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Original article

Anti-trypanosomal compounds. I. *In vivo* and *in vitro* activity of inhibitors of glycolysis in trypanosomes

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Summary — Carboxylated and phosphorylated esters of glycerol, possible inhibitors of the atypical glycolysis in trypanosomes have been synthesised and tested on *Trypanosoma equiperdum in vitro* and *Trypanosoma brucei*, on mice, and some of them also on glycerol phosphate dehydrogenase (GDH). Two of these compounds which showed no *in vitro* activity behaved as good competitive inhibitors of GDH; 3 other compounds were shown to have a good activity on cultures of trypanosomes, but owing to their weak solubility, tests on the enzyme could not be conclusive. Indications for the design of other compounds have been given.

Résumé — Composés à activité anti-trypanosomiale. I. Activités *in vitro* et *in vivo* d'inhibiteurs de la glycolyse chez les trypanosomes. *Des esters carboxyliques et phosphoriques du glycérol, inhibiteurs possibles de la glycolyse chez les trypanosomes ont été synthétisés et étudiés sur* Trypanosoma equiperdum *en culture et sur* Trypanosoma brucei *chez la souris et, pour certains d'entre eux, sur l'enzyme glycérol phosphate déshydrogenase (GDH). Deux de ces composés, bons inhibiteurs compétitifs de la GDH, ne montrent pas d'activité avec les tests* in vitro*; trois autres composés montrent une activité intéressante sur des cultures de trypanosomes mais, en raison de leur faible solubilité, les tests sur l'enzyme n'ont pu être réalisés dans des conditions satisfaisantes. Des conclusions sont tirées pour la définition d'autres composés à préparer.*

trypanocids, phospho-trioses / glycerol phosphate dehydrogenase / glycolysis in trypanosomes

Introduction

Despite recent but limited progress in the therapy of human sleeping sickness [1] more investigation is needed in this area. This need is illustrated by the recent increase in damage of this parasitic disease [2]. A new area of concern has also arisen from the growing number of infection sites, the transport of the disease *via* wildlife, and also the extension of fatalities in cattle. Every year three million cattle on the African continent are infected with the animal form of trypanosomiasis. Also the spread of the cattle disease to the Far East (Vietnam–Philippines) has recently been discovered [2]. The South American continent as well as the Southern part of the United States suffers from a parasitic disease called 'Chagas' disease. This is due to another species of trypanosome. Treatment will largely depend on the development of new drugs as there is a current absence of any efficient remedy [2].

Until the late 1970's, the main drugs used for therapy of the disease were suramin and pentamidine for the bloodstream form, arsenicals such as melarsoprol for the nervous stages and nifurtimox against Chagas disease. Real shortcomings associated with the use of these drugs (kidney damage with suramin, chemoresistance, high toxicity with arsenicals) encouraged more active investigation [3]. A second period of development came after 1980 stemming, from a better knowledge of the biology of trypanosomes [4]. This progress opened access to clearly identified targets in drug design against the disease, based on differences in metabolic pathways between the host and the parasite [5–7].

Two approaches have therefore been investigated: the first, based on differences in activity of polyamine biosynthesis, more active in trypanosomes than in mammals, has led to the development of inhibitors of ornithine decarboxylase. The first compound, difluoromethylornithine (DFMO) proposed by Bacchi

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[8] and now in use, was followed by the use of similar compounds developed by Merell Co [1, 9]. Drugs of this group have interesting activities when used in relatively high concentrations (10^{-3} M for DFMO) and when associated with other compounds such as bleomycin and polyamines [1].

The second approach under active development is the attempt to selectively inhibit the glycolytic pathway in trypanosomes. This target appears to be of great interest for several reasons:

a) Trypanosomes use glucose as their only source of energy; they have no Krebs cycle or oxydative phosphorylation, nor do they store polysaccharides [6, 10]; therefore their glucose consumption is very high [their own dry weight every hour] and interference with glycolysis in the parasite represents a potential target [7].

