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Selective inhibition of *Trypanosoma cruzi* GAPDH by "bi-substrate" analogues

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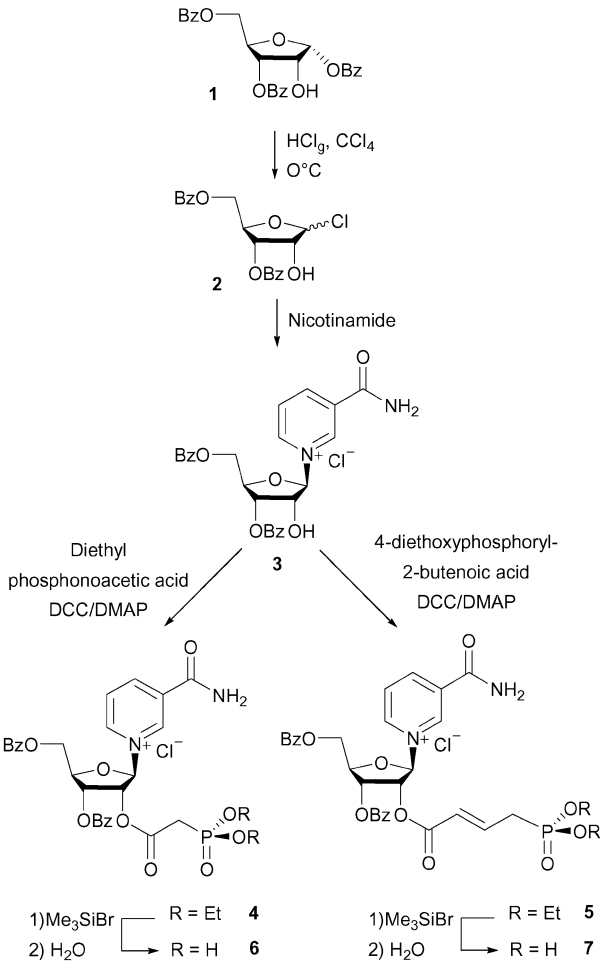
A new series of "bi-substrate" analogues have been synthesized as potential inhibitors of the glyceraldehyde-3-phosphate dehydrogenase and one lead compound has been identified that inhibits the enzyme from *Trypanosoma cruzi* with good affinity and very high (50-fold) specificity.

Trypanosomes are flagellated protozoa responsible for serious parasitic diseases that have been classified by the World Health Organization as tropical diseases of major importance.[1] *Trypanosoma cruzi* in particular is the causative agent of Chagas' disease in South America and is highly dependent on its host's glycolysis in infectious stages for its energy supply. Thus, glycolytic enzymes have been considered as a promising target for the design of new drugs against parasitic trypanosomatid protozoa.[2] Among all glycolytic enzymes, the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) was chosen as our target of interest since (i) its inhibition was reported to significantly reduce the ATP supply of the parasite[3] and (ii) its specific inhibition by adenosine analogues was shown to kill bloodstream-form *Trypanosoma brucei* amastigotes within a few minutes without affecting the growth of fibroblasts.[4]

GAPDH catalyses the reversible oxidative phosphorylation of D-glyceraldehyde-3-phosphate (GAP) into 1,3-*bis*-phospho-D-glyceric acid (1,3-diPG) in the presence of NAD^+ as cofactor and inorganic phosphate. Two highly conserved cationic sites have been identified in GAPDH active site from the co-crystallisation of two sulfate (or phosphate) ions within the protein. These two sites correspond to the interaction sites of the C-3 phosphate of GAP (called Ps) and of inorganic phosphate (called Pi). The Ps site plays a crucial role in the catalytic mechanism since it is occupied by the C-3 phosphate of GAP during the phosphorylation step. The crystal structure revealed that the cofactor NAD^+ participates in the stabilisation of the phosphate anion at the Ps site *via* an electrostatic interaction involving the 3′-hydroxyl of the ribose of the nicotinamide scaffold.[5]

Herein, we describe the design and synthesis of a family of original bi-substrate (GAP + NAD^+) analogues and their detailed inhibition properties tested against the GAPDHs of both mammal and parasite. We report the selective inhibition of *Trypanosoma cruzi* GAPDH with competitive phosphonate inhibitors that were designed to target simultaneously GAP and NAD^+ binding sites.

