Expedited Articles

Rationally Designed Inhibitors of Inosine Monophosphate Dehydrogenase[▽]

Han-Zhong Zhang,[†] Kotesvar Rao,[†] Stephen F. Carr,[‡] Eva Papp,[‡] Kenneth Straub,[‡] John C. Wu,[‡,§] and Josef Fried*,[†]

Department of Chemistry, The University of Chicago, Chicago, Illinois 60637, and Roche Biosciences, Palo Alto, California 94143

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Functionalized 2-alkyl derivatives of inosinic acid have been synthesized to serve as reversible as well as irreversible inhibitors of the human type II enzyme inosine monophosphate dehydrogenase. These compounds were designed to react with Cys-331 of the enzyme to form covalent bonds so as to interfere with the normal enzyme mechanism which involves attack of Cys-331 at C-2 of the substrate. Mass spectrometric analysis of the reaction products after enzymatic degradation confirmed the appropriateness of the inhibitor design.

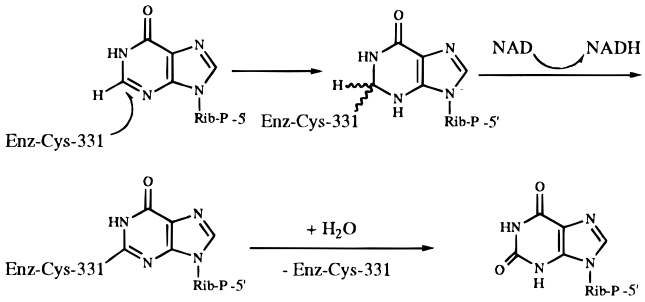
Introduction

Inosine 5′-monophosphate dehydrogenase (IMPDH) catalyzes the NAD-dependent oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP). Because of the critical role of this enzyme in the *de novo* biosynthesis of GMP, there has been considerable interest in the synthesis of inhibitors of this enzyme as anticancer agents[1] and more recently as immunosuppressants.[2−4]

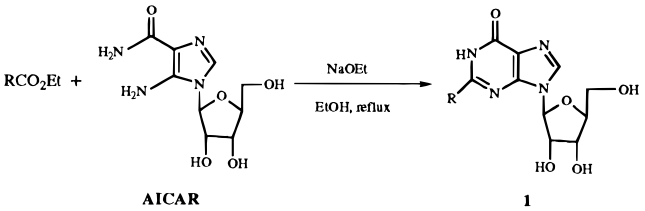
Several lines of evidence favor a mechanism for this reaction in which a nucleophilic species on the enzyme adds at C-2 of the nucleotide substrate with the formation of a tetrahedral intermediate.[5,6] The latter is converted to XMP by hydride transfer to NAD, loss of a proton, and subsequent hydrolysis of the enzyme−substrate adduct. Most recently the nucleophile in human type II IMPDH has been identified as the absolutely conserved Cys-331, by HPLC and tandem MS analysis of tryptic fragments of the covalent adduct (Scheme 1).[7,8] The homologous cysteine-319 has been identified as the catalytic residue in the *Tritrichomonas foetus* enzyme.[9]

This paper describes the development of some potent IMPDH inhibitors, both reversible and irreversible, based on the structural and mechanistic information concerning the chemistry occurring at C-2, the site of the enzyme reaction. It was felt that interfering with these events could best be achieved by substitution of inosinic acid at C-2. However, serious consideration had to be given to the nature of the substituent so as not to interfere with binding of the putative inhibitor to the enzyme. That conclusion seemed to be borne out by the work of Wong and Meyer,[10] who reported IC_{50} data for a number of 2-alkyl- and -arylinosinic acids, consistent with the view that bulky hydrophobic groups are unfavorable for binding to the enzyme.[11] We chose to address this problem by attaching appropriate functionality to the 2-substituent so as to effect increased binding or to cause reaction with the enzyme to occur.

Scheme 1



Scheme 2



1 : R = $CH_3OCH_2OCH_2$, (a); PhCH=CH, (b); CF_3, (c); CHF_2, (d); CH_2F, (e).