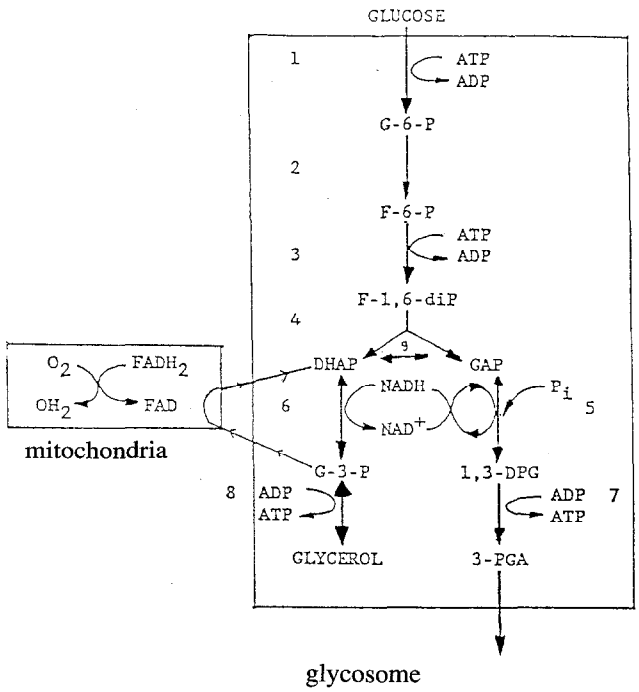


Fig 1. Glycolytic pathway in long slender bloodstream forms of *Trypanosoma brucei*. *Abbreviations:* Substrates: G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; F-1, 6-P: fructose-1,6-diphosphate; DHAP: dihydroxyacetone-3-phosphate; GP: glycerol-3-phosphate; GAP: glyceraldehyde-3-phosphate; di PGA: 1,3-diphosphoglycerate; 3 PGA and 2 PGA: 3- and 2-phosphoglycerate respectively; PEP: phosphoenolpyruvate. Enzymes: 1: hexokinase; 2: phosphoglycoseisomerase; 3: phosphoglucokinase; 4: aldolase; 5: glyceraldehyde phosphate dehydrogenase; 6: glycerol phosphate dehydrogenase; 7: phosphoglycerate kinase; 8: glycerokinase; 9: triose phosphate isomerase.

b) Two enzymes of the glycolytic pathway, hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.90) have recently been purified and their physical properties described [11]; since they display different patterns compared to those of mammals, they have to be considered as possible targets for inhibition.

c) As can be seen in figure 1, the triose part of the glycolytic pathway is different in the parasite when compared to that of the host; the 2 corresponding enzymes glycerol 3P-dehydrogenase (GDH) (EC 1.1.18) and glycerol kinase (GK) (EC 2.7.1.30) might

Table I. Comparative tests, on glycerol phosphate dehydrogenase activity and on a *Trypanosoma equiperdum* population *in vitro* of 12 compounds. *Abbreviations:* C: competitive inhibition; NS: not soluble for enzyme tests; WI: weak inhibition.

