Potent Inhibitors of Human Inosine Monophosphate Dehydrogenase Type II. Fluorine-Substituted Analogues of Thiazole-4-carboxamide Adenine Dinucleotide[1]

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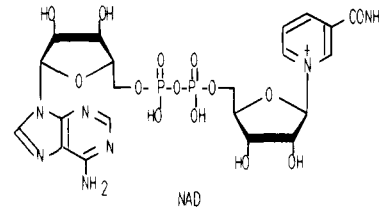
Three analogues of thiazole-4-carboxamide adenine dinucleotide (TAD) (**1-3**) containing a fluorine atom at the C2′ of the adenine nucleoside (in the *ribo* and *arabino* configuration) and at the C3′ (in the *ribo* configuration) were synthesized in high yield from the corresponding 5′-monophosphates of 2′-deoxy-2′-fluoroadenosine (**9**), 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-adenine (**17**), and 3′-deoxy-3′-fluoroadenosine (**14**), respectively. Pure 2′,3′-*O*-isopropylidene-tiazofurin 5′-phosphorimidazolide (**8**) was obtained by phosphorylation of the protected tiazofurin followed by treatment with carbonyldiimidazole and HPLC purification. Reaction of **8** with **9** in DMF-d_7 (monitored by ^1H and ^{31}P NMR) afforded the desired dinucleotide **12**, which after deisopropylidenation gave **1** in 82% yield. Small amounts of symmetrical dinucleotides AppA (**10**, 7.2%) and TRppTR (**11**, 8.0%) were also isolated during HPLC purification of the major product **12**. In a similar manner, compounds **2** and **3** were obtained by coupling of **8** with **14** and **17** in 80% and 76% yield, respectively. All newly prepared fluoro-substituted compounds as well as β-CF_2-TAD, earlier synthesized by us, showed good inhibitory activity against inosine monophosphate dehydrogenase type II, the isozyme which is predominant in neoplastic cells. Binding of **1** (K_{is} = 0.5 μM), **2** (K_{is} = 0.7 μM), and **3** (K_{is} = 2.9 μM) was comparable to that of TAD (K_i = 0.2 μM). The difluoromethylene bisphosphonate analogue, β-CF_2-TAD (K_i = 0.17 μM), was found to be equally effective as the best cofactor-type inhibitor, β-CH_2-TAD (K_i = 0.11 μM). Interestingly, the level of inhibition of horse liver alcohol dehydrogenase by these compounds was found to be much lower (0.1 mM for **1** and **2** and no inhibition up to 10 mM for **3**). These findings show that inhibition of tumor-induced inosine monophosphate dehydrogenase type II is selective and may be of therapeutic interest.

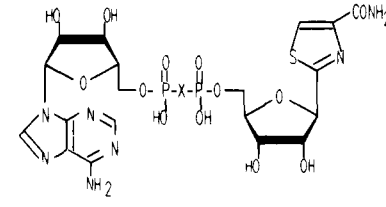
Introduction

Thiazole-4-carboxamide adenine dinucleotide (TAD; Chart 1) is the active metabolite of the oncolytic C-nucleoside 2-(β-D-ribofuranosyl)thiazole-4-carboxamide (tiazofurin, TR). TAD, an analogue of nicotinamide adenine dinucleotide (NAD), was found to be a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), the key enzyme in the *de novo* GTP biosynthesis and an important target in anticancer chemotherapy.[2-13] TAD mimics NAD but cannot function as the coenzyme. It has been discovered[14] recently that human IMPDH exists as two isoforms, type I and type II. In normal cells type I is the predominant isoenzyme, while type II is selectively up-regulated in neoplastic cells and emerges as the dominant species.[15,16] Thus, the development of inhibitors of IMPDH type II with limited interaction with type I would be definitely beneficial in drug design.

The goal in searching for antitumor agents based on IMPDH inhibition is to develop a compound which would not affect numerous cellular dehydrogenases but would act as a selective inhibitor of IMPDH type II. We have assumed that even subtle modifications in the structure of cofactor-based inhibitors, such as TAD, may alter selectivity against targeted dehydrogenases. Thus,

Chart 1





changes in conformation of TAD or alterations in hydrogen bonding between the inhibitor and various dehydrogenases should affect drug–enzyme interactions. As we discuss below, TAD analogues substituted with a fluorine atom at the C2′ or C3′ position of adenosine seem appropriate candidates for such studies.

X-ray structure determination of a number of dehydrogenase–NAD complexes indicate that the 2′- and 3′-hydroxy groups of both furanose rings of the cofactor

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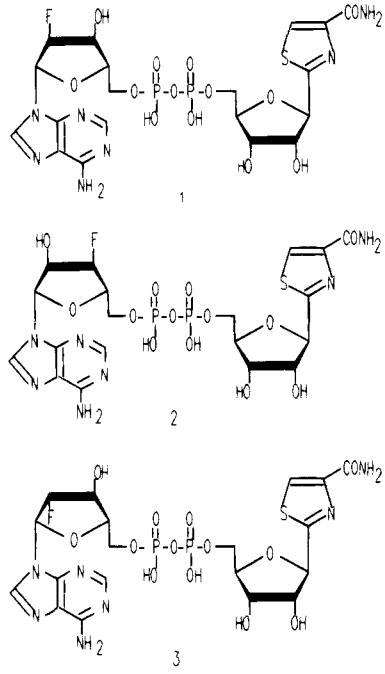
Chart 2



participate in hydrogen bonds which help anchor the ligand within the active site.[17-20] In horse liver alcohol dehydrogenase (LADH), initial recognition of the cofactor appears to occur *via* binding to the adenine end,[21] which requires a C2′-endo conformation of the adenosine ribose.[20] Other dehydrogenases require a C3′-endo conformation at this end of the cofactor.[18,28]

In IMPDH and LADH, TAD displaces NAD binding at the cofactor site.[20,22] We therefore expected that TAD analogues **1**–**3** containing 2′-fluoro- and 3′-fluoro substituted adenine nucleosides (Chart 2) would provide probes of the stereochemical requirements of the adenine end of the cofactor site on IMPDH. It is well known that a fluorine substituent mimics properties of a hydroxyl group in terms of size and polarity.[23] Fluorine acts as even stronger hydrogen bond acceptor than a hydroxyl oxygen but does not serve as hydrogen bond donor. The carbon–fluorine bond length, 1.39 Å, closely resembles the carbon–oxygen bond length, 1.43 Å. However, fluorine is a less sterically demanding substituent than the hydroxyl group having a van der Waals radius (1.35 Å) similar to that of hydrogen (1.29 Å). Replacement of the ribose hydroxyl group by fluorine can constrain the sugar conformation and improve its transport properties. For example, 2′-deoxy-2′-fluoroadenosine is a unique analogue of adenosine, which exhibits a number of interesting biological activities.[25-27] In this analogue, the ribose ring is in a C3′-endo conformation due to the highly electronegative 2′-substituent.[24] In contrast, 3′-deoxy-3′-fluoroadenosine[29-31] favors a C2′-endo sugar pucker.[29]

Similarly, TAD analogues with a fluorine substituent at either the C2′ (**1**) or C3′ (**2**) will demonstrate differences in preferred sugar conformation and hydrogen-bonding capabilities. However, little is known of the importance of the configuration of adenosine hydroxyl groups on the NAD binding in IMPDH. It has been reported that replacement of the nicotinamide riboside of NAD by 2′-deoxy-2′-fluoroarabinose yielded an active cofactor in at least one system.[32] Thus, the relative affinities of all three analogues are of interest. These agents will be expected to show preferential binding to different dehydrogenases, depending on the specific stereochemical demands of the adenosine site. In general, binding sites which place hydrogen-donating residues in close proximity to the fluorine substituent will be favored.

