Ribavirin, Tiazofurin, and Selenazofurin: Mononucleotides and Nicotinamide Adenine Dinucleotide Analogues. Synthesis, Structure, and Interactions with IMP Dehydrogenase

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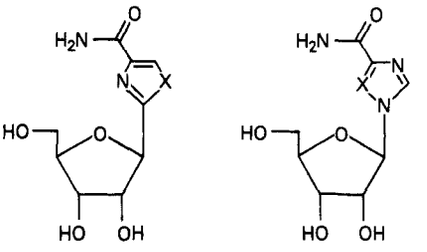
A series of dinucleotides, analogous to nicotinamide adenine dinucleotide but containing the five-membered base nucleosides tiazofurin (**1a**), selenazofurin (**1b**), ribavirin (**2**), and AICAR (**3**) in place of nicotinamide ribonucleoside, were prepared in greater than 50% yield by reacting the corresponding nucleotide imidazolidates (**6a–d**) with adenosine 5′-monophosphate (AMP). The symmetric dinucleotides of tiazofurin (TTD, **8e**) and selenazofurin (SSD, **8f**) were also prepared by a similar methodology. These dinucleotides were characterized by ^{1}H NMR and fast atom bombardment MS and were evaluated for their inhibitory potency against a partially purified preparation of tumoral inosine monophosphate dehydrogenase (IMPD) from P388 cells. The order of potency found was SAD (**8b**) > TAD (**8a**) >> SSD (**8f**) ≃ TTD (**8e**) ≃ RAD (**8c**) >>> ZAD (**8d**). On kinetic analysis none of the dinucleotides produced competitive inhibition of IMPD with NAD as a variable substrate. In addition to their superior IMPD inhibitory activity, SAD and TAD appear to be the only dinucleotides, besides NAD, that are formed naturally by the NAD pyrophosphorylase from P388 lymphoblasts.

The recent observations that the oncolytic C-nucleosides tiazofurin (**1a**) and selenazofurin (**1b**) are metabolized to potent inhibitors of inosine monophosphate dehydrogenase (IMPD) and that the structures of these metabolites are similar to that of the coenzyme NAD raise the important question of exactly how these metabolites interact with the enzyme.[1–5] That this metabolic transformation is essential for a strong inhibition of IMPD was demonstrated by the fact that the monophosphates of **1a** and **1b**, which can be considered structurally similar to the substrate IMP, are 3 orders of magnitude less potent than the corresponding dinucleotides as inhibitors of IMPD.[1,5] By contrast, ribavirin (**2**), a closely related analogue of **1a** and **1b**, exerts very potent inhibition of this target enzyme only as the monophosphate.[6,7] As of this date, in vivo or in vitro formation or chemical synthesis of a ribavirin-adenine dinucleotide has not been documented.

The present work was undertaken with the intent of comparing the IMPD-inhibitory activities of a selected group of related five-membered base nucleotides with their synthetically prepared NAD dinucleotide analogues, in order to discover useful trends for an initial structure–activity model. In addition to the NAD analogues of tiazofurin and selenazofurin, hereafter referred to as TAD (**8a**) and SAD (**8b**), we chose to prepare the dinucleotide analogues of the closely related ribavirin (RAD, **8c**) and the natural purine precursor 5-amino-4-imidazolecarboxamide ribonucleoside (ZAD, **8d**).[8] Also included in this study are the dimeric dinucleotide structures of tiazofurin (TTD, **8e**) and selenazofurin (SSD, **8f**). Finally, a comparative study with intact P388 cells and with NAD pyrophosphorylase from the same source was performed in order to investigate and measure the relative biosynthetic rates of formation of these dinucleotides from the corresponding monophosphates.



1a, X = S
b, X = Se

2, X = N
3, X = C–NH_2

Chemistry. The preparation of TAD (**8a**), SAD (**8b**), RAD (**8c**), and ZAD (**8d**) was initially performed as previously reported for TAD (method A, Scheme I).[2] The tri-*n*-octylamine salts of monophosphates **4a–d** were reacted in pyridine with the activated phosphoromorpholidate form of AMP (**5**) to give the desired dinucleotide. Separation of products was performed chromatographically on a Hamilton HA-X4 anion-exchange resin (HCO_2^- form) developed with a linear gradient of water and ammonium formate/formic acid.[2] The yields obtained with this procedure (method A) were reproducible but low (30%). In the present paper we report an improved method for the synthesis of these dinucleotides (method B). The procedure consisted of activating one of the nucleotides (usually the five-membered base nucleotide) with carbonyldiimidazole in the manner used to synthesize nucleotide triphosphates.[9] The formation of the imidazolidates (**6**) was easily monitored by HPLC and required no isolation. When the reaction was finished,

(1) Cooney, D. A.; Jayaram, H. N.; Gebeyehu, G.; Betts, C. R.; Kelley, J. A.; Marquez, V. E.; Johns, D. G. *Biochem. Pharmacol.* **1982**, *31*, 2133.
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(3) Cooney, D. A.; Jayaram, H. N.; Glazer, R. I.; Kelley, J. A.; Marquez, V. E.; Gebeyehu, G.; Van Cott, A. C.; Zwelling, L. A.; Johns, D. G. *Adv. Enzyme Regul.* **1983**, *21*, 271.
(4) Kuttan, R.; Robins, R. K.; Saunders, P. P. *Biochem. Biophys. Res. Commun.* **1982**, *107*, 862.
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(8) 5-Amino-4-imidazolecarboxamide ribonucleoside (AICAR, **3**) is named ZTP (Bochner, B. R.; Ames, B. N. *Cell* **1982**, *29*, 929) when metabolized to the triphosphate level. In keeping with this simpler nomenclature, we have chosen to use ZR-5′-MP (**4d**) and ZAD (**8d**) to describe AICAR-5′-MP and its corresponding NAD analogue, respectively.

