Synthesis of 8-(Hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine, a Potential Carcinogenic Glycidaldehyde Adduct, and Its Site-Specific Incorporation into DNA Oligonucleotides

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The previously unreported glycidaldehyde adduct, 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (8-HM-ϵdC), has been synthesized for the first time by reaction of 2′-deoxycytidine with bromoacetaldehyde at pH 4.5, followed by reduction with sodium borohydride. The adduct was characterized by UV, MS, and NMR. The compound was stable to neutral and acidic conditions but not in alkaline solution. The corresponding phosphoramidite was synthesized in good yield from the intermediate, 3,N^4-ethenocarbaldehyde-2′-deoxycytidine, using the standard methodology and site-specifically incorporated in both 15- and 25-mer oligonucleotides, for studies on biochemical and biophysical properties. The resulting oligonucleotides were purified using HPLC, and the base composition was verified by HPLC after enzymatic digestion.

Introduction

Glycidaldehyde is a highly reactive alkylating agent that has been shown to be mutagenic in a range of in vitro genotoxicity tests (*1*) and carcinogenic in rodent skin cancer studies (*2*, *3*). It is classified by the International Agency for Research on Cancer as being carcinogenic to experimental animals (*4*, *5*). Glycidaldehyde is a product of P450 monooxygenase action on glycidyl ethers (*6*), an important class of industrial materials. Several glycidyl ethers have also been shown to be carcinogenic in experimental animals (*7*, *8*).

Glycidaldehyde is a bifunctional agent by virtue of its reactive carbonyl and epoxy functionalities and is capable of forming cyclic adducts and cross-links with bases in DNA. Adducts with dA and dG have been synthesized (*9*−*14*), in which the exocyclic etheno ring has a CH_2OH group replacing a hydrogen. The structures of the dA and dG adducts found after glycidaldehyde reaction have been identified and well characterized (*9*−*16*). In vitro at pH 5, the dA adduct, which is fluorescent, is formed to a greater extent than the dG adduct (*9*). At pH 10, the dG adduct is formed to a greater extent than the dA adduct (*9*). However, at pH 7, the dA and dG adducts are formed in similar quantities. The modified dA nucleoside has been identified from mouse skin (*9*, *10*) but not dG or dC adducts which are also likely to be formed. The only report on the formation of dC−glycidaldehyde adducts is from Kohwi (*17*), who observed that glycidaldehyde is highly reactive with dC in non-B DNA. However, no structural information was given.

In this work, we report the large-scale synthesis of 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (8-HM-ϵdC)[1] (Figure 1) and its phosphoramidite for the first time and the site-specific incorporation of the phosphoramidite into DNA oligonucleotides. These oligonucleotides are now being studied for their physical properties and their effect on replication, mutation, and repair.

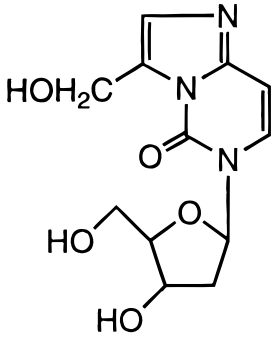

Figure 1. Chemical structure of 8-hydroxymethyl-3,N^4-etheno-2′-deoxycytidine.

Experimental Procedures

Materials and Methods. (1) Chemicals and Reagents. Caution: *Bromomalonaldehyde (BMA) was prepared using the procedure of Trofimenko (18). Bromomalonaldehyde is highly toxic and should be handled carefully. Organic chemicals and solvents used in this work should be stored and used only in a well-ventilated hood.* 2′-Deoxycytidine was purchased from Sigma Chemical Co. (St. Louis, MO). 4,4-Dimethoxytrityl chloride (DMTCl), pyridine, triethylamine, ethanol, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidochloridite, sodium borohydride, 1-acetylimidazole, DMSO, silica gel, sodium hydroxide, sodium chloride, sodium hydrogen carbonate, and toluene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Metha-

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[1] Abbreviations: 8-HM-ϵ-dC, 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine; DMTCl, 4,4′-dimethoxytrityl chloride; BAP, bacterial alkaline phosphatase; SVP, snake venom phosphodiesterase; CPG, controlled-pore glass.

