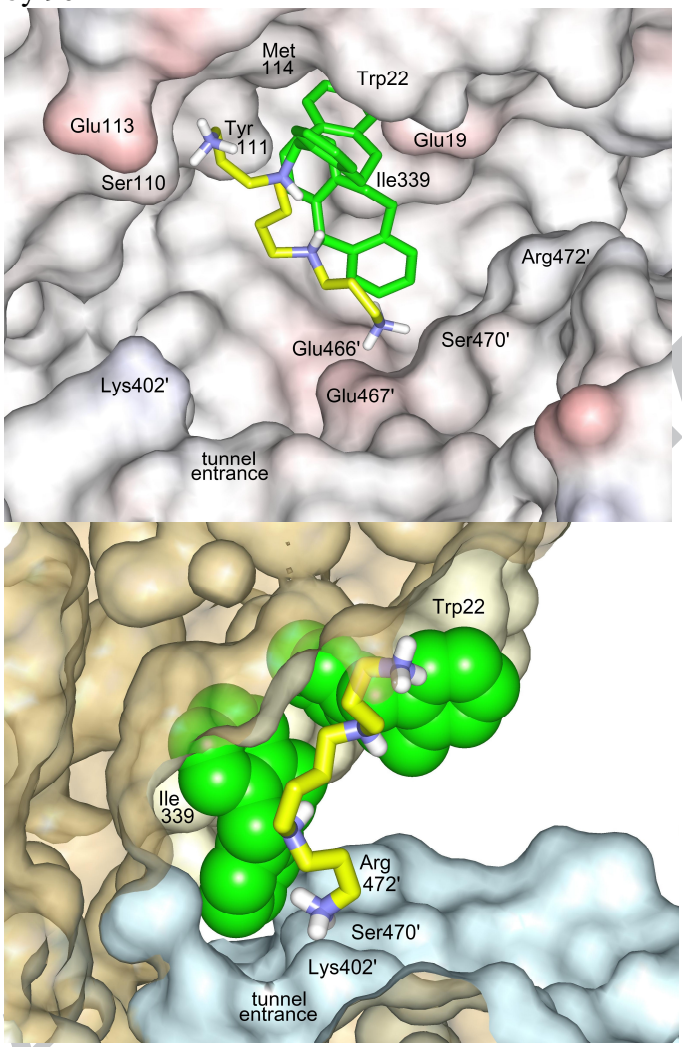
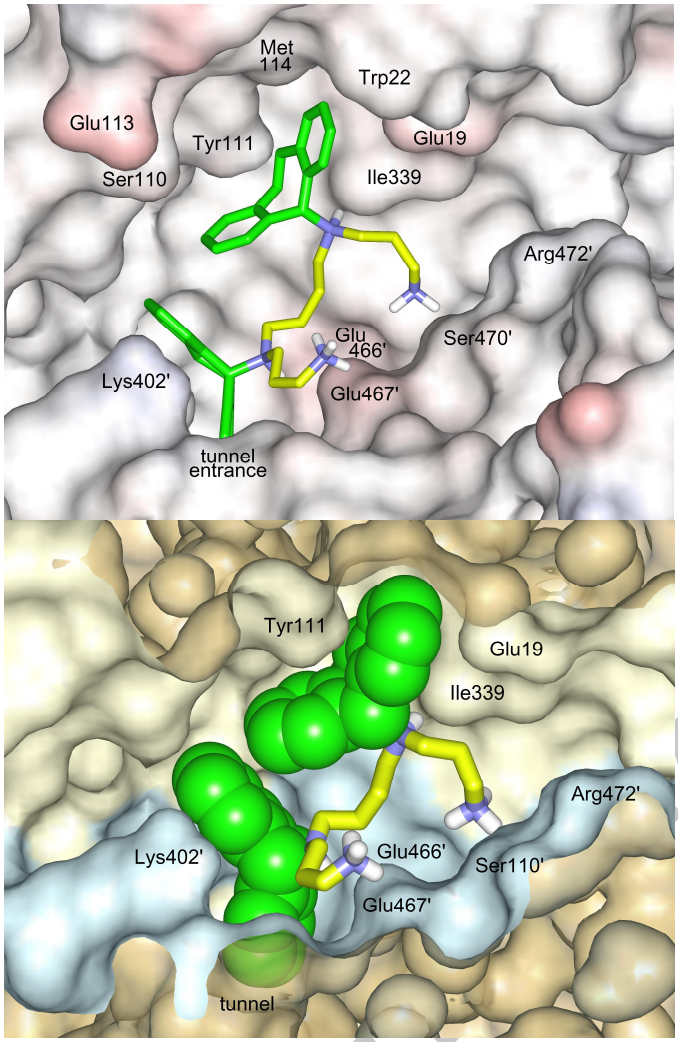


