Thiazole-4-carboxamide Adenine Dinucleotide (TAD). Analogues Stable to Phosphodiesterase Hydrolysis

Victor E. Marquez,* Christopher K. H. Tseng, Gulilat Gebeyehu, David A. Cooney, Gurpreet S. Ahluwalia, James A. Kelley, Maha Dalal, Richard W. Fuller, Yvonne A. Wilson, and David G. Johns

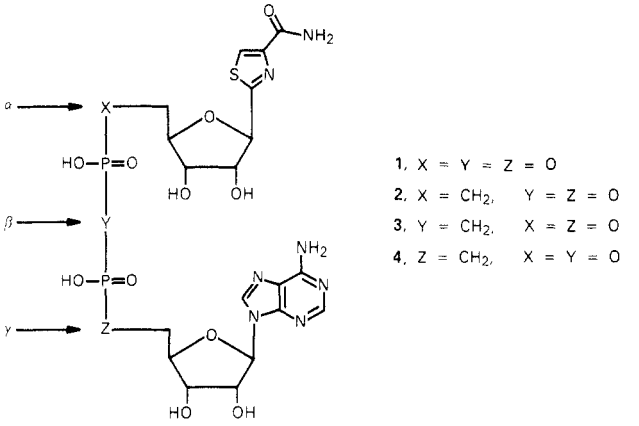
Laboratory of Pharmacology and Experimental Therapeutics, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received October 21, 1985*

Thiazole-4-carboxamide adenine dinucleotide (TAD), the active metabolite of the oncolytic C-nucleoside tiazofurin (TR), is susceptible to phosphodiesteratic breakdown by a unique phosphodiesterase present at high levels in TR-resistant tumors. Since accumulation of TAD, as regulated by its synthetic and degradative enzymes, appears to be an important determinant for sensitivity to the drug, a series of hydrolytically resistant phosphonate analogues of TAD were synthesized with the intent of producing more stable compounds with an ability to inhibit IMP dehydrogenase equivalent to TAD itself. Isosteric phosphonic acid analogues of TR and adenosine nucleotides were coupled with activated forms of AMP and TR monophosphate to give dinucleotides **2** and **4**. Coupling of protected adenosine 5′-(α,β-methylene)diphosphate with isopropylidene-TR in the presence of DCC afforded compound **3** after deprotection. These compounds are more resistant than TAD toward hydrolysis and still retain potent activity against IMP dehydrogenase in vitro. β-Methylene-TAD (**3**), the most stable of the TAD phosphonate analogues, produced a depletion of guanine nucleotide pools in an experimentally induced TR-resistant P388 tumor variant that was superior to that obtained with TR in the corresponding sensitive line.

The antitumor effect of the oncolytic C-nucleoside tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, TR) is directly related to its intracellular anabolism to an analogue of nicotinamide adenine dinucleotide (NAD) in which the thiazole ring of tiazofurin has replaced the pyridine ring of nicotinamide.[1-5] The structure of this dinucleotide, thiazole-4-carboxamide adenine dinucleotide (TAD, **1**), has been confirmed by detailed structural studies and by both enzymatic and chemical syntheses.[1-6] To date, this compound is among the most potent inhibitors of the critical enzyme IMP dehydrogenase.[1,3-6] In two recent studies where the relationship between metabolism and cytotoxicity of the parent drug was examined, it was demonstrated that TAD accumulation was the most important determinant of sensitivity or resistance to tiazofurin in rodent neoplasms and human lung cancer cells.[7,8] This anabolite (TAD) is formed in two steps from tiazofurin (TR), involving first the phosphorylation to the 5′-monophosphate (TRMP), followed by conversion to TAD by NAD-pyrophosphorylase (Figure 1). Since accumulation of TRMP provided no correlation with sensitivity to the drug,[7,8] NAD-pyrophosphorylase was subsequently identified as the critical enzyme in the synthetic arm of the metabolic pathway. However, accumulation of TAD is also regulated by a degradative enzyme, TAD-phosphodiesterase (TAD-ase), and it is rather the ratio between these synthetic and degradative activities which ultimately determines sensitivity or resistance to TR.[7,8] The importance of the synthetic enzyme was illustrated recently by comparing the levels of NAD-pyrophosphorylase in a sensitive line (P388/S) vs. an experimentally induced resistant line (P388/R) of P388 leukemia. The enzymatic activity of NAD-pyrophosphorylase was reduced profoundly in P388/R, while levels of TAD-ase remained constant.[7,9] On the contrary, in naturally resistant lines the levels of both enzymatic activities were variable and for human lung cancer cells it appeared that TAD breakdown alone correlated better with the degree of resistance.[8]

From the standpoint of drug design, it is quite difficult a priori to intervene at the level of the synthetic pathway in order to enhance production of TAD. Furthermore, since it appears that levels of TAD-ase correlate better in some instances with the degree of resistance, it was felt that transformation of TAD into a hydrolytically stable molecule would be an attractive approach to overcoming natural or induced resistance.

