Notes

The Practical Synthesis of a Methylenebisphosphonate Analogue of Benzamide Adenine Dinucleotide: Inhibition of Human Inosine Monophosphate Dehydrogenase (Type I and II)[1]

Krzysztof W. Pankiewicz,*,† Krystyna Lesiak,† Andrzej Zatorski,‡ Barry M. Goldstein,§ Stephen F. Carr,⊥ Marek Sochacki,∥ Alokes Majumdar,† Michael Seidman,† and Kyoichi A. Watanabe†

Codon Pharmaceuticals, Inc., 200 Perry Parkway, Gaithersburg, Maryland 20877, Laboratory of Organic Chemistry, Sloan-Kettering Institute for Cancer Research, New York, New York 10021, Department of Biophysics, University of Rochester Medical Center, Rochester, New York 14642, Department of Biochemistry, Roche Bioscience, Palo Alto, California 94304, and Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, Poland

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β-Methylene-BAD (**8**), a nonhydrolyzable analogue of benzamide adenine dinucleotide (BAD), was synthesized as potential inhibitor of human inosine monophosphate dehydrogenase (IMPDH). Treatment of 2′,3′-*O*-isopropylideneadenosine 5′-methylenebisphosphonate (**15**) with DCC afforded P^1,P^4-bis(2′,3′-*O*-isopropylideneadenosine) 5′-P^1,P^2:P^3,P^4-dimethylenetetrakisphosphonate (**17**). This compound was further converted with DCC to an active intermediate **18** which upon reaction with 3-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)benzamide (**19**) gave, after hydrolysis and deisopropylidenation, the desired β-methylene-BAD (**8**) in 95% yield. In a similar manner, treatment of **18** with 2′,3′-*O*-isopropylidenetiazofurin (**21**) followed by hydrolysis and deprotection afforded β-methylene-TAD (**5**) in 91% yield. Compound **8** (IC_{50} = 0.665 μM) was found to be a 6−8 times less potent inhibitor of IMPDH than **5** (IC_{50} = 0.107 μM) and was almost equally potent against IMPDH type I and type II. Although TAD and β-methylene-TAD were bound by LADH with the same affinity, the binding affinity of **8** toward LADH (K_i = 333 μM) was found to be 50-fold lower than that of the parent pyrophosphate **7** (K_i = 6.3 μM).

Introduction

Among cellular dehydrogenases, inosine monophosphate dehydrogenase (IMPDH), which catalyzes the NAD-dependent conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) is an important target in cancer chemotherapy.[2,3] The oncolytic C-nucleosides, tiazofurin[4,5] (2-β-D-ribofuranosylthiazole-4-carboxamide, TR, **3**, Chart 1) and benzamide riboside[6] (3-β-D-ribofuranosylbenzamide, BR, **6**) are potent inhibitors of IMPDH.[7−10] These compounds require a unique metabolic activation. Both **3** and **6** are converted by cellular enzymes to the corresponding analogues of nicotinamide adenine dinucleotide (NAD, **2**), thiazole-4-carboxamide adenine dinucleotide[11−13] (TAD, **4**), and benzamide adenine dinucleotide[14] (BAD, **7**). TAD and BAD compete with the natural cofactor NAD, but since they cannot participate in hydride transfer, they act as competitive inhibitors of IMPDH. It has been recently discovered that human IMPDH exists as two isoforms, types I and II.[15] Type I is constitutively expressed and is the dominant isoform in normal cells, while type II is selectively up-regulated in neoplastic and replicating cells and emerges as the major species.[16] Thus, the selective inhibition of type II IMPDH is expected to provide significant therapeutic advantage by reducing potential toxicity caused by inhibition of the type I isoform.

The idea to use analogues of NAD in order to selectively inhibit some cellular dehydrogenases emerged long time ago but it has been criticized since its inception. For example, it was emphasized that numerous cellular dehydrogenases depend on NAD as the cofactor, and since the cofactor binding domain in all of these proteins was conserved, a sufficiently selective inhibition of individual cellular dehydrogenases could not be achieved. Contrary to such criticism, potent and selective inhibition of 6-phosphogluconate dehydrogenase by 6-amino-NAD (**10**, K_i = 0.13 μM) was reported as early as in 1971.[17] Later, the synthesis of TAD[18] (**4**) and its methylenebisphosphonate analogue, β-methylene-TAD[19] (**5**) was published. Both **4** and **5** showed potent and selective inhibitory activity against IMPDH with K_i in the range of 0.1 μM. These compounds did not inhibit alcohol, lactate, glutamate, and malate dehydrogenases at concentrations up to 200 μM. Recently, we prepared C-nucleoside analogues of the natural cofactor C-NAD (**12**) and C-PAD (**14**)[20] and found that C-NAD, but not C-PAD, was an extremely potent and specific inhibitor of horse liver alcohol dehydrogenase (LADH) with K_i = 1−2 nM.[21,22]

