Novel Mycophenolic Adenine Bis(phosphonate) Analogues As Potential Differentiation Agents against Human Leukemia[1]

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Novel mycophenolic adenine dinucleotide (MAD) analogues have been prepared as potential inhibitors of inosine monophosphate dehydrogenase (IMPDH). MAD analogues resemble nicotinamide adenine dinucleotide binding at the cofactor binding domain of IMPDH; however, they cannot participate in hydride transfer and therefore inhibit the enzyme. The methylenebis(phosphonate) analogues C2-MAD and C4-MAD were obtained by coupling 2′,3′-O-isopropylideneadenosine 5′-methylenebis(phosphonate) (**22**) with mycophenolic alcohols **20** and **21** in the presence of diisopropylcarbodiimide followed by deprotection. C2-MAD was also prepared by coupling of mycophenolic methylenebis(phosphonate) derivative **30** with 2′,3′-O-isopropylideneadenosine. Compound **30** was conveniently synthesized by the treatment of benzyl-protected mycophenolic alcohol **27** with a commercially available methylenebis(phosphonic dichloride) under Yoshikawa's reaction conditions. C2-MAD and C4-MAD were found to inhibit the growth of K562 cells (IC_{50} = 0.7 μM and IC_{50} = 0.1 μM, respectively) as potently as mycophenolic acid (IC_{50} = 0.3 μM). In addition, C2-MAD and C4-MAD triggered vigorous differentiation of K562 cells an order of magnitude more potently than tiazofurin, and MAD analogues were resistant to glucuronidation in vitro. These results show that C2-MAD and C4-MAD may be of therapeutic interest in the treatment of human leukemias.

Introduction

Many of the strategies for the treatment of neoplasia have focused on the prevention of cell proliferation. Unfortunately, cytotoxic drugs, as well as radiation therapy, often cause debilitating side effects, and frequently, these therapies fail. An attractive alternative to the antiproliferative approach is to induce cell differentiation in order to convert malignant cells into mature resting cells. Not all cells are phenotypically able to differentiate; however, it has been established that leukemia-derived cell lines, such as the human chronic myelogenous leukemia K562 and the human promyelomonocytic leukemia HL-60, easily undergo drug-induced differentiation.[2] Thus, stimulation of cell differentiation is particularly promising for the treatment of human leukemias.

Cellular differentiation, in both prokaryotes and eukaryotes, is controlled by the level of guanine nucleotides.[3] The concentrations of guanine nucleotides in the cell are in turn regulated by the activities of p53 tumor suppressor protein[4] and inosine monophosphate dehydrogenase (IMPDH), the key enzyme in the de novo synthesis of purine nucleotides. Overexpression of wild-type p53 induces differentiation in a variety of cells, resulting in G1 arrest and slow doubling time.[5] Tumor suppressor proteins inhibit cell proliferation, not by killing cells but by activation of tumor cell differentiation.[6] Induction of p53 results in reduced IMPDH expression. Inhibitors of IMPDH such as tiazofurin (**1**, TR; Scheme 2) and mycophenolic acid (MPA, **10**; Scheme 3) cause cell cycle arrest at the G1/S boundary and induce differentiation in several cell lines, including HL-60 and K562 cells.[7] Consequently, it appears that the inhibition of IMPDH mimics the effects of p53 expression[4] and would be of therapeutic importance in cancer treatment.[8]

IMPDH (EC 1.1.1.205) catalyzes the rate-limiting step in the de novo biosynthesis of guanine nucleotides, the nicotinamide adenine dinucleotide (NAD)-dependent conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP; Scheme 1). After substrate addition, nucleophilic attack of an active site cysteine (Cys-331) on the C2 of IMP base forms a covalent intermediate. The binding of cofactor NAD results in hydride transfer to the B-side of the nicotinamide ring, and subsequent hydrolysis leads to the formation of XMP. XMP is then aminated by guanosine 5′-monophosphate (GMP)-syntase in the next step to form GMP.

Two isoforms of IMPDH are found in mammalian cells, each encoded by distinct genes.[9] Type I is expressed in normal cells while the levels of type II are markedly increased in tumor cells and activated lym-

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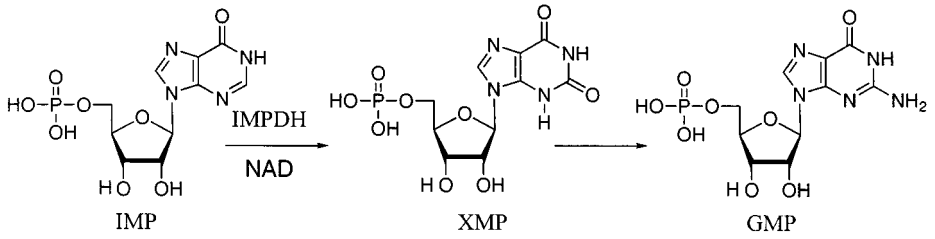
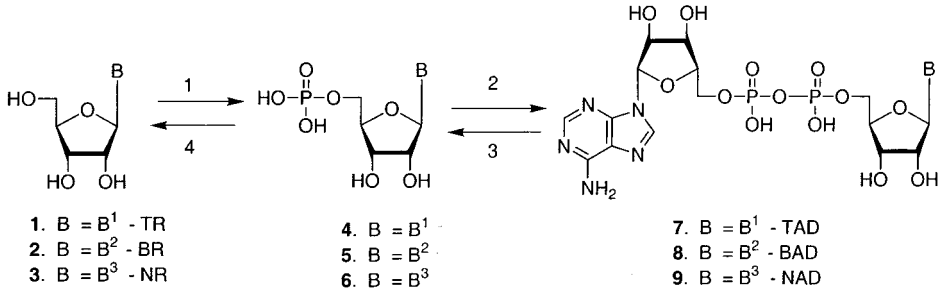
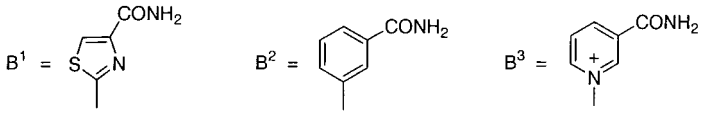
Scheme 1



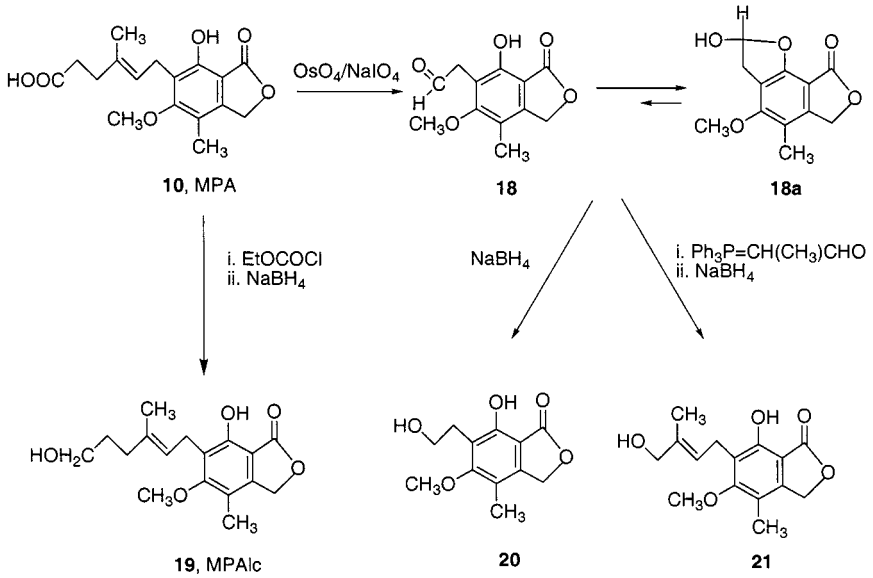
Scheme 2



1. Adenosine kinase, 2. NMN - adenylyl transferase, 3. TADase, phosphodiesterases, 4. Phosphatases



Scheme 3



phocytes. When tumor cells are induced to differentiate, transcripts of type II decline to below those of type I.[10] Therefore, it is expected that specific inhibition of IMPDH type II may induce differentiation while providing a significant therapeutic advantage by eliminating toxicity caused by the inhibition of the type I isoform.

