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Specific Inhibitors for the Glycolytic Enzymes of *Trypanosoma brucei*

Murielle Trinquier,[a] Jacques Perie,[a] Mia Callens,[b] F. Opperdoes[b] and Michele Willson[a]*

[a]*Groupe de Chimie Organique Biologique-URA 470, Université Paul Sabatier, 31062 Toulouse, France*

[b]*Laboratory of Biochemistry, Catholic University of Louvain, Research Unit for Tropical Diseases—International Institute of Cellular and Molecular Pathology, 12 000 Brussels, Belgium*

Abstract—The selective inhibition of four glycolytic enzymes from *Trypanosoma brucei* by α–ω difunctionalized compounds at clusters of amino acids exhibit higher activity than the trypanocidal drug suramin.

Introduction

Glycolysis in the bloodstream from trypanosomes (protozoan parasites existing in humans and livestock) differs from glycolysis in other eukaryotes because it is compartmentalized within the glycosomes.[1] In our search for inhibitors of glycolytic enzymes, we have exploited a unique property of several glycosomal enzymes in *Trypanosoma brucei* (Tb): the positively charged clusters on their enzyme surfaces, called 'Hot-Spots', do not exist on the homologous glycolytic enzymes of mammals.[2]

It has been suggested that the drug Suramin (Fig. 1) (a drug recommended for human trypanosomiasis) acts through interactions with these clusters.[3] We have synthesized a series of symmetrical long-chain compounds with either negative charges or strong dipoles at each end, separated by a spacer of methylene groups of variable length. The inhibition observed is maximal with a spacer of 11–14 methylene groups (a length close to the distance between the two clusters). These compounds inhibit the trypanosomal enzymes selectively, while they do not inhibit their homologous mammalian enzymes.[4]

We formulated here a novel set of anti-Hot-Spot compounds (AHS) that have been designed by combination of structural analogies with suramin and with the previously described AHS.[4] We also wanted to analyze the behavior of these compounds with respect to modifications of the aromatic moiety (size and electronic effects) and investigate the possibility of irreversible inhibition effects.

The effect of each of these compounds was studied on four glycosomal enzymes : hexokinase (HK) and phosphoglucose isomerase (PGI), the first two enzymes of the glycolytic pathway which are important due to their involvement both in glycolysis and in the pentose–phosphate pathway; glycerol kinase (GK), involved in anaerobic glycolysis specific for Tb;[1] and phosphoglycerate kinase (PGK), a monomeric enzyme that reacts according to a sequential mechanism in a thermodynamic quasi-equilibrium which is therefore a suitable kinetic model for inhibition studies.

Results and Discussion

All compounds were prepared by coupling the appropriate aniline derivatives to the *N,N'*-(1,14 tetra decane diyl bis (carbonylamino)) bis (mercapto-thiazoline) (**2**), under stoichiometric conditions, one equivalent of the dicarboxylamide compound with two equivalents of amine (Scheme 1).

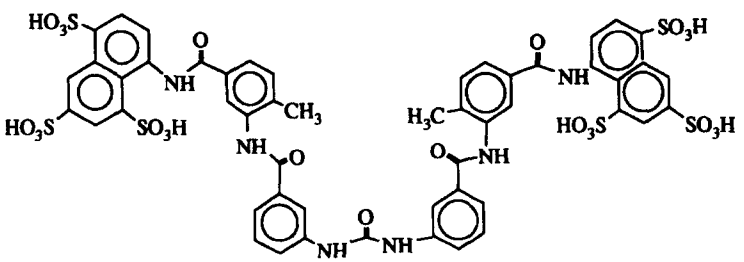
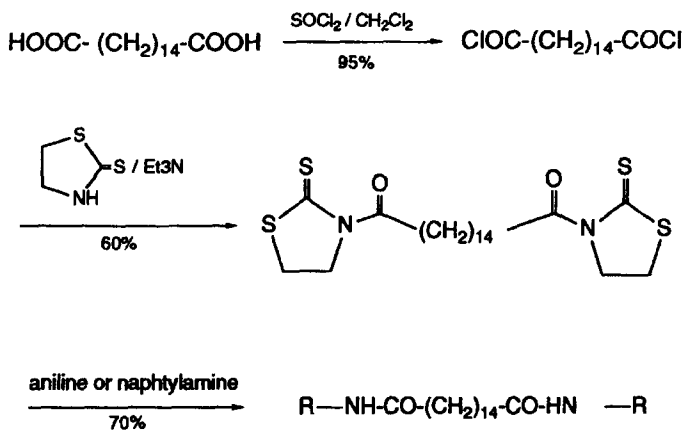