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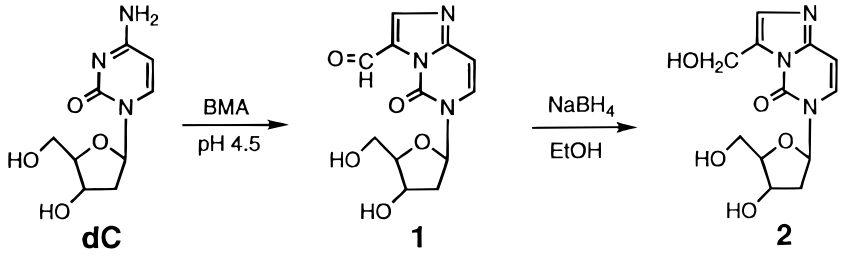
Scheme 1. Synthesis of 8-(Hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine



nol, dichloromethane, acetone, benzene, and acetonitrile were from J. T. Baker (Philipsburg, NJ). Potassium carbonate, phosphorus pentoxide, and thin-layer chromatography silica plates were purchased from EM Science (Gibbston, NJ), and calcium hydride was from Fluka Chemika (Ronkonkoma, NY). Bacterial alkaline phosphatase (BAP) and snake venom phosphodiesterase (SVP) were from Pharmacia Biotech Inc. (Alameda, CA). The PAC phosphoramidites were purchased from Pharmacia (Piscataway, NJ). All solvents were dried by distillation over calcium hydride.

(2) Analysis and Purification. Proton (400 MHz) NMR spectra were recorded with DMSO-d_6 as the solvent, and D_2O exchanges were carried out to assign exchangeable protons, unless otherwise indicated. NMR spectra were recorded using a Bruker AM400 spectrometer and are reported in parts per million (ppm) relative to an internal standard of tetramethylsilane. ^{31}P NMR spectra were recorded on a Bruker AM400 spectrometer, and the chemical shifts are reported relative to an external standard of phosphoric acid. Fast atom bombardment (FAB) spectra were obtained on a VG70 SE Instruments mass spectrometer, and glycerin or thioglycerin was used as a matrix. Electrospray mass spectra were obtained on a VG Bio-Q Instruments mass spectrometer. Ultraviolet spectra were recorded on a Hitachi U-2000 spectrophotometer using 0.5 cm cuvettes. TLC was performed on EM 5735/7 silica gel 60 F_{254} plates. Column chromatography was performed using silica gel 60 with elution under gravity.

(3) HPLC. Analytical and semipreparative HPLC were conducted using a Hewlett-Packard 1050 photodiode array detector and quarternary gradient pump (solvent delivery system). Solvent systems included solvent A (acetonitrile), solvent B [0.1 M triethylammonium acetate (pH 7)], and solvent C [0.01 M potassium phosphate (pH 4.5)]. In system 1, oligonucleotides were purified using a C18 column (300 mm × 3.9 mm, 5 μm, Hamilton PRP-1). The initial concentrations of 15% solvent A and 85% solvent B were maintained for 5 min, and then the concentration of solvent A was increased linearly to 35% over the course of 20 min at a flow rate of 1 mL/min. In system 2, oligonucleotides were analyzed using a C18 column (300 mm × 3.9 mm, 5 μm, Hamilton PRP-1). The initial concentrations of 5% solvent A and 95% solvent B were maintained for 5 min, and then the concentration of solvent A was increased linearly to 35% over the course of 40 min at a flow rate of 1 mL/min. In system 3, analysis of the enzyme digest of the oligonucleotides was performed with a Supelcosil LC-18-DB column (250 mm × 4.6 mm, 5 μm, Supelco Inc.) and 0% solvent A and 100% solvent C. The concentration of solvent A was linearly increased from 0 to 10% over the course of 25 min at a flow rate of 1 mL/min.