The differentiation-inducing activity of potential drugs is commonly studied in vitro in K562 cells, derived from a human chronic myeloid leukemia (CML) cell line, which is a widely used system for differentiation that can be induced by various agents to produce hemoglobin.[11,12] The antitumor agent TR (**1**) has proved to be one of the most effective inducers of differentiation in K562 cells among numerous compounds studied.[13] The biological activity of TR is due to its unique metabolism. TR is phosphorylated in cells to TR monophosphate (**4**; Scheme 2), which is coupled with adenosine 5′-triphosphate by nicotinamide mononucleotide (NMN) adenyltransferase to form the active metabolite, thiazole-4-carboxamide adenine dinucleotide (**7**, TAD).[17]

TR induced complete hematologic remissions in patient's acute myeloid leukemia (AML) and in blast crisis of CML (CML-BC), but the remissions were of short duration. Tricot et al.[14,15] reported that TR was effective in patients with CML-BC in clinical trials. The observation that the therapeutic effect of TR was due to the induction of cell differentiation in humans was confirmed by Wright et al.[16] in their Phase II clinical

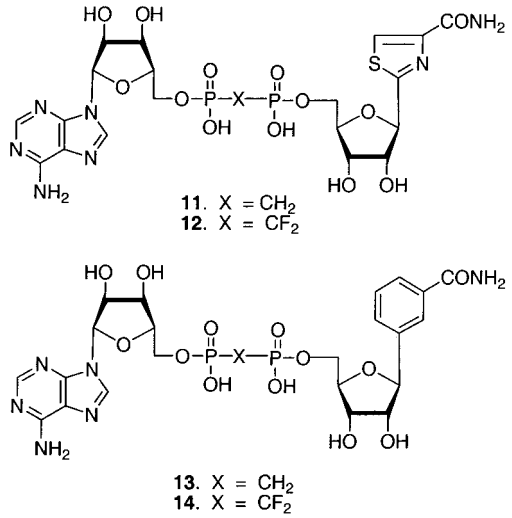


Figure 1.

studies. Unfortunately, TR was found to be too toxic for a broad clinical application.

TAD (**7**) is an analogue of NAD (**9**) in which TR replaces the nicotinamide riboside moiety of NAD. TAD mimics the natural cofactor, but the thiazole ring cannot participate in hydride transfer, resulting in potent inhibition of both isoforms of IMPDH (K_i = 0.1 μM). Unfortunately, TAD, which is a pyrophosphate, is metabolically unstable. Thus, in vivo, TAD is degraded to the corresponding mononucleotides and then to the nucleosides by the action of cellular phosphodiesterases and phosphatases.[18] Resistance to TR is associated both with impaired accumulation of TAD in resistant cells[19] and with excessive degradation of TAD by phosphodiesterases, including a specific TADase.[20] A new inhibitor of IMPDH, benzamide riboside (**2**, BR),[21] is metabolized to benzamide adenine dinucleotide (**8**, BAD)[22] in a manner similar to the conversion of TR to TAD. This compound is currently being examined as an alternative to TR.[23]

In 1986, a methylenebis(phosphonate) analogue of TAD (**11**) was synthesized in which the unstable pyrophosphate moiety (P−O−P) was replaced by a P−CH_2−P linkage that is stable to enzymatic and chemical hydrolysis.[24] This compound inhibited IMPDH as well as TAD and, additionally, showed good activity in TR-resistant cell lines. These findings prompted us to synthesize and examine such bis(phosphonate) inhibitors as potential drugs. We developed[25,26] an efficient method of synthesis of P^1, P^2-disubstituted bis(phosphonate)s and synthesized a number of methylenebis(phosphonate) and difluoromethylenebis(phosphonate) analogues of TAD and BAD[27] (**11**−**14**; Figure 1). We found that these compounds are potent inhibitors of IMPDH, can penetrate cells, and are chemically and metabolically stable in serum and cell extracts.[18,28] We also found that neither TAD nor BAD (pyrophosphates), nor their bis(phosphonate) analogues **11**−**14,** show any specificity against isoforms of IMPDH.[29,30]

Compound **10** (MPA) is one of the few inhibitors to show modest (4-fold) specificity for the type II isoform[9] ($K_i \sim$ 8 and 33 nM for type II and type I, respectively). The crystal structure of the complex of Chinese hamster IMPDH with MPA revealed that MPA binds at the NMN subsite of the cofactor pocket of IMPDH.[31] This