Figure 1. Suramin.



Aniline derivatives R = o-I C_6H_4 **3** ; p-I C_6H_4 **4** ; p-Br C_6H_4 **6** ; p-SO_3Na C_6H_4 **7** p-$CONH_2$ C_6H_4 **8** ; p-SO_2NH_2 C_6H_4 **9** ;

Napthylamine derivatives R = 2-naphtyl **11** ; 7-(1,3-disulfonic acid)-naphtyl **12** ;

Scheme 1.

Compound **2** was synthesized from the acid chloride **1** of the dicarboxylic acid with two equivalents of 2-mercaptothiazoline. The first step of the reaction was carried out by refluxing, in anhydrous methylene chloride, thionyl chloride or oxalyl chloride with the hexadecanoic acid. Two equivalents of 2-mercaptothiazoline were reacted with the diacylchloride in the presence of tertiary amine in THF. Mild aminolysis of the corresponding intermediate amide was carried out in methylene chloride or methyl alcohol at room temperature for several days according to the method proposed by Nagao *et al.*[5]

Compounds **3–6** with two different halogens (either Br or I) at different positions on the aromatic moiety (either the *ortho* or *para* position) have been designed to analyze the electronic and size effects. The acetyl bromo compound **10** had been designed with the purpose of producing a covalent bond with a basic residue on the protein by nucleophilic attack on the electrophilic methylene ($COCH_2Br$). This compound may also be of significance in the analysis of the possible effects of chain length on the aromatic part. The disulfonated naphthyl compound **12** had been designed with the desirability of assaying a compound bearing a functionality similar to that of suramin but with more flexibility in the spacer. The naphthalene compound **11** allows the comparison of the different hydrophobic effects between the iodine atom and the aromatic ring.

The activity measurements of the glycosomal enzymes of *Trypanosoma brucei* (HK, PGI, GK and PGK) were performed on isolated and purified enzymes as described by Misset *et al.* [6] The assays were started by addition of the substrates and the initial rates were calculated from the absorbance change of NADH (ε = 6.22 mM^{-1} cm^{-1}) at 340 nm. Exactly the same conditions were used for the reference enzymes. The concentrations of inhibitor required for 50% inhibition (IC_{50}) are reported in Table 1. These values were determined for each of the enzymes in the presence of their respective substrates: glucose for HK, glycerol for GK, fructose 6-phosphate for PGI, and 3-phosphoglycerate for PGK.

Table 1. The inhibitory effect of compounds **3**, **4**, **7–9**, **11** and **12** and suramin* on glycolytic enzymes from Tb and other organisms

	HK[a]		PGI[b]		GK[c]		PGK[d]	
	Tb	Y[e]	Tb	Y	Tb	Bs[f]	Tb	Rm[g]
S	24	NA[h]	60	NA	35	120	8	55
11	30	NA	50	NA	60	200	40	150
12	58	NA	-	NA	120	300	20	180
7	45	NA	25	NA	1	>200	2.3	120
9	43	57	13	58	3,5	NA	0.5	78
8	5	23	37	NA	12	NA	4	19
3	60	NA	25	NA	3	NA	1.8	480
4	7	NA	5	NA	2	500	5	NA

[a]Hexokinase. [b]Phosphoglucose isomerase. [c]Glycerol kinase. [d]Phosphoglycerate kinase. [e]Yeast . [f]*Bacillus stearothermophilus*. [g]Rabbit muscle. [h]No activity at 500 μM. *IC_{50} μM