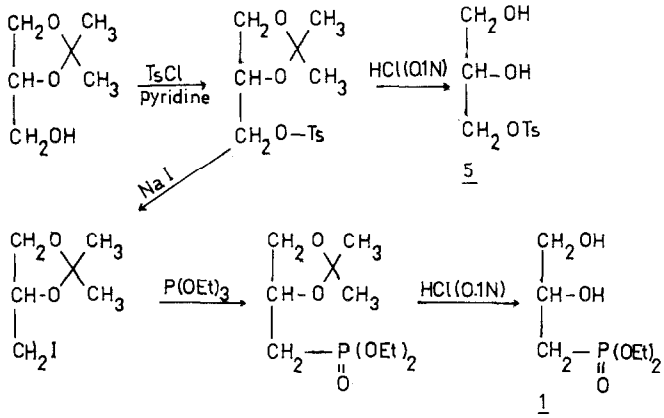
	R	n°	GDH inhibition	in vitro activity
CH_2OH $CHOH$ CH_2R	$-P(O)(OEt)_2$	1	C $K_i = 8$ mM	$>10^{-4}$ M
	$-CH_2P(O)(OEt)_2$	2	$K_i > 10^{-2}$ M	//
	$-OP(S)(OMe)_2$	3	//	//
	$-OP(S)O_2^{--}$	4	//	//
	$-OSO_2-C_6H_4-CH_3$	5	C $K_i = 3$mM	//
	$-O-C_6H_4-CH_2-C_6H_5$	6	NS 10% DMSO	10^{-4} M
	$-O-\overset{O}{\overset{\|}{C}}-CHCl_2$	7	$K_i > 10^{-2}$ M	$>10^{-4}$ M
	$-O-\overset{O}{\overset{\|}{C}}-CH_2Cl$	8	//	//
CH_2 $\|$ CH CH_2R	$P(O)(OEt)_2$	9	//	//
	$CH_2P(O)(OEt)_2$	10	//	//
CH_2O CHO CH_2R	$O\overset{O}{\overset{\|}{C}}CHCl_2$	11	W.I. 10% DMSO	10^{-4} M
	$O\overset{O}{\overset{\|}{C}}CH_2Cl$	12	W.I. 10% DMSO	10^{-4} M

therefore be selectively inhibited, possibly without significant drawbacks for the host.

The present work is a contribution to the investigation of this subject, and is devoted to the study of the possible inhibition of glycerol 3P-dehydrogenase, the second enzyme of this atypic glycolysis. Indeed its location in the pathway makes it a valuable target. The compounds that we synthesized were tested as inhibitors of this enzyme, on trypanosome cultures and for some of the compounds, on mice infected with *Trypanosoma brucei*.

Chemistry

The tosylate **5** and the phosphonate **1** (table I) were synthesized according to Baer and Basu [12] as indicated in scheme 1, but starting from glycerol. Therefore all compounds are racemic mixtures.

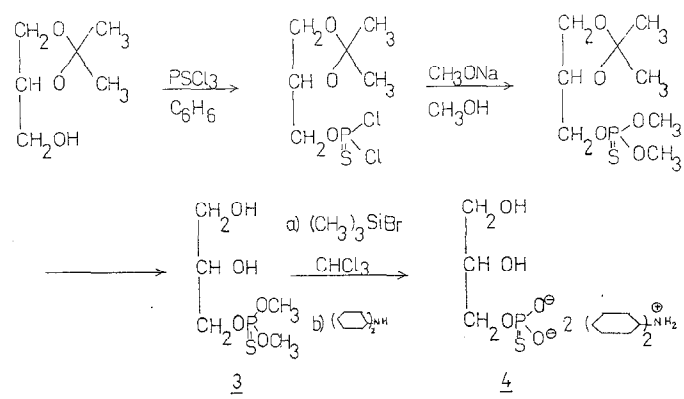


Scheme 1.

The protection of a vicinal diol of a glycerol by an isopropylidene group allowed the tosylation of the primary hydroxyl group; exchange of the tosylate with sodium iodide followed by an Arbuzov reaction and removal of the isopropylidene group led to the phosphonate **1**.

The thiophosphate of glycerol 4 was obtained following the sequence represented in scheme **2**.