In spite of the promising initial results, progress toward biomedical utilization of TAD and BAD has been hampered due mainly to the lack of efficient synthetic procedures for these compounds. Recently, we developed a modified procedure for imidazolide activation of

* Author for correspondence and reprints (phone 301-527-2063, FAX 301-208-6997).
† Codon Pharmaceuticals, Inc.
‡ Sloan-Kettering Institute for Cancer Research.
§ University of Rochester Medical Center.
⊥ Roche Bioscience.
∥ Polish Academy of Sciences.
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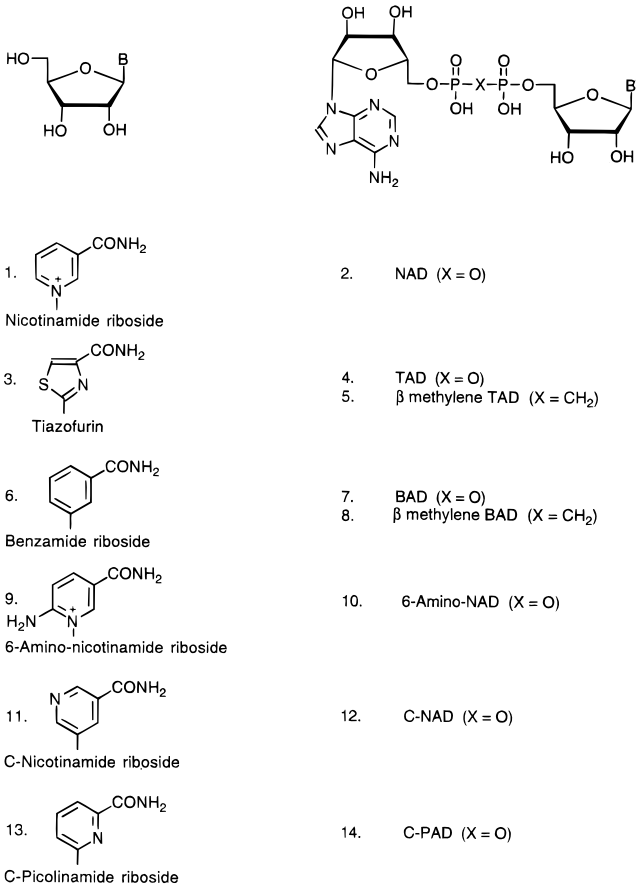
Chart 1



nucleoside 5′-monophosphates for the efficient synthesis of dinucleoside pyrophosphates.[23] Using this method, we prepared BAD[24] in yields over 90%. In this paper we report an efficient synthesis of a methylenebisphosphonate analogue of BAD (**8**), which could be prepared in yields over 90% by our new method of DCC activation of adenosine 5′-methylenebisphosphonate. We also report inhibitory effects of a newly synthesized **8** against the two isoforms of human IMPDH. In addition, inhibition of horse liver alcohol dehydrogenase (LADH) by both BAD (**7**) and β-methylene BAD (**8**) was examined. Inhibition of cell growth by TAD, BAD, and their methylenebisphosphonate analogues was also studied. The new compound was tested as potential inducer of differentiation in human K562 erythroid leukemia cells.