Results and Discussion

Most of the required nucleotides were prepared directly in two steps by cyclization of 5-aminoimidazole-4-carboxamide riboside (AICAR) with the requisite esters to form the nucleosides **1**[12] (Scheme 2). This was followed by 5′-phosphorylation with $POCl_3$ in triethyl phosphate[13] (Scheme 3). In three of the cases (**3**, **4**, and **10**) protected intermediates were required as shown in Schemes 4, 5 and 6. 2-Vinylinosinic acid (**2f**) was prepared from 2-vinylinosine.[14]

We reasoned that substitution of the highly electron-withdrawing CF_3 or CHF_2 group at C-2 would favor nucleophilic addition at that site competing with sulfhydryl addition to the substrate. Such an approach had been employed successfully in the design of inhibitors of proteolytic enzymes.[15,16] (Table 1) However, the fluoro derivatives **2c,d** were as ineffective as enzyme inhibitors as the methyl or other alkyl derivatives,

[▽] Dedicated to Prof. Nelson Leonard on the occasion of his 80th birthday.
[†] The University of Chicago.
[‡] Roche Biosciences.
[§] Present address: Berlex Biosciences, P.O. Box 4099, Richmond, CA 94804.
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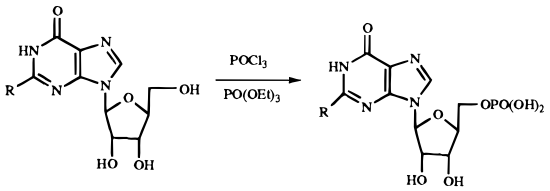
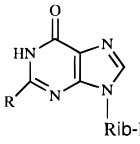
Scheme 3



1 2

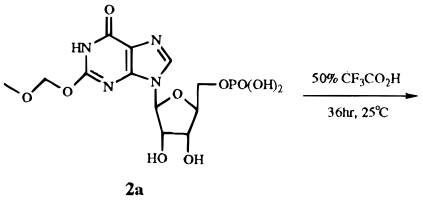
2: R = $CH_3OCH_2OCH_2$, (a); PhCH=CH, (b); CF_3, (c); CHF_2, (d); CH_2F, (e); CH_2=CH, (f); $PhCH_2CH_2$, (g).

Table 1. IMPDH Inhibition by 2-Substituted Inosinic Acids

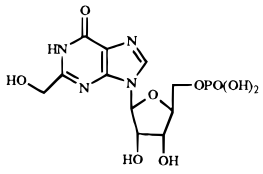


R	IC_{50} (μM)	R	IC_{50} (μM)
CH_3	>1000	CH_2Cl **(10)**	1.32
CF_3 **(2c)**	>1000	CH_2F **(2e)**	1.2
CHF_2 **(2d)**	>1000	PhCH=CH **(2b)**	37
CH_2OH **(3)**	132	$PhCH_2CH_2$ **(2g)**	460
$CH(OH)_2$ **(4)**	0.44	CH_2=CH **(2f)**	1.0

Scheme 4



2a



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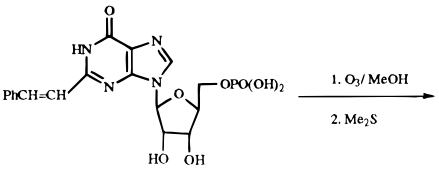
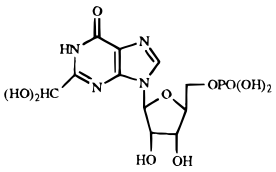
indicating that the favorable electronic effects of the fluoroalkyl groups were unable to overcome their steric effects on thiol addition at C-2.

Significant inhibitory activity was observed when a CH_2OH substituent **(3)** was introduced with the intent of directing NAD^+ to abstract hydride from the neighboring CH_2 group to form the electrophilic aldehyde group. Reaction of the latter with the sulfhydryl group of Cys-331 forming a thiohemiacetal could produce significant inhibition. Although the 2-hydroxymethyl compound **3** was indeed active at $IC_{50} = 132$ μM, the original rationale may have been faulty, since incubation of compound **3** with the enzyme and NAD^+ did not result in measurable NADH formation. The observed inhibition is therefore ascribed to a hydrogen-bonding interaction between this hydroxyl group and a nearby functional group on the enzyme. An additional factor for improved binding of this compound may be its functioning as a transition state analog in which the oxygen of the CH_2OH group occupies the site of the heteroatom at C-2 in the tetrahedral intermediate.[17] When the 2-formyl derivative **4** was prepared by ozonolysis of **2b** (Scheme 5), it was shown to possess $IC_{50} = 0.44$ μM, 300 times more potent than **3** and the most potent compound of this investigation. In light of this large difference in the activities of **3** and **4**, we cannot

Scheme 5



2b



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exclude the possibility that the observed inhibition by **3** is due to the formation of the formyl compound **4** in an amount too small to be detected by our assay. The inhibition was found to be reversible and probably involved in the above-conjectured formation of a thiohemiacetal. NMR data indicate that 2-formylinosine[18] and its 5′-phosphate exist essentially in the form of their hydrates, a fact which would indicate increased stability for such a covalent acetalic enzyme−inhibitor complex. There is ample analogy for the formation of aldehyde hemiacetals and thiohemiacetals in the case of serine[19] and cysteine[20] protease inhibitors.

