Probing Binding Requirements of Type I and Type II Isoforms of Inosine Monophosphate Dehydrogenase with Adenine-Modified Nicotinamide Adenine Dinucleotide Analogues

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Received May 15, 2007

Novel tiazofurin adenine dinucleotide (TAD) analogues **25**−**33** containing a substituent at C2 of the adenine ring have been synthesized as inhibitors of the two isoforms of human IMP-dehydrogenase. The 2-ethyl TAD analogue **33** [K_i = 1 nM (type I), K_i = 14 nM (type II)] was found to be the most potent. It did not inhibit three other cellular dehydrogenases up to 50 μM. Mycophenolic adenine bis(phosphonate)s containing a 2-phenyl (**37**) or 2-ethyl group (**38**), were prepared as metabolically stable compounds, both nanomolar inhibitors. Compound **38** [K_i = 16 nM (type I), K_i = 38 nM (type II)] inhibited proliferation of leukemic K562 cells (IC_{50} = 1.1 μM) more potently than tiazofurin (IC_{50} = 12.4 μM) or mycophenolic acid (IC_{50} = 7.7 μM).

Introduction

Inosine monophosphate dehydrogenase (IMPDH[a]), a nicotinamide adenine dinucleotide (NAD)-dependent enzyme, is a key enzyme in *de novo* synthesis of purine nucleotides and a major therapeutic target.[1] It catalyzes the oxidation of IMP to xanthosine monophosphate (XMP), which is further converted into guanosine monophospahte (GMP) by GMP synthase. The mechanism of action of the enzyme has been recently reviewed.[2] Inhibition of IMPDH results in depletion of guanine nucleotides, including GTP and dGTP. This G-restriction affects DNA and RNA metabolism as well as the activity of GTP-binding proteins involved in transduction pathways.[3] IMPDH inhibitors have antiproliferative activity and are used as immunosuppressive, anticancer, and antiviral agents.

Two isoforms of the human enzyme, type I and type II, are known.[4,5] Their genes are located on different chromosomes, they are regulated differently, and their products (isoforms) are not redundant.[6−8] This raises the question of the role of the isoforms in intracellular processes and their importance as potential targets for therapeutic intervention. Most tissues express both isozymes, although type I predominates in thymus, brain, retina, and lung while type II is the major form in liver and testis.[8] IMPDH is a tetramer;[9] the type II protein contains 514 residues and is 84% identical to type I. The canonical form of type I is also 514 residues, although recently higher molecular alternative splice forms have been discovered in the retina. Knockout of the IMPDH2 gene in mice is embryonic lethal, indicating that type I isoform cannot replace the function of the type II.[7] Deletion of the type I gene has no apparent effect on mouse development but decreases activation of lymphocytes.[8] With the exceptions mentioned above, the type I isoform is expressed in normal cells at a lower concentration than type II.[8] The type II enzyme is up-regulated in neoplastic cells while the level of expression of type I is not affected. For example, the level of IMPDH2 in myelogenous leukemia is considerably higher (15−42 fold) as compared to normal leukocytes.[10] Induction of both isoforms has been observed in *normal* replicating lymphocytes.[11] It was discovered very recently that mycophenolic acid (MPA) showed inhibition of endothelial cell proliferation *in vitro* and inhibition of tumor-induced angiogenesis *in vivo*.[12] These effects were credited to inhibition of the type I isoform as proved by RNA interference knockdown of its gene. It was therefore suggested that IMPDH1 might be an attractive antiangiogenic drug target. In contrast to the type II isoform, inhibition of type I would not lead to undesired suppression of the patients' immune system. Thus, selective inhibitors of the type I or the type II isoform would be of interest for studies of antiproliferative and/or antiangiogenic effects in cancer, particularly in hematological cancers.

Four inhibitors of IMPDH are currently used clinically and *none show significant selectivity* against the IMPDH isoforms.

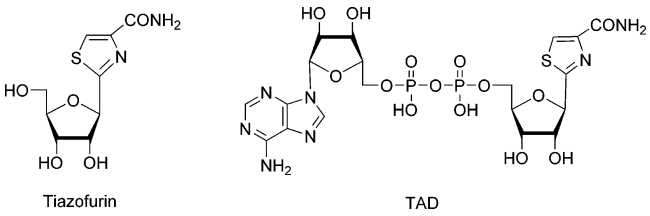

Figure 1.

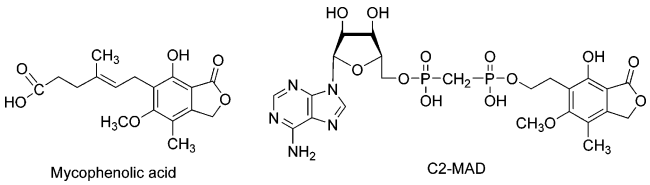

Figure 2.

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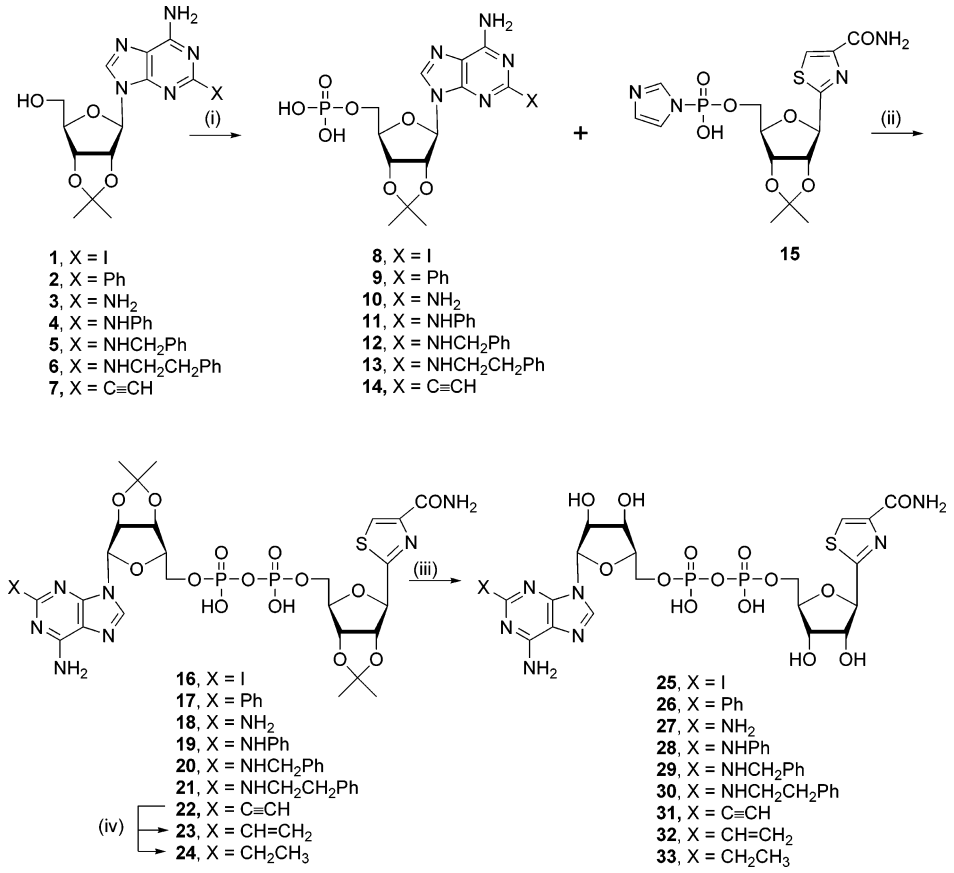
[a] Abbreviations: MPA, mycophenolic acid; IMPDH, inosine monophosphate dehydrogenase; IMP, inosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophospahte; CML, chronic myelogenous leukemia; BC, blast crisis; NMN, nicotinamide mononucleotide; TR, tiazofurin; TAD, tiazofurin adenine dinucleotide; MAD, mycophenolic adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; ADH, alcohol dehydrogenase; MDH, malate dehydrogenase; LDH, lactate dehydrogenase.

10.1021/jm070568j CCC: $37.00
Published on Web 10/24/2007

Scheme 1[a]



[a] Reaction conditions: (i) $P(O)Cl_3$, $(EtO)_3P(O)$, 0 °C overnight; (ii) DMF, rt, 20−24 h; (iii) Dowex 50 (H^+ form), water, and then Dowex 50 (Na^+ form); (iv) Pd/C, MeOH or Lindlar catalyst, MeOH.