Chemical Synthesis

We report herein an efficient method of dicyclohexylcarbodiimide (DCC) activation of the adenosine 5′-methylenebisphosphonate[25] (**15**, Scheme 1) for coupling with another nucleoside. It has been generally accepted that DCC forms an amidine intermediate (such as **16**) which is subsequently displaced by the 5′-OH group of the suitably protected nucleoside to give the desired product **20**. We found, however, that upon admixture of 3−4 equiv of DCC to the pyridine solution of **15** and 3-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)benzamide[6] (**19**), the early product of this reaction was the P^1,P^2,P^3,P^4-bismethylene analogue of adenosine tetraphosphate (**17**, Ap_4A),[26] which could be isolated in good yield. In the absence of **19**, addition of DCC to the pyridine solution of **15** or to the solution of isolated Ap_4A analogue **17** resulted in formation of an intermediate **18**, showing numerous new phosphorus resonances in the ^{31}P NMR spectrum. Intermediate **18** may consist of compound(s) resulting from further DCC-promoted dehydration of **17** to monocyclic, bicyclic, and/or macrocyclic derivatives of **17**. Compound(s) **18** cannot be isolated due to its (their) susceptibility to hydrolysis. Multisignal resonances in ^{31}P NMR either are due to polyphosphate composition of **18** or represent a mixture of diastereomers formed during cyclization of **17** (phosphorus atoms of **18** become chiral upon dehydration). Whatever the structure of **18** is, when nucleoside **19** was added at this stage of reaction, the number of phosphorus resonances was reduced to two broad singlets, and hydrolysis with water afforded the desired dinucleotide **20** in 95% yield. Similarly, addition of 2′,3′-*O*-isopropylidenetiazofurin (**21**) to intermediate **18** gave after hydrolysis the protected β-methylene-TAD (**22**) in excellent yield. Deisopropylidenation of **20** and **22** with Dowex 50/H^+ afforded compounds **8** and **5**, respectively. This procedure is amenable to gram-scale synthesis of compounds such as **5** and **8**; the only limitation is the availability of the corresponding starting nucleosides.

Biological Results

We examined the inhibitory effects of **8** and **5** against IMPDH type I and type II. The IC_{50}'s of these compounds for each isoform were measured as reported previously.[24] β-Methylene-TAD was equally potent against IMPDH type I (IC_{50} = 0.107 μM) and type II (IC_{50} = 0.109 μM), showing an inhibitory potency identical to that of TAD.[19] β-Methylene-BAD was found to be 6−8 times less potent than β-methylene-TAD and slightly less potent against type II (IC_{50} = 0.948 μM) than type I IMPDH (IC_{50} = 0.665 μM). These are similar relative potencies to those reported for BAD versus TAD.[24] Thus, *both* β-methylene-TAD and β-methylene-BAD do not exhibit any significant selectivity against IMPDH type II.

We also examined inhibition of LADH by both BAD and β-methylene-BAD. The pattern of inhibition with respect to NAD is competitive in both cases. Measured K_i's for inhibition of LADH with NAD as the variable substrate are 6.3 ± 1.43 μM for BAD and 333 ± 62 μM for β-methylene-BAD. Thus, *replacement of the pyrophosphate oxygen with a* $-CH_2-$ *linkage results in a 50-fold decrease in binding affinity*, comparable to a *ca.* 2.4 kcal/mol difference in free energy. LADH follows an ordered sequential mechanism, the binding of cofactor preceding that of substrate.[27] The observed pattern of inhibition is consistent with binding of the inhibitor at the normal cofactor site.[28] The pyrophosphate oxygen in NAD does not participate in any specific intermolecular interactions in either open or closed complexes with LADH.[22,27] Thus, the observed difference in K_i's is surprising. However, adjacent phosphorus oxygens do participate in a number of polar interactions.[27] Subtle steric and/or electronic effects of the methylene substitution may perturb these interactions,[29] resulting in the weaker binding of β-methylene-BAD. Crystallographic studies of the inhibitor−LADH complexes are underway to address this question.

Analysis of the influence of the compounds on growth of K562 cells showed that the methylene derivatives of BAD and TAD were active as inhibitors of K562 cell growth, although concentrations higher than TR or TAD

