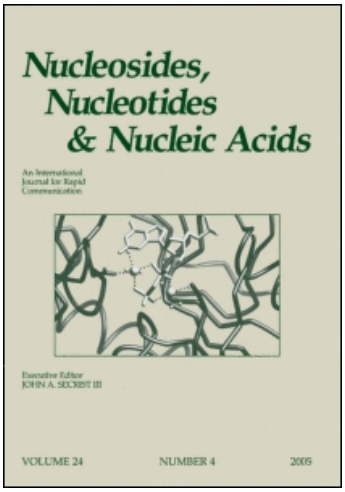

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The Controlled Stereospecific Reduction of Cyclopentenyl Cytosine (CPE-C) to Carbodine and Isocarbodine

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THE CONTROLLED STEREOSPECIFIC REDUCTION OF CYCLOPENTENYL CYTOSINE (CPE-C) TO CARBODINE AND ISOCARBODINE

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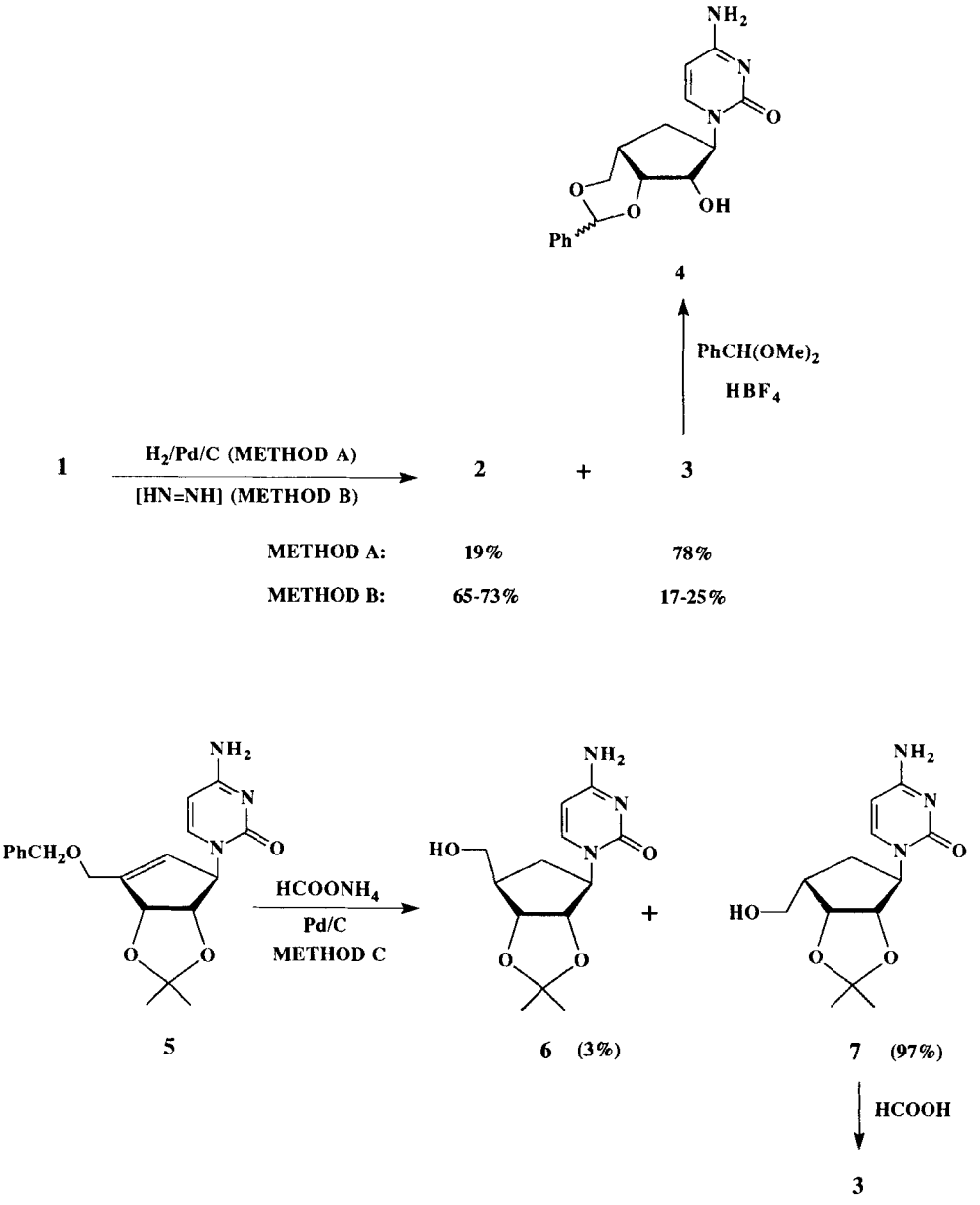
Abstract. The preferential *cis*-addition of hydrogen to either face of the carbocyclic double bond of enantiomerically pure cyclopentenyl cytosine (1) was achieved. The resulting saturated carbocyclic nucleosides carbodine (2) and isocarbodine (3) were evaluated against human influenza virus. Carbodine showed the greater potency against this virus but the activity of isocarbodine was still substantial.

