Journal of Medicinal Chemistry



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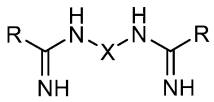
*J. Med. Chem.*, **2005**, 48 (15), 4793-4802• DOI: 10.1021/jm050027z • Publication Date (Web): 25 June 2005






X = 4-phenoxyphenol, 4-(cyclohexyl)methyl-cyclohexane, 1,2-Diethoxy-ethane, octane
R = thiophene, pyrimidine, 2-methoxy-4-methyl-1-(phenylmethoxy)benzene

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# Inhibitors of *Trypanosoma cruzi* Trypanothione Reductase Revealed by Virtual Screening and Parallel Synthesis

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*Received January 11, 2005*

In an approach to discover new inhibitors of trypanothione reductase from *Trypanosoma cruzi*, the causative agent of Chagas' disease, a virtual high-throughput screening was performed. Two structurally new types of inhibitors emerged, the antimicrobial chlorhexidine {1,1′-hexamethylenebis[5-(4-chlorophenyl)biguanide]}, a linear competitive inhibitor ($K_i = 2 \pm 1\ \mu M$), and a piperidine derivative acting as mixed inhibitor ($K_i = 6.2 \pm 2\ \mu M$ and $K_i' = 8.5 \pm 2\ \mu M$). Neither compound interferes with human glutathione reductase. Based on chlorhexidine, different series of compounds were synthesized and studied as inhibitors of *T. cruzi* trypanothione reductase. Most efficient derivatives were three bis(amidines) showing mixed type inhibition with $K_{i,slope}$ and $K_{i,int}$ values of 2−5 $\mu$M and 16−47 $\mu$M, respectively. Although these compounds did not exert an improved inhibitory potency compared to chlorhexidine, the change from competitive to mixed-type inhibition is advantageous, since substrate accumulation does not overcome inhibition. Remarkably, all three derivatives carried two copies of an identical 2-methoxy-4-methyl-1-(phenylmethoxy)benzene substituent.

## Introduction

Protozoan parasites of the genus *Trypanosoma* and *Leishmania* are the causative agents of African sleeping sickness (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*), Chagas' disease (*Trypanosoma cruzi*), Nagana cattle disease (*Trypanosoma brucei brucei* and *Trypanosoma congolense*), Kalar-azar (*Leishmania donovani*), and Oriental sore (*Leishmania tropica*). All these parasitic protozoa lack the ubiquitous enzyme glutathione reductase (GR). To maintain a reducing intracellular redox milieu, trypanosomes and leishmania rely on the flavoenzyme trypanothione reductase (TR),[1,2] which keeps their main thiols, bis(glutathionyl)spermidine [trypanothione, $T(SH)_2$)][3] and mono(glutathionyl)spermidine (Gsp), in the thiol state:

$$TS_2 + NADPH + H^+ \rightarrow T(SH)_2 + NADP^+$$

In addition to trypanosomatids, trypanothione reductase has been found so far in *Entamoeba histolytica*[4] and—together with glutathione reductase—in *Euglena gracilis*.[5] TR shares many mechanistic and structural properties with glutathione reductase, the closest related mammalian enzyme. In contrast to GR, the active site of TR shows an overall negative charge and is much wider and more hydrophobic.[6−9] The opposed charge distribution forms the basis for the mutually exclusive specificity of the two enzymes toward their respective disulfide substrates.[10] Genetic approaches revealed that TR is essential for the parasites. TR-deficient *T. brucei* are avirulent and show increased sensitivity against oxidative stress.[11]

The absence of the enzyme from mammalian cells and its essential role in the antioxidant defense of the parasite render trypanothione reductase an attractive target molecule for a rational drug design. Several recent reviews discuss distinct aspects of TR inhibitors as potential leads.[12−15] A large number of compounds have been detected that inhibit TR but not human GR, but a compound suitable for clinical developments is still pending.

Information-based methods are becoming increasingly useful in the search for new enzyme inhibitors.[16] The adaptive screening technology 4Scan[17] allows an efficient screening of large virtual molecular libraries (e.g. commercially available compounds). With the aid of fast assessment engines, the molecules are ranked by a score. Visual inspection and heuristic filter techniques have been applied to about 1000 compounds leading to a reduced list of less than 100 compounds which then have been purchased and submitted to biological testing. The flexible docking programs FlexX[18,19] and ProPose[20] were used in this screening. An advantage of using two different but similar docking tools is that if they create consistent results the data are highly reliable. The principle of docking is to fit a possible ligand into a previously defined cleft of the enzyme, thereby identifying favorable and unfavorable molecular interactions. This selection procedure aims at optimizing the binding mode and binding energy. The docking algorithm is applied in combination with a scoring function through which free binding energy between ligand and receptor can be calculated. Binding affinities are ranked by this quantification (scoring function). A successful structure-based drug design with modern high-throughput technologies requires: (1) a validated target protein, essen-

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10.1021/jm050027z CCC: $30.25 
Published on Web 06/25/2005

tial for the survival and/or replication of the organism; (2) the knowledge of the three-dimensional structure and mechanistic properties of the target; and (3) adequate differences between the target structure and that of corresponding host enzymes to ensure selectivity.

TR fulfils all these requirements, and the parasite enzyme seems to be a born target for the application of new computer-based methods. Despite this, virtual screening approaches provided only moderate results so far.[21] The active site of TR is extremely wide and obviously allows for multiple binding modes.[22−24] A quantitative docking approach on 23 phenothiazine ligands showed, at an overall low clustering, one-half of the structures in either of two conformations.[24] Very bulky ligands are easily accommodated,[25] or more than one inhibitor molecule can bind simultaneously.[23] In addition, the binding mode of even structurally very similar compounds can be unpredictable, as seen in the case of 9-aminoacridines and 9-thioacridines,[23] as well as $N^1$,$N^8$-bis(dihydrocaffeoyl)spermidine and its respective spermine derivative kukoamine A.[26] The large hydrophobic cavity makes the design of inhibitors using directional interactions such as hydrogen bonds really difficult.[24] For these reasons, TR is an exceptional challenge for the target-based drug discovery and modern high-throughput screening technologies. Here we report on the detection of two novel types of inhibitors by a virtual screening approach. Based on chlorhexidine as lead structure, in a parallel synthesis approach different series of compounds have been synthesized and studied as inhibitors of *T. cruzi* TR. The results will be discussed in the light of the predicted binding modes.

