Isosteric Analogues of Nicotinamide Adenine Dinucleotide Derived from Furanfurin, Thiophenfurin, and Selenophenfurin as Mammalian Inosine Monophosphate Dehydrogenase (Type I and II) Inhibitors

Palmarisa Franchetti,* Loredana Cappellacci, Paolo Perlini, Hiremagalur N. Jayaram,‡ Adrian Butler,‡ Bryan P. Schneider,‡ Frank R. Collart,† Eliezer Huberman,† and Mario Grifantini

Dipartimento di Scienze Chimiche, Università di Camerino, Via S. Agostino, 1, 62032 Camerino, Italy, Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis, Indiana 46202, and Argonne National Laboratory, Argonne, Illinois 60439

Received November 13, 1997

Dinucleotides TFAD (**6**), FFAD (**7**), and SFAD (**8**), isosteric NAD analogues derived, respectively, from C-nucleosides 5-β-D-ribofuranosylthiophene-3-carboxamide (thiophenfurin, **1**), 5-β-D-ribofuranosylfuran-3-carboxamide (furanfurin, **2**), and 5-β-D-ribofuranosylselenophene-3-carboxamide (selenophenfurin, **5**), were synthesized as human inosine monophosphate dehydrogenase (IMPDH) type I and II inhibitors. The synthesis was carried out by imidazole-catalyzed coupling of the 5′-monophosphate of **1**, **2**, and **5** with AMP. These dinucleotides, which are also analogues of thiazole-4-carboxamide adenine dinucleotide (TAD) and selenazole-4-carboxamide adenine dinucleotide (SAD), the active metabolites of the oncolytic C-nucleosides 2-β-D-ribofuranosylthiazole-4-carboxamide (tiazofurin) and 2-β-D-ribofuranosylselenazole-4-carboxamide (selenazofurin), were evaluated for their inhibitory potency against recombinant human IMPDH type I and II. The order of inhibitory potency found was SAD > SFAD = TFAD = TAD ≫ FFAD for both enzyme isoforms. No significant difference was found in inhibition of IMPDH type I and II.

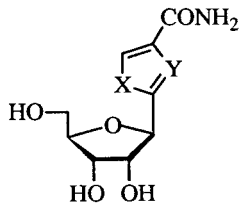
Introduction

Thiophenfurin (5-β-D-ribofuranosylthiophene-3-carboxamide, **1**) (Chart 1) and furanfurin (5-β-D-ribofuranosylfuran-3-carboxamide, **2**) are isosteric analogues of tiazofurin (**3**), a *C*-glycosylthiazole nucleoside endowed with potent antineoplastic activity in human tumor systems. While thiophenfurin was found to be active as an antitumor agent both in vitro and in vivo, furanfurin proved to be inactive.[1] The mechanism of action of both tiazofurin and thiophenfurin appears to be inhibition of inosine 5′-monophosphate dehydrogenase (IMPDH), the enzyme which catalyzes the conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP).[1,2a−c] The resulting decrease in GTP and dGTP biosynthesis produces the inhibition of tumor cell proliferation. In sensitive cells, tiazofurin is metabolized to an analogue of nicotinamide adenine dinucleotide (NAD), called TAD, which is a strong inhibitor of IMPDH.[3a,b]

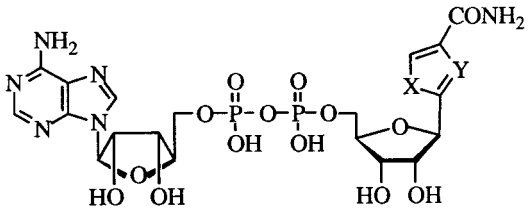
Selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide, **4**), the selenium analogue of tiazofurin, is a widely studied agent with a diverse array of biological effects. These include potent antitumor and antiviral activity, as well as efficacy as a maturation-inducing agent.[4a−d] Selenazofurin is 5−10-fold more potent than tiazofurin in several antitumor screens and in vitro studies.[2b,4b] In addition the antiproliferative and maturation-inducing effects of this nucleoside appear to be due to inhibition of IMPDH after its conversion in sensitive cells to an NAD analogue (SAD).[2b] Recently, we have reported that selenophenfurin (5-β-D-ribofuranosylselenophene-3-carboxamide, **5**), the selenophene analogue of selenazofurin, showed good antitumor activity similar to that of the parent compound and higher cytotoxicity compared to tiazofurin and thiophenfurin to a pannel of cell lines derived from leukemias and lymphomas.[5]

Crystal structures of tiazofurin, and selenazofurin demonstrated close intramolecular contacts between the

Chart 1



1, Thiophenfurin (X = S, Y = CH)
2, Furanfurin (X = O, Y = CH)
3, Tiazofurin (X = S, Y = N)
4, Selenazofurin (X = Se, Y = N)
5, Selenophenfurin (X = Se, Y = CH)