The selective *cis*-addition of hydrogen to opposite sides (α or β) of the double bond of cyclopentenyl cytosine (1, CPE-C) was considered of interest as a way of preparing carbodine (2) and its corresponding 4′-epimer (sugar numbering) isocarbodine (3). Since CPE-C has been synthesized in optically pure form,[1] regiochemical control of this reduction was anticipated to generate, for the first time, enantiomerically pure carbodine and isocarbodine.

Racemic carbodine was prepared initially by Shealy and coworkers and has been shown to possess significant antitumor and antiviral activity.[2,3] It was therefore of interest to compare the biological differences between enantiomerically pure carbodine and its racemate, as well as to investigate the biological properties of the new carbocyclic nucleoside isocarbodine.

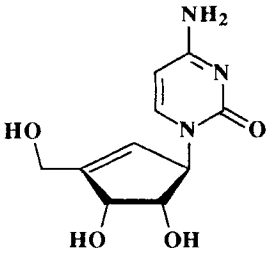
Results and Discussion. The results from the attempted catalytic reduction (Scheme 1, Method A) of CPE-C demonstrated the existence of a

This work is dedicated to the memory of Professor Tohru Ueda.

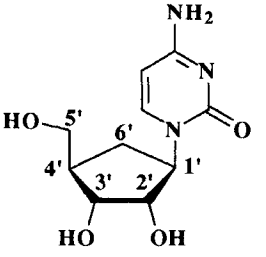


SCHEME 1

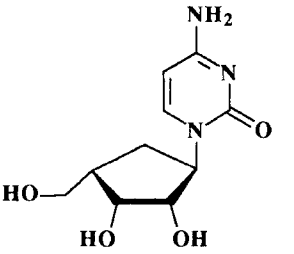
significant bias of the molecule to accept hydrogen preferentially from the top, or β-side. This preference is illustrated by the ca. 4/1 ratio of isocarbodine (3) to carbodine (2) measured by HPLC analysis of the reaction mixture. The β-side selectivity was further enhanced by



1, CPE-C



2, Carbodine



3, Isocarbodine

protecting the *cis*-diol function of CPE-C with an isopropylidene moiety. Thus, when either catalytic hydrogenation or catalytic transfer hydrogenation (Scheme 1, Method C) were attempted on fully protected CPE-C (5), the reaction proceeded to give, almost exclusively, compound 7, the isopropylidene protected form of isocarbodine. Removal of the benzyl group occurred simultaneously with the reduction of the double bond. Direct HPLC analysis of this reaction mixture, or analysis of an aliquot after treatement with formic acid to remove the isopropylidene moiety, indicated a ratio of 97% isocarbodine (3) to 3% carbodine (2). Separation of these two components at the protected stage was accomplished by successive recrystallizations of 7 from the mixture. After four recrystallizations from acetone, an analytical sample of 7 (99.6% pure by HPLC) was obtained. Deblocking of 7 with formic acid afforded enantiomerically pure isocarbodine (3, Scheme 1).