Figure 3. Representative low energy poses for each of the three binding modes of compound (**7**) from the docking of all ligand structures of (**7**) to the *T. cruzi* TR active site using AutoDock Vina. The DBS groups are shown in green; spermine chain in yellow; ligand nitrogen atoms are blue-violet; hydrogen atoms on protonated nitrogens are white. The second view of each binding mode is a cut-plane view with the DBS carbon atoms in spacefill (Van der Waals radii). Residues from protein subunit B are labeled with a prime (').

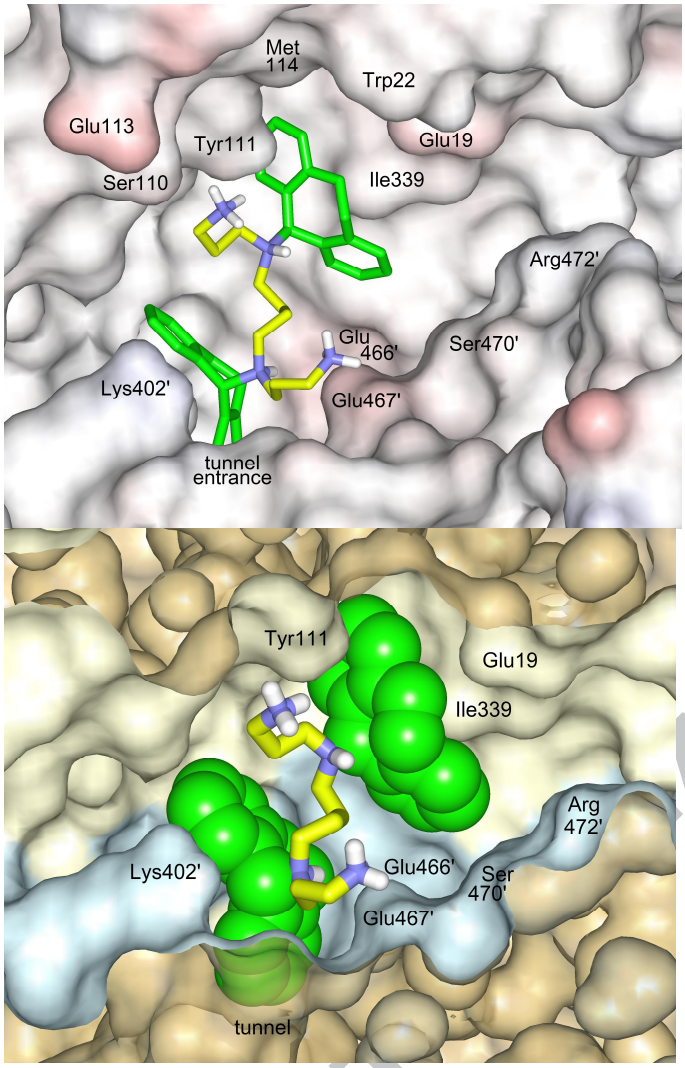
Binding mode 1 for (**7**) with DBS in BR 1 (Ile339) and BR 4 (Trp22), cut-plane view is rotated by 90°



