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A Disubstituted NAD$^+$ Analogue is a Nanomolar Inhibitor of Trypanosomal Glyceraldehyde-3-Phosphate Dehydrogenase

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Abstract—N^6-naphthalenemethyl-2′-methoxybenzamido-β-NAD$^+$, a derivative of a low micromolar first-generation inhibitor of trypanosomal glyceraldehyde phosphate dehydrogenase (GAPDH), was synthesized, taking advantage of methodology for the selective phosphitylation of nucleosides. The compound was found to be a poor alternate cosubstrate for GAPDH, but an extremely potent inhibitor. Although intended for use in crystallization trials, the analogue presents possibilities for further drug-design.

Introduction

Parasitic infections are a serious global health problem, affecting millions of people annually. Specifically, trypanosomiasis, those diseases caused by the haemoflagellates of the genus *Trypanosoma*, and leishmaniasis, the disease caused by protozoa of the related genus *Leishmania*, are of major concern. However, despite the public threat posed by these diseases, currently available treatments are inadequate and are limited by invasive delivery routes, toxicity and ineffectiveness.[1]

One way of finding new treatments for these diseases is by identifying biochemical processes which are unique to parasite physiology. One such feature which offers an opportunity for exploitation is the reliance of trypanosomes on glycolysis for energy production. Specifically, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate with concomitant reduction of NAD$^+$, offers a target which may be exploited to obtain inhibitors of trypanosomal and leishmanial glycolysis and growth.[2] Guided by this hypothesis, earlier efforts in this laboratory resulted in the N^6-, 2′-disubstituted adenosine analogue **1**, which is a selective, low micromolar inhibitor of trypanosomatid GAPDH, and which effectively blocks in vitro growth of the bloodstream form of *T. brucei* ($ED_{50} = 30\,\mu M$) and *T. cruzi* amastigotes ($ED_{50} = 20\,\mu M$).[3] This compound was obtained by rational design, exploiting structural differences between the human and parasite enzymes around the adenosyl moiety of the bound NAD$^+$ cosubstrate. These differences are reflected in a 10-fold lower affinity for NAD$^+$ measured in trypanosomal GAPDH ($K_m = 0.45\,\mu M$) as compared to the human enzyme ($K_m = 0.04\,\mu M$)[4] (Fig. 1).

Compound **1** does not inhibit human GAPDH at its solubility limit of 50 μM, and this selectivity is believed to arise from binding of the 2′-methoxybenzamido group into a narrow hydrophobic cleft which is found in trypanosomal GAPDH but occluded in the human enzyme. In order to initiate another round of structure-based drug design aimed at improving the potency of this first-generation inhibitor, it is necessary to obtain a crystal structure of **1** in complex with GAPDH. However, attempts to crystallize with inhibitor **1** have thus

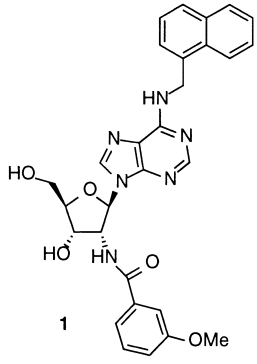

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Figure 1.