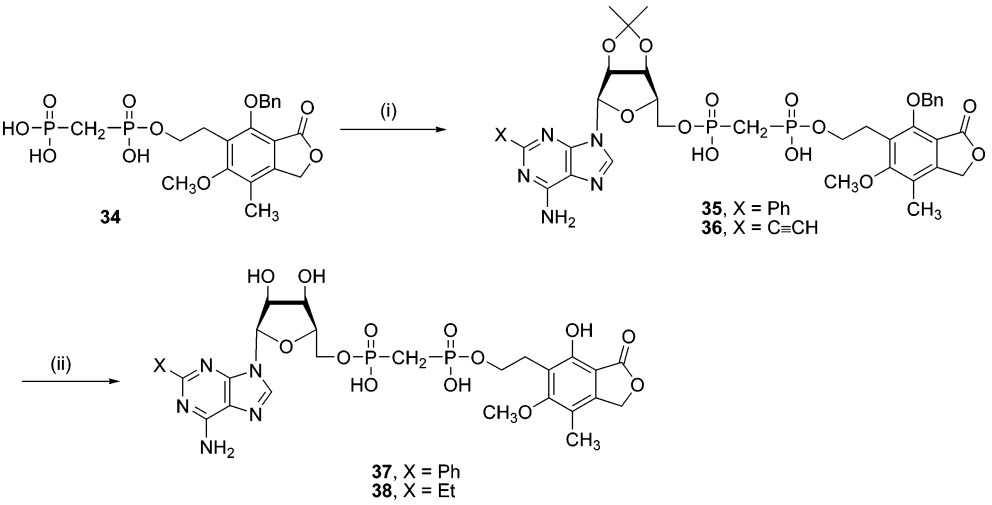
MPA (in the form of prodrug mycophenolic mofetil) and mizoribine are used as immunosuppressants, ribavirin is a broad spectrum antiviral agent, and tiazofurin is approved as an orphan drug for treatment of patients in blast crisis of chronic myelogenous leukemia (CML).[13] Tiazofurin is not active and requires an unusual metabolic activation. It is phosphorylated by adenosine kinase to the tiazofurin-5′-monophosphate which is coupled with ATP by NMN adenylyltransferase to give tiazofurin adenine dinucleotide (TAD, Figure 1), an analogue of NAD, in which tiazofurin replaces the nicotinamide riboside moiety of NAD.[14] TAD mimics the natural cofactor but cannot participate in the hydride transfer, resulting in potent inhibition of IMPDH (K_i = 100 nM). MPA is one of the most potent and specific inhibitors of IMPDH with higher activity against the type II (K_i = 7 nM) than the type I isoform (K_i = 33 nM).[15] MPA does not require metabolic activation and binds at the cofactor (NAD) binding domain mimicking the interaction of nicotinamide mononucleotide (NMN) moiety of NAD with the enzyme.[16] MPA's therapeutic potential is limited by its undesirable metabolism. In humans, the compound is rapidly metabolized into the inactive glucuronide, and as much as 90% of the drug circulates in this inactive form.[17,18] In recent years we synthesized a number of NAD derivatives in which the mycophenolic moiety replaces nicotinamide riboside (Figure 2) to give mycophenolic adenine dinucleotides (MAD) which are remarkably attractive as potential anti CML agents.[19−26] C2-MAD inhibited IMPDH at nanomolar levels (K_i = 250−330 nM), was resistant to glucuronidation, and showed activity in tiazofurin resistant cell line.[22] It was more potent than tiazofurin (8 fold) as a differentiation inducer of K562 cells and less toxic and more efficient than tiazofurin in a murine model of CML.[25] Several other IMPDH inhibitors are under development at major pharmaceutical companies[27−32] and in academia.[24] Our group is evaluating cofactor type inhibitors such as TAD and MAD analogues as potential anticancer agents.[22,24,26]

In this paper we report the synthesis of TAD and MAD analogues containing a substituent at C2 of the adenine ring as probes for studies of binding requirements of IMPDH isoforms. Our computer modeling based on crystal structures of type II isoform[33] in a complex with our TAD or MAD analogue suggested that substitution at C2 position would create isozyme-selective inhibitors. The TAD analogue containing an ethyl group at C2 (**38**) was found to be 14-fold more selective against the type I (K_i = 1 nM) than type II isoform (K_i = 14 nM). It is also the most potent cofactor inhibitor of the IMPDH1 ever reported.

Results and Discussion

Synthesis of TAD Analogues. A key intermediate, 2′,3′-*O*-isopropylidene-2-iodoadenosine **1**[34] was prepared from guanosine[35] and used for further conversion into the C2-substituted adenine analogues **2**, **4**−**7** (Scheme 1). Thus, palladium coupling of **1** with phenylboronic acid afforded 2′,3′-*O*-isopropylidene-2-phenyladenosine (**2**) in 60% yield. Isopropylidenation of commercially available 2,6-diaminopurine riboside under conditions similar to those reported by Matsuda for isopropylidenation of 2-iodoadenosine[34] afforded the protected nucleoside **3**. Palladium coupling of **1** with aniline gave the corresponding 2-phenylamino derivative **4**. Treatment of **1** with benzylamine or phenylethylamine in a sealed tube at 125 °C led to the formation of 2-benzylamino- or 2-phenylethylamino derivatives **4** and **5** in high yield. Finally, coupling of **1** with ethynyltrim-

Scheme 2[a]



[a] Reaction conditions: (i) pyridine, DIC, **2** or **7**, H_2O/Et_3N; (ii) **35** or **36**, H_2, Pd/C, and then Dowex 50 (H^+ form).

Table 1. Inhibition of IMPDH Type I and Type II, Alcohol, Malate, and Lactic Dehydrogenase, as Well as Inhibition of K562 Cell Proliferation, by TAD and TAD Analogues, as well as by MPA and MAD Analogues

inhibitors	IMPDH type I K_i (nM)	IMPDH type II K_i (nM)	ADH IC_{50} (μM)	MDH IC_{50} (μM)	LDH IC_{50} (μM)	K562 cell proliferation IC_{50} (μM)
TAD	110	110				12.4[a]
25, X = I	19 ± 2	84 ± 8	ND	>50	>50	
26, X = Ph	20 ± 6	143 ± 22	ND	>50	>50	
27, X = NH_2	7 ± 9	27 ± 3	>50	>50	>50	
28, X = NHPh	45 ± 5	153 ± 9	>50	>50	>50	
29, X = $NHCH_2Ph$	45 ± 4	169 ± 34	ND	30.0	>50	
30, X = $NHCH_2CH_2Ph$	73 ± 5	255 ± 28	ND	12.7	>50	
31, X = C≡CH	10 ± 2	49 ± 2	>50	>50	>50	
32, X = CH═CH_2	96 ± 39	94 ± 6	39.0	>50	>50	
33, X = CH_2CH_3	1 ± 4	14 ± 2	>50	>50	>50	
MPA	33	7				7.7
*C*2-MAD	330	250				5.7
37, MAD; X = Ph	66 ± 16	108 ± 19	>50	14.3	>50	1.1
38, MAD; X = CH_2CH_3	16 ± 4.4	38 ± 15	>50	>50	>50	1.0

[a] This IC_{50} value is based on TR, which is converted into TAD through enzymatic reactions.

ethylsilane afforded the 2′-3′-*O*-isopropylidene-2-ethynyladenosine (**7**). The reaction conditions for preparation of **4, 5**, and **7** were similar to those reported for the corresponding unprotected nucleosides.[36,37]

Compounds **1**−**7** were phosphorylated using Yoshikawa's procedure[38] to give the corresponding 5′-monophosphates **8**−**14** in moderate to good yield. Reaction of tiazofurin-5′-monophosphate imidazolide (**15**)[39] with monophosphates **8**−**14** afforded the corresponding isopropylidene-protected dinucleotides **16**−**22**. Under mild hydrogenolytic conditions (Pd/C, MeOH), compound **22** was converted into a mixture of 2-ethenyl **23** and 2-ethyl **24**-substituted adenosines. These were separated by HPLC and deprotected to give TAD analogues **32** and **33**, respectively.

Synthesis of MAD Analogues. Even though TAD analogues showed highly potent inhibition of human IMPDH enzymes (Table 1), such pyrophosphate-linked molecules are readily cleaved by cellular enzymes such as pyrophosphatases and phosphodiesterases. Therefore, we chose to synthesize MAD analogues in which two moieties are connected by a methylenebis(phosphonate) linker. Previous studies by us and other groups have clearly demonstrated that methylenebis(phosphonate)-linked TAD and MAD analogues are remarkably stable either in the presence of phosphodiesterases or in serums. They also exhibited potent antiproliferative activities against human cancer cell lines, indicating that these TAD and MAD analogues can penetrate the cell membrane in spite of the ionic nature of their methylenebis(phosphonate) linkers. We synthesized two MAD analogues **37** and **38** (Scheme 2) containing a phenyl and an ethyl group, respectively, based on the fact that a TAD analogue with either of these two substituents (Table 1) showed highly potent inhibition of isoforms of IMPDH. We used our earlier reported C2-mycophenolic bis(phosphonate) **34**[22] for a DIC-mediated coupling with nucleosides **2** and **7** to give intermediate **35** and **36**, respectively. Hydrogenation (Pd/C) of the protected intermediate **35** (X = Ph) followed by treatment with Dowex 50 (H^+ and then Na^+ form) resulted in removal of the 7-benzyl group and deisopropylidenation to give MAD analogue **37**. A similar treatment of **36** (X = C≡CH) afforded analogue **38**.