The bromo compounds **5, 6** and **10** are only active in the range of 150–300 μM and are not shown in Table 1. Apart from compounds **11** and **12**, all the inhibitors exhibit a higher inhibitory activity against glycosomal enzymes from *Trypanosoma brucei* than on the reference enzymes in a similar manner to the drug suramin. The weak activity displayed by **12** may be due to the self association of this compound (critical micellar concentration = 350 μM) with, as a consequence, a lower concentration of free compound than expected. No significant difference was found between the IC_{50} values for the charged molecule **7** and for compound **9** which is neutral. The best inhibitions were obtained with the *p*-iodo compound **4** and the *p*-amido derivative **8.** These compounds displayed IC_{50} values in the range of 5–12 μM on HK, GK and PGK, with an important selectivity towards the Tb enzymes, as compared to the homologous enzymes from other organisms.

The kinetic inhibition studies of the most active compounds were performed on glycosomal PGK from *Trypanosoma brucei* and analyzed by Eadie–Scatchard treatment. The Eadie–Scatchard plot (v/[S] versus v) gives information on the behavior of the enzyme with the substrate in the presence of the inhibitor.

The equation rate is $v/[S] = -1/K_m\ (v - 1/V_m)$; therefore, with increasing inhibitor concentration, the slope $-1/K_m$ decreases and the intercept on the x-axis (V_m) is constant, showing competitive inhibition. If the slope is constant (invariable K_m) for varying inhibitor concentrations and the intercept on the x axis shows a decrease of V_m when the inhibitor concentration is increasing, the inhibition is noncompetitive. For a mixed inhibition pattern, both V_m and K_m values change, they decrease when the inhibitor concentration is increased.

Table 2. The nature of the inhibition of trypanosomal phosphoglycerate kinase as analyzed by Eadie–Scatchard plots

Compound	3-PG[a]	ATP[b]
Suramine	C[c]	C
8	M[d]	M
5	NC[e]	M
7	NC	NC
6	NC	NC
3	NC	M
4	M	NC

[a]3-Phosphoglycerate, [b]Adenosine-tri-phospate, [c]Competitive inhibition, [d]Mixed inhibition, [e] Noncompetitive inhibition.

All the compounds bearing a sulfate group are competitive or mixed inhibitors with respect to the substrates. The analogous tetrahedral structures of SO_3 to PO_4 could account for the interaction of the SO_3 group with the ATP or 3-PG binding sites. For compounds exhibiting a noncompetitive pattern, the interaction out of the active site could correspond to an allosteric inhibition which may prevent the hinge bending of the enzyme. In accordance with this proposal, it is known that this hinge bending is particurlarly significant with kinases.[7]

Contrary to our expectations, this work emphasizes the significant contribution of hydrophobic interactions in the affinity of this set of inhibitors. This effect implies the importance of not only the spacer, and the aromatic moiety as shown earlier,[4] but also the substituents on the ring: the best inhibition is indeed obtained with the most hydrophobic substituent in the set, the iodo group. From a practical point of view, owing to their selectivity on the Tb enzymes, these compounds might be of interest in the design of biologically active molecules that can be used against human trypanosomiasis.

Experimental

All of the reactions were carried out under an argon atmosphere using dry solvents. The solvents were distilled according to known procedures. IR spectra were recorded on a Perkin Elmer 1610 FTIR spectrometer (ν in cm^{-1}), NMR spectra on a Bruker AC250 (^1H: 250 MHz, ^{13}C: 62.5 MHz). Chemical shifts δ are reported in ppm relative to the solvents $CDCl_3$ or DMSO, the following abbreviations are used: singlet (*s*), doublet (*d*), triplet (*t*), multiplet (*m*). Mass spectra were determined on a quadripole Nermag R10/10 spectrometer. Chromatographic purifications were performed using silica gel (Kieselgel $60F_{254}$ Merck). Kinetic studies were performed on a UV Beckman DU-7 and on a UV Perkin Elmer Lambda 15 spectrophotometer.