First generation molecules were synthesized that consist of a phosphonate group (mimic of the C-3 phosphate of GAP) associated to a truncated NAD^+ (limited to its ribose–nicotinamide moiety) through linkers of different length (Scheme 1). While more than 200 NADH analogues have been already described and studied for their ability to work as hydride donor in various dehydrogenases, most of these cofactor modifications have been carried out on the adenine moiety. Only a few ribosyl-β-nicotinamides have been synthesized by displacing an anomeric halide with nicotinamide.[6] In order to selectively phosphorylate the ribofuranose at the 2′ position, 3,5-di-O-benzoyl-D-ribofuranosyl chloride (**2**) was used as starting material. Initially prepared by selective chlorination (HClg, CCl_4) of the 2,3,5-tri-O-benzoyl-α-D-ribofuranose (**1**), the ribofuranosyl chloride quantitatively and stereoselectively reacts with nicotinamide, leading exclusively to the β-anomer of the resulting 3-carbamoyl-1-ribofuranosyl-pyridinium chloride **3**.[7] On this modified ribose, two phosphonates were introduced by formation of an ester linkage with the 2′ hydroxyl. Due to the high unstability of the ribonicotinamide under alkaline conditions, this esterification was carried out under neutral conditions by coupling the appropriate carboxylic acids, activated with the DCC–DMAP couple, on the 2′ hydroxyl. By following such a strategy, diethyl-2-phosphonoacetic acid and diethyl-4-phosphonocrotonic acid were coupled, thus leading to compounds **4** and **5** with 75 and 65% yields, respectively. The cleavage of the diethoxyphosphoric



Scheme 1

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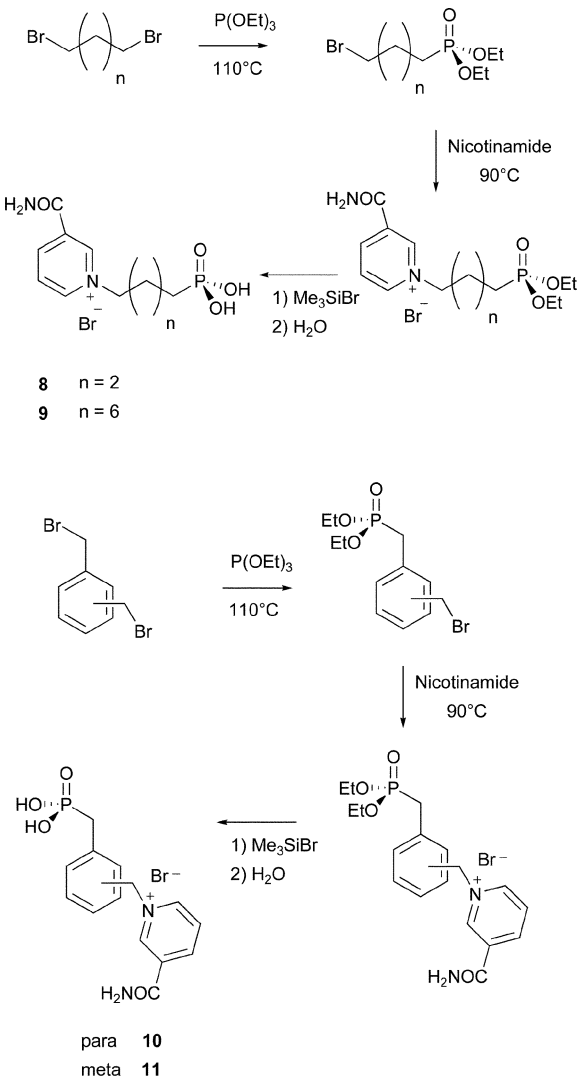
protecting groups by a standard and specific procedure (Me_3SiBr and then H_2O) led to the expected phosphonic acids **6**† and **7**‡. Both 3′ and 5′ benzoyl protecting groups were kept for all inactivation studies in order to prevent an acyl migration of the phosphonic chain towards the 3′ position and to replace the pyrophosphate at the 5′ position as in the entire NAD^+.

Second generation inhibitors were synthesized in which the ribose ring was replaced by either an aliphatic spacer or a more conformationally constrained aromatic ring. These new inhibitors (Scheme 2) were synthesized from the dibromoalkanes and dibromomethylbenzenes by an Arbuzov condensation followed by addition of nicotinamide on the remaining halide. In a last step, the deprotection of the diethoxy phosphoric esters by a standard procedure (Me_3SiBr and then H_2O) led to the expected phosphonic acids. In these simplified "phosphononicotinamides", the phosphonyl and the nicotinamide moities are joined either by flexible and aliphatic spacers (compounds **8** and **9**) or by constrained and hydrophobic aromatic linkers (compounds **10** and **11**).