Molecular Modeling. We modeled **37** and **38** using the X-ray structure of C2-MAD bound to type II IMPDH. As expected, the 2-substituents packed against the CG2 atom of Thr45 (Figure 3). Because of the close proximity of the Thr45 side chain, the phenyl group was not coplanar with the adenine ring but was 30° out of plane. A QM energy scan of this torsion angle showed that deviation from planarity by 45° carries an energy penalty of 1 kcal/mol, but a deviation of 30° carries only a minimal energy penalty of ∼0.3 kcal/mol, and therefore the adjustment observed in the docking is not significantly unfavorable. In both cases, the modeling suggests the hydrophobic C2 substituents may be beneficial because they are sterically of reasonable size and they prefer interaction with the hydrophobic portion of Thr45 over interaction with the solvent.

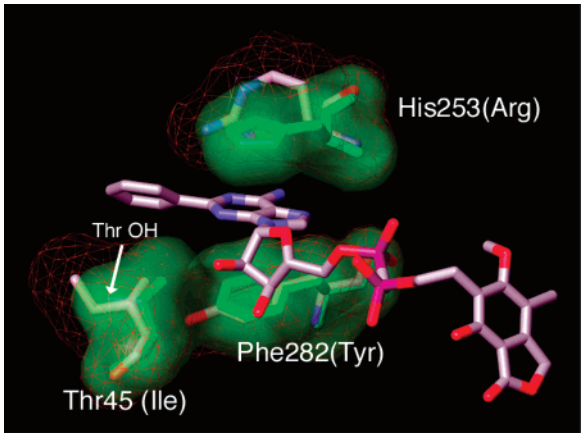


Figure 3. Compound **37** docked to IMPDH2 (PDB entry 1NF7). Residues Thr45, His253, and Phe282 of IMPDH2 are shown in green, with a transparent green surface. Docking shows that both ethyl atoms of **38** also pack against Thr45, whose hydroxyl is indicated by the arrow. IMPDH1 (PDB entry 1JCN) is superimposed with the corresponding residues Ile45, Arg253, and Tyr282 colored by atom and labeled in parentheses. A red mesh surface for the IMPDH1 residues emphasizes the increased hydrophobic surface area of IMPDH1 near the *C*2-substituent.

Biological Evaluation and Conclusions

All new compounds reported herein were evaluated as inhibitors of the two isoforms of human IMPDH. The obtained K_i values (nM) are presented in the first two columns of Table 1. All these compounds were also tested against three other cellular dehydrogenases, namely horse liver alcohol dehydrogenase (ADH), porcine heart malate dehydrogenase (MDH), and bovine heart lactate dehydrogenase (LDH). The corresponding IC_{50} values are provided (in μM) in Table 1. Finally, inhibition of proliferation of human leukemic K562 cells by metabolically stable compounds was also examined. All modifications at the 2 position of adenine ring resulted in more potent inhibitors of IMPDH1 than the parent compounds. The majority (**25**, **27**, **31**−**33**, **37**, **38**), but not all, also showed higher affinity for the type II isoform. In general, the inhibitory activity against the type I enzyme was higher than that against the type II enzyme. The two most potent inhibitors of IMPDH, compounds **33** and **38** in TAD and MAD series, respectively, were found to be as potent as MPA, one of the most potent inhibitors of IMPDH. As expected, compounds reported herein were much less effective (3−4 orders of magnitude) as inhibitors of three other cellular dehydrogenases. We have previously reported that C-NAD (5-β-D-ribofuranosylnicotinamide),[40] an isosteric analogue of NAD, binds ADH (K_i = 4 nM) 3−5 orders of magnitude more potently than other cellular dehydrogenases such as malate, lactate, and glutamate dehydrogenase.[41−43] A number of examples of specific or highly selective NAD-like molecules[44,45] or compounds bound at the NAD pocket could be found in literature.[46,47] Collectively, it is reasonable to believe that an NAD analogue can be designed to selectively inhibit a particular NAD-dependent enzyme, regardless of the conservation of the cofactor binding domain of NAD dependent enzymes.

In this paper we demonstrated that TAD analogues could be used as models for probing binding requirements of MAD analogues for type I and type II isoforms of IMPDH. TAD analogues as pyrophosphates are more readily accessible by chemical synthesis than MAD analogues; however, they are metabolically unstable and cannot be used as potential drugs. The adenine ring of TAD and MAD analogues is positioned at the same place at the AMP subdomain of isoforms. Therefore, specific modification of the adenine ring of TAD or MAD affects binding affinity in the similar manner. Indeed, substitution at the C2 position with ethyl or phenyl groups afforded more potent compounds in both TAD and MAD series. The MAD analogue **38** was found to be 20-fold more potent inhibitor of type I and 6-fold more potent inhibitor of the type II isoform than parent C2-MAD. As expected, MAD-**38** did not inhibit other cellular dehydrogenases up to 50 μM, showing a 3.1×10^3 selectivity index. It is also a 4-fold more potent inhibitor of proliferation of K562 cells and serves as a promising lead compound for our future studies of MAD analogues as potential therapeutic agents against CML.

Experimental Section

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_2O, and CH_2Cl_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 column (C18, 5 μm, 4.6 × 250 mm) with a flow rate of 0.5 mL/min while preparative HPLC was performed on a Varian Dynamax column (C18, 8 μm, 41.4 × 250 mm) with a flow rate of 40 mL/min. An isocratic or linear gradient of 0.04 M Et_3N H_2CO_3 (TEAB) and aqueous MeCN (70%) was used. Nuclear magnetic resonance spectra were recorded on a Varian 300 or 600 MHz with Me_4Si, DDS, or signals from residual solvent as the internal standard for ^1H or ^{13}C, and external H_3PO_4 for ^{31}P. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), brs (broad singlet), and dd (double doublet). 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.

Preparation of Nucleosides 2, 4−7. 2′,3′-*O*-Isopropylidene-2-phenyladenosine (2). To 2′,3′-*O*-isopropylidene-2-iodoadenosine (**1**, 109 mg, 0.25 mmol) in a sealed tube were added phenylboronic acid (47 mg, 0.38 mmol), palladium acetate (6.0 mg, 0.027 mmol), (2-biphenyl)dicyclohexylphosphine (13.1 mg, 0.037 mmol), and potassium phosphate (106 mg, 0.50 mmol). After addition of anhydrous dioxane (1.5 mL), the sealed tube was evacuated and then flushed and filled with nitrogen. The tube was sealed and heated at 100 °C for 24 h. After cooling to rt, the mixture was diluted with EtOAc (10 mL) and filtered through a pad of Celite. The filtrate was concentrated, and the resulting residue was purified on a Chromatotron (1 mm, 0−6% MeOH/CH_2Cl_2) to give 2′,3′-*O*-isopropylidene-2-phenyladenosine (**2**) as a pale solid (57.4 mg, 60%). ^1H NMR (CD_3OD, 300 MHz) δ 8.40−8.29 (m, 2H), 8.26 (s, 1H), 7.48−7.37 (m, 3H), 6.23 (d, J = 2.7 Hz, 1H), 5.51 (dd, J = 6.3, 3.0 Hz, 1H), 5.13 (dd, J = 6.2, 2.8 Hz, 1H), 4.33 (ddd, J = 4.6, 4.6, 2.9 Hz, 1H), 3.75 (dd, J = 11.8, 4.4 Hz, 1H), 3.69 (dd, J = 11.8, 5.2 Hz, 1H), 1.62 (s, 3H), 1.40 (s, 3H). HRMS calcd for $C_{19}H_{22}N_5O_4$ 384.1671 $(M + H)^+$, found 384.1665.

2′,3′-*O*-Isopropylidene-2-phenylaminoadenosine (4). A similar reaction of **1** (325 mg, 0.75 mmol) with palladium acetate (17 mg, 0.075 mmol), *rac*-BINAP (70 mg, 0.11 mmol), cesium carbonate (370 mg, 1.1 mmol), and aniline (103 μL, 1.1 mmol) in anhydrous 1,4-dioxane (2 mL) in a sealed tube at 100 °C for 18 h afforded after filtration and evaporation a residue, which was purified by flash chromatography with CH_2Cl_2/MeOH (2−6%) as the eluent to give **4** (53 mg, 53%) as a pale solid. ^1H NMR (CD_3OD, 300 MHz) δ 8.01 (s, 1H), 7.67 (d, J = 8.1 Hz, 2H), 7.26 (t, J = 8.0 Hz, 2H), 6.94 (t, J = 7.5 Hz, 1H), 6.08 (d, J = 3.0 Hz, 1H), 5.44 (dd, J = 6.2, 2.8 Hz, 1H), 4.96 (dd, J = 6.2, 2.8 Hz, 1H), 4.32−4.23 (m, 1H), 3.70−3.58 (m, 2H), 1.60 (s, 3H), 1.39 (s, 3H). HRMS calcd for $C_{19}H_{23}N_6O_4$ 399.1780 $(M + H)^+$, found 399.1774.

