Synthesis of 3,N^4-Etheno, 3,N^4-Ethano, and 3-(2-Hydroxyethyl) Derivatives of 2′-Deoxycytidine and Their Incorporation into Oligomeric DNA

Weifeng Zhang, Robert Rieger, Charles Iden, and Francis Johnson*

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

Received June 10, 1994[⊗]

3,N^4-Etheno, 3,N^4-ethano, and 3-(2-hydroxyethyl)derivatives of 2′-deoxycytidine arise in mammalian DNA that has been exposed to the metabolic products of either vinyl chloride or the antitumor drug bis(chloroethyl)nitrosourea. These chemically-related adducts are thought to be associated with both mutagenesis and carcinogenesis. In this paper we report reliable syntheses of these deoxynucleosides and incorporation of the latter into oligodeoxynucleotides by the phosphoramidite route, using automated methods. It was found that 3-(2-hydroxyethyl)-2′-deoxycytidine is unstable in aqueous solution and undergoes an autoinduced hydrolysis to 3-(2-hydroxyethyl)-2′-deoxyuridine. The rate of this hydrolysis was found to be pH-dependent, having a maximum around pH 8, and a half-life of approximately 5 h. At higher or lower acidities, the reaction rate falls, indicating that the process involves a general acid–base catalysis. Thus in this case, oligomers were obtained that possessed 3-(2-hydroxyethyl)-2′-deoxyuridine residues, rather than the cytidine analogue. It is likely that the former represents the longer-lived species in DNA under physiological conditions. Representative oligomers containing these chemical lesions were analyzed by mass spectrometric and enzymatic degradation methods to confirm their structures.

Introduction

Vinyl chloride is produced in large quantities by the chemical industry and is widely used for the production of polymers, mainly PVC (*1*). Exposure to this volatile compound has been associated with the development of liver sarcomas in humans. The connection between cancer induction and vinyl chloride was first recognized in 1974 by Creech and Johnson (*2*). However, vinyl chloride itself is not carcinogenic but is metabolized in vivo by the microsomal monooxygenases of the liver to chloroethylene oxide (CEO)[1] (*1*) and chloroacetaldehyde (CAA), both of which are highly efficient mutagenic agents (*1*, *3*). They both react with DNA to form a variety of exocyclic deoxynucleoside adducts, including 3,N^4-etheno-2′-deoxycytidine (**1**; Etheno-dCyd) (Chart 1), 1,N^6-etheno-2′-deoxyadenosine, and N^2,3-etheno-2′-deoxyguanosine (*4*–*6*).

Closely related in chemical structure to these substances are the adducts formed from DNA and the nitrosourea antitumor agents. Typical of the latter is 1,3-bis(2-chloroethyl)nitrosourea (BCNU) whose cytotoxic mechanism has been studied extensively (*7*–*9*). The formation of deoxynucleoside adducts and cross-links has been found to play a key role in the expression of its biological activity.

BCNU decomposes spontaneously under physiological conditions to form 2-chloroethanediazohydroxide (*10*), a powerful electrophilic chloroethylating agent. The initial attack on 2′-deoxycytidine residues appears to occur mainly at the N-3 position and leads to DNA containing 3-(2-chloroethyl)-2′-deoxycytidine (**2**). Spontaneously, the latter cyclizes to the "exocyclic adduct" 3,N^4-ethano-2′-deoxycytidine (**3**; Ethano-dCyd) (*11*). In addition, **2** also undergoes a hydrolytic reaction to produce 3-(2-hydroxyethyl)-2′-deoxycytidine (**4**; 3HE-dCyd) (*11*). By a second pathway, 2-hydroxyethanediazohydroxide, a minor BCNU decomposition product, can also react with 2′-deoxycytidine to generate 3HE-dCyd. Compound **4** is unstable on standing in aqueous solution. This spontaneous hydrolysis, involving the loss of ammonia, occurs rapidly even under physiological conditions. It is mediated by the 2-hydroxyethyl group and leads to 3-(2-hydroxyethyl)-2′-deoxyuridine (**5**; 3HE-dUrd) as the final stable product (*12*–*14*).