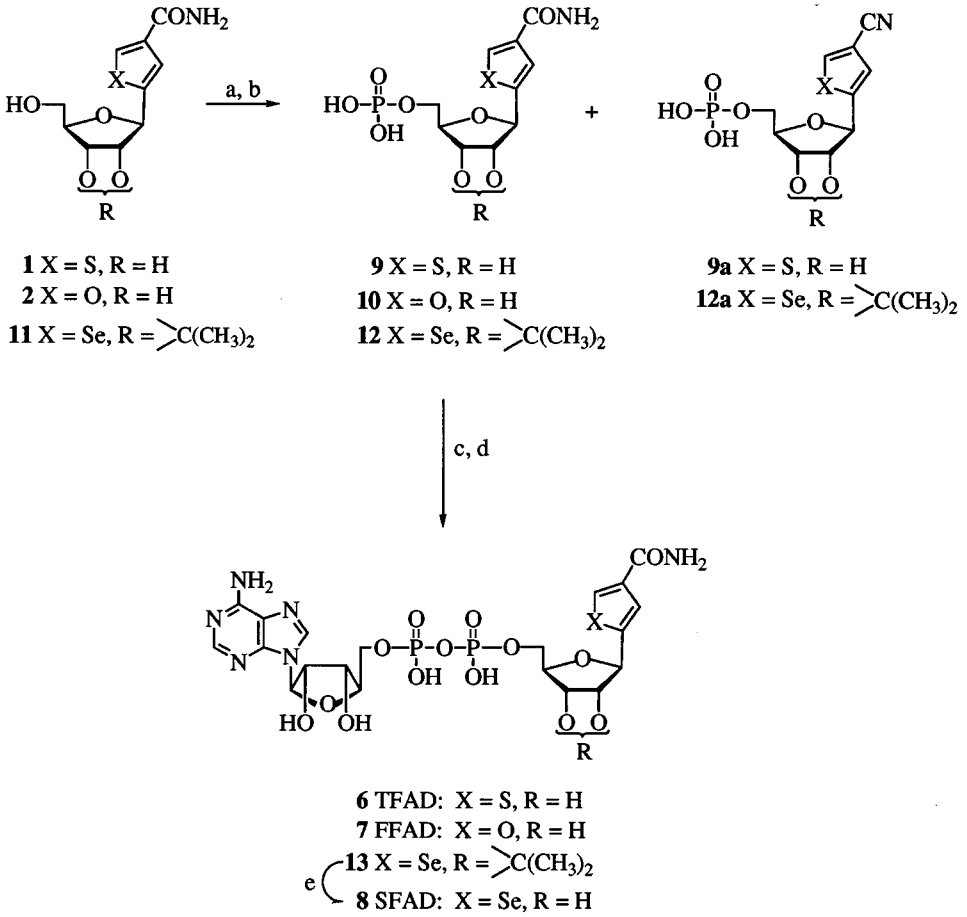


6, TFAD (X = S, Y = CH)
7, FFAD (X = O, Y = CH)
8, SFAD (X = Se, Y = CH)
TAD (X = S, Y = N)
SAD (X = Se, Y = N)

* Author for correspondence. Tel: (+39)737-402233. Fax: (+39)-737-637345. E-mail: grima@camserv.unicam.it.
‡ Indiana University School of Medicine.
† Argonne National Laboratory.

S0022-2623(97)00772-3 CCC: $15.00
Published on Web 04/21/1998

Scheme 1[a]



[a] Reagents: (a) $POCl_3$, $(CH_3O)_3PO$, then 2 M TEAB; (b) (in the case of **9** and **10**) Dowex 50W/H^+; (c) carbonyldiimidazole; (d) tri-*n*-butylamine, DMF; (e) Dowex 50W/H^+.

thiazole S or selenazole Se heteroatoms and the furanose ring oxygen O1′.[6] Similar close contacts were also found in thiophenfurin and selenophenfurin.[1,5] These close contacts have been attributed to an attractive electrostatic interaction between positively charged thiophene sulfur or selenium and the lone pair of electrons on the furanose oxygen, as confirmed by molecular orbital calculations. This interaction would be expected to constrain rotation of the C-glycosidic bond in the active NAD analogues, influencing the binding of these dinucleotide inhibitors to the target enzyme. Concerning furanfurin, its inactivity was attributed to the O−O repulsion between the negatively charged furan and furanose oxygens which forced the glycosyl torsional angle to adopt a different conformation.[1]

It has recently been discovered that IMPDH exists as two isoforms, type I and type II.[7a,b] Type I is ubiquitously expressed and is the prevalent species in normal cells, whereas the type II isoform is upregulated and predominates in neoplastic and replicating cells.[8a−c] Thus, the selective inhibition of type II IMPDH may provide improved selectivity against target cells in anticancer chemotherapy.

We found that thiophenfurin was more easily converted to the NAD analogue (thiophene-3-carboxamide adenine dinucleotide, TFAD) than tiazofurin in myelogenous leukemia K562 cells, whereas furanfurin was converted with difficulty to furan-3-carboxamide adenine dinucleotide, FFAD.[1] Thus, the inactivity of furanfurin may be due to its poor conversion to the dinucleotide in target cells and/or to the failure of the dinucleotide to inhibit the enzyme. To check these hypotheses, we synthesized the NAD analogues of thiophenfurin (TFAD, **6**), furanfurin (FFAD, **7**), and selenophenfurin (SFAD, **8**) and examined their inhibitory effects against mammalian IMPDH type I and II.

Chemistry

NAD analogues of thiophenfurin (**6**, TFAD) and furanfurin (**7**, FFAD) were synthesized by the imidazole-catalyzed coupling of the corresponding 5′-monophosphates **9** and **10** with AMP as shown in Scheme 1. Compounds **9** and **10** were prepared by Yoshikawa phosphorylation of C-nucleosides **1** and **2**.[9] In the case of compound **1**, 5-β-D-ribofuranosylthiophene-3-carbonitrile 5′-monophosphate (**9a**) was formed as a byproduct. To reduce the production of the nitrile derivative, a lower amount of $POCl_3$ than that reported in the literature[9] was used. Monophosphates **9** and **10** were then activated with carbonyldiimidazole[10a,b] and converted to the desired dinucleotides TFAD and FFAD by reaction of in situ formed imidazolides with AMP. The purity of TFAD and FFAD was checked by HPLC, and their structures were confirmed by ^1H NMR spectra in D_2O which show the heterocyclic protons as singlets at δ 7.25, 7.72, 8.1, and 8.33 (TFAD) and at δ 6.61, 7.83, 8.17, and 8.40 (FFAD).