## Results

**Detection of New Inhibitors of *T. cruzi* Trypanothione Reductase by Virtual Screening Approaches.** In the search for new classes of trypanothione reductase inhibitors, a virtual high-throughput screening (vHTS) of different databases was performed using the software package 4Scan.[17] Screening of about one million commercially available chemicals revealed a list of 25 in silico hits that were subsequently subjected to kinetic analyses. The piperidine derivative **1** and the antimicrobial chlorhexidine **2** (Figure 1) proved to be strong inhibitors of TR, yielding more than 90% inhibition at each 100 $\mu$M inhibitor and substrate ($TS_2$). Both compounds have structural features of typical TR inhibitors, namely extended hydrophobic parts together with an overall positive charge. The latter property especially plays a crucial role for ligands binding in the trypanothione disulfide substrate pocket of the enzyme.[10]

**Compound 1 Is a Mixed Type Inhibitor of TR.** Compound **1** has a complex structure composed of hydrophobic nitrophenyl and naphthyl ligands and a piperidine moiety that confers the molecule a positive charge under physiological conditions. **1** is a mixed type inhibitor of TR versus $TS_2$, as shown by the double reciprocal plot, where the lines intersect on the left of the $1/v$ axis and above the baseline[27,28] (Figure 2a). Replots of the slopes and intercepts on the vertical axis of the reciprocal plot versus the inhibitor concentration yielded straight lines in accordance with pure mixed-type inhibition with an inactive ESI-complex (Figure 2b,c).[27] The $K_i$ and $K_i'$ values derived from the replots are 6.2 ± 2 and 8.5 ± 2 $\mu$M, respectively. To study a probable binding of **1** at the NADPH binding site of the enzyme, kinetics were conducted at a fixed concentration of 180 $\mu$M $TS_2$ (corresponding to 10 × $K_m$)[29] and varying concentrations of 7−95 $\mu$M NADPH in the presence of 5, 10, and 15 $\mu$M and the absence of **1**. The Lineweaver−Burkplot yielded a noncompetitive type of inhibition with a $K_i$ value of 9 ± 2 $\mu$M (data not shown). Thus, a direct competition between compound **1** and NADPH can be excluded.

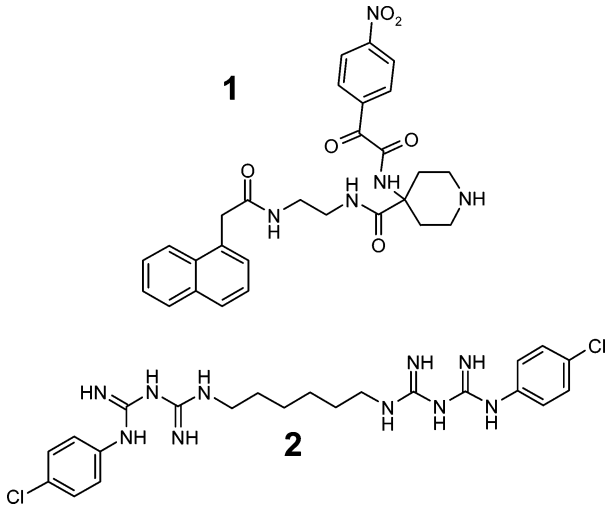



**Figure 1.** Compound **1** and chlorhexidine (**2**) revealed as inhibitors of *T. cruzi* trypanothione reductase by virtual high throughput screening.

**Chlorhexidine Is a Competitive Inhibitor of TR.** Chlorhexidine {1,1′-hexamethylenebis[5-(4-chlorophenyl)biguanide]} contains two biguanide groups, rendering **2**, a strong base ($pK_a$ = 11.73, SciFinder Scholar) that at physiological pH values occurs as a dication. Chlorhexidine salts have been used as antiseptic solutions in dentistry and oral health for decades as well as for the treatment of infections with *Acanthamoeba*.[30] **2** is a strong competitive inhibitor of *T. cruzi* TR with a $K_i$ value of 2 ± 1 $\mu$M (Figure 3a). The replot of the slopes of the reciprocal plot versus the inhibitor concentration yielded a straight line, corresponding to a pure competitive inhibitor with an inactive EI-complex (Figure 3b).[27] In contrast, toward human GR, **2** acts as a weak mixed type inhibitor with $K_i$ and $K_i'$ values of 192 ± 20 and 210 ± 20 $\mu$M, respectively. Based on **2** as lead structure, different series of derivatives have been synthesized and studied as inhibitors of *T. cruzi* TR. The results are presented in the following sections.

**Chlorophenyl-Substituted Bis(amides) and Bis(amidines) as Inhibitors of TR.** A combinatorial parallel-synthesis approach was chosen to create different series of derivatives (Scheme). The reaction products from the parallel synthesis libraries were purified by preparative LC/MS. The purity of the compounds was at least 85%. In the first series of compounds, the 4-chlorophenyl groups were retained and the biguanide groups were replaced by amidines, inverse amidines, or amides. Furthermore, the central linker—a hexyl moiety in chlorhexidine—was varied using a series of unbranched alkyl chains or cyclic systems. All of these symmetrical compounds exhibited