As mentioned above, these exocyclic nucleoside adducts are the keys to understanding the cytotoxic mechanisms of BCNU, vinyl chloride, and other α,β-unsaturated compounds. For this reason we have focused our attention on the synthesis of Etheno-dCyd (**1**), Ethano-dCyd (**3**), 3HE-dCyd (**4**), and 3HE-dUrd (**5**), verified their structures, studied their properties, and incorporated them into oligodeoxynucleotides for structural and mutagenesis studies.

Results and Discussion

Deoxynucleoside Synthesis, Chemical Characterization, and Conversion to Their Protected 4,4′-Dimethoxytritylphosphoramidites. Etheno-dCyd (1). The synthesis of the 2′-hydroxy homologue 3,N^4-ethenocytidine was originally reported by Barrio et al. (*15*), who prepared it by the reaction of chloroacetaldehyde with cytidine in aqueous solution. We simply adopted this method in allowing chloroacetaldehyde to react with 2′-deoxycytidine in aqueous solution, which

* Tel: (516)-632-8865; Fax: (516)-632-7394.

[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1994.

[1] Abbreviations: 3HE-dCyd, 3-(2-hydroxyethyl)-2′-deoxycytidine; 3HE-dUrd, 3-(2-hydroxyethyl)-2′-deoxyuridine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CAA, chloroacetaldehyde; CEO, chloroethylene oxide; CPG, controlled-pore glass; DMF, *N,N*-dimethylformamide; DMT, 4,4′-dimethoxytrityl; Ethano-dCyd, 3,N^4-ethano-2′-deoxycytidine; Etheno-dCyd, 3,N^4-etheno-2′-deoxycytidine; FAB, fast atom bombardment; TBDMS, *tert*-butyldimethylsilyl.

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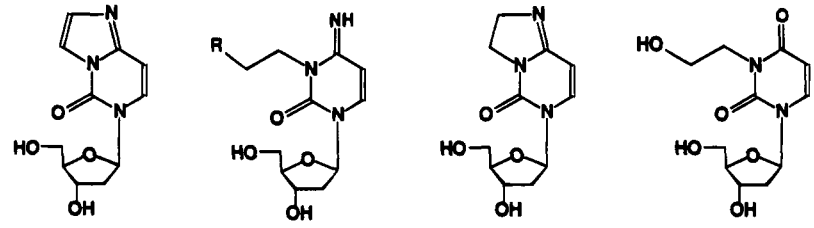
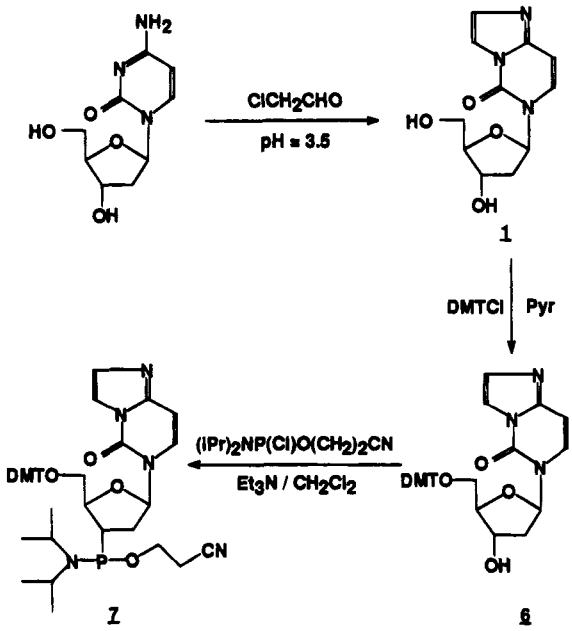
Chart 1