In order to change the facial selectivity of the reduction, diimide was considered as a possible reagent. This reagent, generated from potassium azadicarboxylate (PADA), is an important reducing agent in synthetic chemistry[4-6] but has been sparsely utilized in nucleic acid chemistry.[7] Merely on the basis of its size, diimide was at least expected to deliver hydrogen with equal ease from both sides of the double bond. However, the results from the experiments showed that

diimide appeared to favor delivery of hydrogen from the α-side of CPE-C since the ratio of carbodine (2) to isocarbodine (3) was consistently between 3/1 and 4/1 (Scheme 1, Method B). Such preference of diimide for the α-side of CPE-C could result from transiently formed hydrogen bonds between the *cis* form of diimide (the actual reducing agent) and the *cis*-diol of the substrate. Despite the observed selectivity, the reaction was somewhat sluggish. This was possibly due to the relative hindered nature of the double bond of CPE-C which required several additions of PADA to drive the reaction to completion. After four additions, the yields fluctuated between 65% to 73% for carbodine (2), 17% to 25% for isocarbodine (3) and 1% to 17% of unreacted CPE-C (1), as determined by HPLC analysis. Treatment of the mixture with benzaldehyde dimethyl acetal in order to preferentially trap the *syn*-3′,5′-diol system of isocarbodine (compound 4, Scheme 1) afforded a new mixture in which unreacted 1 and 2 were separated from 4 by preparative TLC. Despite the fact that the new resulting mixture was enriched in carbodine (2), complete removal of isocarbodine was not achieved and the composition of the new reaction mixture improved to only 87% carbodine (2), 12% isocarbodine (3) and ca. 1% of unreacted CPE-C (Figure 1, panels a and b). Separation of this mixture by preparative HPLC was difficult due to the closeness of the k′ values of carbodine and isocarbodine. Panels c and d in Figure 1 illustrate the results of the semi-preparative HPLC purification of carbodine from this mixture to produce enantiomerically pure compound.

The ^{1}H and ^{13}C-NMR spectra of (-)carbodine (2) were superimposable to those obtained from a sample of authentic racemic carbodine kindly supplied to us by Dr. Shealy from Southern Research Institute, Alabama. All NMR assignments were made with the aid of ^{1}H-^{1}H-COSY, DEPT and published data for cytidine nucleosides.[8] In addition, one dimensional NOE difference spectroscopy revealed important differences between carbodine and its 4′-epimer, isocarbodine. In isocarbodine (3), a strong NOE effect observed at H-2′ and H-3′, when H-4′ was irradiated, established the all *cis* relationship of these three hydrogens. This, coupled to the absence of NOE between H-1′ and H-4′, corroborated the α-orientation of the hydroxymethyl side chain in isocarbodine. In