Synthesis of hexadecanedioyl chloride (1)

To a solution of hexadecanedioic acid (4 g, 0.014 mol) in CH_2Cl_2 (200 mL) was added dropwise thionyl chloride (2.1 mL, 0.028 mol). The solution was heated to reflux overnight. Infrared spectroscopy (Cl–C=O 1805 cm^{-1}) was used to follow the formation of acid chloride.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (mercaptothiazoline) (2)*

2-Mercaptothiazoline (3.33 g, 0.028 mol) in THF (100 mL) and Et_3N (8 mL; 0.056 mol) were added dropwise while stirring at room temperature to **1**. The solution was refluxed for 4 h. The solvent was removed by evaporation and the crude product was dissolved in CH_2Cl_2, washed with brine and dried over $MgSO_4$. After the evaporation of the solvent and purification by chromatography on silica gel (eluant CH_2Cl_2), compound **2** was obtained as a yellow powder (2.85 g, 53% yield). IR (pellet KBr), $\nu_{C=O}$ 1681 $\nu_{C=S}$ 1310, mp 120 °C; ^1H NMR ($CDCl_3$), 4.7 (2H, *t*), 3.25 (4H, *m*), 1.66 (2H, *m*), 1.22 (10H, *m*); ^{13}C NMR ($CDCl_3$), 201.5 (C=S), 175 (C=O), 56 (CH_2–N), 38.5 (CH_2–S), 29.3 (CH_2), 24.75 (CH_2).

The same method was utilized for compounds **3–12**: compound **2** (1 equivalent) in CH_2Cl_2 was added to the

corresponding aromatic amine (2 equivalents) dissolved in CH_2Cl_2, or methanol for compounds **7**, **9**, or **12**. The solution was stirred at room temperature for several days. After filtration, all compounds were washed with water, dried and recrystallized from a mixture of DMF and acetone. All compounds were obtained as a white powder.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (2-benzene iodide) (3)*

Mp 202 °C; IR (pellet KBr), ν_{NH} 3390, ν_{CH} 2960–2850, $\nu_{C=O}$ 1660; ^{1}H NMR (DMSO-d_6), 8.2 (1H, *d*), 7.76 (1H, *d*), 7.34 (1H, *m*), 6.63 (1H, *t*), 2.42 (2H, *t*), 1.76 (2H, *m*), 1.26 (10H, *s*); ^{13}C NMR (DMSO-d_6), 136.7, 129.3, 125.3 and 121.9 (CH aromatic), 36.06 (CH_2), 29.3 (CH_2), 25.6 (CH_2); mass (+H) = 689.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (4-benzene iodide) (4)*

Mp 248 °C; IR (pellet KBr), ν_{NH} 3380, ν_{CH} 2990, 2850, $\nu_{C=O}$ 1660; ^{1}H NMR (DMSO-d_6), 7.45 and 7.58 (4H, 2*d*), 2.24 (2H, *t*), 1.6 (2H, *m*), 1.23 (10H, *s*); mass (+H) = 689.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (2-benzene bromide) (5)*

IR (pellet KBr), ν_{NH} 3264, ν_{CH} 2919 and 2848, $\nu_{C=O}$ 1666; mass (+H) = 595.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (4-benzene bromide) (6)*

IR (pellet KBr), ν_{NH} 3300, ν_{CH} 2917-2849, $\nu_{C=O}$ 1662; ^{1}H NMR (DMSO-d_6), 9.9 (1H, *s*), 7.47 and 7.54 (4H, 2*d*), 2.28 (2H, *t*, CH_2), 1.56 (2H, *m*), 1.22 (10H, *m*); mass (+H) = 595.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (4-benzene sulfonic acid) (7)*