1 2; R = Cl 4; R = OH 3 5

Scheme 1



1 7 6

directly provided the desired product **1** in 81% yield (Scheme 1). Although the reaction requires acidic conditions (pH = 3.5) to achieve this yield, no cleavage of the glycosydic bond was observed with either starting material or product. Prior to conversion to the DMT-phosphoramidite derivative (**7**) needed for DNA oligomer synthesis, **1** was subjected to the reagents and reaction conditions of automated DNA synthesis (*16*) to assess the stability of the imidazopyrimidine moiety of the nucleoside. No instability was observed except in the case of prolonged treatment with concentrated ammonia at 55 °C. This caused a very slow decomposition with complete loss of the ultraviolet spectrum above 220 nm. However, this decomposition, which appears to be due to the implicit presence of an *N*-acylimidazole within the heterocyclic moiety, is slow enough so as not to interfere seriously with the synthesis of the desired oligomers.

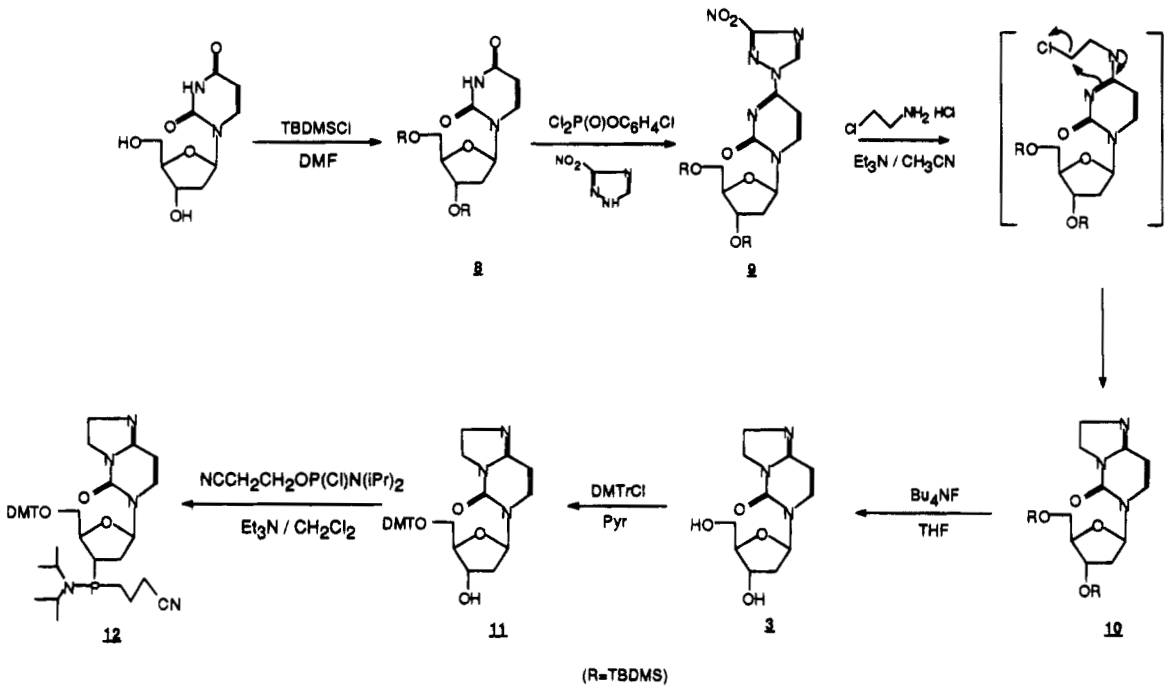
Conversion of **1** to the desired DMT-phosphoramidite **7** was accomplished first by treatment of **1** with 4,4′-dimethoxytrityl chloride in pyridine to give **6** in 90% yield (*17*) (Scheme 1). The latter compound when treated with bis(diisopropylamino)chlorophosphine in dry CH_2Cl_2 in the presence of triethylamine according to a previously described procedure (*18*, *19*) led to **7** as a 1:1 mixture of the expected diastereomers. Spectroscopic and TLC analyses indicated that this material was essentially free of contaminants. When used as such in the automated synthesis of a series of DNA oligomers (see below), high coupling efficiency was observed and excellent yields of the desired products were obtained. The incorporation of Etheno-dCyd into oligomeric DNA by essentially the same route has been reported recently by Dosanjh et al. (*20*). However, no experimental details were reported.