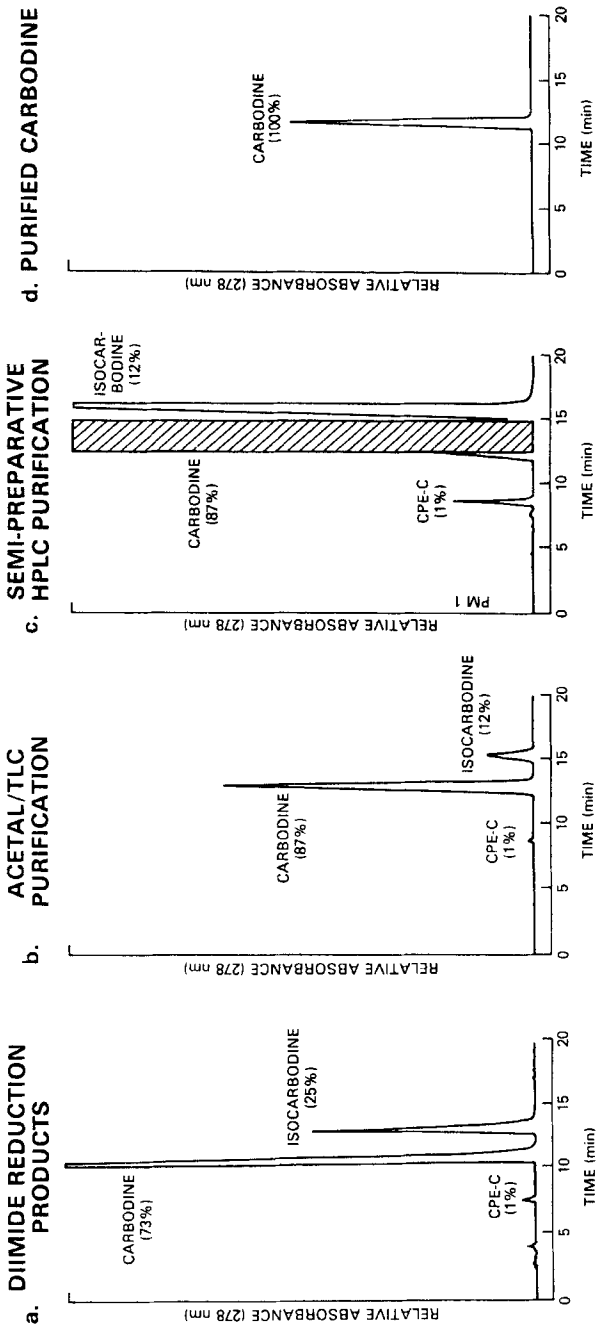


FIGURE 1. Analytical (a, b, and d) and preparative (c) HPLC profiles. The hatched rectangle represents the collected sample.

TABLE 1. Inhibition of L1210 Cell Growth[a] by (-)CPE-C, (-)Carbodine, and (-)Isocarbodine.

	% Inhibition		
Conc. (μM)	CPE-C	Carbodine	Isocarbodine
	IC_{50}=0.03 μM	IC_{50}=0.30 μM	IC_{50}=N.D.
0.1	>90	<10	<10
0.2	>90	<10	<10
0.25	-	<10	<10
0.3	-	85	<10
0.5	-	95	<10
1.0	-	-	<10

[a]L1210 cells were seeded at 1.5×10^4 cell/mL in Fisher's medium containing 10% heat-inactivated horse serum. Cells were exposed to the drugs continuously for 48 h, and the cell number was determined by the use of a Coulter counter. Percent inhibition was calculated relative to control cultures without drug.

carbodine, the NOE effect observed between H-1′ and H-4′ after irradiation of H-1′ was not very diagnostic due to the overlap that exists between the H-4′ signal and the signal of one of the H-6′ protons. Interestingly, in isocarbodine, a strong NOE interaction observed at H-6 when H-1′ is irradiated - and vice versa - is probably the result of a significant contribution from the *syn* conformer in solution.

Biological Activity. The in vitro cytotoxicities displayed by enantiomerically pure CPE-C (1), carbodine (2) and isocarbodine (3) are shown in Table 1. In these experiments L1210 cells were treated with several doses of the test drugs to compare their relative potencies. CPE-C exhibited the greatest cytotoxicity achieving greater than 90% inhibition of growth at 100 nM. Carbodine showed no cytotoxicity at concentrations less or equal to 250 nM, yet it achieved approximately 85% growth inhibition at 300 nM and 95% inhibition at 500 nM. Isocarbodine was essentially non-toxic at concentrations of 1 μM or less.