2′,3′-*O*-Isopropylidene-2-benzylaminoadenosine (5). A solution of **1** (167 mg, 0.39 mmol) and benzylamine (0.43 mL, 3.9 mmol)

in 2-methoxyethanol (0.50 mL) was heated in a sealed tube at 125 °C for 24 h. After cooling to rt, the mixture was directly purified on a Chromatotron [1 mm, CH_2Cl_2/MeOH (0−6%)] to give 2′,3′-*O*-isopropylidene-2-benzylaminoadenosine (**5**) as a tan solid (136 mg, 85%). ^{1}H NMR (CD_3OD, 300 MHz) δ 7.86 (d, J = 0.6 Hz, 1H), 7.40−7.14 (m, 5H), 5.96 (d, J = 2.7 Hz, 1H), 5.26 (dd, J = 6.0, 3.0 Hz, 1H), 4.86−4.78 (m, 1H), 4.62 (d, J = 15.6 Hz, 1H), 4.53 (d, J = 15.6 Hz, 1H), 4.24−4.16 (m, 1H), 3.84 (s, 1H), 3.67−3.52 (m, 2H), 1.54 (s, 3H), 1.27 (s, 3H). HRMS calcd for $C_{20}H_{25}N_6O_4$ 413.1937 (M + H)$^+$, found 413.1937.

2′,3′-*O*-Isopropylidene-2-phenethylaminoadenosine (6). In a similar manner **1** (218 mg, 0.50 mmol) under treatment with phenylethylamine (0.32 mL, 2.54 mmol) afforded **6** (62.2 mg, 74%) as a pale solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.90 (s, 1H), 7.32−7.22 (m, 4H), 7.22−7.12 (m, 1H), 6.03 (d, J = 3.0 Hz, 1H), 5.39 (dd, J = 6.0, 3.0 Hz, 1H), 5.03 (dd, J = 6.0, 2.7 Hz, 1H), 4.33−4.27 (m, 1H), 3.79−3.42 (m, 4H), 3.36 (s, 1H), 2.90 (t, J = 7.4 Hz, 2H), 1.59 (s, 3H), 1.36 (s, 3H). HRMS calcd for $C_{21}H_{27}N_6O_4$ 427.2093 (M + H)$^+$, found 427.2098.

2′,3′-*O*-Isopropylidene-2-ethynyladenosine (7). A solution of compound **1** (879 mg, 2.03 mmol), $PdCl_2(PPh_3)_2$ (142 mg, 0.20 mmol), CuI (79 mg, 0.41 mmol), and triethylamine (0.60 mL, 4.30 mmol) in DMF (3 mL) in a sealed tube was evacuated and backfilled with nitrogen five times. After addition of ethynyltrimethylsilane (2.0 mL, 14.4 mmol), the resulting mixture was heated at 80 °C for 18 h. The mixture was cooled to rt, diluted with toluene (200 mL), and washed with water (2 × 50 mL). The aqueous layer was extracted with Et_2O (2 × 50 mL), and the combined organic layer was stirred with Celite and activated carbon. After filtration, the filtrate was concentrated and the residue was passed through a silica gel column. The combined fractions was concentrated, and the residue was dissolved in CH_3CN (6 mL) and treated with TBAF (645 mg, 2.04 mmol) for 1.5 h. The reaction mixture was then concentrated, and the residue was purified by flash chromatography with CH_2Cl_2/MeOH (0−6%) as the eluent to give **7** (518 mg, 77%) as a pale solid. ^{1}H NMR ($CDCl_3$/CD_3OD, 300 MHz) δ 8.02 (s, 1H), 5.90 (d, J = 4.8 Hz, 1H), 5.12 (pseudo t, J = 5.2 Hz, 1H), 5.03 (dd, J = 5.7, 1.5 Hz, 1H), 4.48−4.40 (m, 1H), 3.90 (dd, J = 12.6, 2.1 Hz, 1H), 3.74 (dd, J = 12.6, 2.4 Hz, 1H), 3.34 (s, 1H), 1.59 (s, 3H), 1.33 (s, 3H). HRMS calcd for $C_{15}H_{18}N_5O_4$ 332.1353 (M + H)$^+$, found 332.1371.

Preparation of Mononucleotides (8−14). 2′,3′-*O*-Isopropylidene-2-iodoadenosine 5′-Monophosphate (8). To a solution of compound **1** (217 mg, 0.50 mmol) in triethyl phosphate (1 mL) at 0 °C was added a solution of phosphorus oxychloride (0.10 mL, 0.11 mmol) in triethyl phosphate (1 mL, precooled with ice−water). The resulting solution was kept in a refrigerator for 20 h. The mixture was then added to a solution of TEAB (0.5 M, 20 mL, cooled with ice−water). After stirring for 10 min, the mixture was extracted with EtOAc (3 × 20 mL). The aqueous layer was concentrated and lyophilized. The residue was dissolved in water (5 mL) and purified by RP-HPLC to give **8** (165.8 mg, 54%, Et_3N salt) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.46 (s, 1H), 6.18 (d, J = 3.6 Hz, 1H), 5.36 (dd, J = 5.8, 3.4 Hz, 1H), 5.18 (dd, J = 6.0, 1.8 Hz, 1H), 4.60 (pseudo brs, 1H), 4.00−3.82 (m, 2H), 3.08 (q, J = 7.4 Hz, (CH_3CH_2)$_3$NH^+), 1.66 (s, 3H), 1.44 (s, 3H), 1.21 (t, J = 7.4 Hz, (CH_3CH_2)$_3$NH^+). ^{31}P NMR (CD_3OD, 121 MHz) δ 4.23. HRMS calcd for $C_{13}H_{16}N_5O_7PI$ 511.9832 (M − Et_3N − H)$^-$, found 511.9838.

2′,3′-*O*-Isopropylidene-2-phenyladenosine 5′-Monophosphate (9). In a similar manner compound **2** (48.8 mg, 0.13 mmol) was phosphorylated to give **9** (25.0 mg, 35%) as a white solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.41 (s, 1H), 8.39−8.31 (m, 2H), 7.50−7.37 (m, 3H), 6.32 (d, J = 2.7 Hz, 1H), 5.48 (dd, J = 6.0, 3.0 Hz, 1H), 5.18 (dd, J = 6.2, 2.2 Hz, 1H), 4.55−4.46 (m, 1H), 4.08−3.98 (m, 2H), 3.09 (q, J = 7.2 Hz, (CH_3CH_2)$_3$NH^+), 1.64 (s, 3H), 1.40 (s, 3H), 1.23 (t, J = 7.2 Hz, (CH_3CH_2)$_3$NH^+). ^{31}P NMR (CD_3OD, 121 MHz) δ 1.89. HRMS calcd for $C_{19}H_{21}N_5O_7P$ 462.1184 (M − Et_3N − H)$^-$, found 462.1173.

2′,3′-*O*-Isopropylidene-2-aminoadenosine 5′-Monophosphate (10). Isopropylidenation of commercially available 2-aminoadenosine with 2,2-dimethoxypropane (3.0 equiv) in acetone in the presence of perchloric acid (70% solution, 5.5 equiv) afforded 2′,3′-*O*-isopropylidene-2-aminoadenosine (**3**). Compound **3** (323 mg, 1.00 mmol) was phosphorylated as described above to give mononucleotide **10** (303 mg, 60%, Et_3N salt) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.11 (brs, 1H), 6.08 (d, J = 3.0 Hz, 1H), 5.34 (dd, J = 6.2, 2.8 Hz, 1H), 5.16 (dd, J = 6.0, 2.1 Hz, 1H), 4.61 (pseudo brs, 1H), 4.12−3.98 (m, 2H), 3.18 (q, J = 7.3 Hz, (CH_3CH_2)$_3$NH^+), 1.63 (s, 3H), 1.42 (s, 3H), 1.26 (t, J = 7.2 Hz, (CH_3CH_2)$_3$NH^+). ^{31}P NMR (D_2O, 121 MHz) δ 1.06. HRMS calcd for $C_{13}H_{18}N_6O_7P$ 401.0974 (M − Et_3N − H)$^-$, found 401.0976.

