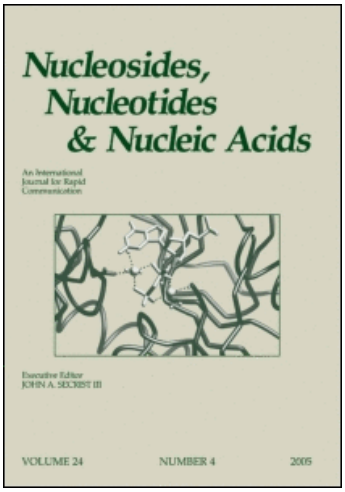

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Synthesis and Biological Study of the Cyclopentenyl Carbocyclic Nucleoside Analogue of 5-Azacytidine

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SYNTHESIS AND BIOLOGICAL STUDY OF THE CYCLOPENTENYL CARBOCYCLIC NUCLEOSIDE ANALOGUE OF 5-AZACYTIDINE

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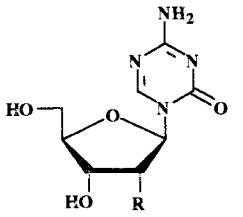
Abstract. Cyclopentenyl cytosine (CPE-C, 3) possesses excellent antitumor and antiviral activity. The synthesis of the analogous cyclopentenyl triazine nucleoside, 5-aza-CPE-C (4), was accomplished by a novel approach that utilized a key 1-cyclopentenyl-4-methylisobiuret intermediate (7) produced from the corresponding cyclopentenylamine 5. 5-Aza-CPE-C was more than six-hundred times less potent than CPE-C both in its capacity to reduce CTP levels as well as in its antitumor and antiviral activity.

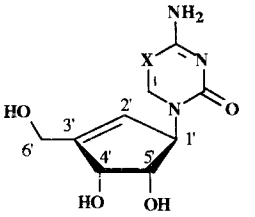
5-Azacytidine (5-Aza-Cyd, 1) and 5-aza-2'-deoxycytidine (5-Aza-dCyd, 2) are analogues of cytidine and deoxycytidine in which a nitrogen atom replaces the carbon at position five of the cytosine ring. Since the syntheses of these compounds were first reported in 1964[1,2] they have been the subject of extensive work due to their promising antileukemic activity.[3,4] In addition to their clinical utility, one of the more interesting features of these two drugs is their use as tools to study the role of DNA methylation on gene activation and expression.[5-7] By means of different biochemical pathways, these drugs lead to the effective incorporation of 5-azacytosine residues in DNA which results in a direct loss of DNA methyltransferase activity.[5,6] This inhibitory activity is due to the formation of either a highly stable noncovalent complex,[8] or a covalent complex linking the enzyme to the 6-position of the triazine ring.[9] Such reactivity of the 5-azacytosine moiety is a consequence of the inherent electrophilicity of the triazine ring[10] which causes DNA methyltransferase, or other nuclear proteins, to

attack DNA segments containing the modified base.[9] It is precisely this property of the triazine ring that prompted us to consider the synthesis



1, R = OH (5-Aza-Cyd)

2, R = H (5-Aza-dCyd)



3, X = CH (CPE-C)

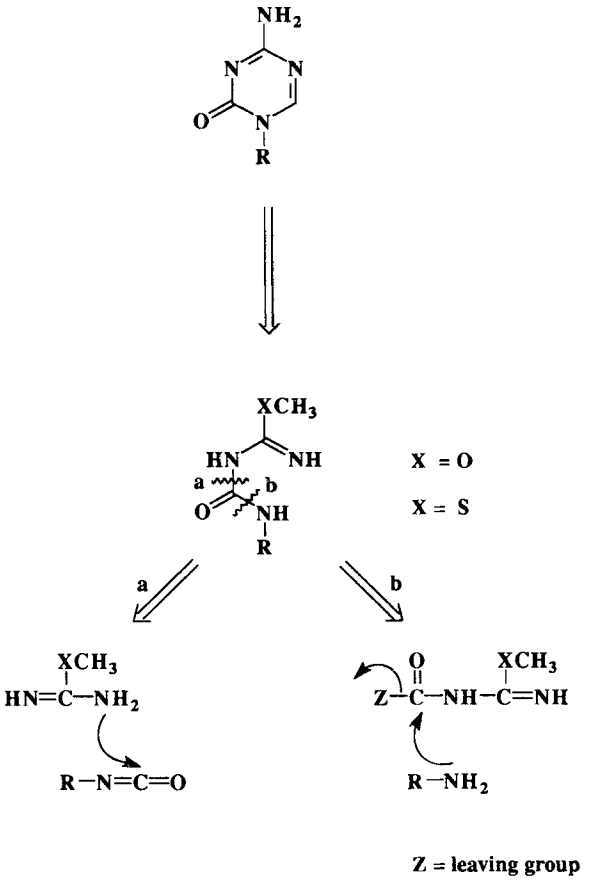
4, X = N (5-Aza-CPE-C)