TABLE 2. Antiviral Activity of (-)Carbodine and (-)Isocarbodine Against Human Influenza Virus Type A_o/PR/8/34 in Cell Culture[a].

	VR[b]	ID_{50}[c]	MTC[d]	TI[e]
(-)Carbodine	5.1	0.4	24.1	60
rac-Carbodine	4.9	0.7	25.0	36
(-)Isocarbodine	2.6	21.2	241	11

[a]Madin-Darby Canine Kidney (MDCK) cells. [b]VR is a weighted measurement of antiviral activity that takes into account both the degree of inhibition of virus-induced cytopathogenic effects and the degree of cytotoxicity produced by the test compound. A VR of 1.0 or greater is indicative of statistically significant antiviral activity.[c]The minimum drug dose (μg/mL) that inhibited cytophatogenic effects by 50%.[d]The minimum drug concentration (μg/mL) causing any cytotoxicity.[e]Therapeutic index = MTC/ID_{50}.

The wide spectrum antiviral activity of CPE-C has been thoroughly documented.[1] Data on racemic carbodine, however, is more limited although it has been reported to be very effective against influenza viruses.[3] A comparative study of our enantiomerically pure compounds carbodine (2) and isocarbodine (3) versus *rac*-carbodine was performed against human influenza virus (type A_o/PR/8/34) in cell culture by the cytopathic effect inhibition assay. The results from this study are shown in Table 2. From these data, one can see that (-)carbodine is experimentally 1.75-fold more potent (theoretically 2-fold) than *rac*-carbodine. In addition, since their respective minimum toxic concentrations (MTC) were virtually identical, the pure enantiomer had a greater therapeutic index (TI). This was reflected in the better viral rating (VR) obtained for the enantiomeric form. Quite unexpectedly, isocarbodine showed significant activity against this virus with a VR of 2.6 and a TI of 11. Such an unexpected result suggests that if nucleotide formation from these analogues is a prerequisite for activity against this virus, the unusual conformation of this 4'-epimer of carbodine is nevertheless accepted by a kinase present in the assay mixture. The antiherpetic activity of isocarbodine, however, was negligible with a VR of 0.71 (data not shown).

This study demonstrates that the preferential reduction of CPE-C from either side of the double bond is feasible. The resulting enantiomerically pure isomers, carbodine and isocarbodine, are both endowed with interesting biological activities. The unexpected activity of isocarbodine against human influenza virus may provide a new lead for the development of future drugs in this area.

EXPERIMENTAL SECTION

General. Melting points were taken on a Mel-Temp II apparatus or Fisher-Johns melting point apparatus and are uncorrected. UV spectra were obtained on a Beckman DU-70 spectrophotometer. Optical rotations were recorded on a Perkin-Elmer 727B polarimeter at the sodium D line. Proton and carbon NMR spectra were run on a Varian XL-200 spectrometer at 200 Mz and 50 MHz, respectively. Chemical shifts are given in ppm (δ) relative to Me_4Si and are referenced to the solvents in which the samples were run. NOE experiments were run on a Bruker AC-250 at 250 MHz and carbon multiplicity was determined with the same instrument by a DEPT-45 sequence in the automated mode at 62.9 MHz. Analytical TLC was performed on Uniplate GHLF silica gel (Analtech, 250 μ). Purification by cation exchange chromatography was carried out using a 15 x 3 cm column (50 mL glass syringe body) packed with 15 mL Dowex 50W-X4 resin (Bio-Rad, 50-100 mesh, H^+ form). Azodicarbonamide (98%) was purchased from Fluka Chemical Corp. while other reagents were of the highest commercially available grade. Positive ion fast atom bombardment mass spectra were obtained on a VG 7070E mass spectrometer operated at an accelerating voltage of 6 kV and a resolution of 1000. Glycerol was used as the sample matrix and xenon fast atoms were produced by charge-exchange neutralization of a 1.2-1.3 mA beam of xenon ions accelerated through 8.4-9.2 kV. Spectra were acquired under the control of a VG 11/250 J^+ data system at a scan speed of 10 s/decade and the background due to the glycerol matrix was automatically subtracted. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