For the synthesis of the NAD analogue derived from

Table 1. Inhibition Constants for NAD Analogues TFAD, FFAD, SFAD, TAD, and SAD Using Mammalian Type I and II IMPDH

	IMPDH−I K_i[a] (μM)		IMPDH−II K_i[a] (μM)	
compd	IMP	NAD	IMP	NAD
TFAD	0.37	0.34	0.32	0.34
FFAD	37.97	135.12	57.90	99.10
SFAD	0.58	0.26	1.10	0.27
TAD	0.71	0.47	0.43	0.44
SAD	0.033	0.062	0.021	0.026

[a] Mean of determinations at three inhibitor concentrations. In no case did values differ from these means by more than 20%.

selenophenfurin (SFAD, **8**), we have used a similar method starting from acetonide-protected selenophenfurin (**11**). Phosphorylation of **11** by the Yoshikawa method gave a mixture of acetonide-protected selenophenfurin 5′-monophosphate (**12**) and the corresponding nitrile **12a**, which were separated as ammonium salts by chromatography on a silica gel column eluting with ^{i}PrOH−NH_4OH−H_2O. Activation of **12** with carbonyldiimidazole and reaction of the imidazolide intermediate with AMP gave protected dinucleotide **13**. Deisopropylidenation of **13** with Dowex 50W/H^+ resin in water afforded SFAD (**8**). Selenophenfurin 5′-monophosphate (**14**) was also prepared by deprotection of isopropylidene derivative **12**.

Biological Evaluation and Discussion

We examined the inhibitory effects of TFAD, FFAD, and SFAD against recombinant human IMP dehydrogenase type I and II.[8] Dinucleotides TAD and SAD were used as reference compounds. The K_i values (Table 1) of these compounds for each isoform of IMPDH were determined as described by Magasanik et al.,[11] except that 10 mM 2-mercaptoethanol was included in the reaction mixture which provided a 2−3-fold increase in enzyme activity.

All compounds exhibited uncompetitive type of inhibition toward IMP and NAD substrates of both enzymes isoforms. SAD was the most potent inhibitor (type I IMPDH, K_i toward IMP and NAD utilization of 0.033 and 0.062 μM, respectively; type II IMPDH, K_i toward IMP and NAD utilization of 0.021 and 0.026 μM, respectively), showing a slight selectivity for the inhibition of type II. SFAD demonstrated similar inhibitory potency toward IMPDH type I (K_i toward IMP and NAD utilization of 0.58 and 0.26 μM, respectively) and type II IMPDH (K_i toward IMP and NAD utilization of 1.1 and 0.27 μM, respectively). TFAD was slightly less potent than SFAD and at the same time more potent than TAD exhibiting equal affinity toward IMPDH type I and II. So, the ranking of the inhibitory activity against IMPDH of these dinucleotides is SAD > SFAD = TFAD = TAD ≫ FFAD, which is similar to the action of the corresponding C-nucleosides in inhibiting IMPDH activity in human myelogenous leukemia K562 cells.[5] FFAD proved to be a weak inhibitor of IMPDH: type I, K_i toward IMP and NAD utilization of 37.9 and 135.1 μM, respectively; type II, K_i toward IMP and NAD utilization of 57.9 and 99.1 μM, respectively. No significant differences were found in inhibition of type I and II isoforms.

The corresponding monophosphates of the respective NAD analogues were expected to be weak inhibitors of IMPDH similar to the monophosphates of the parent compounds, tiazofurin and selenazofurin.[2a,b,3a,b]

The data of the enzymes' inhibition demonstrate that the inactivity of furanfurin as an antitumor agent is due not only to its poor ability to be converted to the anabolite FFAD in target cells[1] but also to the low potency of this anabolite as an IMPDH inhibitor.

In a recent paper on the conformation of tiazofurin analogues, Makara and Keserû have confirmed that the conformational properties of furanfurin are unique leading to an inactive NAD analogue.[12] Our finding that FFAD has poor affinity for IMPDH corroborates the hypothesis that conformational properties are important for the antitumor activity of tiazofurin and its analogues.[1,13] The fact that the recently synthesized benzamide adenine dinucleotide (BAD)[14a,b] was found to be a potent inhibitor of IMPDH, although the rotation around its benzamide−riboside glycosyl bond is not restricted at all, might be explained by the flexibility about the C-glycosidic bond which facilitates the adoption in the active site of the target enzyme of a conformation suitable to bind the enzyme.

Experimental Section

Melting points were determined on a Büchi apparatus and are uncorrected. Elemental analyses were determined on a EA 1108 CHNS-O (Fisons Instruments) analyzer. The analytical samples of nucleotides were lyophilized and dried under vacuum over P_2O_5. Thin-layer chromatography (TLC) was run on silica gel 60 F_{254} and RP-18 plates; silica gel 60 (70−230 and 230−400 mesh; Merck) for column chromatography was used. Nuclear magnetic resonance spectra were recorded on a Varian VXR-300 spectrometer with Me_4Si or DDS as the internal standard for ^{1}H NMR and external H_3PO_4 for ^{31}P NMR. Chemical shifts are expressed in δ values (parts per million). All exchangeable protons were confirmed by addition of D_2O. Tiazofurin and selenazofurin were obtained through the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). TAD and SAD were synthesized by the methods described by Gebeyehu et al.[10b]