of the corresponding cyclopentenyl 5-azacytosine nucleoside analogue (4, 5-aza-CPE-C).

Cyclopentenyl cytosine (3, CPE-C)[11] has an entirely different mechanism of action compared to the triazine nucleosides since its antitumor activity appears to correlate with the drug's ability to strongly inhibit cytidine triphosphate synthetase (CTP-synthetase).[12,13] After conversion to the active triphosphate anabolite, CPE-CTP, the compound effectively blocks CTP-synthetase causing intracellular levels of cytidine triphosphate (CTP) to drop precipitously.[13-15] The rationale for the synthesis of 5-aza-CPE-C (4) was the idea that in addition to the affinity towards CTP-synthetase afforded by CPE-C, one could additionally capitalize on the reactivity of the triazine ring in the hope of forming a tight complex with the enzyme. Formation of such a complex, whether covalent or not, was anticipated to lead to the irreversible inactivation of CTP-synthetase. The present report describes the synthesis and the biological evaluation of this compound.

Chemistry

5-Aza-Cyd (1) and 5-Aza-dCyd (2) have been prepared by two different methods. The most recent published syntheses proceed via the direct glycosylation of silylated 5-azacytosine,[16-20] while the earlier versions involve a multistep approach that starts with the peracylated 1-glycosyl isocyanates.[1,2,21] These glycosyl isocyanates when converted to the 4-methylisobiuret or 4-methylisothiobiuret intermediates serve as direct precursors to the s-triazine nucleosides (Scheme 1, path a).[1,2,21]