It is well-known that phosphonic acids and their derivatives can be considered as biological analogues of naturally occurring phosphates but with the added advantage that the carbon–phosphorus bond is stable toward the phosphodiesterases involved in the cleavage of phosphate linkages.[10] On the basis of such reasoning, the α-, β-, and γ-methylene analogues of TAD (**2**, **3**, and **4**) were selected



as targets for potential drugs capable of overcoming cleavage by TAD-ase. Additionally, these compounds were

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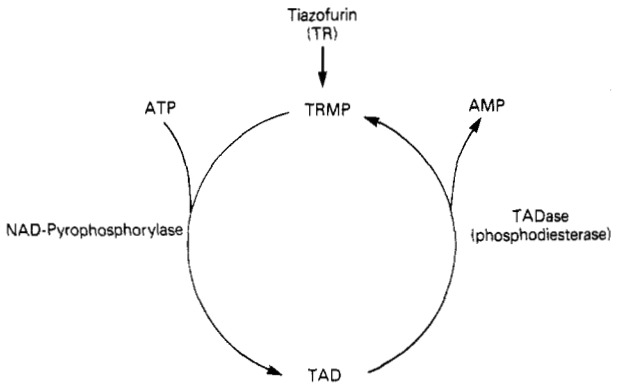
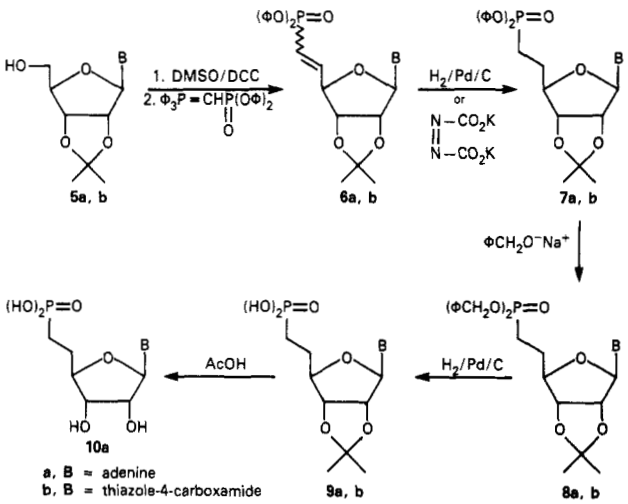


Figure 1. Mechanism of formation and hydrolysis of TAD.

Scheme I

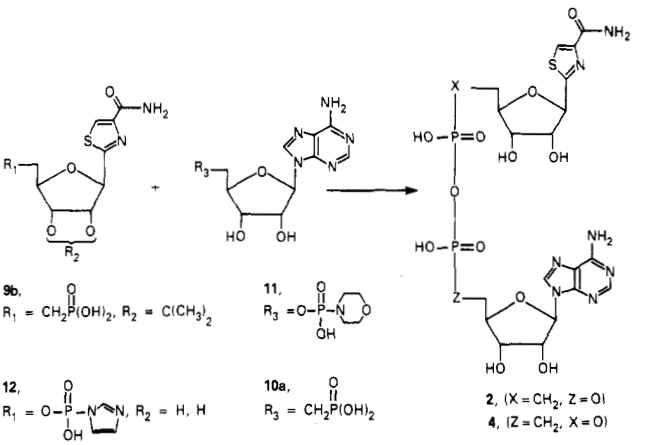


to serve as probes to study the exact site of cleavage along the 5′ → 5′ pyrophosphate bridge of TAD. The present work describes the synthesis, inhibitory activity against the target enzyme IMP dehydrogenase, and stability toward phosphodiesteratic cleavage of the three isomeric TAD phosphonate analogues.

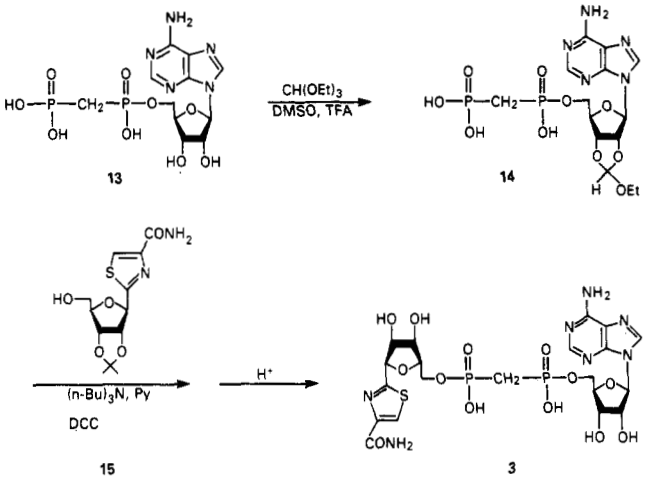
Chemistry. TAD phosphonate derivates **2** and **4**, in which either the α- or the γ-oxygen atom of the pyrophosphate bridge was replaced by a methylene group, required the prior preparation of the isosteric phosphonic acid analogues of AMP and TRMP. Following the standard procedure of Jones and Moffatt, 6′-deoxyhomoadenosine-6′-phosphonic acid (**10a**) was prepared as originally reported[11] (Scheme I). Similarly, the preparation of previously unknown 6′-deoxyhomotiazofurin-6′-phosphonic acid (**9b**), proceeded with the very effective catalytic reduction of **6b** to **7b**. This avoided the use of dipotassium azodicarboxylate required in the adenosine case.[11]

The two isosteric phosphonic acid analogues **9b** and **10a** were coupled with activated forms of AMP and TRMP (respectively the morpholidate **11** and the imidazolidate **12**) under identical reaction conditions developed earlier for the synthesis of TAD and analogues (Scheme II).[6,12] In this case however the yields of TAD phosphonates **2** and **4** were quite low (3–5%). On the contrary, the symmetric β-methylene-TAD (**3**) was prepared in 36% yield via the dicyclohexylcarbodiimide (DCC)-catalyzed coupling between protected adenosine 5′-(α,β-methylene)diphosphate (**14**) and 2′,3′-*O*-isopropylidenetiazofurin (**15**) as shown in Scheme III. Following a similar workup, all three dinucleotides were isolated in pure form from their reaction mixtures by anion exchange chromatography (HCO_2^- form) developed with a linear gradient of aqueous ammonium formate/formic acid as indicated in the Experimental Section.