Binding mode 2 for (**7**) with DBS in BR 2 (tunnel) and BR 3 (Tyr 111)



Binding mode 3 for (**7**) with DBS in BR 1 (Ile339) and BR 2 (tunnel)



Scheme 1. Reagents and conditions: (i) CF_3COOEt, H_2O, CH_3CN, reflux; (ii) 5-chlorodibenzosuberane, triethylamine, CH_3CN; (iii) 1:1 CH_3OH : NH_4OH, reflux; for preparation of (**7**) 1:1 pyridine : 8 M NaOH(aq), reflux.

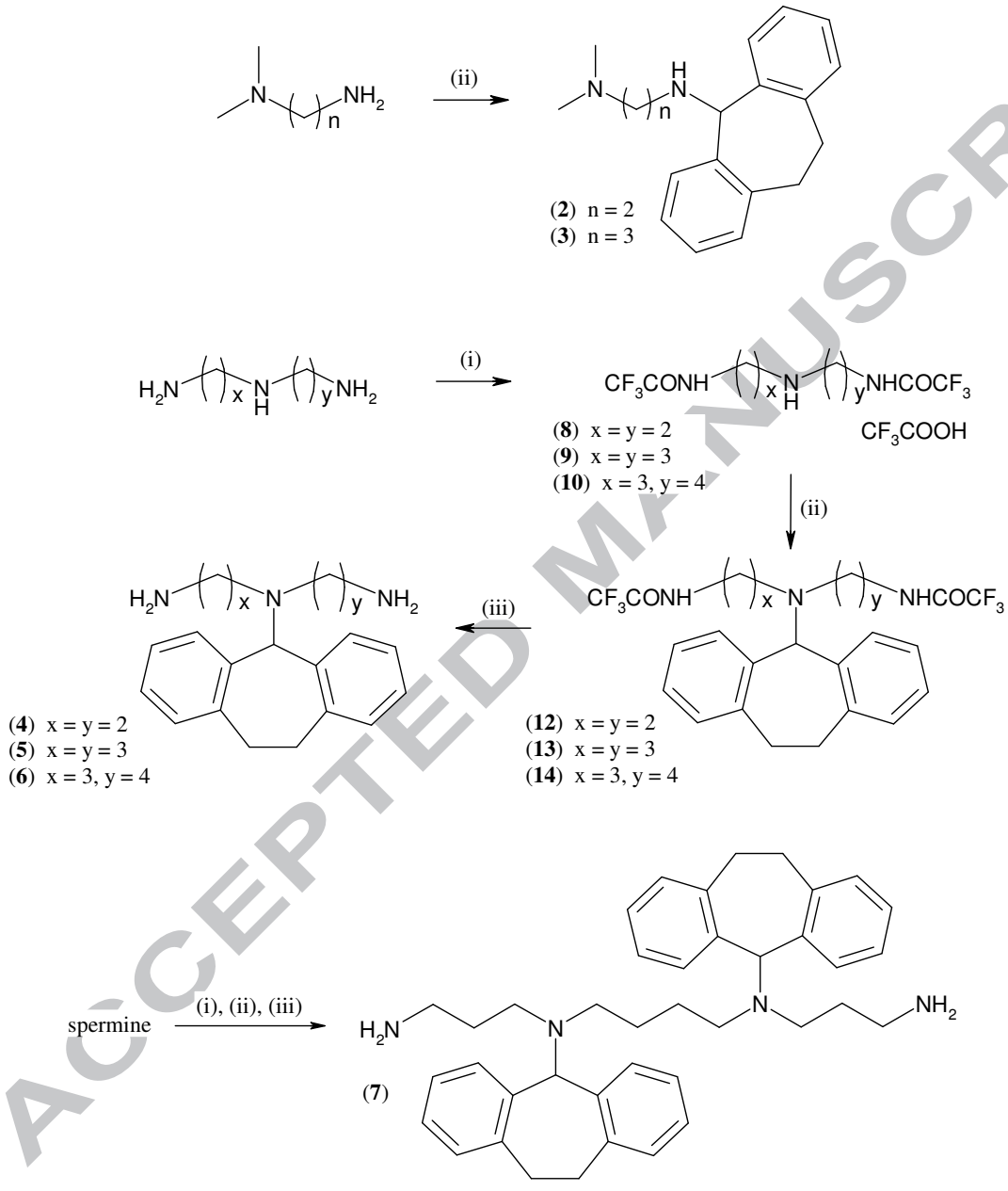
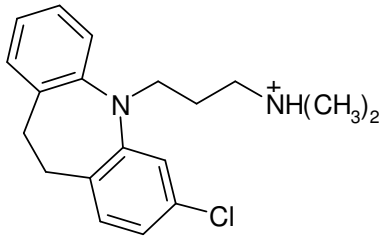
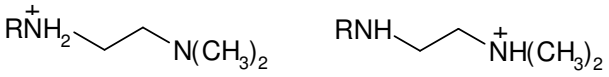
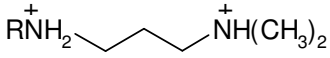
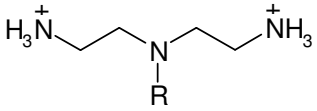
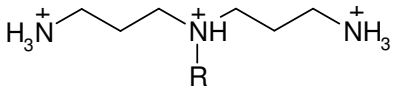
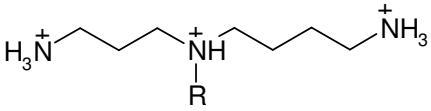
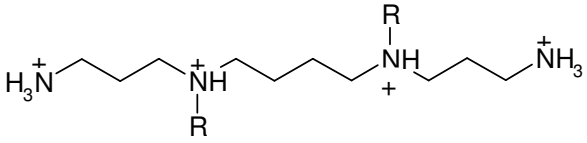
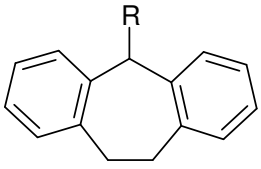