TAD analogues substituted with fluorine in the ribose moiety of adenosine are also more hydrophobic than their corresponding hydroxy congeners. The increased liphophilicity of fluorine-substituted TAD analogues in comparison with unmodified TAD is well expressed by their substantially longer retention times on reverse phase HPLC. This feature would be crucial in the activity of TAD analogues, which must be able to penetrate cell membranes. The β-methylene TAD[33] (β-CH_2-TAD; Chart 1) as well as β-difluoromethylene TAD[34] (β-CF_2-TAD), recently synthesized by us, can penetrate cell membranes although not as efficiently as tiazofurin.[35] Lastly, fluorinated TAD derivatives **1** and **3** are potentially less toxic than the parent compound. NAD analogues **1** and **3** do not contain the 2′-hydroxyl function of the adenosine moiety and therefore cannot be converted into the corresponding NADP analogue by cellular enzyme(s). Thus TAD analogues **1** and **3** are expected to be less toxic than TAD itself, since the former should be harmless toward numerous NADP-dependent cellular enzymes.

In this paper we report the synthesis of three TAD analogues (**1**–**3**) containing a fluorine atom at the C2′ (in the *ribo* and *arabino* configuration) or C3′ (in the *ribo* configuration) of the adenine nucleoside. We also report the preliminary results of inhibitory activity studies of our TAD analogues (**1**–**3**) as well as β-CF_2-TAD against the tumor-dominant IMPDH type II.

Results and Discussion

We have recently developed a new and simple method of direct introduction of a fluorine atom at C2′ in the β-configuration of purine nucleosides[36,37] as well as efficient methods for the synthesis of 2′-deoxy-2′-fluoro-[34] and 3′-deoxy-3′-fluoroadenosine from adenosine.[29] Thus, the fluoro-substituted adenine nucleosides were prepared according to these procedures and converted into their 5′-monophosphates by the Yoshikawa phosphorylation.[38]

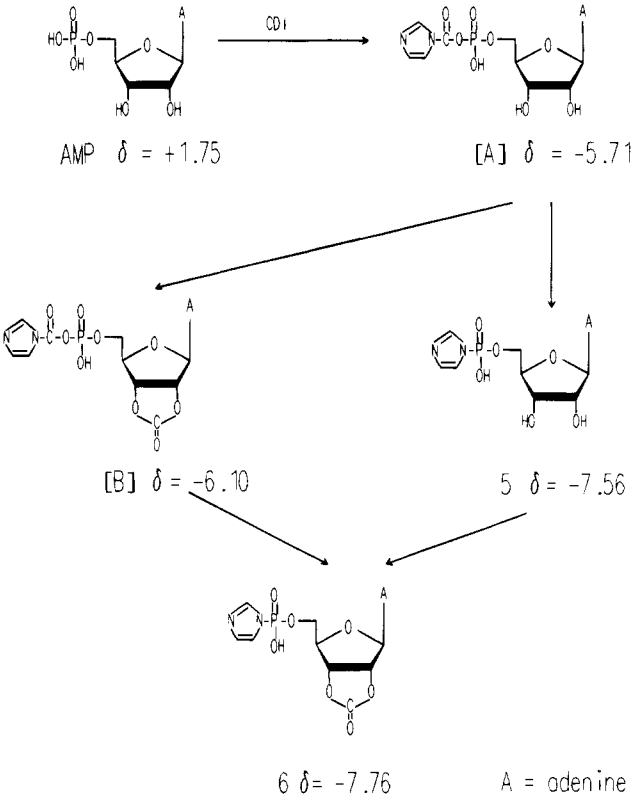
Although the first successful synthesis of NAD was described by Todd[39] in 1957, a number of recent papers discuss different approaches to the chemical synthesis of dinucleotide pyrophosphates. Oppenheimer *et al.*[32] synthesized 2′-fluoroarabinose by the procedure developed in our laboratory,[40] and then they synthesized 2′-fluoronicotinamide arabinoside 5′-monophosphate which was coupled with adenine mononucleotide (AMP). Using diphenyl phosphorochloridate as the coupling agent (according to Michelson's procedure[41]), the desired dinucleotide was obtained in 22% yield. The same procedure gave only 9% yield in Slama's preparation of the carbocyclic NAD analogue.[42] The alternative coupling of adenosine 5′-(dibutylphosphinothioylphosphoric) anhydride and carbanicotinamide 5′-mononucleotide (Furusawa's method[43]) afforded carbocyclic NAD in 50% yield. Finally, Marquez *et al.*[4,44] reported several different methods of preparation of TAD. Coupling of tiazofurin 5′-monophosphate (TRMP) with AMP in the presence of excess dicyclohexylcarbodiimide (DCC) gave

only low yields of TAD. A 16% yield, which was attained when activated AMP was coupled with TRMP according to Furusawa's procedure, was also disappointing. Activation of AMP as its phosphoromorpholidate followed by reaction with TRMP afforded TAD in up to 31% yield, depending on the temperature and time of reaction. Although it was believed that, in the morpholidate route, no symmetrical side products were formed (since only one nucleotide was activated), the formation of P^1,P^2-diadenosine 5′-pyrophosphate (AppA) in amounts almost equal to TAD was observed. The authors[44] explained that traces of water hydrolyzed the activated AMP back to AMP which in turn would compete with TRMP for the remaining activated AMP.

The best results were achieved[44] by carbonyldiimidazole (CDI) activation[45] of TRMP, and reaction *in situ* formed the 2′-carbonate derivative of the nucleotide imidazolide with AMP. The progress of the coupling reaction was followed by HPLC. In the same manner, several other NAD analogues were synthesized by these authors. The yields of isolated dinucleotides were generally in the range of 50%, and formation of AppA was not reported. Following Marquez's procedure, we have recently synthesized[34] the TAD analogue **1** in 50% yield by CDI-catalyzed coupling of TRMP with 2′-deoxy-2′-fluoroadenosine 5′-monophosphate (**4**).