Scheme II



Scheme III



Complete characterization of these dinucleotides was achieved by the use of negative ion fast atom bombardment (FAB) mass spectrometry and by high resolution ^1H NMR spectroscopy. As observed in Figure 2 for β-methylene-TAD (**3**), the three low-field aromatic singlets corresponding to H-5 of the thiazole ring and H-2 and H-8 of the adenine ring, are characteristic for these dinucleotides and they show nearly identical chemical shifts as in the spectrum of TAD.[12] Similarly, the corresponding anomeric protons of the adenosine and tiazofurin residues were observed at the expected values. In addition to these signals, the methylene protons of the phosphonate linkage were observed around δ 2.00. This signal appeared as an ill-defined triplet in compounds **2** and **4**; whereas in the symmetric TAD phosphonate **3**, it appeared as a sharply defined triplet reflecting only the presence of phosphorus to proton coupling.

Mass spectral analysis, which in this case is a more definitive tool for structural assignment, clearly indicates the extent and site of structural modification in these TAD isosteres.[12] This is illustrated by the negative ion FAB spectrum of the symmetrical TAD phosphonate **3** shown in Figure 3. The molecular weight is indicated by an intense M–H peak at m/z 666 and corroborated by a weak peak corresponding to the glycerol adduct at m/z 758 (not

(11) Jones, G. H.; Moffatt, J. G. *J. Am. Chem. Soc.* **1968**, *90*, 5337.
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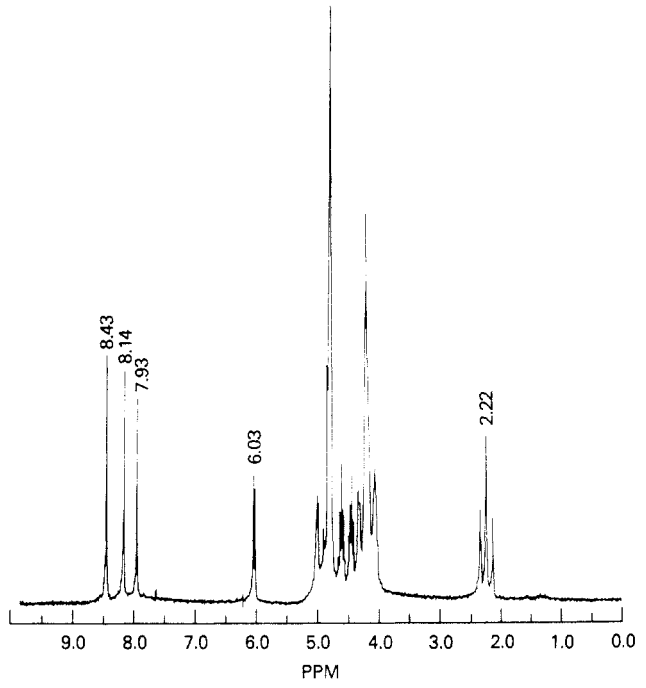
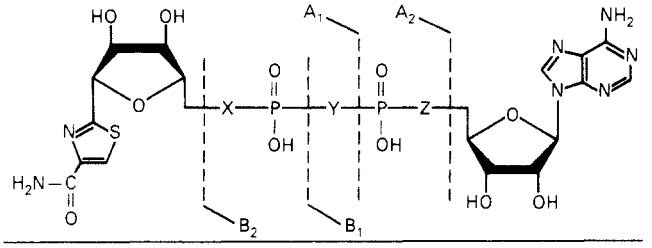


Figure 2. ^1H NMR spectrum of β-methylene-TAD in D_2O.

Table I. Principal Mass Spectral Fragments of TAD and TAD Phosphonates



m/z	TAD (1): X = Y = Z = O	α-TAD (2): X = CH_2, Y = Z = O	β-TAD (3): Y = CH_2, X = Z = O	γ-TAD (4): Z = CH_2, X = Y = O
M - H	668 (64)	666 (100)	666 (33)	666 (42)
A_2	419 (27)	417 (42)	417 (12)	
A_1	339 (13)	337 (20)		339 (100)
B_2	426 (31)		424 (15)	424 (18)
B_1	346 (14)	346 (72)		344 (6.7)
PP[a]	177 (69)		175 (24)	
PP - H_2O	159 (100)		157 (100)	

[a] PP stands for either P–O–P or P–CH_2–P in TAD and β-TAD, respectively.