2′,3′-*O*-Isopropylidene-2-phenylaminoadenosine 5′-Monophosphate (11). Phosphorylation of nucleoside **4** (136 mg, 0.34 mmol) under the above conditions gave monophosphate **11** (87.4 mg, 44%) as a white solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.12 (s, 1H), 7.77−7.66 (m, 2H), 7.30−7.18 (m, 2H), 6.97−6.86 (m, 1H), 6.12 (d, J = 3.0 Hz, 1H), 5.41 (dd, J = 6.0, 3.3 Hz, 1H), 5.09 (dd, J = 6.3, 2.1 Hz, 1H), 4.49−4.40 (m, 1H), 4.09−3.90 (m, 2H), 3.00 (q, J = 7.1 Hz, (CH_3CH_2)$_3$NH^+), 1.62 (s, 3H), 1.39 (s, 3H), 1.21 (t, J = 7.2 Hz, (CH_3CH_2)$_3$NH^+). ^{31}P NMR (CD_3OD, 121 MHz) δ 2.38. HRMS calcd for $C_{19}H_{22}N_6O_7P$ 477.1293 (M − Et_3N − H)$^-$, found 477.1297.

2′,3′-*O*-Isopropylidene-2-benzylaminoadenosine 5′-Monophosphate (12). As above the reaction of nucleoside **5** (116 mg, 0.28 mmol) with $P(O)Cl_3$ afforded nucleotide **12** (59.1 mg, 35%) as a white powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.96 (s, 1H), 7.42−7.32 (m, 2H), 7.32−7.22 (m, 2H), 7.21−7.12 (m, 1H), 5.99 (d, J = 2.7 Hz, 1H), 5.24 (dd, J = 6.0, 3.0 Hz, 1H), 4.63 (d, J = 15.3 Hz, 1H), 4.51 (d, J = 15.3 Hz, 1H), 4.42−4.33 (m, 1H), 4.10−3.90 (m, 2H), 3.10 (q, J = 7.3 Hz, (CH_3CH_2)$_3$NH^+), 1.52 (s, 3H), 1.30−1.18 (m, isopropylidene methyl and (CH_3CH_2)$_3$NH^+). ^{31}P NMR (CD_3OD, 121 MHz) δ 1.99. HRMS calcd for $C_{20}H_{24}N_6O_7P$ 491.1449 (M − Et_3N − H)$^-$, found 491.1452.

2′,3′-*O*-Isopropylidene-2-phenethylaminoadenosine 5′-Monophosphate (13). In a similar manner compound **6** (82.5 mg, 0.19 mmol) was converted into nucleotide **13** (49.5 mg, 42%, Et_3N salt) ^{1}H NMR (CD_3OD, 300 MHz) δ 8.07 (brs, 1H), 7.32−7.21 (m, 4H), 7.21−7.10 (m, 1H), 7.21−7.12 (m, 1H), 6.08 (d, J = 3.0 Hz, 1H), 5.40 (dd, J = 6.0, 3.0 Hz, 1H), 5.11 (dd, J = 5.8, 2.2 Hz, 1H), 4.48−4.38 (m, 1H), 4.04−3.92 (m, 2H), 3.64−3.50 (m, 2H), 2.96−2.78 (m, NHCH$_2$*CH$_2$*Ph and (CH_3CH_2)$_3$NH^+), 1.58 (s, 3H), 1.36 (s, 3H), 1.16 (t, J = 7.4 Hz, (CH_3CH_2)$_3$NH^+). ^{31}P NMR (CD_3OD, 121 MHz) δ 2.99. HRMS calcd for $C_{21}H_{26}N_6O_7P$ 505.1606 (M − Et_3N − H)$^-$, found 505.1612.

2′,3′-*O*-Isopropylidene-2-ethynyladenosine 5′-Monophosphate (14). As above nucleoside **7** (389 mg, 1.17 mmol) was phosphorylated to give mononucleotide **14** (365.3 mg, 61%, Et_3N salt)) as off-white solid. ^{1}H NMR (D_2O, 600 MHz) δ 8.56 (s, 1H), 6.25 (d, J = 3.0 Hz, 1H), 5.48 (dd, J = 5.4, 3.6 Hz, 1H), 5.23 (d, J = 4.2 Hz, 1H), 4.68 (pseudo brs, 1H), 4.08−3.98 (m, 2H), 3.56 (s, 1H), 3.22 (q, J = 7.2 Hz, (CH_3CH_2)$_3$NH^+), 1.72 (s, 3H), 1.50 (s, 3H), 1.30 (t, J = 7.2 Hz, (CH_3CH_2)$_3$NH^+). ^{31}P NMR (D_2O, 243 MHz) δ 3.02. HRMS calcd for $C_{15}H_{17}N_5O_7P$ 410.0865 (M − Et_3N − H)$^-$, found 410.0872.

Coupling of Mononucleotides (8−14) with 2′,3′-*O*-Isopropylidenetiazofurin-5′-monophosphate Imidazolide (15). *P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-iodoadenosin-5′-yl) Pyrophosphate (16). Tiazofurin 5′-phosphoimidazolide (**15**, 60.6 mg, 0.099 mmol) and nucleotide **8** (27.6 mg, 0.057 mmol) were dissolved in DMF-d_7 (0.9 mL). The resulting mixture was kept at rt, and the progress of reaction was monitored by ^{31}P NMR. After 8 days, the mixture was lyophilized. The residue was dissolved in a TEAB solution, and the resulting solution was purified by RP-HPLC to give pyrophosphate **16** (14.5 mg, 24%, Et_3N salt) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.29 (brs, 1H), 8.04 (s, 1H), 6.13 (d, J = 3.3 Hz, 1H), 5.38 (dd, J = 6.2, 3.4 Hz, 1H), 5.22 (dd, J = 6.2, 2.0 Hz, 1H), 5.04 (d, J = 4.5 Hz, 1H), 4.88 (dd, J = 6.2, 3.0 Hz, 1H), one signal buried under D_2O, 4.62 (pseudo brs, 1H), 4.38−4.30 (m, 1H), 4.22−4.05 (m, 2H), 3.96 (pseudo brs, 2H), 3.18 (q, J = 7.3 Hz, (CH_3CH_2)$_3$NH^+), 1.66 (s, 3H), 1.61 (s, 3H), 1.44 (s, 3H), 1.37 (s, 3H), 1.26

(t, J = 7.4 Hz, (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 121 MHz) δ −10.61. HRMS calcd for $C_{25}H_{31}IN_7O_{14}P_2S$ 874.0169 (M − Et_3N − H)$^-$, found 874.0168.

***P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-phenyladenosin-5′-yl) Pyrophosphate (17)**. In a similar manner coupling of mononucleotide **9** (17.5 mg, 0.033 mmol) with imidazolide **15** (23.1 mg, 0.041 mmol) afforded pyrophopshate **17** (10.6 mg, 31%) as a white powder. ^{1}H NMR (D_2O, 300 MHz) δ 8.28 (s, 1H), 7.98−7.88 (m, 2H), 7.84 (s, 1H), 7.50−7.36 (m, 3H), 6.26 (d, J = 2.7 Hz, 1H), 5.41 (dd, J = 6.0, 2.7 Hz, 1H), 5.26 (dd, J = 6.2, 2.6 Hz, 1H), 4.77−4.70 (m, 2H), 4.65−4.54 (m, 2H), 4.20−4.04 (m, 3H), 3.85 (pseudo brs, 2H), 3.17 (q, J = 7.3 Hz, (CH_3CH_2)$_3$NH$^+$), 1.67 (s, 3H), 1.55 (s, 3H), 1.46 (s, 3H), 1.29 (s, 3H), 1.25 (t, J = 7.4 Hz, (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 121 MHz) δ −10.63. HRMS calcd for $C_{31}H_{36}N_7O_{14}P_2S$ 824.1516 (M − Et_3N − H)$^-$, found 824.1519.

***P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-aminoadenosin-5′-yl) Pyrophosphate (18).** Tiazofurin 5′-phosphoimidazolide (**15**, 50.2 mg, 0.10 mmol) and mononucleotide **10** (35.7 mg, 0.067 mmol) were coupled as described above to give **18** (43.8 mg, 67%) as a white solid. ^{1}H NMR (D_2O, 300 MHz) δ 8.03 (s, 1H), 8.01 (s, 1H), 6.02 (d, J = 3.6 Hz, 1H), 5.30 (dd, J = 6.3, 3.6 Hz, 1H), 5.19 (dd, J = 6.0, 2.4 Hz, 1H), 5.14−5.08 (m, 1H), 4.90−4.86 (m, 2H), 4.58−4.50 (m, 1H), 4.38 (pseudo brs, 1H), 4.13 (pseudo brs, 2H), 4.00 (pseudo brs, 2H), 2.71 (q, J = 7.2 Hz, (CH_3CH_2)$_3$NH$^+$), 1.63 (s, 3H), 1.59 (s, 3H), 1.41 (s, 3H), 1.35 (s, 3H), 1.06 (t, J = 7.2 Hz, (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 121 MHz) δ −10.54. HRMS calcd for $C_{25}H_{33}N_8O_{14}P_2S$ 763.1312 (M − Et_3N − H)$^-$, found 763.1317.