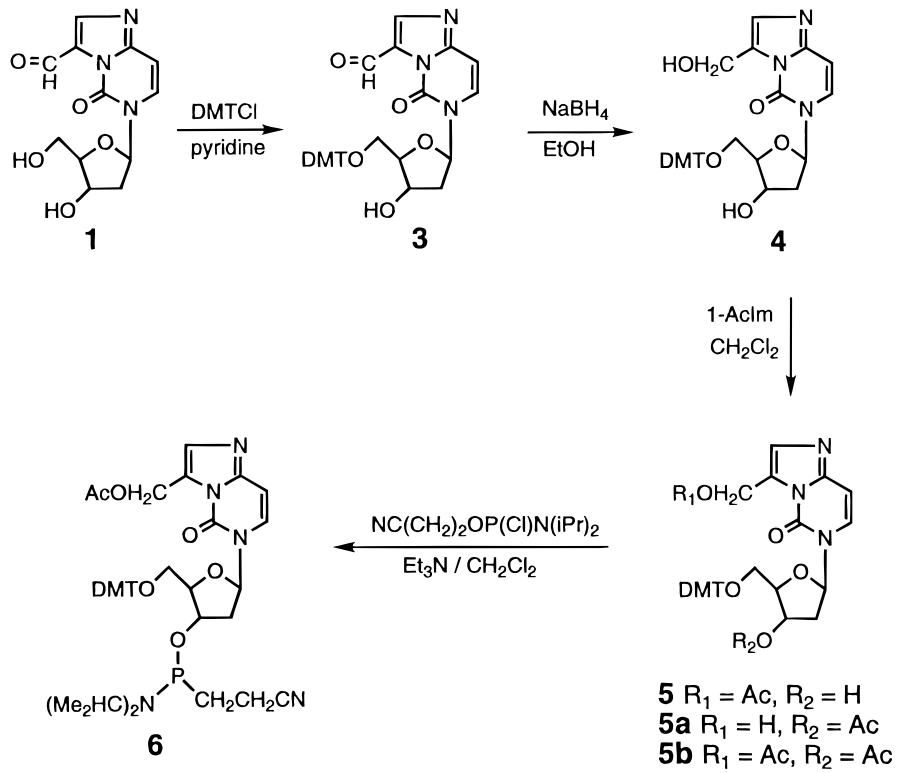
Chemical Syntheses. (1) Synthesis of the dC−Glycidaldehyde Adduct (Scheme 1). (A) Preparation of 3,N^4-Ethenocarbaldehyde-2′-deoxycytidine (1). Bromomalonaldehyde (0.622 g, 4.07 mmol, 1.0 equiv) was added to a solution of 2′-deoxycytidine (1.00 g, 4.07 mmol) in water (100 mL). The pH of the reaction mixture was then adjusted to 4.5 with a 2 M sodium hydroxide solution. The reaction mixture was stirred at 60 °C for 30 h. Further bromomalonaldehyde (0.200 g, 1.30 mmol, 0.3 equiv) was added after this time. The reaction mixture was stirred at 60 °C for an additional 18 h (TLC analysis indicated that the dC had been completely consumed and that other products were present, including 3,N^4-etheno-dC). The solvent was removed in vacuo to afford a yellow gum. The desired product (**1**) was isolated using silica gel column chromatography eluting with 40% acetone and 60% dichloromethane. A mixture of more polar byproducts which included 3,N^4-etheno-dC was also eluted from the column. The product was dried to afford a yellow gum with a 28% yield (0.321 g). UV (water): λ_{max} 272, 283, 326 nm. ^1H NMR (DMSO-d_6): δ 10.53 [1H, s, C(O)H], 8.14 (1H, s, H-7), 8.02 (1H, d, J = 8 Hz, H-6), 6.89 (1H, d, J = 8 Hz, H-5), 6.39 (1H, t, J = 6 Hz, H-1′), 5.33 (1H, d, J = 4 Hz, 3′-OH), 5.15 (1H, t, J = 6 Hz, 5′-OH), 4.29−4.27 (1H, m, H-4′), 3.89−3.86 (1H, m, H-3′), 3.70−3.60 (2H, m, 2 × H-5′), 2.32−2.18 (2H, m, 2 × H-2′). FAB-MS (rel intensity): m/z 280 (41%, MH^+), 164 (100%, base H^+)

(B) Preparation of 8-(Hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (2). Sodium borohydride (0.004 g, 0.10 mmol, 2.0 equiv) was added to a solution of 3,N^4-ethenocarbaldehyde-2′-deoxycytidine (0.014 g, 0.05 mmol) in ethanol (4 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature, then stirred for 2 h, and then diluted with 10 volumes of dichloromethane. The solution was washed with saturated sodium chloride and water, dried (Na_2SO_4), filtered, and concentrated in vacuo to afford a white foam. The desired product (**2**) was isolated using silica gel column chromatography eluting with 4% methanol and 96% dichloromethane. The resulting yellow solid was obtained in 84% yield (0.012 g). UV (water): λ_{max} 278 (pH 7, ϵ_{max} = 7700 mol^{-1} cm^2, ϵ_{260} = 4900 mol^{-1} cm^2), 278 (pH 14), 296 nm (pH 1). ^1H NMR (DMSO-d_6): δ 7.75 (1H, d, J = 8 Hz, H-6), 6.82 (1H, s, H-7), 6.53 (1H, t, J = 6 Hz, H-1′), 6.28 (1H, d, J = 8 Hz, H-5), 5.53−5.51 (2H, m, CH_2), 4.23−4.09 (2H, m, H-4′, H-3′), 3.55−3.42 (2H, m, 2 × H-5′), 2.19−2.08 (2H, m, 2 × H-2′). FAB-MS (rel intensity): m/z 282 (50%, MH^+), 166 (23%, base H^+), 135 (100%, base H^+ − Ome).