1,2-*o*-isopropylidene glycerol was phosphorylated with thiophosphorylchloride; reaction of the intermediate thiophosphochloridate with sodium methylate then removal of the isopropylidene group allowed the formation of the thioester **3**. The structure of **3** was confirmed by the simplification of the multiplet shape of the resonance peak in phosphorus NMR; this multiplet centered at 70.8 ppm ($CDCl_3$) due to the coupling of the phosphorus atom with the 2 protons on the CH_2 group and the 6 protons on the methyl

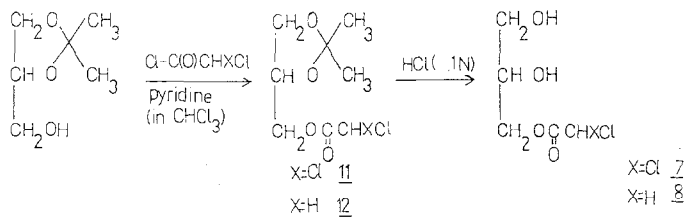


Scheme 2.

groups, was simplified to a triplet by irradiation of the methyl signals; this triplet as only signal means that the phosphorus atom remains linked to the CH_2 of the glycerol framework. Therefore a migration of the thiophosphate group from position 1 to position 2 on glycerol which may occur during removal of the isopropylidene group is not significant since **3** is the only isolated product. This rearrangement, well documented for phosphoric esters [13], might be less favourable with thiophosphoric esters owing to the weaker electrophilic character at phosphorus. Reaction with **3** of trimethylsilyl bromide allowed through the formation of a silyl ester [14] the formation of **4** as its dicyclohexylammonium salt. The structure of **4** was also confirmed by ^{31}P NMR (poorly resolved triplet centered at 70.1 ppm in D_2O).

1 → 2 migration of the thiophosphate group does not occur either during reaction with dicyclohexylamine.

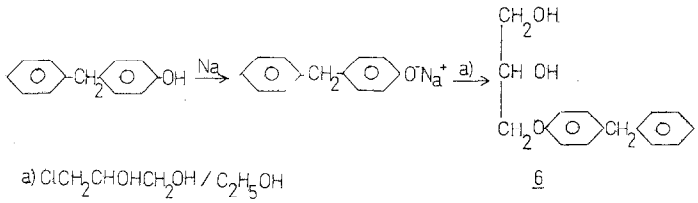
Compounds **7, 8, 11** and **12** were synthesized according to scheme 3.



Scheme 3.

Reaction of mono- or dichloro-acetyl chloride with 1,2 *o*-isopropylidene glycerol in the presence of pyridine led to the formation of **11** or **12** respectively; removal of the isopropylidene group in acidic conditions provoked no significant hydrolysis of the ester part as checked by IR and NMR spectroscopies and in agreement with previous determinations [15].

Finally compound **6** was obtained as the only product according to scheme 4.



Scheme 4.

Pharmacology

Table I indicates the results obtained with 12 different compounds divided into 3 sets.

In the first set compounds are included with a diol structure, owing to the fact that it was claimed by Whitesides [16] that this part is needed for recognition by glycerol kinase, one of the 2 enzymes on which the compounds that we tested were capable of this action.

The 2 first compounds tested **1** and **2**, are phosphonates; it is known that a structure such as that found in (S) 3,4-dihydroxybutylphosphonate is a substrate for glycerol 3P-dehydrogenase [17]; this means that the replacement of an oxygen atom by a CH_2 group does not suppress recognition or activity. Therefore diesters such as **2** were potential substrates or inhibitors. In fact, no activity was found for **2**. But a similar diester structure in compound **1**, with a shorter chain length produces an activity as a competitive inhibitor of GDH (fig 2); one interesting point is the fact that the corresponding acid, dihydroxypropyl phosphonic acid, is not active, either as substrate or inhibitor [17]. Possibly, a similarity in size of the chain length with the normal substrate of GDH is required for activity.

Then 2 thio compounds **3** and **4** were tested: **3** the corresponding thiotriester and **4** analogous to the normal substrate; neither was active. The replacement of an oxygen atom in the substrate of GDH by a bigger and less electronegative sulfur atom makes the compound unrecognizable as a substrate, in contrast to observations with phosphorothioates analogues of nucleotides which are substrates for kinases and RNases [18].