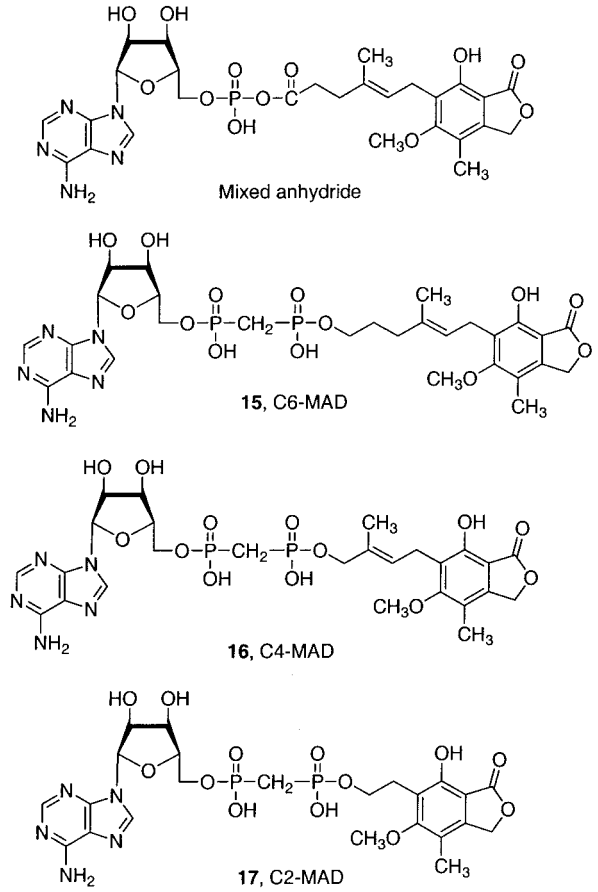


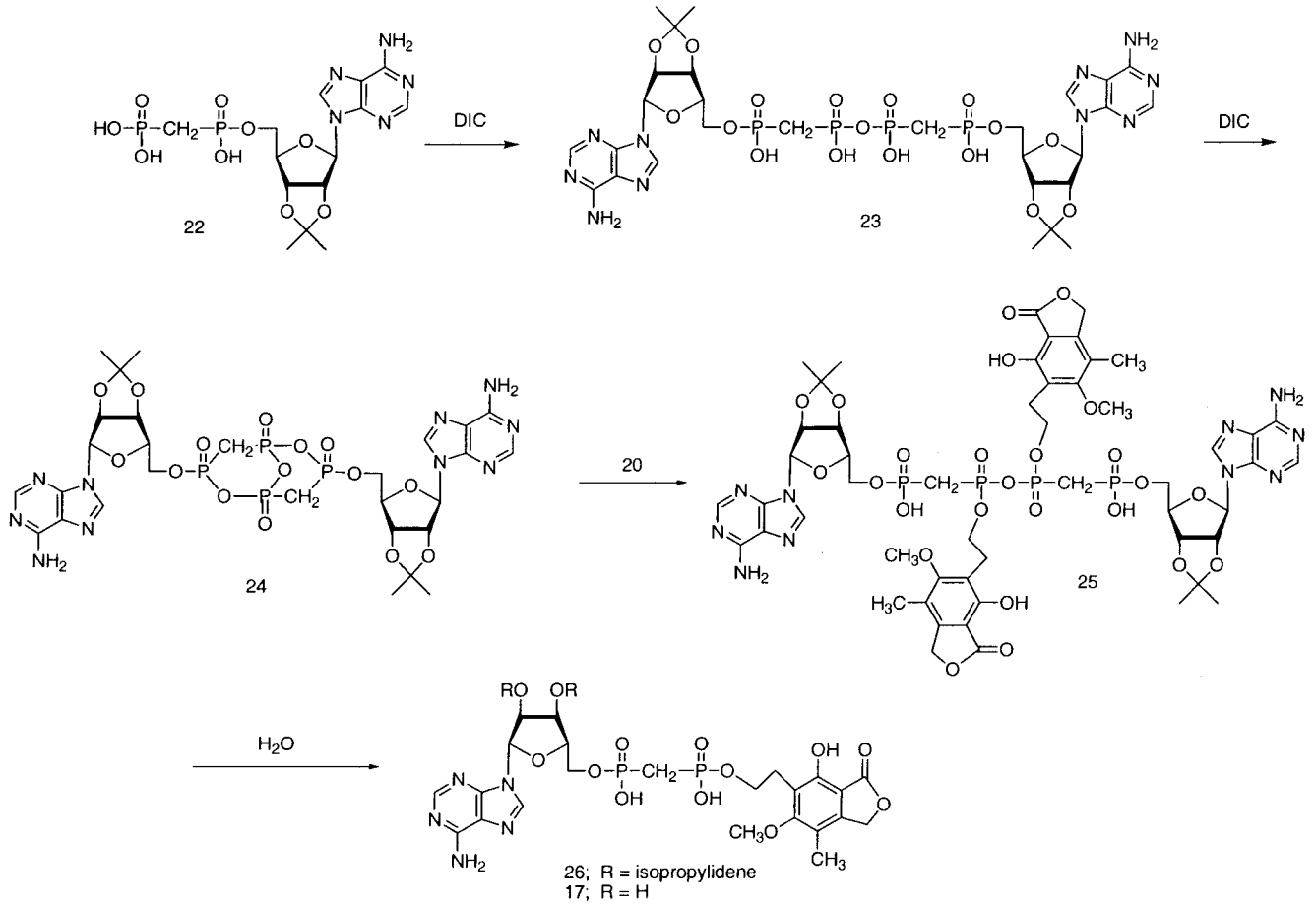
Figure 2.

leaves the adenosine 5′-monophosphate (AMP) subsite of the inhibitor−enzyme complex empty.[31] Thus, we assumed that attachment of AMP to MPA would result in a mixed anhydride (Figure 2) that extends into the AMP binding site. This may offer the possibility of enhancing binding affinity. Furthermore, addition of the bulky AMP to the side chain of MPA as well as introduction of the negatively charged phosphorus group was expected to reduce or prevent the undesired glucuronidation of MPA (below).

Despite these potential advantages, a mixed anhydride would not be stable in protic solvents. Therefore, we replaced the unstable P(O)−O−C(O) anhydro group of the mixed anhydride with a stable methylenebis(phosphonate) [P(O)−CH_2−P(O)] linkage. Thus, reduction of the carboxyl group of MPA afforded the known mycophenolic alcohol (**19**, MPAlc; Scheme 2),[32] which was then coupled with 2′,3′-O-isopropylideneadenosine 5′-methylenebis(phosphonate) to give, after deprotection, the desired bis(phosphonate) analogue of mycophenolic adenine dinucleotide (**15**, C6-MAD; Figure 2).[33] This analogue of MAD contains six carbon atoms and the oxygen in the linker between the aromatic ring of mycophenolic alcohol and the β-phosphorus atom of the adenosine bis(phosphonate) moiety. C6-MAD is a potent inhibitor of IMPDH type II (K_i = 0.3 μM); however, it is 30-fold less potent than MPA (K_i = 0.01 μM).[33] Its linker is two atoms longer than that in the ideal mixed anhydride.

In an attempt to optimize the efficacy of this hybrid compound, we synthesized two MAD analogues with shorter linkers (**16**, C4-MAD and **17**, C2-MAD; Figure

Scheme 4



2). We report here the syntheses of these analogues and their ability to inhibit both isoforms of IMPDH. In addition, we examine both the in vitro cell differentiation-inducing and the growth inhibitory activities of the MAD analogues in K562 cells. These results are compared with activities of TR (**1**), BR (**2**), and the bis(phosphonate) analogues of TAD (**11** and **12**) and BAD (**13**) prepared by us earlier.[27,30,33]

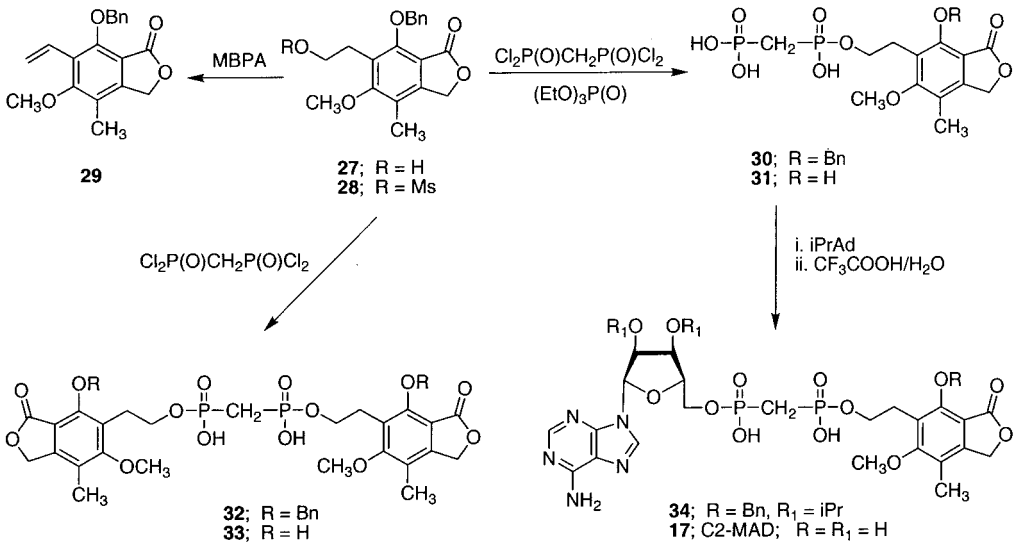
Results and Discussion

1. Chemical Synthesis. In our synthetic strategy, we used MPA (**10**) as the starting material for the preparation of mycophenolic alcohols **20** and **21** with side chains containing two and four carbon atoms, respectively (Scheme 3). We modified the known procedure[32] of synthesis of the aldehyde **18**. Instead of ozonolysis of **10**, which worked poorly in our hands, we used a one pot OsO_4 oxidation of the double bond of **10** followed by oxidative cleavage ($NaIO_4$). The desired product precipitated from the reaction mixture in 96% yield. The product was found to be an approximately 2.5:1 mixture of the aldehyde **18** and its hemiacetal **18a** formed by intramolecular attack of the neighboring 7-OH group. The ^1H nuclear magnetic resonance (NMR) of the product showed all resonances characteristic for both **18** and **18a**. The ratio of **18**:**18a** was determined by a relative integration of the resonance signals of the corresponding 4-methyl groups of both compounds. Reduction of the mixture of **18** and **18a** with $NaBH_4$ gave the alcohol derivative **20** in high yield, whereas treatment of the mixture with 2-(triphenylphosphoranylidene)propionaldehyde followed by reduction with $NaBH_4$ afforded the desired alcohol **21**.[32] The aldehyde precursor of **21** (see Experimental Section) and consequently the alcohol **21** were obtained exclusively as *E* isomers. Irradiation of the aldehyde proton (at 9.36 ppm) resulted in a significant nuclear Overhauser effect increasing the intensity of the olefinic proton (triplet at 6.51 ppm) of the precursor. This indicates that the olefinic proton and the aldehyde group are in cis configuration, e.g., *E* stereochemistry of the double bond.