(2) Synthesis of the Protected Phosphoramidite Derivative of the dC−Glycidaldehyde Adduct (Scheme 2). (A) Preparation of 5′-*O*-(4,4′-Dimethoxytrityl)-3,N^4-ethenocarbaldehyde-2′-deoxycytidine (3). 4,4′-Dimethoxytrityl chloride (0.530 g, 1.56 mmol, 1.5 equiv) was added to a solution of 3,N^4-ethenocarbaldehyde-2′-deoxycytidine (0.291 g, 1.04 mmol) in dry pyridine (20 mL). The reaction mixture was stirred at room temperature for 16 h. Further 4,4-dimethoxytrityl chloride (0.352 g, 1.04 mmol, 1.0 equiv) was added, and then the reaction mixture was stirred for an additional 4 h. The solvent was removed in vacuo, and the residue was dissolved in dichloromethane. The solution was washed with 5% sodium hydrogen carbonate solution and with saturated sodium chloride solution, dried (Na_2SO_4), and filtered. The solvent was removed by forming the azeotrope with toluene in vacuo to afford a yellow gum. The desired product (**3**) was isolated using silica gel column chromatography eluting with 2% methanol and 98% dichloromethane. The resulting yellow foam was obtained in 69% yield (0.418 g). ^1H NMR ($CDCl_3$): δ 10.67 [1H, s, C(O)H], 8.17 (1H, s, H-7), 7.91 (1H, d, J = 8 Hz, H-6), 7.39−6.76 (13H, m, DMTr), 6.55 (1H, t, J = 6 Hz, H-1′), 6.38 (1H, d, J = 8 Hz, H-5), 4.64−4.62 (1H, m, H-4′), 4.10−4.07 (1H, m, H-3′), 3.56−3.49 (2H, m, 2 × H-5′), 2.58−2.55 (1H, m, H-2′), 2.39−2.36 (1H, m, H-2′). FAB-MS (rel intensity): m/z 582 (68%, MH^+), 303 (100%, $DMTH^+$), 164 (8%, base H^+).

(B) Preparation of 5′-*O*-(4,4′-Dimethoxytrityl)-8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (4). Sodium boro-

Scheme 2. Synthesis of the Protected Phosphoramidite Derivative of 8-(Hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine



hydride (0.036 g, 0.94 mmol, 2.0 equiv) was added to a solution of 5′-*O*-(4,4′-dimethoxytrityl)-3,N^4-ethenocarbaldehyde-2′-deoxycytidine (0.275 g, 0.47 mmol) in 10 mL of ethanol at 0 °C. The reaction mixture was allowed to warm to room temperature and was then stirred for 1 h and diluted with dichloromethane. The organic solution was washed with saturated sodium chloride solution and water, dried (Na_2SO_4), filtered, and concentrated in vacuo to afford a white foam. The desired product (**4**) was isolated using silica gel column chromatography eluting with 4% methanol and 96% dichloromethane. The resulting white foam was obtained in 89% yield (0.245 g). ^{1}H NMR ($CDCl_3$): δ 7.62 (1H, d, J = 8 Hz, H-6), 7.41−6.82 (14H, m, DMTr, H-7), 6.52 (1H, t, J = 6 Hz, H-1′), 6.29 (1H, d, J = 8 Hz, H-5), 4.82−4.80 (2H, m, CH_2), 4.61−4.59 (1H, m, H-4′), 4.07−4.06 (1H, m, H-3′), 3.59−3.44 (2H, m, 2 × H-5′), 2.53−2.47 (1H, m, H-2′), 2.37−2.32 (1H, m, H-2′). FAB-MS (rel intensity): m/z 584 (50%, MH^+), 303 (100%, $DMTH^+$), 166 (23%, base H^+).