A logical extension of the above experiments to probe the active site was the conversion of the hydrogen-bonding hydroxyl group to a leaving group with the intention of irreversibily inactivating the enzyme by alkylation of the active site. Following the now classical work of Shaw,[21] the hydroxymethyl group was converted to chloromethyl, which indeed produced the irreversible inhibitor **10** (Scheme 6) with $IC_{50} = 1.32$ μM. Incubation of a 10-fold excess of **10** (20 μM) with enzyme showed time-dependent inactivation at 37 °C with $k_{obs} = 3.16 \pm 0.14$ min^{-1}, which was decreased at least 15-fold in the presence of substrate, indicating that the inactivation reaction occurred at the active site (Figure 1 in Supporting Information). Incubation of **10** with Tris buffer alone showed no reaction until enzyme was added.

The corresponding 2-monofluoromethyl compound **2e**, in contrast to the difluoro **(2d)** and trifluoromethyl **(2c)** derivatives, showed irreversible inhibition at $IC_{50} = 1.2$ μM and reaction with the enzyme at $k_{obs} = 1.0 \pm 0.3$ min^{-1}. This difference in reactivity is not surprising in view of the stabilization of the di- and tri- fluoro derivatives by no-bond resonance.

In light of the inactivation of the enzyme by the 2-halomethyl derivatives, we reexamined the work by Wong and Meyer,[10,11] who had reported without comment the surprising finding that 2-styrylinosinic acid **(2b)** was 11 times more potent than the corresponding phenethyl derivative **2g**. Using the human enzyme instead of the *Escherichia coli* enzyme, we found similar differences in the activities of the two compounds (see Table 1). Reasoning that the styryl compound might serve as a Michael acceptor, we prepared 2-vinylinosinic acid **(2f)** which, lacking the phenyl group, should be considerably more reactive toward nucleophiles. Indeed, **2f** showed inhibition of the enzyme at $IC_{50} = 1.0$ μM. Moreover, incubation of the enzyme with the

Scheme 6

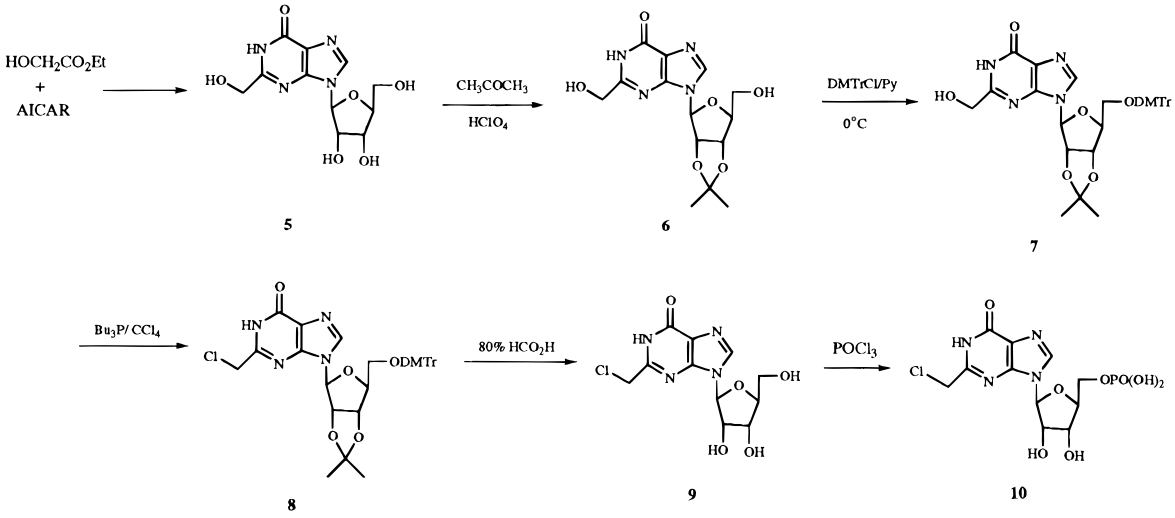

Table 2. *m*/*z* Values of Eicosaheptapeptides T38 from Reaction of IMPDH-II with Inhibitors

inhibitor	retention time (min)	*m*/*z*
control	57.1	2853.5 ± 0.5
10	56.3	3157.4 ± 0.5
2e	56.6	3157.6 ± 0.9
2f	56.5	3170.1 ± 0.6

inhibitor at 37 °C led to enzyme inactivation at a first-order rate of 0.86 ± 0.12 min^{-1}.