R = 2,3,5-tri-O-Acyl-β-D-ribofuranosyl

R = 3,5-di-O-Acyl-2-deoxy-β-D-ribofuranosyl

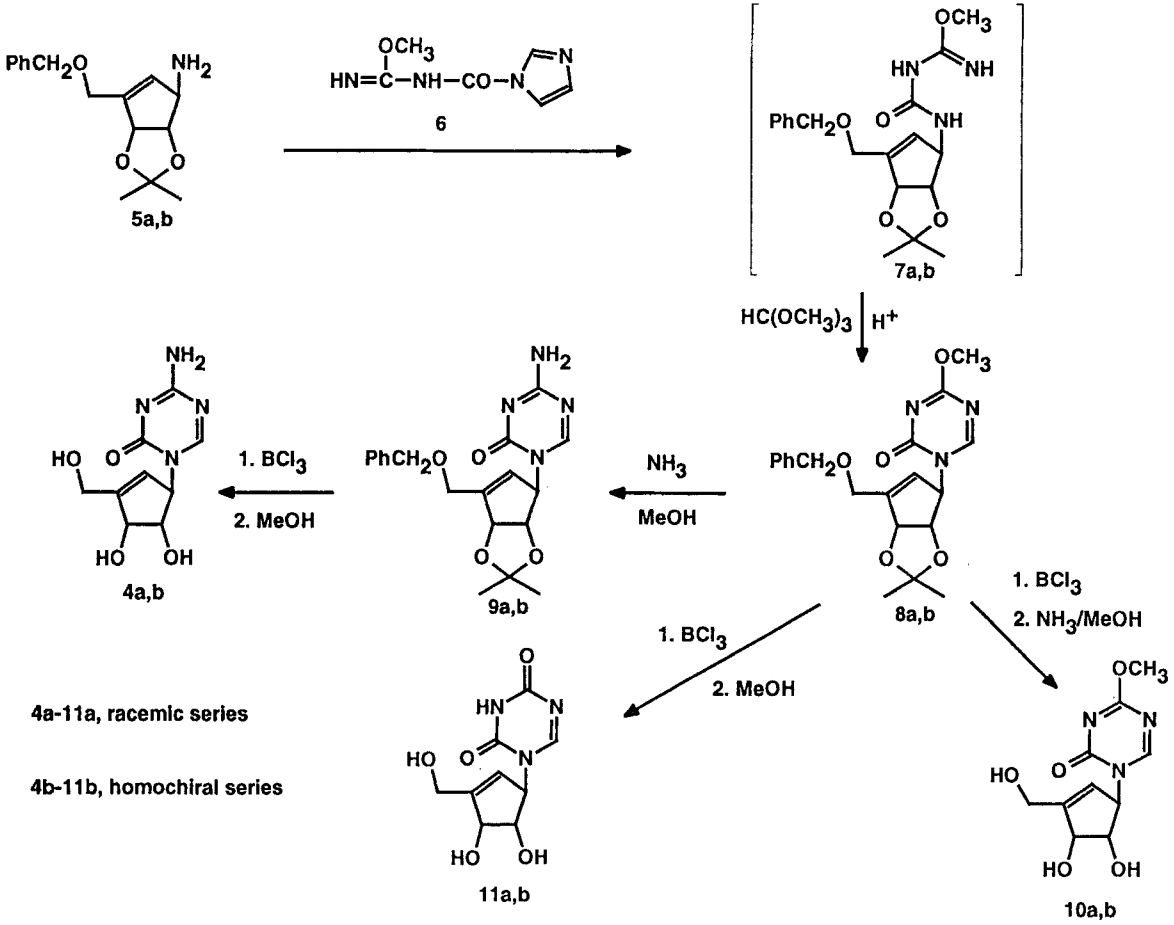
R = 3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopenten-1-yl

Scheme 1

After considering these two approaches and their possible adaptation to the synthesis of carbocyclic cyclopentenyl triazine nucleosides, the second approach was selected. Therefore, the corresponding 1-cyclopentenyl-4-methylisobiuret was sought as an immediate precursor to the target compound. Since the carbocyclic cyclopentenylamine was a readily available precursor from our earlier work, we envisioned that a reaction of this amine with a sufficiently electrophilic methylisobiuret would

generate essentially the same intermediate methylisobiuret that would have been formed between 2-methylisourea and the corresponding cyclopentenyl isocyanate (Scheme 1, path b). A very good electrophilic methylisobiuret (6) was synthesized through the reaction of 1,1'-carbonyldiimidazole and 2-methylisourea. To our satisfaction, this compound reacted well with cyclopentenylamine (5) and the resulting crude cyclopentenyl 4-methylisobiuret intermediate (7) cyclized readily to the desired s-triazine 8 (Scheme 2). This approach represents an alternative procedure for the construction of the triazine nucleus that might be applicable in cases where the gycosyl isocyanate is not readily accessible. Ammonolysis of 8 produced the desired penultimate intermediate 9, which was deprotected with BCl_3 to afford the desired target, 5-aza-CPE-C (4). It was possible to control the deprotection of the methoxytriazine intermediate 8 to generate either the extremely unstable 5-aza-CPE-U (11), or the more stable 4-methoxy analogue 10 (see Experimental). Initially, the synthesis was performed with the less expensive racemic cyclopentenylamine 5a and later with the pure enantiomer 5b. Both racemic and homochiral series are reported.