***P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-phenylaminoadenosin-5′-yl) Pyrophosphate (19).** As described above coupling of nucleotide **11** (68.1 mg, 0.13 mmol) with **15** (91.7 mg, 0.16 mmol) gave pyrophosphate **19** (40.2 mg, 30%) as a white powder. ^{1}H NMR (D_2O, 300 MHz) δ 7.92 (brs, 1H), 7.90 (s, 1H), 7.43 (d, J = 8.1 Hz, 2H), 7.23 (t, J = 7.6 Hz, 2H), 6.96 (t, J = 7.2 Hz, 1H), 5.98 (d, J = 2.1 Hz, 1H), 5.25 (dd, J = 5.8, 2.2 Hz, 1H), 4.99 (dd, J = 6.0, 2.4 Hz, 1H), 4.77−4.68 (m, 2H), 4.52−4.42 (m, 1H), 4.26−4.16 (m, 1H), 4.04 (pseudo brs, 2H), 3.88 (pseudo brs, 2H), 3.14 (q, J = 7.3 Hz, (CH_3CH_2)$_3$NH$^+$), 1.60 (s, 3H), 1.52 (s, 3H), 1.39 (s, 3H), 1.28 (s, 3H), 1.23 (t, J = 7.4 Hz, (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 121 MHz) δ −10.62. HRMS calcd for $C_{31}H_{37}N_8O_{14}P_2S$ 839.1625 (M − Et_3N − H)$^-$, found 839.1629.

***P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-benzylaminoadenosin-5′-yl) Pyrophosphate (20).** The similar coupling of nucleotide **12** (35.1 mg, 0.066 mmol) with imidazolide **15** (50.7 mg, 0.085 mmol) afforded pyrophosphate **20** (19.4 mg, 28%) as a white powder. ^{1}H NMR (D_2O, 300 MHz) δ 7.87 (s, 1H), 7.79 (s, 1H), 7.40−7.24 (m, 4H), 7.18 (t, J = 6.9 Hz, 1H), 5.94 (d, J = 2.4 Hz, 1H), 5.10−5.00 (m, 2H), 4.90−4.84 (m, 1H), 4.56−4.32 (m, 5H), 4.16−3.94 (m, 5H), 3.16 (q, J = 7.2 Hz, (CH_3CH_2)$_3$NH$^+$), 1.57 (s, 3H), 1.55 (s, 3H), 1.32 (s, 3H), 1.30−1.18 (m, isopropylidene methyl and (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 121 MHz) δ −10.57. HRMS calcd for $C_{32}H_{39}N_8O_{14}P_2S$ 853.1781 (M − Et_3N − H)$^-$, found 853.1787.

***P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-phenethylaminoadenosin-5′-yl) Pyrophosphate (21).** As above nucleotide **13** (36.7 mg. 0.069 mmol) was converted into the corresponding pyrophosphate **21** (16.6 mg) in 22% yield. ^{1}H NMR (D_2O, 300 MHz) δ 7.95 (s, 1H), 7.90 (s, 1H), 7.30−7.10 (m, 5H), 6.00 (s, 1H), 5.26−5.12 (m, 2H), 5.04 (d, J = 4.8 Hz, 1H), 4.88−4.82 (m, 1H), 4.76−4.70 (m, 1H), 4.61 (pseudo brs, 1H), 4.37 (pseudo brs, 1H), 4.15 (pseudo brs, 2H), 3.98 (pseudo brs, 2H), 3.40 (t, J = 6.8 Hz, 2H), 3.16 (q, J = 7.2 Hz, (CH_3CH_2)$_3$NH$^+$), 2.82−2.66 (m, 2H), 1.58 (s, 3H), 1.54 (s, 3H), 1.35 (s, 3H), 1.29 (s, 3H), 1.25 (t, J = 7.4 Hz, (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 121 MHz) δ −10.40 (d, J = 20.7 Hz), −10.81 (d, J = 20.7 Hz). HRMS calcd for $C_{33}H_{41}N_8O_{14}P_2S$ 867.1938 (M − Et_3N − H)$^-$, found 867.1944.

***P*^{1}-(2′,3′-*O*-Isopropylidenetiazofurin-5′-yl)-*P*^{2}-(2′,3′-*O*-isopropylidene-2-ethynyladenosin-5′-yl) Pyrophosphate (22).** Compound **22** (227.3 mg, 42%)) was obtained as a white powder by conversion of nucleotide **14** (295 mg, 0.55 mmol) as described above. ^{1}H NMR (D_2O, 600 MHz) δ 8.46 (s, 1H), 8.07 (s, 1H), 6.19 (d, J = 3.6 Hz, 1H), 5.42 (dd, J = 6.3, 3.6 Hz, 1H), 6.26 (dd, J = 6.0, 2.4 Hz, 1H), 5.06 (d, J = 4.8 Hz, 1H), 4.91 (dd, J = 6.3, 2.7 Hz, 1H), 4.88−4.84 (m, 1H), 4.78 (brs, 1H), 4.66 (brs, 1H), 4.36 (brd, J = 3.6 Hz, 1H), 4.20 (ddd, J = 11.4, 4.2, 4.2 Hz, 1H), 4.15 (ddd, J = 11.4, 3.0, 3.0 Hz, 1H), 4.04−3.94 (m, 2H), 3.19 (q, J = 7.2 Hz, (CH_3CH_2)$_3$NH$^+$), 1.70 (s, 3H), 1.64 (s, 3H), 1.48 (s, 3H), 1.39 (s, 3H), 1.29 (t, J = 7.2 Hz, (CH_3CH_2)$_3$NH$^+$). ^{31}P NMR (D_2O, 243 MHz) δ 10.49 (d, J = 20.4 Hz), 10.61 (d, J = 20.9 Hz). HRMS calcd for $C_{27}H_{32}N_7O_{14}P_2S$ 772.1197 (M − Et_3N − H)$^-$, found 772.1157.

Deprotection of TAD Analogues 16−22. General Procedure. To a solution of isopropylidene-protected pyrophosphate derivative (0.030 mmol, Et_3N form) in water (2 mL) was added Dowex 50 WX8−200 (H^+ form, ca. 500 mg). The mixture was stirred for 15−20 h at rt and then passed through a column of Dowex 50 WX8−200 (Na^+ form). After elution with water, UV active fractions were collected and lyophilized to give deprotected pyrophosphate (sodium salt) as a white powder in yields 35−96%.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-iodoadenosin-5′-yl) Pyrophosphate (25).** White powder (96%). ^{1}H NMR (D_2O, 300 MHz) δ 8.31 (s, 1H), 7.98 (s, 1H), 5.97 (d, J = 5.7 Hz, 1H), 4.99 (d, J = 4.8 Hz, 1H), 4.68 (pseudo t, J = 5.2 Hz, 1H), 4.53−4.46 (m, 1H), 4.38 (pseudo brs, 1H), 4.30−4.02 (m, 7H). ^{31}P NMR (D_2O, 121 MHz) δ −10.24. HRMS calcd for $C_{19}H_{23}IN_7O_{14}P_2S$ 793.9543 (M − H)$^-$, found 793.9546.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-phenyladenosin-5′-yl) Pyrophosphate (26).** White powder (60%). ^{1}H NMR (D_2O, 300 MHz) δ 8.37 (s, 1H), 8.04−7.94 (m, 2H), 7.79 (s, 1H), 7.56−7.42 (m, 3H), 6.15 (d, J = 5.4 Hz, 1H), 4.87−4.83 (m, 1H), 4.77−4.72 (m, 1H), 4.57−4.50 (m, 1H), 4.42−3.90 (m, 8H). ^{31}P NMR (D_2O, 121 MHz) δ −10.24. HRMS calcd for $C_{25}H_{28}N_7O_{14}P_2S$ 744.0890 (M − H)$^-$, found 744.0892.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-aminoadenosin-5′-yl) Pyrophosphate (27).** White powder (64%). ^{1}H NMR (D_2O, 300 MHz) δ 8.05 (s, 1H), 7.96 (s, 1H), 5.86 (d, J = 5.7 Hz, 1H), 5.03 (d, J = 4.5 Hz, 1H), 4.65 (pseudo t, J = 5.6 Hz, 1H), 4.44 (dd, J = 4.8, 3.9 Hz, 1H), 4.31 (pseudo brs, 1H), 4.26−4.02 (m, 7H). ^{31}P NMR (D_2O, 121 MHz) δ −10.27. HRMS calcd for $C_{19}H_{25}N_8O_{14}P_2S$ 683.0686 (M − H)$^-$, found 683.0693.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-phenylaminoadenosin-5′-yl) Pyrophosphate (28).** White powder (64%). ^{1}H NMR (D_2O, 300 MHz) δ 8.04 (s, 1H), 7.87 (s, 1H), 7.49−7.41 (m, 2H), 7.34−7.24 (m, 2H), 7.06−6.97 (m, 1H), 5.90 (d, J = 5.1 Hz, 1H), 4.93 (d, J = 5.4 Hz, 1H), 4.66 (pseudo t, J = 5.2 Hz, 1H), 4.44 (pseudo t, J = 4.6 Hz, 1H), 4.33−3.93 (m, 8H). ^{31}P NMR (D_2O, 121 MHz) δ −10.24. HRMS calcd for $C_{25}H_{29}N_8O_{14}P_2S$ 759.0999 (M − H)$^-$, found 759.1007.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-benzylaminoadenosin-5′-yl) Pyrophosphate (29).** White powder (40%). ^{1}H NMR (D_2O, 300 MHz) δ 7.96 (s, 1H), 7.88 (s, 1H), 7.42−7.21 (m, 5H), 5.85 (d, J = 5.7 Hz, 1H), 5.02 (d, J = 3.3 Hz, 1H), 4.60 (pseudo t, J = 5.2 Hz, 1H), 4.50 (pseudo brs, 2H), 4.38 (pseudo t, J = 4.4 Hz, 1H), 4.31−3.99 (m, 8H). ^{31}P NMR (D_2O, 121 MHz) δ −10.26. HRMS calcd for $C_{26}H_{31}N_8O_{14}P_2S$ 773.1155 (M − H)$^-$, found 773.1162.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-phenethylaminoadenosin-5′-yl) Pyrophosphate (30).** White powder (35%). ^{1}H NMR (D_2O, 300 MHz) δ 7.98 (s, 1H), 7.91 (s, 1H), 7.37−7.14 (m, 5H), 5.87 (d, J = 5.4 Hz, 1H), 5.03−4.98 (m, 1H), 4.68−4.60 (m, 1H), 4.47 (pseudo t, J = 4.4 Hz, 1H), 4.34−3.98 (m, 8H), 3.54 (t, J = 6.8 Hz, 2H), 2.85 (t, J = 6.8 Hz, 2H). ^{31}P NMR (D_2O, 121 MHz) δ −10.28. HRMS calcd for $C_{27}H_{33}N_8O_{14}P_2S$ 787.1312 (M − H)$^-$, found 787.1319.