Scheme 1

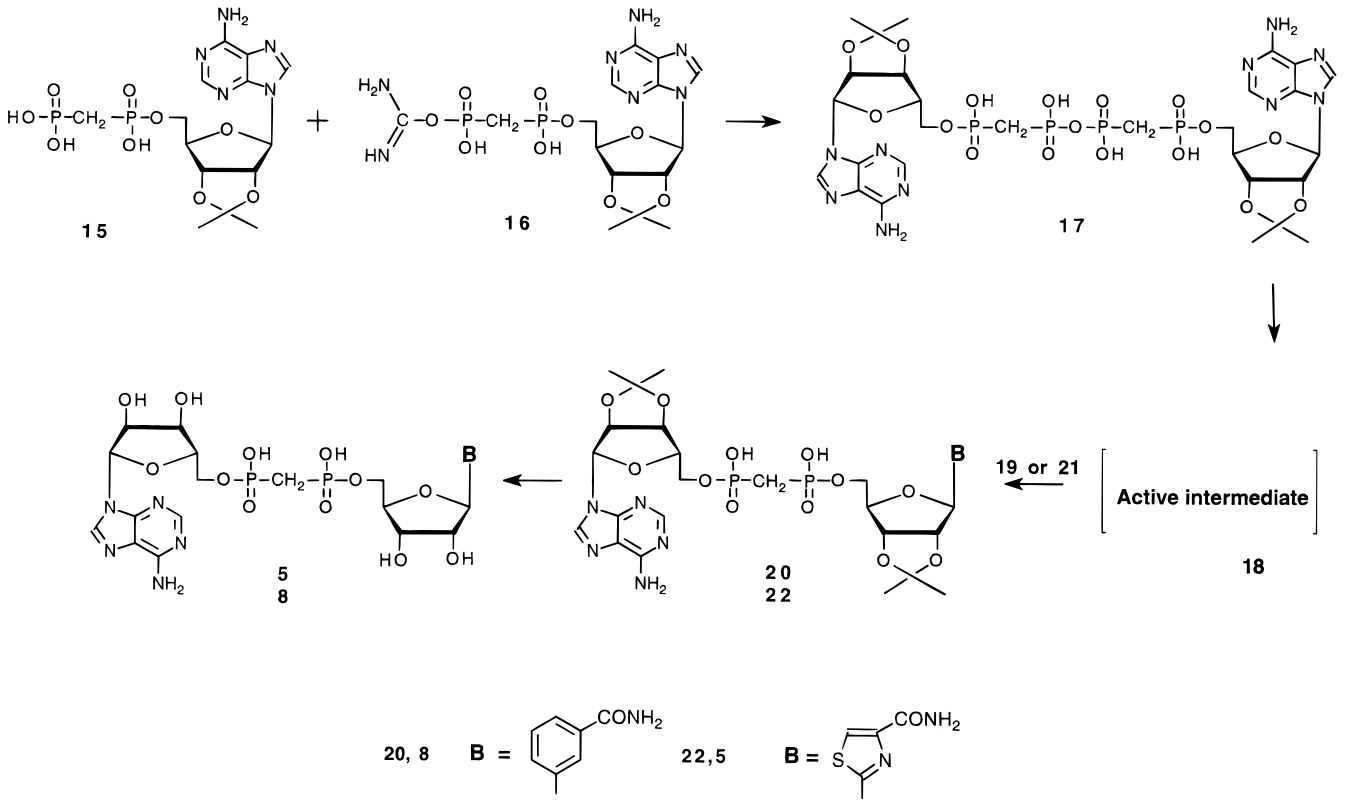


Table 1. Inhibition of Growth of K562 Cells by β-Methylene-BAD as Compared with Inhibitory Activity of TR, TAD, and β-Methylene-TAD

compound	IC_{50} (μM)	growth (% of untreated control)
TR	3	90
TAD	3.7	80
β-methylene- BAD	60	90
β-methylene-TAD	18	90

Table 2. Differentiation of K562 Cells by β-Methylene-BAD, TR, TAD, and β-Methylene-TAD without and with Coadministration of Guanosine

		% benzidine positive cells	
compound	concn (μM)	−Gua	+Gua
TR	3.8	36	12
	19	80	40
TAD	7.5	50	15
	15	70	30
β-methylene-BAD	60	45	15
	120	75	17
β-methylene-TAD	15	34	23
	30	85	30

were required (Table 1). Coadministration of guanosine blocked the inhibition of cell growth by all compounds, indicating that IMPDH was the target of both methylene derivatives.

Cell differentiation was monitored by analysis of hemoglobin expression. Hemoglobin was induced by all compounds in the same concentration ranges as the IC_{50}. Guanosine partially blocked the production of benzidine positive cells (Table 2).

These results indicate that the methylene derivatives are not as active as the TR or the dinucleotide derivative of TR, TAD. Since TAD shows activity at concentrations similar to TR, it is unlikely that the activity of TAD is due to breakdown and recycling of the TR moiety in the cell. Instead, it seems probable that TAD is active itself. This implies that it is taken up by the cells despite the pyrophosphate linkage. The lower activity of the methylene BAD derivative is in accord with the reduced activity against IMPDH.