The ^1H NMR spectra of 4 in D_2O remained unchanged after 24 h at room temperature. This is in sharp contrast to 5-azacytidine (1) which after 24 h under the same conditions was converted into a mixture of starting material, N-(formylamidino)-N'-β-D-ribofuranosyl urea and 1-β-D-ribofuranosyl-3-guanylurea.[22] However, under strong alkaline conditions (pH 12), a progressive change in the ^1H NMR spectrum of 5-aza-CPE-C (4) was observed which, in the same manner as the nucleoside 1,[23] appeared to have been converted to the corresponding 3-guanylurea analogue during the course of one hour. Despite this base-catalyzed degradation, 5-aza-CPE-C is nevertheless significantly more stable than 5-azacytidine, since under the same basic conditions, 1 underwent an almost instantaneous decomposition. Contrary to 5-aza-CPE-C, 5-aza-CPE-U (11) was very unstable in aqueous solution and after the removal of the methoxy, isopropylidene, and benzyl protective groups, only a mixture of compounds was observed by ^1H NMR. Although signals corresponding to 5-aza-CPE-U (11) were clearly discernible, the solution deteriorated with time and no attempt was made to isolate the products.



Scheme 2

TABLE 1. L1210 Cytotoxicity and CTP Levels After Drug Treatment.

	5-Aza-CPE-C		CPE-C	
Cell line	IC_{50} (μM)[a]	CTP(%)[b]	IC_{50} (μM)[a]	CTP (%)[b]
L1210[c]	50	70-73	0.075	60-84
Molt-4[d]	80	44-56	0.150	67-72

[a]Inhibitory concentration that causes 50% inhibition of cell growth. [b]Percent depression of CTP levels relative to control levels after a 24 h incubation with the IC_{50} concentration of each drug (duplicate experiment). At the conclusion of the incubation period the cells were pelleted and extracted with 60% methanol and the extracts were analyzed by ion-exchange HPLC to determine effects on CTP pools. [c]L1210 cells were seeded at 0.15 x 10^5 cell/mL in Fishers' medium containing 5% heat-inactivated horse serum. Cells were exposed to various concentrations of drugs in the range of 1-100 μM continuosly for 48 h, and the cell number was determined by use of a Coulter counter. [d]Molt-4 cells (0.34 x 10^5 cell/mL) were incubated with various concentrations of drugs in the range of 1-100 μM continuously for 24 h, and the cell number was counted.

Biological Activity

The cytotoxicity of enantiomerically pure 5-aza-CPE-C (4b) to L1210 tumor cells and Molt-4 cells was investigated in direct comparison with the parent antitumor-active compound CPE-C (3) (Table 1). The potency of the racemate 4a was approximately 50% lower (data not shown). Additionally, since CPE-C is capable of inducing a significant reduction of CTP pools, CTP levels were measured after exposure to both drugs. As shown in Table 1, the incorporation of the nitrogen atom at position-five of the pyrimidine ring caused a dramatic reduction in cytotoxicity relative to CPE-C. Concomitantly, the potency of 5-aza-CPE-C to lower CTP levels was reduced more than six hundred times relative to CPE-C. The 4-methoxy CPE-U analogue, 10, was not inhibitory (data not shown).

5-Aza-CPE-C (4b) and the 5-aza-4-methoxy CPE-U (10b) were also evaluated for antiviral activity. 5-Aza-CPE-C possesses the same spectrum of antiviral activity as CPE-C[24] but is much less potent. Only those viruses for which a distinct response was obtained are included in Table 2. A recent evaluation of CPE-C against the same viruses[24] indicates that CPE-C is approximately 100 to 1000-fold more potent than 4b. 4-Methoxy CPE-U, 10, was devoid of any antiviral

TABLE 2. Antiviral Activity Spectrum of 5-Aza-CPE-C

	5-Aza-CPE-C		Positive Control	
Virus[a]	Host Cell	IC_{50}[b]	Compound	IC_{50}[b]
HSV-1(KOS)	E_6SM[c]	300	BVDU[g]	0.02
HSV-1(F)	E_6SM[c]	100	BVDU[g]	0.02
HSV-1(McIntyre)	E_6SM[c]	150	BVDU[g]	0.007
CMV(AD-169)	HEL[d]	8.69	DHPG[h]	0.52
Vaccinia	E_6SM[c]	70	BVDU[g]	0.07
VSV	HeLa[e]	20	C-C^3Ado[i]	2
Parainfluenza-3	Vero[f]	20	C-C^3Ado[i]	2
Reovirus-1	Vero[f]	20	C-C^3Ado[i]	2
Sindbis	Vero[f]	20	C-C^3Ado[i]	40
Cytotoxicity	MCC[j] (5-Aza-CPE-C) > 400 mg/mL for all host cells.			