***P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-ethynyladenosin-5′-yl) Pyrophosphate (31).** White powder (88%). ^{1}H NMR (D_2O, 600 MHz) δ 8.59 (s, 1H), 8.03 (s, 1H), 6.06 (d, J = 6.0 Hz, 1H), 5.02 (d, J = 6.0 Hz, 1H), 4.73 (pseudo t, J = 5.4 Hz, 1H), 4.53 (pseudo t, J = 4.2 Hz, 1H), 4.42 (brd, J = 1.8 Hz, 1H), 4.32−4.22 (m, 5H), 4.20−4.14 (m, 1H), 4.14−4.08 (m, 1H), 3.78 (s, 1H). ^{31}P NMR (D_2O,

121 MHz) δ −10.27. HRMS calcd for $C_{21}H_{24}N_7O_{14}P_2S$ 692.0582 $(M - H)^-$, found 692.0521.

Hydrogenolysis and Deprotection of TAD Analogue 22. *P*^{1}-(Tiazofurin-5′-yl)-*P*^{2}-(2-ethyladenosin-5′-yl) Pyrophosphate (33). A solution of pyrophosphate **22** (54.5 mg, 0.056 mmol, Et_3N salt) in MeOH (3 mL) was hydrogenated (1 atm) in the presence of 10% Pd/C (10.2 mg) for 5 h. After the reaction was complete, the reaction mixture was filtered through a pad of Celite, and the filtrate was concentrated. The residue was dissolved in water (3 mL), and Dowex 50WX8−200 (H^+ form, ca. 850 mg) was added. The mixture was stirred for 18 h at rt and filtered through a pad of Celite. After the filtrate was lyophilized, the residue was dissolved in water (2 mL) and then passed through a column of Dowex 50WX8−200 (Na^+ form). After elution with water, UV active fractions were collected and lyophilized to give **33** (35.4 mg, 86% for two steps) as the white powder sodium salt. ^{1}H NMR (D_2O, 600 MHz) δ 8.53 (s, 1H), 8.03 (s, 1H), 6.15 (d, J = 5.4 Hz, 1H), 5.07 (d, J = 5.4 Hz, 1H), 4.57 (pseudo t, J = 4.5 Hz, 1H), 4.42 (brd, J = 3.0 Hz, 1H), 4.36−4.24 (m, 6H), 4.24−4.10 (m, 2H), 2.93 (q, J = 7.2 Hz, 1H), 2.90 (q, J = 7.8 Hz, 1H), 1.36 (t, J = 7.5 Hz, 3H). ^{31}P NMR (D_2O, 121 MHz) δ −10.28. HRMS calcd for $C_{21}H_{28}N_7O_{14}P_2S$ 696.0895 $(M - H)^-$, found 696.0844. Alternatively, the similar hydrogenation of **22** (57.0 mg, 0.058 mmol) in the presence of Lindlar catalyst (10.5 mg) afforded two compounds **33** (14.8 mg, 34%) and **32** (8.8 mg, 20%). They were separated on the RP-HPLC column and converted into their corresponding sodium salts by passing through a column of Dowex 50WX8−200 (Na^+ form). The pyrophosphate **32** was obtained as a white powder. ^{1}H NMR (D_2O, 600 MHz) δ 8.54 (s, 1H), 8.00 (s, 1H), 6.71 (dd, J = 17.4, 10.8 Hz, 1H), 6.59 (d, J = 17.4 Hz, 1H), 6.15 (d, J = 5.4 Hz, 1H), 5.99 (d, J = 10.8 Hz, 1H), 5.03 (d, J = 5.4 Hz, 1H), 4.56 (pseudo t, J = 4.5 Hz, 1H), 4.42 (brd, J = 3.0 Hz, 1H), 4.35−4.23 (m, 6H), 4.22−4.17 (m, 1H), 4.15−4.09 (m, 1H). ^{31}P NMR (D_2O, 121 MHz) δ −10.28. HRMS calcd for $C_{21}H_{26}N_7O_{14}P_2S$ 694.0739 $(M - H)^-$, found 694.0703.

Synthesis of *C*2-MAD Analogues. *P*^{1}-(2-Phenyladenosin-5′-yl)-*P*^{2}-[7-*O*-benzyl-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis(phosphonate) (35). To a solution of **34** (338 mg, 0.50 mmol) in anhydrous pyridine (5 mL) was added DIC (310 μL, 2 mmol), and the mixture was left overnight until the intermediate was formed (multisignal resonances in ^{31}P NMR). Compound **2** (230 mg, 0.6 mmol) was then added, and the reaction mixture was heated at 55−65 °C for 60 h. At that time, the ^{31}P NMR of the reaction mixture showed two broad signals at 8 and 25 ppm. Then, a mixture of water (9 mL) and Et_3N (1 mL) was added, and the reaction mixture was kept at 55−65 °C for 27 h. After removal of the solvent, the residue was dissolved in MeOH and purified on HPLC (50% to 100% buffer B in 20 min, gradient). Compound **35** (92 mg, 22%) obtained as the triethylammonium salt. ^{1}H NMR (D_2O): δ 1.25 (t, CH_3 from Et_3N), 1.41 and 1.64 (two s, 3H each, isopropylidene), 2.14 (s, 3H, CH_3), 2.96 (m, 2H, PCH_2−P-), 3.02 (m, CH_2− from Et_3N), 3.80 (m, OCH_3), 4.02 (m, 2H), 4.15 (m, 2H), 4.46 (brs, 1H), 5.22 (s, 4H), 5.23 (m, 1H), 5.45 (m, 1H), 6.31 (d, 1H, J = 3.0 Hz, 1′-H), 7.31 (t, 1H, benzyl), 7.35 (t, 2H, benzyl), 7.43 (m, 3H, phenyl), 7.54 (d, 2H, benzyl), 8.38 (d, 2H, phenyl), 8.54 (s, 1H, H8-adenine). ^{31}P NMR (D_2O): δ 16.58 (d, J = 24.0 Hz), 17.46 (d, J = 24.0 Hz). LC-MS (ESI): m/z 850 (M − H).