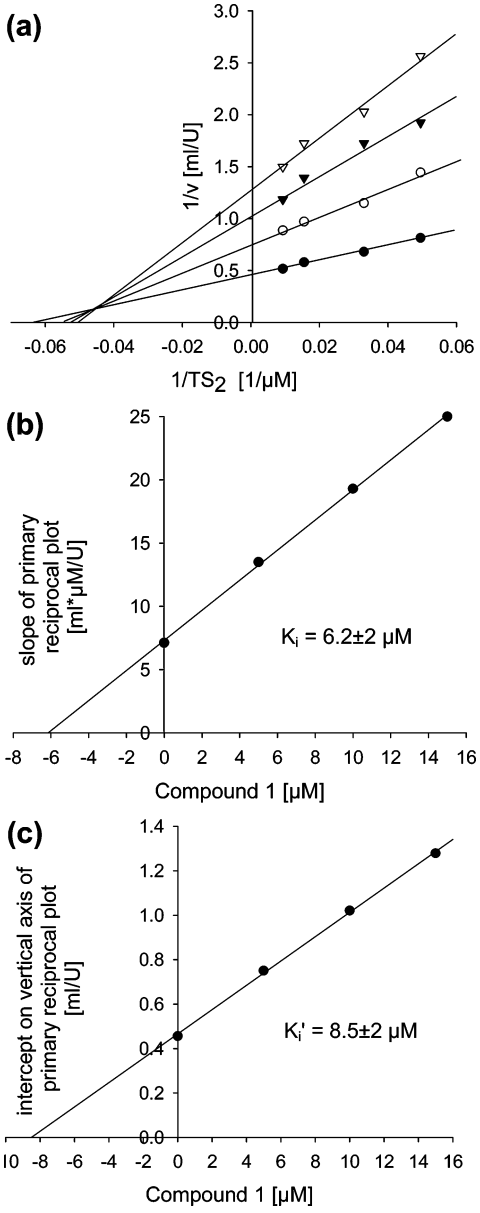



**Figure 2.** Inhibition of *T. cruzi* TR by **1**. The kinetics was measured at 25 °C as described under Experimental Procedures at a constant concentration of 100 $\mu$M NADPH. Each set of kinetics was measured twice whereby the single data points differed by ≤15%. (a) Lineweaver−Burk plot. The $TS_2$ concentration was varied in the absence (●) and presence of (○) 5 $\mu$M, (▼) 10 $\mu$M, and (▽) 15 $\mu$M **1**. (b) Replot of the slopes of the reciprocal plot versus inhibitor concentration. (c) Replot of the intercepts on the vertical axis versus inhibitor concentration. The plots directly give the inhibitor constants: The intercepts on the horizontal axis correspond to $[I] = -K_i$ (b) and $[I] = -K_i'$ (c). The deviations given for the $K_i$ and $K_i'$ values represent the differences in inhibitor constants calculated for each inhibitor concentration.

zero or negligible inhibition of *T. cruzi* trypanothione reductase (data not shown).

**Amidine Derivatives as Inhibitors of Trypanothione Reductase.** In the second series, unsymmetrically substituted amidines with different aromatic and saturated ring structures were synthesized (Table 1). **3** and **6** have a thiophene as substituent R and a pyridine ring or piperazine ring as substituent R′. Compounds **4** and **7** have a bromothiophene as substituent R and again a pyridine or piperazine ring as substituent R′.

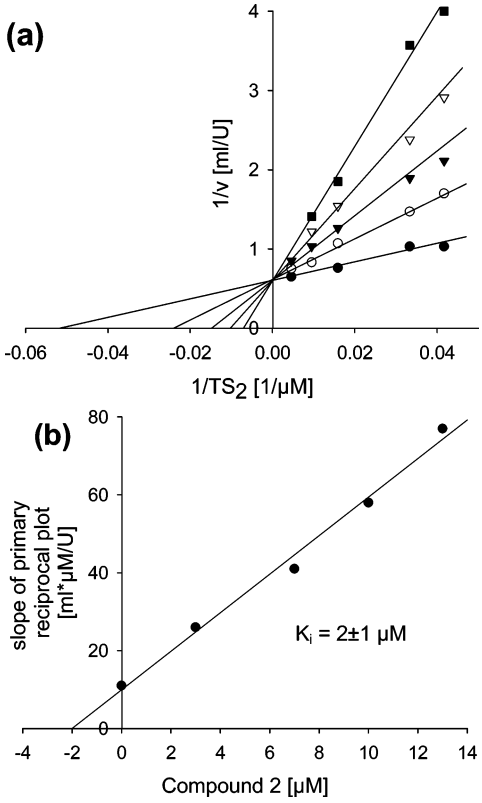
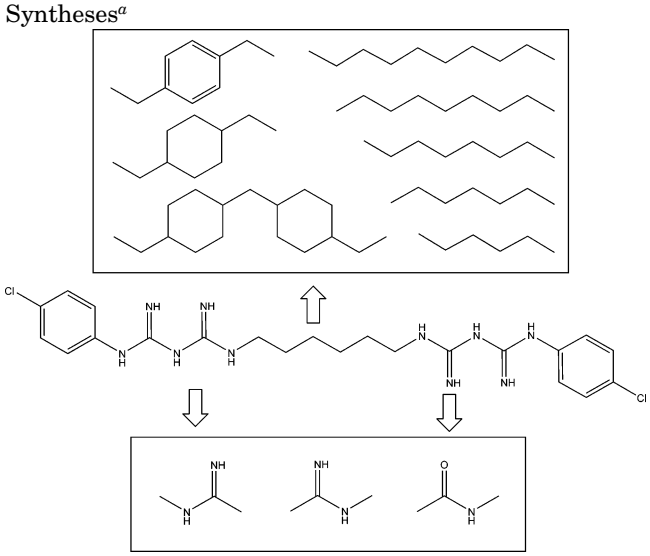



**Figure 3.** Inhibition of *T. cruzi* TR by chlorhexidine **(2)**. The kinetics was measured at 25° C as described under Experimental Procedures at a constant concentration of 100 $\mu$M NADPH. Each set of kinetics was measured twice whereby the single data points differed by ≤10%. (a) Lineweaver−Burk plot. The $TS_2$ concentration was varied in the absence (●) and in the presence of (○) 3 $\mu$M, (▼) 7 $\mu$M, (▽) 10 $\mu$M, and (■) 13 $\mu$M inhibitor. (b) Replot of the slopes of the reciprocal plot versus inhibitor concentration. The line intersects the horizontal axis at a point where $[I] = -K_i$. The deviations for the $K_i$ value represent the variation in inhibitor constants calculated for the different inhibitor concentrations.

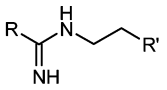
**Scheme 1.** Derivatization of Chlorhexidine by Parallel Syntheses[a]

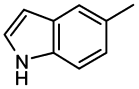
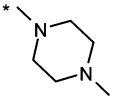
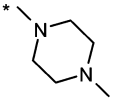
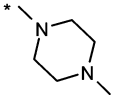


[a] The biguanide groups were replaced by amidines or amides, and the central hexyl chain was replaced by various linkers. None of these compounds showed significant inhibition of *T. cruzi* trypanothione reductase.

Independent of the combination of substituents, the compounds showed only weak inhibition of TR. The

**Table 1.** Inhibition of *T. cruzi* TR by Amidine Derivatives



| compound | substituent R | substituent R' | % inhibition caused by 100 μM inhibitor at [$TS_2$] | |
|---|---|---|---|---|
| | | | 110 μM | 40 μM |
| **3** |  |  | ≤ 10 | ≤ 10 |
| **4** |  |  | 15 | 14 |
| **5** |  |  | 31 | 39 |
| **6** |  |  | ≤ 10 | ≤ 10 |
| **7** |  |  | ≤ 10 | ≤ 10 |

most effective derivative of this series was **5** with an indole ring as substituent R and a piperazine as substituent R′. Compound **5** inhibits *T. cruzi* TR activity by 30% when 100 $\mu$M of each disulfide substrate and inhibitor are present in the assay.

