Selective inhibition of nicotinamide adenine dinucleotide kinases by dinucleoside disulfide mimics of nicotinamide adenine dinucleotide analogues

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A B S T R A C T

Diadenosine disulfide (**5**) was reported to inhibit NAD kinase from *Lysteria monocytogenes* and the crystal structure of the enzyme–inhibitor complex has been solved. We have synthesized tiazofurin adenosine disulfide (**4**) and the disulfide **5**, and found that these compounds were moderate inhibitors of human NAD kinase (IC_{50} = 110 μM and IC_{50} = 87 μM, respectively) and *Mycobacterium tuberculosis* NAD kinase (IC_{50} = 80 μM and IC_{50} = 45 μM, respectively). We also found that NAD mimics with a short disulfide (–S–S–) moiety were able to bind in the folded (compact) conformation but not in the common extended conformation, which requires the presence of a longer pyrophosphate (–O–P–O–P–O–) linkage. Since majority of NAD-dependent enzymes bind NAD in the extended conformation, selective inhibition of NAD kinases by disulfide analogues has been observed. Introduction of bromine at the C8 of the adenine ring restricted the adenosine moiety of diadenosine disulfides to the syn conformation making it even more compact. The 8-bromoadenosine adenosine disulfide (**14**) and its di(8-bromoadenosine) analogue (**15**) were found to be the most potent inhibitors of human (IC_{50} = 6 μM) and mycobacterium NAD kinase (IC_{50} = 14–19 μM reported so far. None of the disulfide analogues showed inhibition of lactate-, and inosine monophosphate-dehydrogenase (IMPDH), enzymes that bind NAD in the extended conformation.

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1. Introduction

Until recently, between the two pyridine nucleotides, nicotinamide adenine dinucleotide (NAD) has received much more attention than its phosphorylated counterpart, nicotinamide adenine dinucleotide 2′-phosphate (NADP).[1,2] NAD plays a crucial role in cellular catabolic conversions,[3,4] whereas the major role of NADPH is to control the oxidative state of the cell and stand guard against the 'oxidative stress'.[5,6]

NAD kinase is a key enzyme that regulates supply of NADP in the cell.[7] Human NAD kinase catalyzes a magnesium-dependent phosphorylation of the 2′-hydroxyl group of the adenosine ribose moiety of NAD using adenosine triphosphate (ATP) as phosphoryl donor to produce NADP (Scheme 1). Thus, in contrast to the family of ATP-dependent protein kinases that phosphorylate serine, threonine, or tyrosine residues, NAD kinases bind both ATP and NAD. Majority of protein kinase inhibitors have been designed to bind at the ATP-binding domain,[8] however, this domain is not a good

target for the design of potential NAD kinase inhibitors. It is solvent exposed and does not interact with the ATP closely. Chelation with Mg^{2+} is needed to engage ATP at the catalytic domain. In contrast, the NAD binding domain, as a substrate binding site, is ideal as it plays a crucial role in molecular recognition of NAD and its analogues.

Bacterial enzymes can use inorganic polyphosphates as phosphoryl donors (Scheme 1) in addition to ATP. An extensive biochemical, enzymatic, and structural characterization of *Mycobacterium tuberculosis* NAD kinase have been recently reported.[9–11] This kinase is of special interest as a new target for the development of potential drugs against multi-drug resistant (MDR) tuberculosis (TB). The mycobacterium enzyme requires a millimolar concentration of NAD (K_m = 3.3 mM), which is sixfold higher than that of human enzyme (0.5 mM). Thus, *low micromolar inhibitors would be sufficiently competitive to suppress NAD kinase activity, especially that of the M. tuberculosis enzyme.*

Inhibitors of the human enzyme are also of interest. They should indirectly reduce critical supply of NADPH.[1,6] Although NAD kinase provides mainly oxidized form NADP+ (phosphorylation of NADH by the human enzyme is much less efficient), this

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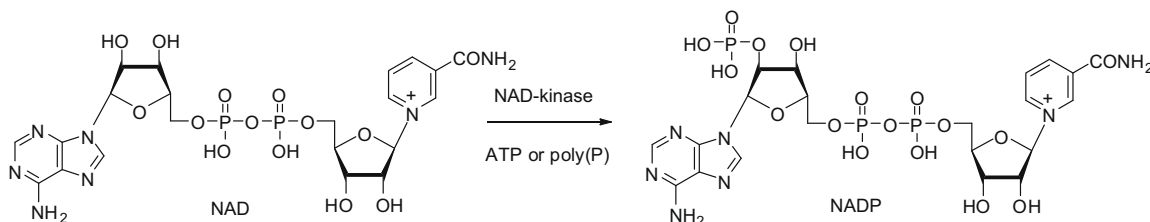
Scheme 1. Phosphorylation of NAD by NAD kinase.

nucleotide is immediately reduced into NADPH by several cellular enzymes, such as NADP+-, isocitrate-, and glucose-6-phosphate dehydrogenases, as well as malic enzyme.[5,12] Interestingly these enzymes are markedly increased during oxidative stress and in cancer cells.[5,13] Recently, NADPH oxidase has been suggested as a target for anticancer therapy.[14] It plays an important role in production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide that are found in large number of tumors.[14] It is highly expressed in human colon, prostate cancer[15] as well as in melanoma and ovarian, cancer.[16–18] It was suggested, therefore, that suppression of NADPH oxidase activity could result in anti-angiogenesis and anticancer activities.[14,18] If cancer cells require higher supply of NADPH than normal cells, then inhibitors of human NAD kinase may show some anticancer effect.[14]

Consequently, we report herein novel selective inhibitors of NAD kinases that bind at the NAD-binding domain of NAD kinases, but *do not affect the majority of other NAD-dependent enzymes.*

2. Results and discussion

2.1. Selectivity at the NAD binding domain

NAD kinases are enzymes which bind both ATP and NAD and this distinguishes them from protein kinases that are almost exclusively ATP-dependent.[8] A majority of protein kinase inhibitors have been designed to bind at the ATP-binding domain and numerous small-molecule kinase inhibitors have been approved by US Food and Drug Administration (FDA).[8] The structure of the ATP catalytic domain of all protein kinases is *highly conserved*, therefore, selective inhibition of a single ATP-dependent enzyme, which causes metabolic disorder or serves an unwanted biochemical pathway(s), is an attractive therapeutic strategy. For example, imatinib (Gleevec) was designed to bind at the ATP cleft of tyrosine kinase encoded by aberrant gene (Bcr/Abl) expressed only in chronic myelogenous leukemia (CML), and became the first effective and selective (rationally designed) agent for treatment of cancer.[19]

The NAD binding domain of NAD-dependent enzymes is *conserved* and it was believed that it would be difficult, if not impos-

sible, to design any NAD-like molecules with good selectivity against a particular enzyme. However, we and others clearly demonstrated that this is not the case.[20–25] It was reported that tiazofurin (Fig. 1), an anticancer C-nucleoside, is converted in the cell into an active metabolite tiazofurin adenine dinucleotide (TAD),[26] an analogue of NAD.

It inhibits IMPDH (K_i = 100 nM) without affecting other cellular enzymes up to concentration of 100 μM. We found that TAD analogues substituted at the C2 of the adenine moiety are even more potent (low nanomolar) and selective inhibitors of IMPDH.[27] Earlier we reported an analogue of NAD that showed specificity against alcohol dehydrogenase (ADH, K_i = 1 nM).[20,21] Recently, selective inhibitors of *Cryptosporidium parvum* IMPDH that bind at the NAD-binding domain and do not inhibit the human enzyme were identified.[28,29]

NAD kinases belong to a large family of NAD-utilizing enzymes.[3] Although, the substrate binding domain of NAD kinases is also conserved it is likely that NAD mimics may show some selectivity against these enzymes without inhibition of majority of cellular NAD-dependent enzymes.