Ethano-dCyd (3). Initially, we attempted to synthesize this compound by the method of Ludlum (*21*), a procedure that is similar to the one that had proven successful in the case of the synthesis of 1,N^2-(1,3-propano)-2′-deoxyguanosine (*22*). However, when the 3′,5′-*O*-bis(*tert*-butydimethysilyl) derivative of 2′-deoxycytidine was heated with 1,2-dibromoethane in DMSO in the presence of anhydrous potassium carbonate, little (less than 1%) of the desired product was formed. No better results were obtained when either ethylene glycol di-*p*-tosylate or 2-fluoroethyl triflate were substituted for 1,2-dibromoethane. The selective hydrogenation of Etheno-dCyd over a 10% platinum-on-carbon catalyst was also attempted, but only starting material was recovered. Attempts to cyclize the 3HE-dCyd to **3** by conversion of the β-hydroxyl group to a leaving group (Cl or *p*-TsO) also failed. Thus a longer but more secure route (Scheme 2), which utilized the known (*23*) nitrotriazolodeoxyuridine derivative **9** as the key intermediate, was followed. In repeating the synthesis of this compound, we found that much superior yields (66% vs 5%) could be obtained by utilizing 4-chlorophenyl dichlorophosphate as the coupling agent (*24*, *25*) rather than the originally recommended 2-chlorophenyl isomer (*23*). In addition, the isolation procedure was simplified, requiring only a flash chromatographic workup.

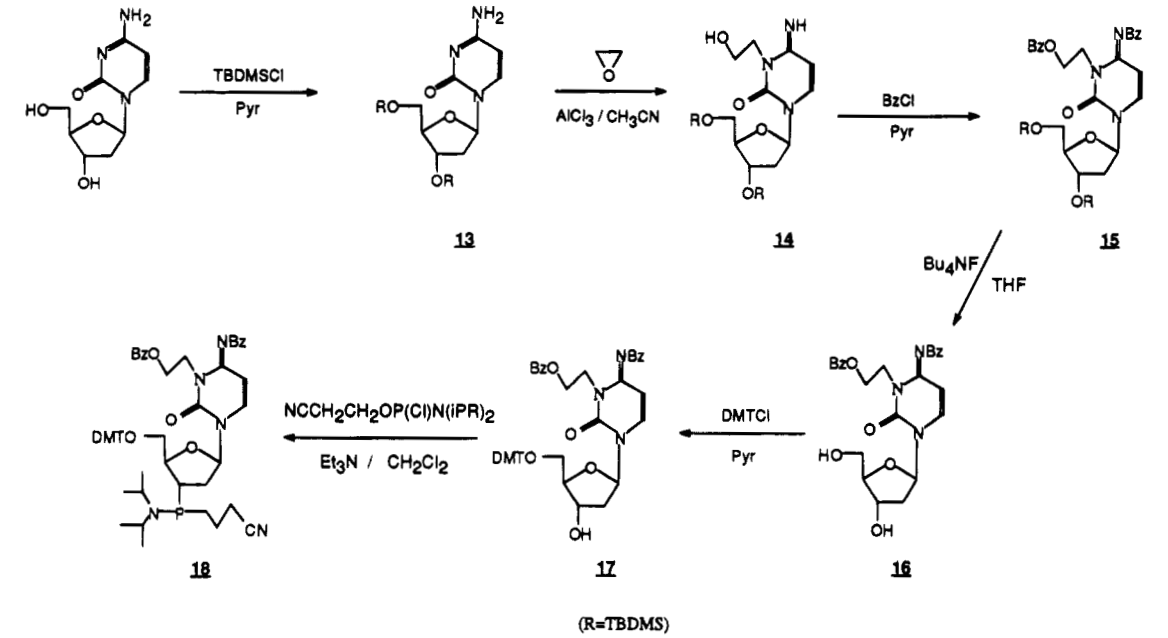
The substitution of the 3-nitro-1,2,4-triazolo group of **9** by 2-chloroethylamine occurred smoothly and was accompanied by spontaneous ring closure to give **10** in 86% yield. Removal of the blocking silyl groups was then accomplished by means of tetrabutylammonium fluoride in THF to give Ethano-dCyd (**3**) in almost quantitative yield. This compound proved to be stable to all of the reagents and conditions of automated DNA synthesis. The conversion of **3** to its DMT derivative and the latter to the desired phosphoramidite derivative **12** was carried out using the conditions described previously for the conversion of Etheno-dCyd to the corresponding derivatives **6** and **7**. Good coupling efficiency was observed when **12** was used without further purification in the synthesis of DNA oligomers. Other aspects of these oligomer syntheses are discussed in the section entitled "Synthesis of DNA Oligomers", below.