**Inhibition of Trypanothione Reductase by Bis(amidine) Derivatives.** As none of the chlorhexidine derivatives retaining the *p*-chlorophenyl moieties (Scheme) was a potent inhibitor, the ligand was replaced by other aromatic substituents. The third series of compounds obtained by parallel syntheses were bis(amidines) (Table 2). Compounds **8**, **11**, and **14** proved to be efficient mixed type inhibitors of TR, resulting in ≥85% inhibition in the presence of both 100 $\mu$M inhibitor and trypanothione disulfide. All three derivatives carry the same phenyl-substituted diether group as terminal substituents R but vary in the central substituent (X in Table 2), which is a diphenyl ether in **8**, a bis(cyclohexyl) methylene group in **11**, and an octyl chain in **14**, respectively. Although compound **14** shows slightly weaker inhibition than **8** and **11**, it is obvious that the nature of the central part plays only a minor role for the binding mode and binding strength of these compounds.

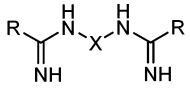
The inhibitor constants were calculated for different inhibitor concentrations (10 and 20 $\mu$M in the case of **8**), (5, 10, and 20 $\mu$M compound **11**) from the slope and the intercepts of the *x*-axis of the Lineweaver−Burk plot (Figure 4a) as described under Experimental Procedures. They do not represent true dissociation constants for the EI complex, since they were dependent on the respective inhibitor concentration. In any case, lower inhibitor constants resulted at higher inhibitor concentrations. The $K_i$ and $K_i'$ values given in Table 2 are $K_{i,slope}$ and $K_{i,int}$ values, respectively. Subsequent variation of the concentrations of compounds **8** and **11** at fixed concentrations of trypanothione disulfide resulted in Dixon plots that curved upward parabolically (Figure 4b). This type of inhibition curve occurs when more than one molecule of inhibitor can bind to the same form of the enzyme.[27,28] $K_i$ and $K_i'$ for compound **14** were determined using a single inhibitor concentration (20 $\mu$M). Toward human glutathione reductase, **8** and **11** proved to be modest inhibitors. At each 100 $\mu$M inhibitor and GSSG, the activity was diminished by 45% and 35%, respectively.

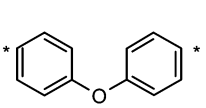
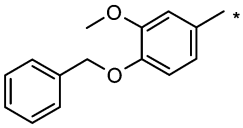
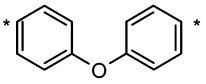
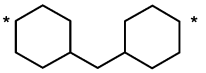
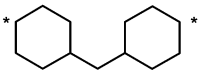
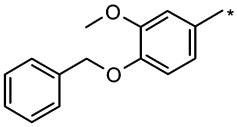
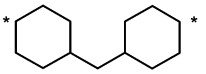
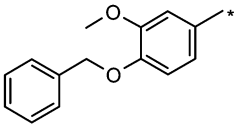
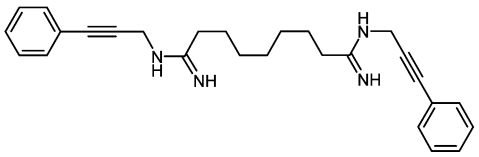
The inverse bis(amidine) **17** with two 1-phenyl-1-propyne moieties was another effective inhibitor. The inhibitor constant of 6 $\mu$M was derived from Lineweaver−Burk plots and replots versus [I] (not shown). It was independent of the inhibitor concentration, in accordance with a pure competitive type of inhibition. All other bis(amidine) derivatives with thiophene or pyrazine substituents showed only weak inhibition of *T. cruzi* TR. Compound **17** does not interfere with human glutathione reductase. **17** (100 $\mu$M), in the presence of 100 $\mu$M GSSG, lowered the activity by only 5%.

## Discussion

A virtual high-throughput screening approach with the program 4Scan[17] revealed chlorhexidine (**2**) and compound **1** as new inhibitor types of *T. cruzi* trypanothione reductase. The flexible docking programs FlexX and ProPose provided low-energy models for binding chlorhexidine in the active site of TR, in accordance with the pure competitive type of inhibition observed (Figure 5a). Both programs predict an identical placement for the central part of **2**, including the guanidino moieties, whereas the aromatic terminal groups are positioned

**Table 2.** Inhibition of *T. cruzi* TR by Bis(amidine) Derivatives



| compd | substituent X | substituent R | % inhibition caused by 100 μM inhibitor at [$TS_2$] 110 μM | 40 μM | $K_i$ (μM) | $K_i'$ (μM) | type of inhibition |
|---|---|---|---|---|---|---|---|
| **8** |  |  | 90 | 95 | 2 | 22 | mixed[a] |
| **9** |  |  | 12 | 11 | | | |
| **10** |  |  | ≤ 10 | ≤ 10 | | | |
| **11** |  |  | 97 | 94 | 2 | 16 | mixed[a] |
| **12** |  |  | 16 | 17 | | | |
| **13** | $(CH_2)_8$ |  | ≤ 10 | 15 | | | |
| **14** | $(CH_2)_8$ |  | 85 | 92 | 5 | 47 | mixed[a] |
| **15** | $-(CH_2)_2O(CH_2)_2O(CH_2)_2-$ |  | ≤ 10 | 11 | | | |
| **16** | $-(CH_2)_2O(CH_2)_2O(CH_2)_2-$ |  | ≤ 10 | 14 | | | |
| **17** |  | | 69 | 80 | 6±3 | | competitive |

[a] The $K_i$ and $K_{i'}$ values for **8**, **11**, and **14** are actually $K_{i,slope}$ and $K_{i,int}$ values, calculated from the data obtained at 20 $\mu$M inhibitor to allow direct comparison (for details, see the text).

in different orientations. The predicted extended conformation of **2** when bound to the enzyme allows interactions with rather remote active site residues. The necessity of an inhibitory ligand to undergo multiple interactions with the protein is corroborated by the inability of the antimalarial 1-(4-chlorophenyl)-5-isopropylbiguanide (chloroguanide, proguanil), which structurally resembles one-half of the symmetrical chlorhexidine molecule, to inhibit TR. The presence of 250 $\mu$M proguanil in an assay containing 70 $\mu$M $TS_2$ lowered the activity only by 20% (E. Jacoby and R. L. Krauth-Siegel, unpublished data). Interestingly, the FlexX model gives interactions of the inhibitor with three out of five active site residues that are not conserved in the host GR (Glu18, Trp21, and Met113 in TR are replaced by Ala34, Arg37, and Asn117 in human GR). These interactions may explain the specificity of chlorhexidine to inhibit TR but not human GR. The same residues have been shown to be responsible for binding the competitive inhibitor mepacrine as well as an alkylating derivative of it in the active site of TR.[31,32] In contrast, ProPose predicts another orientation of the terminal substituents, resulting in interactions of the chlorophenyl group with Tyr110 instead of Trp21 and Met113. The rather hydrophobic and negatively charged (Glu18, Trp21, Ser109, Tyr110, and Met113) region in TR also fixes the spermidine moiety of trypanothione disulfide and is thus responsible for the mutually exclusive substrate specificities when comparing the parasite TR and human GR.[33] Phenothiazines, dibenzazepines (based on imi-