We have examined 147 protein co-crystal structures containing NAD or close NAD analogs, with the proteins selected for sequence diversity.[3] Most proteins bind the cofactor in an elongated (*extended*) conformation, such that the distance between the adenine 6-amino group and the nicotinamide amide carbon is 16 Å or more (Figs. 2 and 3). However, there are clear exceptions to this general trend. Several bacterial reductases bind NAD in extremely *folded* conformations, where this distance is less than 6 Å (PDB entries 1RZ1, 1ZPT, and 2BKJ). Human and bacterial NAD kinases accommodate NAD in folded, bent (twisted) conformations forming a cluster in the histogram around 12 Å.

As these large conformational differences illustrate (Fig. 3), the cofactor binding pockets of these enzymes recognize a variety of geometrically different conformations of NAD. Thus, compact (short) NAD analogs, that do not satisfy the optimal distance (16–21 Å) requirement, would unlikely inhibit a majority of NAD-dependent enzymes. A selective binding can be achieved by distinguishing between NAD analogues fixed in the extended, compact or folded conformation, as well as adopting the *syn* or *anti* conformation [around the glycosyl bond of nicotinamide mononu-
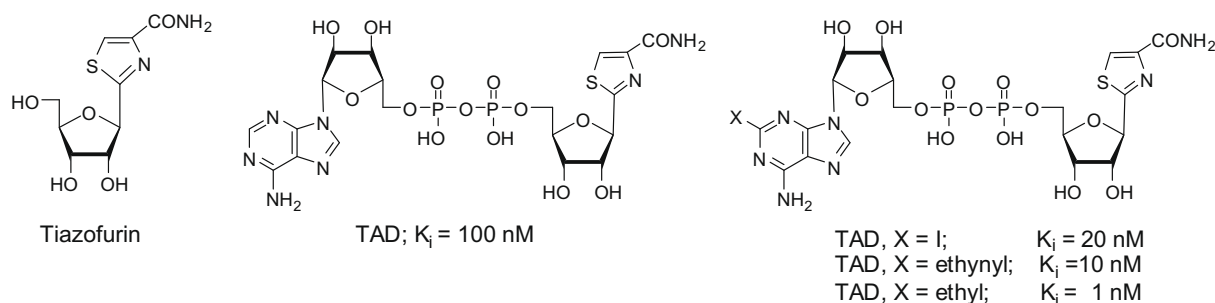
Figure 1. Inhibitors of IMPDH; tiazofurin, tiazofurin adenine dinucleotide (TAD), and 2-substituted TAD analogues.
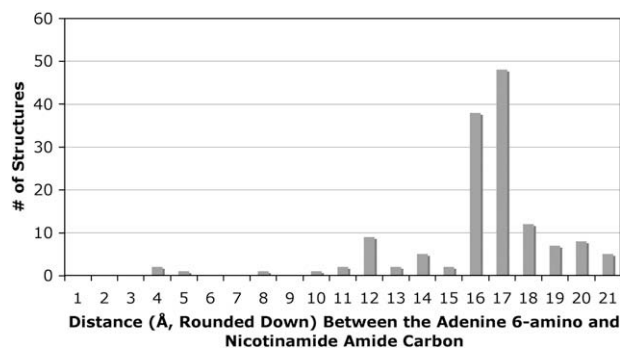
Figure 2. Histogram of distances between the adenine 6-amino and nicotinamide amide carbon in 147 NAD-containing protein X-ray structures, selected for protein sequence diversity.
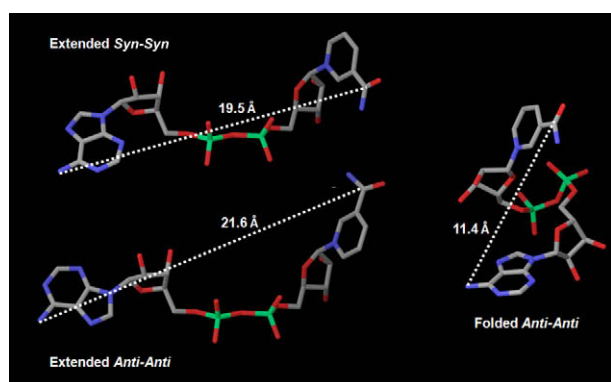
Figure 3. Extended conformations of NAD (*syn–syn* and *anti–anti*) and folded (*anti–anti*) conformation of nucleotide components of NAD.

cleotide (NMN) and adenosine monophosphate (AMP)], or 'north' or 'south' conformation of nucleotide sugars components. Such preferred steric interactions should result in selective or even specific inhibition of a single protein.

Recently, crystal structures of NAD kinase from the human pathogen *Listeria monocytogenes* (*Lm*NADK1) in its free state, and also bound to NAD or NADP have been solved.[30] The authors found that di-5′-thio-adenosine (DTA) inhibits the *Listeria* enzyme (K_i = 20 μM) and their X-ray analysis showed that DTA was bound at the NAD-binding domain. Apparently, nicotinamide riboside sub-domain of the NAD binding pocket is quite promiscuous and tolerates well the substitution of nicotinamide riboside by adenosine. Remarkably, DTA bound to the *Listeria* enzyme adopted a sim-

ilar folded (twisted, bent) conformation as NAD bound at NAD kinases, with a characteristic distance between the two 6-amino groups of about 12 Å.

Since the crystal structure of *M. tuberculosis* NAD kinase is known[10,31] we docked DTA at its binding domain and found as expected that the distance between the two amino groups was in the same range (e.g., 12.3 Å, Fig. 4) as reported for the *Listeria* enzyme.

2.2. Inhibitors design, syntheses, and enzymatic activities

Although the important role of NADP(H) in cellular processes is well recognized, no potent inhibitors of human or bacterial NAD kinase has ever been reported.[6] It was suggested that modifications of either nicotinamide or adenine moiety of NAD prevent binding and phosphorylation.[32] The X-ray structures of the mycobacterium NAD kinase as well as NAD–*Listeria* complexes (the structure of the human enzyme is not known) provide a good starting point in the design process. However, due to their moderately low resolution (2.1–2.6 Å), details are not sharply resolved. We, therefore, believe that the additional information about the enzyme–NAD interactions should emerge from studies of binding affinities of NAD analogues at the NAD-binding domain. We assumed that dinucleoside disulfide NAD mimics could serve well as molecular probes since they can be easily synthesized, modified in the sugar and adenine moiety, and prepared as conformationally restricted derivatives.

Since TAD is known to inhibit IMPDH (IC_{50} = 0.1 μM) by binding in the extended conformation we expected that shorter disulfide analogue **4** would not fit to IMPDH (or other NAD-dependent enzymes that bind the cofactor in the extended conformation) but may inhibit NAD kinase. Thus, tiazofurin adenine disulfide (**4**, Scheme 2) was prepared in 55% yield by in situ deacetylation (MeOH/NH₃) of 5′-acetylthiotiazofurin (**1**) and 5′-acetylthioadenosine (**2**)[33] followed by iodine treatment of the mixture of the corresponding di-5′-thionucleosides, and chromatographic separation. In this process we also isolated symmetric by-products such as ditiazofurin disulfide (**3**) and diadenosine disulfide (DTA, **5**), and evaluated these compounds against mycobacterium and human NAD kinase (Table 1).