(C) Preparation of 5′-*O*-(4,4′-Dimethoxytrityl)-8-(acetoxymethyl)-3,N^4-etheno-2′-deoxycytidine (5). 1-Acetylimidazole (0.037 g, 0.34 mmol, 1.0 equiv) was added to 5′-*O*-(4,4′-dimethoxytrityl)-8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (0.195 g, 0.33 mmol) in dichloromethane. The reaction mixture was dried by evaporation (vacuo) and allowed to stand for 72 h at 4 °C. The reaction produced three products (**5**, **5a**, and **5b**). The desired product (**5**) was isolated using silica gel column chromatography eluting with 15% acetone and 85% dichloromethane. The resulting white foam was obtained in 35% yield (0.076 g). ^{1}H NMR ($CDCl_3$): δ 7.62 (1H, d, J = 8 Hz, H-6), 7.41−6.82 (14H, m, DMTr, H-7), 6.53 (1H, t, J = 6 Hz, H-1′), 6.28 (1H, d, J = 8 Hz, H-5), 5.53−5.51 (2H, m, CH_2), 4.59−4.57 (1H, m, H-4′), 4.05−4.04 (1H, m, H-3′), 3.53−3.47 (2H, m, 2 × H-5′), 2.53−2.47 (1H, m, H-2′), 2.36−2.30 (1H, m, H-2′), 2.06 (3H, s, OAc). FAB-MS (rel intensity): m/z 626 (60%, MH^+), 303 (100%, $DMTH^+$), 208 (23%, base H^+).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-acetyl-8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (**5a**) was obtained as a byproduct in 38% yield (0.082 g). ^{1}H NMR ($CDCl_3$): δ 7.58 (1H, d, J = 8 Hz, H-6), 7.39−6.82 (14H, m, DMTr, H-7), 6.58 (1H, t, J = 6 Hz, H-1′), 6.27 (1H, d, J = 8 Hz, H-5), 5.46−5.45 (1H, m, H-3′), 4.83−4.81 (2H, m, CH_2), 4.20−4.19 (1H, m, H-4′), 3.53−3.47 (2H, m, 2 × H-5′), 2.56−2.47 (2H, m, 2 × H-2′), 2.10 (3H, s, OAc). FAB-MS (rel intensity): m/z 626 (71%, MH^+), 303 (100%, $DMTH^+$), 166 (29%, base H^+).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-acetyl-8-(acetoxymethyl)-3,N^4-etheno-2′-deoxycytidine (**5b**) was obtained as a byproduct in 9% yield (0.023 g). ^{1}H NMR ($CDCl_3$): δ 7.61 (1H, d, J = 8 Hz, H-6), 7.45−6.84 (14H, m, DMTr, H-7), 6.62 (1H, t, J = 6 Hz, H-1′), 6.26 (1H, d, J = 8 Hz, H-5), 5.57−5.55 (2H, m, CH_2), 5.50−5.48 (1H, m, H-3′), 4.22−4.21 (1H, m, H-4′), 3.57−3.49 (2H, m, 2 × H-5′), 2.61−2.56 (1H, m, H-2′), 2.49−2.44 (1H, m, H-2′), 2.12 (3H, s, OAc), 2.10 (3H, s, OAc). FAB-MS (rel intensity): m/z 668 (71%, MH^+), 303 (100%, $DMTH^+$), 208 (37%, base H^+).

(D) Preparation of 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[2-cyanoethyl (*N*,*N*-diisopropyl)phosphinyl]-8-(acetoxymethyl)-3,N^4-etheno-2′-deoxycytidine (6). Triethylamine (7 mL, 0.121 g, 1.20 mmol, 10 equiv) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (29 μL, 0.042 g, 0.17 mmol, 1.4 equiv) were added to a solution of 5′-*O*-(4,4′-dimethoxytrityl)-8-(acetoxymethyl)-3,N^4-etheno-2′-deoxycytidine (0.075 g, 0.12 mmol) in dry dichloromethane (7 mL). The reaction mixture was stirred for 2 h and then was diluted with dry dichloromethane. The organic solution was washed with 5% sodium hydrogen carbonate solution and saturated sodium chloride solution, dried (Na_2SO_4), filtered, and evaporated in vacuo (azeotrope/benzene) to afford an off-white gum in 92% yield (0.097 g). This material (**6**) was dried (P_2O_5, NaOH, in vacuo, 60 h) and then used in oligonucleotide synthesis without further purification. ^{31}P NMR ($CDCl_3$): δ 148.0, 147.6 (1:1).