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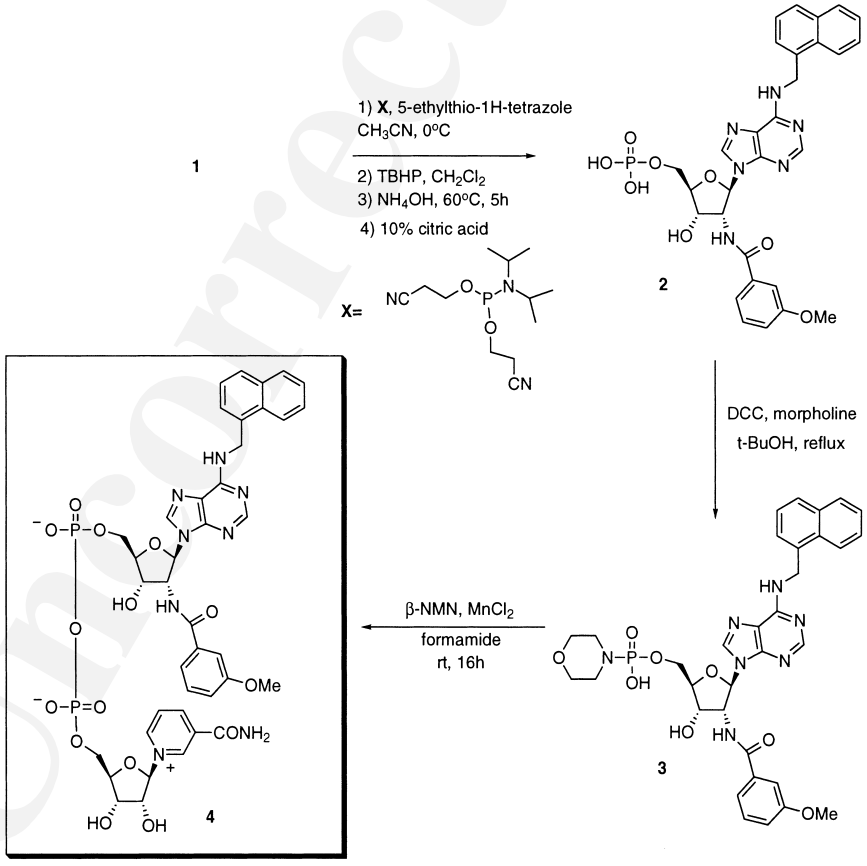
far proved unsuccessful, probably because it does not induce the same conformational change as does the binding of NAD^+ to GAPDH (GAPDH without bound NAD^+ also fails to crystallize). A structure of N^6-benzyl-NAD^+ bound to GAPDH has been solved, a further indication that the full dinucleotide structure is necessary for crystallization.[3] We therefore reasoned that coupling inhibitor **1** to β-nicotinamide mononucleotide (β-NMN), and thereby more closely mimicking the normal NAD^+ cosubstrate, would increase the chance of obtaining co-crystals. The synthesis of this analogue, which extends methodology for selective phosphitylation to a novel nucleoside substrate, is described. Also, inhibition data is presented which show the compound to be an extremely potent and selective GAPDH inhibitor.

Chemistry

The initial step of the synthesis involves the selective phosphorylation of the 5′-hydroxyl of **1**. This is commonly accomplished by employing a protection-deprotection strategy, first using a bulky protecting group (typically dimethoxytrityl) to block the sterically less hindered primary hydroxyl, followed by protection of the secondary groups; the 5′-hydroxyl can then be selectively deprotected and phosphorylated. Recently however, a useful method for the direct phosphitylation of the primary hydroxyl group in these unprotected nucleosides has been described.[5] Although these authors applied this chemistry to the anomers of only one mono-substituted riboside, we expected that the disubstituted nucleoside **1** would serve equally well as a substrate. Therefore, following this methodology, selective phosphitylation of the 5′-hydroxyl of **1** was accomplished using the phosphoramidite, bis-(2-cyanoethoxy)(diisopropylamino)phosphine,[6] activated with a substituted tetrazole. Following oxidation, the resulting phosphotriester, as well as unreacted starting material, were readily purified by silica gel chromatography. Deprotection of the product gave 5′-phosphorylated compound **2** (57% based on limiting phosphoramidite, 80% based on total riboside recovered), which was isolated as the free acid by precipitation with 10% citric acid. Coupling of the 5′-phosphate to β-NMN was accomplished by first forming the activated phosphoromorpholidate **3** (62%),[7] and then subsequently reacting with β-NMN in formamide with added Lewis acid catalyst.[8] The final product **4** was isolated by reverse-phase HPLC (19% isolated yield, 10% overall from **1**)[9] (Scheme 1).

Results

Compound **4** is an analogue of the NAD^+ cosubstrate normally used during catalysis by GAPDH and so could potentially serve as an alternate cosubstrate of the enzyme. However, when substituted for NAD^+ at the K_m concentration (0.45 mM), **4** was used by *L. mexicana* GAPDH at 5% of the rate of NAD^+ utilization. This is in contrast to N^6-benzyl-NAD^+ which was found to be a good alternate substrate for *L. mexicana* GAPDH,



Scheme 1. Synthesis of disubstituted NAD^+ analogue **4**.

with a K_m of 0.25 mM.[3] Either the additional 2′ substitution on the adenosine of **4** induces a binding geometry such that the nicotinamide moiety is no longer optimally aligned for hydride transfer, or the rate of product (NADH) release is slowed by the additional binding energy from the added adenosyl substituents. The analogue does fit nicely in the NAD^+ binding pocket and shows high affinity for the enzyme, a fact confirmed by its potency as an inhibitor. These results, in comparison to the inhibition data for the first-generation antagonist **1**, are shown in Table 1.