Compounds **5** and **6** provide an interesting indication since the introduction of a hydrophobic part in the molecule renders them active as inhibitor of GDH for the sulfonic ester **5** (table I) and on *in vitro* activity even at low concentration for the diol **6** (table I); activity on GDH of this last compound could not be tested due to the insolubility in DMSO-water mixtures, compatible with GDH activity.

The results given in figures 2 and 3 are obtained with compounds **1** and **5** respectively, which behave as competitive inhibitors with K_i of 8 and 3 mM. For

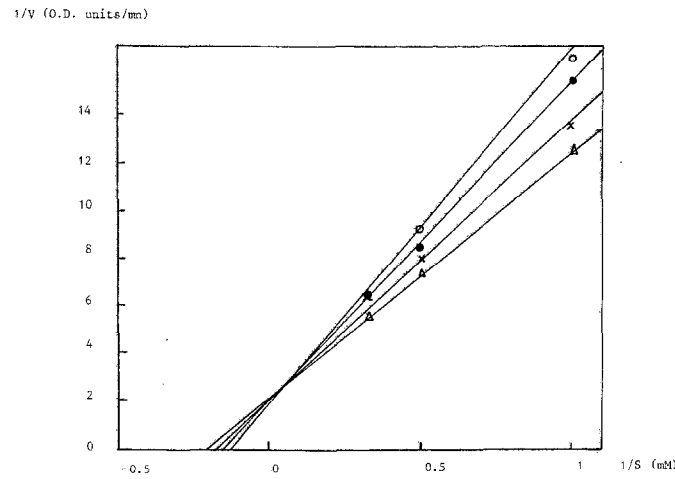


Fig 2a. LINEWAEVER curve on glycerol phosphate dehydrogenase (EC 1.1.18) with different inhibitor concentrations $(EtO)_2P(O)CH_2CHOHCH_2OH$ compound **1**. Δ, I = 0 mM; x, I = 0.33 mM; •, I = 1 mM; ○, I = 2 mM.

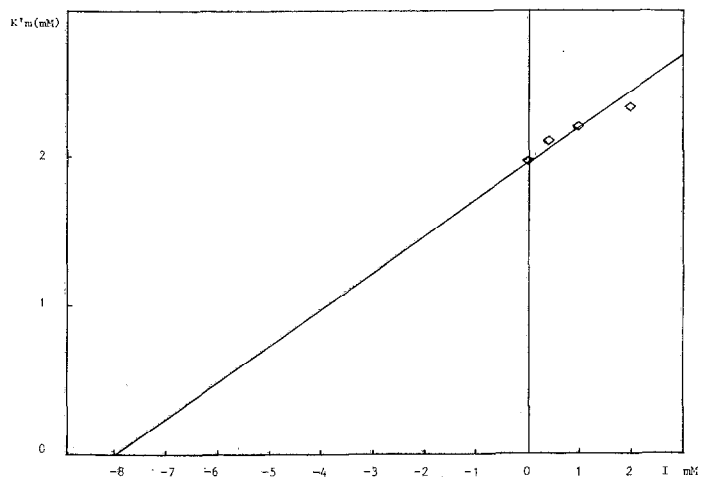


Fig 2b. Inhibition constant determination for DL-dihydroxypropylphosphonic acid diethyl ester 1. $K'm = Km(1 + 1/K_I)$, $K_I = 8$ mM.

the DL glycerol phosphate sodium salt, the K_m in the same conditions (pH = 9, temperature 30°C) was found equal to 1.43 mM.

Since compounds **7** and **8** are described as non competitive inhibitors of glycerol kinase [15] they were also tested on GDH: they showed no activity.

Compounds of the second set were designed first with a phosphonic diester structure owing to positive results obtained with **1** but also because bearing an sp^2 center as the normal substrate of GDH; no activity was observed.