[a]HSV-1 = herpes simplex type 1; CMV = cytomegalovirus; VSV = vescicular stomatitis virus. [b]Inhibitory concentration in μg/mL required to reduced virus-induced cytopathogenicity (or plaque formation for CMV) by 50%. [c]Human embryonic skin-muscle fibroblast. [d]Human embryonic lung fibroblast. [e]Human epithelial cell derived from a cervix carcinoma. [f]Simian fibroblast cell derived from African Green Monkey kidney. [g](E)-5-(2-bromovinyl)-2′-deoxyuridine. [h]g-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir). [i]Carbocyclic 3-deazaadenosine. [j]Minimum cytotoxic concentration to cause a microscopically detectable alteration of the normal cell morphology.

activity. Neither this compound nor 5-aza-CPE-C showed any activity against HIV-1 or HIV-2 replication in MT-4 cells (data not shown).

An important conclusion from this work, in conjunction with other recent findings from our laboratory, is the reduced tolerance that exists for the class of pyrimidine cyclopentenyl nucleosides to structural variations.[25,26] Modifications of cytidine that would normally lead to effective antitumor agents, such as 5-aza-Cyd and ara-C, do not appear to extrapolate well to the cyclopentenyl carbocyclic series.

EXPERIMENTAL SECTION

General Procedures. All chemical reagents were commercially available. Melting points were determined on a Mel-Temp II apparatus, Laboratory

Devices USA, and are uncorrected. Normal-phase column chromatography was performed on silica gel (silica gel 60, 230-400 mesh, E. Merck) and analytical TLC was performed on Analtech Uniplates silica gel GF with the solvents indicated for the individual experiments. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR instrument. Proton and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, in a Varian XL-200 spectrometer. Proton chemical shifts are expressed as δ values and referenced to the Me_4Si scale. Specific rotations were measured in a Perkin-Elmer Model 241 polarimeter. Positive-ion fast atom bombardment (FAB) mass spectra were obtained by using samples dissolved in a glycerol matrix, and ionization was effected by a beam of xenon atoms derived by neutralizing xenon ions accelerated through 8.6 kV. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta GA, or by Galbraith Laboratories, Inc., Knoxville, TN.

2-Methyl-1-(1-imidazoylcarbonyl)-isourea (6). 1,1′-Carbonyldiimidazole (1.7 g, 10.1 mmol) was added to a solution of 2-methylisourea (0.7g, 9.4 mmol) in THF (20 mL) and the resulting solution was stirred for 3 h at room temperature. The reaction mixture was concentrated to a solid which was collected by filtration with the aid of a small amount of THF. Recrystallization from acetonitrile afforded 6 (1.3 g, 82%) as a crystalline solid, mp 144.3-145.3° C; IR (KBr) 3341 (amide NH), 3125 (imide NH), 1684 (C=O), 1654 (C=NH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.95 (s, 3H, OCH_3), 6.02 (br s, 1H, NH), 7.02 (s, 1H, imidazole), 7.56 (s, 1H, imidazole), 8.33 (s, 1H, imidazole), 8.59 (br s, 1H, NH). Anal. Calcd for $C_6H_8N_4O_2$: C, 42.85; H, 4.80; N, 33.32. Found C, 42.94; H, 4.79; N, 33.40.