5-β-D-Ribofuranosylthiophene-3-carboxamide 5′-Monophosphate (9) and 5-β-D-Ribofuranosylthiophene-3-carbonitrile 5′-Monophosphate (9a). To a suspension of 5-β-D-ribofuranosylthiophene-3-carboxamide (**1**; 200 mg, 0.77 mmol) in $(C_2H_5O)_3PO$ (3 mL) was added $POCl_3$ (140 mg, 0.92 mmol) at 0 °C, and the mixture was stirred at 0 °C for 5 days. The reaction was quenched by addition of water (0.3 mL), and the mixture was stirred at 0 °C for 12 h and then added dropwise into a solution of 2 M Et_3N−H_2CO_3 (TEAB) (4 mL) in water (30 mL). Extraction with EtOAc (2 × 20 mL) and concentration of the aqueous layer in vacuo gave a residue which was chromatographed on a silica gel column eluting with ^{i}PrOH−^{n}BuOH−NH_4OH−H_2O (5.5:1:2.5:1, v/v/v/v). Two compounds were separated. Faster migrating **9a** (ammonium salt, 32 mg, 11%): ^{31}P NMR (D_2O) δ 0.06 (s); ^{1}H NMR (Me_2SO-d_6) δ 4.82 (d, 1H, H1′, $J_{1',2'}$ = 6.2 Hz), 7.39 (s, 1H, H4), 8.47 (s, 1H, H2); ^{1}H NMR (D_2O) δ 3.44−4.10 (m, 5H, H2′, H3′, H4′, H5′, H5″), 4.82 (d, 1H, H1′, $J_{1',2'}$ = 6.2 Hz), 7.4 (s, 1H, H4), 8.45 (s, 1H, H2). Anal. ($C_{10}H_{15}N_2O_7PS\cdot 2H_2O$) C, H, N.

Slower migrating **9** (ammonium salt, 94.7 mg, 31.3%): ^{31}P NMR (D_2O) δ −0.22 (s); ^{1}H NMR (Me_2SO-d_6) δ 4.78 (d, 1H, H1′), 7.09, 8.16 (2s, 2H, NH_2), 7.65 (s, 1H, H4), 7.97 (s, 1H, H2); ^{1}H NMR (D_2O) δ 3.52−4.10 (m, 5H, H2′, H3′, H4′, H5′, H5″), 4.80 (d, 1H, H1′, $J_{1',2'}$ = 6.2 Hz), 7.57 (s, 1H, H4), 8.0 (s, 1H, H2). Anal. ($C_{10}H_{17}N_2O_8PS\cdot 2H_2O$) C, H, N.

Compound **9** as the free acid was obtained by passage of the ammonium salt through a Dowex 50W-X8 (H^+) column eluting with water. After lyophilization a white powder was obtained: ^{31}P NMR (D_2O) δ −0.47 (s); ^{1}H NMR (Me_2SO-d_6) δ 3.78−4.03 (m, 5H, H2′, H3′, H4′, H5′, H5″), 4.81 (d, 1H, H1′,

$J_{1',2'}$ = 6.6 Hz), 5.65 (br s, 8H, OH, H_2O), 7.20, 7.73 (2s, 2H, NH_2), 7.40 (s, 1H, H4), 8.02 (s, 1H, H2). Anal. ($C_{10}H_{14}NO_8PS\cdot 2H_2O$) C, H, N.

5-β-D-Ribofuranosylthiophene-3-carboxamide 5′,5″-Adenosine Pyrophosphate (TFAD, 6). Compound **9** (free acid, 120 mg, 0.36 mmol) was dried by coevaporation with anhydrous pyridine (3 × 5 mL) and DMF (4 × 5 mL) and then dissolved in dry DMF (1 mL). Carbonyldiimidazole (291.9 mg, 1.8 mmol) was added, and the progress of reaction was followed by TLC (^{i}PrOH−^{n}BuOH−NH_4OH−H_2O, 5.5:1:2.5:1, v/v/v/v). After 5 h at room temperature the excess of CDI was hydrolyzed by addition of MeOH (100 μL). To the imidazolide intermediate was added a solution of AMP (197.1 mg, 0.54 mmol) in dry DMF (6 mL) containing Bu_3N (128 μL, 0.54 mmol). The reaction mixture was stirred for 5 days at room temperature. Water (10 mL) was added, and the mixture was concentrated in vacuo. The gummy residue was dissolved in water containing NaOAc (90 mg in 15 mL) and extracted with $CHCl_3$ (2 × 30 mL) and then with Et_2O (2 × 30 mL). The aqueous layer was treated with Et_3N (20 mL) and then concentrated in vacuo. The residue was purified by chromatography on a silica gel column eluting with ^{i}PrOH-^{n}BuOH−NH_4OH−H_2O (5.5:1:2.5:1, v/v/v/v) to give **6** as the diammonium salt (131 mg, 48%): ^{31}P NMR (D_2O) δ −10.7 (br s); ^{1}H NMR (D_2O) δ 4.11−4.10 [m, 8H, H3′(T), H4′(T), H5′(T), H5″(T), H3′-(A), H4′(A), H5′(A), H5″(A)], 4.28−4.43 [m, 1H, H2′(T)], 4.54−4.64 [m, 1H, H2′(A)], 4.89 [d, 1H, H1′(T), $J_{1',2'}$ = 6.3 Hz], 6.02 [dd, 1H, H1′(A), $J_{1',2'}$ = 5.1 Hz], 7.25 [s, 1H, H4(T)], 7.72 [s, 1H, H2(T)], 8.10 [s, 1H, H2(A)], 8.33 [s, 1H, H8(A)]. Anal. ($C_{20}H_{32}N_8O_{14}P_2S\cdot 2H_2O$) C, H, N.

5-β-D-Ribofuranosylfuran-3-carboxamide 5′-Monophosphate (10). Nucleoside **2** (190 mg) was treated with $POCl_3$ to give the 5′-monophosphate as described above. Evaporation of EtOAc extracts in vacuo gave a residue which was chromatographed on a silica gel column eluting with ^{i}PrOH−NH_4-OH−H_2O (6:3:1, v/v/v). The fractions containing **10** as the ammonium salt (R_f = 0.25) were collected and lyophilized. The salt was converted into the free-acid form by passing it through a column of Dowex 50W-X8 (H^+). The yield of **10** was 80 mg (33.3%): ^{31}P NMR (D_2O) δ 0.72 (s); ^{1}H NMR (D_2O) δ 3.75 (m, 2H, H5′, H5″), 3.83 (m, 1H, H4′), 3.95−4.05 (m, 2H, H3′, H2′), 4.59 (d, 1H, H1′, $J_{1',2'}$ = 5.8 Hz), 6.88 (s, 1H, H4), 8.12 (s, 1H, H2). Anal. ($C_{10}H_{14}NO_9P\cdot 2H_2O$) C, H, N.