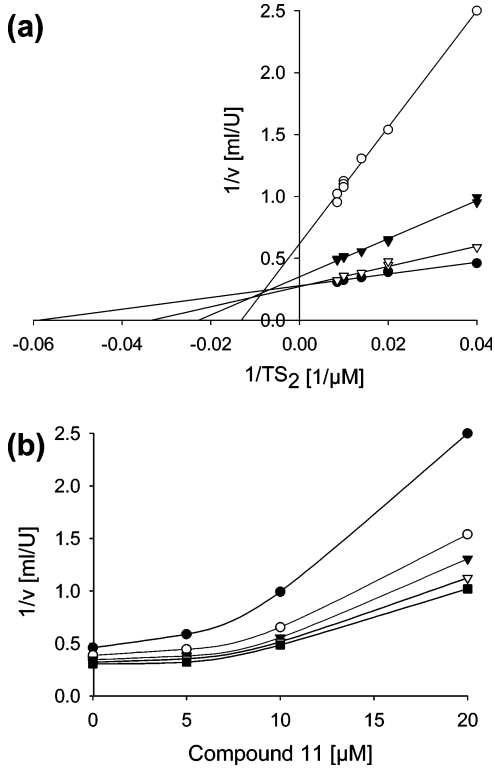



**Figure 4.** Inhibition of *T. cruzi* TR by compound **11**. The kinetics was measured at 25 °C as described under Experimental Procedures at a constant concentration of 100 $\mu$M NADPH. (a) Lineweaver−Burk plot. The $TS_2$ concentration was varied in the absence (●) and in the presence of (▽) 5 $\mu$M, (▼) 10 $\mu$M, and (○) 20 $\mu$M **11**. (b) Dixon plot. The inhibitor concentration was varied in the presence of fixed concentrations of (■) 118 $\mu$M, (▽) 100 $\mu$M, (▼) 71 $\mu$M, (○) 50 $\mu$M, and (●) 25 $\mu$M $TS_2$. The complete data set was measured at least in duplicate.

pramine), and other tricyclic compounds are also competitive inhibitors of TR.[22,34] Modeling studies resulted in multiple binding modes of the tricycles at the hydrophobic wall formed by Trp21 and Met113 and an accessory interaction between their alkylamino side chain and Glu465′ and Glu466′, active site residues that are provided by the other subunit of the homodimeric protein. Further modeling studies suggested a second hydrophobic region, mainly formed by Phe395′, Pro397′, and Leu398′ (called the Z-site), which can accommodate the extended hydrophobic substituents of the compounds[35] as well as the tricycle itself.[24] The proposed binding mode of chlorhexidine (Figure 5a) in TR is mainly based on lipophilic interactions between the chlorophenyl groups and protein residues of both hydrophobic pockets as well as the electrostatic anchoring of the biguanide groups by the side chains of Glu18 and Glu465′. Since Glu465′, in contrast to Glu18, is conserved in the host GR, the latter interaction is unlikely to account for the selective binding of chlorhexidine to TR.

Using chlorhexidine as starting structure, a different series of bis(amides), bis(amidines), and aromatic mono(amidines) were synthesized and studied as inhibitors of the parasite enzyme. Several antitrypanosomal drugs, such as pentamidine and berenil, are diamidines or contain closely related structural elements, such as the melaminophenyl arsenical melarsorpol.[36] The amidine group serves as recognition motif for the uptake by the parasite purine transporter P2. A general problem of positively charged aromatic compounds is their DNA-binding capacity, which can give rise to host toxicity but also be the basis for their therapeutic action. In the case of pentamidine, the selective accumulation to millimolar concentrations in the parasite and binding in the DNA minor groove correlate with its antitrypanosomal effect. Recently, pentamidine has been reported also to inhibit trypanothione reductase.[37] Another obstacle is that compounds with positively charged groups, such as amidine or guanidine moieties, are in general poorly orally available. This problem can be overcome using a prodrug approach. Amidoximes or alkylated amidoxines can be converted in vivo to amidines and show reasonable oral availability.[38] In fact, this strategy was used for the development of DB75, an antitrypanosomal agent currently in clinical phase IIb.[39]

None of the newly synthesized compounds showed an improved inhibitory potency compared to chlorhexidine. Replacement of the two biguanide groups of chlorhexidine by amides or amidines strongly weakens the interaction with the enzyme. The amidine derivatives showed a slightly better inhibition than the corresponding amides, which agrees with the finding that positive charges play a crucial role for ligands binding in the active site of TR.[10] Other positively charged inhibitors of the enzyme are polyamine derivatives with terminal hydrophobic substituents. Most of them are spermine or spermidine derivatives, such as Kukoamine A[26] or substrate analogues. Among them are competitive as well as mixed and noncompetitive inhibitors.[26,40−42] The much stronger inhibition of TR by chlorhexidine in comparison to the respective amidine cannot be attributed to charge conditions. Both compounds should carry two positive charges at physiological pH values. The superiority of chlorhexidine may be due to the fact that the biguanide groups allow an extended delocalization of the charges, which may facilitate interactions with the protein.

The three most effective inhibitors of the newly synthesized compounds were **8**, **11**, and **14**. They are derived from a series of symmetrical bis(amidines) where the *p*-chlorophenyl moieties of chlorhexidine were also replaced. The compounds possess terminal 2-methoxy-4-methyl-1-(phenylmethoxy)benzene substituents but differ in the nature of the linker connecting the two amidine moieties. Irrespective of the central substituent, **8**, **11**, and **14** showed an identical mixed type inhibition pattern with very similar inhibitor constants. Thus, the central part plays only a minor role for TR inhibition. In contrast, replacement of the 2-methoxy-4-methyl-1-(phenylmethoxy)benzene by thiophene or pyrimidine caused a dramatic decrease in the inhibitory potency of the compounds. In a series of bisbenzylisoquinoline alkaloids, cepharantine was the most effective inhibitor of TR, showing mixed-type kinetics with $K_i$ and $K_i'$ values of 7.6 and 51.6 $\mu$M, respectively.[43] Interestingly, cepharantine contains a brenzcatechine diether motif that structurally resembles the terminal substituents of **8**, **11**, and **14**. As indicated by the parabolic Dixon plot (Figure 4b), more than one molecule of **11** can bind to the enzyme simultaneously.[27] Other examples where more than one inhibitor molecule binds to the enzyme at the same time are 9-aminoacridines such as mepa-