(+/-)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopenten-1-yl]-4-methoxy-s-triazin-2(1H)-one (8a). A solution of racemic cyclopentenyl amine 5a[27] (1.5 g, 5.5 mmol) and 6 (1.1 g, 6.5 mmol) in acetonitrile (60 mL) was heated at reflux under nitrogen for 2 h. After the reaction mixture was evaporated to a thick oil, trimethyl orthoformate (35 mL) was added and the resulting solution was heated at reflux under nitrogen for 2 h in the presence of a catalytic amount of trifluoroacetic acid (0.1 mL, 1.3 mmol). The solvent was removed under reduced pressure and the residual oil was purified by silica gel column

chromatography with a mixture of EtOAc:hexanes (1:1) as eluant. The fractions containing product [TLC, R_f 0.19, toluene:EtOAc (3:1)] were evaporated to a thick oil which solidified on standing. This solid was collected by filtration with the aid of a small amount of ether and was recrystallized from 5% benzene in ether to give 8a (0.8 g, 38%) as a white solid, mp 129-130° C; IR (KBr) 1705 cm^{-1} (C=O); ^1H NMR ($CDCl_3$) δ 1.34 and 1.42 (2s, 6H, $2CH_3$), 3.99 (s, 3H, OCH_3), 4.23 (s, 2H, H-6′$_{a,b}$), 4.59 (s, 2H, OCH_2Ph), 4.65 (d, J = 5.4 Hz, 1H, H-5′), 5.23 (d, 1H, partially hidden H-4′), 5.26 (s, 1H, H-1′), 5.62 (s, 1H, H-2′), 7.34 (m, 5H, Ph), 8.00 (s, 1H, H-6). Anal. Calcd for $C_{20}H_{23}N_3O_5$: C, 62.32; H, 6.01; N, 10.90. Found: C, 62.24; H, 6.03; N, 10.88.

(-)-1-[(1′R,4′R,5′S)-3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cylopenten-1-yl]-4-methoxy-s-triazin-2(1H)-one (8b). This compound was prepared from enantiomerically pure cyclopentenyl amine 5b[27] as described above to give 8b as a white crystalline solid, mp 135-136° C;$[\alpha]^{23}_D$ -16.5° (c 1.67, $CHCl_3$).

(+/-)-1-[3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopenten-1-yl]-4-amino-s-triazin-2(1H)-one (9a). A solution of 8a (0.15 g, 0.40 mmol) in saturated methanolic ammonia (25 mL) was stirred at room temperature in a closed container for 3 h. The reaction mixture was evaporated to a solid which was collected by filtration with the aid of some ether. This solid was recrystallized from EtOAc to give 9a (0.14 g, 94%) as white crystals, mp 172-173° C; IR (KBr) 1684 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.34 and 1.42 (2s, 6H, $2CH_3$), 4.22 (s, 2H, H-6′$_{a,b}$), 4.59 (s, 2H, CH_2Ph), 4.67 (d, J = 5.8 Hz, 1H, H-5′), 5.22 (s, 1H, H-1′), 5.23 (d, J = 5.7 Hz, 1H, H-4′), 5.52 (br s, 1H, NH), 5.61 (s, 1H, H-2′), 6.55 (br s, 1H, NH), 7.34 (m, 5H, Ph), 7.83 (s, 1H, H-6). Anal. Calcd for $C_{19}H_{22}N_4O_4$: C, 61.61; H, 5.99; N, 15.13. Found: C, 61.52; H, 6.00; N, 15.13.

(-)-1-[(1′R,4′R,5′S)-3-[(Benzyloxy)methyl]-4,5-(isopropylidenedioxy)-2-cyclopenten-1-yl]-4-amino-s-triazin-2(1H)-one (9b). This compound was prepared from 8b as described above to give 9b as an amorphous foam. This material was used for the preparation of 4b after several futile attempts to crystallize it.