The coupling of **20** with the 2′,3′-O-isopropylideneadenosine 5′-methylenebis(phosphonate) (**22**; Scheme 4) was accomplished by treatment of **22** with 3.5 equiv of diisopropylcarbodiimide (DIC) in dry pyridine followed by the addition of the mycophenolic alcohol **20** according to our method for preparation of P^1, P^2-disubstituted methylenebis(phosphonate)s.[25,26] Thus, dehydration of **22** afforded the active bicylic trisanhydride intermediate **24**, which under reaction with **20** gave a product **25**. Hydrolysis of **25** with water afforded the desired derivative **26** in 65% yield, after high-performance liquid chromatography (HPLC) separation. A small amount of unreacted tetraphosphonate derivative **23** (5% yield) was also obtained along with some other unidentified products. Compound **23** can be recycled, e.g., used as a starting material for the reaction with DIC and **20**. Deisopropylidenation of **26** with trifluoroacetic acid afforded the desired C2-MAD (**17**).

The assignment of the structure of **17** was confirmed by ^1H and ^{31}P NMR. The ^1H NMR resonance signal of

Scheme 5



the $-CH_2-$ of the side chain at 3.73 ppm was recorded as a quartet (J_{H-P} = 7.0 Hz, J_{H-H} = 7.0 Hz) showing the coupling with the β-phosphorus atom of the bis(phosphonate) moiety. Heteronuclear shift correlation experiments also confirmed the $P-CH_2$ coupling with phosphorus.

Because mycophenolic alcohol **20** contains a free phenolic group at C7, the formation of 7-O-linked regioisomer and/or double-substituted derivative (at the O7 and the C2′) could be expected. Therefore, the protection of the phenol function may be necessary in order to improve efficiency of the coupling procedure. We used a benzyl group for this protection. The reaction of mycophenolic alcohol **20** with benzyl bromide in a 1 M solution of tetrabutylammonium fluoride in tetrahydrofuran (THF) afforded the desired 7-O-benzyl derivative **27** (Scheme 5) in crystalline form and high yield. Contrary to expectations, we found that the coupling of adenosine bis(phosphonate) **22** with the protected mycophenolic alcohol **27** required a longer reaction time and the yield was similar to that observed for the coupling with the unprotected compound **20**.

We also examined an alternative synthetic strategy by using 7-O-benzyl derivative **27** for the preparation of the methylenebis(phosphonate) analogue **30** and the subsequent coupling of **30** with 2′,3′-O-isopropylideneadenosine (Scheme 5). Mesylation of alcohol **27** afforded a good yield of mesylate **28**. However, further reaction with the tris(tetra-*n*-butylammonium) salt of methylenebis(phosphonic) acid according to Poulter's procedure[34] gave exclusively the elimination product **29**. Nevertheless, the desired bis(phosphonate) was obtained in very good yield by the reaction of **27** with commercially available methylenebis(phosphonic dichloride) in conditions of Yashikawa's phosphorylation in $(EtO)_3P(O)$. Although a small amount of bis(mycophenolic)bis(phosphonate) derivative **32** (e.g., the P^1, P^2-disubstituted analogue) was also formed in the reaction, compounds **30** and **32** were easily separated by preparative HPLC. The benzyl protecting group was then removed quantitatively from **30** and **32** by hydrogenolysis on Pd/C to give bis(phosphonate) derivatives **31** and **33**, respectively. These compounds were also examined as potential inhibitors of IMPDH.

Table 1. Inhibition of IMPDH and K562 Cell Growth by MPA (**10**), TR (**1**), BR (**2**), and Methylenebis(phosphonate) Analogues of TAD (**11**, **12**), BAD (**13**), and MAD (**15**−**17**)

inhibitors	IMPDH type I K_i (μM)	IMPDH type II K_i (μM)	K562 cell growth IC_{50} (μM)
MPA (**10**)	0.04	0.01	0.3
TR (**1**)	not active	not active	3.0
BR (**2**)	not active	not active	1.5
TAD (**11**)	0.11	0.11	18.0
TAD (**12**)	0.30	0.30	11.0
BAD (**13**)	0.66	0.95	60.0
C6-MAD (**15**)	0.33	0.29	1.5
C4-MAD (**16**)	0.52	0.38	0.1
C2-MAD (**17**)	0.33	0.26	0.7
bis(phosphonate) **31**	0.89	0.40	43.2
bis(phosphonate) **33**	2.3	0.47	2089.7

Finally, DIC coupling of the 7-O-benzyl-protected bis(phosphonate) derivative **30** with the 2′,3′-O-isopropylideneadenosine (^{i}PrAd) afforded, after hydrogenolytic debenzylation (Pd/C) and deisopropylidenation (CF_3COOH/H_2O), the desired analogue **17**. We found, however, that the overall yield of this new procedure was not superior to that depicted in Scheme 4. Both synthetic approaches were almost equally efficient.

The similar coupling of the 7-unprotected mycophenolic alcohol derivative **21** with bis(phosphonate) **22** gave, after hydrolysis and deisopropylidenation with CF_3COOH/H_2O, the desired C4-MAD (**16**; Figure 2) in moderate yield. The ^1H NMR resonance signal of the $-CH_2-$ group of the side chain at 4.20 ppm was recorded as a doublet (J_{H-P} = 7.0 Hz) showing the coupling with the β-phosphorus atom of the bis(phosphonate) moiety. The similar P−H coupling confirming the attachment of the bis(phosphonate) moiety to the $-CH_2-$ group of the side chain of the mycophenolic alcohol in compounds **30**−**34** has been also recorded.

2. Biological Activity. The MAD analogues **16** and **17** as well as the bis(phosphonate)s **31** and **33** were assayed for inhibitory activity against human IMPDH type I and type II. The pattern of inhibition in each case is uncompetitive. K_i values are given in Table 1. It is worthwhile to note that K_m values for NAD are much higher and similar for the type I and the type II isoforms, −46 and 32 μM, respectively.[9] Antiprolifera-

tive activity of MAD analogues against K562 cells was also evaluated, and the corresponding IC_{50} values are given in Table 1. For comparison, previously published K_i and IC_{50} values of MPA (**10**), TR (**1**), BR (**2**), and the bis(phosphonate) analogues of TAD (**11**, **12**) and BAD (**13**) are also presented in Table 1.[27,30]

Comparison of these data indicates that MAD analogues are potent inhibitors of IMPDH but show little specificity against the two isoforms of the human enzyme. Regardless of the linker size, MAD analogues are an order of magnitude less potent than MPA. Contrary to expectations, the attachment of the adenosine moiety to MPA did not enhance binding affinity. Indeed, a bis(phosphonate) derivative **31**, which does not contain the adenosine moiety, binds IMPDH with comparable affinity to the dinucleotide analogues. Interestingly, this compound still showed a 2-fold better inhibitory activity against IMPDH type II than type I. Surprisingly, bis(mycophenolic)bis(phosphonate) analogue **33**, which contains a mycophenolic moiety attached to the P^1 and the P^2, showed a similar selectivity (5-fold) in the inhibition of the type II isoform to that of MPA (4-fold).