Table 1. K_i values for the competitive inhibition by compounds of trypanothione disulfide reduction by recombinant TR from *T. cruzi*

	Compound[a]	K_i (μM) ± SD
1		8.40 ± 1.43
2		136 ± 34.3
3		81.6 ± 23.6
4		27.9 ± 8.29
5		7.62 ± 1.80
6		4.00 ± 1.93
7		0.255 ± 0.06
	R represents dibenzosuberyl group 	

[a]The predominant protonation state(s) of each amino group at pH 7.2 is shown as predicted by MarvinSketch 6.3.0[118] and based on a structural comparison with the experimentally determined pK_a values of similar ammonium ions.[119, 120]

Table 2. Trypanocidal activities of compounds against four *T. brucei* ssp. strains *in vitro*

Compound	IC_{50} (μM)			
	Lab 110[a]	K 243[b]	K 269[b]	K 243-As-10-3[c]
1	(5.05)[d]			
2	1.68	2.0		
3	1.38			
4	2.8	1.6	2.15	4.0
5	not determined			
6	0.62	4.5	4.3	8.3
7	2.55	1.5	1.69	22

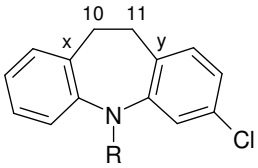
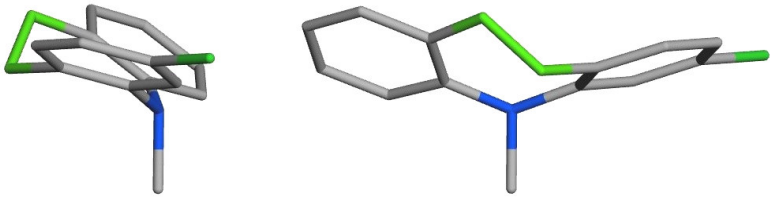
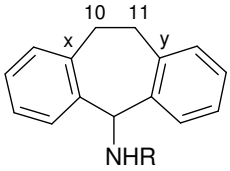
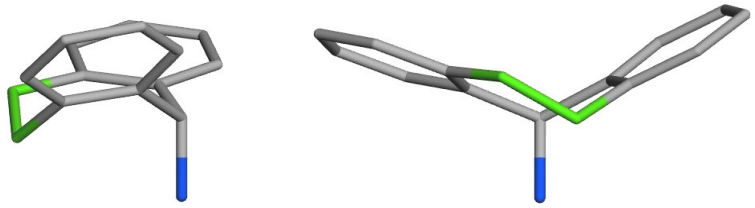
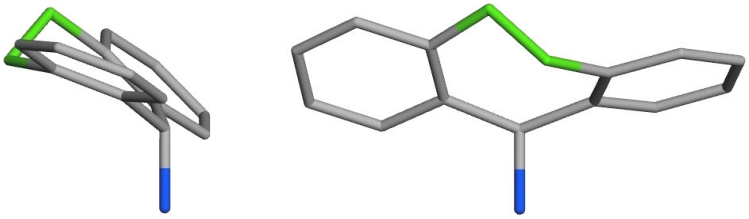
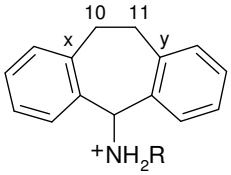
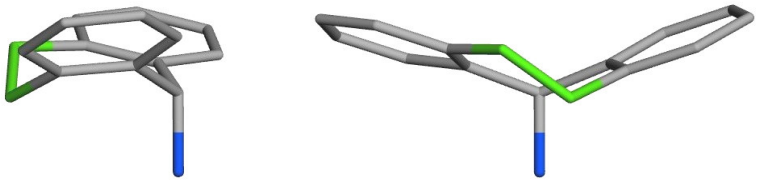
[a]*T. b. brucei* Lab 110 EATRO is a drug-sensitive strain.
[b]KETRI 243 and KETRI 269 are uncloned clinical isolates of *T. b. rhodesiense*.
[c]KETRI 243-As-10-3 is a pentamidine and melarsoprol-resistant clone of *T. b. rhodesiense* KETRI 243.
[d]Richardson *et al.*[39] reported an EC_{50} value of 5.05 μM for clomipramine against a bloodstream form of *T. brucei*.

Table 3. Protonation states of compounds used in docking studies, their relative occurrence at pH 7.2, and the pK_a values of aminium ions as predicted by MarvinSketch[a]

Compound and structure abbreviation	pK_a of protonated N[a]					protonation states used in modelling studies (% occurrence at pH 7.2)[a]	
	endo-cyclic	N_A	N_B	N_C	N_D		
clomipramine **1** DBA–N_A	-1.06	9.20				DBA–N_AH^+	(99.0)
compound **2** DBS–N_A–N_B		9.11	4.93			DBS–N_AH^+–N_B DBS–N_A–N_BH^+	(63.9) (34.3)
compound **3** DBS–N_A–N_B		7.33	9.88			DBS–N_AH^+–N_B DBS–N_A–N_BH^+ DBS–N_AH^+–N_BH^+	(14.3) (28.1) (57.5)
compound **4** N_A-N_B(DBS)-N_C		9.02	4.58	9.72		N_AH^+–N_B(DBS)–N_CH^+	(97.9)
compound **5** N_A-N_B(DBS)-N_C		9.78	7.65	10.59		N_AH^+–N_B(DBS)–N_CH^+ N_AH^+–N_BH^+(DBS)–N_CH^+	(23.9) (73.8)
compound **6** $N_A(CH_2)_3N_B$(DBS)$(CH_2)_4N_C$		10.01	8.64	10.62		$N_AH^+(CH_2)_3N_BH^+$(DBS)$(CH_2)_4N_CH^+$	(96.5)
compound **7** N_A-N_B(DBS)-N_C(DBS)-N_D		8.94	10.13	8.35	10.72	N_AH^+-N_BH^+(DBS)-N_CH^+(DBS)-N_DH^+	(93.2)