(9) Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785.

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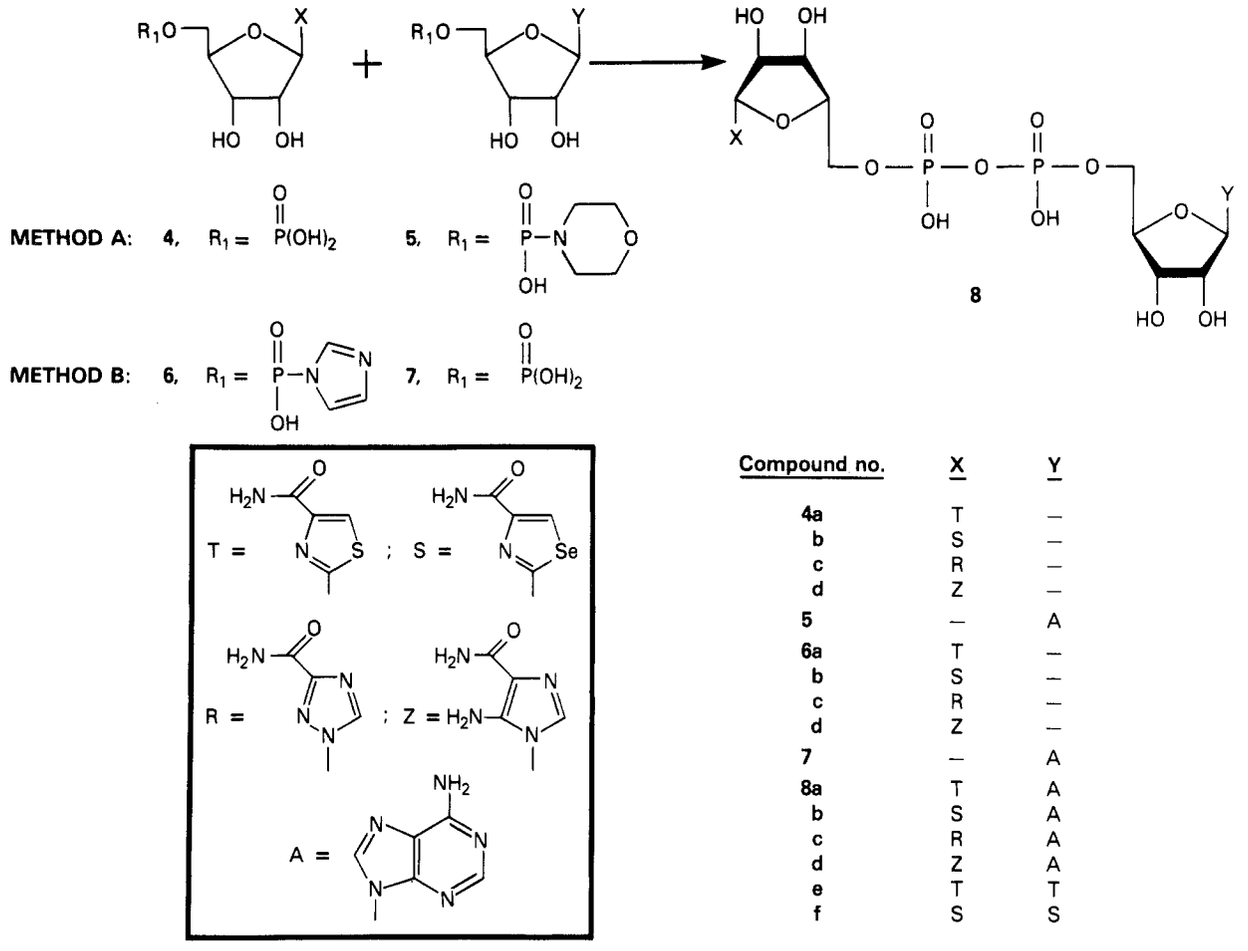
Scheme I



Compound no.	X	Y
4a	T	–
b	S	–
c	R	–
d	Z	–
5	–	A
6a	T	–
b	S	–
c	R	–
d	Z	–
7	–	A
8a	T	A
b	S	A
c	R	A
d	Z	A
e	T	T
f	S	S

Table I. Comparative Results from the Two Methods of Synthesis of Dinucleotides

reactants	method	product	yield,[a] %	formula	anal.
4a + 5	A	**8a**	31	$C_{19}H_{25}N_7O_{14}P_2S\cdot2.5H_2O$	C, H, N, P
6a + 7	B	**8a**	61		
4b + 5	A	**8b**	18	$C_{19}H_{25}N_7O_{14}P_2Se\cdot5H_2O$	C, H, N, P,[b] Se[b]
6b + 7	B	**8b**	63		
4c + 5	A	**8c**	41	$C_{18}H_{25}N_9O_{14}P_2\cdot3.5H_2O$	C, H, N,[b] P[b]
6c + 7	B	**8c**	49		
4d + 5	A	**8d**	39	$C_{19}H_{27}N_9O_{14}P_2\cdot5.5H_2O$	C, H, N,[b] P
6d + 7	B	**8d**	52		
6a + 4a	B	**8e**	78	$C_{18}H_{24}H_7O_{15}P_2S_2\cdot H_2O$	C, H, N, P
6b + 4b	B	**8f**	79	$C_{18}H_{24}N_4O_{15}P_2Se,2\cdot2H_2O$	C, H, N,[b] P, Se[b]