In order to confirm that the enzyme had undergone reaction with the inhibitors **10** and **2e,f** and which of the eight cysteine residues was involved, the composition of the three reaction products was compared with that of the native enzyme IMPDH-II used in this work.[22] Tryptic digests were prepared following reduction and alkylation with DTT and iodoacetamide, and the resulting reaction mixtures were subjected to HPLC−MS. A portion of the column effluent was analyzed by Edman microsequencing and tandem MS as previously reported.[8] This resulted in 58 fully analyzed peptides and their respective *m*/*z* values. Analysis of IMPDH that had undergone reaction with the substrate analogs **10** and **2e**,**f** resulted in HPLC profiles that were identical with that for the native enzyme except for the region between 55 and 60 min. For each modified IMPDH-II a new peak (T38*) with a retention time of *ca.* 56.5 min appeared (Figure 3 in Supporting Information), which contains the eicosaheptapeptide T38 (VGMGSGSIC-ITQEVLACGRPQGTAVYK, MW 2853.5) corresponding to residues 323−349 of the native enzyme.[22] The new peaks appearing for the three IMP analogs showed the *m*/*z* values shown in Table 2. Each *m*/*z* value corresponds to the molecular weight of peptide T38 with substitution of a cysteine-IMP adduct for a *S*-acetamidocysteine. Since peptide T38 contains two cysteines (Cys-331 and Cys-339), the modified T-38's (T38*) obtained from each of the three substrate analogs were collected and further characterized by Edman microsequencing and tandem MS in order to ascertain which of the two cysteines was involved in the reaction. The results showed that the amino acid in position 9 of T38 corresponding to Cys-331 of IMPDH-II had been alkylated by the substrate analogs **10** and **2e**,**f**.

Experimental Section[23]

General Procedure for Cyclization of RCO_2Et with 5-Aminoimidazole-4-carboxamide Riboside (AICA-riboside). To a solution of ethanolic sodium ethoxide [prepared from sodium (1.1 g, 48 mmol) in 25 mL of ethanol] was added AICA-riboside (1.03 g, 4 mmol), ester (24 mmol) was then added, the mixture was refluxed for 36 h. The ethanolic solution was evaporated to dryness and the residue redissolved in water. After the pH was adjusted to 3.0 with 1 N HCl, the solution was stirred at room temperature for 1 h and then adjusted to pH 7 with 1 N NaOH and concentrated to dryness *in vacuo*. The crude product was purified by silica gel chromatography (eluant, EtOAc/MeOH) and crystallization from EtOAc/MeOH to give pure product.

2-[[(Methoxymethylene)oxy]methyl]inosine (1a): 93%; mp 108−110 °C; ^{1}H NMR (D_2O) δ 3.3 (1H, s, CH_3O-), 3.74 (1H, dd, J_{gem} = 13.2 Hz, J = 3.5 Hz, H-5′), 3.83 (1H, dd, J_{gem} = 13.1 Hz, J = 3.4 Hz, H-5′), 4.22 (1H, m, H-4′), 4.38 (1H, m, H-3′), 4.72 (1H, m, H-2′), 4.67 (2H, s, -O-CH_2O-), 4.81 (2H, s, OCH_2-inosine), 5.98 (1H, d, H-1′, J = 5.68 Hz), 8.21 (1H, s, H-8).

2-(Phenylvinyl)inosine (1b): 76%; mp 258−259 °C; ^{1}H NMR (CD_3OD) δ 3.80 (1H, m, J_{gem} = 12.8 Hz, J = 3.3 Hz, H-5′), 3.91 (1H, m, J_{gem} = 12.8 Hz, J = 3.3 Hz, H-5′), 4.10 (1H, m, H-4′), 4.34 (1H, m, H-3′), 4.64 (1H, m, H-2′), 6.14 (1H, d, J = 5.60 Hz, H-1′), 6.92 (1H, d, J = 16.4 Hz, PhCH=CH-), 7.40−7.68 (5H, m, Ph), 7.88 (1H, d, J = 16.4 Hz), 8.25 (1H, s, H-8).

2-(Trifluoromethyl)inosine (1c): 61%; mp 236 °C dec; ^{1}H NMR (CD_3OD) δ 3.72 (1H, m, J_{gem} = 12.6 Hz, J =3.4 Hz, H-5′), 3.84 (1H, m, J_{gem} = 12.6 Hz, J = 3.4 Hz, H-5′), 4.13 (1H, m, H-4′), 4.33 (1H, m, H-3′), 4.73 (1H, m, H-2′), 5.93 (1H, d, J = 5.8 Hz, H-1′), 8.16 (1H, s, H-8).