In retrospect, the fact that the MAD analogues do not show improved binding over MPA is perhaps not surprising. These compounds have more degrees of freedom than MPA. Thus, any gain in binding enthalpy is likely offset by the entropic penalty of constraining the longer dinucleotide analogue. The penalty for the constraint of even a single rotational degree of freedom in a ligand can be severe.[35] Furthermore, Digits and Hedstrom[36] have shown recently that binding between the nicotinamide and the adenosine 5′-diphosphate components of the NAD site in human IMPDH type II is not synergistic.

The data in Table 1 also show that MAD analogues are approximately as potent inhibitors of IMPDH as methylenebis(phosphonate) analogues of TAD and BAD (**11**−**13**). However, MAD analogues were found to be superior to the bis(phoshonate)s of TAD and BAD **11**−**13** in the inhibition of the growth of K562 leukemic cells by 2 orders of magnitude. Compounds **11**−**13** were less effective than their parent nucleosides (**1** or **2**) in the inhibition of K562 cells growth (IC_{50} values). In contrast, the most potent MAD analogue **16** shows more potent growth inhibition than MPA itself, although it is less active than MPA as an inhibitor of the isolated enzyme. This is probably due to the efficient (active) transport of MAD analogues into the cells. Indeed, all MAD analogues inhibit cell growth much more potently than analogues of TAD and BAD, or even TR.

Although MPA is the most potent inhibitor of IMPDH, it is not active against cancer.[37] This drug's therapeutic potential is limited by its undesirable metabolism. In humans, MPA is rapidly metabolized (via its C7-phenolic function) into the inactive glucuronide (MPAG; Figure 3), and as much as 90% of the drug circulates in this inactive form, which is then excreted into the bile.[38] In addition, MPAG is deglucuronidated by colonic bacteria, and MPA is adsorbed in the lower gastrointestinal track causing local damage to the intestinal epithelium. Protection of the C7 function or replacement of the phenol group with a fluorine atom, amino, or nitrile group in order to prevent glucuronidation resulted in inactive compounds.[39] In the form of a prodrug, mycophenolic mofetil (MMF), MPA is used in clinics as an immunosuppressant. Although there is no glucuronidation in activated lymphocytes, high doses (2−3 g per day) of MMF are needed in order to maintain the therapeutic level of MPA. Because the level of glucuronyltransferase activity is rather high in many cancer cells (in fact, human colorectal carcinoma cells were used in vitro as a means to assess the metabolism of analogues of MPA),[39] it is not surprising that MPA lacks any significant anticancer activity.

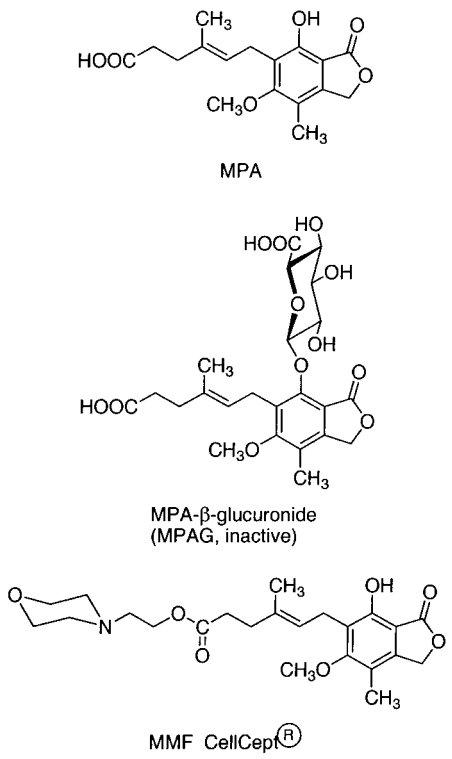


Figure 3.

We found that in contrast to MPA, MAD analogues were resistant to glucuronidation in vitro.[33] The incubation of MAD analogues or MPA with uridine 5′-diphosphoglucuronyltransferase and uridine 5′-diphosphoglucuronic acid resulted in extensive (70%) glucuronidation of MPA, whereas no formation of the corresponding MAD glucuronides was observed under similar conditions.[33] These results indicate that if MAD analogues are not glucuronidated in vivo, they would have a good therapeutic potential against cancer.

Interestingly, MAD analogues, specifically C2-MAD and C4-MAD, were also found to be potent inducers of K562 cell differentiation (Table 2), an order of magnitude more potent than TR. Taken together, their resistance to glucuronidation, potent inhibition of cell growth, and differentiation-inducing abilities make the MAD analogues potentially valuable antineoplastic agents. Synthesis of gram amounts of the above methylenebis(phosphonate) analogues of MAD for pharmacokinetic and toxicological studies is now in progress.

3. Conclusions. Known inhibitors of IMPDH such as MMF, mizoribine, and ribavirin were introduced to the drug market some time ago, and they continue to serve as clinically useful immunosuppressants or antiviral drugs. However, no anticancer drug, based on the inhibition of IMPDH, has been approved for clinical use.

Table 2. Effect of TR (**1**), TAD (**7**), Methylenebis(phosphonate) Analogues of TAD and BAD (**11**−**13**), and MAD Analogues **15**−**17** on Differentiation of K562 Cells

inhibitors	concn (μM)	% of benzidine positive cells	% of benzidine positive cells at 1.0 μM
TR (**1**)	15.0	80.0	5.3
TAD (**7**)	14.0	35.0	2.5
TAD (**11**)	14.3	35.0	2.4
TAD (**12**)	14.0	48.0	3.4
BAD (**13**)	15.0	9.5	0.6
C6-MAD (**15**)	10	90.0	9.0
C4-MAD (**16**)	1.5	95.0	63.3
C2-MAD (**17**)	1.6	65.4	40.9
bis(phosphonate) **31**	20.0	67.0	3.3
bis(phosphonate) **33**	100.0	4.4	0.4

TR is still in clinical trials; however, it was found to be too toxic for a broad clinical application. MAD analogues show interesting potential as anticancer drug candidates. Their resistance to glucuronidation and potent differentiation-inducing activity indicate that MAD analogues may be of therapeutic interest in the treatment of human leukemias.

MPA, MAD analogues, and other bis(phosphonate)s discussed herein do not show significant selectivity against an isoform of the human enzyme. However, an extension of the inhibitor into the AMP site would offer the advantage to design the isoform-specific inhibitor. The crystal structure of the type II isoform complexed with the NAD analogue selenazole-4-carboxamide adenine dinucleotide revealed the location of the adenosine end of the cofactor binding site.[40] In this region, the adenine moiety interacts with several residues that are not conserved between isoforms. Thus, modification of the adenine moiety of the MAD analogue would allow for exploitation of these isoform-dependent interactions to enhance specificity for the type II isoform. The synthesis of such analogues is now under way in our laboratory.

Experimental Section

General Methods. Preparative HPLC was performed on a Dynamax-300A C18-83-243-C column with a flow rate 20 or 25 mL/min of 0.1 M triethylammonium bicarbonate (TEAB) by a linear gradient of 0.1 M TEAB−aqueous MeCN (70%). The homogeneity of dinucleotides was determined by analytical HPLC (reverse phase column) in the linear gradient and/or in the isocratic mixture of 0.1 M TEAB−aqueous MeCN (70%) in the ratio of 4:1. Analytical thin-layer chromatography (TLC) was performed on Analtech Uniplates with short wavelength UV light for visualization. Column chromatography was performed on silica gel G60 (70−230 mesh, ASTM, Merck). NMR spectra were recorded on a JEOL Eclipse EX-270 or Varian 400 MHz instrument. Chemical shifts are reported in ppm (δ), and signals are described as s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), m (multiplet), or bs (broad singlet). Values given for coupling constants are first-order. Chemical shifts for ^{31}P NMR are referred to H_3PO_4. All exchangeable protons were detected by disappearance on the addition of D_2O. Elementary analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Negative-ion fast atom bombardment mass spectroscopy was performed by Emory University Mass Spectrometry Center.