In this paper we report a simple and effective synthesis of NAD analogues (70–80% yield) by modification of the CDI-catalyzed coupling reaction. We found that preparation of *pure nucleotide imidazolides* (known but not used) prior to the reaction with the corresponding nucleotides gave much better results than commonly used *in situ* generation (CDI) of the nucleotide imidazolides. The latter are sufficiently stable at about pH 7 for isolation by chromatographic purification.[45,46] Thus, we treated the commercially available AMP monohydrate with CDI (4 equiv) in Me_2SO-d_6 and followed the course of reaction (Scheme **1**) by ^{31}P (Figure **1**) and ^1H NMR. Immediately after addition of CDI, a complete disappearance of the resonance signal of **4** (δ 1.75) and the formation of intermediate **A** (δ −5.71) were observed in the ^{31}P NMR spectrum. The ^1H NMR of **A** (see the Experimental Section) revealed a downfield shift of approximately 0.15 ppm for the H5″ and the presence of three imidazolide protons at δ 6.99, 7.24, 7.48. After 20 min, the resonance signal of the intermediate **A** (70%) was still present but two new resonances, a singlet of intermediate **B** (16%) at δ −6.10 and another singlet of the AMP imidazolide (**5**; 14.5%) at δ −7.76, were detected. After 1 h the reaction mixture still contained **A** (7%), **B** (12%), and **5** (26%) but formation of a significant amount of 2′-cyclic carbonate derivative **6** (δ −7.75; 55%) was observed. Finally, all the reactants and intermediates disappeared, and compound **6** was the only product detected in the ^{31}P NMR spectrum of the reaction mixture (approximately 2 h). HPLC purification of **6** removed the excess imidazole but did not afford the pure AMP imidazolide 2′-cyclic carbonate (**6**) due to its hydrolysis to AMP imidazolide (**5**) in triethylammonium bicarbonate (TEAB) used as the eluent. Compounds **5** and **6** were well separated on the column, but after workup cyclic carbonate **6** was always contaminated with unprotected imidazolide **5**. Therefore compound **6** was treated with Et_3N–water to give pure imidazolide **5**

Scheme 1



quantitatively. This compound was quite resistant to hydrolysis at neutral pH. Even traces of AMP were undetectable in a sample of **5** which was kept in water solution at room temperature for more than 2 weeks.

Since the nucleotide imidazolide 2′-cyclic carbonates were not stable enough for HPLC purification, we synthesized the acetonide-protected tiazofurin 5′-phosphorimidazolide **8**, (Scheme 2) for further (NMR-monitored) reaction with our fluoro-substituted adenosine nucleotides. Thus, treatment of 2′,3′-*O*-isopropylidenetiazofurin 5′-monophosphate[4,44,47] (**7**) with CDI (1.1 equiv) in DMF-d_7 at room temperature for 1 h afforded the imidazolide **8** in quantitative yield. Again, the corresponding intermediate **A** was formed first and then converted into the desired imidazolide **8** as judged by ^{31}P NMR (disappearance of signal at δ 1.63 and appearance of the resonance peak of **A** at δ −6.53, which vanished with time with simultaneous formation of the final peak of **8** at δ −7.79). Imidazolide **8** was isolated by lyophilization of the reaction mixture and then separated from the excess imidazole by preparative HPLC and isolated as the triethylammonium salt, which was stable in neutral and alkaline conditions. Compound **8** was then dissolved in DMF-d_7 and allowed to react with nucleotide **9**[34] (1.5 equiv, as monotriethylammonium salt) at room temperature. After 7 days, the ^{31}P NMR analysis showed the disappearance of the imidazolide **8** singlet and the formation of a major group of resonance signals at δ 9.15–9.76. The resonance signal of unreacted **9** (20%) was also detected at δ 2.39. After lyophilization, the residue was chromatographed on a HPLC column to give nucleotide **9** (26 mg), P^1,P^2-bis(2′-deoxy-2′-fluoroadenosin-5′-yl) pyrophosphate (**10**; 8 mg), P^1-(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) P^2-(2′-deoxy-2′-fluoroadenosin-5′-yl) pyrophosphate (**12**; 105 mg), and P^1,P^2-bis(2′,3′-*O*-isopropylidenetiazofurin-5′-yl)

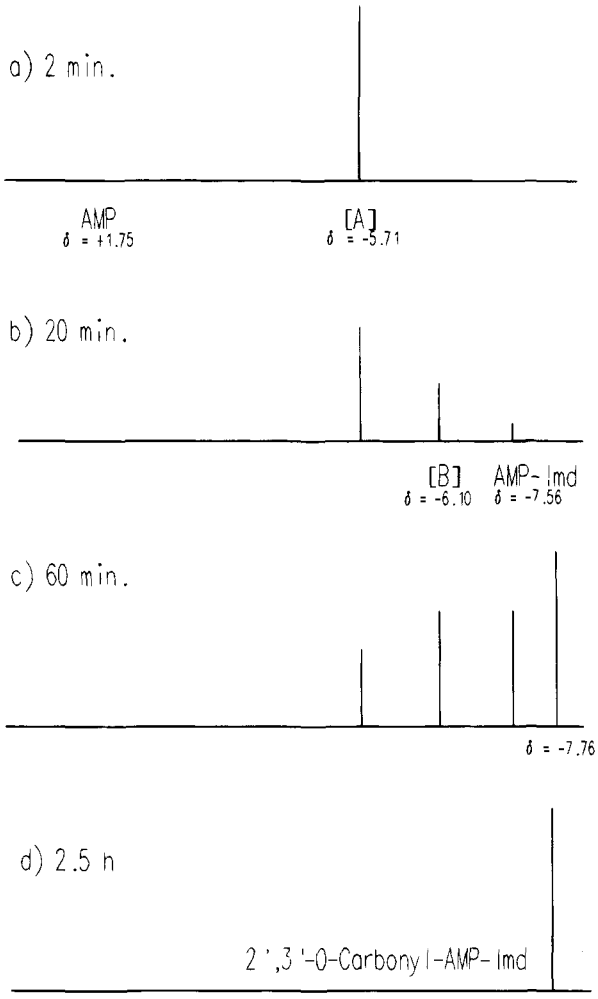


Figure 1. ^{31}P NMR spectra of reaction of AMP with CDI.

pyrophosphate (**11**; 15 mg). Treatment of **11** and **12** with Dowex 50W-X8 (H^+) in water gave the dinucleotide **13**[44] and the desired TAD analogue **1**, respectively. The overall yield of **1** was 56 mg (82%).

Reaction of 3'-deoxy-3'-fluoroadenosine 5'-monophosphate (**14**) with tiazofurin imidazolide derivative **8** required **11** days for completion, affording the TAD analogue **2** in 80% yield. Again the symmetrical pyrophosphate derivatives, namely P^1,P^2-bis(3'-deoxy-3'-fluoroadenosin-5'-yl) pyrophosphate (**15**) and bis(tiazofurin) pyrophosphate derivative **11**, were obtained in 6% and 5% yield, respectively.

In a similar manner, 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine 5'-monophosphate (**17**) was converted (5 days) into the corresponding TAD analogue **3** in 76% yield. The symmetrical dinucleotide **18** (6%) and ditiazofurin pyrophosphate **11** (5%) were also isolated.