2-(Difluoromethyl)inosine (1d): 72%; mp 185 °C; ^{1}H NMR (CD_3OD) δ 3.70 (1H, m, J_{gem} = 11.50 Hz, J = 3.40 Hz, H-5′), 3.83 (1H, m, J_{gem} = 11.50 Hz, J = 3.40 Hz, H-5′), 4.11 (1H, m, H-4′), 4.28 (1H, m, H-3′), 4.65 (1H, m, H-2′), 5.93 (1H, d, J = 5.5 Hz, H-1′), 6.47 (1H, t, J = 55 Hz, CHF_2), 8.20 (1H, s, H-8).

2-(Fluoromethyl)inosine (1e). This compound was separated from the ethoxymethyl derivative, which was formed as a byproduct and moved more slowly on TLC: ^{1}H NMR (CD_3OD) δ 3.69 (1H, m, J_{gem} = 12.0 Hz, H-5′), 3.81 (1H, m, J_{gem} = 12.0 Hz), 4.08 (1H, m, H-4′), 4.24 (1H, m, H-3′), 4.60 (1H, m, H-2′), 5.23 (2H, d, CH_2F, J = 46.76 Hz), 5.89 (1H, d, J = 5.88 Hz, H-1′), 8.14 (1H, s, H-8).

2-(Hydroxymethyl)inosine (5): 81%; mp 160−162 °C; ^{1}H NMR (CD_3OD) δ 3.70 (1H, m, J_{gem} = 11.50 Hz, J = 3.45 Hz, H-5′), 3.81 (1H, m, J_{gem} = 11.50, J = 3.50 Hz, H-5′), 4.07 (1H, m, H-4′), 4.29 (1H, m, H-3′), 4.53 (2H, s, −OCH_2-inosine), 4.55 (1H, m, H-2′), 5.92 (1H, d, J = 5.80 Hz, H-1′), 8.21 (1H, s, H-8).

General Procedure for 5′-Phosphorylation of Nucleosides.[13] A suspension of the 2-substituted inosine **1** or **9** (0.8

mmol) in dry triethyl phosphate (0.5 mL) was cooled to 0 °C, phosphorus oxychloride (2 mmol) was added, and the mixture was stirred at 0 °C for 6 h. Ether was added, and the resulting precipitate was separated by centrifugation. Ice water (5 mL) was added to the residual solid, and the resulting solution was extracted with $CHCl_3$. The aqueous layer was adjusted to pH 8 with sodium bicarbonate and the solution concentrated and passed through a Dowex-50 column (formate form). Elution with aqueous formic acid gave the 2-substituted inosinic acids **2** and **10**. For enzyme inhibition assays the solution was adjusted to pH 8.0 and lyophilized.

2-[[(Methoxymethylene)oxy]methyl]inosinic acid (2a): 30%; ^{1}H NMR (CD_3OD) δ 3.32 (3H, s, CH_3O-), 4.01 (2H, m, H-5′), 4.13 (1H, m, H-4′), 4.31 (1H, m, H-3′), 4.52 (2H, -OCH_2-inosine), 4.58 (1H, t, H-2′), 4.70 (2H, s, -OCH_2O-), 5.97 (1H, d, J = 5.40 HZ, H-1′), 8.32 (1H, s, H-8).

2-(Phenylvinyl)inosinic acid (2b): 43%; ^{1}H NMR (CD_3OD) δ 4.07 (2H, m, H-5′), 4.22 (1H, m, H-4′), 4.39 (1H, t, H-3′), 4.68 (1H, t, H-2′), 6.11 (1H, d, J = 5.0 Hz, H-1′), 6.93 (1H, d, J = 15.6 Hz, vinyl), 7.40−7.62 (5H, m, Ph), 7.90 (1H, d, J = 15.6 Hz, vinyl), 8.41 (1H, s, H-8). Anal. ($C_{18}H_{19}N_4O_8P\cdot H_2O$): C, H, N.

2-(Trifluoromethyl)inosinic acid (2c): 59%; ^{1}H NMR (D_2O) δ 4.08 (2H, m, H-5′), 4.28 (1H, br, H-4′), 4.42 (1H, m, H-3′), 4.68 (1H, H-2′), 6.07 (1H, d, J = 4.07 Hz, H-1′), 8.52 (1H, s, H-8). Anal. ($C_{11}H_{12}F_3N_4O_8P\cdot 2H_2O$) C, H, N.