Mp > 300 °C; IR (pellet KBr), ν_{NH} 3300, ν_{CH} 3000–2850, $\nu_{C=O}$ 1660; ^{1}H NMR (DMSO-d_6), 9.8 (1H, *s*), 7.51 (4H, *m*), 2.27 (2H, *m*), 1.5 (2H, *m*), 1.24 (10H, *m*); ^{13}C NMR (DMSO-d_6), 171.3 (C=O), 142.9 and 129.3 (C aromatic), 125.9 and 117.7 (CH aromatic), 36.3 (CH_2), 26.7 (CH_2), 24.9 (CH_2); mass (M–2Na$^+$) = 594.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (4-benzene amide) (8)*

Mp 226 °C; IR (pellet KBr), ν_{NH} 3320–3290, ν_{CH} 2919–2849, $\nu_{C=O}$ 1650; ^{1}H NMR (DMSO-d_6), 7.42 and 7.53 (4H, 2*d*), 1.451 (4H, *m*), 1.18 (10H, *m*); mass (M–4 H) (+ NH_4^+) = 536.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (4-benzene sulfamide) (9)*

Mp 292 °C; IR (pellet KBr), ν_{NH} 3280–3490, ν_{CH} 2920–2852, $\nu_{C=O}$ 1670; ^{1}H NMR (DMSO-d_6), 7.48 and 7.61 (4H, 2*d*), 1.5 (4H, *m*), 1.21 (10H, *m*); mass M$^+$ = 594.

N,N'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (3-benzene* N *acetyl bromide) (10)*

^{1}H NMR (DMSO-d_6), 10.32 (1H, *s*), 9.85 (1H, *s*), 7.94 and 7.24 (4H, *m*), 4.00 (2H, *s*), 2.31 (2H, *m*), 1.56 (2H, *m*) and 1.24 (10H, *m*); ^{13}C NMR (DMSO-d_6), 183.5 (CO), 172 (CO-NH), 140.1 and 130.2 (C aromatic), 129.4, 125.6, 122, 120 (CH aromatic), 47.3 (CH_2), 36.6 (CH_2), 27.2 (CH_2), 20.1 (CH_2); mass (+ NH_4) = 726.

7,7'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis naphthalene (11)*

^{1}H NMR (DMSO-d_6), 8.30 (2H, *d*), 7.81 (1H, *d*), 7.74 (1H, *s*), 7.21 (1H, *dd*), 2.12 (2H, *t*), 1.51 (2H, *m*), 1.20 (10H, *m*); ^{13}C NMR (DMSO-d_6), 142.1, 134.4, 126.3 125.6, 124.7, 123.7, 120.8, 119.0, 109.7 (C naphthalene), 37.1 (CH_2), 32.0 (CH_2), 29.78 (CH_2), 22.7 (CH_2).

7,7'-[1,14 *Tetra decane diyl bis (carbonylamino)] bis (1,3 naphthalene disulfonic acid) (12)*

Mp > 300 °C ; IR (pellet KBr), ν_{OH} 3500 ν_{CH} 2960–2850, $\nu_{C=O}$ 1632 , $\nu_{S=O}$ 1190–1024; ^{1}H NMR (D_2O), 8.3 (2H, *d*), 7.8 (1H, *d*), 7.74 (1H, *s*), 7.2 (1H, *dd*), 2.12 (2H, *t*), 1.51 (2H, *m*), 1.2 (10H, *m*); ^{13}C NMR (D_2O), 169.2 (C=O), 150.7, 136.9, 136.6 and 133.2 (CH naphthalene), 134, 133.2, 125.1, 122.6 and 106.6 (C naphthalene), 40.21 (CH_2), 31.3 (CH_2), 28.5 (CH_2).

Inhibition studies

The glycolytic enzymes from Tb were isolated and purified as described by Misset *et al.*[6] Commercially available homologous enzymes and substrates were purchased from Boehringer Mannheim or from Sigma Chemical Company.

All inhibitions of enzyme activity were determined after 5 min of preincubation of the respective enzyme with the appropriate amount of buffer and inhibitor in the reaction cuvette at room temperature. The percentage of remaining activity was calculated from comparison with a control experiment in which the inhibitor was replaced by the same amount of solvent. Possible effects of inhibitors on the absorbance of NADH and NADPH were checked by reaction assays without enzyme.

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