Finally, we studied the possibility of imidazolide activation of our fluoro-substituted nucleotides (**9**, **14**, and **17**) and subsequent coupling of these imidazolides with TRMP. Although such a procedure did not require any protection to avoid formation of 2'-cyclic carbonates in reaction with CDI and further coupling was equally efficient, we encountered serious difficulties in HPLC separation of unprotected TAD analogues from symmetrical dinucleotides (AppA analogues and TRppTR). The procedure with acetonide-protected TRMP was found to be the most efficient.

Since the "activated" nucleotide **5** was found to be resistant to hydrolysis in water, disproportionation of

Scheme 2

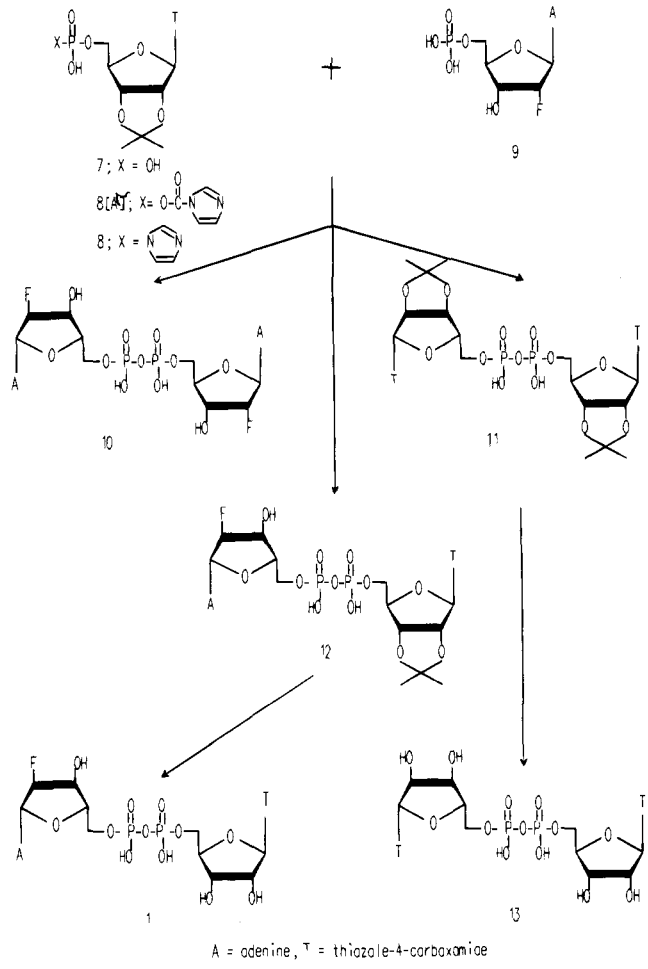


Table 1. Inhibition of Human IMPDH Type II by TAD Analogues[a]

inhibitor	K_{ii} (μM)	K_{is} (μM)
1	0.5 ± 0.1	6.0 ± 12.0
2	0.7 ± 0.1	1.5 ± 0.6
3	2.9 ± 0.5	13 ± 10
β-CF_2-TAD	0.17 ± 0.03	0.3 ± 0.2
β-CH_2-TAD	0.11 ± 0.02	0.2 ± 0.1
TAD	0.19 ± 0.03	0.3 ± 0.1
NADH	120 ± 7	175 ± 22

[a] NAD is the variable substrate, with IMP constant at 100 μM. The pattern of inhibition in each case is noncompetitive. Values were obtained at 37 °C.

unsymmetrical dinucleotides rather than hydrolysis of the imidazolides was apparently responsible for the formation of symmetrical pyrophospates. The low yields of coupling reactions reported in the literature were probably due to poor control of reaction conditions in *in situ*-generated reacting species.

Inhibition of inosine monophosphate dehydrogenase type II (IMPDH type II) by the fluorinated TAD analogues **1**–**3**, the parent compound TAD, and the phosphodiesterase resistant analogues β-CH_2-TAD and β-CF_2-TAD were examined. As noted IMPDH type II is the target isozyme, predominant in neoplastic cells.[15,16]

Interestingly, all the fluorinated compounds showed good inhibitory activity against IMPDH type II, generally binding in the 10^{-7} M range (Table I). The pattern of inhibition with respect to NAD is noncompetitive in each case. Like type I, IMPDH type II follows an ordered bi–bi mechanism, the binding of substrate

preceding that of cofactor.[14] Thus, this pattern of inhibition is consistent with binding of the inhibitors to both free enzyme and the enzyme–substrate complex.[48]

In most cases, binding is tighter than or comparable to that seen for binding of TAD to human IMPDH type II (Table 1), as well as to IMPDH from a variety of sources.[4,8,12,22] Binding of β-CF_2-TAD is equivalent to that of both TAD and the phosphorodiesterase resistant analogue β-CH_2-TAD,[33] itself a biologically effective inhibitor.[9] Compounds **1**–**3** showed similar affinity, the *ara*-fluorinated derivative **3** showing the weakest binding. However, in all cases, binding is significantly tighter than that of NADH.

In a preliminary effort to examine specificity of the fluorinated compounds, inhibition of horse LADH by analogues **1**–**3** was also examined. Although limited quantities of material prevented precise measurements, initial results showed the fluorine-substituted analogues to be relatively weak LADH inhibitors. K_i's for **1**, **2**, and β-CF_2-TAD were estimated to be no less than 10^{-4} M. No inhibition of LADH was observed for the 2′-*ara*-substituted compound **3** up to 10^{-2} M.

Conclusions

Results suggest that fluorine substituents at C2′ and C3′ of the sugar can effectively mimic the functional role of the hydroxyl group in IMPDH type II. Displacement of this functionality to the 2′-*ara* position is less successful, although still surprisingly effective in this enzyme. These findings suggest either the presence of the key electrophilic residues at the adenine pocket and/or sufficient flexibility in this site to adopt to functional changes at the adenine end of the inhibitor. This flexibility is apparently not present in LADH, whose site is dominated by the nucleophilic Asp 223.[18,23] Although, these questions will be addressed by further studies, current findings do indicate that the newly synthesized compounds are potent inhibitors of IMPDH type II, show some selectivity, and may be of therapeutic interest. Examination of transport, metabolism, and toxicity of these fluoro-substituted analogues is now in progress.

Experimental Section

General Methods. Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. HPLC was performed on a Dynamax-60A C18–83–221-C column with flow rate of 5 mL/min or Dynamax-300A C18–83–243-C column with a flow rate of 20 mL/min of 0.1 M Et_3N H_2CO_3 (TEAB) followed by a linear gradient of 0.1 M TEAB–aqueous MeCN (70%). Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Nuclear magnetic resonance spectra were recorded on Bruker AMX-250 and –400 spectrometers with Me_4Si or DDS as the internal standard for ^1H and ^{13}C NMR and external H_3PO_4 for ^{31}P NMR. Chemical shifts are reported in ppm (δ), and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), and dd (double doublet). Values given for coupling constants are first order.