[a] Isolated yield. [b] Values with greater than ±0.40% deviation.

methanol was added to hydrolyze the excess reagent, followed immediately by the addition of AMP (**7**), or another nucleoside monophosphate, as a DMF solution containing tri-*n*-butylamine. Formation of the dinucleotides was normally complete after 48 h (method B, Scheme I). Following the same procedure as in method A, the aqueous extract containing both the product (**8**) and its 2′,3′-cyclic carbonate analogue (**9**) was treated with triethylamine to convert **9** to the deblocked product **8**. In those cases where this last step was omitted, significant amounts of the corresponding cyclic carbonate dinucleotide **9** was obtained as verified by MS analysis (vide infra). The formation of a 2′,3′-cyclic carbonate has been reported in connection with the use of carbonyldiimidazole.[10] After the triethylamine treatment and following an identical chromatographic separation as in method A, the yields of isolated dinucleotides were generally in excess of 50%. The results from both methods A and B are compared in Table I. The symmetric dinucleotides TTD (**8e**) and SSD (**8f**) were prepared only by method B and gave isolated yields of almost 80%.

NMR Studies. Table II summarizes the NMR spectral characteristics of the six syntesized dinucleotides. Some interesting features which may be relevant to the conformation of these dinucleotides in solution became apparent, especially when the spectra are compared with those of mixtures of the constituent mononucleotides. There is a consistent upfield shift of approximately 0.2 ppm for both the H-2 and H-8 protons of the adenosine portion of TAD (**8a**), SAD (**8b**), RAD (**8c**), and ZAD (**8d**) in comparison with AMP (**7**). Likewise, the anomeric proton of the adenosine component experiences an upfield shift of ca. 0.1 ppm relative to AMP. The chemical shift of the single proton in the five-membered base moiety of dinucleotides **8a–d** also showed a consistent 0.2–0.3 ppm upfield shift relative to the detached nucleotide monophosphate (only the NMR data for tiazofurin 5′-monophosphate is included

(10) Maeda, M.; Patel, A. D.; Hampton, A. *Nucleic Acids Res.* **1977**, *4*, 2843.

Table II. Proton NMR Spectral Characteristics of Dinucleotide Analogues of NAD

compd	adenosine: H-1′	X-ribonucleoside: H-1′	adenosine H-2	adenosine H-8	X-ribonucleoside H-2	X-ribonucleoside H-5
TAD (**8a**)	6.06 (d), J = 6.0 Hz	5.04 (d), J = 4 Hz	8.17 (s)	8.40 (s)		7.96 (s)
SAD (**8b**)	6.06 (d), J = 6.0 Hz	4.94 (d), J = 4 Hz	8.18 (s)	8.42 (s)		8.62 (s)
RAD (**8c**)	6.06 (d), J = 6.0 Hz	5.88 (d), J = 4 Hz	8.20 (s)	8.40 (s)		8.60 (s)
ZAD (**8d**)	6.07 (d), J = 6.0 Hz	5.48 (d), J = 6.5 Hz	8.16 (s)	8.38 (s)	7.26 (s)	
TTD (**8e**)		5.12 (d), J = 5 Hz				8.18 (s)
SSD (**8f**)		5.04 (d), J = 4 Hz				8.86 (s)
tiazofurin 5′-monophosphate (**4a**)		5.18 (d), J = 5.5 Hz				8.24 (s)
AMP (**7**)	6.18 (d), J = 5.5 Hz		8.40 (s)	8.60 (s)		
4a + **7**	6.19 (d), J = 5.5 Hz	5.20 (d), J = 5.5 Hz	8.44 (s)	8.62 (s)		8.26 (s)

in Table II for comparison with TAD). In contrast, no trend was observed for the anomeric proton of the five-membered base nucleotide component: in TAD (**8a**) and ZAD (**8d**), a ca. 0.1 ppm upfield shift was observed, whereas in SAD (**8b**) and RAD (**8c**), a negligible downfield shift was detected (0.03–0.06 ppm).

The consistency of the adenosine signals in TAD (**8a**), SAD (**8b**), RAD (**8c**), and ZAD (**8d**) observed in the present study indicate that our previous assignments made for TAD and SAD in this region of the spectrum were not correct.[1,5] The revised assignments are listed in Table II.

In general, the observed mutual shielding of the aromatic signals from both heterocycles, which translates into the corresponding upfield shifts relative to the NMR of mixtures of the individual components, lends support to the idea of a folded or stacked conformation in solution for these dinucleotides.[11] A stacked conformation has also been suggested for NADH,[11] which by virtue of its lack of positive charge, may be a better analogue to the dinucleotides examined in this study. The possibility of a folded conformation for TAD (**8a**) was further confirmed by studying the effects of increasing concentrations of a less polar destacking agent, such as methanol, on the chemical shifts of the H-2 and H-8 protons of the adenine moiety and the H-5 proton of the thiazole moiety of TAD (**8a**). This study was conducted in a similar fashion as performed previously with NAD and NADH (Figure 1).[12,13] Clearly, the direction of these shifts is one consistent with a progressive unfolding which causes proton deshielding. Similarly, as observed in the case of NAD and NADH, the adenine H-2 proton is less affected by the unfolding than the adenine H-8 proton.[13] This similarity in the behavior of the H-2 and H-8 protons of adenine in NAD, NADH, and TAD (**8a**) gives additional support to the assignments listed in Table II. It should be mentioned, however, that these chemical shifts are highly dependent on concentration, a fact that is also consistent with an equilibrium mixture between folded and unfolded forms.

