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Cell permeation of a *Trypanosoma brucei* aldolase inhibitor: Evaluation of different enzyme-labile phosphate protecting groups

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Abstract—A series of four prodrugs directed against *Trypanosoma brucei* aldolase bearing various transient enzyme-labile phosphate protecting groups was developed. Herein, we describe the synthesis and evaluation of cell permeation of these prodrugs. The oxymethyl derivative was the most efficient prodrug with a good recovering of the free drug (IC_{50} = 20 μM) and without any measurable cytotoxicity.

Trypanosomiases, parasitic diseases, are endemic in many countries of Africa and South America.[1] Besides the animal form, two human forms of the disease occur: *Trypanosoma cruzi* in South America and *Trypanosoma brucei* in Africa. These parasites have developed resistance to chemotherapy and there is an urgent need for the development of new drugs.[2,3] It was found that the bloodstream form of the African trypanosome is exclusively dependent on glycolysis for energy production and therefore this metabolic pathway represents a good target for the development of new inhibitors.[4–6]

In this field, our group has developed a mechanism-based inhibitor of fructose-1,6-bisphosphate aldolase.[7–9] Incubation of the glycolytic enzyme in the presence of various dihydroxybenzaldehyde phosphate derivatives resulted in a slow-binding inhibition of the aldolase.[8,10] Interestingly, we observed that 2-hydroxybenzaldehyde-5-phosphate inhibited the aldolase from *T. brucei* more efficiently than the aldolase from mammalian origin. The selectivity toward *T. brucei* was also observed for an hydroxynaphthaldehyde phosphate inhibitor synthetized in our group.[11] Unfortunately, the benzaldehyde derivative, bearing a phosphate group which is negatively charged at physiological pHElsevier Ltd, did not readily undergo passive diffusion across cellular membranes. Accordingly, the inhibitor presented no activity on parasitic cultures.

This kind of problem has been widely studied during the last twenty years, and various approaches were developed in order to enhance cell permeation of these potential drugs.[12–17] Our interest focused on the use of enzyme-labile transient phosphate protecting groups, which mask the negative charges of the phosphate by esterification, giving triester derivatives with increased lipophilicity.[18–20] This modification is known to facilitate the passive diffusion of the prodrug through the cell membrane, followed by the delivery of the active form of the drug by taking advantage of intracellular enzymes such as the widely present esterases, phosphodiesterases or reductases (Fig. 1).

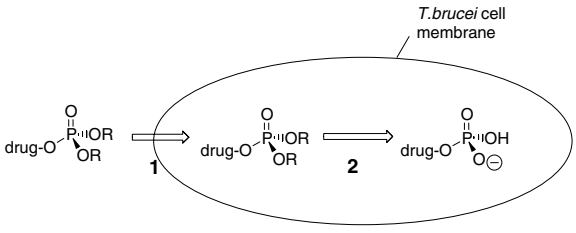

Figure 1. Protection of the phosphate group allows for passive diffusion through the cell membrane (arrow 1), before deprotection by suitable cytosolic enzymes (arrow 2).

Keywords: *Trypanosoma brucei*; Aldolase; Phosphate; Inhibitor; Enzyme-labile protecting group; Drug delivery.
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Herein, we report the synthesis of selected enzyme-labile phosphate protecting groups and their evaluation for their ability to liberate the active drug in the presence of suitable enzymes or parasite cell extracts. Studies on parasite cultures are also reported. The different protecting groups under study are indicated in Figure 2.

Prodrugs **1** and **2**, bearing a bis(acyloxymethyl) protecting group,[21,22] are sensitive to esterases which are present in the targeted parasite. Prodrug **3**, which incorporates the *S*-acyl 2-thioethyl protecting group, is sensitive to the same kind of enzyme, but releases an episulfide instead of formol as a by-product.[23] Finally, prodrug **4** incorporates the *S*-[(2-hydroxyethyl)sulfidyl]-2-thioethyl group as a reductase-labile phosphate protection.[24–28]

Two different synthetic pathways have been developed to obtain compounds **1**–**4**. Prodrugs **1**, **2**, and **4** were synthesized by the reaction sequence described in Scheme 1. Selective protection of 2,5-dihydroxybenzaldehyde (2,5-DHBA) on position 2 was achieved by using pivaloyl chloride in the presence of sodium hydride.[29] Subsequent phosphorylation on position 5 by using triethylphosphite in the presence of iodine gave compound **5** in a good yield.[30] Phosphate deprotection with trimethylsilylbromide was followed by hydrolysis of the pivaloic ester, thus affording compound **6** in quantitative yield. The silver salt of derivative **6** was prepared by using silver nitrate. Then the phosphate derivative was allowed to react with compounds **9**–**11** (Scheme 2) to afford compounds **1**, **2**, and **4** in reasonable yields.