As expected, the disulfide **4** inhibited *M. tuberculosis* (IC_{50} = 80 μM) and the human (IC_{50} = 110 μM) NAD kinase. DTA (**5**) was a little more potent competitive inhibitor of both *M. tuberculosis* (IC_{50} = 45 μM) and the human enzyme (IC_{50} = 87 μM). The symmetric ditiazofurin analogue **3** was not active. Remarkably, none of these compounds inhibited human IMPDH or lactate dehydrogenase, enzymes known to bind NAD in an extended conformation.

As is illustrated in Figure 3, if the conformation of nucleoside components of NAD is restricted to *syn* or *anti*, it would decrease
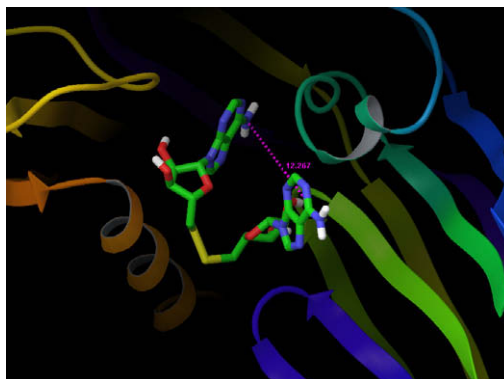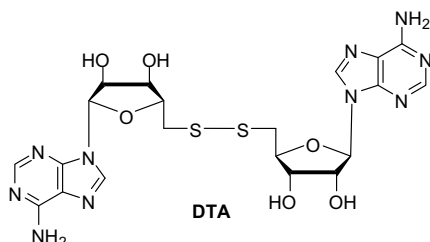
Figure 4. Structure of DTA and its folded (bent, twisted) shape when docked into *M. tuberculosis* NAD kinase.
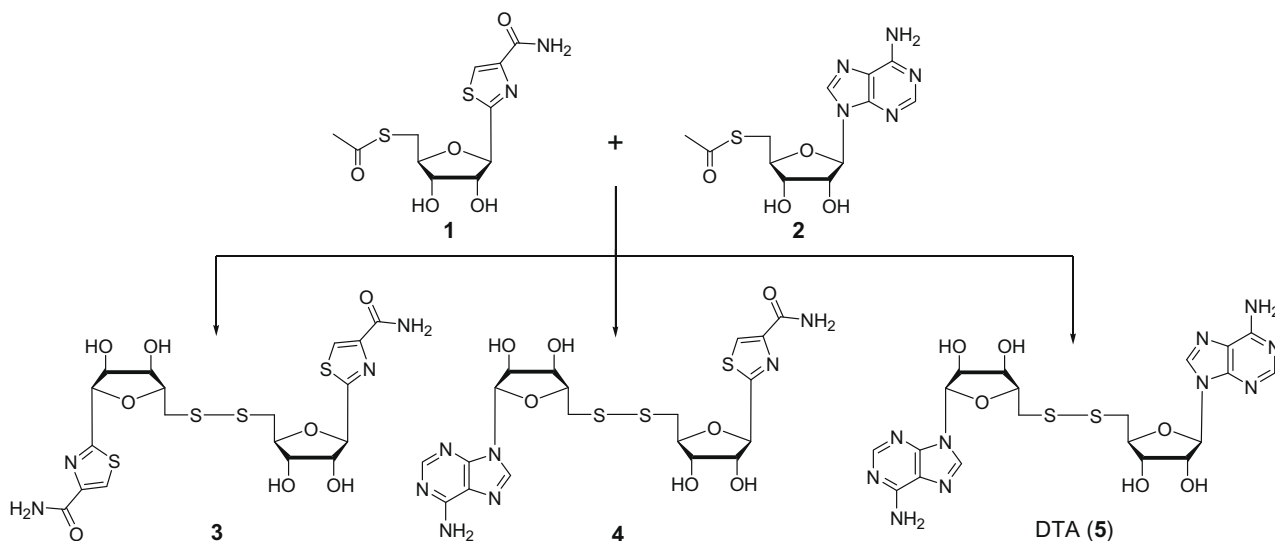
Scheme 2. Synthesis of ditiazofurin disulfide (**3**), tiazofurin adenine disulfide (**4**), and dithioadenosine (DTA, **5**).

Table 1
Inhibition of *M. tuberculosis* and human NAD kinase by dinucleoside disulfides

Structure	*M. tuberculosis* NAD kinase IC$_{50}$ (μM)	Human NAD kinase IC$_{50}$ (μM)
Ditiazofurin disulfide (**3**)	NA[a]	NA
Tiazofurin adenosine disulfide (**4**)	80	110
Diadenosine disulfide (DTA, **5**)	45	87
8-Bromoadenosine adenosine disulfide (**14**)	14	6
Di(8-Bromoadenosine) disulfide (**15**)	19	6
Di(8-phenyladenosine) disulfide (**16**)	NA	45
Di(2′-*C*-methyladenosine) disulfide (**23**)	500	280
Di-(3′-*C*-methyladenosine) disulfide (**24**)	NA	NA
Benzamide adenine dinucleotide (BAD)	200	90

[a] NA—no activity.

or increase, respectively, the crucial distance between the adenine 6-amino group and the nicotinamide amide carbon of NAD. Conformations around the glycosyl bond of the two adenosines of **5** are not restricted allowing NAD kinases to accommodate the inhibitor in any conformation the enzyme requires. However, fixing the conformation of the inhibitor to the one the enzyme prefers might be considerably beneficial. An excellent illustration of such lowering of the entropic barrier by restriction of conformational flexibility is a cyclic inhibitor of penicillopepsin, which due to restricted rotation around the one of the peptide bonds showed 420-fold increase in binding affinity over its free rotational isomer (Fig. 5).[34]
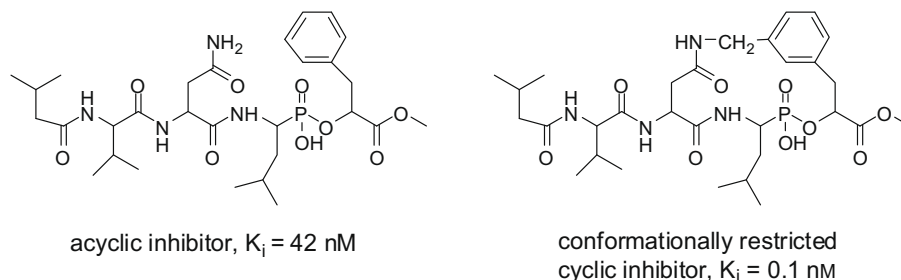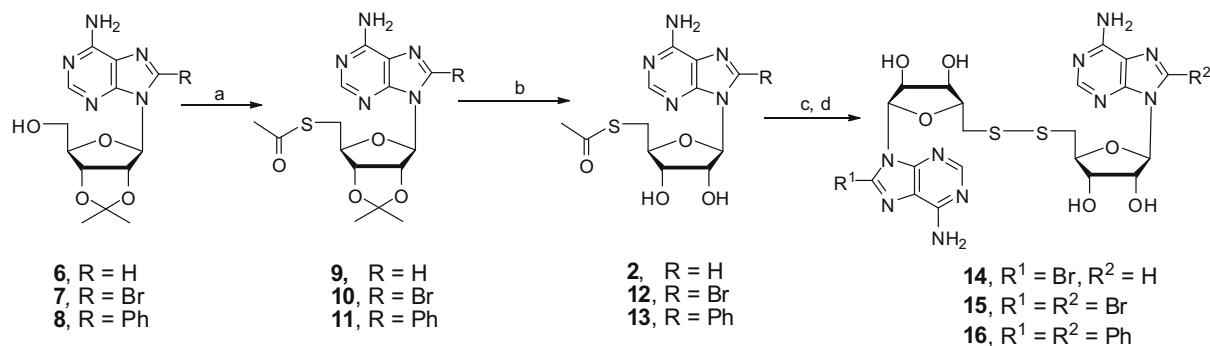
With this in mind, we decided to insert a bulky bromine atom at the position 8 of the one or two adenine rings of DTA as in **14** or **15**, respectively (Scheme 3), which is known to force the adenosine to adopt the *syn* conformation (there is not enough space for bromine to be close to and above the ribose ring)[35] (Scheme 3).

