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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 1512–1515

Probing binding requirements of NAD kinase with modified substrate (NAD) analogues

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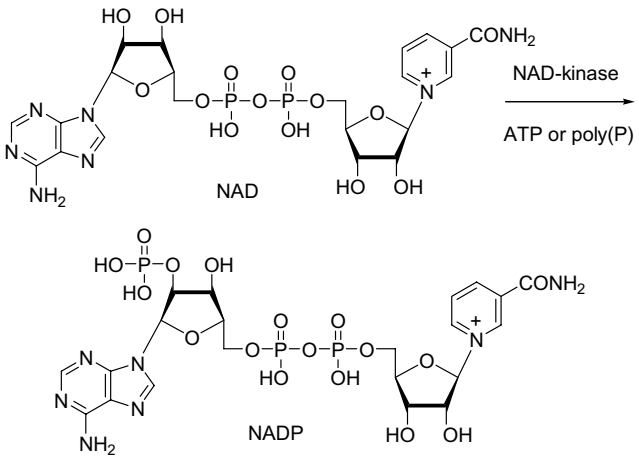
Received 16 November 2006; revised 2 January 2007; accepted 3 January 2007
Available online 17 January 2007

Abstract—Synthesis of novel NAD^+ analogues that cannot be phosphorylated by NAD kinase is reported. In these analogues the C2′ hydroxyl group of the adenosine moiety was replaced by fluorine in the *ribo* or *arabino* configuration (**1** and **2**, respectively) or was inverted into *arabino* configuration to give compound **3**. Compounds **1** and **2** showed inhibition of human NAD kinase, whereas analogue **3** inhibited both the human and *Mycobacterium tuberculosis* NAD kinase. An uncharged benzamide adenine dinucleotide (BAD) was found to be the most potent competitive inhibitor (K_i = 90 μM) of the human enzyme reported so far.

NAD kinase catalyzes a magnesium-dependent phosphorylation of the 2′-hydroxyl group of the adenosine ribose moiety of nicotinamide adenine dinucleotide (NAD) using ATP or inorganic polyphosphates as phosphoryl donors to give NADP (Scheme 1).[1] There are two classes of the enzyme, one which is specific for NAD^+ and one which also phosphorylates NADH. The *Mycobacterium tuberculosis* enzyme falls into the latter category. No other pathway of NADP biosynthesis has been found in prokaryotic or eukaryotic cells. Although NAD kinase was discovered as early as in 1937, little was known about the structure, function, and mode of action of this enzyme until very recently.[2] NAD kinase plays a crucial role in controlling the cellular redox state by regulating the ratio of reduced coenzymes NADH/NADPH.[2,3] More recently, it has become clear that NADP-mediated signal transduction affects numerous metabolic pathways and there is a growing body of evidence that NADP serves as an important component in the control of essential cellular processes. For example, NADP is converted into nicotinic acid adenine dinucleotide phosphate (NAADP) by the exchange of nicotinamide with nicotinic acid catalyzed by CD38 and ADP-ribosyl cyclase.[4–6] NAADP is the most potent Ca^{2+} mobilizing agent in mammalian cells. Interestingly, human NAD-kinase is highly selective and does not phosphorylate NAAD into NAADP.[7]



Scheme 1. NAD phosphorylation by NAD-kinase.

In contrast to NAD, which serves as a substrate for a number of degradation enzymes such as ADP-ribosyl transferases,[8] poly-ADP-ribose polymerases,[9]

Keywords: NAD kinase; *M. tuberculosis*; Benzamide adenine dinucleotide (BAD); NAD analogues.
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doi:10.1016/j.bmcl.2007.01.012

ADP-ribosyl cyclase,[10] and NAD-dependent histone deacetylases,[11] NADP is not known to be significantly degraded. The affinities of ATP (K_m = 3.3 mM) and NAD (K_m = 0.54 mM) for the human enzyme *are low* and close to the physiological concentration of these nucleotides.[7]

NAD-kinase is expressed in both prokaryotic and eukaryotic cells. NADP is essential for the growth of several microorganisms, including multi-drug resistant *M. tuberculosis*, *Bacillus subtillis*, *Escherichia coli*, and others.[12–14] Bacterial NAD kinases showed substantial differences from the human and mammalian enzymes, and therefore they have been suggested as attractive targets for the development of novel antibiotics.[12,15]

The first crystal structure of NAD kinase was published in 2004[12] describing the apo-enzyme from *M. tuberculosis* followed by the structure of the same kinase in complex with NAD.[16] An X-ray structure of NAD kinase from *Archeoglobus fulgidus* in complex with ATP, NAD, or NADP[17] revealed more details of these enzymes' architecture. Studies of site-directed mutagenesis of the *M. tuberculosis* enzyme[15] and evidence of its allosteric inhibition provided a wealth of potentially useful information for drug design.