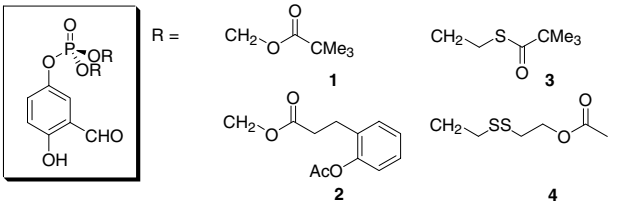
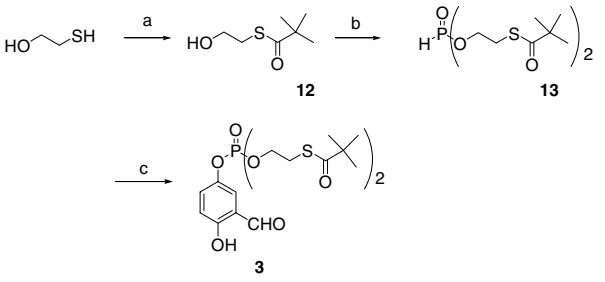


Figure 2. Structure of the compounds with four different phosphate protecting groups, studied herein.

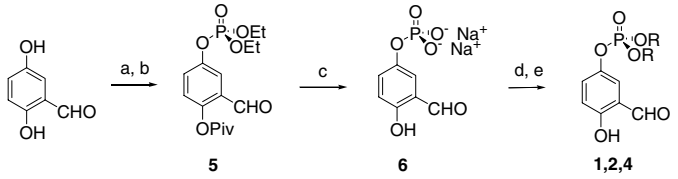
Whereas the synthesis of compound **10** was described elsewhere,[23,31–33] an original synthesis has been developed for compound **9**, as shown in Scheme 2.

The first step is the mono-acetylation of dithioethanol using acetic anhydride to afford derivative **7**, which was subsequently tosylated, leading to the new ester **8**. Nucleophilic substitution of the tosylate group by iodine afforded precursor **9**, which was used directly for the next step (Scheme 1).

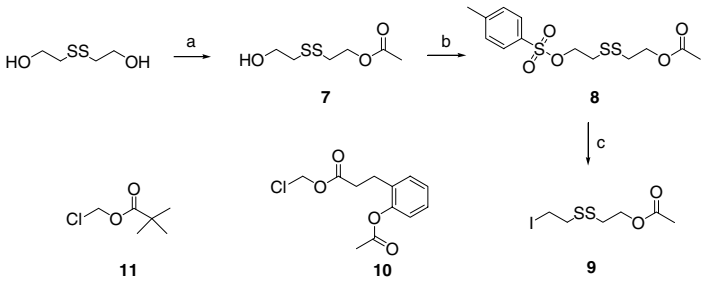
Scheme 3 describes the synthesis of the *S*-acyl 2-thioethyl derivative **3**.



Scheme 3. Synthesis of compound **3**. Reagents and conditions: (a) NaH, Et_2O, 3 h, 0 °C then Piv-Cl, 3 h, 78%; (b) PCl_3, 1-*H* tetrazole, pyridine, 3 h, then **12**, pyridine, 12 h, then H_2O, 30 min, 0 °C, 52%; (c) 2,5-DHBA, CCl_4, DIPEA, DMAP, acetonitrile, 15 min, 0 °C, then **13**, 10 min, 0 °C, 55%.



Scheme 1. Synthesis of the potential prodrugs **1**, **2**, and **4**. Reagents and conditions: (a) NaH, DMF, 3 h, then Piv-Cl, 12 h, 55%; (b) $P(OEt)_3$, I_2, CH_2Cl_2, pyridine, 30 min, 98%; (c) Me_3SiBr, 12 h, then H_2O, NaOH, 10 min, 97%; (d) $AgNO_3$, H_2O, 12 h; (e) **9**, **10** or **11**, toluene, reflux, 12 h, 17–44%.



Scheme 2. Synthesis of compound **9**. Reagents and conditions: (a) Ac_2O, DIPEA, CH_2Cl_2, 12 h, 51%; (b) TsCl, pyridine, 12 h, 75%; (c) NaI, acetone, reflux, 12 h.

The first step is the selective esterification of mercaptoethanol using pivaloyl chloride and sodium hydride to afford compound **12**.[24] Reaction of phosphorus tritetrazolide (formed in situ) with the thioester **12** afforded the *H*-phosphonate derivative **13**. The last step was the condensation of derivative **13** with 2,5-dihydroxybenzaldehyde through a Todt reaction to obtain the expected prodrug **3**, in good yield.[34]

The stability of the prodrugs **1**–**4** was assessed in 50 mM TEA–HCl buffer, 5% DMSO at pH 7.0 and 37 °C. The degradation of these compounds (monitored by UV–vis spectroscopy, data not shown) is ascribed to the formation of the mono-protected form of the free drug.[23] The half-lives for hydrolysis of compounds **1**–**4** were examined. Compound **2** is the most sensitive prodrug, with an half-life of 28 h. The second oxymethyl type prodrug **1** is slightly more stable, with an half-life of 32 h. Even though it is a sensitive protecting group, it is enough to be used to deliver the drug into cell.

The thioester derivative **3** appears to be much more stable, with an half-time of 49 h. Finally, compound **4** is the most stable and only the hydrolysis of the ester group is observed (half-time of 45 h) without formation of the free drug.