3HE-dCyd (4). The synthesis of this material as its bis-TBDMS derivative **13**, was accomplished by the treatment of **8** with ethylene oxide in the presence of a catalytic quantity of aluminum chloride (Scheme 3), following a modification of the procedure of Maggio (*23*). However, in order to obtain **13** in good yield (69%), it was necessary to deactivate the catalyst by exposure to air for a brief period (3 h). A small sample of **4** was obtained by the deprotection of **13** by means of tetrabutylammonium fluoride in THF. When **4** was exposed to the

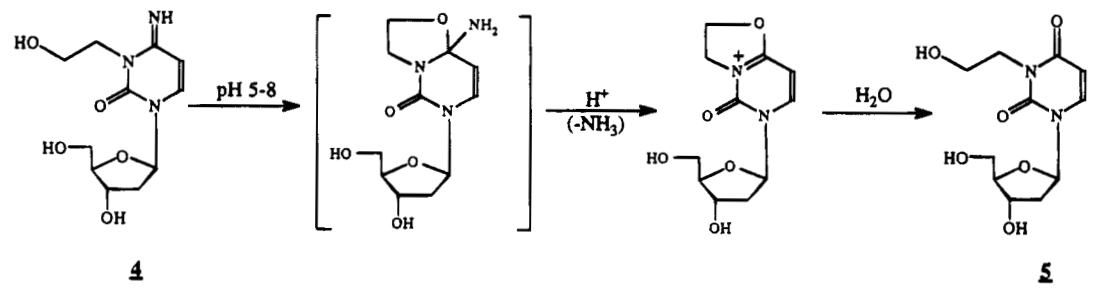
Scheme 2



Scheme 3



Scheme 4



various reagents and conditions of DNA synthesis, it proved to be stable except to aqueous solution around pH 8. Under the latter conditions at 37 °C, it was converted completely to 3-(2-hydroxyethyl)-2′-deoxyuridine (3HE-dUrd; **5**) within 20 h, in agreement with the recent work of Solomon (*12*, *26*) who has suggested a mechanism (Scheme 4) to account for this conversion. A broader study of the effects of pH on the stability of **4** revealed that at either higher or lower pH values, the hydrolysis proceeds much more slowly (Figure 1). Thus it appears that the hydrolysis of **4** to **5** involves general acid–base catalysis and is in agreement with the work