2-(Difluoromethyl)inosinic acid (2d): 60%; ^{1}H NMR (D_2O) δ 4.03 (2H, m, H-5′), 4.31 (1H, m, H-4′), 4.48 (1H, m, H-3′), 6.10 (1H, d, J = 4.0 Hz, H-1′), 6.68 (1H, t, CHF_2, J = 51 Hz), 8.44 (1H, s, H-8). Anal. ($C_{11}H_{13}F_2N_4O_8P\cdot H_2O$) C; H: calcd, 3.63; found, 2.78. N: calcd, 13.45; found, 12.57.

2-(Fluoromethyl)inosinic acid (2e): 42%; ^{1}H NMR (D_2O) δ 4.10 (2H, m, H-5′), 4.31 (1H, br, H-4′), 4.42 (1H, m, H-3′), 5.45 (1H, d, J = 45 Hz), 6.09 (1H, d, J = 4.5 Hz, H-1′), 8.67 (1H, s, H-8). Anal. ($C_{11}H_{12}FN_4Na_2O_8P\cdot H_2O$) C, H, N.

2-Vinylinosinic acid (2f): 35%; ^{1}H NMR (CD_3OD) δ 4.20 (2H, m, H-5′), 4.33 (1H, br, H-4′), 4.61 (1H, br, H-3′), 5.85 (1H, dd, J = 11, <1 Hz, C*H*═CH_2), 6.09 (1H, d, J = 4.0 Hz), 6.55−6.67 (2H, m, CH═*C*H_2), 8.80 (1H, s, H-8). Anal. ($C_{12}H_{13}N_4Na_2O_8P\cdot 4H_2O$): C, N; H: calcd, 4.28; found, 3.55.

2-(Phenylethyl)inosinic acid (2g): 45%; ^{1}H NMR (D_2O) δ 3.01 (4H, m, -CH_2CH_2-), 3.90−4.24 (2H, m, H-5′), 4.25 (1H, m, H-4′), 4.28 (1H, m, H-3′), 4.40 (1H, m, H-2′) 6.00 (1H, d, J = 4.5 Hz, H-1′), 7.05−7.20 (5H, m, Ph), 8.8 (1H, s, H-8). Anal. ($C_{18}H_{21}N_4O_8P\cdot 1.5H_2O$) C, H, N.

2-(Chloromethyl)inosinic acid (10): 46%; ^{1}H NMR (CD_3OD) δ 4.03 (2H, m, H-5′), 4.17 (1H, m, H-4′), 4.37 (1H, m, H-3′), 4.51 (2H, s, CH_2Cl), 4.64 (1H, t, H-2′), 6.00 (1H, d, J = 4.8 Hz, H-1′), 8.41 (1H, s, H-8). Anal. ($C_{11}H_{12}ClN_4Na_2O_8P\cdot H_2O$) C, H, N.

2-(Hydroxymethyl)inosinic Acid (3). A solution of MOM ether **2a** (24 mg, 0.056 mmol**)** in 50% trifluoroacetic acid (1.5 mL) was stirred at 25 °C for 36 h. After evaporation of the solution to dryness, the residual oil was neutralized with sodium bicarbonate to pH 7.5 and applied to a column of Dowex 1 × 6 (formate, 100−200 mesh). After washing with distilled water, the column was eluted with increasing concentrations of formic acid (0.2−1 N). The product was removed by 0.5 N formic acid, and the combined fractions were evaporated to give 12 mg of pure 2-(hydroxymethyl)inosinic acid **(3)**: 49%; dec at 150 °C; ^{1}H NMR (D_2O) δ 4.10 (2H, m, H-5′), 4.31 (1H, br, H-4′), 4.39 (1H, m, H-3′), 4.65 (2H, s, *C*H_2-OH), 6.11 (1H, br, H-1′), 8.89 (1H, s, H-8). Anal. ($C_{11}H_{13}N_4Na_2O_9P\cdot 0.5H_2O$) C, H; N: calcd, 12.96; found, 12.38.