In a similar manner as described for synthesis of **15**, an analogue **16** with 8-phenyl group was prepared (Scheme 3). Di(8-phenyladenosine) disulfide (**16**) also adopts the *syn–syn* conformation but contains a large 'flat' aromatic ring. Disulfides **14**–**16** were prepared from 2′,3′-di-*O*-isopropylidene protected adenosine derivatives **6**–**8** in a similar manner as published for the synthesis of **5**.[30,33]

In ^{1}H NMR the chemical shift of the H2′ signal can be used as an indicator of the *syn/anti* equilibrium of nucleosides;[36] a significant downfield/upfield shift of the H2′ signal is indicative of the predominance of the *syn* conformer or *anti* conformer, respectively.[37] A typical Δ value for H2′ is δ 5.4 ppm for compounds restricted in *syn* conformation, exactly what we observed in the ^{1}H NMR of disulfides **14**–**16** (see Section 4).

We found that the monobromo analogue **14** and its dibromo derivative **15** showed identical potency (IC$_{50}$ = 6 μM) against the human NAD kinase and similar potency against the mycobacterium enzyme (IC$_{50}$ = 14 μM and IC$_{50}$ = 19 μM, respectively, Table 1). The di(8-phenyl) disulfide (**16**) inhibited human enzyme (IC$_{50}$ = 45 μM) but was not active against *M. tuberculosis* NAD kinase. These results indicate that although *syn–syn* binding of 8-bromo and 8-phenyl substituted derivatives **15** and **16** is most likely preferred by both enzymes, the substitution with large phe-

acyclic inhibitor, K$_i$ = 42 nM

conformationally restricted cyclic inhibitor, K$_i$ = 0.1 nM

Figure 5. Acyclic and conformationally restricted (cyclic) peptide-phosphonates.

Scheme 3. Synthesis of 8-bromoadenosine adenosine disulfide (**14**), di(8-bromoadenosine) disulfide (**15**), and di(8-phenyladenosine disulfide (**16**). Reagents and conditions: (a) DEAD, PPh$_3$, AcSH, THF, 0 °C to rt; (b) HCO$_2$H, H$_2$O (c) NH$_3$, CH$_3$OH, H$_2$O (d) I$_2$.

nyl moieties is not beneficial in the case of the mycobacterium enzyme.

The encouraging activities of the conformationally restricted compounds **14–16** prompted us to take one more step forward and examine the activity of DTA analogues that prefer to adopt a specific conformation around the glycosyl bond in addition to 'south' or 'north' conformation of their sugar moiety. Consequently, we focused our attention on 2′-*C*-methyl 'up'- and 3′-*C*-methyl 'up' adenosine.[38,39] The ribose of these compounds is known to be restricted to the 'north' and 'south' conformation, respectively. In addition, the bulky methyl group in the 2′-'up' configuration enforced the adenine ring to adopt the *anti* conformation, whereas the 3′ 'up' methyl group keeps the adenine moiety in the opposite *syn* conformation.[38,39] Thus, we prepared compounds **23** and **24** (Scheme 4) and evaluated them against the two NAD kinases (Table 1).

Unfortunately, disulfide **23** showed only a weak inhibitory activity against mycobacterium (IC$_{50}$ = 500 μM) and human NAD kinase (IC$_{50}$ = 250 μM) whereas compound **24** was inactive against both enzymes. Possibly, the insertion of methyl group either at the C2′ or the C3′ resulted in steric hindrance that significantly diminished or revoked binding affinity of these analogues.

We reported earlier that benzamide adenine dinucleotide (BAD), a close 1-deaza analogue of NAD, which is a potent inhibitor of IMPDH (IC$_{50}$ = 0.8 μM),[40] showed moderate competitive inhibition of human NAD kinase (IC$_{50}$ = 90 μM).[41] BAD like NAD can adopt the extended conformation or be bent and twisted to be bound in the folded conformation required by NAD kinases. DTA, also a competitive inhibitor of NAD kinases, can fit the folded but not the extended NAD domain.

M. tuberculosis and human NAD kinase were purified to homogeneity as described earlier.[41] IC$_{50}$ values for compounds showing an inhibitory effect were calculated and are summarized in Table 1.

All new disulfide compounds were evaluated against two isoforms of human IMPDH and against lactate dehydrogenase as described earlier.[27] None of these compounds inhibited these enzymes up to concentration of 100 μM.

3. Conclusions

Dinucleoside disulfides such as tiazofurin adenosine disulfide (**4**) and diadenosine disulfide (**5**) were found to be moderate inhibitors of *M. tuberculosis* and human NAD kinase (IC$_{50}$'s = 45–110 μM). A restriction of the conformation of adenine moiety to *syn* by substitution with bromine atom at the C8 to give compound **14** or **15** resulted in a 3–15-fold increase of potency against mycobacterium (IC$_{50}$ = 14–19 μM) and human enzyme (6 μM), respectively. These results support our hypothesis that compact NAD analogues that are too short to mimic NAD binding in the extended conformation are expected to show selective inhibition of NAD ki-
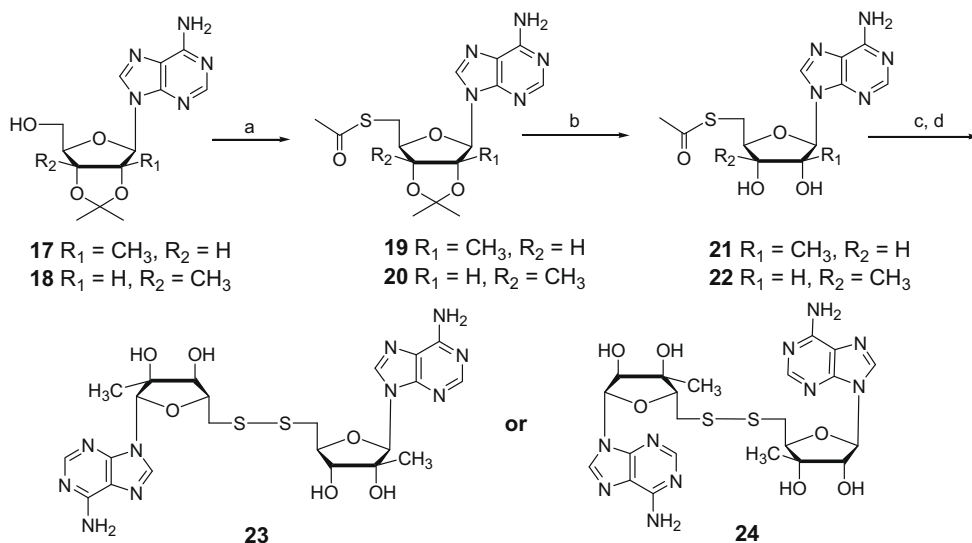
Scheme 4. Synthesis of di(2′-*C*-methyladenosine) disulfide (**23**) and di(3′-*C*-methyladenosine) disulfide (**24**). Reagents and conditions as in Scheme 3.

nases. Bacterial reductases that bind NAD in extremely folded conformations could be especially good targets for drug design based on compact NAD derivatives. Further studies with analogues in which nicotinamide and adenine moieties are connected by short linkers are in progress.