The addition of methanol did not affect the chemical shift of the H-8 proton of adenine and H-5 proton of the thiazole ring when an equimolar mixture of AMP and tiazofurin 5′-monophosphate (**4a**) was examined. In changing from 0% to 80% methanol, the $\Delta\delta$ for these proton signals was 0.05 ppm in the shielding direction. On the contrary, the H-2 proton of adenine changed from δ 8.44 to δ 8.23 ($\Delta\delta$ = 0.21) again in the shielding direction. This may explain why this proton signal in the dinucleotides was less sensitive to the deshielding effects caused by destacking. The fact that increased methanol concentrations per se had either a negligible or opposite effect to that caused by the unfolding of the dinucleotides allows straightforward interpretation of the above results.

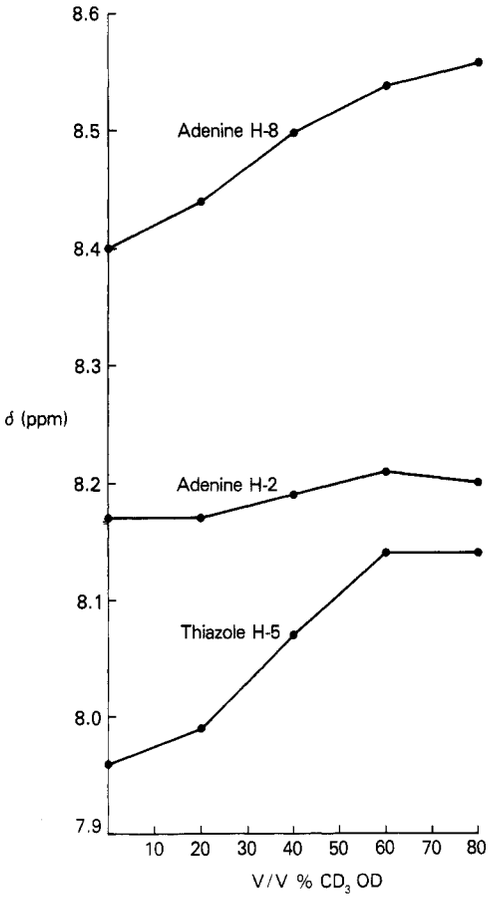


Figure 1. The effect of the concentration of methanol (CD_3OD) on the chemical shifts for the indicated protons of TAD.

Not surprisingly, the dimeric structures of TTD (**8e**) and SSD (**8f**) showed almost no change when compared with the spectra of the free nucleotides (see for example tiazofurin 5′-monophosphate vs. TAD in Table II), which would indicate that there is little or no interaction between the two identical heterocycles in these dinucleotide structures.

Mass Spectral Analysis. Fast atom bombardment mass spectrometry (FAB/MS) has been extensively employed for the structural analysis of nonvolatile, thermally labile molecules of biological interest.[14,15] As a class of compounds, underivatized nucleosides and nucleotides are well-suited to this mode of analysis.[16,17] FAB/MS has

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(12) Oppenheimer, N. J.; Arnold, L. J., Jr.; Kaplan, N. O. *Biochemistry* **1978**, *17*, 2613.
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(14) Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. *Nature (London)* **1981**, *293*, 270.
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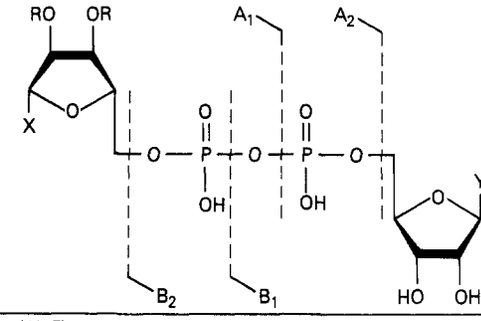
Table III. Selected Mass Spectral Data for Dinucleotide Analogues of NAD[a]

8 (R = H); 9 (R,R = C=O)	X	Y
a	T	A
b	S	A
c	R	A
d	Z	A
e	T	T
f	S	S



	m/z (% I)						
	TAD (8a)	SAD (8b)	RAD (8c)	ZAD (8d)	TTD (8e)	SSD (8f)	RAD carbonate (9c)
M + G[b] - H	760 (2.6)		744 (3.6)	758 (1.5)			
M - H	668 (100)	716 (100)	652 (100)	666 (100)	661 (99)	757 (51)	678 (100)
M - YH_2	553 (3.1)			531 (2.8)	533 (1.8)		
M - XH_2			540 (2.1)	540 (2.9)			
A_2 + 42	461 (6.3)	509 (5.8)	445 (7.0)	459 (1.0)	461 (3.0)	509 (13)	471 (14)
A_2	419 (38)	467 (44)	403 (53)	417 (37)	419 (94)	467 (84)	429 (86)
A_2 - H_2O	401 (15)	449 (16)	385 (14)	399 (11)	401 (15)	449 (18)	411 (38)
A_1	339 (15)	387 (44)	323 (25)	337 (16)	339 (100)	387 (100)	349 (29)
A_1 - H_2O	321 (7.6)	369 (7.4)	305 (1.6)	319 (8.0)	321 (3.3)	369 (7.0)	331 (1.6)
B_2 + 42	468 (8.3)	468 (19)	468 (6.7)	468 (4.7)			468 (6.5)
B_2	426 (41)	426 (91)	426 (43)	426 (39)			426 (68)
B_2 - H_2O	408 (13)	408 (30)	408 (9.4)	408 (9.6)			408 (26)
B_1	346 (16)	346 (35)	346 (9.7)	346 (12)			346 (28)
B_1 - H_2O	328 (7.4)	328 (17)	328 (4.7)	328 (3.6)			328 (10)