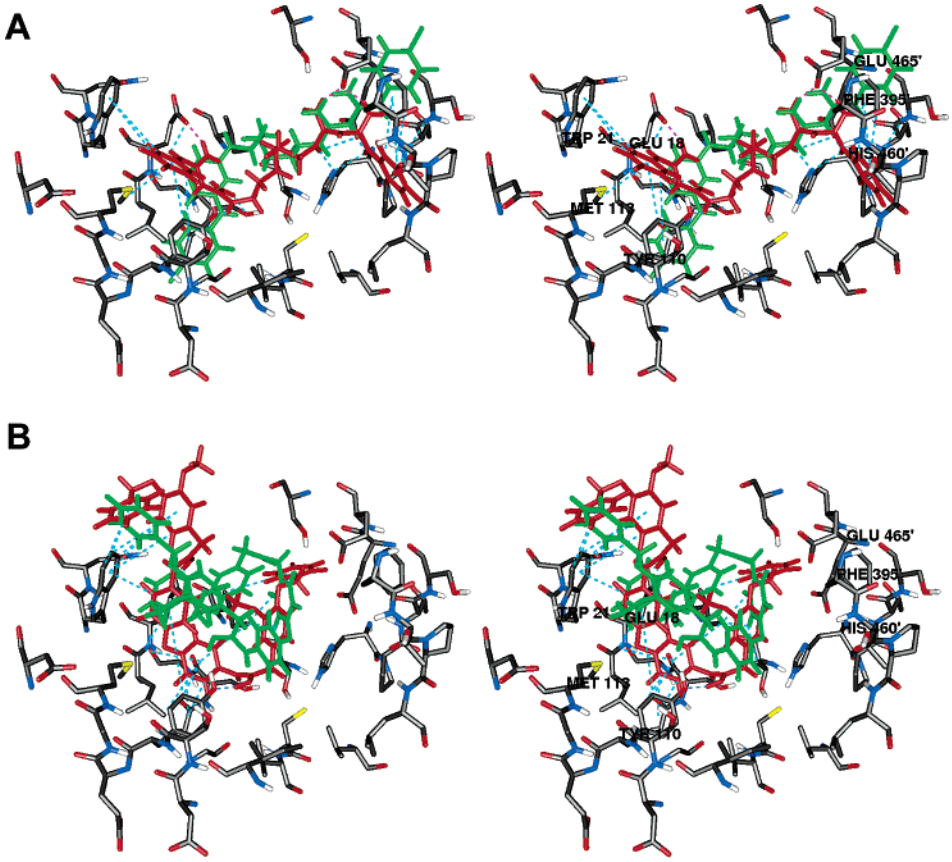



**Figure 5.** (a) Stereoview of possible binding modes of chlorhexidine (**2**) in the active site of TR calculated with the docking programs FlexX (red) and ProPose (green). Residues of the active site are shown in atomic colors. The dotted lines represent strong interactions detected in the FlexX solution scheme. (b) Stereoview of possible binding modes of **8** in the active site of TR calculated with FlexX (red) and ProPose (green). Residues of the active site are shown in atomic colors. The thiol group (yellow) is that of the redox active Cys52. The dotted lines represent strong interactions obtained in the FlexX solution scheme.

crine.[23] The recent crystal structure of TR irreversibly inactivated by an alkylating mepacrine analogue revealed two inhibitor molecules that stacked in the trypanothione disulfide binding site.[32] Photoaffinity labeling of trypanothione reductase by fluphenazine also indicated the incorporation of 2−5 equiv of the ligand per enzyme subunit.[44] Previous modeling approaches on TR suggested a second hydrophobic pocket in the outer region of the active site formed by Phe395′, Pro397′, and Leu398′ that may accommodate ligands.[24] The binding site(s) of compounds **8**, **11**, and **14** in TR are not known. They are probably not identical with those of the mepacrine analogues, which are irreversibly binding ligands. In addition, **8**, **11**, and **14** show mixed-type inhibition patterns. A third possible binding site of ligands in TR is a large cavity at the interface of the homodimeric protein. In human GR, this cavity has been shown to accommodate different types of ligands that inhibit the enzyme with non/uncompetitive or mixed-type kinetics.[14]

Docking of **8** by both programs placed the compound at the trypanothione disulfide binding site lined by Trp21, Glu18, and Met113, but the exact binding modes were distinct (Figure 5b). FlexX found a solution with a salt bridge between one imidino group and Glu18 and neutral H-bonds to Tyr110 and Ser14. In the ProPose solution, the imidino groups only interact with the uncharged side chains of Ile338′ and Tyr110. Hydrophobic interactions, although from different aromatic rings of **8**, occur in both solutions to the side chains of Leu17, Trp21, Tyr110, and Ile338′. A further analogy of both solutions are the missing interactions with Glu465′ and Glu466′. The different localization of **8** obtained by the two docking programs and the large difference to slightly higher ranking placements (not shown) in the solution sets of both methods indicate that several energetically similar binding modes are possible.

Chlorhexidine and the newly synthesized inhibitors have in common that they are symmetrical extended structures with two positive charges in their cores and aromatic terminal substituents. A strong improvement of TR inhibition upon dimerization of a ligand has been found for aminodiphenylsulfides[25] as well as naphthoquinones.[45] This is in accordance with the unusually wide disulfide substrate binding site of TR, which may allow the binding of more than one bulky ligand at the same time. Another efficient inhibitor was **17**, an inverse bis(amidine) with terminal phenylpropyne moieties. In contrast to **8**, **11**, and **14**, compound **17** showed, like chlorhexidine, pure competitive inhibition toward trypanothione disulfide.

Taken together, the bis(amidines) with the most bulky and hydrophobic terminal moieties were the most efficient TR inhibitors studied here. The change in inhibition type from competitive to mixed type inhibition can be regarded as an advantage although the inhibitor constants did not improve when compared to chlorhexidine. For mixed inhibitors, accumulation of substrate

due to blockage of the pathway cannot overcome inhibition as easily as is the case with purely competitive inhibitors.