Results

Compound **4** was found to be a nanomolar inhibitor of *L. mexicana* GAPDH, 100 times more potent than the first generation antagonist **1**. For purposes of structure-based drug design, this is the enzyme of choice as it is easiest to express in *E. coli* and provides the best diffracting crystals. However, the sequence homology between all three trypanosomatid enzymes is high, and the NAD^+ binding pocket is virtually identical for each, with the exception of the substitution of a serine (Ser-40) in *L. mexicana* GAPDH by asparagine (Asn-40) in *T. brucei*/*T. cruzi* GAPDH. This difference occurs in the hydrophobic selectivity cleft in which the 2′-methoxybenzamido substituent is thought to bind, and this may account for the slight difference in affinity of **1** for the three parasite enzymes. Compound **4** is also a potent inhibitor of *T. brucei* and *T. cruzi* GAPDH but, consistent with the presence of the amino acid substitution in the selectivity cleft, slightly less potent than against the *L. mexicana* enzyme. However, the relative difference in potency between the three enzymes is not as pronounced in **4** as compared to **1**, presumably because the sizeable increase in affinity provided by the addition of NMN attenuates the contribution to binding from the selectivity cleft interaction. Finally, in accordance with the selectivity of 2′-substituted adenosines for trypanosomal GAPDH, **2** and **4** did not inhibit mammalian GAPDH at 100 and 500 μM, respectively.

In order to gain insight into the binding interactions responsible for the high affinity of **4**, the two joined fragments of the analogue were also tested for GAPDH inhibition. Compound **2**, the 5′-phosphate of **1**, was found to be roughly threefold more potent than the parent compound against each of the three enzymes. The small increase in potency indicates that there are not significant electrostatic interactions gained from addition of the negatively charged phosphate. This was expected, as the pyrophosphate of bound NAD^+ is surface exposed and thus the charged linker is largely solvated. This is in contrast with other nucleotide-binding enzymes which make important electrostatic contacts with the nucleotide 5′-phosphate group, and thus bind corresponding nucleosides with much lower affinity.[10]

As the phosphate substituent does not contribute much to the increased affinity of **4**, it was thought that the nicotinamide mononucleotide half of the compound might have appreciable affinity for the enzyme. However, β-NMN did not inhibit *L. mexicana* GAPDH when tested as high as 500 μM. The additional 30-fold increase in potency gained when this moiety is coupled to **2** must thus be due to entropic contributions. That is, although the NMN moiety itself has low affinity for its binding pocket, the binding of the N^6-napthalenemethyl and 2′-methoxybenzamido substituents in the adenosine pocket serves to lock the inhibitor in place, and allows the NMN group to bind with little additional loss of entropy. Presumably it would be possible to take further advantage of the entropic contribution by coupling the two nucleotide moieties with a more rigid linker.

The high molecular weight and charged nature of **4** would seem to preclude its use as an effective therapeutic agent against live parasites. As expected, when tested against the bloodstream form of *T. brucei*, **4** and the phosphorylated analogue **2** both showed lower activity than the lead inhibitor (Table 1). However, against *T. cruzi* amastigotes both compounds were approximately 3-fold more potent than **1**. Although only a modest increase, this result is an indication that despite their charged nature, these analogues are able to penetrate parasitic cells. Thus, in addition to being a tool for use in crystallization trials, compound **4** provides insight towards further drug design based upon lead inhibitor **1**. Even in the absence of a crystal structure, the potential for substitution at the 5′-hydroxyl of the lead inhibitor is readily apparent. A small set of 5′-substituted analogues of **1** have previously been synthesized with no resultant increase in affinity, but these compounds do not fill the NMN cavity.[11] A substituent which could fill this pocket connected with a linker of suitable length could result in an analogue with equal or greater potency than

Table 1. Effect of analogues on trypanosomatid and mammalian GAPDH (IC_{50}) and growth of cultured parasites (ED_{50})[d]