Experimental Section

General Methods. HPLC was performed on a Dynamax-60A C18-83-221-C column with flow rate of 5 mL/min or Dynamax-300A C18-83-243-C column with a flow rate of 20 mL/min of 0.1 M Et_3N H_2CO_3 (TEAB) followed by a linear gradient of 0.1 M TEAB−aqueous MeCN (70%). Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Nuclear magnetic resonance spectra were recorded on a JEOL Eclipse 270 and Bruker-AMX-400 spectrometer with Me_4Si or DDS as the internal standard for ^1H and external H_3PO_4 for ^{31}P. Chemical shifts are reported in ppm (δ), and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), and dd (double doublet). Values given for coupling constants are first order. Negative ion mass spectra were recorded using Finnigan TSQ instrument equipped with electronspray ion source.

***P*^{1},*P*^{4}-Bis(2′,3′-*O*-isopropylideneadenosine) 5′-*P*^{1},*P*^{2}:*P*^{3},*P*^{4}-dimethylenetetrakisphosphonate (17).** 2′,3′-*O*-Isopropylideneadenosine 5′-methylenebisphosphonate (62 mg, 0.11 mmol) as monotriethylammonium salt was dissolved in pyridine (0.5 mL) containing DCC (25 mg, 0.12 mmol), and the mixture was stirred at room temperature for 1 h and concentrated *in vacuo*. The residue was dissolved in 0.1 M Et_3N H_2CO_3 (TEAB) and chromathographed on HPLC to give **17** (36 mg, 59%) as the ditriethylammonium salt (after lyophilization): ^1H NMR (D_2O) δ 1.22−1.26 (two t, 18H, Et_3N), 1.44 (s, 6H, iPr), 1.67 (s, 6H, iPr), 2.31−2.41 (m, 4H, two PCH_2P), 3.02−3.16 (two q, 12H, Et_3N), 4.05, 4.08 (dt, 4H, H5′, $J_{4',5'}$ = 4.2 Hz, $J_{5',5''}$ = 11.4 Hz, $J_{5',P}$ = 4.2 Hz), 4.12−4.17 (m, 4H, H5″), 4.58−4.59 (m, 2H, H4′), 5.23 (dd, 2H, H3′, $J_{2',3'}$ = 6.1 Hz, $J_{3',4'}$ = 1.9 Hz), 5.36 (dd, 2H, H2′, $J_{1',2'}$ = 3.7 Hz), 6.14 (d, 2H, H1′), 8.11, 8,41 (two 2H singlets, H2, H8); ^{31}P NMR (D_2O) δ 10.51 (P^2, P^3), 21.35 (P^1, P^4); MS (ES) *m*/*z* 911 $(M - H)^-$, 455 $(M - 2H)^{2-}$.