5-β-D-Ribofuranosylfuran-3-carboxamide 5′,5″ Adenosine Pyrophosphate (FFAD, 7). Monophosphate **10** (142 mg) was activated with carbonyldiimidazole and coupled with AMP as described above (reaction time 10 days). The product was purified as the diammonium salt by chromatography on a silica gel column eluting with ^{i}PrOH−NH_4OH−H_2O (7.5:1.5:1, v/v/v) (55 mg, 20%): ^{31}P NMR (D_2O) δ −10.7 (br s); ^{1}H NMR (D_2O) δ 4.0−4.33 [m, 8H, H3′(F), H4′(F), H5′(F), H5″(F), H3′-(A), H4′(A), H5′(A), H5″(A)], 4.42 [pseudo t, 1H, H2′(F)], 4.61 [pseudo t, 1H, H2′(A)], 4.70 [d, 1H, H1′(F), $J_{1',2'}$ = 6.7 Hz], 6.02 [d, 1H, H1′(A), $J_{1',2'}$ = 5.2 Hz], 6.61 [s, 1H, H4(F)], 7.83 [s, 1H, H2(F)], 8.17 [s, 1H, H2(A)], 8.40 [s, 1H, H8(A)]. Anal. ($C_{20}H_{32}N_8O_{15}P_2\cdot 2H_2O$) C, H, N.

5-(2,3-*O*-Isopropylidene-β-D-ribofuranosyl)selenophene-3-carboxamide (11). To a suspension of 5-β-D-ribofuranosylselenophene-3-carboxamide (**5**) (400 mg, 1.28 mmol) and triethyl orthoformate (200 mg, 1.36 mmol) in acetone (8 mL) was added 168 μL of 1 N HCl in ethyl ether, and the reaction mixture was stirred at room temperature for 36 h. After neutralization with concentrated ammonium hydroxide, the mixture was concentrated in vacuo and then dissolved in 20 mL of water. The aqueous solution was extracted with ethyl acetate (4 × 50 mL), and the resulting organic solution was dried (Na_2SO_4) and concentrated in vacuo. The recovered oil was purified by chromatography on a silica gel column eluting with $CHCl_3$−MeOH (95:5) to give **11** as a foam (352 mg, 77.8%): ^{1}H NMR (Me_2SO-d_6) δ 1.3, 1.52 (2s, 6H, CH_3), 3.45−3.60 (m, 2H, H5′, H5″), 3.97−4.08 (m, 1H, H4′), 4.57 (pseudo t, 1H, H2′), 4.71 (dd, 1H, H3′, $J_{2',3'}$ = 6.6 Hz, $J_{3',4'}$ = 3.2 Hz), 4.95 (d, 1H, H1′, $J_{1',2'}$ = 5.1 Hz), 4.99 (t, 1H, OH), 7.18 (br s, 1H, NH_2), 7.65 (s, 1H, H4), 7.75 (br s, 1H, NH_2), 8.75 (s, 1H, H2). Anal. ($C_{13}H_{17}NO_5Se$) C, H, N.

5-(2,3-*O*-Isopropylidene-β-D-ribofuranosyl)selenophene-3-carboxamide 5′-Monophosphate (12) and 5-(2,3-*O*-Isopropylidene-β-D-ribofuranosyl)selenophene-3-carbonitrile 5′-Monophosphate (12a). To a suspension of **11** (209 mg, 0.6 mmol) in $(EtO)_3PO$ (1.3 mL) was added a mixture of $(EtO)_3PO$ (1.3 mL) containing water (12 μL) and $POCl_3$ (180 μL). The mixture was kept at 10 °C for 14 h and then added dropwise into a solution of 2 M TEAB (7 mL) in water (60 mL). Extraction with EtOAc (2 × 50 mL) and concentration of the aqueous layer in vacuo gave a residue which was chromatographed on a DEAE Sephadex A25 anion-exchange column (HCO_3^- form, 0.8 × 28 cm) eluting with water. The fractions containing a mixture of **12** and **12a** were evaporated in vacuo, and the residue was chromatographed on a silica gel column eluting with ^{i}PrOH−NH_4OH−H_2O (7:2:1, v/v/v). After evaporation two compounds were separated. Faster migrating **12a** (ammonium salt, 82.2 mg, 28.4%): ^{31}P NMR (D_2O) δ 0.02 (br s); ^{1}H NMR (Me_2SO-d_6) δ 1.3, 1.55 (2s, 6H, CH_3), 3.60−3.85 (m, 2H, H5′, H5″), 4.12−4.22 (m, 1H, H4′), 4.66 (pseudo t, 1H, H2′), 4.78 (dd, 1H, H3′, $J_{2',3'}$ = 6.3 Hz, $J_{3',4'}$ = 2.2 Hz), 5.06 (d, 1H, H1′, $J_{1',2'}$ = 4.8 Hz), 7.60 (s, 1H, H4), 9.19 (s, 1H, H2). Anal. ($C_{13}H_{19}N_2O_7PSe\cdot 3H_2O$) C, H, N.

Slower migrating **12**, as a hygroscopic powder (ammonium salt, 141 mg, 47%): ^{31}P NMR (D_2O) δ 0.7 (br s); ^{1}H NMR (Me_2SO-d_6) δ 1.30, 1.53 (2s, 6H, CH_3), 3.60−3.82 (m, 2H, H5′, H5″), 4.12−4.22 (m, 1H, H4′), 4.63 (pseudo t, 1H, H2′), 4.80 (dd, 1H, H3′, $J_{2',3'}$ = 6.3 Hz, $J_{3',4'}$ = 2.2 Hz), 5.12 (d, 1H, H1′, $J_{1',2'}$ = 4.8 Hz), 7.10 (br s, 1H, NH_2), 7.72 (s, 1H, H4), 8.18 (br s, 1H, NH_2), 8.72 (s, 1H, H2). Anal. ($C_{13}H_{21}N_2O_8PSe\cdot 3H_2O$) C, H, N.