***P*^{1}-[2′,3′-*O*-Isopropylidene(2-ethynyladenosin-5′-yl)-*P*^{2}-[7-*O*-benzyl-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis(phosphonate) (36).** In a similar manner **34** (338 mg, 0.50 mmol) was converted into **36** (112 mg, 25%, triethylammonium salt). ^{1}H NMR (D_2O): δ 1.25 (t, 9H, Et_3N adduct on ethynyl formed a 2-vinyl salt), 1.35 (t, 9H, Et_3N), 1.38 and 1.59 (two s, 3H each, isopropylidene), 2.17 (t, 2H, PCH_2−P, J = 18.6 Hz), 2.19 (s, 3H, CH_3), 3.02 (m, 8H, CH_2 and Et_3N), 3.85 (s, 3H, OCH_3), 4.02 (m, 8H, OCH_2 and Et_3N adduct on ethynyl formed a 2-vinyl salt), 4.08 (m, 2H), 4.16 (brs, 2H), 4.48 (brs, 1H), 5.21 (s, 2H), 5.23 (m, 1H), 5.24 (s, 2H), 5.31 (m, 1H), 6.05 (d, 1H, J = 10.6 Hz, 2-vinyl Et_3N salt), 6.14 (d, 1H, J = 2.4 Hz, 1′-H), 6.80 (d, 1H, J = 10.6 Hz, 2-vinyl Et_3N salt), 7.29 (t, 1H, benzyl), 7.34 (t, 2H, benzyl), 7.52 (d, 2H, benzyl), 8.66 (s, 1H, H8-adenine). ^{31}P NMR (D_2O): δ 16.82 (d, J = 24.6 Hz), 17.50 (d, J = 24.6 Hz). LC-MS (ESI): m/z 899 (M − H), 901 (M + H).

***P*^{1}-[7-Hydroxy-6-(hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]-*P*^{2}-(2-phenyladenosin-5′-yl)methylenebis(phosphonate) (37).** Compound **35** (53 mg, 0.05 mmol, as the triethylammonium salt) was dissolved in MeOH (10 mL) containing a catalytic amount of $Pd(OH)_2$ (20% Pd/C, 5−10 mg). The mixture was stirred at room temperature under H_2 for 36 h. Methanol was removed in vacuo, and the residue was mixed with small amount of Dowex 50WX8−200 (H^+) overnight. This mixture was directly pass a column of Dowex 50WX8−200 (Na^+) to give the final product as sodium salt (15 mg, 42%). ^{1}H NMR (D_2O): δ 1.88 (s, 3H, CH_3), 2.23 (t, 2H, PCH_2−P, J = 20 Hz), 2.79 (m, 2H), 3.71 (s, 3H, OCH_3), 3.98 (m, 1H), 4.21−4.30 (m, 2H), 4.39 (d, 1H, J = 3.0 Hz), 4.61 (t,1H, J = 4.8 Hz), 4.78 (t, 1H, J = 4.8 Hz), 4.93 (d, 2H, J = 1.6 Hz), 5.21 (d, 1H, J = 7.8 Hz), 6.17 (d, 1H, J = 4.8 Hz, 1′-H), 7.58 (m, 3H, phenyl), 8.10 (d, 2H, benzyl), 8.43 (s, 1H, H8-adenine). ^{31}P NMR (D_2O): δ 18.0 (d, J = 27.6 Hz), 18.80 (d, J = 27.6 Hz). HRMS (ESI−): calcd for $C_{29}H_{32}N_5O_{13}P_2$ (M − H) 720.1471, found 720.1418.

***P*^{1}-[7-Hydroxy-6-(hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]-*P*^{2}-(2-ethyladenosin-5′-yl)methylenebis(phosphonate) (38).** Compound **36** (50 mg, 0.05 mmol, as the triethylammonium salt) was dissolved in MeOH (10 mL) and deprotected as described above to give **38** as a sodium salt (15 mg, 42%). ^{1}H NMR (D_2O): δ 1.29 (t, 3H, J = 7.2, 7.8 Hz, CH_3), 2.13 (s, 3H, CH_3), 2.24 (t, 2H, PCH_2−P, J = 19.8 Hz), 2.74 (dd, 2H, CH_2 J = 7.2, 7.8 Hz), 2.86 (m, 2H), 3.75 (s, 3H, OCH_3), 4.03 (m, 1H), 4.23(m, 1H), 4.28 (m, 1H), 4.36 (brs, 1H), 4.53 (t,1H, J = 4.8 Hz), 4.64 (t, 1H, J = 4.8 Hz), 5.02 (s, 2H), 5.23 (s, 1H), 6.03 (d, 1H, J = 4.8 Hz, 1′-H), 8.36 (s, 1H, H8-adenine). ^{31}P NMR (D_2O): δ 18.16 (d, J = 29 Hz), 18.55 (d, J = 29 Hz). HRMS (ESI−): calcd for $C_{25}H_{32}N_5O_{13}P_2$ (M − H) 672.1471, found 672.1398.

Molecular Modeling. The X-ray structure of human IMPDH2 with C2-MAD bound (PDB entry 1NF7) was used for modeling in MacroModel[48] with the MMFFs force field and GB/SA solvation model. The structure was first minimized with only hydrogens free to move and then with heavy atoms restrained by a 1.0 kcal/mol-Å^2 force. Residues with no atoms within 16 Å of the ligand were deleted, and truncated portions of the backbone were methyl-capped. Modeling was validated by docking mycophenolic acid (MPA) into the structure using the MacroModel MCMM and LMCS methods, resulting in a 1.2 Å ligand rmsd relative to the X-ray structure of MPA bound to Chinese hamster IMPDH (PDB entry 1JR1). Simple models for the binding of **37** and **38** were generated by modifying the 2 position of the minimized C2-MAD and carrying out conformational sampling on the adenosine portion of the ligand (atoms up to the 5′ OH of adenosine were free to move, and the remainder were not allowed to move). The glycosidic bond and the C2-phenyl or C2-ethyl torsion were selected for sampling. The simulations had converged by 1000 search steps. A relaxed torsion energy scan around the phenyl group/adenine torsion angle was calculated at 15° increments at the B3LYP/6-311G(d,p) level in Jaguar 6.5 using the SCRF model for water solvation and was performed on a model compound consisting of adenine, the 2-substituent, and an N9 methyl cap.

IMPDH Type I and Type II. Human IMPDH type I and type II was expressed and purified as previously described.[49,50] Inhibition assays were performed as previously described.[51] Briefly, assays were set up in duplicate using two different concentrations of IMPDH type I (87 and 155 nM) and type II (33 and 66 nM) and varying concentrations of inhibitor. IMPDH and inhibitors were added to 1 mL of reaction buffer (50 mM Tris, pH 8.0, 100 mM KCL, 1 mM DTT, 100 μM IMP, 100 μM NAD) at 25 °C and mixed gently while the production of NADH was monitored by following changes in absorbance at 340 nm on a Hitachi U-2000 spectrophotometer. Steady-state velocities were used to determine K_i (app) values by fitting the velocities vs inhibitor concentration to a simple binding model with Dynafit.[52]

Cellular Dehydrogenases: Horse Liver Alcohol Dehydrogenase (ADH) Malate (MAD) and Lactate Dehydrogenase (LAD). Inhibition studies were modified from previously described continuous assays (Goldstein 1994). Enzymes and substrates were purchased from Sigma. Enzymes were diluted in 100 mM sodium phosphate buffer pH 7.8 to give a final concentration of 50 μU/μL as determined by monitoring the generation of NADH at A_{360} under the assay conditions used. For ADH reactions, 180 mM EtOH and 100 μM NAD were used as substrates. LDH and MDH reactions contained 5 mM lactate and 5 mM malate, respectively, as well as 400 μM NAD. Enzymes (1 μL) were added to 100 μL of reaction buffer (enzyme specific substrates in 100 mM sodium phosphate buffer pH 7.8) and added to 96 half-volume plates containing inhibitors (1 μL). Generation of NADH was monitored at A_{360} over 5 min at 25 °C using a M5e plate reader (Molecular Devices). IC_{50} curves were generated for compounds that showed activity against ADH by fitting to a dose−response curve using Prism 4 (GraphPad Software, Inc.).

Cytotoxicity of MAD Analogues 37 and 38 to Human Myelogenous Leukemia K562 Cells. Logarithmically growing K562 cells in RPMI medium supplemented with 10% fetal bovine serum were plated in 96-well plates at a density of 2×10^3 cells/0.1 mL and incubated at 37 °C in an atmosphere of air and 5% CO_2. Twenty-four hours later, various concentrations of the compounds were added in 3 μL volume, mixed, and further incubated for 72 h. At the end of the incubation period, 20 μL of cellTiter 96 reagent (Promega, Madison, WI) containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS] was added and incubated at 37 °C in an atmosphere of air and 5% CO_2, the color developed was read at 490 nm using SpectraMax software in a microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA), and the data were analyzed by GraphPad Prism 4 software (GraphPad Software, San Diego, CA) as described earlier.[53]

Acknowledgment. This research was supported by USDOD Army grant W81XWH-05-01-0216 and by the Center for Drug Design in the Academic Health Center of the University of Minnesota. We thank Drs. Takashi Tsuji and Kunisuke Izawa of Ajinomoto Co., Inc., Japan, for a gift of MPA.

Supporting Information Available: HPLC profiles of compounds **25**−**33**, **37**, and **38**. This material is available free of charge via Internet at http://pubs.acs.org.

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JM070568J