High-Performance Liquid Chromatography. HPLC analyses were carried out on a Beckman model 324 gradient liquid chromatograph consisting of a Model 210 sample injection valve with a 250 μL injection loop, two Model 100A pumps, a Model 421 pump controller, and a Waters

994 photodiode array detector. Carbocyclic cytosine nucleosides were detected by monitoring UV absorbance at 278 nm, and the resulting peaks were integrated and recorded on a Spectra-Physics 4400 computing integrator. For analytical separations, a 10-20 μL sample injection was made onto a Beckman 250 x 4.6 mm 5-μm ODS Ultrasphere column, which was protected by a 30 x 4.6 mm 5-μm Brownlee RP-18 reverse phase cartridge precolumn. This analytical column system was eluted at a flow rate of 1.0 mL/min with a mobile phase consisting of either 15% MeOH in pH 5, 0.1 M ammonium formate buffer (mobile phase A), pH 5, 0.1 M ammonium formate buffer (mobile phase B); or deionized distilled water (mobile phase C). All mobile phase components were degassed by vacuum filtration through 0.22 μm Durapore or Fluoropore filters before mixing and use. For semipreparative isolation of carbodine, 50-100 μL of concentrated reaction mixture was injected onto a column system consisting of a Waters guard column filled with 37-50 μm Vydac 201SC reverse phase packing and a Beckman 250 x 10 mm 5-μm ODS ultrasphere column. Mobile phase C was used at a flow rate of 4.0 mL/min.

(1R,2S,3R,4S)-1-[2,3-(isopropylidenedioxy)-4-(hydroxymethyl)-cyclopentyl]cytosine (7). Fully protected CPE-C[1] (5, 0.304 g, 0.822 mmol) was refluxed in methanol (45 mL) for 4 h in the presence of a mixture of 10% Pd/C (0.608 g) and ammonium formate (5 g). After such time, monitoring of the reaction by TLC (silica gel, 20% MeOH/CH_2Cl_2) indicated complete comsumption of starting material. The catalyst was removed by vacuum filtration and the filtrate was concentrated under vacuum. The residue was solidified by trituration with 15 mL of acetone to give 0.210 g (91%) of a white solid. HPLC analysis of this solid (mobile phase A), or of the resulting mixture after treatment with formic acid (*vide infra*) to remove the isopropylidene moieties (mobile phase B), indicated that the composition of the mixture was 97% 7 and 3% 6. Four successive recrystallizations from acetone were required to give an analytical pure sample of 7, mp 213-214° C (99.6% pure by HPLC, mobile phase A, $k' = 6.71$); ^1H NMR (Me_2SO-d_6) δ 1.20 (s, 3 H, CH_3), 1.35 (s, 3 H, CH_3), 1.62-1.93 (m, 2 H, H-6′$_{a,b}$), 2.20-2.45 (m, 1 H, H-4′), 3.32 (m, 1 H, H-5′$_a$), 3.53 (m, 1 H, H-5′$_b$), 4.40 (m, 2 H, H-2′, H-3′), 4.67 (m, 2 H, OH, H-1′), 5.60 (d, J = 7.5 Hz, 1 H, H-5), 7.00 (br s, 2

H, $N\underline{H}_2$), 7.40 (d, J = 7.5 Hz, 1 H, H-6) . Anal. Calcd for $C_{13}H_{19}N_3O_4$: C, 55.49; H, 6.81; N, 14.94. Found: C, 55.57; H, 6.85; N, 14.96.