7-Hydroxy-6-(formylmethyl)-5-methoxy-4-methylphthalan-1-one (18) and Its Hemiacetal 18a. MPA (30 mmol, 9.60 g) and 4-methylmorfoline-*N*-oxide (60 mmol, 7.03 g) was dissolved in a 3:1 (v/v) mixture of THF/H_2O (120 mL), and a solution of OsO_4 (250 mg) in acetone (10 mL) was added. The reaction mixture was stirred for 3.5 h, and the disappearance of MPA was monitored by TLC with CH_2Cl_2−MeOH (20%) as the eluent. The mixture was diluted with water (300 mL) and cooled in an ice bath, and a solution of $NaIO_4$ (21.4 g) in water (200 mL) was added in portions. The formed precipitate was filtered, washed with cold water (3 × 50 mL), and dried to give a 2.5:1 mixture of **18** and **18a** (6.8 g, 96.0%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 2.04 (**18a**, s, 3H, CH_3), 2.16 (**18**, s, 3H, CH_3), 3.29 (**18a**, dd, 1H, H1, J_{1-2} = 2.0 Hz, $J_{1-1'}$ = 16.2 Hz), 3.52 (**18a**, dd, 1H, H1′, $J_{1'-2}$ = 6.5 Hz), 3.72 (**18**, s, 3H, OCH_3), 3.76 (**18**, d, 2H, CH_2, J_{1-2} = 6.0 Hz), 3.98 (**18a**, s 3H, OCH_3), 5.13 (**18a**, s, 2H, CH_2−lactone), 5.23 (**18**, s, 2H, CH_2−lactone), 6.31 (**18a**, dd, 1H, H2), 7.71 (**1**, s, 1H, 7-OH, exchange), 9.75 (**18**, t, 1H, aldehyde). Anal. ($C_{12}H_{12}O_5$) C, H.

7-Hydroxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one (20). The solution of a mixture of **18** and **18a** (10 mmol, 2.36 g) in EtOH (100 mL) was treated with $NaBH_4$ (1.0 g) in portions over 0.5 h. The mixture was kept at room temperature until TLC (a small sample for TLC analysis was neutralized with 3 N HCl, extracted with EtOAc, and chromatographed with CH_2Cl_2 (2%) as the eluent) showed complete reduction (approximately 2 h). The mixture was concentrated, and the residue was treated with 3 N HCl (50 mL) and extracted with EtOAc to give after concentration the alcohol **20** (2.36 g). ^{1}H NMR ($CDCl_3$): δ 2.15 (s, 3H, CH_3), 2.98 (t, 2H, CH_2, J = 6.4 Hz), 3.79 (s, 3H, OCH_3), 3.85 (t, 2H, CH_2, J = 6.4 Hz), 5.20 (s, 2H, CH_2−actone), 7.71 (s, 1H, 7-OH, exchange). Anal. ($C_{12}H_{14}O_5$) C, H.

7-Hydroxy-6-(4-hydroxy-3-methylbut-2-enyl)-5-methoxy-4-methylphthalan-1-one (21). The mixture of **18** and **18a** (4.0 g) and Ph_3P=C(Me)CHO (6.0 g) in benzene (300 mL) was heated under reflux for 24 h. The reaction mixture was concentrated, and the residue was chromatographed on a silica gel column with *n*-hexanes−EtOAc (20%), followed by *n*-hexanes−EtOAc (30%) affording a white solid, which was crystallized from EtOH to give an aldehyde (3.6 g, 77%). ^{1}H NMR ($CDCl_3$): δ 1.90 (s, 3H, CH_3−side chain), 2.15 (s, 3H, CH_3), 3.71 (d, 2H, CH_2, J = 7.0 Hz), 3.77 (s, 3H, OCH_3), 5.21 (s, 2H, CH_2−lactone), 6.51 (t, 1H, H2), 7.75 (s, 1H, 7-OH, exchange), 9.36 (s, 1H, aldehyde).

A solution of the aldehyde (3.6 g) in EtOH (220 mL) was treated with $NaBH_4$ (900 mg) added in portions over 45 min. The reaction mixture was kept overnight, concentrated, and treated with a mixture of 3 N HCl and EtOAc. The organic layer was washed with water and concentrated, and the residue was chromatographed on a silica gel column with CH_2Cl_2−acetone (2.5%) as the eluent to give a white solid. Crystallization from benzene−petrol ether afforded **21** (3.4 g). ^{1}H NMR ($CDCl_3$): δ 1.83 (s, 3H, CH_3−side chain), 2.14 (s, 3H, CH_3), 3.43 (d, 2H, CH_2 (1), J = 7.0 Hz), 3.78 (s, 3H, OCH_3), 3.98 (s, 2H, CH_2 (4)), 5.19 (s, 2H, CH_2−lactone), 5.48 (t, 1H, H2, J = 7.0 Hz), 7.68 (s, 1H, 7-OH, exchange). Anal. ($C_{15}H_{18}O_5$) C, H.

P^1-(2′,3′-O-Isopropylideneadenosin-5′-yl)-P^2-[7-hydroxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis(phosphonate) (26). To a solution of **22** (226 mg, 0.34 mmol) in pyridine (2 mL) was added DIC (156 mL, 2 mmol), and the mixture was left overnight until the intermediate **24** was formed (multisignal resonances in ^{31}P NMR).[25] Compound **20** (95 mg, 0.4 mmol) was then added, and the reaction mixture was heated at 55−60 °C for 14 h and at 70 °C for 6 h. At that time, the ^{31}P NMR of the reaction mixture showed two broad signals at 8 and 25 ppm, characteristic for the presence of tetrasubstituted derivative **25**. Then, a mixture of water (400 μL) and Et_3N (200 μL) was added, and the reaction mixture was kept at 75 °C for 30 h. HPLC purification afforded a faster migrating tetraphosphonate **23** (19 mg, 5%), ^{1}H NMR identical to that of the original sample,[25] and a slower migrating compound **26** (200 mg, 65%) as the triethylammonium salt. ^{1}N NMR (D_2O): δ 1.24 (t, 18H, Et_3N), 1.35 and 1.60 (two s, 3H each, isopropylidene) 1.72 (s, 3H, CH_3), 2.17 (t, 2H, P−CH_2−P, J = 19.8 Hz), 2.65 (t, 2H, CH_2 (1), J = 7.2 Hz),

3.08 (q, 12H, Et_3N), 3.52 (s, 3H, OCH_3), 3.75 (q, 2H, CH_2 (2), $J_{H,H}$ = 7.0 Hz, $J_{H,P}$ = 7.0 Hz), 4.07 (m, 1H, H5′, $J_{5',5''}$ = 12.4 Hz), 4.10 (m, 1H, H5″), 4.19 (m, 1H, H4′), 4.34 (t, 1H, H3′, $J_{2',3'}$ = 4.8 Hz, $J_{3',4'}$ = 5.8 Hz), 4.49 (t, 1H, H2′, $J_{1',2'}$ = 4.4 Hz), 4.79 (s, 2H, CH_2−lactone), 5.77 (d, 1H, H1′), 7.90, 8.21 (two 1H singlets, H2, H8−adenine). ^{31}P NMR (D_2O): δ 16.20−17.50 (AB system, J = 13.8 Hz). MS (FAB): m/z 684 $(M - H)^-$ 688 (100%) and the sodium adduct (644 + 22).