shown). Because the β-oxygen has been replaced by a methylene moiety in **3**, fragmentation about this locus to produce the stable monophosphate anions observed in TAD (e.g., A_1 and B_1 fragments, Table I) does not occur. However, the corresponding diphosphonate anions (A_2, m/z 417; B_2, 424) are of good intensity and shifted by 2 mass units to indicate the substitution of methylene for oxygen. That methylene substitution occurs at the β-oxygen is further substantiated by the diphosphonate anion at m/z 175 which loses the elements of H_2O to give rise to the base peak at m/z 157. These ions are analogous to the m/z 177 and 159 pyrophosphate anions in TAD and are not observed in diphosphonate isomers **2** and **4**. Because the α- or γ-oxygen of the pyrophosphate bridge has been replaced by a methylene group in these latter isosteres, the corresponding diphosphonate fragment (B_2 in **2**, A_2 in **4**) is also not observed. Thus, the absence of fragmentation across the phosphonate linkage, coupled with the mass shift of the fragments that are observed, clearly indicates the extent and locus of the structural modifications (Table I).

Biological Activity. Using the standard procedure developed previously in this laboratory for measuring IMP dehydrogenase activity,[13] the three TAD phosphonate analogues were tested for their in vitro effectiveness in inhibiting this target enzyme. As seen in Table II, all the compounds were active as IMP dehydrogenase inhibitors with the β-methylene-TAD analogue (**3**) being virtually indistinguishable from TAD. Subsequently, using the partially purified TAD-ase isolated from naturally resistant colon-38 carcinoma cells[14] and the more general phosphodiesterase from snake venom, the compounds were evaluated for their stability against these enzymes following a 30-min incubation period (Table II). As a positive control for this assay TAD hydrolysis was set to 100% (specific activity = 7.5 μmol/h/mg TAD-ase protein).[7,14] Depending on the substrate, the rate of disappearance of the parent dinucleotide or alternatively the rate of formation of products of cleavage (i.e., AMP or TRMP) was measured by HPLC to determine the stability of the compounds. Since TAD engenders an equimolar mixture of AMP and TRMP after enzymatic hydrolysis, the enzymes are probably cleaving at the site of the middle oxygen of the pyrophosphate bridge. Both α- and γ-methylene-TAD, which have this oxygen intact, were expected therefore to be susceptible to enzymatic cleavage to some extent. Indeed, this was observed but to a significantly lesser degree than with TAD, indicating perhaps a loss in substrate capacity resulting from an overall change in the conformation of the molecule. Not surprisingly, both compounds behaved quite similarly as substrates for TAD-ase and venom phosphodiesterase. On the contrary, and consistent with the expected mechanism of hydrolysis, the β-methylene analogue **3** was completely inert toward both phosphodiesterase even after a long incubation period of 48 h.

The interpretation of the cytotoxicity data (Table II) requires taking into account the hydrolytic stability of these dinucleotides during this 48-h experiment.[19] TAD, which is equitoxic to TR on a molar basis,[6,19] rapidly hydrolyzes to TRMP which in turn hydrolyzes to TR. The nucleoside (TR) readily enters the cell, and if NAD pyrophosphorylase is present, it can reform the active metabolite TAD which accounts for the cytotoxicity.[6] With the α- and γ-methylene-TAD analogues, **2** and **4**, the eventual breakdown of the molecules after 48 h would generate TRMP and TR only from the γ-methylene-TAD (**4**). This would explain the nearly identical ID_{50} for both **4** and TAD and the much lower cytotoxicity displayed by the α-methylene analogue **2**. The β-methylene-TAD (**3**), on the contrary, was completely stable after 48 h, and therefore the ID_{50} value of 45 μM appeared to indicate that the intact molecule was entering the cell. However, since the possibility existed that other forms of cleavage, undetected in these experiments, would generate TR from **3**, it became imperative to test this compound against one of the available TR-resistant cell lines. The experimentally induced TR-resistant (P388/R) line, which has extremely low levels of the enzyme responsible for the formation of TAD (NAD-pyrophosphorylase), was selected for the experiment. In the event that cleavage of **3** occurred, by some presently unknown mechanism, the cells would have