[a] The largest peak above *m/z* 300 has been normalized to 100%. [b] G stands for glycerol.

proven very useful for rapidly sequencing the triethylammonium salts of synthetic oligodeoxyribonucleotides[18,19] and has been applied to examine the nucleoside and nucleotide adducts of nitrogen mustards[20] and chemical carcinogens.[21] In our own laboratory FAB/MS has been used for the characterization of biologically and synthetically derived dinucleotide pyrophosphate analogues of NAD.[1-3,5] Negative ion FAB/MS, in particular, has permitted detailed structural analysis of these dinucleotide pyrophosphates (**8a-f**, **9**) and allowed a rapid, nonchromatographic assessment of product purity.[22]

For each dinucleotide, a clear indication of molecular weight (M - H) and two series of ions (A and B) corresponding to cleavage of the phosphodiester linkage from either side of the molecule were observed. This is clearly illustrated by the spectrum of RAD (**8c**, Table III) where the negative ion at *m/z* 652 formed by proton abstraction from the intact molecule was the base peak. This ion, together with its less abundant but commonly encountered glycerol adduct, defined a molecular weight of 653 (glycerol adducts were not observed for fragment ions). The A series phosphate anions at *m/z* 323 and 403 indicated 1,2,4-triazole-3-carboxamide as one base, while the fragments at *m/z* 346 and 426 (B series) defined adenine as the other base.

These phosphate anions were also observed to undergo dehydration (*m/z* 385, A_2 - H_2O), possibly to cyclic phosphates, and to incorporate some elements of the other sugar (*m/z* 445, A_2 + 42). In the case of dimeric dinucleotides, **8e** and **8f**, only one series of phosphate anions was observed since both bases were the same. Although the structure of these dinucleotides can be defined by their mass spectra above *m/z* 300 (Table III), where there is little interference from the glycerol matrix or other contaminants, other structurally important ions were also observed at lower mass. In those dinucleotides containing a C-N glycosidic bond, fragmentation of this bond occurred to give ions directly indicative of the base. For "X" or "Y" equal to A, R, or Z, moderately intense (30-60%) peaks were observed at *m/z* 134, 111, and 125, respectively. Little or no cleavage of this glycosidic bond was seen, however, for the thiazole (T, *m/z* 127) or selenazole (S, *m/z* 175) bases because of the greater stability of a C-C glycosidic bond. Thus the base anion was not always diagnostic and in the case of thiazole-4-carboxamide was obscured by the glycerol adduct of chloride ion. Additionally, the phosphodiester linkage was indicated in the spectra of all dinucleotides by a set of very abundant ions at *m/z* 177 ($H_3P_2O_7^-$) and *m/z* 159 (177 - H_2O).

The above self-consistent set of fragment ions allowed easy determination of modification to either side of the dinucleotide. This can be seen in Table III where the B series ions for **8a-d** are all the same since "Y" (adenine) never changes. However, the A series ions and M - H vary as "X", the carboxamide-containing base, is varied. When dinucleotide products from method B, which employed carbonyldiimidazole activation, were initially analyzed, the FAB spectra showed a shift of 26 amu for one series of phosphate fragments and the M - H ion. This mass shift occurred for the side of the molecule corresponding to the nucleotide activated as the phosphorimidazolidate. Ap-

(16) Hogg, A. M.; Kelland, J. G.; Vederas, J. C. Presented at the 31st Annual Conference on Mass Spectrometry and Allied Topics, Boston, MA, May 8-13, 1983.
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Table IV. IMPD Inhibition and Cytotoxicity of Nucleotide 5′-Monophosphates

compd	P388 cytotoxicity ID_{50}, μM	IMPD inhibn		
		av K_i,[a] μM	variable substrate	type of inhibn[b]
tiazofurin 5′-monophosphate (**4a**)	10	265	IMP	noncompetitive
		405	NAD	uncompetitive
selenazofurin 5′-monophosphate (**4b**)	1.4	170	IMP	noncompetitive
		470	NAD	uncompetitive
ribavirin 5′-monophosphate (**4c**)	65	4.18	IMP	competitive
		2.24	NAD	noncompetitive
ZR-5′-MP (**4d**)	>220	inactive[c]	IMP	
		inactive[c]	NAD	

[a] Mean of determinations at three inhibitor concentrations. In no case did individual values differ from these means by more than 20%. [b] As defined in ref 23. [c] A compound was regarded as inactive when the concentration causing 50% inhibition of IMPD (IC_{50}) was greater than 1 mM.

parently, normal workup conditions were not sufficient to destroy completely the cyclic 2′,3′-carbonate which was formed upon carbonyldiimidazole treatment. Thus, a spectrum such as that of **9c** (Table III) was observed. Here ribavirin monophosphate was activated by carbonyldiimidazole after reaction with AMP. Since anion-exchange chromatography did not completely separate **9** from **8**, the FAB mass spectra of isolated reaction products often indicated a mixture of the cyclic carbonate and fully deprotected dinucleotide. After treatment of **9c** with aqueous triethylamine, only **8c** could be detected by negative ion FAB/MS.