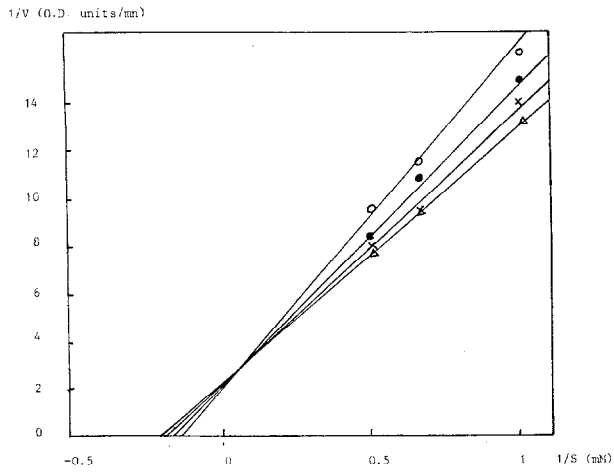


Fig 3a. Linewaever curve on glycerolphosphate dehydrogenase (EC 1.1.18) with different inhibitor concentrations ($CH_3OSO_2OCH_2CHOHCH_2OH$ compound **5**. Δ, I = 0; x, I = 0.5 mM; •, I = 1 mM; ○, I = 2 mM.

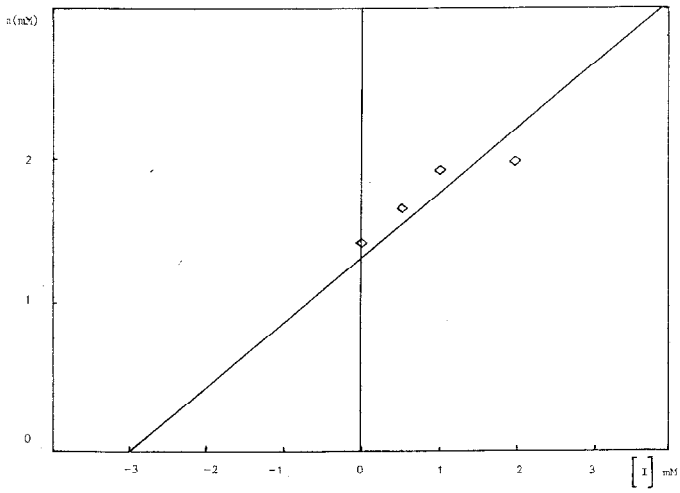


Fig 3b. Inhibition constant determination for compound **5**. $K'm = Km(1 + 1/K_I)$, $K_I = 3$ mM.

Compounds **11** and **12** of the third set were designed with the aim of replacing the phosphate group by an α-chloroester structure with a more hydrophobic contribution from the rest of the chain. These last 2 compounds gave promising results : they showed a weak inhibition of GDH, when tested in 10% DMSO required for solubility, conditions where the efficiency of the enzyme remains significant. It is interesting to note that both exhibit a high activity on trypanosome cultures and therefore merit further development.

Discussion

Concerning first the enzyme inhibition test, we notice that two of the compounds, **1** and **5**, behave as competitive inhibitors of rabbit GDH. The validity of this test might be questionable, but the similarity of this enzyme with the trypanosome GDH which has been recently established [19] allows some confidence.

Secondly, the correlation between enzyme inhibition and *in vitro* activity does not appear clearly : not only for compound **1** but also for compounds **7** and **8**, which have been identified as inhibitors of GK [15] but show no activity on trypanosomes. This contrasts with compounds **11** and **12**, which behave as weak inhibitors and are highly efficient against trypanosomes. Inhibition tests with these 2 compounds will be carried out with glycerolkinase. Compounds **6, 11, 12**, are of some interest since they exhibit a good activity on trypanosome cultures. These results were indeed obtained with a medium containing high glucose concentration (2 $g \cdot l^{-1}$) where competition between the tested compounds and the normal products of glucose metabolism occurs.