2-Formylinosinic Acid Hydrate (4). A solution of 2-styrylinosinic acid **(2b)** (18 mg) in 25 mL of dry methanol was cooled to −78 °C. Ozone was introduced with stirring at −78 °C until the solution retained the blue color. Excess ozone was removed with N_2, and dimethyl sulfide (0.5 mL) was added. The solution was allowed to stir at room temperature for 3 h, the solvent was removed under reduced pressure, and the residual material was purified by ion-exchange chromatography (Dowex-50, formate); 6.7 mg of pure product **4** was obtained: 41%; ^{1}H NMR (D_2O) δ 4.01 (1H, m, J_{gem} = 13.5 Hz, H-5′), 4.14 (1H, m, J_{gem} = 13.0 Hz), 4.31 (1H, br, H-4′), 4.41 (1H, t, J = 4.7 Hz, H-3′), 5.86 (1H, s, $CH(OH)_2$), 6.13 (1H, d, J = 4.0 Hz), 8.74 (1H, s, H-8). Anal. ($C_{11}H_{13}N_4Na_2O_{10}P\cdot 3.5H_2O$): C, H; N: calcd, 11.12; found, 9.91.

Preparation of 2-(Chloromethyl)inosine (9). 2-(Hydroxymethyl)-2′,3′-isopropylideneinosine (6). Perchloric acid (70%) (8 mL) was added to a suspension of 2-(hydroxymethyl)inosine **(5)** (2.98 g, 10 mmol) in acetone (150 mL) at 0 °C, the mixture was stirred overnight, and the solution was neutralized with 30% NH_3. The solvent was removed *in vacuo,* and the residue was purified by column chromatography (silica gel, MeOH/EtOAc, 4:1). Recrystallization gave 2.8 g of pure **6**: 72%; mp 196−198 °C; ^{1}H NMR (CD_3OD) δ 1.39 (3H, s, CH_3), 1.61 (3H, s, CH_3), 3.69 (1H, dd, J_{gem} = 11.5 Hz, J = 3.5 Hz, H-5′), 3.75 (1H, dd, J_{gem} = 11.50 Hz, J = 3.5 Hz, H-5′), 4.31 (1H, m, H-4′), 4.58 (2H, s, CH_2O), 5.00 (1H, m, H-3′), 5.22 (1H, m, H-2′), 6.12 (1H, d, J = 4.0 Hz), 8.22 (1H, s, H-8).

2-(Hydroxymethyl)-2′,3′-isopropylidene-5′-(4,4′-dimethoxytrityl)inosine (7). To a solution of **6** (0.1 g, 0.3 mmol) in dry pyridine (0.8 mL) was added 4,4-dimethoxytrityl chloride (0.14 g, 0.42 mmol) at 0 °C. After 5 h the reaction mixture was poured into ice water, and the mixture was extracted with methylene chloride. The combined organic extracts, after drying over Na_2SO_4 and chromatography, gave 0.11 g of pure **7**: 58%; mp 140−142 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (3H, s, CH_3), 1.62 (3H, s, CH_3), 3.30 (2H, m, H-5′), 3.75 (6H, s, $2CH_3O$), 4.46 (1H, br, H-4′), 4.52 (2H, m, CH_2O), 4.75 (1H, br, OH), 4.83 (1H, m, H-3′), 5.19 (1H, m, H-2′), 6.02 (1H, d, J = 3 Hz), 6.68−7.4 (14H, m, 3Ar), 7.82 (1H, s, H-8), 11.17 (1H, br, NH).

2-(Chloromethyl)-2′,3′-isopropylidene-5′-(4,4′-dimethoxytrityl)inosine (8). To a suspension of **(7)** (0.26 g, 0.41 mmol) in carbon tetrachloride was added tributylphosphine (0.2 mL, 0.81 mmol), and the reaction mixture was stirred at 25 °C for 40 min. After evaporation of the solvent *in vacuo* at room temperature, the residue was purified by preparative TLC (EtOAc as eluant). The product, 0.145 g, which was contaminated with a small amount of tributylphosphine was used for the following reaction without further purification: ^{1}H NMR ($CDCl_3$) δ 1.38 (3H, s, CH_3), 1.61 (3H, s, CH_3), 3.31 (2H, m, H-5′), 3.75 (6H, s, $2CH_3O$), 4.33 (1H, d, CH_2Cl, J = 11 Hz), 4.38 (1H, d, CHCl, J = 11.0 Hz), 4.48 (1H, br, H-4′), 4.90 (1H, m, H-3′), 5.20 (1H, m, H-2′), 6.06 (1H, d, J = 3.0 Hz), 6.65−7.35 (13H, m, Ar), 7.87 (1H, s, H-8), 11.73 (1H, br, NH).