4. Experimental

4.1. General methods

All commercial reagents (Sigma–Aldrich, Acros) were used as provided unless otherwise indicated. An anhydrous solvent dispensing system (J. C. Meyer) using two packed columns of neutral alumina was used for drying THF, Et$_2$O, and CH$_2$Cl$_2$, while two packed columns of molecular sieves were used to dry DMF. Solvents were dispensed under argon. Flash chromatography was performed with Ultra Pure silica gel (Silicycle) with the indicated solvent system. Analytical HPLC was performed on a Varian Microsorb (C18, 5 μm, 4.6 × 250 mm) with a flow rate of 0.5 mL/min. An isocratic or linear gradient of 0.04 M Et$_3$N H$_2$CO$_3$ (TEAB) and aqueous MeCN (70%) was used. Nuclear magnetic resonance spectra were recorded on a Varian 600 MHz with Me$_4$Si, DDS, or signals from residual solvent as the internal standard for ^{1}H or ^{13}C. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br s (broad singlet), dd (double doublet), and dt (double triplet). Values given for coupling constants are first order. High-resolution mass spectra were recorded on an Agilent TOF II TOF/MS instrument equipped with either an ESI or APCI interface. Elemental analyses were determined on an EA 1108 CHNS–O (Fisons Instruments) analyzer.

4.1.1. Di(tiazofurin-5′-yl) disulfide (3), (tiazofurin-5′-yl) (adenosin-5′-yl) disulfide (4), and di(adenosin-5′-yl) disulfide (DTA, 5)

The 5′-thioacetyltiazofurin (1) was prepared according to the published procedure for synthesis of adenosine derivative 2.[33] Thus, 1 (51 mg, 0.157 mmol) was dissolved in 7 N methanolic ammonia (4 mL) and stirred at 0 °C for 30 min. In another flask the 5′-thioadenosine (2, 50 mg, 0.157 mmol) was dissolved in 7 N methanolic ammonia and stirred at 0 °C for 30 min. The resulting reaction mixtures were mixed together and a catalytic amount of iodine (5 mg, 0.012 mmol) in ethanol (0.5 mL) was added dropwise. The resulting mixture was allowed to stir for 12 h at rt and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography with EtOAc/acetone/EtOH/H$_2$O (6:1:1:0.5) as the eluent affording the fast (R_f = 0.35) migrating disulfide 3 (28 mg, 32%) as a white solid ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.97 (dd, 1H, J = 7.3, 13.8 Hz, H-5′), 3.18 (dd, 1H, J = 4.4, 13.8 Hz, H-5″), 3.85 (m, 1H, H-4′), 4.06–4.14 (m, 2H, H-2′, H-3′), 4.95 (d, 1H, J = 4.4 Hz, H-1′), 5.22 (d, 1H, OH-exchangeable), 5.45 (d, 1H, OH- exchangeable), 7.53 (s, 1H, CONH$_2$, exchangeable), 7.65 (s, 1H, CONH$_2$, exchangeable), 8.11 (s, 1H, H-5). HRMS (ESI+) calcd for C$_{18}$H$_{23}$N$_4$O$_8$S$_4$ 51.0398 (M+H)$^+$ found 551.0409. Anal. Calcd for C$_{22}$H$_{26}$N$_4$O$_{10}$S$_2$ (570.59): C, 39.2; H, 4.0; N, 10.1. Found: C, 39.3; H, 4.1; N, 10.0, followed by tiazofurin adenosine disulfide 4 (R_f = 0.25) as a white solid (48 mg, 55%) ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.86 [dd, 1H, J = 7.9 Hz, J = 14.1 Hz, H-5′ (T)], 3.05 [m, 2H, H-5″ (T), H-5′ (A)], 3.16 [dd 1H, J = 13.8 Hz, J = 5.4 Hz, H-5′ (A)], 3.82 [m, 1H, H-4′ (T)], 4.10–4.19 [m, 4H, H-4′ (A), H-3′ (T), H-2′ (T), H-3′ (A)], 4.78 [m, 1H, H-2′(A)], 4.94 [d, 1H, J = 4.1 Hz, H-1′ (T)], 5.22 [br s, 1H, OH-exchangeable], 5.37 [br s, 1H, OH-exchangeable], 5.45 [d, 1H, OH-exchangeable], 5.51 [d, 1H, OH-exchangeable], 5.88 [d, 1H, J = 5.8 Hz, H-1′ (A)], 7.25 [br s, 2H, NH$_2$-exchangeable], 7.52 and 7.65 [two brs, 1H each, CONH$_2$(T)-exchangeable], 8.12 (s, 1H, H-5), 8.16 (s, 1H, H-2), 8.32 (s, 1H, H-

8). HRMS (ESI+) calcd for C$_{19}$H$_{24}$N$_7$O$_7$S$_3$ 558.0899 (M+H)$^+$ found 558.0910. Anal. Calcd for C$_{19}$H$_{23}$N$_7$O$_7$S$_3$ (557.62): C, 40.9; H, 4.1; N, 17.5. Found: C, 40.8; H, 4.0; N, 17.6 and the slowest migrating (R_f = 0.15) DTA (5, 11 mg, 12%), ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.06 (dd, 1H, J = 7.6 Hz, J = 13.8 Hz, H-5′), 3.31 (dd, 1H, J = 5.6 Hz, J = 13.8 Hz, H-5″), 4.08 (m, 1H, H-4′), 4.13 (m, 1H, H-3′), 4.76 (pseudo t, 1H, J = 5.6 Hz, H-2′), 5.38 (d, 1H, OH-exchangeable), 5.49 (d,1H, OH-exchangeable), 5.86 (d, 1H, J = 5.6 Hz, H-1′), 7.24 (br s, 2H, NH$_2$-exchangeable), 8.11 (s, 1H, H-2), 8.31 (s, 1H, H-8). HRMS calcd for C$_{20}$H$_{25}$N$_{10}$O$_6$S$_2$ 365.1399 (M+H)$^+$, found 365.1384. Anal. Calcd for C$_{20}$H$_{24}$N$_{10}$O$_6$S$_2$ (364.6): C, 42.5; H, 4.2; N, 24.8. Found: C, 42.4; H, 4.3; N, 24.6.