Reaction of AMP with 1,1′-Carbonyldiimidazole. To a solution of adenosine 5′-monophosphate monohydrate (**4**; 36.5 mg, 0.1 mmol) in Me_2SO-d_6 (1 mL) was added CDI (76 mg, 0.4 mmol), and the reaction mixture was monitored using ^{31}P and ^1H NMR. After 3 min, the ^{31}P resonance signal of **4** at δ 1.75 disappeared and a singlet of intermediate **A** emerged at δ –5.71. ^1H NMR of **4**: δ 3.97 (dd, 1H, H5″, $J_{4',5''}$ = 6.10 Hz, $J_{5'5''}$ = 12.20 Hz), 4.04–4.08 (m, 2H, H4′, H5′), 4.16–4.20 (m, 1H, H3′), 4.59 (pseudo t, 1H, H2′), 5.42 (bs, 4H, 4 x OH), 5.92 (d, 1H, H1′, $J_{1',2'}$ = 5.75 Hz), 7.43 (s, 2H, NH_2), 8.17, 8.35 (two 1H singlets, H2, H8). ^1H NMR of **A**: δ 4.10–4.12 (m, 3H, H4′, H5′, H5″), 4.22 (m, 1H, H3′), 4.62 (pseudo t, 1H, H2′), 5.95 (d, 1H, H1′, $J_{1',2'}$ = 5.96 Hz), 6.69 (bs, 3 x OH), 6.99, 7.27, 7.48 (three 1H singlets, imidazole), 7.34 (s, 2H, NH_2), 8.14, 8.43 (two 1H singlets, H2, H8), 7.20, 7.78, 8.36 (three singlets of excess CDI).

After 20 min, intermediate **A** remained a major component (70%) of the reaction mixture but two new resonance signals, i.e., a singlet of intermediate **B** (16%) at δ –6.09 and a singlet of **5** (14%) at δ –7.76, appeared. After 1 h, the reaction mixture contained four compounds: **A** (7%), **B** (12%), **5** (26%), and **6** (55%; singlet at δ –7.75), and after 2.5 h, compound **6** was the only product detected. ^1H NMR: δ 3.72–3.83 (m, 2H, H5′, H5″), 4.41–4.45 (m, 1H, H4′), 5.44 (dd, 1H, H3′, $J_{2',3'}$ = 7.7 Hz, $J_{3',4'}$ = 3.2 Hz), 5.93 (dd, 1H, H2′, $J_{1',2'}$ = 2.3 Hz), 6.46 (dd, 1H, H1′), 6.92, 7.19, 7.77 (three 1H singlets, imidazole), 7.48 (s, 2H, NH_2), 8.12, 8.32 (two 1H singlets, H2, H8), 7.10, 7.84 (two singlets of the free imidazole).

The mixture was then lyophilized, and the residue was chromatographed on an HPLC column to give **5** (10 mg, 20%) and an approximately 9:1 mixture of **6** and **5** (45 mg). This mixture was dissolved in water–Et_3N (pH 9), kept at room temperature for 2 h, and concentrated *in vacuo* to give **5** (33 mg). Total yield of **5** was 43 mg, 100%. ^1H NMR (Me_2SO-d_6): δ 1.00 (t, 9H, Et_3N), 2.70 (q, 6H, Et_3N), 3.60–3.76 (m, 2H, H5′, H5″), 3.89–3.91 (m, 1H, H4′), 3.99–4.06 (m, 1H, H3′), 4.57 (pseudo t, 1H, H2′), 5.88 (d, 1H, H1′, $J_{1',2'}$ = 6.1 Hz), 6.85, 7.10, 7.65 (three 1H singlets, imidazole), 7.25 (s, 2H, NH_2), 8.12, 8.40 (two 1H singlets, H2, H8).

2′,3′-*O*-Isopropylidenetiazofurin 5′-Monophosphate (7) and 2-(2,3-*O*-Isopropylidene-β-D-ribofuranosyl)thiazole-4-carbonitrile 5′-Monophosphate (7a).[47] To a suspension of 2′,3′-*O*-isopropylidenetiazofurin[4] (300 mg, 1 mmol) in triethyl phosphate (2 mL) was added a mixture of $(EtO)_3PO$ (2 mL) containing water (20 μL) and $P(O)Cl_3$ (200 μL). The mixture was kept at 5–10 °C for 20 h and then added dropwise into a solution of 2 M TEAB (6 mL) in water (100 mL). Extraction with EtOAc (2 × 50 mL) and concentration of the aqueous layer *in vacuo* gave the residue which was further purified on a HPLC column. After lyophilization two compounds were obtained. Faster migrating **7**, as monotriethylammonium salt (240 mg, 50%): ^{31}P NMR (D_2O) δ 0.64 (t, $J_{5',5'',P}$ = 5.5 Hz); ^1H NMR (D_2O) δ 1.29 (t, 9H, Et_3N), 1.46 (s, 3H, iPr), 1.66 (s, 3H, iPr), 3.19 (q, 6H, Et_3N), 3.99 (pseudo t, 2H, H5′, H5″), 4.56–4.60 (m, 1H, H4′), 5.02 (dd, 1H, H3′, $J_{2',3'}$ = 6.1 Hz, $J_{3',4'}$ = 2.3 Hz), 5.20 (dd, 1H, H2′, $J_{1',2'}$ = 4.0 Hz), 5.38 (d, 1H, H1′), 8.27 (s, 1H, H5). ^{13}C NMR (D_2O) δ 11.11 (Et_3N), 27.32 (iPr), 29.10 (iPr), 49.51 (Et_3N), 67.74 (d, C5′, $J_{C,P}$ = 4.9 Hz), 85.00 (C2′), 86.87 (C3′), 87.14 (C4′, d, $J_{C,P}$ = 9.0 Hz), 87.77 (C1′), 117.70 (iPr), 129.47 (C5), 151.00 (C4), 168.28 (C=O), 173.76 (C2). Slower migrating **7a** (138 mg, 30%; triethylammonium salt): ^{31}P NMR (D_2O) δ 0.58 (dt, $J_{5',5'',P}$ = 5.6 Hz, $J_{4',P}$ = 1.8 Hz); ^1H NMR (D_2O) δ 1.29 (t, 9H, Et_3N), 1.45 (s, 3H, iPr), 1.65 (s, 3H, iPr), 3.19 (q, 6H, Et_3N), 3.98 (pseudo t, 2H, H5′, H5″), 4.59–4.62 (m, 1H, H5′), 5.03 (dd, 1H, H3′, $J_{2',3'}$ = 6.0 Hz, $J_{3',4'}$ = 2.0 Hz), 5.20 (dd, 1H, H2′, $J_{1',2'}$ = 3.8 Hz), 5.38 (d, 1H, H1′), 8.48 (s, 1H, H5); ^{13}C NMR (D_2O) δ 11.11 (Et_3N), 27.30 (iPr), 29.10 (iPr), 49.51 (Et_3N), 67.94 (d, C5′, $J_{C,P}$ = 4.8 Hz), 85.21 (C2′), 87.30 (C3′), 87.42 (C4′, d, $J_{C,P}$ = 9.2 Hz), 87.94 (C1′), 117.00 (CN), 117.55 (iPr), 127.82 (C4), 136.99 (C5), 176.37 (C2).