Finally, the same compounds **6, 11, 12** were also tested on mice infected with *Trypanosoma brucei* at the Institute for Veterinary Medicine in Berlin; no activity was found. This difference might depend on the transportation of the drug. Metabolisation of the drug before it reaches it target might also explain this discrepancy between results *in vitro* and *in vivo*. We are therefore considering the synthesis of pro-drugs of these compounds as a possible drug delivery route.

Experimental protocols

Chemistry

Melting points were obtained using a Büchi melting point apparatus. The IR spectra were recorded on Perkin–Elmer 284 and 68, the ^{31}P NMR spectra on Bruker AC 80 and AM 300 WB spectrometers operating at 32.4 MHz and 121.49 MHz respectively, using H_3PO_4 (85%) as external reference. Elemental analysis for C, H, N, S were performed by Microanalytical service of CNRS Vernaison, France.

Synthesis

Compounds **1, 2, 5, 7–12**, were synthesized according to described procedures; they were purified by distillation, their purity checked by TLC analysis and their spectra (IR, ^1H NMR) were in agreement with the assigned structures.

Complementary characterization is given by their chemical shifts; compounds **1** and **5** were prepared according to Baer and Basu [12]: δ ^{31}P (D_2O) = 31.4 ppm, for compound **1**; compounds **2, 9, 10** synthesized as Kabak [20], δ ^{31}P ($CDCl_3$) respectively 28.9; 26.9; 30.0 ppm; compounds **7, 8, 11, 12** were prepared according to Tisdale [15].

(2,3-Isopropylidene-glyceryl)-dimethyl-thiophosphate (scheme 2)
10 g of racemic isopropylidene-glycerol (0.075 mol) in benzene were added dropwise to a cold solution of 12.8 g of thiophosphorylchloride (0.075 mol) and 6 g of pyridine at –20°C. After slow warming to room temperature, the mixture was stirred for one day. After filtration of the formed chlorhydrate, the mixture was distilled; the only product (Eb 80°C/l mmHg) was dichloro(2,3-isopropylidene glyceryl) thiophosphate (18 g; 90%).

The corresponding ester was prepared by the reaction of sodium methylate in methanol (4 g, 0.074 mol) on a solution of 10 g (0.0377 mol) of dichloro(2,3-isopropylidene glyceryl) thiophosphate in 100 ml of benzene at –20°C. After warming to room temperature and stirring 24 h, the solution was concentrated and distilled (80°C/0.1 mmHg) to give 7.82 g (81%) of product. ^{31}P NMR ($CDCl_3$) = 71.8 ppm; ^{1}H NMR ($CDCl_3$, 80 MHz) δ 1.40 (d, 6H, $J_{H,H}$ = 6 Hz); δ = 3.95 (d, 6H, $J_{H,P}$ = 13 Hz, P-OCH_3) δ 3.5–4.1 (m, 5H, –CH_2–CH–CH_2–) mass spectrum (E.I) 241 (M-CH_3); 181 (I = 100).
Anal for $C_8H_{17}O_5PS$: C, H, O.

Glyceryl-dimethyl thiophosphate **3**
Hydrolysis of the ester (2,3-isopropylidene-glyceryl)-dimethyl-thiophosphate by 1 N HCl at room temperature for 2 days gave quantitatively **3**; analysis after purification (chromatography on silica gel) gave: δ ^{31}P NMR = 70.8 ppm ($CDCl_3$); with coupling and selective irradiation of the CH_3O– signal, gives triplet ($J_{H,P}$ = 6 Hz, –CH_2OP); mass spectrum (EI) 217 (M + 1); 185 (M–CH_3O).
^{1}H NMR (D_2O, 80 MHz) δ 3.75 (d, 6H, $J_{H,P}$ = 13 Hz, $POCH_3$); δ 3.5–4.0 (m, 5H, –CH_2–CH–CH_2–).