P^1-(Adenosin-5′-yl)-P^2-[7-hydroxy-6-(2-hydroxyethyl)-5-methoxy-4-methyl-phthalan-1-one-2-yl]methylenebis-(phosphonate) (17, C2-MAD). (A) From **26**. Compound **26** (90 mg, 0.1 mmol, as the triethylammonium salt) was dissolved in a mixture of MeOH (4 mL) and water (4 mL) containing CF_3COOH (2 mL). The mixture was kept at room temperature for 1 h and was heated at 50 °C for 30 min. Methanol was removed in vacuo, and the mixture was diluted with water (8 mL), neutralized with concentrated ammonia, and concentrated. The residue was chromatographed on HPLC to give **17** as the trietylammonium salt. This compound was converted into a sodium salt (57 mg, 83%) by passing through a column of Dowex 50WX8/Na^+ form. ^{1}H NMR (D_2O): δ 1.69 (s, 3H, CH_3), 2.07 (t, 2H, P−CH_2−P, J = 20.0 Hz), 2.61 (t, 2H, CH_2 (1), J = 6.8 Hz), 3.52 (s, 3H, OCH_3), 3.73 (q, 2H, CH_2 (2), $J_{H.H}$ = 6.4 Hz, $J_{H.P}$ = 6.4 Hz), 4.02 (m, 1H, H5′, $J_{5',5''}$ = 12.4 Hz), 4.10 (m, 1H, H5″), 4.17 (m, 1H, H4′), 4.34 (t, 1H, H3′, $J_{2',3'}$ = 4.8 Hz, $J_{3',4'}$ = 5.8 Hz), 4.47 (t, 1H, H2′, $J_{1',2'}$ = 4.4 Hz), 4.74 (s, 2H, CH_2−lactone), 5.77 (d, 1H, H1′), 7.85, 8.21 (two 1H singlets, H2, H8−adenine). ^{31}P NMR (D_2O): δ 17.20−17.50 two overlapping doublets. MS (FAB): m/z 644 $(M - H)^-$ (55%).

(B) From **34**. Compound **34** (94 mg, 0.1 mmol) was dissolved in MeOH (20 mL), palladium on activated carbon (10%) was added, and the mixture was shaken under a hydrogen atmosphere (20 psi) in a Parr hydrogenation apparatus for 1 h. The mixture was filtered and concentrated in vacuo to give a white solid (84 mg). This compound was treated with a mixture of MeOH−H_2O−CF_3COOH as described for compound **26** to give **17** (62 mg, 90%), identical to the compound obtained from **26**.

P^1-(Adenosin-5′-yl)-P^2-[7-hydroxy-6-(4-hydroxy-3-methylbut-2-enyl)-5-methoxy-4-methylphthalan-1-one-4-yl]-methylenebis(phosphonate) (16, C4-MAD). In a similar manner as described for the synthesis of **17**, compound **21 (**124 mg, 0.5 mmol) was coupled with bis(phosphonate) **22** (300 mg, 0.45 mmol, triethylammonium salt). The hydrolysis and formation of the isopropylidene-protected derivative (major compound) were monitored by analytical HPLC. The reaction mixture was concentrated and treated with a mixture of MeOH−H_2O−CF_3COOH as described for compound **26.** HPLC purification followed by conversion into the sodium salt afforded **16** (148 mg, 45%). ^{1}H NMR (D_2O): δ 1.78 (s, 3H, CH_3− side chain), 2.08 (s, 3H, CH_3), 2.14 (t, 2H, P−CH_2−P, J = 19.5 Hz), 3.27 (d, 2H, CH_2 (1), J = 7.0 Hz), 3.68 (s, 3H, OCH_3), 4.12 (m, 2H, H5′,5″), 4.20 (d, 2H, CH_2 (4), J_{H-P} = 6.8 Hz), 4.27 (M, 1H, H4′), 4.42 (m, 1H, H3′), 4.65 (m, 1H, H2′), 5.19 (s, 2H, CH_2−lactone), 5.28 (t, 1H, (2), J = 7.0 Hz), 5.81 (d, 1H, H1′, $J_{1'-2'}$ = 5.0 Hz), 8.00 and 8.35 (2 1H singlets, H2, H8). ^{31}P NMR (D_2O): δ 17.80 and 18.10 (AB system, J = 11.5 Hz). MS (FAB): m/z 684 $(M - H)^-$.

7-Benzyloxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one (27). A solution of compound **20** (3.0 g, 12.7 mmol,) in 1 M Bu_4NF in THF (26 mL) was treated with benzyl bromide (1.65 mL). The mixture was stirred for 1 h and analyzed by TLC with CH_2Cl_2−EtOH (3%). An additional amount of benzyl bromide (0.3 mL) was added to complete the reaction. After saturated $NaHCO_3$ was added, the mixture was extracted with EtOAc, washed with water, dried (Na_2SO_4), and concentrated. The residue was chromatographed on a silica gel column with CH_2Cl_2−EtOH (1%) to give **27** (4. 0 g, 97%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 2.20 (s, 3H, CH_3), 2.90 (t, 2H, CH_2 (1), J = 6.5 Hz), 3.70 (t, 2H, CH_2 (2), J = 6.5 Hz), 3.79 (s, 3H, OCH_3), 5.17 (s, 2H, CH_2−lactone), 5.32 (s, 2H, CH_2-Ph), 7.39−7.49 (m, 5H, Ph). Anal. ($C_{19}H_{20}O_5$) C, H.

7-Benzyloxy-6-(2-mesyloxyethyl)-5-methoxy-4-methylphthalan-1-one (28). To a mixture of compound **27** (1.95 g, 6.0 mmol), Et_3N (1.67 mL, 12.0 mmol), and 4-(dimethylamino)pyridine (DMAP, 732 mg, 6.0 mmol) in CH_2Cl_2 (15 mL), a solution of mesyl chloride (MsCl, 930 μL, 12.0 mmol) in CH_2Cl_2 (5 mL) was added dropwise. The reaction mixture was stirred for 10 min, diluted with MeOH (20 mL), and concentrated. The residue was chromatographed on a silica gel column with hexane−EtOAc (30%), followed by hexanes−EtOAc (40%), and then hexanes−EtOAc (50%) as the eluent to give **28** (2.2 g, 91%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 2.20 (s, 3H, CH_3), 2.82 (s, 3H, Ms), 3.04 (t, 2H, CH_2 (1), J = 7.2 Hz), 3.80 (s, 3H, OCH_3), 4.25 (t, 2H, CH_2 (2), J = 7.2 Hz), 5.18 (s, 2H, CH_2−lactone), 5.32 (s, 2H, CH_2Ph), 7.35−7.49 (m, 5H, Ph). Anal. ($C_{20}H_{22}O_7S$), C, H.

7-Benzyloxy-6-(2-ethene)-5-methoxy-4-methylphthalan-1-one (29). A mesyl derivative **28** (404 mg, 1.0 mmol) was dissolved in CH_3CN (1 mL) and treated with the tris(tetrabutylammonium) salt of methylenebis(phosphonic) acid according to Poulter's procedure.[34] The reaction mixture was partitioned between EtOAc−water, and the organic layer was separated, dried, and concentrated to give **29** as a white solid (200 mg, 91%). ^{1}H NMR ($CDCl_3$): δ 2.19 (s, 3H, CH_3), 3.74 (s, 3H, OCH_3), 5.16 and 5.19 (two 2H s, CH_2−lactone, CH_2Ph), 5.54 (dd, 1H, H1, J_{1-2} = 2.4 Hz, $J_{1-2'}$ = 12.0 Hz), 6.11 (dd, 1H, H2, $J_{2-2'}$ = 18.0 Hz), 6.79 (dd, 1H, H2′), 7.35−7.55 (m, 5H, Ph). Anal. ($C_{19}H_{18}O_4$) C, H.