4.1.2. (8-Bromoadenosine-5′-yl) (adenosine-5′-yl) disulfide (14)

2′,3′-O-Isopropylidene-adenosine (6) was thioacetylated to give 9 and then the isopropylidene group was removed to give 5′-thioacetyladenosine (2) as described before.[30] To an ice-cold solution of triphenylphosphine (296 mg, 1.771 mmol) in dry THF (10 mL), diethyl azodicarboxylate (0.41 mL, 1.771 mmol) was added over 5 min. After stirring for 30 min, 2′,3′-O-isopropylidene 8-bromoadenosine (7)[42] (300 mg, 0.776 mmol) was added, and stirring was continued for 10 min. To the resulting deep yellow suspension a solution of thioacetic acid (121 μL, 1.771 mmol) in dry THF (1 mL) was added dropwise and stirring was continued for another 1 h at 0 °C. At the end of the reaction the solvent was removed under reduced pressure, and the resulting yellowish residue was purified by flash chromatography on silica gel column eluting with CHCl$_3$/MeOH (0–10%). The fractions containing the product were combined, the solvent was removed under reduced pressure, and the residue was dried in vacuo to yield pure 2′,3′-O-isopropylidene-5′-acetylthio-5′deoxy-8-bromoadenosine (10, 175 mg, 50%) as a white solid: R_f 0.2 (95:5 CHCl$_3$/MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.35 (s, 3H), 1.37 (s, 3H), 2.35 (s, 3 H, COCH$_3$), 3.08 (dd, 1H, J = 6.7 Hz, J = 13.7 Hz, H-5′), 3.19 (dd, 1H, J = 7.0 Hz, J = 13.7 Hz, H-5″), 4.12–4.16 (m, 2H, H-4′), 4.93 (m, 1H, H-3′), 5.48 (dd, 1H, J = 2.1 Hz, J = 6.2 Hz, H-2′), 6.15 (d, 1H, J = 2.1 Hz, H-1′), 6.73 (br s, 2H, NH$_2$), 8.12 (s, 1H, H-2). HRMS (ESI+) calcd for C$_{15}$H$_{19}$BrN$_5$O$_4$S 445.0231 (M+H)$^+$, found: 445.0239. Anal. Calcd for C$_{15}$H$_{18}$BrN$_5$O$_4$S (444.30): C, 40.5; H, 4.1; N, 15.7. Found: C, 40.4; H.

A solution of 10 (150 mg, 0.337 mmol) in a mixture of formic acid and water (10 mL, 7:3) was stirred at room temperature. After 5 h reaction time the solvent was evaporated under reduced pressure, and traces of formic acid were removed by co-evaporation (five times) with anhydrous ethanol. The obtained white powder was purified by flash chromatography with CHCl$_3$/MeOH (0–10%) to give 5′-acetylthio-5′-deoxy-8-bromoadenosine (12, 92 mg, 66%) as a white solid: R_f 0.15 (90:10 CHCl$_3$/MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.25 (s, 3 H, COCH$_3$), 3.47 (m, 2H, H-5′, H-5″), 4.17 (m, 1H, H-4′), 4.33 (m, 1H, H-3′), 5.28 (m, 1H, H-2′), 5.35 (d, 1H, OH-exchangeable), 5.48 (d, 1H, OH-exchangeable), 5.77 (d, 1H, J = 5.6 Hz, H-1′), 7.42 (br s, 2H, NH$_2$), 8.09 (s, 1H, H-2). HRMS (ESI+) calcd for C$_{12}$H$_{15}$BrN$_5$O$_4$S 405.0028 (M+H)$^+$, found: 405.0036. Anal. Calcd for C$_{12}$H$_{14}$BrN$_5$O$_4$S (404.24): C, 35.6; H, 3.5; N, 17.3. Found: C, 35.6; H, 3.4; N, 17.2.

The thionucleoside 12 (60 mg, 0.148 mmol) was dissolved in methanolic ammonia 7 N (6 mL) and stirred at 0 °C for 30 min. In another flask nucleoside 2 (50 mg, 0.148 mmol) was dissolved in methanolic ammonia 7 N and stirred at 0 °C for 30 min. The resulting reaction mixtures are mixed together and iodine (5 mg, 0.018 mmol) in ethanol (0.5 mL) was added dropwise. The resulting mixture was allowed to stir at rt for 14 h and the solvent was evaporated under reduced pressure. The residue was purified by flash chromatography with EtOAc/acetone/EtOH/H$_2$O (6:1:1:0.5) to give (8-bromoadenosine-5′-yl) (adenosine-5′-yl) disulfide 14 as a white solid (52 mg, 55%): R_f 0.25 (6:1:1:0.5 EtOAc/acetone/

EtOH/H$_2$O), ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.05–3.12 (m, 2H, H-5′, H-5″), 3.25–3.36 (m, 2H, H-5′, H-5″), 4.09 (m, 1H, H-4′), 4.13 (m, 1H, H-4′), 4.24 (m, 1H, H-3′), 4.35 (m, 1H, H-3′), 4.76 (m, 1H, H-2′), 5.31 (m, 1H, H-2′), 5.34 (d, 1H, OH-exchangeable), 5.41 (d, 1H, OH-exchangeable), 5.49 (d, 1H, OH-exchangeable), 5.51 (d, 1H, OH-exchangeable), 5.82 (d, 1H, J = 5.8 Hz, H-1′), 5.87 (d, 1H, J = 5.5 Hz, H-1′), 7.25 (br s, 2H, NH$_2$), 7.58 (br s, 2H, NH$_2$), 8.12 (s, 1H, H-2), 8.14 (s, 1H, H-2), 8.31 (s, 1H, H-2). HRMS (ESI+) for C$_{20}$H$_{24}$BrN$_{10}$O$_6$S$_2$ 644.3145 (M+H)$^+$ found 644.3162. Anal. Calcd for C$_{20}$H$_{23}$BrN$_{10}$O$_6$S$_2$ (643.39): C, 42.5; H, 4.3; N, 21.7. Found: C, 42.6; H, 4.2; N, 21.8; followed by di(8-bromoadenosine-5′-yl) disulfide (**15**) as a white solid (10 mg, 21%): R_f 0.4 (6:1:1:0.5 EtOAc/acetone/EtOH/H$_2$O) and **DTA** (9 mg, 23%): R_f 0.15 (6:1:1:0.5 EtOAc/acetone/EtOH/H$_2$O).

4.1.3. Di(8-bromoadenosine-5′-yl) disulfide (15)

Deacetylation of **12** (40 mg, 0.098 mmol) with methanolic ammonia 7 N followed by reaction with iodine overnight (4 mg, 0.011 mmol) and purification by flash chromatography with EtOAc/acetone/EtOH/H$_2$O (6:1:1:0.2) afforded the desired di(8-bromoadenosine-5′-yl) disulfide **15** (33 mg, 51%) as a white solid: R_f 0.25 (6:1:1:0.5 EtOAc/acetone/EtOH/H$_2$O). ^{1}H NMR (600 MHz, DMSO-d_6): δ 3.20-3.38 (m, 2H, H-5′, H-5″), 4.13 (m, 1H, H-4′), 4.35 (m, 1H, H-3′), 5.31 (m, 1H, H-2′), 5.32 (d, 1H, OH-exchangeable), 5.47 (d, 1H, OH-exchangeable), 5.97 (d, 1H, J = 5.5 Hz, H-1′), 7.45 (br s, 2H, NH$_2$), 8.12 (s, 1H, H-2). HRMS (ESI+) calcd for C$_{20}$H$_{23}$Br$_2$N$_{10}$O$_6$S$_2$ 723.2643 (M+H)$^+$, found: 723.2635. Anal. Calcd for C$_{20}$H$_{22}$Br$_2$N$_{10}$O$_6$S$_2$ (722.39): C, 33.2; H, 3.1; N, 19.4. Found C, 33.3; H.