In spite of this no inhibitors of the human or bacterial enzyme have ever been reported and still little is known about the mechanism of action of NAD kinase.[17] No crystal structure of the human NAD kinase has been reported. Clearly further studies are needed to understand the interactions of NAD kinase with ATP and NAD as substrates, and other ligands as potential inhibitors. In this paper, we present our approach to drug design through examination of interactions of modified NAD analogues with NAD kinases to complement information available from site-directed mutagenesis.[15,18]

We report herein the synthesis[19] and NAD kinase inhibitory activity[20] of novel analogues of NAD (**1**–**3**). These analogues were prepared by replacement of the 2′-hydroxyl group of the adenine ribose of NAD by fluorine in the 'down' *ribo* or 'up' *arabino* configuration or by inversion of the 2′-OH into the *arabino* configuration (Figs. 1 and 2).

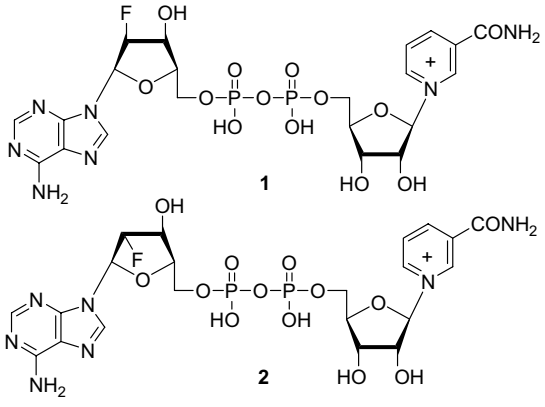


Figure 1. 2′-fluoro *ribo*- and 2′-fluoro *arabino* NAD.

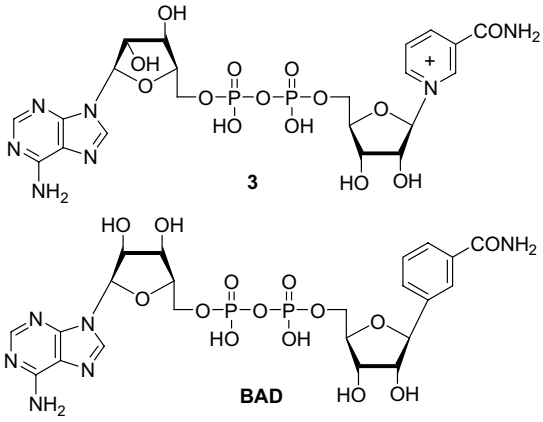


Figure 2. NAD with an inverted (*arabino*) configuration and benzamide adnine dinucleoside (BAD).

In addition, the activity of a known uncharged NAD analogue, benzamide adenine dinucleotide (BAD or 1- deaza-NAD, Fig. 2)[21] was also examined.

The 2′-fluoro analogue **1** is structurally very similar to NAD but cannot be phosphorylated by NAD kinase to the corresponding NADP analogue. A fluorine atom is a good mimic of a hydroxyl group in terms of size and polarity, and it preserves the same C3′-endo conformation of the adenosine ribose as that of NAD.[22] We synthesized analogue **1** by coupling of the commercially available nicotinamide mononucleotide (NMN) with 5′-monophosphate imidazolide of 2′-deoxy-2′-fluoroadenosine[23] and found that at 1 mM concentration it inhibited 43% of activity of the human but not the bacterial enzyme (Table 1).

Next, we investigated the importance of the *ribo* configuration of the analogue **1** for the enzymatic activity of NAD kinase. Using (2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl) adenine[22] and similar coupling with NMN we prepared NAD analogue **2** in which the 2′-fluoro atom is in the *arabino* configuration. The adenine sugar of this compound is in the C2′-endo conformation, which is preferred in 2′-deoxynucleosides rather than in ribonucleosides. In spite of this different conformation, the NAD analogue **2** showed a similar activity to that of **1**. It inhibited the human enzyme (37% inhibition at 1 mM) and was inactive against *M. tuberculosis* NAD kinase.