(+/-)-1-[3-(Hydroxymethyl)-4,5-dihydroxy-2-cyclopenten-1-yl]-4-amino-s-triazin-2(1H)-one (4a). Under a nitrogen atmosphere, a 1 M BCl_3 solution in methylene chloride (7 mL) was added slowly to a solution of 9a (0.38 g, 1.0 mmol) in 70 mL of methylene chloride previously cooled to -78° C. After stirring at that temperature for 3.5 h, the reaction mixture was warmed to 0° C, treated with methanol (30 mL), and evaporated to dryness. The resultant glassy material was dissolved in 10 mL of ethanol from which a precipitate formed on standing. This solid was recrystallized from 30% aqueous ethanol to give 4a (0.12 g, 49%) as a white solid, mp 228° C (dec.); IR (KBr) 1625 (C=O) cm^{-1}; ^1H NMR (Me_2SO-d_6/D_2O) δ 3.97 (t, J = 5.4 Hz, 1H, H-5′), 4.02 (s, 2H, H-$6'_{a,b}$), 4.32 (d, J = 5.4 Hz, 1H, H-4′), 5.10 (br s, 1H, H-1′), 5.53 (d, J = 1.8 Hz, 1H, H-2′), 8.00 (s, 1H, H-6); ^{13}C NMR (D_2O) δ 61.16 (C-6′), 69.55 (C-1′), 75.16 (C-5′), 78.70 (C-4′), 126.92 (C-2′), 141.19 (C-4), 147.45 (C-3′), 151.42 (C-2), 160.53 (C-6); FAB mass spectrum, m/z (relative intensity) 241 (MH^+, 64), 113 (b + 2 H, 100); Anal. Calcd for $C_9H_{12}N_4O_4$: C, 45.00; H, 5.05; N, 23.32. Found C, 45.27; H, 5.05; N, 23.41.

(-)-1-[(1′R,4′R,5′S)-3-(Hydroxymethyl)-4,5-dihydroxy-2-cyclopenten-1-yl]-4-amino-s-triazin-2(1H)-one (4b). This compound was prepared from 9b as described above. Recrystallization from 5% aqueous ethanol afforded 4b as a white crystalline solid, mp 205.5-206.5° (dec.); $[\alpha]^{23}_D$ -105.3° (c 1.02, H_2O).

(-)-1-[(1′R,4′R,5′S)-3-(Hydroxymethyl)-4,5-dihydroxy-2-cyclopenten-1-yl]-4-methoxy-s-triazin-2(1H)-one (10b). Under a nitrogen atmosphere, a 1 M solution of BCl_3 in methylene chloride (6.3 mL) was added slowly to a solution of 8b (0.36 g, 0.97 mmol) in 30 mL of methylene chloride previously cooled to -78° C. After stirring at that temperature for 3.5 h, the cooling bath was removed and the solution was treated with concentrated methanolic ammonia to pH 9.[28] Additional methanol (20 mL) was added and the solution was filtered. The filtrate was evaporated to dryness in the presence of ca. 2 g of silica gel. The collected solid material was then placed atop a silica gel column and eluted with a mixture of $CHCl_3$:MeOH (4:1). The fractions containing the desired product [TLC, R_f 0.39, $CHCl_3$:MeOH (3:1)] were combined and evaporated to

give a solid residue. This solid was recrystallized from 5% aqueous EtOH to give 10b (0.11 g, 44%) as white crystals, mp 164.5-165.2° C (dec); $[\alpha]^{23}_{D}$ -76.8° (c 1.03, H_2O); IR (KBr) 1697 (C=O) cm^{-1}; ^1H NMR (D_2O) δ 3.80 (s, 3H, OCH_3), 4.13 (m, 3H, H-6′$_{a,b}$, H-5′), 4.48 (d, J = 5.7 Hz, 1H, H-4′), 5.17 (br s, 1H, H-1′), 5.68 (s, 1H, H-2′), 8.23 (s, 1H, H-6); ^{13}C NMR (D_2O) δ 56.13 (OCH_3), 58.64 (C-6′), 67.72 (C-1′), 72.74 (C-5′), 76.10 (C-4′), 123.61 (C-2′), 149.85 (C-3′), 156.87 (C-2), 159.82 (C-6), 170.07 (C-4). Anal. Calcd for $C_{10}H_{13}N_3O_5$: C, 47.06; H, 5.13; N, 16.46. Found: C, 47.12; H, 5.18; N, 16.44.

Antiviral Activity. Inhibition of virus-induced cytopathogenicity was measured following well-established procedures.[29,30] In all viral cytopathogenicity assays the virus inoculum was 100 $CCID_{50}$ per microtiter well (1 $CCID_{50}$ corresponding to the virus stock dilution that proved infective for 50% of the cell cultures). In the cytomegalovirus assay, plaque formation was measured instead of viral cytopathogenicity. In this assay the virus inoculum was 20 PFU (plaque forming units).

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