Solid-Phase Synthesis of the Oligonucleotides. Synthesis of the oligonucleotides was carried out on an Applied Biosystem 394 automated DNA synthesizer on a 1 μmol scale using phosphoramidite chemistry on a controlled-pore glass (CPG) support with an aminopropyl succinate linker. Phenoxyacetyl phosphoramidites (PAC) were used to synthesize the DNA oligomers. The synthesis followed the standard protocol of the DNA synthesizer for phosphoramidite chemistry, except for the step in which the modified deoxynucleoside was inserted. In this step, the time of coupling was increased to 900 s, to maximize

the coupling efficiency which was 89%. The 5′-dimethoxytritylated oligonucleotides were base deprotected and cleaved from the resin with 0.05 M potassium carbonate/anhydrous methanol (8 h, room temperature). The 5′-dimethoxytritylated oligonucleotides were then purified by HPLC (system 1), detritylated (80% acetic acid/water), purified by HPLC (system 2), and desalted (Sep-Pak Cartridges, Waters Associates).

The following DNA oligomers were prepared: **1**, 5′-CCG CTA GXG GGT ACC GAG CTC GAA T [HPLC, t_R, 25.69 min in system 1 and 24.23 min in system 2]; **2**, 5′-AGC GGT TXT TGA GCT [HPLC t_R, 29.13 min in system 1 and 24.66 min in system 2]; and **3**, 5′-AGC GGC CXC CGA GCT [HPLC t_R, 29.04 min in system 1 and 24.61 min in system 2] [X = 8-(hydroxymethyl)-3,N^4-etheno-dC].

Enzyme Hydrolysis of the Oligonucleotides. The defined oligonucleotides (0.5 A_{260} unit) were dissolved in 80 μL of freshly deionized water, 10 μL of 0.1 M $MgCl_2$, and 10 μL of 0.5 M Tris-HCl buffer (pH 7.5) and digested with snake venom phosphodiesterase (5 μL) and bacterial alkaline phosphatase (5 μL) at 37 °C for 1 h. The digested mixture was analyzed on a a Supelcosil LC-18-DB HPLC column (250 mm × 4.6 mm, 5 μm, Supelco Inc.) using system 3. Quantitation of the deoxynucleosides was on the basis of integration of the peak areas at 260 nm.

Results and Discussion

The glycidaldehyde adduct of dC, 8-HM-ϵdC, and its phosphoramidite were synthesized (Schemes 1 and 2). In 1984, Nair and co-workers (*19*) reported that the etheno carboxaldehyde derivatives of ribonucleosides can be obtained in high yield and purity using bromomalonaldehyde (BMA). This methodology was used to prepare 3,N^4-ethenocarbaldehyde-dC **1** (Scheme 1) in 28% yield, which could then be converted to the phosphoramidite for oligonucleotide synthesis.

3,N^4-Ethenocarbaldehyde-dC was found to have the same UV parameter (λ_{max} = 326 nm) as the analogous ribocytidine analogue reported by Nair and Kronberg (*19, 20*). The molecular ion in its mass spectrum at *m/z* 280 suggested the formation of a 1:1 adduct in which bromine is not present. The ^{1}H NMR spectrum showed the presence of an aldehydic proton at δ 10.53 (s). The desired product (Scheme 1) was separated from the biologically significant byproduct, 3,N^4-etheno-dC, using silica gel column chromatography twice eluted with acetone and dichloromethane (see Experimental Procedures). It was found that the rate of formation of 3,N^4-etheno-dC was lower in acidic buffer [potassium phosphate (pH 4.5)] than under neutral conditions. 3,N^4-Ethenocarbaldehyde-dC is not stable in methanol; i.e., it is slowly converted to the hemiacetal at room temperature with an estimated half-life of about 18 h. Therefore, methanol was not used in its purification.

A small portion of **1** (Scheme 1) was reduced using sodium borohydride to afford an analytical sample of 8-HM-ϵdC **2** as a marker for HPLC to verify the incorporation of the adduct in oligonucleotides.

The primary 5′-OH of **1** (Scheme 2) was protected with dimethoxytrityl chloride in 69% yield. The expected DMTr signals were found to be present in the ^{1}H NMR spectrum at δ 7.41−6.82 (m). The aldehyde moiety of the 5′-dimethoxytritylated nucleoside **3** (Scheme 2) was reduced using sodium borohydride to afford the 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine adduct **4** in 89% yield. The C$\underline{H}_2$OH protons were observed in the ^{1}H NMR spectrum at δ 4.82−4.80 (m).