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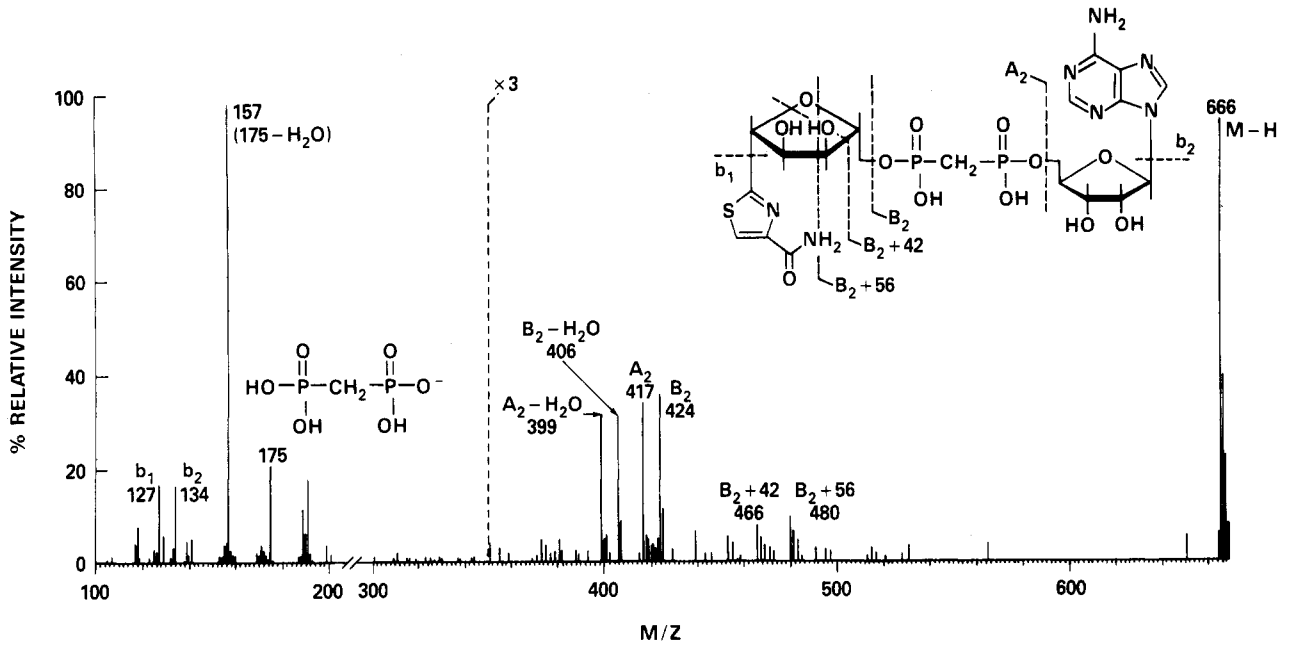

Figure 3. Negative ion FAB mass spectrum of β-methylene-TAD (3). Peaks from the glycerol matrix have been computer subtracted.

Table II. Biological Activity of TAD Phosphonates

dinucleotide	cytotxcty (P388) ID_{50}, μM	IMPD-inhibitn		degradatn (% TAD)	
		K_i,[a] μM	type of inhibitn	TAD-ase	venom phosphodiesterase
TAD (1)	2.1	0.24	not competitive	100[c]	100[d]
α-methylene-TAD (2)	100.0	1.97	not competitive	10.4	27.2
β-methylene-TAD (3)	45.0	0.23	not competitive	0	0
γ-methylene-TAD (4)	2.8	17[b]	ND	13.0	37.6

[a] With NAD as variable substrate. [b] ID_{50}. [c] TAD hydrolysis (specific activity) = 7.5 μmol/h per mg protein was set at 100%. [d] TAD hydrolysis (specific activity) = 170 μmol/h per mg protein was set at 100%.

remained insensitive to 3 as they already were to TR. Conversely, a significant cytotoxic effect of 3 against P388/R was to be interpreted as evidence that the drug was entering the cell.

The results showed that β-methylene-TAD had identical effectiveness against either P388/S or P388/R with an ID_{50} of approximately 50 μM. Furthermore, when this dose was tested for its effect on guanine nucleotide pools, the evidence for the effectiveness of 3 became very clear (Table III). This information was very important because it demonstrated not only that the compound was able to penetrate the cell but that it also appeared to block IMP dehydrogenase effectively. In fact, the data showed that, in the resistant cells, guanine nucleotide pools were even more effectively reduced than in the sensitive line. TR, used as a control, was effective against P388/S in reducing guanine nucleotide pools and ineffective, as expected, against P388/R. The resistant line began to respond to TR only at doses 2000 times higher than those inhibiting growth of the parent strain (Table III).

When analyzing the results obtained for the β-methylene-TAD it appears that the principal objectives of the present investigation have been realized. β-Methylene-TAD (3) is a compound that (a) is enzymatically stable, (b) is equipotent to TAD as inhibitor of IMP dehydrogenase, (c) does not require the presence of NAD-pyrophosphorylase for its formation, and (d) is capable of penetrating the cell.

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 280 nm. ^1H NMR spectra were recorded on a Varian XL-200 spectrometer in D_2O. 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 7.2–9.0 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 substracted. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Table III. Influence of β-Methylene-TAD on Guanine Nucleotide Pools of Natural P388 Cells (P388 Sensitive) and a Variant Line (P388 Resistant) Refractory to Tiazofurin Because of a Substantial Loss of NAD Pyrophosphorylase

treatment (concn, μM)	guanine nucleotide concn (% of control)		
	GMP	GDP	GTP
P388 sensitive line			
β-CH_2TAD (50)	14	24	24
β-CH_2TAD (100)	6	19	39
tiazofurin (2.5)	12	41	51
tiazofurin (5.0)	8	36	34
P388 resistant line			
β-CH_2TAD (50)	3	24	5
β-CH_2TAD (100)	3	34	8
tiazofurin (2.5)	98	88	111
tiazofurin (5000)	0	74	52

2-[5′,6′-Dideoxy-2′,3′-*O*-(1-methylethylidene)-6′-phosphono-β-D-*ribo*-hex-5′-enofuranosyl]-4-thiazolecarboxamide, Diphenyl Ester (6b). 2′,3′-*O*-Isopropylidenetiazofurin (**5b**,[15] 0.9 g, 3 mmol) was dissolved in 10 mL of Me_2SO.