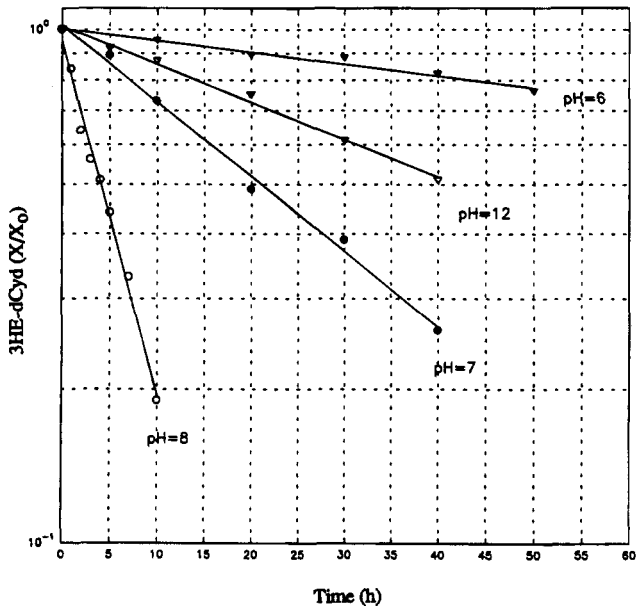


Figure 1. Stability of 3HE-dCyd at different pH values. 3HE-dCyd was incubated at 37 °C in aqueous solution at a specific pH value. Samples were analyzed by means of HPLC periodically. The ratio of the initial concentration of 3HE-dCyd over the final concentration (X_0/X) was plotted as the ordinate. Rates of hydrolysis are maximal around pH 8 and diminish at lower or higher acidity values.

Table 1. Oligonucleotides Containing Adducts That Have Been Synthesized

5′-d(ACXGT)-3′	**19a**	X = Etheno-dCyd
5′-d(CCATAXGTACTTC)-3′	**19b**	
5′-d(CCTTCXCTACTTTCCTCT)-3′	**19c**	
5′-d(CCTTCXCTACTTTCCTCTCAATTT)-3′	**19d**	
5′-d(TTXTT)-3′	**20a**	X = Ethano-dCyd
5′-d(CCATAXGTACTTC)-3′	**20b**	
5′-d(CCTTCXCTACTTTCCTCT)-3′	**20c**	
5′-d(CCTTCXCTACTTTCCTCTCAATTT)-3′	**20d**	
5′-d(CCXAC)-3′	**21a**	X = 3HE-dUrd
5′-d(CCTTCXCTACTTTCCTCT)-3′	**21b**	
5′-d(CCTTCXCTACTTTCCTCTCAATTT)-3′	**21c**	

by Sowers et al. (*27*), who found that N^3-methyl-dCyd undergoes facile hydrolytic deamination in aqueous solution to the deoxyuridine analogue.

When **13** was benzoylated in pyridine it afforded **14** in excellent yield. Removal of the protecting groups by tetrabutylammonium fluoride then gave the desired dibenzoyl derivative **15** of **4**. This was then converted first to its 5′-*O*-DMT derivative **16** and then to the ultimate 3′-*O*-phosphoramidite **17** by conventional methods. When the latter compound was used in the synthesis of a series of oligomers, high coupling efficiency was observed at the point of introduction of the 3HE-dCyd residue and good yields of oligomeric products were obtained (see discussion below).

Synthesis of DNA Oligomers. After completion of the synthetic work on the deoxynucleosides, the DMT phosphoramidites **7**, **12**, and **18** were incorporated individually into a series (Table 1) of oligodeoxynucleotides **19**–**21**, using the normal automated DNA synthesis and purification procedures. All couplings involving these phosphoramidites proceeded with good to excellent results as judged by DMT cation release in the subsequent synthetic cycle.