Compounds	*L. mexicana* IC_{50}	*T. brucei* IC_{50}[b]	ED_{50}[c]	*T. cruzi* IC_{50}	ED_{50}	Rabbit muscle IC_{50}	3T3 Fibroblasts[a] ED_{50}
1	6	25	30	12	20	>50	>50
2	2	7	32	5	6	>100	90
4	0.06	0.10	63	0.10	8	>500	>150
β-NMN	>500	—	—	—	—	>500	–
Pentamidine	—	—	0.008	—	—	—	–
Benznidazole	—	—	—	—	0.6	—	>50

[a]Noninfected host cells used to support growth of *T. cruzi* amastigotes.
[b]IC_{50} values determined by measuring inhibition at 3-5 inhibitor concentrations (10% error).
[c]ED_{50} values determined in triplicate (15% error limit) for bloodstream *T. brucei* and *T. cruzi* amastigotes according to previous procedure..[3]
[d]All values are in μM; data for **1** is from reference 3; pentamidine and benznidazole values provided for reference.

4. As noted, the phosphate moiety does not contribute significantly to binding affinity, so variation of the pyrophosphate linker, which is susceptible to cleavage by non-specific phospho-hydrolyases, would be possible. Other groups have examined using more stable phosphonate[12] or methylenebisphosphonate linkers[13] to make NAD^+ analogues, but in this case a simple ether-type linkage without phosphates could also be feasible. Our expectation is that attempts to design analogues which exploit these additional binding regions will result in even more potent inhibitors of glycolysis and potential treatments for the devastating diseases of trypanosomiasis and leishmaniasis.

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9. The detailed synthesis of compound **4** is as follows: 40 mg (0.074 mmol) of **1** and 5-ethylthio-tetrazole (0.17 mmol) were dissolved in 3 mL dry CH_3CN and stirred at −10 °C under argon atmosphere. Bis-(2-cyanoethoxy)-(diisopropylamino)-phosphine (0.45 M solution in dry CH_3CN, 0.06 mmol 133 μL) was added slowly. After complete addition the solution was stirred at −10 °C for 30 min and then *tert*-butyl hydroperoxide (0.67 mmol) was added, the solution was warmed to room temp. and stirred for an additional 20 min. Solvent was evaporated, the residue was diluted with $CHCl_3$ and the product was purified by silica gel chromatography eluting with 9:1 $CHCl_3$:EtOH. The purified phosphate triester was deprotected by stirring at room temperature in methanolic ammonium hydroxide for 17 h. Solvent was removed to a small volume and **2** was precipitated as its free acid by the addition of 10% citric acid solution. The 5′-phosphate (26 mg, 0.04 mmol) was converted to the phosphoromorpholidate by dissolving with morpholine (0.17 mmol, 15 μL) in 0.4 mL *t*-BuOH and 0.4 mL H_2O and heating at reflux. DCC (0.17 mmol, 35 mg) in 0.6 mL *t*-BuOH was added slowly over 4 h, and after complete addition reflux continued for 16 h. The cooled solution was filtered, evaporated to dryness and washed with ether. The crude material was purified by silica gel chromatography, eluting with 7:3 CH_2Cl_2:MeOH ($R_f = 0.4$) to give **3**. Pyrophosphate coupling was accomplished by dissolving **3** (16 mg, 0.023 mmol) with β-NMN (0.025 mmol, 8.3 mg) in 275 μL formamide containing $MnCl_2$ (0.15 mmol) and $MgSO_4$ (0.05 mmol), and stirring at room temperature for 24 h. The crude product was precipitated by the addition of acetone, and the final product **4** was purified as the triethylammonium salt by reverse-phase HPLC using a CH_3CN:H_2O (with 0.01 M triethyl- ammonium acetate) gradient. ^1H NMR (CD_3CN/D_2O, 300 MHz), δ 1.18 (t, 9H, $J = 7.3$ Hz), 3.05 (q, 6H, $J = 7.3$ Hz), 3.67 (s, 3H), 4.05–4.60 (m, 9H), 5.00–5.15 (m, 3H), 5.98 (d, 1H, $J = 5.2$ Hz), 6.23 (d, 1H, $J = 8.3$ Hz), 7.00–8.20 (m, 13H), 8.45 (s, 1H), 8.79 (d, 1H, $J = 5.2$ Hz), 9.10 (d, 1H, $J = 9.4$ Hz), 9.38 (s, 1H); ^{31}P NMR (CD_3CN/D_2O, 1 M H_3PO_4 external standard) δ −10.97; ESI-MS (H_2O) 935.24 $[M\text{-}H^+]^-$.
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