[7-Benzyloxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis(phosphonic) Acid (30) and P^1-[7-Benzyloxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]-P^2-[7-benzyloxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]-methylenebis(phosphonate) (32). A cold solution of **27** (587 mg, 1.65 mmol) in triethyl phosphate (2 mL) was added to a cold solution of methylenebis(phosphonic) dichloride (450 mg, 1.8 mmol) in triethyl phosphate (2 mL), and the reaction mixture was kept in the refrigerator overnight and then at room temperature for 4 h, and the reaction mixture was added dropwise into the solution of 1 M TEAB (16 mL). The mixture was diluted with water and extracted with EtOAc, and the water layer was concentrated. The residue was chromatographed on an HPLC column to give a faster migrating bis-(phosphonic) acid **30** (985 mg, 87%) as a triethylammonium salt. A small sample of this compound was converted into a sodium salt by passing through a column of Dowex 50/Na^+ form. ^{1}H NMR (D_2O): δ 1.85 (t, 2H, P−CH_2P, J = 19.6 Hz), 2.07 (s, 3H, CH_3), 2.76 (t, 2H, CH_2 (1), J_{1-2} = 6.8 Hz), 3.69 (s, 3H, OCH_3), 3.79 (q, 2H, CH_2 (2), $J_{H,H}$ = $J_{H,P}$ = 6.8 Hz), 5.06 and 5.20 (two 2H s, CH_2−lactone, CH_2Ph), 7.29−7.37 (m, 5H, Ph). ^{31}P NMR (D_2O): δ 15.84 (d, 1P, J = 7.7 Hz), 17.71 (d, 1P). MS (FAB): m/z 485 $(M - H)^-$.

The slower migrating **32** (73 mg, 9%) was then eluted and converted into a sodium salt. ^{1}H NMR (D_2O): δ 1.96 (s, 3H, CH_3), 2.04 (t, 2H, P−CH_2P, J = 20.0 Hz), 2.82 (t, 2H, CH_2 (1), J = 6.8.0 Hz), 3.69 (s, 3H, OCH_3), 3.88 (q, 2H, CH_2 (2), $J_{H,H}$ = $J_{H.P}$ = 6.8 Hz), 4.87 and 4.92 (two 2H s, CH_2−lactone, CH_2-Ph), 7.11−7.26 (m, 5H, Ph). ^{31}P NMR (D_2O): δ 17.21 (s).

[7-Hydroxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis(phosphonic) Acid (31). Compound **30** (69 mg, 0.1 mmol) was dissolved in MeOH (20 mL), palladium on activated carbon (10%) was added, and the mixture was shaken under a hydrogen atmosphere (20 psi) in a Parr hydrogenation apparatus for 1 h. The mixture was filtered and concentrated in vacuo, and the residue was dissolved in water and passed through a column of Dowex 50WX8/Na^+ form to give **31** (45 mg, 99%). ^{1}H NMR (D_2O): δ 2.07 (t, 2H, P−CH_2−P, J = 19.6 Hz), 2.07 (s, 3H, CH_3), 2.76 (t, 2H, CH_2 (1), J_{1-2} = 7.0 Hz), 3.69 (s, 3H, OCH_3), 3.79 (q, 2H, CH_2 (2′), $J_{H,H}$ = $J_{H,P}$ = 7.0 Hz), 5.20 (s, 2H, CH_2−lactone, CH_2Ph). ^{31}P NMR (D_2O): δ 15.84 (d, 1P, J = 7.7 Hz), 17.71 (d, 1P). MS (FAB): m/z 395 $(M - H)^-$ (10%); the sodium adduct: m/z 417 (5%).

P^1-[7-Hydroxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]-P^2-[7-hydroxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis-

(phosphonate) (33). Compound **32** (101 mg, 0.1 mmol) was dissolved in MeOH (20 mL), palladium on activated carbon (10%) was added, and the mixture was shaken under a hydrogen atmosphere (20 psi) in a Parr hydrogenation apparatus for 1 h. The mixture was filtered and concentrated in vacuo, and the residue was dissolved in water and passed through a column of Dowex 50WX8/Na^+ form to give **33** (65 mg, 95%). ^{1}H NMR (D_2O): δ 2.10 (s, 3H, CH_3), 2.14 (t, 2H, P−CH_2−P, J = 16.6 Hz), 2.93 (t, 2H, CH_2 (1), J = 7.2 Hz), 3.85 (s, 3H, OCH_3), 4.03 (q, 2H, CH_2 (2), $J_{H,H} = J_{H,P}$ = 7.2 Hz), 5.11 (s, 2H, CH_2−lactone). ^{31}P NMR (D_2O): δ 17.72 (s). MS (FAB): m/z 615 $(M - H)^-$ (55%) and the sodium adduct 637 (100%).

P^1-(2′,3′-O-Isopropylideneadenosin-5′-yl)-P^2-[7-benzyloxy-6-(2-hydroxyethyl)-5-methoxy-4-methylphthalan-1-one-2-yl]methylenebis(phosphonate) (34). (A) From **30**. To a solution of compound **30** (345 mg, 0.5 mmol) in pyridine (2 mL) was added DIC (156 mL, 2 mmol), and the mixture was left overnight until the multisignal resonances were observed in ^{31}P NMR.[23] The 2′,3′-O-isopropylideneadenosine (153 mg, 0.5 mmol) was then added, and the reaction mixture was heated at 55−60 °C for 48 h. At that time, the ^{31}P NMR of the reaction mixture showed two broad signals at 8 and 25 ppm, characteristic for the presence of the tetrasubstituted derivative. Then, a mixture of water (400 μL) and Et_3N (200 μL) was added, and the reaction mixture was kept at 75 °C for 30 h. HPLC purification afforded compound **34** (297 mg, 60%) as the triethylammonium salt. ^{1}N NMR (D_2O): δ 1.18 (t, 18H, Et_3N), 1.45 and 1.60 (two s, 3H each, isopropylidene), 2.05 (s, 3H, CH_3), 2.51 (dt, 2H, P−CH_2−P, J = 21.2 Hz, J = 8.8 Hz), 3.10 (q, 12H, Et_3N), 3.40−3.48 (m, 2H, H5′, 5″), 3.60 (t, 2H, CH_2 (1), J = 6.4 Hz), 3.62 (s, 3H, OCH_3), 4.10−4.12 (m, 3H, CH_2 (2), H4′), 4.54 (m, 1H, H3′), 4.67 (m, 1H, H2′), 4.95 (s, 2H, CH_2−lactone), 5.14, (s, 2H, CH_2−benzyl), 6.01 (d, 1H, H1′, $J_{1',2'}$ = 2.4 Hz), 7.94 (t, 2H, phenyl, J = 7.6 Hz), 7.96, 8.27 (two 1H singlets, H2, H8−adenine), 8.42 (t, 1H, phenyl, J = 7.6 Hz), 8.69−8.71 (m. 2H, phenyl). ^{31}P NMR (D_2O): δ 16.06−17.79 (AB system, J = 16.6 Hz).

(B) The coupling of compounds **22** and **27**. The reaction of **22** (332 mg, 0.5 mmol) and **27** (197 mg, 0.6 mmol) was performed as described for the coupling of **22** with **20**, except the reaction mixture was heated at 55−60 °C for 2 days. After hydrolysis and HPLC purification, compound **34** (314 mg, 67%) was obtained as a triethylammonium salt. Proton and phosphorus NMR were identical as reported above.

Enzyme Assays. The new compounds were tested as inhibitors of IMPDH type I and type II as described by us previously.[29] Briefly, substrate, cofactor, and inhibitor are mixed, and the reaction started by the addition of the enzyme. The course of the reaction is followed by measuring the changing absorbance at 340 nm of the absorbance peak of NADH.

Measurement of Cellular Differentiation. Human leukemia K562 cells were treated with TR, C2-MAD, C4-MAD, and bis(phosphonate) analogues **31** and **33** and then examined for their differentiating activity. K562 cells were grown and assayed as described by us earlier.[30]

Resistance to Glucuronidation. In the same manner as described by us previously,[33] the C2-MAD and C4-MAD were examined as potential substrates for glucuronidation in vitro. Under conditions in which MPA was completely glucoronidated, MAD analogues did not produce any glucuronides.

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