The most difficult step in the synthesis was the selective acylation of the primary hydroxymethyl on the etheno ring which unexpectedly had a reactivity similar to that of the secondary 3′-OH. The hydroxymethyl on the etheno ring could not be acylated with acetic anhydride. Low-temperature acylation (4 °C) with 1-acetylimidazole (*21*) (Scheme 2) afforded a mixture of the intended base-acylated product **5** in 35% yield, a sugar-3′-*O*-acylated byproduct **5a** in 38% yield, and a diacylated byproduct **5b** in 9% yield. These products were separated using silica gel column chromatography. There are important differences between the spectra of **5** and **5a**. The mass spectrum of **5** has a peak (*m/z* 208) corresponding to an acylated base. This signal is not present in the mass spectrum of **5a**. The sugar protons have higher chemical shifts in the ^{1}H NMR spectrum of **5a** (δ 5.46−5.45, m, H-3′; δ 4.20−4.19, m, H-4′) than in the spectrum of **5** (δ 4.59−4.57, m, H-3′; δ 4.05−4.04, m, H-4′).

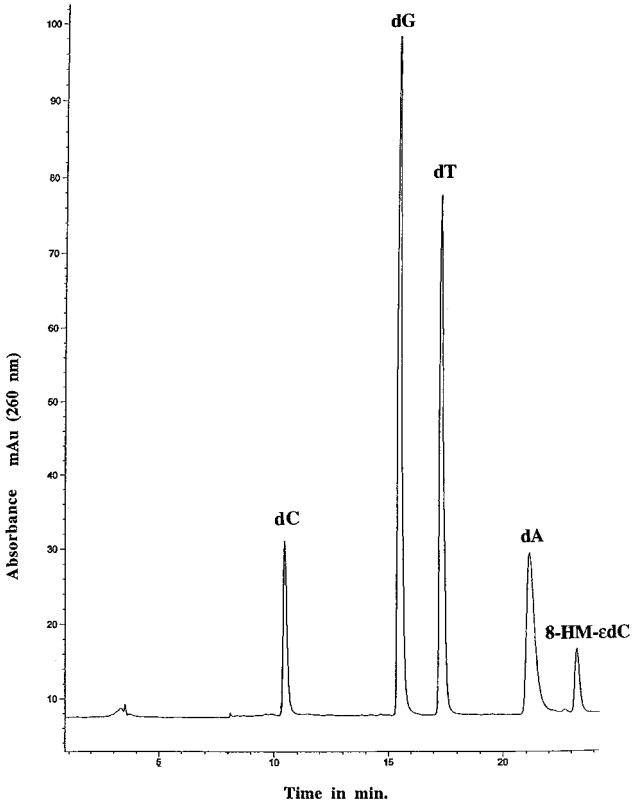


Figure 2. HPLC profile of 2′-deoxynucleosides obtained as a result of enzymatic digestion of oligonucleotide **2**, which contains a single 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine (see Materials and Methods). HPLC was performed using system 2. The retention times of the deoxynucleosides are as follows: dC, 10.5 min; dG, 15.5 min; dT, 17.4 min; dA, 21.2 min; and 8-(hydroxymethyl)-3,N^4-etheno-2′-dC, 23.7 min.

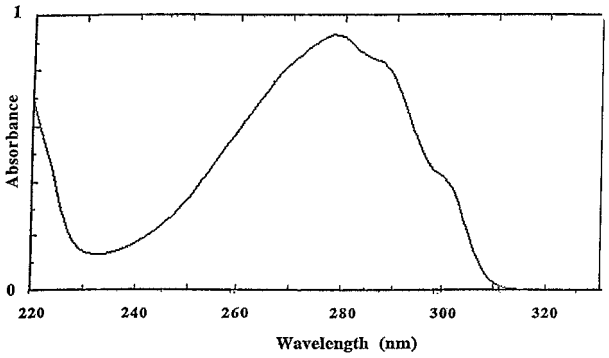


Figure 3. UV spectrum of 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine at pH 7.