***P*¹-(3-*β*-D-Ribofuranos-5-ylbenzamide) *P*²-Adenosine-5′-(Methylenebisphosphonate) (8, *β*-Methylene-BAD). (A) Synthesis from 15.** 2′,3′-*O*-Isopropylideneadenosine 5′-methylenebisphosphonate (62 mg, 0.11 mmol) as the monotriethylammonium salt was dissolved in pyridine (1.5 mL) containing DCC (49 mg, 0.24 mmol), and the mixture was stirred at room temperature for 20 h. ^{31}P NMR analysis showed disappearance of resonance signals of **15** (δ 15.46, singlet) and formation of an intermediate **18** with characteristic multisignal resonances. At this time nucleoside **19** (32 mg, 0.11 mmol) was added and the reaction mixture was kept at 65 °C (2 days) until the ^{31}P spectrum of the reaction showed the formation of a simple AB system (δ = 16.90). Then water was added, and the mixture was stirred at room temperature for 3 h and concentrated *in vacuo*. The residue was chromatographed on HPLC column to give *P*¹-[3-(2,3-*O*-isopropylidene-*β*-D-ribofuranos-5-yl)benzamide] *P*²-(2′,3′-*O*-isopropylideneadenosine) 5′-methyelenebisphosphonate **(20)** as the ditriethylammoniun salt (102 mg): ^{1}H NMR (D_2O) δ 1.27−1.31 (t, 18H, Et_3N), 1.40 (s, 3H, iPr), 1.46 (s, 3H, iPr), 1.64 (s, 3H, iPr), 1.68 (s, 3H, iPr), 2.05−2.20 (m, 2H, PCH_2P), 3.21 (q, 12H, Et_3N), 4.09−4.12 [m, 4H, H5′,H5″(B) and (A)], 4.26−4.28 [m, 1H, H4′-(B)], 4.59−4.64 [m, 2H, H4′(A), H2′(B)], 4.80 [1H, H1′(B)], 4.88 [dd, 1H, H3′(B), $J_{1',2'}$ = 5.7 Hz, $J_{2',3'}$ = 6.5 Hz), 5.20 [m, 1H, H3′(A)], 5.30 [dd, 1H, H2′(A), $J_{1',2'}$ = 3.0 Hz, $J_{2',3'}$ = 6.5 Hz], 6.12 [d, 1H, H1′(A)], 7.39 [pseudo t, 1H, H5(B)], 7.46 [d, 1H, H4(B), $J_{4,5}$ = 7.8 Hz], 7.64 [d, 1H, H6(B), $J_{5,6}$ = 7.8 Hz], 7.67 (s, 1H, H2(B)], 8.15, 8.40 [two 1H singlets, H2(A), H8(A)]. Compound **20** was treated with Dowex 50-X8/H^+ in water and purified by passing through the column of Dowex 50-X8/H^+ to give *β*-methylene-BAD (**8**, 69 mg, 95%): ^{1}H NMR (D_2O) δ 2.27 (pseudo t, 2H, PCH_2P), 4.05 [dd, 1H, H2′(B), $J_{1',2'}$ = 7.0 Hz, $J_{2',3'}$ = 5.1 Hz], 4.17−4.20 [m, 4H, H5′,5″(A) and (B)], 4.25−4.27 [m, 2H, H3′(B), H4′(B)], 4.37 [pseudo t, 1H, H3′(A)], 4.66 [pseudo t, H2′(A)], 4.80 [d, 1H, H1′(B)], 6.06 [d, 1H, H1′(A), $J_{1',2'}$ = 4.9 Hz], 7.41 [pseudo t, 1H, H5(B)], 7.56 [d, 1H, H4(B), $J_{4,5}$ = 7.8 Hz], 7.63 [d, 1H, H6(B), $J_{5,6}$ = 7.8 Hz], 7.69 (s, 1H, H2(B)], 8.32, 8.57 [two 1H singlets, H2(A), H8(A)], ^{31}P NMR (D_2O) δ 20.94 (s); MS (ES) *m*/*z* 659 $(M - H)^-$.

(B) Synthesis from 17. Compound **17** (30 mg, 0.027 mmol) as the ditriethylammonium salt was dissolved in pyridine (0.5 mL) containing DCC (20 mg, 0.1 mmol), and the mixture was stirred at room temperature for 3 h. ^{31}P NMR analysis showed the disappearance of resonance signals of **17** with simultaneous formation of intermediate **18** with characteristic multisignal resonances. At this time nucleoside **19** (19 mg, 0.06 mmol) was added, and the reaction mixture was kept at 65 °C (2 days) until the ^{31}P spectrum of the reaction showed the formation of the AB system (δ = 16.90). Water was added, and the mixture was stirred at room temperature for 3 h and then concentrated *in vacuo*. The residue was chromatographed on HPLC column to give **20** (21 mg, 91%) as the ditriethylammoniun salt. The analytical data of this compound was identical to that of **20** reported above.