2-(Chloromethyl)inosine (9). A solution of **8** (0.18 g, 0.27 mmol) in 80% aqueous formic acid (2 mL) was stirred at 0 °C for 10 min, warmed to room temperature, and stirred for an additional 2.5 h. After evaporation to dryness *in vacuo,* the residue was purified by column chromatography (silica gel, EtOAc/MeOH, 5:1, as eluant) to give **9**; recrystallization from MeOH/EtOAc gave pure material (40 mg, 46.3%), which decomposed at 150 °C without melting: ^{1}H NMR (CD_3OD) δ 3.81 (1H, dd, J_{gem} = 12.5 Hz, J = 2.8 Hz, H-5′), 3.93 (1H, dd, J_{gem} = 12.5 Hz, J = 2.8 Hz, H-5′), 4.20 (1H, m, H-4′), 4.48 (1H, m, H-3′), 4.56 (2H, s, CH_2Cl), 4.73 (1H, t, H-2′), 6.00 (1H, d, J = 5.69 Hz, H-1′), 8.26 (1H, s, H-8).

Assay and Inhibition of IMPDH Activity. IMPDH activity was determined by a spectrophotometric assay as described previously.[8,24] The assay measures the enzyme-catalyzed increase in absorbance of NADH at 340 nm as NADH and XMP are formed from NAD and IMP. Reactions to determine inhibition by the compounds were performed in 1 cm path length spectrophotometer cells. Standard reactions contained 50 μM IMP, 100 μM NAD, and varying concentrations of inhibitor in a buffer composed of 0.1 M potassium phosphate, pH 7.4, 0.1 M potassium chloride, 3 mM EDTA, 1 mM DTT, and 100 μg/mL bovine serum albumin. The reactions were initiated by the addition of enzyme to a final concentration of 25 nM in a total volume of 1 mL. Rate data were collected at 37 °C in a spectrophotometer equipped with a temperature-regulated multicell transporter. IC_{50} values were determined by nonlinear regression curve fit of the rate data to the following equation (eq 1) using the Macintosh program Systat:

$$f = \frac{1}{1 + \frac{I}{IC_{50}}} \quad (1)$$

where f is the fractional activity relative to the uninhibited rate and I is the concentration of inhibitor.

Inactivation of IMPDH by 2-(Chloromethyl)- (10), 2-(Fluoromethyl)- (2e), and 2-Vinyl-IMP (2f). Rates of inactivation of IMPDH by the 2-halomethyl and -vinyl analogs of IMP were measured by incubation of the enzyme in the presence of compounds followed by determination of enzyme activity in an assay with saturating concentrations of substrates as described previously.[8] IMPDH (2 μM) was incubated at 23 °C in the presence of 20 μM compound in 0.1 M potassium phosphate, pH 7.9, 0.5 M potassium chloride, 3 mM EDTA, 10 mM DTT, and 100 μg/mL bovine serum albumin in a total volume of 200 μL. At various times of incubation, 20 μL samples were assayed for remaining enzyme activity in a 1 mL reaction mixture containing 200 μM IMP and 400 μM NAD (8 × K_m of both substrates) in the same buffer solution. Apparent rates of inactivation (k_{inact}) were determined from the collected rate data by nonlinear regression curve fit to the following first-order equation (eq 2) using the Macintosh program Systat:

$$v = V_o \cdot e^{k_{inact} t} \quad (2)$$

where v is the observed activity remaining, V_o is the uninhibited velocity at time zero, and t is time of incubation with compound.

The time course of inactivation of human type II IMPDH by **2e**,**f** and **10** was determined by incubation of the enzyme with a 10-fold excess of compound at 20 μM. IMPDH was irreversibly inactivated with apparent rate constants k_{obs} of 3.16 ± 0.14, 0.98 ± 0.26, and 0.86 ± 0.12 min^{-1} for the chloromethyl **(10)**, fluoromethyl **(2e)**, and vinyl **(2f)** compounds, respectively. IMP at 200 μM (*ca.* 10 × K_m) protected the enzyme from inactivation by all three compounds; at least a 15-fold decrease in the rates of inactivation in the presence of IMP was observed (Figure 1 in Supporting Information). These results are consistent with a competitive interaction with respect to IMP, indicating that they compete for the same enzyme species that bind IMP. These compounds most likely associate with IMPDH at the IMP binding site of the enzyme.

HPLC and MS Analysis of Tryptic Peptides of Native and Inactivated IMPDH. These analyses performed as described previously.[8] Trypsinolysis was performed following reduction and alkylation with DTT and iodoacetamide. See Supporting Information for chromatograms obtained via reversed phase HPLC of the resulting mixture of peptides (Figures 2 and 3).

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Supporting Information Available: Time course of inactivation of IMPDH-II by inhibitors **2e**,**f** and **10** (Figure 1) and HPLC tracings (Figures 2 and 3) for tryptic digests of native and inactivated enzyme (4 pages). See any current masthead page for ordering information.

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