The inhibitory activities of all "bi-substrate" analogues were tested against the GAPDHs from *Trypanosoma cruzi* and rabbit muscle as previously reported by us by spectrophotometrically monitoring the reduction of NAD^+ into NADH at 340 nm, using the substrate GAP and cofactor (NAD^+) at saturating concentrations of 0.8 and 2 mM, respectively. The inhibitory activities were measured after preincubation of the enzyme with the ligand for 5 min followed by addition of the substrates and cofactor. The reaction was then monitored by following the absorbance change of NADH at 340 nm using a SAFAS spectrophotometer equipped with a kinetic accessory unit. Initial reaction rates were calculated from the slopes of the curves recorded during the first 3 min of the reaction. The concentration of inhibitor required for 50% inhibition (IC_{50}) was calculated from the percentage of remaining enzyme activity by comparison with an inhibitor free control experiment and based on measurements at five different concentrations of inhibitor.[8]

The inhibition pattern and inhibition constants (K_i) were subsequently determined from Lineweaver–Burk plots. The inhibitions with respect to GAP and then NAD^+ were studied using at least five different concentrations of substrate/cofactor and at least three concentrations of ligand. The inhibition results are summarized in Table 1. Initial experiment carried out using these molecules in replacement of the natural cofactor NAD^+ showed no enzyme activity, even at very high concentration of ligand. This confirmed that these molecules are not recognised as an enzyme pseudo-substrate and do not interfere with the enzyme activity measurements.

The non-phosphorylated intermediate **3** was also tested as a potential inhibitor of GAPDH but appeared to have no effect on the enzyme activity at a 3 mM concentration. However,



Scheme 2

molecules based on a similar scaffold but functionalized at position 2′ of the sugar with a phosphonate group (compounds **6** and **7**) were shown to inhibit *Trypanosoma cruzi* GAPDH with an IC_{50} value of 5 and 75 μM, respectively. Even more interesting is the fact that these molecules appeared to be specific for the parasite's enzyme and exhibit a very moderate inhibitory effect of the mammal's enzyme (IC_{50} values *ca.* mM). Detailed inactivation kinetics studies were then carried out which showed that these inhibitors are competitive with respect to the GAP,

Table 1 Inhibitory effect of "bi-substrate" analogues with respect to either GAP or NAD^+ against GAPDHs from *Trypanosoma cruzi* (Tc) and rabbit muscle (Rm)

Compound		IC_{50}/μM	Inhibition of GAP/μM	Inhibition of NAD^+/μM
3	Rm	—[a]	—[a]	—[a]
	Tc	—[a]	—[a]	—[a]
6	Rm	1300	$K_i = 200 \pm 10$ (competitive)	$K_i = 900 \pm 50$ (mixed)
	Tc	5	$K_i = 4 \pm 1$ (competitive)	$K_i = 10 \pm 1$ (non competitive)
7	Rm	650	$K_i = 180 \pm 15$ (competitive)	$K_i = 40 \pm 2$ (mixed)
	Tc	75	$K_i = 6 \pm 1$ (competitive)	$K_i = 33 \pm 2$ (mixed)
8	Rm	1200	ND[b]	ND[b]
	Tc	1000	ND[b]	ND[b]
9	Rm	—[a]	—[a]	—[a]
	Tc	1100	$K_i = 1050 \pm 50$ (competitive)	$K_i = 950 \pm 40$ (non competitive)
10	Rm	850	ND[b]	ND[b]
	Tc	750	ND[b]	ND[b]
11	Rm	1800	ND[b]	ND[b]
	Tc	2000	ND[b]	ND[b]

[a] No inhibition observed at a ligand concentration of 3 mM. [b] Not determined.

thus suggesting an interaction of the phosphonate group at the binding site of the C3 phosphate of GAP. However, these compounds also show a mixed-type or non-competitive inhibition with respect to the cofactor, suggesting that they do not fully occupy the NAD^+ binding site or do not completely prevent its binding. This is particularly remarkable for compound **6**, the most potent inhibitor of this series, which was non-competitive with regard to NAD^+ for *T. cruzi* GAPDH, indicating that the ligand binds to the enzyme–cofactor binary complex and to the free enzyme with the same affinity. This could be a consequence of the favourable interaction of one or both benzoyl protecting groups in a hydrophobic pocket within the active site preventing the inhibitor ribose nicotinamide from binding to the cofactor natural binding site. These results demonstrate that, although the phosphonate groups of compounds **6** and **7** probably have the same binding sites in *T. cruzi* and Rm GAPDH (*i.e.* the so-called Ps site), the ribose–nicotinamide moiety interacts specifically with *T. cruzi* GAPDH, thus leading to a highly specific (50-fold) inhibition of the parasite's enzyme. Adenosine analogues were previously reported to selectively inhibit the GAPDH of *Trypanosomatidae* by targeting a hydrophobic cleft around the adenosyl binding site, far from the catalytic cysteine.[4,9] Here, we demonstrate that it is possible to inhibit the enzyme from parasite with a comparable specificity by targeting the enzyme catalytic site. Co-crystallisation experiments of these inhibitors with *Trypanosoma cruzi* GAPDH are in progress to elucidate their mode of binding.