5-(2,3-*O*-Isopropylidene-β-D-ribofuranosyl)selenophene-3-carboxamide 5′,5″ Adenosine Pyrophosphate (13). To a solution of **12** (130 mg, 0.26 mmol) in DMF (1.5 mL) was added carbonyldiimidazole (231 mg, 1.43 mmol), and the reaction mixture was stirred for 2 h at room temperature. The excess of CDI was hydrolyzed by addition of MeOH (94 μL). To the reaction mixture containing the imidazolide intermediate was added a solution of AMP (161 mg, 0.43 mmol) in dry DMF (6 mL) containing Bu_3N (105 μL, 0.44 mmol). The mixture was stirred for 3 days and then concentrated in vacuo. The residue was purified by chromatography on a silica gel column eluting with ^{i}PrOH−NH_4OH−H_2O (8:1:1, v/v/v). The fractions containing **13** as the diammonium salt were collected and evaporated in vacuo to give a white solid (173 mg, 76.7%): ^{31}P NMR (D_2O) δ −10.7 (br s); ^{1}H NMR (D_2O) δ 1.32−1.57 (2s, 6H, CH_3), 4.01−4.65 [m, 8H, H2′(Se), H4′(Se), H5′-(Se), H5″(Se), H3′(A), H4′(A), H5′(A), H5″(A)], 4.88 [dd, 1H, H3′(Se), $J_{2',3'}$ = 6.4 Hz, $J_{3',4'}$ = 2.8 Hz], 5.0 [d, 1H, H1′(Se), $J_{1',2'}$ = 4.84 Hz], 6.02 [d, 1H, H1′(A), $J_{1',2'}$ = 5.38 Hz], 7.45 [s, 1H, H4(Se)], 8.17 [s, 1H, H2(A)], 8.45 [s, 1H, H8(A)], 8.55 [s, 1H, H2(Se)]. Anal. ($C_{23}H_{36}N_8O_{14}P_2Se\cdot 4H_2O$) C, H, N.

5-β-D-Ribofuranosylselenophene-3-carboxamide 5′,5″-Adenosine Pyrophosphate (SFAD, 8). Compound **13** was deisopropylidenated by treatment with Dowex 50W-X8 (H^+) in water overnight and purified by chromatography on a silica gel column eluting with ^{i}PrOH−NH_4OH−H_2O (8:1:1, v/v/v) to give dinucleotide **8** as the diammonium salt (yield 47%): ^{31}P NMR (D_2O) δ −10.7 (br s); ^{1}H NMR (D_2O) δ 3.99−4.39 [m, 8H, H3′(Se), H4′(Se), H5′(Se), H5″(Se), H3′(A), H4′(A), H5′-(A), H5″(A)], 4.47 [pseudo t, 1H, H2′(A)], 4.62 [pseudo t, 1H, H2′(Se)], 5.01 [d, 1H, H1′(Se), $J_{1',2'}$ = 6.18 Hz], 6.03 [d, 1H, H1"(A), $J_{1',2'}$ = 5.38 Hz], 7.61 [s, 1H, H4(Se)], 8.17 [s, 1H, H2-(A)], 8.42 [s, 1H, H8(A)], 8.72 [s, 1H, H2(Se)]. Anal. ($C_{20}H_{32}N_8O_{14}P_2Se\cdot 2H_2O$) C, H, N.

5-β-D-Ribofuranosylselenophene-3-carboxamide 5′-Monophosphate (14). The title compound was obtained from **12** which was applied on a Dowex 50W-X8 (H^+) column eluting with water. The eluate was evaporated to dryness and purified by chromatography on a silica gel column with ^{i}PrOH−NH_4-OH−H_2O (7:2:1, v/v/v) to give **14** (ammonium salt) as a white solid (yield 51%): ^{31}P NMR (D_2O) δ −0.001 (s); ^{1}H NMR (Me_2SO-d_6) δ 3.66−3.82 (m, 3H, H4′, H5′, H5″), 3.91 (dd, 1H,

H3′, $J_{2',3'}$ = 8.6 Hz, $J_{3',4'}$ = 5.0 Hz), 4.02 (pseudo t, 1H, H2′), 4.78 (d, 1H, H1′, $J_{1',2'}$ = 6.2 Hz), 7.10 (s, 1H, NH_2), 7.65 (s, 1H, H4), 7.88 (s, 1H, NH_2), 8.68 (s, 1H, H2). Anal. ($C_{10}H_{17}N_2O_8PSe \cdot 2H_2O$) C, H, N.

Purification of Inosine 5′-Monophosphate Dehydrogenase (IMPDH) Type I and II. The coding region of IMPDH type I and II was cloned into a pET23a (Novagen) expression vector and used to transform BL21 (DE3)lysS bacterial cells. Expression of the IMPDH enzymes was induced by the addition of IPTG to 0.5 mM. The enzymes were purified using a modification of a procedure cited.[15] Briefly, induced cells from 10 L of broth culture were lysed in 10 mM Tris-HCl, pH 8.8, 50 mM NaCl, 1 mM dithiothreitol (DTT), 20 μg/mL lysozyme, sonicated for 1 min to reduce viscosity, and centrifuged at 18000*g* at 4 °C for 30 min. The supernatant was applied to a Fast Q Sepharose (Pharmacia) column (3 × 30 cm) at a flow rate of 5 mL/min. The column was washed with 50 mM Tris-HCl, pH 8.8, 1 mM DTT, and the enzyme was eluted with a linear gradient of 0.2−0.7 M NaCl in column washing buffer. Peak fractions were pooled, and the enzyme was precipitated by the addition of ammonium sulfate to 50% saturation. The ammonium sulfate pellet was resuspended in 25 mM Tris-HCl, pH 7.2, containing 20 mM NaCl and 1 mM DTT (buffer A) and applied to a Blue Sepharose (Pharmacia) column (2.5 × 8 cm) equilibrated with buffer A. After washing with buffer A, the column was eluted with a linear gradient of 0.1−1.0 M NaCl in buffer A. Peak fractions were pooled, dialyzed against buffer A, and applied to a Mono Q 10/10 FPLC column (Pharmacia) equilibrated with buffer A. The enzyme was eluted with a linear gradient of 0.1−1.0 M NaCl in buffer A. Peak fractions were concentrated, glycerol was added to 50% (v/v), and aliquots were stored at −70 °C until used for IMPDH kinetic studies.