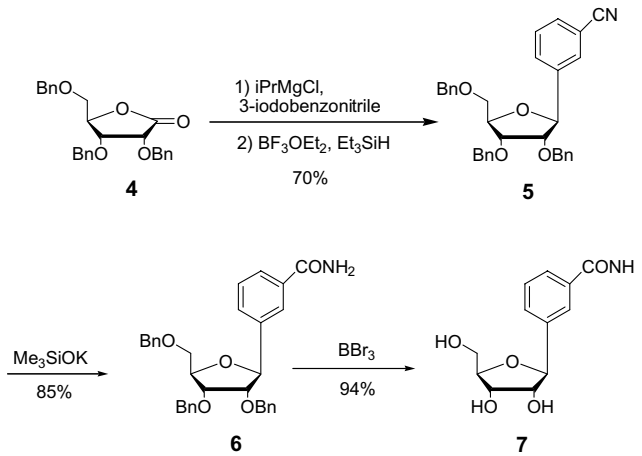
Table 1. Effects of NAD analogues **1**–**3**, BAD, and bromoacetyl pyridines **11**–**13** on inhibition of human and *M. tuberculosis* NAD kinase

NAD analogue concn 1 mM	Inhibition (%)		K_i (μM) human enzyme
	Human enzyme	*M. tuberculosis*	
1	43	<10	ND
2	37	<10	ND
3	40	40	ND
BAD	90	<10	90
BAD (at 2 mM)	90	37	90
11	60	63	ND
12	44	30	ND
13	64	40	ND

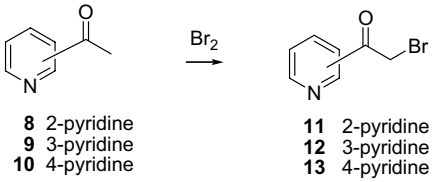
Finally, we studied whether or not NAD analogues with an inverted configuration of the 2′-OH group or lacking the positive charge of the nicotinamide ring would serve as substrates.[24] To answer these questions, we synthesized NAD analogue **3** and prepared uncharged benzamide adenine dinucleotide (BAD or 1-deaza-NAD, Fig. 2). Interestingly, NAD analogue **3** inhibited both the human and *M. tuberculosis* NAD kinase (40% inhibition at 1 mM for both enzymes). Since the 2′-OH group can act as a donor or as an acceptor in hydrogen bonding, whereas fluorine has only acceptor ability, this result may indicate that the 2′-substituent should act as a hydrogen donor in order to inhibit the bacterial enzyme. Such a requirement is consistent with the X-ray structure of the *M. tuberculosis* enzyme, in which the 2′-OH hydrogen bonds to Asp85; however, this residue is also conserved in the human enzyme. In general, the inhibitory activity of NAD analogues **1**–**3** was low; however, these compounds may compete with NAD^+ whose affinity to the enzyme is also in the low millimolar range.[7]

In contrast to the charged NAD^+ analogues **1**–**3**, the uncharged BAD was found to be a more potent inhibitor of human NAD kinase (90% inhibition at 1 mM) than compounds **1**–**3**. It showed *competitive* inhibition of the human enzyme with K_i = 90 μM. In higher concentration (2 mM) it also inhibited the bacterial enzyme (36%). The TB enzyme does have NADH kinase activity.[18]

BAD is an active metabolite of benzamide riboside (**7**, BR, Scheme 2), the C-nucleoside which showed similar anticancer activity to that of tiazofurin, an orphan drug used clinically for treatment of patients with chronic myelogenous leukemia.[25] The laborious and inefficient (10 step) synthesis of BR[26] hampered research on biological activities of this compound in the last decade. Recently, we developed a new, simple, 4-step procedure for stereoselective synthesis of BR. Thus, Grignard reaction of commercially available 3-iodobenzonitrile with ribonolactone (**4**, Scheme 2) followed by 'one pot' removal of the anomeric hydroxyl group, conversion



Scheme 2. A simple, stereoselective synthesis of benzamide riboside (BR).



Scheme 3. Bromoacetyl pyridines, irreversible NAD kinase inhibitors.

of the cyano group of nucleoside **5** into the carboxyamido function of **6**, and debenzylation afforded BR in 56% overall yield. Using this new methodology we prepared gram amounts of BR and converted it into BAD.[21]

As a positive control we synthesized three bromoacetyl pyridines (**11**–**13**, Scheme 3) since one of them, 3-bromoacetyl pyridine **12**, was reported as a potent irreversible inhibitor of NAD kinase from pigeon liver.[27] Apparently compound **12** alkylates a histidine residue present in the active site of NAD kinase. It is a non-specific inhibitor which was reported to alkylate the active site of other NAD-dependent enzymes.[29,30] These compounds were prepared in high yield (95–98%) from the corresponding acetyl pyridines **8**, **9**, **10**, by direct bromination as previously reported.[28] All three compounds showed inhibitory activity against both the human and the bacterial enzyme (Table 1).