(1R,2S,3R,4S)-1-[2,3-dihydroxy-4-(hydroxymethyl)cyclopentyl]cytosine (isocarbodine 3). A solution of 7 (0.105 g, 0.374 mmol) in 3 mL of distilled water was treated with formic acid (Sigma, 99%, 100 μL) and heated at 60° C in a closed vial. Comsumption of 7, as monitored by HPLC, (mobile phase A) indicated that a small amount of starting material was still present after 24 h. However, additional formic acid (30 μL) and continued heating for 24 h afforded complete deprotection. The reaction mixture was diluted with 20 mL of methanol and reduced to dryness to afford crude 3 (90 mg, 100%) which was 99.7% pure by HPLC (mobile phase B, k′ = 4.06). Recrystallization from absolute ethanol produced 0.043 g (48%) of 3 as a white crystalline solid (a second crop of 0.020 mg was also obtained), mp 236-238° C; $[\alpha]^{25}_D$ -53.6° (c 0.097, H_2O); UV (0.01 M phosphate buffer, pH 7) λ_{max} 275 nm (log ϵ 3.93); ^{1}H NMR (D_2O) δ 1.78 (m, 2 H, H-6′$_{a,b}$), 2.32 (m, 1 H, H-4′), 3.40 (dd, J = 12, 6 Hz, 1 H, H-5′$_a$), 3.57 (dd, J = 12, 8 Hz, 1 H, H-5′$_b$), 4.00 (t, J = 4 Hz, 1 H, H-3′), 4.30 (dd, J = 8, 4 Hz, 1 H, H-2′), 4.45 (distorted q, 1 H, H-1′), 5.82 (d, J = 7.5 Hz, 1 H, H-5), 7.45 (d, J = 7.5 Hz, 1 H, H-6); ^{13}C NMR (Me_2SO-d_6) δ 29.47 (t, C-6′), 41.33 (d, C-4′), 60.88 (t, C-5′), 63.05 (d, C-1′), 72.03 (d, C-2′), 75.82 (d, C-3′), 93.19 (d, C-5), 144.80 (d, C-6), 156.00 (s, C-2), 165.32 (s, C-4); FAB MS *m/z* (relative intensity) 242 (MH^+, 100), 112 (b + 2H, 42). Anal. Calcd for $C_{10}H_{15}N_3O_4$: C, 49.77; H, 6.27; N, 17.42. Found: C, 49.83; H, 6.28; N, 17.34.

Potassium Azodicarboxylic Acid (PADA). This reagent was prepared in 87% yield according to literature procedures by treating azodicarboxamide with 50% KOH at 0° C. This yellow solid was stored desiccated over anhydrous $CaSO_4$ (Drierite) prior to use.

(1R,2S,3R,4R)-1-[2,3-dihydroxy-4-(hydroxymethyl)cyclopentyl]cytosine (carbodine 2). CPE-C[1] (0.480 g, 2.0 mmol) was dissolved in 20 mL of DMF at room temperature. PADA (3.2 g) was added to the stirred solution followed by the dropwise addition of 2 mL of glacial acetic acid. The reaction mixture temperature was slowly increased to 55° C and stirred at this temperature for 12-16 h until all of the PADA was consumed as