2′,3′-*O*-Isopropylidenetiazofurin 5′-Phosphoimidazolide (8). Nucleotide **7** (96 mg, 0.20 mmol as monotriethylammonium salt) was dissolved in DMF-d_7 (1 mL), CDI (33 mg, 0.22 mmol) was added, and the progress of the reaction was monitored by ^{31}P NMR. The resonance signal of **7** (δ 1.63) disappeared, and the new signal of intermediate **A** emerged at δ –6.53, which diminished in time with simultaneous formation of the resonance of the imidazolide derivative **8** (δ –7.79). After 30–40 min, the reaction was completed, the mixture was lyophilized, and the residue was dissolved in 0.1 M TEAB (3 mL) and purified by HPLC to give nucleotide imidazolide **8** (100 mg, 94%; triethylammonium salt): ^{31}P NMR (D_2O) δ –7.80; ^1H NMR (D_2O) δ 1.42 (s, 3H, iPr), 1.61 (s, 3H, iPr), 3.92–3.97 (m, 2H, H5′, H5″), 4.42–4.57 (m, 1H, H4′),

4.79–4.81 (m, 1H, H3′), 5.18 (dd, 1H, H2′, $J_{1',2'}$ = 3.4 Hz, $J_{2',3'}$ = 6.0 Hz), 5.33 (d, 1H, H1′), 7.03, 7.11, 7.75 (three 1H singlets, imidazole), 8.17 (s, 1H, H5).

2′-Deoxy-2′-fluoroadenosine 5′-Monophosphate (9).[49] 2′-Deoxy-2′-fluoroadenosine (269 mg, 1 mmol) was treated with $P(O)Cl_3$ in the same manner as 2′,3′-*O*-isopropylidenetiazofurin to give **9**, as its triethylammonium salt, in 78% yield: ^{31}P NMR (D_2O) δ 0.90; ^{1}H NMR (D_2O) δ 1.27 (t, 9H, Et_3N), 3.19 (q, 6H, Et_3N), 4.15 and 4.27 (dd of AB system, 2H, H5′, H5″, $J_{5',5''}$ = 12.0 Hz, $J_{4',5'}$ = 3.0 Hz, $J_{5',P}$ = 5.5 Hz, $J_{4',5''}$ = 2.4 Hz, $J_{5'',P}$ = 4.5 Hz), 4.34–4.42 (m, 1H, H4′), 4.69 (ddd, 1H, H3′, $J_{2',3'}$ = 4.4 Hz, $J_{3',4'}$ = 7.4 Hz, $J_{3',F}$ = 20.0 Hz), 5.40 (ddd, 1H, H2′, $J_{1',2'}$ = 2.15 Hz, $J_{2',F}$ = 52.0 Hz), 6.39 (dd, 1H, H1′, $J_{1',F}$ = 16.2 Hz), 8.22 and 8.41 (two 1H singlets, H2, H8). Anal. ($C_{16}H_{28}FN_6O_6P\cdot 3H_2O$) C,H,N.

3′-Deoxy-3′-fluoroadenosine 5′-Monophosphate (14).[50] **14** was prepared, as the triethylamonium salt, as above from 3′-deoxy-3′-fluoroadenosine (269 mg, 1 mmol) in 65% yield: ^{31}P NMR (D_2O) δ 0.74; ^{1}H NMR (D_2O) δ 1.26 (t, 9H, Et_3N), 3.18 (q, 6H, Et_3N), 4.08 and 4.16 (m and dd of AB system, 2H, H5′, H5″, $J_{5',5''}$ = 11.8 Hz, $J_{4',5''}$ = 2.5 Hz, $J_{5'',P}$ = 5.2 Hz), 4.62–4.78 (d of m, 1H, H4′, $J_{4',F}$ = 27.4 Hz), 4.92 (ddd, 1H, H2′, $J_{1',2'}$ = 8.2 Hz, $J_{2',3'}$ = 4.5 Hz, $J_{2',F}$ = 25.0 Hz), 5.31 (dd, 1H, H3′, $J_{3',F}$ = 53.7 Hz), 6.16 (d, 1H, H1′), 8.19 and 8.48 (two 1H singlets, H2, H8). Anal. ($C_{16}H_{28}FN_6O_6P\cdot 3H_2O$) C,H,N.

9-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)adenine 5′-Monophosphate (17). **17** was prepared, as its triethylammonium salt, as above from 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (269 mg, 1 mmol) in 82% yield: ^{31}P NMR (D_2O) δ 0.80; ^{1}H NMR (D_2O) δ 1.22 (t, 9H, Et_3N), 3.12 (q, 6H, Et_3N), 4.02–4.06 (m, 2H, H5′, H5″), 4.21 (q, 1H, H4′, $J_{3',4'}$ = $J_{4',5'}$ = $J_{4',5''}$ = 4.95 Hz), 4.69 (ddd, 1H, H3′, $J_{2',3'}$ = 4.4 Hz, $J_{3',F}$ = 19.0 Hz), 5.34 (dt, 1H, H2′, $J_{2',F}$ = 51.7 Hz), 6.48 (dd, 1H, H1′, $J_{1',2'}$ = 4.4 Hz, $J_{1',F}$ = 14.5 Hz), 8.18 (s, 1H, H2), 8.48 (d, 1H, H8, $J_{8,F}$ = 2.1 Hz). Anal. ($C_{16}H_{28}FN_6O_6P\cdot 3H_2O$) C,H,N.

TAD Analogues. P^1-(Tiazofurin-5′-yl) P^2-(2′-Deoxy-2′-fluoroadenosin-5′-yl) Pyrophosphate (1). To a solution of **8** (53 mg, 0.1 mmol) in DMF-d_7 (1 mL) was added the nucleotide **9** (70 mg, 0.155 mmol; monotriethylammonium salt), and the mixture was kept at room temperature for 7 days. After that time, ^{31}P NMR analysis showed a complete disappearance of the imidazolide **8** resonance, the presence of an excess of **9** (δ 2.39), and a group of signals at δ 9.15–9.75. The reaction mixture was then lyophilized, and the residue was chromatographed by HPLC to give nucleotide **9** (t_R = 31.3 min; 26 mg), P^1,P^2-bis(2′-deoxy-2′-fluoroadenosin-5′-yl) pyrophosphate (**10**) [t_R = 35.1 min; 8 mg; ^{1}H NMR (D_2O) δ 1.28 (t, 9H, Et_3N), 3.20 (q, 6H, Et_3N), 4.22–4.42 (m, 4H, 5′,5″), 4.44–4.54 (m, 2H, H4′), 4.57 (ddd, 2H, H3′, $J_{3',F}$ = 21.5 Hz, $J_{2',3'}$ = 3.4 Hz, $J_{3',4'}$ = 7.8 Hz), 5.21 (dd, 2H, H2′, $J_{2',F}$ = 52.2 Hz), 6.18 (d, 2H, H1′, $J_{1',F}$ = 15.6 Hz), 8.03, 8.14 (two 2H singlets, H2, H8), ^{31}P NMR (D_2O) δ −10.75], P^1-(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) P^2-(2′-deoxy-2′-fluoroadenosin-5′-yl) pyrophospate (**12**) (t_R = 43.7 min; 105 mg), and P^1,P^2-bis(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) pyrophosphate (**11**) (t_R = 51.2 min; 15 mg).