Bis-dicylclohexylammonium glyceryl-thiophosphate **4**
To a solution of 750 mg of **3** in 10 ml of chloroform were added dropwise 1.5 ml of trimethylsilylbromide [14]. The mixture was stirred overnight at room temperature, then concentrated *in vacuo* and the residue dissolved in dry ethanol before the stoichiometric amount of dicyclohexylamine was added. The precipitate was filtered off to give 450 mg (24%) of **4**. Purification was achieved by crystallization in methanol.
^{31}P NMR (D_2O) = 70.4 ppm (triplet $J_{H,P}$ = 5 Hz–CH_2OP) mp > 260° IR (KBr pellet) 3580 cm^{-1} ν OH; 1150 ν P-O.
Anal for $C_{27}H_{55}N_2O_5PS$: C, H, N.

3-[4(Benzyl)phenoxy]-1,2-propanediol **6** *(scheme 4)*
0.25 g (0.010 mol) of sodium was added to a solution of 2 g (0.010 mol) of 4-hydroxydiphenylmethane in ethanol and refluxed for an hour. A solution of 1.2 g (0.010 mol) of chloro-1,2-propanediol in ethanol (15 ml) was then added dropwise and the mixture refluxed for one hour. After filtration of the salt, concentration *in vacuo* and crystallisation in hexane gave 1.5 g (53%) of **6**.
Mass spectrum E.I M^+ 258; 184. Anal for $C_{16}H_{18}O_3$: C, H.

Kinetic determinations

Glycerol-3-phosphate dehydrogenase (extracted from rabbit muscle), β nicotinamide adenine dinucleotide from yeast grade IV and DL glycerol (P) sodium salt hexahydrate were purchased from Sigma. Initial rates of enzyme reactions were followed by determining the formation of NADH from NAD^+. The increase in ε_{340} was measured with a Pye Unicam PV 8800 UV spectrophotometer.

The reaction was initiated by the addition of the enzyme to a solution of 1 M diethanolamine (HCl), pH 9.0, EDTA = 1 mM, 2 mercaptoethanol 1 mM, 6 mM NAD^+ and different concentrations of DL glycerol-3-phosphate, sodium salt (1, 1.5, 2, 3 mM) and different concentrations of inhibitor in a total volume of 1 ml.

A standard assay for glycerol-3-phosphate dehydrogenase activity with 6 mM NAD^+ and different concentrations of DL-glycerolphosphate sodium salt was performed before and after each series of experiments to check the stability of the enzyme.

Tests with 10% DMSO
Solution: 1 M diethanolamine pH 9.0, 10% DMSO, EDTA 1 mM, 2-mercaptoethanol 1 mM, 6 mM NAD^+, and different concentrations of DL glycerol-3-phosphate, sodium salt (1, 1.5, 2 and 3 mM).

Biological tests

In vitro *tests*
These were performed in a semi-defined medium for the cultivation of bloodstream forms of the african *Trypanosoma equiperdum* [21]. The cultured trypanosomes had all the characteristics of the *in vivo* bloodstream forms including morphology, infectivity, antigenic variation and glucose metabolism. The standard medium consisted in Hepes buffered minimum essential medium with Earle's salt supplemented with 0.2 mM 2-mercaptoethanol, 2 mM pyruvate and 10% inactivated rabbit serum. Full details are given in [21].

The values indicated for trypanocidal activity correspond to the minimum amount required to obtain complete depletion of a *Trypanosoma equiperdum* population. The test was run on different samples and the results were read by counting on the grid of a microscope.

In vivo *tests*
These were carried out at the Institute of Veterinary Medecine of Berlin. Parasite: *Trypanosoma brucei* (strain 8/18) on the mouse (NMRI); infection dose: 10^5 trypanosomes/mouse; treatment: subcutaneous 2 h after infection. The experimental preparation of products was: 400/100/25 mg/kg body weight. In these conditions the products tested were inactive.

Acknowledgments

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