It was often possible to identify the components of simple synthetic mixtures and to obtain a qualitative assessment of compound purity by using FAB/MS. The well-defined nature of dinucleotide fragmentation (vide supra) and the fact that similar compounds were being examined made this type of nonchromatographic mixture analysis quite reliable. Thus, components unresolved by anion-exchange chromatography could be identified, and incomplete removal of protecting groups could be easily assessed (e.g., 2′,3′-carbonates and isopropylidenes). It was also possible to determine the presence of inorganic contaminants and phosphorylating agents.[22] FAB/MS is quite sensitive to the presence of preformed ions in the glycerol matrix. Both Cl^- and $H_2PO_4^-$ can be detected at masses corresponding to G_n + 35, 37 (e.g., m/z 127, 129) and G_n + 97 (e.g., m/z 189), respectively, where G_n is the mass of the appropriate glycerol adduct (n = 0, 1, 2, ...). Organic phosphate contaminants could also be seen since they formed adducts with glycerol or mixed dimers with other phosphates.

Biology. All of the synthesized dinucleotides and their precursor monophosphates were tested for their cytotoxicity against P388 cells in culture, as well as for their inhibitory properties against IMPD from the same source. The inhibitory activity was measured with use of both IMP and NAD as variable substrates in an attempt to understand the mechanism of inhibition. As seen in Table IV, ribavirin 5′-monophosphate (**4c**) was the most potent inhibitor among the monophosphates, showing competitive inhibition with respect to IMP. The other monophosphates ranked from inactive (ZR-5′-MP, **4d**) to moderately active (tiazofurin 5′-monophosphate (**4a**) and selenazofurin 5′-monophosphate (**4b**)). With the latter two nucleotides, the inhibition was clearly not of a competitive type and the resulting K_i's were similar in value.[23] The order of activity in this series was ribavirin 5′-monophosphate >> tiazofurin 5′-monophosphate selenazofurin 5′-monophosphate >> ZR-5′-MP. IMPD inhibitory activity was dramatically reversed in the case of three of the four corresponding dinucleotides (Table V). The inactive monophosphate ZR-5′-MP (**4d**) engendered an equally inactive dinucleotide (ZAD, **8d**), whereas the intermediately active monophosphates, tiazofurin 5′-monophosphate (**4a**) and selenazofurin 5′-monophosphate (**4b**), respectively generated very powerful dinucleotide inhibitors (TAD and SAD) that were 3–4 orders of magnitude more potent than their constituent monophosphates. Conversely, the very active ribavirin 5′-monophosphate (**8c**) gave rise to a rather weak inhibitor (RAD, **8c**) that was 100 times less active than its parent monophosphate. The order of potency for the dinucleotides was clearly defined as SAD > TAD >> RAD >> ZAD. The inhibitory activity of the symmetric dinucleotides TTD and SSD was decisively inferior to that of the adenine-containing dinucleotides TAD and SAD. Surprisingly, the very powerful dinucleotides, TAD (**8a**) and SAD (**8b**), did not show competitive inhibition with respect to NAD; in fact, the pattern of inhibition produced by these agents resembled that reported for NADH.[24]

In addition to generating dinucleotides with greater IMPD inhibitory potency, tiazofurin (**1a**) and selenazofurin (**1b**) are the only ribonucleosides in this series which are converted to dinucleotides in vivo. As indicated in Table VI, neither RAD nor ZAD were formed in detectable amounts in the cell culture assay nor with NAD pyrophosphorylase extracted from these cells. These results also suggest that there is a definite preference for the formation of SAD as demonstrated in our earlier studies.[5]

Table V. IMPD Inhibition and Cytotoxicity of Dinucleotide Analogues

compd	P388 cytotoxicity ID_{50}, μM	av K_i,[a] μM	variable substrate	type of inhibn[b]
TAD (**8a**)	7.5	0.13	IMP	uncompetitive
		0.24	NAD	noncompetitive
SAD (**8b**)	9.8	0.05	IMP	uncompetitive
		0.04	NAD	noncompetitive
RAD (**8c**)	40	148	IMP	noncompetitive
		235	NAD	noncompetitive
ZAD (**8d**)	78	inactive[c]	IMP	
		inactive[c]	NAD	
TTD (**8e**)	4.6	140	IMP	uncompetitive
		370	NAD	uncompetitive (mixed)
SSD (**8f**)	0.6	190	IMP	uncompetitive
		240	NAD	uncompetitive (mixed)

[a] See footnote *a* in Table IV. [b] As defined in ref 23. [c] A compound was regarded as inactive when the IC_{50} > 1 mM (see Table IV).

(23) The definition of the type of inhibition observed in these studies is taken from Webb, J. L. "Enzyme and Metabolic Inhibitors"; Academic Press: New York, 1963; pp 49–104.