Compounds **11** and **12** were deisopropylidenated by treatment with Dowex 50-X8 (H^+) in water overnight and purified by passing through a column of Dowex 50-X8 (H^+) to give dinucleotide **13** (6 mg, 8.0%; the ^{1}H NMR spectrum of this compound was identical with that of authentic sample[44]) and **1** (56 mg, 82%), respectively. TAD analogue **1** was identical with that reported by us earlier.[34] Compound **10** was also converted into free acids by passing through a column of Dowex 50-X8 (H^+). The yield of **10** was 5 mg (7.2%).

P^1-(Tiazofurin-5′-yl) P^2-(3′-Deoxy-3′-fluoroadenosin-5′-yl) Pyrophosphate (2). To a solution of **8** (53 mg, 0.1 mmol) in DMF-d_7 (1 mL) was added 3′-deoxy-3′-fluoroadenosine 5′-monophosphate (**14**; 70 mg, 0.155 mmol; monotriethylammonium salt), and the mixture was kept at room temperature for 10 days. The ^{31}P NMR analysis showed a complete disappearance of the imidazolide **8** resonance, the presence of an excess of **14** (δ 2.32), and the group of signals at δ −9.57 to −9.09. The reaction mixture was then lyophilized, and the residue was applied on a preparative HPLC column to give nucleotide **14** (t_R = 30.6 min; 21 mg), P^1,P^2-bis(3′-deoxy-3′-fluoroadenosin-5′-yl) pyrophosphate (**15**) [t_R = 34.8 min; 11 mg; ^{1}H NMR (D_2O) δ 1.27 (t, 9H, Et_3N), 3.19 (q, 6H, Et_3N), 4.28 (pseudo s, 4H, 5′,5″), 4.70 (d, 2H, H4′, $J_{4',F}$ = 27.0 Hz), 4.82 (ddd, 2H, H2′, $J_{2',F}$ = 27.0 Hz), 5.30 (dd, 2H, H3′, $J_{3',F}$ = 54.0 Hz, $J_{2',3'}$ = 4.25 Hz), 6.02 (d, 2H, H1′, $J_{1',2'}$ = 8.0 Hz), 8.05, 8.20 (two 2H singlets, H2, H8); ^{31}P NMR (D_2O) δ −10.8], P^1-(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) P^2-(3′-deoxy-3′-fluoroadenosin-5′-yl) pyrophospate (**16**) (t_R = 42.6 min; 98 mg), and P^1,P^2-bis(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) pyrophosphate (**11**) (t_R = 51.2 min; 13 mg).

Compounds **11** and **16** were deisopropylidenated by treatment with Dowex 50-X8 (H^+) in water overnight and purified by passing through a column of Dowex 50-X8 (H^+) to give dinucleotide **13** (5 mg, 7.5%) and TAD analogue **2** (54 mg, 80%), respectively. Compound **2**: ^{31}P NMR (D_2O) δ −10.75 (bs); ^{1}H NMR δ 4.21–4.34 [m, 7H, H2′(T), H3′(T), H4′(T), H5′(T), H5″(T), H5′(A), H5″(A)], 4.68–4.74 [m, 1H, H4′(A)], 4.91–5.05 [m, 1H, H2′(A)], 5.09 [d, 1H, H1′(T), $J_{1',2'}$ = 5.2 Hz], 5.36 [dd, 1H, H3′(A), $J_{2',F}$ = 53.5 Hz, $J_{2',3'}$ = 4.2 Hz], 6.22 [d, 1H, H1′(A), $J_{1',2'}$ = 7.9 Hz], 8.05 [s, 1H, H5(T)], 8.40 [s, 1H, H2(A)], 8.63 [s, 1H, H8(A)]. Anal. ($C_{19}H_{24}FN_7O_{13}P_2S\cdot 5H_2O$) C,H,N.

Compound **15** was also converted into its free acid form by passing it through a column of Dowex 50-X8 (H^+). The yield of **15** was 4 mg (5.8%): ^{31}P NMR δ 10.75 (bs); ^{1}H NMR (D_2O) δ 4.27–4.31 (m, 3H, H4′, H5′, H5″), 4.7 (dt, 1H, H2′, $J_{1',2'}$ = 8.1 Hz, $J_{2',F}$ = 27.8 Hz, $J_{2',3'}$ = 4.4 Hz), 5.30 (dd, 1H, H3′, $J_{3',F}$ = 53.7 Hz), 6.03 (d, 1H, H1′), 8.06, 8.19 (two 1H singlets, H2, H8).

P^1-(Tiazofurin-5′-yl) P^2-[9-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)adenin-5′-yl] Pyrophosphate (3). To the solution of **8** (53 mg, 0.1 mmol) in DMF-d_7 (1 mL) was added the nucleotide **17** (70 mg, 0.155 mmol; monotriethylammonium salt), and the mixture was kept at room temperature for 5 days. The ^{31}P NMR analysis showed a complete disappearance of the imidazolide **8** resonance, the presence of the unreacted excess of **17** at δ 2.45, and the group of signals at δ −9.82 to −9.36. The reaction mixture was lyophilized, and the residue was purified by HPLC to give nucleotide **17** (t_R = 32.4 min; 27 mg), P^1,P^2-bis[9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenin-5′-yl] pyrophosphate (**18**) [t_R = 36.3 min; 10 mg; ^{1}H NMR (D_2O) δ 1.28 (t, 9H, Et_3N), 3.19 (q, 6H, Et_3N), 4.18–4.22 (m, 6H, 4′,5″), 4.62 (dt, 2H, H3′, $J_{3',F}$ = 18.35 Hz, $J_{2',3'}$ = 3.8 Hz), 5.21 (dt, 2H, H2′, $J_{2',F}$ = 51.7 Hz, $J_{1',2'}$ = 4.0 Hz), 6.23 (dd, 2H, H1′, $J_{1',F}$ = 14.0 Hz), 7.99, 8.17 (two 2H singlets, H2, H8); ^{31}P NMR (D_2O) δ −10.6], P^1-(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) P^2-[9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenin-5′-yl] pyrophospate (**19**) [t_R = 43.3 min; 100 mg; ^{31}P NMR δ −10.50 (P^1), −11.10 (P^2, AB system, $J_{P,P}$ = 21.3 Hz); ^{1}H NMR δ 1.22 (t, 18H, 2 x Et_3N), 1.31 (s, 3H, iPr), 1.60 (s, 3H, iPr), 3.03 (q, 12H, 2x Et_3N), 4.08–4.12 [m, 2H, H5′(A), H5″(A)], 4.25–4.30 [m, 3H, H5′(T), H5″(T), H4′(A)], 4.44–4.47 [m, 1H, H4′(T)], 4.64–4.90 [m, 3H, H3′(A), H3′(T), H2′(T)], 5.14 [d, 1H, H1′(T), $J_{1',2'}$ = 3.2 Hz], 5.35 [double pseudo t, 1H, H2′(A), $J_{2',F}$ = 51.8 Hz], 6.49 [dd, 1H, H1′(A), $J_{1',2'}$ = 4.6 Hz, $J_{1',F}$=13.2 Hz], 8.08 [s, 1H, H5(T)], 8.18 [s, 1H, H2(A)], 8.43 [d, 1H, H8(A), $J_{8,F}$=1 Hz]], and P^1,P^2-bis(2′,3′-*O*-isopropylidenetiazofurin-5′-yl) pyrophosphate (**11**) (t_R = 51.3 min; 9 mg).