The drug release efficiency of compounds **1**–**3** sensitive to esterases (and then also to phosphodiesterases, see below) was subsequently tested and monitored by HPLC. Relative peak integrations permitted the estimation of fully and mono-protected compounds present in the mixture, the half-lives are summarized in Table 1. As it is not possible to normalize these data, these experiments give us an insight about the relative efficiency of the prodrug for the release of the free drug. Compounds **1**–**3** were tested with a mixture of commercially available pig liver esterase and crotale venom phosphodiesterase II. Compounds **1** and **2** gave the same pattern, with an accumulation of the mono-protected drug, before the concentration of free drug increased. These profiles are consistent with the proposed mechanism where a first deprotection step catalyzed by an esterase is followed by a second deprotection by phosphodiesterase or esterase.[16] Compound **2** releases the free drug at a higher rate, probably because the cleavage site for esterase is distant from the first created charge, allowing a second attack by esterase. Compound **3** presents a slightly different profile. No accumulation of the mono-protected compound was observed, suggesting that the phosphodiester intermediate is a very good substrate for a phosphodiesterase.

Different behaviors occurred in the presence of the *T. brucei* cell extract. Table 1 summarizes these results. Compounds **1** and **2** released the active form of the drug, while compound **3** was not fully enzymatically degraded (data not shown). Indeed, we observed that the deprotection reaction stopped after around 20% of mono-deprotected compound formation. This suggests that the by-product (episulfide) may act as an inhibitor of the esterase of the parasite. Since compound **4** was unable to release the free drug in the in vitro assay, it is concluded that it was not a proper substrate of the reductases found in the *T. brucei* cell extract.

Finally, the prodrugs were tested in vitro, against different parasitic systems (Table 2). Compound **2** did not present any activity. This could stem from the nature of the protection: poor stability in the serum used for the assay on the one hand, and high aromaticity, leading to a stacking effect in the membrane on the other hand. Compound **1** was active only against *T. brucei* and showed no cytotoxicity. This means that this system is able to release the drug into the cell and that the formol (the by-product of the deprotection reaction) is not toxic for the human cell for tested concentrations. This result is consistent with the fact that in this study only *T. brucei* grows in a free-macrophage medium. With the other parasites, the prodrug has to diffuse across two cellular membranes (at the level of the macrophage and of the parasite) to be efficient. The second step is not made possible by the activity of the enzymes of the macrophage. Compound **3** was active against both *T. brucei* and the other systems studied, and was cytotoxic at the

Table 2. IC_{50} (μM) determined in the presence of different parasitic systems with compounds **1**–**4**[35]

		Compound			
	6[c]	**1**	**2**	**3**	**4**
T.b[a]	>50	20	>50	16	>50
T.c[a]	>50	>50	>50	15	>50
L.i[a]	>50	>50	>50	25	>50
P.f[a]	>50	>50	>50	12	>50
Cytotoxicity[b]	>50	>50	>50	6	>50

[a] Abbreviation: T.b: *Trypanosoma brucei*; T.c: *Trypanosoma cruzi*; L.i: *Leishmania infantilis*; and P.f: *Plasmodium falciparum*.
[b] The cytotoxicity is determined on MRC-5 cells.
[c] Control.

Table 1. Half-life time of the release of the mono-protected drug (diester) and of the free drug in the presence of a mixture of standard esterase and phosphodiesterase (mixed enzymes) or in the presence of *Trypanosoma brucei* (cell extract)[a]

	Compound:	**1**	**2**	**3**	**4**
Mixed enzymes	Diester	35 min	10 min	4 h 40 min	nd[b]
	Free drug	8 h 10 min	1 h 30 min	5 h	nd[b]
Cell extract	Diester	8 h 20 min	2 h 30 min	>25 h	Stable
	Free drug	>25 h	20 h	>25 h	Stable

[a] [**1**–**4**] = 500 μM, in 50 mM TEA–HCl, pH 7, at 37 °C; PLE: 80 UI; crotale venom phosphodiesterase II: 0.5 UI; *T. brucei* cells: 0.2×10^{19} per assay.
[b] nd, not determined.

concentration used. This system of drug delivery is then useful only if the drug is active at lower concentration (typically at the nanomolar range).[12] Finally, compound **4** presented no effect; the disulfide bridge was probably not reduced in the cells, due to the strong specificity of the intracellular reductase, as expected from previous experiments.

In this paper, we report the first study concerning the cell permeation of an inhibitor directed against the aldolase of *T. brucei* using four different enzyme-labile phosphate protecting groups. We show that the pivaloyl oxymethyl protection (compound **1**) is especially suitable for the *T. brucei* parasite, with a good recovery of the free inhibitor, without measured cytotoxicity. However, in the presence of a cell extract (Table 1), half of the free drug was delivered in more than 8 h with compound **1**, while the doubling time of the parasite population was about 7 h. Future work will then be the optimization of the prodrug, by varying the pivaloyl moiety, to enhance the recognition of the system by the *T. brucei* esterase.

Acknowledgments

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Supplementary data

Experimental details for all the syntheses, drug delivery studies, and biological evaluation are available in Supplementary data. Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.04.010.

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