To this solution were added dicyclohexylcarbodiimide (DCC, 3.69 g, 18 mmol) and 1.5 mL of a 1 M solution of H_3PO_4 in Me_2SO, and the reaction mixture stirred overnight for 18 h. Addition of pyridine (1 mL) was followed by 1.74 g (3 mmol) of diphenyl triphenylphosphoranylidene methylphosphonate dissolved in 5 mL of Me_2SO, and the mixture stirred again for 18 h. An additional amount of the Wittig reagent (1.0 g) was necessary to complete the reaction with continuous stirring for 18 h. The reaction mixture was diluted with 100 mL of $CHCl_3$ and extracted twice (2 × 250 mL) with water. The organic layer was dried ($MgSO_4$) and subjected to two succesive flash chromatographies (2.5 × 15 cm) over silica gel and developed with ethyl acetate–hexane (3:1) and $CHCl_3$–ethyl acetate (1:1), respectively. The desired compound with a R_f value of 0.2 on silica gel TLC plates (developed with $CHCl_3$–ethyl acetate) was collected to give 0.850 g (57%) of pure material which was used for the next step without further purification. The complexity of the NMR spectrum in the δ 4.50–6.50 region indicated that the compound was a mixture of cis and trans isomers.

2-[5′,6′-Dideoxy-2′,3′-*O*-(1-methylethylidene)-6′-phosphono-β-D-*ribo*-hexofuranosyl]-4-thiazolecarboxamide, Diphenyl Ester (7b). The vinylphosphonate **6b** (0.7 g, 132 mmol) was dissolved in 100 mL of methanol and hydrogenated over Pd/C (5%) for 1 h at 1 atm pressure. The reaction mixture was filtered through Celite and concentrated to give 0.62 g (88%) of a fluffy homogeneous solid which was used directly for the next step.

2-[5′,6′-Dideoxy-2′,3′-*O*-(1-methylethylidene)-6′-phosphono-β-D-*ribo*-hexofuranosyl]-4-thiazolecarboxamide (9b). Sodium hydride (0.12 g) was added to 10 mL of dry benzyl alcohol which was kept under dry nitrogen. After 30 min, this reagent was added to the diphenyl phosphonate **7b** (0.5 g, 0.94 mmol) dissolved in 5 mL of dry benzyl alcohol. The reaction was stirred under nitrogen for 1.5 h. After diluting with ethyl ether and bubbling CO_2 gas into the solution, the precipitate was filtered off, and the filtrate was applied to a flash chromatography column (2.5 × 15 cm) over silica gel and eluted with ethyl acetate (300 mL) to remove all of the benzyl alcohol. The column was then eluted with 10% methanol in ethyl acetate to obtain the desired compound **8b** (0.36 g, 68%) as an oil. The NMR spectrum of this product showed the expected signals for the two carbon chain connecting the sugar and the phosphonate at δ 1.4–2.2. Hydrogenation of this compound over 5% Pd/C in 100 mL of methanol at 1 atm pressure afforded, after removal of the catalyst and solvent, 0.14 g (69%) of pure **9b**: mp 85 °C; NMR (Me_2SO-d_6) δ 1.30 (s, 3 H), 1.60 (s, 3 H), 2.00 (m, 4 H), 4.25 (m, 2 H), 4.55 (m, 1 H), 5.10 (d, J = 2.6 Hz, 1 H), 8.20 (s, 1 H). Anal. Calcd for $C_{13}H_{19}N_2O_7PS$: C, 41.26; H, 5.02; N, 7.40; P, 8.20; S, 8.46. Found: C, 41.06; H, 5.23; N, 7.28; P, 8.02; S, 8.30.

5′-Adenylic Acid, Monoanhydride with 2-(5′,6′-Dideoxy-6′-phosphono-β-D-*ribo*-hexofuranosyl)-4-thiazolecarboxamide (2, α-Methylene-TAD). A solution of **9b** (0.4 g, 1.05 mmol) in 50% aqueous pyridine (80 mL) was treated with an equimolar amount of tri-*n*-octylamine (0.370 g, 1.05 mmol) and subsequently lyophilized. The residue obtained was dissolved in dry pyridine and reduced to dryness again under vacuum. The new residue was treated with a solution of adenosine 5′-monophosphomorpholidate[16] (**11**, 0.70 g, 0.98 mmol) in pyridine (100 mL), and the resulting mixture stirred at 40 °C for 20 h. At this point, HPLC analysis revealed that the morpholidate **11** had been consumed. The reaction mixture was then reduced to dryness and partitioned between ether (50 mL) and water (50 mL). The aqueous layer was treated with sodium acetate (0.24 g) and extracted twice with ether (2 × 50 mL) after which it was lyophilized to give a white fluffy solid. The compound was dissolved in 50 mL of 10% acetic acid and heated at 70 °C for 1 h to remove the isopropylidene group. After a second lyophilization, the residue was charged on a Benson (BA-X4) anion exchange resin column (HCO_2^- form, 0.9 × 50 cm). The mobile phase consisted of a 7-h linear gradient from water to 1 M HCO_2NH_4 + 2 M HCO_2H, at a flow rate of 3 mL/min. Fractions (7.5 mL) were monitored by UV at 280 nm and checked by analytical HPLC. The product-containing fractions were combined, lyophilized, and rechromatographed twice in the same system until HPLC analysis indicated that two single peaks were resolved in the area of the dinucleotides. One of the peaks corresponded to diadenosine 5′→5′-pyrophosphate and the other was the desired product as confirmed by ^{1}H NMR analysis. The desired fractions were collected and treated with cation-exchange resin (AG50W-X8, H^+ form), and after removal of the resin and lyophilization of the filtrate, 18 mg (3% yield) of the target α-methylene TAD (**2**) was obtained as the free acid in the form of a white lyophilized powder. The purity and identity of the product was confirmed by HPLC, MS, and ^{1}H NMR analyses.