4.1.4. Di(8-Phenyladenosine-5′-yl) disulfide (16)

In a similar manner as described above for 8-bromo analogue **7**, compound **8** (180 mg, 0.469 mmol) was converted into 5′-acetyl-thio-2′,3′-O- isopropylidene 8-phenyladenosine **11**. Purification by flash chromatography with CH$_2$Cl$_2$/MeOH (0–3%) afforded **11** (190 mg, 92%) as a white solid: R_f 0.2 (98:2 CH$_2$Cl$_2$/MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.35 (s, 3H, CH$_3$), 1.56 (s, 3H, CH$_3$), 2.44 (s, 3H, COCH$_3$), 3.42 (m, 1H, H-5′), 3.53 (m, 1H, H-5″), 3.77 (m, 1H, H-4′), 4.11 (m, 1H, H-3′), 5.19 (m, 1H, H-2′), 5.79 (d, 1H, J = 7.1 Hz, H-1′), 7.44 (br s, 2H, NH$_2$), 7.61 (m, 3H, Ph), 7.82 (m, 2H, Ph), 8.13 (s, 1H, H-2). HRMS (ESI+) for C$_{21}$H$_{24}$N$_5$O$_4$S calcd for 441.1545 (M+H)$^+$, found: 441.1552.

A solution of **11** (70 mg, 0.158 mmol) in a mixture of formic acid and water (12 mL, 2:1) was stirred at room temperature. After 8 h reaction time the solvent was evaporated under reduced pressure, and traces of formic acid were removed by co-evaporation (five times) with anhydrous ethanol. Purification by flash chromatography with CHCl$_3$/MeOH (0–5%) afforded de-isopropylidenated compound **13** (45 mg, 72%) as a white solid: R_f 0.2 (95:5 CHCl$_3$/MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 2.47 (s, 3H, COCH$_3$), 3.28 (m, 1H, H-5′), 3.39 (m, 1H, H-5″), 4.01 (m, 1H, H-4′), 4.09 (m, 1H, H-3′), 5.27 (m, 1H, H-2′), 5.77 (d, 1H, J = 6.6 Hz, H-1′), 7.39 (br s, 2H, NH$_2$), 7.58 (m, 3H, Ph), 7.73 (m, 2H, Ph), 8.19 (s, 1H, H-2). HRMS (ESI+) for C$_{18}$H$_{20}$N$_5$O$_4$S calcd for 401.1278 (M+H)$^+$, found: 401.1284.

Deacetylation of **13** (32 mg, 0.079 mmol) with methanolic ammonia 7 N followed by reaction with iodine (4 mg, 0.009 mmol) overnight and purification by flash chromatography with EtOAc/acetone/EtOH/H$_2$O (6:1:1:0.2) afforded the title compound **16** (34 mg, 62%) as a white solid: R_f 0.15 (6:1:1:0.1 EtOAc/acetone/EtOH/H$_2$O).^{1}H NMR (600 MHz, DMSO-d_6): δ 3.18 (m, 1H, H-5′), 3.34 (m, 1H, H-5″), 4.09–4.19 (m, 2H, H-4′, H-3′), 5.31 (br s, 1H, OH-exchangeable), 5.41 (br s, 1H, OH-exchangeable), 5.45 (m, 1H, H-2′), 5.71 (d, 1H, J = 6.2 Hz, H-1′), 7.34 (br s, 2H, NH$_2$), 7.52 (m, 3H, Ph), 7.67 (m, 2H, Ph), 8.15 (s, 1H, H-2). HRMS (ESI+) for C$_{32}$H$_{33}$N$_{10}$O$_6$S$_2$ calcd for 717.1923 (M+H)$^+$, found: 717.1935. Anal.

Calcd for C$_{32}$H$_{32}$N$_{10}$O$_6$S$_2$ (716.79): C, 53.6; H, 5.5; N, 19.5. Found: C, 53.5; H, 4.4; N, 19.6.

4.1.5. Di(2′-C-methyladenosine-5′-yl) disulfide (23)

To an ice-cold solution of triphenylphosphine (140 mg, 0.819 mmol) in abs THF (10 mL), diethyl azodicarboxylate (0.19 mL, 0.819 mmol) was added over 5 min. After stirring for 30 min, 2′-C-methyl-2′,3′-O-isopropylidene-adenosine **17**[38] (120 mg, 0.373 mmol) was added, and stirring was continued for 10 min. To the resulting yellow suspension, a solution of thioacetic acid (6 μL, 0.819 mmol) in abs THF (0.5 mL) was added dropwise and stirring was continued for another 4 h at 0 °C. During this time the yellow suspension cleared, and an orange solution was obtained. At the end of the reaction the solvent was removed under reduced pressure, and the resulting yellowish residue was purified by flash chromatography on silica gel CHCl$_3$/MeOH (0–5%). The fractions containing the product were combined, the solvent was removed under reduced pressure, and the residue was dried in vacuo to yield pure 5′-thionucleoside **19** (105 mg, 75%) as a white solid: R_f 0.25 (95:5 CHCl$_3$/MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.18 (s, 3H, CH$_3$), 1.39 (s, 3H, CH$_3$), 1.53 (s, 3H, CH$_3$), 2.41 (s, 3H, COCH$_3$), 3.63 (m, 2H, H-5′, H-5″), 4.39 (m, 1H, H-4′), 4.58 (d, 1H, J = 2.64 Hz, H-3′), 6.17 (s, 1H, H-1′), 7.53 (br s, 2H, NH$_2$), 8.14 (s, 1H, H-2), 8.35 (s, 1H, H-8). HRMS (ESI+) calcd for C$_{16}$H$_{22}$N$_5$O$_4$S 380.1392 (M+H)$^+$, found 380.1405. A solution of 5′-thionucleoside **19** (70 mg, 0.184 mmol) in a mixture of formic acid and water (10 mL, 2:1) was stirred at room temperature. After 12 h reaction time the solvent was evaporated under reduced pressure, and traces of formic acid were removed by co-evaporating five times with abs ethanol. The obtained white powder was purified by flash chromatography on silica gel with CHCl$_3$/MeOH (5–20%). The fractions containing the product were combined, the solvent was removed under reduced pressure, and the product was dried in vacuo to yield **21** (58 mg, 92%) as a white powder. R_f 0.15 (90:10 CHCl$_3$/MeOH). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.08 (s, 3H, CH$_3$), 2.32 (s, 3H, COCH$_3$), 3.41 (m, 2H, H-5′, H-5″), 4.21 (m, 1H, H-4′), 4.58 (d, 1H, J = 2.64 Hz, H-3′), 5.12 (d, 1H, OH-exchangeable), 5.28 (d, 1H, OH-exchangeable), 6.11 (s, 1H, H-1′), 7.23 (br s, 2H, NH$_2$), 8.11 (s, 1H, H-2), 8.27 (s, 1H, H-8). HRMS (ESI+) calcd for C$_{13}$H$_{18}$N$_5$O$_4$S 340.1079 (M+H)$^+$, found 340.1087.