Inosine 5′-Monophosphate Dehydrogenase Assay and Kinetic Studies. The enzyme activity was measured by a modification of a direct spectrophotometric assay based on the method described previously.[11] Purified human IMPDH type I or II was mixed with various inhibitors and kept in the cold at 4 °C for 10 min, and this was added last to initiate the reaction. Reaction mixture in a total volume of 1 mL contained 100 μL of 1 M Tris-HCl, pH 8.0, 100 μL of 1M KCl, 100 μL of 1 mM NAD, 100 μL of 1mM IMP, 200 μL of 10 mM 2-mercaptoethanol, 100 μL of 0.3 M EDTA, and enzyme or enzyme mixed with inhibitor. The reaction was carried out in a 1-cm^2 cuvette at 37 °C in a Cary spectrophotometer at a wavelength of 340 nm. The production of NADH indicated by an increase in adsorbance was recorded at timed intervals. The enzyme activity is expressed as μmol of NADH produced/min at 37 °C. Kinetic constants were calculated by using a computer program.[16] Experimental values were fed into the program, and plots were generated for competitive, uncompetitive and noncompetitive, types of inhibition. The inhibitory constants were subsequently determined from output generated from the appropriate computer-based inhibition fit. Kinetic values were established using the simple Michaelis−Menton equation, and inhibition constants were calculated from replots of the reciprocal graph. A mean value of three separate determinations is presented in Table 1.

Acknowledgment. The research has been supported by the Ministry of University and Scientific-Technological Research (40% funds), by a grant from the Italian National Research Council (CNR), and by the Project Development Program, Research and Sponsored Programs, Indiana University at Indianapolis, IN.

References

(1) Franchetti, P.; Cappellacci, L.; Grifantini, M.; Barzi, A.; Nocentini, G.; Yang, H.; O'Connor, A.; Jayaram, H. N.; Carrell, C.; Goldstein, B. M. Furanfurin and Thiophenfurin: Two Novel Tiazofurin Analogues. Synthesis, Structure, Antitumor Activity, and Interactions with Inosine Monophosphate Dehydrogenase. *J. Med. Chem.* **1995**, *38*, 3829−3837.

(2) (a) Jayaram, H. N.; Smith, A. L.; Glazer, R. I.; Johns, D. G.; Cooney, D. A. Studies on the mechanism of action of 2-β-D-ribofuranosylthiazole-4-carboxamide (NSC-286193). II. Relationship Between Dose Level and Biochemical Effects in P388 Leukemia in vivo. *Biochem. Pharmacol.* **1982**, *31*, 3839−3845. (b) Jayaram, H. N.; Ahluwalia, G. S.; Dion, R. L.; Gebeyehu, G.; Marquez, V. E.; Kelley, J. A.; Robins, R. K.; Cooney, D. A.; Johns, D. G. Conversion of 2-β-D-Ribofuranosylselenazole-4-carboxamide to an Analogue of NAD with Potent IMP Dehydrogenase-inhibitory Properties. *Biochem. Pharmacol.* **1983**, *32*, 2633−2636. (c) Jayaram, H. N.; Dion, R. L.; Glazer, R. I.; Johns, D. G.; Robins, R. K.; Srivastava, P. C.; Cooney, D. A. Initial Studies on the Mechanism of Action of a New Oncolytic Thiazole Nucleoside, 2-β-D-Ribofuranosylthiazole-4-carboxamide. *Biochem. Pharmacol.* **1982**, *31*, 2371−2380.

(3) (a) Cooney, D. A.; Jayaram, H. N.; Gebeyehu, G.; Betts, C. R.; Kelley, J. A.; Marquez, V. E.; Johns, D. G. The Conversion of 2-β-D-Ribofuranosylthiazole-4-carboxamide to an Analogue of NAD with Potent IMP-dehydrogenase-inhibitory Activity. *Biochem. Pharmacol.* **1982**, *31*, 2133−2136. (b) Kuttan, R.; Robins, R. K.; Saunders, P. P. Inhibition of Inosinate Dehydrogenase Metabolites of 2-β-D-Ribofuranosylthiazole-4-carboxamide. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 862−868.

(4) (a) Srivastava, P. C.; Robins, R. K. Synthesis and Antitumor Activity of 2-β-D-Ribofuranosylselenazole-4-carboxamide and Related Derivatives. *J. Med. Chem.* **1983**, *26*, 445−448. (b) Streeter, D.; Robins, R. K. Comparative *in vitro* Studies of Tiazofurin and a Selenazole Analogue. *Biochem. Biophys. Res. Commun.* **1983**, *115*, 544−550. (c) Kirsi, J. J.; North, J.; McKernan, P. A.; Murray, B. K.; Canonico, P. G.; Huggins, J. W.; Srivastava, P. C.; Robins, R. K. Broad-spectrum Antiviral Activity of 2-β-D-Ribofuranosylselenazole-4-carboxamide, a New Antiviral Agent. *Antimicrob. Agents Chemother.* **1983**, *24*, 353−361. (d) Goldstein, B. M.; Leary, J. F.; Farley, B. A.; Marquez, V. E.; Rowley, P. T. Induction of HL60 Cell Differentiation by Tiazofurin and Its Analogue: Characterization and Efficacy. *Blood* **1991**, *78*, 593−598.