9-(5′,6′-Dideoxy-6′-phosphono-β-D-*ribo*-hexofuranosyl)-adenine (10a). This compound was prepared essentially as reported by Jones and Moffatt.[11] The material obtained was homogeneous by HPLC, and its NMR and MS were in agreement with the structure.

9-(5′,6′-Dideoxy-6′-phosphono-β-D-*ribo*-hexofuranosyl)-adenine 6′→5′-Ester with 2-β-D-Ribofuranosyl-4-thiazolecarboxamide (4, γ-Methylene-TAD). Tiazofurin monophosphate[17] (0.035 g, 0.1 mmol) was rigorously dried and suspended in DMF (0.5 mL). Carbonyldiimidazole (0.081 g, 0.5 mmol) was added, and the reaction became homogeneous in a few minutes. After stirring for 3 h, methanol (33 μL) was added, and stirring continued for 30 min. Immediately after **10a**[11] (0.051 g, 0.15 mmol), dissolved in 2 mL of anhydrous DMF containing 0.14 mmol of tri-*n*-butylamine, was added. The reaction mixture was stirred at room temperature for 48 h after which time it was treated with 5 mL of water and reduced to dryness. The 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 was chromatographed under identical conditions as above. The desired fractions were collected and treated with cation-exchange resin (AG50W-X8, H^+ form) to afford the solid free acid γ-methylene-TAD (**4**) after lyophilization (5 mg, 5% yield). The purity and identity of the product was confirmed by HPLC, MS, and ^{1}H NMR analyses.

Adenosine 5′-[Hydrogen (phosphonomethyl)phosphonate] 5′→5′-Ester with 2-β-D-Ribofuranosyl-4-thiazolecarboxamide (3, β-Methylene-TAD). Adenosine 5′-methylenediphosphonate[18] (**13**, 0.1 g, 0.235 mmol) was stirred with triethyl orthoformate (0.78 mL, 4.69 mmol) and trifluoroacetic acid (1.08 mL, 14 mmol) in anhydrous Me_2SO (4 mL) at room temperature for 16 h. The solution was evaporated under reduced pressure to remove most of the trifluoroacetic acid. Diethyl ether (120 mL) was added to the residue, and the precipitate formed was collected by centrifugation. The ether was discarded, and the precipitate was washed 3 more times in the same manner. After drying under reduced pressure, 0.112 g (99% yield) of **14** was obtained and used directly without further purification [negative ion FABMS, m/z 480 (M–H)].

The protected adenosine 5′-(α,β-methylene)diphosphate (**14**, 0.0275 g, 0.057 mmol) was stirred together with anhydrous pyridine (0.85 mL) and tributylamine (0.19 mL) at 65 °C until the reaction mixture was homogeneous. To this solution were added 2′,3′-*O*-isopropylidenetiazofurin (**15**, 0.018 g, 0.06 mmol) and dicyclohexylcarbodiimide (0.054 g, 0.26 mmol), and the reaction mixture was stirred at 65 °C overnight for 21 h. Most of the pyridine was removed under reduced pressure, and the residue was partitioned between diethyl ether (25 mL) and water (25 mL). The ethereal layer was discarded, and the aqueous layer was washed twice more with diethyl ether. The aqueous layer was lyophilized, and the white residue was treated while stirring with cation-exchange resin (AG50W-X8, H^+ form). After 1 h the solution was filtered, and the filtrate was lyophilized. The residue was dissolved in 2 mL

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(16) Adenosine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt is available from Sigma Chemical Co.

(17) Srivastava, P. C.; Revankar, G. R.; Robins, R. K. *J. Med. Chem.* **1984**, *27*, 266.

(18) Dixit, V. M.; Poulter, C. D. *Tetrahedron Lett.* **1984**, *25*, 4055. This compound is also available from P-L Biochemicals, Inc., Milwaukee, WI.

of water and chromatographed under the same conditions as for 2 and 4. The desired fractions were collected, treated again with cation exchange resin, and lyophilized to give 14 mg (36%) of the desired free acid dinucleotide 3 as a white fluffy solid. The purity and identity of the product was confirmed by HPLC, MS, and ^{1}H NMR analyses.

IMP Dehydrogenase Inhibition. Kinetics of inhibition of a partially purified preparation of IMPD was examined as described previously[13] by using 3 concentrations of inhibitor and 6 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 nucleoside TR with use of P388 cells in log phase growth.[19]

Degradation of TAD and TAD Phosphonates. TAD and its phosphonate analogues were subjected to hydrolysis by either partially purified TAD phosphodiesterase or snake venom phosphodiesterase at a substrate concentration of 7.5 mM in 0.03 M Tris-$MgCl_2$ buffer, pH 7.6. Quantitation of AMP or TRMP by HPLC was taken as a measure of dinucleotide degradation after a 30-min incubation period.