## Conclusions

The parasite-specific flavoenzyme trypanothione reductase is an attractive target molecule for an antitrypanosomal drug development. A virtual high throughput screening based on the active site structure of *T. cruzi* trypanothione reductase revealed the antimicrobial chlorhexidine as a new type of inhibitory ligand. The bis(biguanide) is an efficient competitive inhibitor ($K_i$ = 2 $\mu$M), whereas the antimalarial proguanil—which structurally resembles one-half of chlorhexidine—does not interfere with the enzyme. This corroborates previous findings that dimerization of a ligand strongly improves inhibition of the enzyme. In several parallel syntheses, the chlorophenyl and/or the biguanide groups of chlorhexidine were replaced. The most effective derivatives obtained were three bis(amidines), each carrying two bulky 2-methoxy-4-methyl-1-(phenylmethoxy)benzene ligands. They inhibited *T. cruzi* trypanothione reductase with mixed-type kinetics, and probably more than one molecule is bound to the enzyme simultaneously. This in accordance with the extremely wide trypanothione disulfide binding site and underlines the difficulty to set up quantitative structure−activity relations in the case of trypanothione reductase. Future work will include the design of compounds composed of bis(biguanide) cores and terminal 2-methoxy-4-methyl-1-(phenylmethoxy)benzene substituents.

## Experimental Procedures

**Materials.** Recombinant *T. cruzi* trypanothione reductase[46] and human glutathione reductase[47] were prepared according to published procedures. Trypanothione disulfide ($TS_2$) was purchased from Bachem. Chlorhexidine was from Sigma, compound **1** from Tripos Inc. (St. Louis, MO). Stock solutions (4 mM) of the inhibitors were made in DMSO and $H_2O$, respectively, and stored at −20 °C.

**TR Inhibition Assays.** TR activity was measured spectrophotometrically at 25 °C in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5) as described.[29] The assay mixture (1 mL) contained 100 $\mu$M NADPH and 10−15 mU TR. The enzyme activity was followed at two fixed concentrations of 40 and 110 $\mu$M $TS_2$ in the absence and presence of 100 $\mu$M inhibitor. The structural formula of the compounds studied are given in Tables 1 and 2. Control assays containing the respective amount of DMSO were carried out where appropriate. The reaction was started by adding $TS_2$, and the absorption decrease at 340 nm was followed.

**GR Inhibition Assays.** GR activity was measured in GR assay buffer (20.5 mM $KH_2PO_4$, 26.5 mM $K_2HPO_4$, 200 mM KCl, 1 mM EDTA, pH 6.9) at 25 °C as described.[47] The assay mixture (1 mL) contained 100 $\mu$M NADPH, 5−10 mU GR and 40−1000 $\mu$M GSSG. The kinetic data for **2** were obtained at two fixed concentrations of inhibitor (120 and 240 $\mu$M) with variation of the GSSG concentration. In the case of **8**, **11**, and **17**, the assays contained each 100 $\mu$M GSSG and inhibitor. Control assays with the respective amount of DMSO were carried out. The reaction was started by adding GSSG, and the absorption decrease at 340 nm was followed.

**Determination of Inhibitor Constants.** The activity of *T. cruzi* TR was followed in the absence and presence of different fixed concentrations of inhibitor varying the $TS_2$ concentration between 20 and 218 $\mu$M. The type of inhibition was derived from Lineweaver−Burk and Dixon plots. The inhibitor constants were either obtained graphically from secondary plots or were calculated from the expressions for the slopes and intercepts on the vertical axis of the Lineweaver−Burk plots.[27]

For mixed-type inhibition, the equations are

$$\text{slope} = K_s\,(1 + i/K_i)/V$$

$$\text{intercept} = (1 + i/K_i')/V$$

where $K_s$ is equated with $K_m$ assuming equilibrium conditions.

For competitive inhibition:

$$\text{intercept on baseline} = 1/(K_m(1 + i/K_i)).$$

**In Silico Screening.** The structure of *Crithidia fasciculata* trypanothione reductase (pdb entry 1TYP)[48] has been used for modeling the active site for the docking approaches. All residues in a distance of less than 3 Å to the substrate $N^1$-glutathionylspermidine disulfide were included. Energy-minimized hydrogen positions were calculated with MOLOC.[49] The default parametrizations of ProPose, FlexX, and 4SCan were used for the docking and screening studies, respectively.

**General Procedure of the Combinatorial Chemistry.** All chemicals were purchased from Sigma or Acros and were of the highest available purity. The respective nitrile was dissolved at 0 °C in 3 mL of toluene. The amide and trimethylaluminum were added in 1 mL of toluene, and the reaction mixture was heated for 8 h at 100 °C. The reaction was quenched with water and extracted three times with ethyl acetate, and the combined organic layers were washed with brine, dried over $MgSO_4$, and filtered. The solvent was removed in a vacuum and the product was purified by preparative LC/MS.

The HPLC system consisted of a Waters 600 Multisolvent Delivery System, a Waters 2700 Sample Manager (Waters, Eschborn) and a LabPRO module (Rheodyne). Column eluate was split with a Graduated Micro-Splitter Valve (Upchurch Scientific) between a Waters 2487 Dual $\lambda$ Absorbance detector set at 254 nm and the MS interface. For preparative separations, column eluate was split 1:1000 between detectors and a Waters Fraction Collector II employing an Accurate splitter (LC Packings) and makeup solvent was delivered using a Reagent Manager pump (Waters).

ESI mass spectra were collected on a platform LCZ mass spectrometer (Micromass, UK) in the positive ion mode scanning *m/z* 50−500 in 1 s. Capillary and cone potentials were set at 3.5 kV and 20 V, respectively, with a multiplier voltage of 400 V; probe and desolvation gas temperature were 120 and 350 °C, respectively.

Preparative chromatography was performed using an XTerra RP18 Column (19 × 150 mm, 7 $\mu$m, Waters) equipped with a 19 × 10 mm guard column. The crude material dissolved in water−acetonitrile−formic acid (400−150−0.5 (v/v/v)) was applied onto the column at a flow rate of 0.5 mL/min. The mobile phases consisted of 0.1% formic acid in water ((v/v) solvent A) and acetonitrile (solvent B). For analytical separations, a gradient of 5% to 100% B within 10 min was used, followed by 100% B for 8 min and reequilibration in solvent A for 10 min at a flow rate of 1 mL/min. In the case of preparative runs, the gradient was 10 to 100% B in 10 min, 100% B for 5 min, and 5 min reequilibration at a flow rate of 20 mL/min. All separations were carried out at room temperature. Data recording and processing were performed on a PC using MassLynx 3.4 software (Waters).