evidenced by the disappearance of the yellow solid. The reaction mixture was cooled to room temperature and the extent of the reduction and formation of 2 was monitored by HPLC (mobile phase B, k′ = 7.27). The inorganic solids were removed by vacuum filtration and the filtrate was reduced to dryness under vacuum. The residue was redissolved in 20 mL of DMF and reacted with additional PADA (3.2 g) and 2 mL of acetic acid as above. This reaction cycle was repeated for a total of five additions of PADA after which time HPLC analysis revealed a mixture consisting of 65% 2 (carbodine), 17% 3 (isocarbodine) and 17% unreacted 1 (CPE-C). Residual inorganic salts were removed from this mixture by cation exchange chromatography (Bio-Rad, 50W-X4 Dowex resin, 50-100 mesh, H^+ form) monitored at 280 nm. An aqueous solution of the entire reaction mixture was loaded onto the column (50 mL glass syringe body) packed with 15 mL of resin and exhaustively washed with water. Carbocyclic nucleosides were then eluted with 2 N NH_4OH. Concentration of the eluant and reconcentration with MeOH under vaccum gave 0.440 g of solid. Semipreparative HPLC isolation of the reduction products using mobile phase C produced 0.190 g of 2 (carbodine) and 0.048 g of 3. Recrystallization of 90 mg of 2 from absolute ethanol gave 59 mg of 2 as white needles, mp 218-219° C (99.1% pure by HPLC, mobile phase B); $[\alpha]^{20}_D$ -76.0° (c 0.107, H_2O); UV (0.01 M phosphate buffer, pH 7) λ_{max} 275 (log ϵ 3.99); ^1H NMR (D_2O) δ 1.35 (m, 1 H, H-6′$_a$), 2.05 (m, 2 H, H-6′$_b$, H-4′), 3.49 (m, 2 H, H-5′$_{a,b}$), 3.80 (dd, J = 6, 4 Hz, 1 H, H-3′), 4.08 (dd, J = 8, 6 Hz, H-2′), 4.53 (distorted q, 1 H, H-1′), 5.90 (d, J = 8 Hz, 1 H, H-5), 7.61 (d, J = 8 Hz, 1 H, H-6); ^{13}C NMR (Me_2SO-d_6) δ 28.52 (t, C-6′), 44.93 (d, C-4′), 61.33 (d, C-1′), 62.95 (t, C-5′), 71.72 (d, C-2′), 73.42 (d, C-3′), 93.38 (d, C-5), 143.34 (d, C-6), 156.14 (s, C-2), 165.19 (s, C-4); FAB MS *m/z* (relative intensity) 242 (MH^+, 100), 112 (b + 2H, 34). Anal. Calcd for $C_{10}H_{15}N_3O_4$: C, 49.77; H, 6.27; N, 17.42. Found: C, 49.67; H, 6.31; N, 17.43.

Catalytic Reduction of CPE-C. CPE-C (0.050 g, 0.21 mmol) was dissolved in 25 mL of distilled water and shaken in a Parr hydrogenator at 20 psi of hydrogen in the presence of 5% Pd/$BaSO_4$ (0.010 g) for 48 h. At that time HPLC analysis (mobile phase B) indicated that all starting material had been consumed. The ratio of isocarbodine (3) to carbodine (2) as determined by HPLC was 4:1.

Enrichment of the Mixture in Carbodine (2) by Reaction with Benzaldehyde Dimethyl Acetal. After performing a similar diimide reduction of CPE-C (1) as described for the preparation of carbodine (2), a mixture (0.057 g) consisting of 73% carbodine (2), 25% isocarbodine (3) and 1% unreacted CPE-C (HPLC analysis, mobile phase B, Figure 1a) was obtained. This mixture was dissolved in DMF (2 mL) and treated with benzaldehyde dimethyl acetal (40 μl, 0.26 mmol) and HBF_4 (5 drops) at room temperature overnight. After three successive additions of HBF_4 and continued stirring for 6 h, no further changes were noted by TLC analysis. The reaction mixture was then neutralized with Et_3N and reduced to dryness under vacuum. The material obtained was dissolved in a small amount of MeOH and applied to a preparative TLC tapered plate (Analtech, silica gel GF) which was developed with a mixture of $CHCl_3$:MeOH:NH_3 (300:100:10). The band that remained at the origin was extracted with MeOH (3 x 5 mL) and reduced to dryness to give a solid which by HPLC analysis (mobile phase B) was 87% carbodine, 12% isocarbodine and 1% CPE-C (Figure 1b). This mixture was separated by semiprerative HPLC chromatography (mobile phase C) as illustrated in Figure 1c.

Antiviral Evaluations. Antiviral evaluations were carried out under NIH purchase order 263-MD-610174 at Southern Research Institute, Birmingham, AL, under the direction of Dr. William Shannon and Ms. Gussie Arnett.

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