Tiazofurin-Resistant P388/R Line. The P388 resistant cell line used in this study was developed by exposing P388 cells to gradually increasing concentrations of TR until the population could grow almost normally in the presence of a 1 mM concentration of the drug; the doubling time was 17 h as opposed to 15 h for the parent line. To compare the metabolism of TR in these two lines, they were incubated with [^{14}C]-labeled drug for 5 h after which the cells were washed, and the pellets were extracted with 10% TCA and neutralized with tri-*n*-octylamine in freon. Using 90% methanol as solvent, paper chromatography (Whatman 3MM) was carried out on the resultant extracts. Spots coincident with TAD were then cut out, eluted with water, and counted for radioactivity. TAD levels in the sensitive and resistant cells were 14.3 and 0.98 pmol/10^6 cells, respectively.

Influence of β-Methylene-TAD (3) on the Guanine Pools of P388/S Cells and TR-Resistant P388/R Mutants. Logarithmically growing P388/S or P388/R cells were incubated with the indicated concentration of β-methylene-TAD or TR for 18 h (Table III). The cells were then washed once with cold normal saline, extracted with cold 10% TCA (0.4 mL), and finally neutralized with an equal volume of tri-*n*-octylamine in freon. The resultant extracts were then analyzed by HPLC utilizing a radial compression column of Partisil-10 SAX resin (Waters Co., Milford, MA) pre-equilibrated and isocratically eluted with 0.03 M ammonium phosphate, pH 3.5, for 10 min. A 30-min gradient to 0.6 M ammonium phosphate, pH 3.8, followed, and the column was washed with the 0.6 M buffer for 10 min. In sensitive control cells, the levels of GMP, GDP, and GTP were 0.14, 0.20, and 0.39 nmol/10^6 cells, respectively. In resistant control cells the same nucleotides were 0.18, 0.28, and 1.0 nmol/10^6 cells, respectively.

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Registry No. 1, 83285-83-0; 2, 102977-54-8; 3, 102977-57-1; 4, 102977-55-9; 5b, 60084-11-9; *cis*-6b, 102977-49-1; *trans*-6b, 102977-50-4; 7b, 102977-51-5; 8b, 102977-52-6; 9b, 102977-53-7; 10a, 22257-15-4; 11, 7331-13-7; 13, 3768-14-7; 14, 102977-56-0; TRMP, 83161-83-5; IMP dehydrogenase, 9028-93-7; diphenyl triphenylphosphoranylidene methylphosphonate, 22400-41-5.

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Synthesis of 11*H*-Pyridocarbazoles and Derivatives. Comparison of Their DNA Binding and Antitumor Activity with Those of 6*H*- and 7*H*-Pyridocarbazoles

Elie Lescot,*† Gabriel Muzard,† Judith Markovits,† Joël Belleney,‡ Bernard P. Roques,‡ and Jean-Bernard Le Pecq†

Laboratoire de Pharmacologie Moléculaire, LA 147 (CNRS), U 140 (INSERM), Institut Gustave-Roussy, 94800 Villejuif, France, and Département de Chimie Organique, ERA 613 (CNRS), U 266 (INSERM), UER des Sciences Pharmaceutiques et Biologiques, 75006 Paris, France. *Received July 22, 1985*

The 8-methoxy- and 8-hydroxy-11*H*-pyrido[2,3-*a*]-, -[3,4-*a*]-, -[4,3-*a*]-, and [3,2-*a*]carbazoles were synthetized as potential DNA intercalating antitumor drugs. The structure of these compounds was confirmed by ^{1}H NMR study including NOE experiments. The DNA binding properties of substituted and unsubstituted (8-*H*) heterocycles were determined by using their hydrochlorides or methiodides. These derivatives are able to bind to DNA with an affinity varying from 2.0 × 10^4 to 1.0 × 10^6 M^{-1}, but most of them are unable to intercalate in contrast with the behavior of 6*H*- and 7*H*-pyridocarbazole analogues. The cytotoxicity of 11*H*-pyridocarbazoles, measured on L1210 cells in vitro, is much lower than those of 6*H*- and 7*H*-pyridocarbazole analogues.

The antitumor drug 2-methyl-9-hydroxyellipticinium acetate has recently been introduced in the treatment of breast cancer.[1] It is a DNA intercalating compound derived from the ellipticine alkaloid,[2] which belongs to the 6*H*-pyridocarbazole series (Chart I). 9-Methoxyellipticine also exhibits antitumor properties.[3] The antitumor activity of these derivatives is in part related to their high DNA binding affinity.[2] The size and the shape of the 6*H*-pyridocarbazole ring lead to an almost perfect overlapping of the aromatic ring with that of a DNA base pair.[4] Therefore, the pyridocarbazole ring appears as an appropriate skeleton to design DNA intercalating drugs. Accordingly, various 7*H*-pyridocarbazole derivatives have recently been synthesized[5] and have led to antitumor drugs in the series of bifunctional intercalators.[6] Furthermore, the antitumor potency of these heterocycles seems to be enhanced by the introduction of a hydroxy or a methoxy group in the para position with respect to the indolic NH

† Institut Gustave-Roussy.
‡ UER des Sciences Pharmaceutiques et Biologiques.

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