In conclusion, we found that the replacement of the 2′-OH group of the adenosine moiety of NAD^+ by fluorine (analogues **1** and **2**) afforded only weak inhibitors of human NAD kinase, which were inactive against the *M. tuberculosis* NAD kinase. In contrast, inversion of the configuration of the OH (such as in analogue **3**) led to the inhibition of both the human and the bacterial enzyme. BAD was found to be a competitive inhibitor of the human enzyme with an affinity 6-fold higher than that of NAD^+ (K_i = 90 μM vs K_m = 540 μM, respectively). Apparently elimination of the positive charge of the NAD^+ increased the inhibitory activity. In higher concentration BAD also inhibited the bacterial enzyme. Our studies indicate that the analogue of BAD with the inverted (*arabino*) configuration of the 2′-OH of adenosine moiety is likely to show increased inhibition of *M. tuberculosis* NAD kinase. The preparation of this and other NAD analogues is now in progress.

Acknowledgment

These studies were funded by the Center for Drug Design, University of Minnesota.

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19. NAD analogues **1**–**3** were prepared by coupling of the 5′-phosphoimidazolides of the corresponding adenine sugars with nicotinamide mononucleotide (NMN) and characterized by ^{1}H, ^{31}P NMR and MS. The synthesis of P^{1}-(nicotinamide-ribos-5′-yl)-P^{2}(adenine-arabinofuranos-5′-yl) pyrophosphate (**3**) serves as an example: NMN (82 mg, 0.24 mmol) was added to a 0.2 M $MnCl_2$/formamide solution (1.5 mL, 0.30 mmol), which was dried with molecular sieves for several days before use. To this mixture were added adenine-arabinofuranosyl 5′-phosphoimidazolide (101 mg, 0.20 mmol) and anhydrous $MgSO_4$ (50 mg, 0.42 mmol). The reaction mixture was stirred for 24 h at rt and the crude product was precipitated by adding CH_3CN (6 mL). After washing with CH_3CN, the solid was dissolved in 1 M TEAB solution (10 mL), filtered, and the filtrate was used directly for purification by RP-HPLC. The resulting Et_3N salt was applied on a column of Dowex50WX8-200 (Na^+ form) at 4 °C and eluted with water to give the desired sodium salt. From about one-fourth of the crude product compound **3** (11 mg, sodium salt) was obtained as a white solid (yield 32%). ^{1}H NMR (D_2O, 600 MHz) δ 9.35 (s, 1H), 9.21 (d, J = 6.0 Hz, 1H), 8.87 (d, J = 7.8 Hz, 1H), 8.38 (s, 1H), 8.27-8.22 (m, 1H), 8.19 (s, 1H), 6.32 (d, J = 6.6 Hz, 1H), 6.10 (d, J = 5.4 Hz, 1H), 4.60 (pseudo t, J = 6.6 Hz, 1H), 4.58–4.55 (m, 1H), 4.51 (pseudo t, J = 5.4 Hz, 1H), 4.48–4.44 (m, 2H), 4.40 (ddd, J = 11.8, 4.0, 2.6 Hz, 1H), 4.36 (ddd, J = 11.7, 5.1, 2.7 Hz, 1H), 4.31–4.25 (m, 2H), 4.19–4.15 (m, 1H). ^{31}P NMR (D_2O, 243 MHz) δ −10.13 (d, J = 20.6 Hz), −10.56 (d, J = 21.1 Hz). MS ESI calcd $(M-H)^-$ 662.1018, found 662.0819.
20. NAD kinase[14,15] activity was determined by an HPLC assay. The assay mixture (150 μL) consisted of 50 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 5 mM ATP, 1 mM NAD, and an appropriate amount of inhibitor and enzyme. After incubation for 30 min at 37 °C, an aliquot (70 μL) was withdrawn and added to ice-cold 1.2 M $HClO_4$ (35 μL) and kept for 10 min. The mixture was centrifuged for 1 min at 16,000*g*. An aliquot (100 μL) of the $HClO_4$ supernatant was neutralized by addition of 0.8 M K_2CO_3 (28 μL), kept at −20 °C for 15 min, and centrifuged. An aliquot of the supernatant was loaded onto a Supelcosil LC-18 reversed-phase column (250 × 4.6 mm, 5 μm). The elution conditions: 9 min at 100% buffer A (0.1 M potassium phosphate, pH 6.0), 6 min at up to 12% buffer B (buffer A, containing 20% methanol), 2.5 min at up to 45% buffer B, 2.5 min at up to 100% buffer B, and hold at 100% buffer B for 5.5 min; finally the gradient returns to 100% buffer A in 5 min. The column was flushed with buffer A for 4.5 min prior to the next run. The flow rate was 1.3 ml/min, and the column was thermostated at 25 °C. The eluate absorbance was monitored at 260 nm.
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