Compound **19** was deisopropylidenated by treatment with Dowex 50-X8 (H^+) in water overnight and purified by passing through a column of Dowex 50-X8 (H^+) to give dinucleotide **3** (51 mg, 76%): ^{31}P NMR δ −10.71 (bs); ^{1}H NMR δ 4.21–4.34 [m, 8H, H2′(T), H3′(T), H4′(T), H5′(T), H5″(T), H4′(A), H5′(A), H5″(A)], 4.72 [two pseudo t, 1H, H3′(A), $J_{3',F}$=18.3 Hz], 5.09 [d, 1H, H1′(T), $J_{1',2'}$ = 4.4 Hz], 5.37 [two pseudo t, 1H, H2′(A), $J_{2',F}$ = 51.7 Hz], 6.56 [dd, 1H, H1′(A), $J_{1',2'}$ = 4.6 Hz, $J_{1',F}$ = 12.5 Hz], 8.05 [s, 1H, H5(T)], 8.40 [s, 1H, H2(A)], 8.52 [d, 1H, H8(A), $J_{8,F}$ = 1.6 Hz]. Anal. ($C_{19}H_{24}FN_7O_{13}P_2S\cdot 4H_2O$) C,H,N.

Inhibition of IMPDH Type II and LADH. Purified human recombinant IMPDH type II was obtained from Dr. George D. Markham of the Institute for Cancer Research, Fox Chase Cancer Center, Fox Chase, PA. Horse liver ADH was obtained in crystalline form from Boehringer Mannheim Biochemicals, IN. β-Nicotinamide adenine dinucleotide (NAD) in crystalline free acid form was also obtained from Boe-

hringer. Inosine 5′-monophosphate (IMP) was purchased from Sigma Chemical Co., St. Louis, MO, as the disodium salt. All solutions were prepared using nanopure water.

Kinetic constants of the inhibitors with respect to NAD were obtained by monitoring spectrophotometrically the rate of production of NADH during the reaction. The course of the reaction was followed by measuring the changing absorbance of the reaction mixture at 340 nm, the absorbance peak for NADH, using a Beckman DU-65 spectrophotometer and an extinction coefficient of 6.22 Å mM^{-1} cm^{-1} for the reduced cofactor.[51]

Human IMPDH type II was stored at −70 °C in buffer containing 20 mM KCl, 1 mM EDTA, 1 mM DTT, and 20 mM Tris-HCl adjusted to pH 8 at 23 °C. In preparation for the assays, a frozen aliquot was thawed rapidly and filtered through a 0.45 μm acetate filter. Protein concentration was determined from a Coomassie assay. In some cases the enzyme was diluted 4:1 in similar 20 mM Tris buffer. NAD and IMP solutions were prepared on the basis of molecular weight. The IMP concentration at the beginning of each run was kept at 100 μM, approximately 10 times the published K_m for IMPDH.[14]

Combination of the reactants was performed as follows: An aliquot of substrate solution was pipetted down one side of a cuvet, one of cofactor down another side, and inhibitor down a third. The volume was brought to 990 μL with reaction buffer, and the cuvet was agitated. This mixture was allowed to incubate in the spectrophotometer sample holder for 5 min before the reaction was started, the temperature held at 37 °C by a circulating water bath. The reaction was initiated by the introduction of 10 μL of enzyme solution on a mixing plunger, and NADH production was monitored for between 2 and 10 min, depending on the activity of the protein solution.

The uninhibited rate of reaction as a function of cofactor concentration was first established. Velocities at four NAD concentrations and saturating substrate concentrations were measured, and K_m for NAD was obtained. Inhibitor was then introduced to the reaction mixture with an NAD concentration in the vicinity of K_m. The initial trial concentration of inhibitor was 100 μM. If no reaction was observed, the inhibitor solution underwent serial 10:1 dilutions until reasonable velocities were obtained.

Once a range of inhibitor concentrations resulting in measurable velocities was established, runs were performed with NAD concentrations of 20, 40, 80, and 160 μM, with two or three inhibitor concentrations. Lineweaver–Burk plots were used to assess the quality of the data and initially characterize the mode of inhibition with respect to NAD. However, values of inhibition constants and patterns of inhibition were ultimately obtained by direct least-squares fits to the nonreciprocal forms of the Michaelis–Menten rate equation.[52] The pattern of inhibition considered to best account for the observed data was that giving both the smallest residuals between observed and calculated values and the smallest standard errors in the computed kinetics constants. Data for all compounds tested were most consistent with a noncompetitive pattern of inhibition of IMPDH type II with respect to NAD at saturating IMP concentrations. Consequently, values of K_{ii} (intercept) and K_{is} (slope) reported in Table 1 were obtained from direct least-squares fits to the expression: $v_o = V_m[\mathbf{A}]/\{K_m(1 + [I]/K_{is}) + [\mathbf{A}](1 + [I]/K_{ii})\}$, where v_o is the measured initial rate, V_m is the maximum rate, K_m is the Michaelis constant of NAD, and **A** is concentration of NAD.

Preliminary assays of alcohol dehydrogenase were performed in a similar manner to that described above. LADH in crystalline suspension was spun down to a pellet and dissolved in 100 mM Tris-HCl buffer containing 100 mM KCl adjusted to pH 8 at 23 °C. Stock solutions of NAD and ethanol were prepared in buffer on the basis of molecular weight and volume, respectively. Ethanol was introduced as an aliquot of stock solution, and its concentration was kept constant at 1.2 mM while NAD concentration was varied by serial dilution of the stock solution. If the initial trial concentration did not influence the velocity measurably, larger amounts of inhibitor were used to established at least a lower bound for K_i, given the limited amounts of inhibitors available.

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(50) The long range coupling of the fluorine with H5′ ($J_{5',F}$ = 1.2 Hz) resulted in the presence of the H5′ multiplet in the simulated ^1H NMR spectrum of 14. Similarly, the multiplet resonance of H4′ was due to the coupling of H4′ with the phosphorus with estimated coupling constant $J_{4',P}$ = 1.8 Hz.
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