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Table VI. Biosynthesis of Dinucleotide Analogues of NAD by P388 Cells and Isolated NAD Pyrophosphorylase

cell culture			NAD pyrophosphorylase			
compd added to the culture	dinucleotide formed	sp act.[a]	starting nucleotide	dinucleotide synthesized	sp act.[b]	% of NAD[c]
tiazofurin	TAD	99.7 ± 2.3	tiazofurin 5′-monophosphate	TAD	49.0	68
selenazofurin	SAD	270.1 ± 4.2	selenazofurin 5′-monophosphate	SAD	54.0	75
ribavirin	(RAD)	ND[d]	ribavirin 5′-monophosphate	(RAD)	0	0
ZR (AICAR)	(ZAD)	ND[d]	ZR-5′-MP	(ZAD)	0	0

[a] Specific activity = nanomoles of dinucleotide/gram of cell ± SD formed after 2 h. [b] Nanomoles of dinucleotide hour^{-1} (miligram of protein)$^{-1}$ (results represent the mean of two determinations with less than 5% variation). [c] The specific activity for NAD (100%) was 72.0. [d] Not detectable.

Discussion

It would appear from the NMR data (Table II) that the conformation of the adenine-containing dinucleotides in aqueous solution is preferentially folded, whereas the conformation of the dimeric dinucleotide structures, TTD and SSD, appears to be extended. It is now generally believed that the conformation of NAD and NADH can be best described as an equilibrium mixture between extended and folded states.[25]

As observed in Table V, none of the dinucleotides showed competitive behavior with respect to NAD. Since all of these new dinucleotides lack the characteristic positive charge of the nicotinamide portion of NAD, it is presumed that these compounds might instead behave as structural analogues of NADH which also lacks this critical charge. In this context it is interesting to note that, with certain dehydrogenases, there appear to be two binding sites for NADH, one catalytic and one regulatory.[26] In both liver and hepatoma IMPD's, NADH was found to inhibit the enzymatic reaction noncompetitively (K_i = 260 μM) with both IMP and NAD, suggesting that perhaps there is also a regulatory site in IMPD.[27]

It was also surprising that the dimeric dinucleotides TTD (**8e**) and SSD (**8f**) showed a measurable degree of inhibition against IMPD. The modest nature of this inhibition probably has to do with the fact that the AMP portion is required for the best possible orientation of the molecule at the receptor site.

Any attempt to correlate some structural feature of the different nucleoside monophosphates and their corresponding dinucleotides with IMPD inhibitory activity has to take into account the relative preference for the syn or anti conformation of the parent five-membered base nucleoside, either free, as a monophosphate, or as a part of a dinucleotide structure. At first glance, and with the use of space-filled molecular models, it would appear that for tiazofurin (**1a**) and selenazofurin (**1b**), the conformation would have to be preferentially syn in order to relieve the severe steric contact between the bulkier S and Se atoms with the furanose ether oxygen. However, a recent X-ray crystallographic study has shown that the preferred conformation, at least for tiazofurin (**1a**), is anti and that the close S···O contact results from an electrostatic attraction between the positive thiazole sulfur and the lone-pair electrons on the furanose oxygen.[28] Despite the fact that such important attractive force may very well determine the overall preference for the anti conformation in the 5′-monophosphates as well as in the dinucleotide structures, there is no experimental evidence to support this prediction at the present time. Additional attractive or repulsive interactions between the carboxamide and phosphate groups in the nucleotide and dinucleotide forms may alter the syn/anti preference significantly.[29]

Since ribavirin 5′-monophosphate (**4c**) was the only compound that showed competitive inhibition of IMPD with respect to IMP, it will likely adopt the corresponding conformation (syn or anti) that will best mimic that of the natural substrate IMP. As reported previously, the rotational barrier for a conformational interconversion anti–syn in ribavirin 5′-monophosphate is relatively small.[29] On the contrary, the more restricted conformational preference for the C-nucleosides tiazofurin (**1a**) and selenazofurin (**1b**)[28] is probably one that does not allow a juxtaposition with IMP, when existing as the 5′-monophosphates, and one that confers the greatest affinity for a yet unknown receptor in the dinucleotide structure. It is possible that this receptor is indeed a regulatory site whose stereochemical preference is independent of the stereoselectivity displayed by IMPD for the syn form of the coenzyme NAD.[30,31] As can be seen with the analogues RAD and ZAD, a mere likeness to NAD is not predictive of a strong inhibitory activity against IMPD.

In summary, this work reveals IMPD's exquisite selectivity for the sulfur- or selenium-containing dinucleotide. This selectivity does not appear to be due solely to the resemblance of these dinucleotides to NAD but rather to very stringent stereochemical and conformational requirements that are uniquely met by these dinucleotides. That their interaction is not competitive with respect to NAD might indicate that these molecules are not binding to the NAD catalytic site. Since changes introduced in the five-membered aglycon or the sugar moiety of tiazofurin have proven fruitless in yielding a more effective compound (with the exception of selenazofurin),[32,33] it appears that future synthetic efforts should be oriented toward the development of lipid-soluble forms or prodrugs of either TAD or SAD in order that these charged molecules could thereby be introduced into the cell. This tactic would obviate the otherwise necessary metabolic activation by NAD pyrophosphorylase.