***P*¹-Tiazofurin *P*²-Adenosine-5′-methylenebisphosphonate (*β*-Methylene-TAD, 5).** 2′,3′-*O*-Isopropylideneadenosine 5′-methylenebisphosphonate (620 mg, 1.1 mmol) as the monotriethylammonium salt was dissolved in pyridine (30 mL) containing DCC (500 mg, 2.5 mmol), and the mixture was stirred at room temperature for 20 h. ^{31}P NMR analysis showed conversion of **15** (δ = 15.46, singlet) into intermediate **18** with characteristic multisignal resonances. At this time nucleoside **21** (330 mg, 1.1 mmol) was added, and the reaction mixture was kept at 65 °C (2 days) until the ^{31}P spectrum became the simple AB system (δ = 17.80). The reaction was quenched by addition of water, and the mixture was stirred at room temperature for 3 h and concentrated *in vacuo*. A small amount of the residue (50 mg) was chromatographed on an HPLC column to give the analytical sample of *P*¹-(2′,3′-*O*-isopropylidenetiazofurine *P*²-(2′,3′-*O*-isopropylideneadenosine) 5′-methylenebisphosphonate (**22**): ^{1}H NMR (D_2O) δ 1.26 (18H, t, Et_3NH^+), 1.38, 1.45, 1.60 and 1.67 (3H each, Me-isopropylidene of tiazofurin and adenosine), 2.07 (2H, t, PCH_2P, J_{P-H} = 20.1 Hz), 3.19 (12H, q, Et_3NH^+), 3.88−3.98 (2H, m, H5′,5″ of tiazofurin), 4.07−4.12 (2H, H5′,5″ of adenosine), 4.35−4.38 (1H, m, H4′ of tiazofurin), 4.59−4.62 (1H, m, H4′ of adenosine), 4.89−4.95 (2H, m, H2′, 3′ of tiazofurin), 5.09 (1H, d, H1′ of tiazofurin, $J_{1'2'}$ = 3.4 Hz), 5.22 (1H, dd, H3′, $J_{2',3'}$ = 6.2 Hz, $J_{3',4'}$ = 2.2 Hz), 5.36 (1H, dd, H2′, $J_{1',2'}$ = 3.5 Hz), 6.18 (1H, d, H1′), 8.06, 8.14, and 8.42 (three 1H singlets, H2, H8 adenosine and H5 tiazofurin). The rest of the residue was dissolved in water (100 mL), treated with Dowex50/H^+, chromatographed on the column of Dowex50/H^+, and concentrated *in vacuo*. Final purification on HPLC afforded **5** (870 mg, 91%). The ^{1}H NMR spectrum of **5** was identical with that of authentic sample.

Inhibition of LADH. Horse liver ADH was obtained in crystalline form from Boehringer Mannheim Biochemicals, Indianapolis, IN. *β*-Nicotinamide adenine dinucleotide in crystalline free acid form was also obtained from Boehringer. BAD was prepared as reported earlier,[24] and *β*-methylene-BAD was synthesized as described above.

LADH in crystalline suspension was spun down to a pellet and dissolved in 100 mM Tris-HCl buffer containing 100 mM KCl adjusted to pH 8 at 23 °C. Kinetic constants of the inhibitors with respect to NAD were obtained by monitoring spectrophotometrically the rate of production of NADH during the reaction. The course of the reaction was followed by measuring the changing absorbance of the reaction mixture at 340 nm, the absorbance peak for NADH, using Beckman DU-65 spectrophotometer, and an extinction coefficient of 6.22 A mM^{-1} cm^{-1} for the reduced cofactor.[30] Substrate, cofactor, and inhibitor were combined and equilibrated at 23 °C for 5 min. The reaction was initiated by the introduction of 10 μL of enzyme solution on a mixing plunger, and NADH production was monitored for between 2 and 4 min, depending on the activity of the protein solution.

Initial velocities were measured at four NAD concentration (25, 50, 100, and 200 μM) at the fixed saturating substrate concentration (1.2 μM). Velocities were measured in the absence of inhibitor and in presence of multiple concentrations of BAD and *β*-methylene-BAD (5−40 μM and 120−300 μM, respectively). Values of inhibition constants and patterns of inhibition were obtained as described by us earlier[23] by direct least-squares fits to the nonreciprocal forms of the Michaelis−Menten rate equations.[31]

Cells, Growth, and Differentiation Assays. Human erythroleukemia K562 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated calf serum, glutamine, and penicillin/streptomycin. Growth assays were performed by suspending cells at a concentration of 5×10^3 mL in a final volume of 200 in a 96-well microtiter plate. The chemicals, with or without added guanosine (30 μM), were added at the start of the assay. After 5 days of incubation, 100 μL of medium was removed from each well and 10 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] was added, and the mixture incubated at 37 °C for 3 h.[32] The insoluble formazan was then dissolved by addition of 150 μL of 2-propanol/0.04 mM HCl. The plates were then read in a plate reader at 550 nm. All points were done in triplicate. The concentration required for 50% inhibition of growth (IC_{50}) was calculated using an Excel program (Microsoft). The results were averaged from four experiments.

The expression of hemoglobin was measured by benzidine staining.[33] Cells in 2 mL were incubated for 5 days in the presence of compounds. An equal volume of cells and freshly prepared staining solution (0.1% benzidine in 0.25% hydrogen peroxide) were mixed and incubated at room temperature for 5 min. A total of 300−400 cells were counted to determine the percentage of benzidine positive cells. The experiments were done in duplicate, and the reported results are the average of three experiments.

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