The thionucleoside **21** (50 mg, 0.147 mmol) was dissolved in methanolic ammonia 7 N (5 mL) and stirred at 0 °C for 30 min. After stirring for 30 min, iodine (5 mg, 0.012 mmol) in ethanol (0.5 mL) was added dropwise. The resulting mixture was allowed to stir at rt for 12 h and the solvent was evaporated under reduced pressure. Purification by flash chromatography with EtOAc/acetone/EtOH/H$_2$O (6:1:1:0.1) afforded **23** as a white solid (54 mg, 62%): R_f 0.15 (6:1:1:0.1 EtOAc/acetone/EtOH/H$_2$O). ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.20 (s, 3H, CH$_3$), 3.16–3.18 (m, 2H, H-5′, H5″), 4.09–4.18 (m, 2H, H-3′, H-4′), 5.22 (br s, 1H, OH-exchangeable), 5.35 (d, 1H, OH-2-exchangeable), 5.95 (s, 1H, H-1′), 7.25 (br s, 2H, NH$_2$), 8.11 (s, 1H, H-2), 8.21 (s, 1H, H-8). HRMS (ESI+) calcd for C$_{22}$H$_{29}$N$_{10}$O$_6$S$_2$ 593.1712 (M+H)$^+$, found 593.1698. Anal. Calcd for C$_{22}$H$_{28}$N$_{10}$O$_6$S$_2$ (592.6): C, 44.5; H, 4.7; N, 23.6. Found: C, 44.4; H, 4.8; N, 23.5.

4.1.6. Di(3′-C-methyladenosine-5′-yl) disulfide (24)

As described above compound **18**[38,39] (100 mg, 0.311 mmol) was converted into thionucleoside **20** (65 mg, 55%) as a white solid: R_f 0.25 (95:5 CHCl$_3$/MeOH). ^{1}HNMR (600 MHz, DMSO-d_6): δ 1.34 (s, 3H, CH$_3$), 1.48 (s, 3H, CH$_3$), 1.58 (s, 3H, CH$_3$), 2.34 (s, 3H, COCH$_3$), 3.01 (dd, 1H, J = 9.4 Hz, J = 13.8 Hz, H-5′), 3.20 (dd, 1H, J = 4.4 Hz, J = 13.8 Hz, H-5″), 4.00 (dd, 1H, J = 4.4 Hz, J = 9.4 Hz, H-4′), 4.94 (d, 1H, J = 2.1 Hz, H-2′), 6.03 (d, 1H, J = 2.1 Hz, H-1′), 7.31 (br s, 2H, NH$_2$), 8.13 (s, 1H, H-2), 8.29 (s, 1H, H-8). HRMS (ESI+) calcd for C$_{16}$H$_{22}$N$_5$O$_4$S 380.1397 (M + H)$^+$, found 380.1407.

A solution of **20** (60 mg, 0.158 mmol) in a mixture of formic acid and water (10 mL, 2:1) was stirred at room temperature. After 6 h reaction time the solvent was evaporated under reduced pressure, and traces of formic acid were removed by co-evaporation (five times) with anhydrous ethanol. The obtained white powder was purified by flash chromatography with $CHCl_3/MeOH$ (0–10%) to give 5′-acetylthio-3′-C-methyl-5′-deoxyadenosine **22** (35 mg, 66%) as a white solid: R_f 0.15 (90:10 $CHCl_3/MeOH$). ^{1}HNMR (600 MHz, DMSO-d_6): δ 1.27 (s, 3H, CH_3), 2.27 (s, 3H, $COCH_3$), 3.20 (dd, 1H, $J = 4.1$ Hz, $J = 13.8$ Hz, H-5′), 3.22 (dd, 1H, J = 9.9 Hz, H-5″), 3.82 (dd, 1H, $J = 4.1$ Hz, 9.9 Hz, H-4′), 4.62 (d, 1H, $J = 7.6$ Hz, H-2′), 5.05 (s, 1H, OH-exchangeable), 5.51 (d, 1H, OH-exchangeable) 5.77 (d, 1H, $J = 7.6$ Hz, H-1′), 7.18 (brs, 2H, NH_2), 8.09 (s, 1H, H-2), 8.33 (s, 1H, H-8). HRMS (ESI+) calcd for $C_{13}H_{18}N_5O_4S$ 340.1084 (M+H)$^+$, found 340.1086.

Deacetylation of **22** (40 mg, 0.114 mmol) with methanolic ammonia 7 N followed by reaction with iodine 12 h and purification by flash chromatography with $EtOAc/acetone/EtOH/H_2O$ (6:1:1:0.1) afforded **24** (38 mg, 56%) as a white solid: R_f 0.15 (6:1:1:0.1 $EtOAc/acetone/EtOH/H_2O$). ^{1}HNMR (600 MHz, DMSO-d_6): δ 1.17 (s, 3H, CH_3), 2.91 (dd, 1H, $J = 2.6$ Hz, J = 13.6 Hz, H-5′), 3.14 (dd, 1H, $J = 10.6$ Hz, $J = 13.6$ Hz, H-5″), 3.97 (dd, 1H, $J = 2.6$, J = 10.6 Hz, H-4′), 4.61 (d, 1H, $J = 7.9$ Hz, H-2′), 4.99 (s, 1H, OH-exchangeable), 5.46 (d, 1H, OH-exchangeable) 5.81 (d, 1H, $J = 7.9$ Hz, H-1′), 7.22 (br s, 2H, NH_2), 8.11 (s, 1H, H-2), 8.33 (s, 1H, H-8). $C_{22}H_{29}N_{10}O_6S_2$ 593.1718 (M+H)$^+$, found 593.1728. Anal. Calcd for $C_{22}H_{28}N_{10}O_6S_2$ (592.6): C, 44.5; H, 4.7; N, 23.6. Found: C, 44.6; H, 4.4; N, 23.7.

4.2. Molecular modeling

Pair wise sequence alignment was carried out using Clustal W v1.8[43] to identify conserved sequence regions. Molecular modelling was carried out using the Schrodinger modelling software package (Schrodinger, LLC, Portland, OR 2007). The structure of human NAD kinase is homology modelled using Prime based on the Clustal W sequence alignment and the recently solved X-ray structure of Mycobacterium tuberculosis NAD kinase–NAD complex (PDB ID: 1Y3I). DTA were docked into the binding site of both human and M. tuberculosis NAD kinase by superposition using the solved X-ray structure of L. monocytogenes NAD kinase-DTA complex (PDB ID:2I2C). All residues within a 9 Å shell radius of each of the top selected bound NAD and DTA conformation were subjected to a series of restraint energy minimizations using the generalized born solvent accessible (GB/SA) continuum solvent model to achieve final optimized bound structure.

4.3. *M. tuberculosis* and human NAD kinase inhibitory activity

The enzymatic activity was determined by a slightly modified HPLC based assay.[44] The assay mixture, consisting of 50 mM Tris/HCl, pH 8.0, 20 mM $MgCl_2$, ATP and NAD substrates, and an appropriate amount of enzymatic preparation, was incubated both in the absence and in the presence of various concentrations of the compounds to be tested. After suitable time incubation at 37 °C, the reaction was stopped by $HClO_4$ treatment, following neutralization with K_2CO_3. HPLC analyses was performed by using the chromatographic separation conditions previously set up by us in order to separate a large number of different nucleotides, nucleosides and bases. Briefly, samples were loaded onto a Supelcosil LC-18 reversed-phase column (250 × 4.6 mm, 5 μm), and elution conditions was as follows: 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 temperature 25 °C. The eluate absorbance was monitored at 260 nm. If necessary, other elution conditions, or other columns, were employed in order to separate the synthesized compounds from the nucleotides of the reaction. Control reaction mixtures have been prepared to assess the stability of the various compounds during the incubation at 37 °C. The possibility that some of the synthesized compounds could be used as alternative substrates in the NAD kinase catalyzed reaction was considered and appropriate reaction mixtures were analyzed.

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