Experimental Section

General Methods. HPLC analyses were performed at ambient temperature on a Waters Associates Model 204W chromatograph by using a 4.6 × 250 mm Partisil 10-SAX column eluted with a linear gradient of 0.01–0.5 M $(NH_4)H_2PO_4$ at 1.0 mL/min over 30 min. Compounds of interest were monitored by their UV absorbance at 254 nm. ^{1}H NMR spectra were recorded on a

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Varian XL-200 spectrometer; 3-(trimethylsilyl)-1-propanesulfonic acid (TSP) in D_2O was used as an internal reference. The effect of CD_3OD on the chemical shift of TAD was studied with D_2O/CD_3OD solutions containing a final concentration of TAD of 1 mg/mL. All spectra were recorded at 25 °C. Concentrations of CD_3OD (v/v) were 0, 20, 40, 60, and 80%. All the changes in chemical shift were referenced to TSP. Positive and negative ion mass spectra were obtained on a VG Analytical 7070E mass spectrometer equipped with a VG fast atom bombardment ion source operated at an accelerating voltage of 6 kV. Glycerol was used as the sample matrix, and ionization was effected by a beam of xenon atoms derived by charge exchange neutralization of a 1-mA beam of xenon ions accelerated through 8.2–8.8 kV. Spectra were acquired at a scan speed of 10 s/decade by using a VG Analytical 2035 data system, and the background due to the glycerol matrix was automatically subtracted. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

General Procedure for the Synthesis of NAD Analogues by the Carbonyldiimidazole Approach (Method B). The corresponding free acid monophosphate (**4**, 0.1 mmol) was rigorously dried and suspended in DMF (0.5 mL). Carbonyldiimidazole (0.5 mmol) was added and the reaction mixture became homogeneous in a few minutes. After 3 h, HPLC analysis showed the disappearance of starting material (t_R = 13–15 min) and the appearance of a peak at t_R = 4–5 min. Methanol (33 μL) was added to hydrolyze excess carbonyldiimidazole and after 30 min, 0.15 mmol of the other monophosphate (usually AMP), dissolved in 2 mL of anhydrous DMF containing 0.15 mmol of tri-*n*-butylamine, was added. The reaction mixture was stirred for 48 h at room temperature and monitored by HPLC. When the reaction was completed, 5 mL of water was added and the solution was reduced to dryness. The gummy residue was dissolved again in 20 mL of water containing sodium acetate (30 mg) and extracted twice with 20 mL each of chloroform and ether. The aqueous layer was treated with triethylamine (~200 μL) until pH 10 was reached and stirred for 8–24 h. After such time, the entire mixture was lyophilized and the residue chromatographed on a Hamilton HA-X4 anion-exchange column (HCO_3^- form, 0.8 × 28 cm). During a 7-h gradient from water to 2 M $(H_4N)HCO_3$, with a flow rate of 2 mL/min, samples of 12 mL were collected and monitored by UV at 290 nm. The desired fractions were collected and passed through a strong cation-exchange resin (AG50W-X8, H^+ form, 1.5 × 15 cm) and eluted with water. After collection of 15-mL fractions from this column, the product was always present in fractions 2–4. These fractions were lyophilized to give the dinucleotides as white fluffy solids in the yields indicated in Table I.

IMP Dehydrogenase Inhibition. Kinetics of inhibition of a partially purified preparation of IMPD was examined as described previously,[3] using three concentrations of inhibitor and six concentrations of the substrates NAD or IMP (0.25, 0.5, 1, 2, 4, and 6 × K_m). Results were subjected to double-reciprocal analysis using a least-squares computer program developed in this laboratory.

Cytotoxicity. These studies were performed as described previously for the parent nucleosides with use of P388 cells in log phase growth.[32]

Biosynthetic Studies. P388 Cell Culture. P388 cells growing in log phase (6 × 10^6 cells/10 mL of RPMI 1640 medium were exposed to 5 μCi of [8-^{14}C]adenosine (54 mCi/mmol, 50 μCi/mL from Amersham Searle Corp.) for 60 min and then incubated with no drug or with tiazofurin, selenazofurin, ribavirin, or ZR (final concentration 100 μM) for 120 min. Cells were quickly spun, washed twice with 1 mL each of cold Hanks balanced salt solution, and extracted with a 250-mL aliquot of 10% trichloroacetic acid. The extract was immediately neutralized with 0.5 M trioctylamine in Freon and an aliquot analyzed on a Partisil 10 SAX column as described earlier.[32]

NAD Pyrophosphorylase. These studies were performed with NAD pyrophosphorylase isolated with P388 cells in culture.[34] The assay mixture contained 3.75 mM of substrate, 2.5 mM of ATP, 0.015 M $MgCl_2$, and 0.03 M Tris-HCl, pH 7.6, in a total volume of 100 μL. The reaction was initiated by the addition of a particulate enzyme (36 μg). After a 30-min incubation at 37 °C, the reaction was terminated by adding Cl_3CCOOH to a final concentration of 10%. The acid extract was neutralized with 0.5 trioctylamine in Freon,[35] and the samples were analyzed on HPLC as described previously.[32] The amount of dinucleotide formed was calculated by using the corresponding chemically synthesized dinucleotides as standards.

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Registry No. **1a**, 60084-10-8; **1b**, 83705-13-9; **2**, 36791-04-5; **3**, 3031-94-5; **4a**, 83161-83-5; **4a** (tri-*n*-octylamine salt), 93135-48-9; **4b**, 88268-53-5; **4b** (tri-*n*-octylamine salt), 93135-49-0; **4c**, 40925-28-8; **4c** (tri-*n*-octylamine salt), 93135-50-3; **4d**, 3031-94-5; **4d** (tri-*n*-octylamine salt), 93135-51-4; **5**, 7331-13-7; **7**, 61-19-8; **8a**, 83285-83-0; **8b**, 88144-98-3; **8c**, 88734-03-6; **8d**, 917-08-8; **8e**, 93135-46-7; **8f**, 93135-47-8; **9c**, 93135-52-5; IMPD, 9028-93-7; carbonyldiimidazole, 530-62-1.

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