In an attempt to simplify the general scaffold of these inhibitors and investigate the influence of the ribose ring on both affinity and selectivity of inhibition, second generation ligands were synthesized in which the ribose had been removed and substituted by either an aliphatic or aromatic spacer. The inhibition results obtained with compounds **8**–**11** are summarized in Table 1. As for compounds **6** and **7**, these new ligands appeared to be very poor inhibitors of Rm GAPDH (IC_{50} values higher than 0.8 mM). Unfortunately, equally poor inhibition effects were observed with these molecules when tested against *T. cruzi* GAPDH. These results demonstrate the importance of the ribose motif for the recognition of these inhibitors by *T. cruzi* GAPDH.

In summary, we have reported the synthesis of an original family of GAPDH inhibitors that inhibit the enzyme of *Trypanosoma cruzi* with good affinity (K_i value up to 4 μM) but also very high specificity (up to 50-fold). Detailed inactivation studies suggested that (i) the phosphonate group interacted with the GAP C3-phosphate binding site, and (ii) the ribose moiety is necessary but not sufficient for inducing a specific inhibition of *T. cruzi* GAPDH. Compound **6** represents a good lead for the design of new potential trypanocide drugs.

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Notes and references

† 3-Carbamoyl-1-(2-(2-oxoethanephosphonate)-3,5-di-*O*-benzoyl-D-ribofuranosyl) pyridinium chloride (**6**). δ_H (CD_3OD) 9.61 (s, 1H, H_2), 9.36 (d, 1H, H_4), 9.02 (d, 1H, H_6), 8.24 (dd, 1H, H_5), 7.25–8.13 (m, 10H, Ph), 6.82 (d, 1H, $H_{1'}$), 5.90 (m, 2H, $H_{5'}$), 5.15 (m, 1H, $H_{4'}$), 4.91 (m, 2H, $H_{2'}$ and $H_{3'}$), 2.96 (ddd, 2H, CH_2–P); δ_C (CD_3OD) 168.2 (d, J_{C-P} = 6.8 Hz), 167.5, 166.8, 164.8, 147.7, 144.1, 142.1, 136.1, 135.2, 134.9, 129.9–131.1, 98.9, 85.3, 78.0, 72.5, 64.8, 37.0 (d, J_{C-P} = 123.0 Hz); δ_P (CD_3OD) 14.8; MS (ES) *m/z* 585 ([M^+]). Purity was > 99% as assessed by HPLC (CH_3CN–H_2O).

‡ 3-Carbamoyl-1-(4-(4-2-enedihydroxyphosphonobutane)-3,5-di-*O*-benzoyl-D-ribofuranosyl) pyridinium chloride (**7**). δ_H (CD_3OD) 9.67 (s, 1H, H_2), 9.40 (d, 1H, H_4), 9.05 (d, 1H, H_6), 8.28 (dd, 1H, H_5), 7.38–8.09 (m, 10H, Ph), 7.12 (m, 1H), 6.85 (d, 1H, $H_{1'}$), 6.12 (dd, 1H), 5.91 (m, 2H, $H_{5'}$), 5.13 (m, 1H, $H_{4'}$), 4.55 (m, 2H, $H_{2'}$ and $H_{3'}$), 2.76 (dd, 2H, CH_2–P); δ_C (CD_3OD) 167.6 (d, J_{C-P} = 6.7 Hz), 166.8, 166.2, 164.9, 147.0, 143.6, 142.4, 136.0, 135.2, 134.9, 127.5–131.8, 123.8 (d, J_{C-P} = 13.1 Hz), 99.4, 84.5, 76.5, 71.7, 64.5, 33.5 (d, J_{C-P} = 132.4 Hz); δ_P (CD_3OD) 20.4; MS (ES) *m/z* 611 ([M^+]). Purity was > 99% as assessed by HPLC (CH_3CN–H_2O).

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