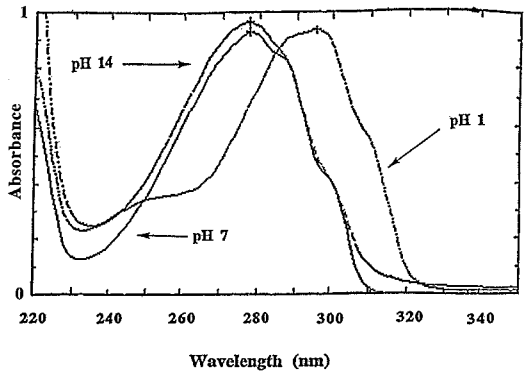


Figure 4. UV spectra of 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine at pH 1, 7, and 14.

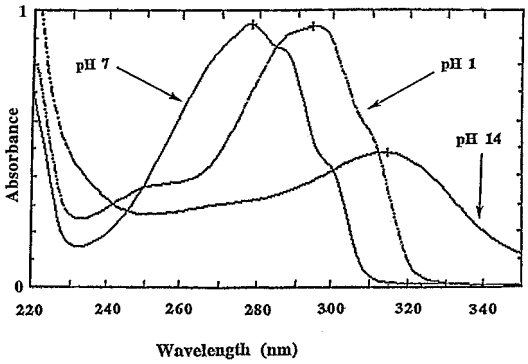


Figure 5. UV spectra of 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine at pH 1, 7, and 14 after 48 h at ambient temperature.

Compound **5** was phosphorylated (Scheme 2) using the standard methodology to afford the phosphoramidite **6** in 92% yield as a 1:1 mixture of diastereoisomers [^{31}P NMR δ 148.0, 147.6 (1:1)].

The phosphoramidite **6** was used to synthesize on a 1 μmol scale three different oligonucleotides: **1**, 5′-CCG CTA GXG GGT ACC GAG CTC GAA T; **2**, 5′-AGC GGT TXT TGA GCT; and **3**, 5′-AGC GGC CXC CGA GCT [X = 8-(hydroxymethyl)-3,N^4-etheno-dC].

The phosphoramidite was incorporated with a coupling efficiency of 89% using a coupling time of 900 s. The oligomers were deprotected using 0.05 M potassium carbonate/methanol. In addition, the use of the PAC protecting group allowed the deprotection of DNA oligomers under mild conditions.

The composition of the 25-mer and the presence of 8-HM-ϵdC were confirmed by enzymatic digestion (Figure 2). This adduct was unstable to the digestion conditions at pH 7.4 (37 °C, 18 h). This problem was prevented by reducing the digestion time to 1 h.

The UV absorption of 8-HM-ϵdC was determined at pH 7 (Figures 3 and 4), pH 1 (Figure 4), and pH 14 (Figure 4). The sample at pH 7 was adjusted to pH 1 with 0.1 N hydrochloric acid and to pH 14 with 0.1 N sodium hydroxide. The protonated base has a significantly different spectrum (λ_{max} = 296 nm) than under neutral (λ_{max} = 278 nm) and alkaline (λ_{max} = 278 nm) conditions. The 8-HM-ϵdC solutions at pH 1, 7, and 14 were allowed to stand at room temperature for 48 h, and then the UV spectra were again recorded (Figure 5). There was no significant change in absorption of the spectra of the samples held at pH 1 (λ_{max} = 294 nm) or at pH 7 (λ_{max} = 278 nm). However, there was a large change when the mixture was held at pH 14 (λ_{max} = 314 nm), which indicates that 8-HM-ϵdC is not stable under alkaline conditions. The instability of the adduct to hydroxide ion was further demonstrated by a slow rate of decomposition at pH 7, which was observed after 3 weeks at room temperature.

However, it is important to distinguish between the stability of the adduct as a monomer and the stability of the adduct within an oligonucleotide. For example, the purity of oligomer **2** is shown by HPLC (system 2, single peak, retention time of 24.61 min) and by its electrospray mass spectrum (*m/z* 4653, M^+). This indicates that the oligomer containing the adduct is stable to deprotection and purification. Furthermore, oligomer **2** was allowed to stand at room temperature at pH 7 for 3 weeks and exhibited no additional peak on digestion. This indicates that the adduct has far greater stability within an oligonucleotide than as a monomer.

In summary, we have prepared 8-(hydroxymethyl)-3,N^4-etheno-2′-deoxycytidine, a potential carcinogenic glycidaldehyde adduct (*1*−*8*), for the first time on a large scale with good yield. The corresponding phosphoramidite was synthesized and site-specifically incorporated into DNA oligonucleotides which are being used for studies of their physical and biochemical properties.

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