***N*-(2-Pyridin-3-ylethyl)thiophene-2-carboxamidine (3).** The product was synthesized by following the general procedure, starting from 25 mg (0.23 mmol) of thiophene-2-carbonitrile, 28 mg (0.23 mmol) of 2-pyridin-3-ylethylamine, and 17 mg (0.23 mmol) of trimethylaluminum. LC/MS: *m/z* = 232.1.

**3-Bromo-*N*-(2-pyridin-3-ylethyl)thiophene-2-carboxamidine (4).** The product was synthesized by following the general procedure, starting from 25 mg (0.13 mmol) of 3-bromothiophene-2-carbonitrile, 16 mg (0.13 mmol) of 2-pyridin-3-ylethylamine, and 10 mg (0.13 mmol) of trimethylaluminum. LC/MS: *m/z* = 310.0.

***N*-[3-(4-Methylpiperazin-1-yl)propyl]-1*H*-indole-5-carboxamidine (5).** The product was synthesized by following the general procedure, starting from 25 mg (0.18 mmol) of 1*H*-indole-5-carbonitrile, 28 mg (0.18 mmol) of 2-(4-methylpiperazin-1-yl)ethylamine, and 13 mg (0.18 mmol) of trimethylaluminum. LC/MS: $m/z = 300.2$.

***N*-[3-(4-Methylpiperazin-1-yl)propyl]thiophene-2-carboxamidine (6).** The product was synthesized by following the general procedure, starting from 25 mg (0.23 mmol) of thiophene-2-carbonitrile, 36 mg (0.23 mmol) of 2-(4-methylpiperazin-1-yl)ethylamine, and 17 mg (0.23 mmol) trimethylaluminum. LC/MS: $m/z = 267.2$.

**3-Bromo-*N*-[3-(4-methylpiperazin-1-yl)propyl]thiophene-2-carboxamidine (7).** The product was synthesized by following the general procedure, starting from 25 mg (0.13 mmol) of thiophene-2-carbonitrile, 21 mg (0.13 mmol) of 2-(4-methylpiperazin-1-yl)ethylamine, and 10 mg (0.13 mmol) of trimethylaluminum. LC/MS: $m/z = 345.1$.

**4,4′-Bis(4-benzyloxy-3-methoxybenzimidoylamino)-diphenyl Ether (8).** The product was synthesized by following the general procedure, starting from 50 mg (0.2 mmol) of 4-benzyloxy-3-methoxyphenyl)acetonitrile, 20 mg (0.1 mmol) of 4,4′-diaminodiphenyl ether, and 14 mg (0,2 mmol) of trimethylaluminum. LC/MS: $m/z = 707.3$.

**4,4′-Bis[(thiophenyl-2-carboximidoyl)amino]diphenyl Ether (9).** The product was synthesized by following the general procedure, starting from 50 mg (0.46 mmol) of thiophene-2-carbonitrile, 46 mg (0.23 mmol) of 4,4′-diaminodiphenyl ether, and 33 mg (0.46 mmol) of trimethylaluminum. LC/MS: $m/z = 419.1$.

**4,4′-Bis[(pyrazinyl-2-carboximidoyl)amino]dicyclohexylmethane (10).** The product was synthesized by following the general procedure, starting from 50 mg (0.48 mmol) of pyrazine-2-carbonitrile, 50 mg (0.24 mmol) of 4,4′-diaminodicyclohexylmethane, and 34 mg (0.48 mmol) of trimethylaluminum. LC/MS: $m/z = 421.3$.

**4,4′-Bis(4-benzyloxy-3-methoxybenzimidoylamino)dicyclohexylmethane (11).** The product was synthesized by following the general procedure, starting from 50 mg (0.2 mmol) of (4-benzyloxy-3-methoxyphenyl)acetonitrile, 21 mg (0.1 mmol) of 4,4′-diaminodicyclohexylmethane, and 14 mg (0.2 mmol) of trimethylaluminum. LC/MS: $m/z = 717.4$.

**4,4′-Bis[thiophenyl-2-carboximidoyl)amino]dicyclohexylmethane (12).** The product was synthesized by following the general procedure, starting from 50 mg (0.46 mmol) of thiophene-2-carbonitrile, 21 mg (0.23 mmol) of 4,4′-diaminodicyclohexylmethane, and 33 mg (0.46 mmol) of trimethylaluminum. LC/MS: $m/z = 429.2$.

**1,8-Bis[(thiophenyl-2-carboximidoyl)amino]octane (13).** The product was synthesized by following the general procedure, starting from 50 mg (0.46 mmol) of thiophene-2-carbonitrile, 33 mg (0.23 mmol) of 1,8-diaminooctane, and 33 mg (0.46 mmol) of trimethylaluminum. [LC/MS: $m/z = 363.2$].

**1,8-Bis(4-benzyloxy-3-methoxybenzimidoylamino)octane (14).** The product was synthesized by following the general procedure, starting from 50 mg (0.2 mmol) of (4-benzyloxy-3-methoxyphenyl)acetonitrile, 14 mg (0.1 mmol) of 1,8-diaminooctane, and 14 mg (0.2 mmol) of trimethylaluminum. LC/MS: $m/z = 651.4$.

***N*-[2-(2-{2-[(Pyrazinyl-2-carboximidoyl)amino]ethoxy}-ethoxy)ethyl]pyrazine-2-carboxamidine (15).** The product was synthesized by following the general procedure, starting from 50 mg (0.48 mmol) of pyrazine-2-carbonitrile, 35 mg (0.24 mmol) of 1,8-diamino-3,6-dioxaoctane, and 34 mg (0.48 mmol) of trimethylaluminum. LC/MS: $m/z = 359.2$.

***N*-[2-(2-{2-[(Thiophenyl-2-carboximidoyl)amino]-ethoxy}ethoxy)ethyl]thiophene-2-carboxamidine (16).** The product was synthesized by following the general procedure, starting from 50 mg (0.46 mmol) of thiophene-2-carbonitrile, 34 mg (0.23 mmol) of 1,8-diamino-3,6-dioxaoctane, and 33 mg (0.46 mmol) of trimethylaluminum. LC/MS: $m/z = 367.1$.

**1,7-Bis[(4-phenylbut-3-ynimidoyl)amino]heptane (17).** The product was synthesized by following the general procedure, starting from 25 mg (0.17 mmol) of nonanedinitrile, 44 mg (0.33 mmol) of 3-phenylprop-2-ynylamine, and 24 mg (0.33 mmol) of trimethylaluminum. LC/MS: $m/z = 413.3$.

**Acknowledgment.** Our work is supported by the Deutsche Forschungsgemeinschaft (SFB 544 "Kontrolle tropischer Infektionskrankheiten", Projekt B3, LKS).

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JM050027Z