(5) Franchetti, P.; Cappellacci, L.; Abu Sheikha, G.; Jayaram, H. N.; Gurudutt, V. V.; Sint, T.; Schneider, B. P.; Jones, W. D.; Goldstein, B. M.; Perra, G.; De Montis, A.; Loi, A. G.; La Colla, P.; Grifantini, M. Synthesis, Structure, and Antiproliferative Activity of Selenophenfurin, an IMP Dehydrogenase Inhibitor Analogue of Selenazofurin. *J. Med. Chem.* **1997**, *40*, 1731−1737.

(6) Burling, F. T.; Goldstein, B. M. Computational Studies of Nonbonded Sulfur−Oxygen and Selenium−Oxygen Interactions in the Thiazole and Selenazole Nucleosides. *J. Am. Chem. Soc.* **1992**, *114*, 2313−2320 and references therein.

(7) (a) Natsumeda, Y.; Ohno, S.; Kawasaki, H.; Konno, Y.; Weber, G.; Suzuki, K. Two Distinct cDNAs from Human IMP Dehydrogenase. *J. Biol. Chem.* **1990,** *265*, 5292−5295. (b) Carr, S. F.; Papp, E.; Wu, J. C.; Natsumeda, Y. Characterization of Human Type I and Type II IMP Dehydrogenase. *J. Biol. Chem.* **1993**, *268*, 27286−27290.

(8) (a) Konno, Y.; Natsumeda, Y.; Nagai, M.; Yamaji, Y.; Ohno, S.; Suzuki, K.; Weber, G. Expression of Human IMP Dehydrogenase Types I and II in Escherichia Coli and Distribution in Human Normal Lymphocytes and Leukemic Cell Lines. *J. Biol. Chem.* **1991**, *266*, 506−509. (b) Nagai, M.; Natsumeda, Y.; Konno, Y.; Hoffman, R.; Irino, S.; Weber, G. Selective Up-regulation of Type II Inosine 5′-Monophosphate Dehydrogenase Messenger RNA Expression in Human Leukemias. *Cancer Res.* **1991**, *51*, 3886−3890. (c) Nagai, M.; Natsumeda, Y.; Weber, G. Proliferation-linked Regulation of Type II IMP Dehydrogenase Gene in Human and Normal Lymphocytes and HL-60 Leukemic Cells. *Cancer Res.* **1992**, *52*, 258−261.

(9) Yoshikawa, M.; Kato, T.; Takenishi, T. A Novel Method for Phosphorylation of Nucleosides to 5′-Nucleotides. *Tetrahedron Lett.* **1967**, *50*, 5065−5068.

(10) (a) Cramer, F.; Neunhoeffer, H. Reaktionen von Adenosine-5′-phosphorsaure-imidazolid-eine neue Synthese von Adenosindiphosphat und Flavin-adenin-dinucleotid. (Reaction of Adenosine 5′-phosphorimidazolide. New Synthesis of Adenosine Diphosphate and Flavin-adenin-dinucleotid.) *Chem. Ber.* **1962**, *95*, 1664−1669. (b) Gebeyehu, G.; Marquez, V. E.; van Cott, A.; Cooney, D. A.; Kelley, J. A.; Jayaram, H. N.; Ahluwalia, G. S.; Dion, R. L.; Wilson, Y. A.; Johns, D. G. Ribavirin, Tiazofurin, and Selenazofurin: Mononucleotides and Nicotinamide Adenine Dinucleotide Analogue. Synthesis, Structure, and Interactions with IMP Dehydrogenase. *J. Med. Chem.* **1985**, *28*, 99−105.

(11) Magasanik, B.; Moyer, H. S.; Gehring, L. B. Enzymes Essential for the Biosynthesis of Nucleic Acid Guanine. I. Inosine-5′-phosphate Dehydrogenase as Aerobacter Aerogenes. *J. Biol. Chem.* **1957**, *226*, 339−350.

(12) Makara, G. M.; Keserû, G. M. On the Conformation of Tiazofurin Analogues. *J. Med. Chem.* **1997**, *40*, 4154−4159.

(13) Goldstein, B. M.; Bell, J. E.; Marquez, V. E. Dehydrogenase Binding by Tiazofurin Anabolites. *J. Med. Chem.* **1990**, *33*, 1123−1127.

(14) (a) Gharehbaghi, K.; Paull, K. D.; Kelly, J. A.; Barchi, J. J., Jr.; Marquez, V. E.; Cooney, D. A.; Monks, A.; Scudiero, D.; Krohn, K.; Jayaram, H. N. Cytotoxicity and Characterization of an Active Metabolite of Benzamide Riboside, a Novel Inhibitor of IMP Dehydrogenase. *Int. J. Cancer* **1994**, *56*, 892−899. (b) Zatorski, A.; Watanabe, K. A.; Carr, S. F.; Goldstein, B. M.; Pankiewicz, K. W. Chemical Synthesis of Benzamide Adenine Dinucleotide: Inhibition of Inosine Monophosphate Dehydrogenase (Types I and II). *J. Med. Chem.* **1996**, *39*, 2422−2426.

(15) Hager, P. W.; Collart, F. R.; Huberman, E.; Mitchell, B. S. Recombinant Human Inosine Monophosphate Dehydrogenase Type I and Type II Proteins. Purification and Characterization of Inhibitor Binding. *Biochem. Pharmacol.* **1995**, *49*, 1323−1329.

(16) Cleland, W. W. Statistical analysis of enzyme kinetics data. *Methods Enzymol.* **1979**, *63*, 103−138.

JM970772E