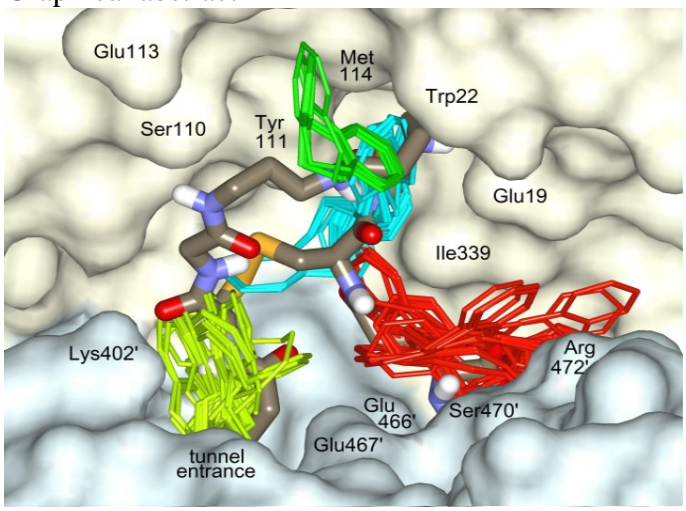
[a]Calculator Plugins were used for physico-chemical property predictions, MarvinSketch 6.3.0[118]

Table 4. Conformations of the 3-chloro-10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepinyl (DBA) ring used in docking studies of compound (**1**), and representative conformations of the dibenzosuberyl (DBS) ring used in docking studies of compounds (**2**) to (**7**).[a]

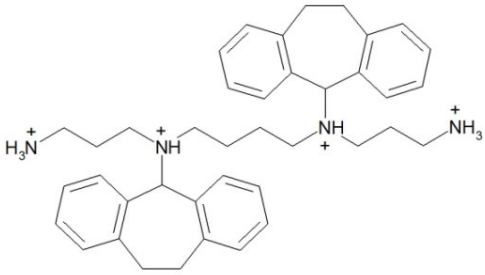
ring structure	structures of conformers[b]	abbreviation and (torsion angle)[c]
		DBA_i–R (+77°)
[additional DBA conformers used: DBA_{ii}–R (-77°), DBA_{iii}–R (+77°) and DBA_{iv}–R (-77°)[a]]		
		DBS_iN–R_2 (+66°)
	mirror image of DBS_iN–R_2	$DBS_{ii}N$–R_2 (-66°)
		$DBS_{iii}N$–R_2 (+86°)
	mirror image of $DBS_{iii}N$–R_2	$DBS_{iv}N$–R_2 (-86°)
		DBS_vNH^+–R_2 (+72°)
	mirror image of DBS_vNH^+–R_2	$DBS_{vi}NH^+$–R_2 (-72°)

[a]Structures of all the conformations used in the docking studies are provided in the Supplemental Information.
[b]The two representations of each conformer shown differ by a 90° rotation around N–R bond axis (the vertical bond).
[c]Torsion angles measured along bonds Cx–C10–C11–Cy these bonds are shown in green (chlorine atom of DBA is also green). For DBS-containing compounds, the torsion angles given are ± 2° of value for the actual structures used in docking studies.

Graphical abstract



binding regions of dibenzosuberyl groups to trypanothione reductase



K_i 0.26 μM against *Trypanosoma cruzi* trypanothione reductase