Prior to utilizing any of the larger oligomers in biological studies, a preliminary investigation was undertaken to confirm the presence and location of the abnormal nucleosides in the DNA. In the case of the Etheno-dCyd residue, the pentamer 5′-ACXGT-3′ (X = Etheno-dCyd residue, **19a**) was synthesized and its structure was

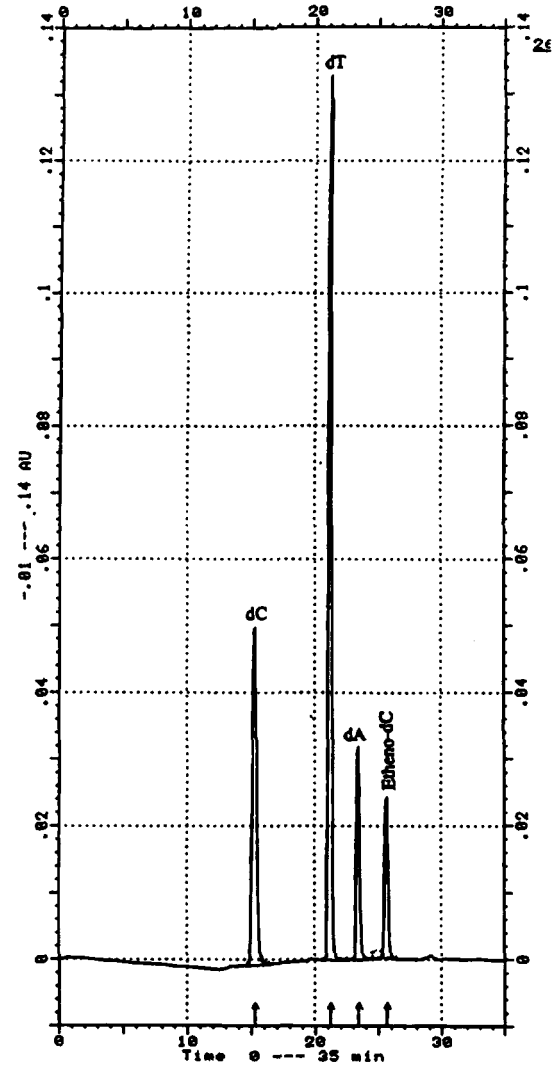


Figure 2. The HPLC profile of 2′-deoxynucleosides obtained as the result of enzymatic digestion of the oligodeoxynucleotide 5′-CCTTCXCTACTTTCCTCT (X = Etheno-dCyd, **19c**). HPLC conditions: elution with water (pH 5.85) and 0–30% acetonitrile (linear gradient) over 30 min at a flow rate of 1.0 mL/min.

Table 2. Nucleoside Content of the Oligomer 5′-d(CCTTCXCTACTTTCCTCT)-3′ Containing Etheno-dCyd[a]

nucleoside	nucleoside content (nmol)[b,c]	ratio[c]
dCyd	3.17 (4.08)	7.94 (8.0)
dThd	3.19 (4.08)	7.97 (8.0)
dAdo	0.40 (0.51)	1.00 (1.0)
Etheno-dCyd	0.37 (0.51)	0.93 (1.0)

[a] Etheno-dCyd 18-mer (3 μg) was used for the analysis of nucleoside content. [b] Nucleoside content was calculated by dividing the integrated HPLC peak area for each nucleoside under 260 nm with the values obtained from standard solutions. [c] Theoretical ratios are given in parentheses.

confirmed by electrospray mass spectroscopy which revealed the correct molecular mass of 1486 (calcd 1486) daltons. The oligomer **19c** then was selected for base composition analysis. When it was subjected to sequential enzymatic digestion with nuclease P1 and then bacterial alkaline phosphatase, the individual deoxynucleosides were found to be present in the expected molecular ratios (Figure 2 and Table 2). Similarly, the structure of the pentamer, containing an Ethano-dCyd residue (5′-TTXTT-3′, **20a**), was confirmed by electrospray mass spectroscopy: mass value 1469 (calcd 1469) daltons. The oligomer **20c** also was selected for base

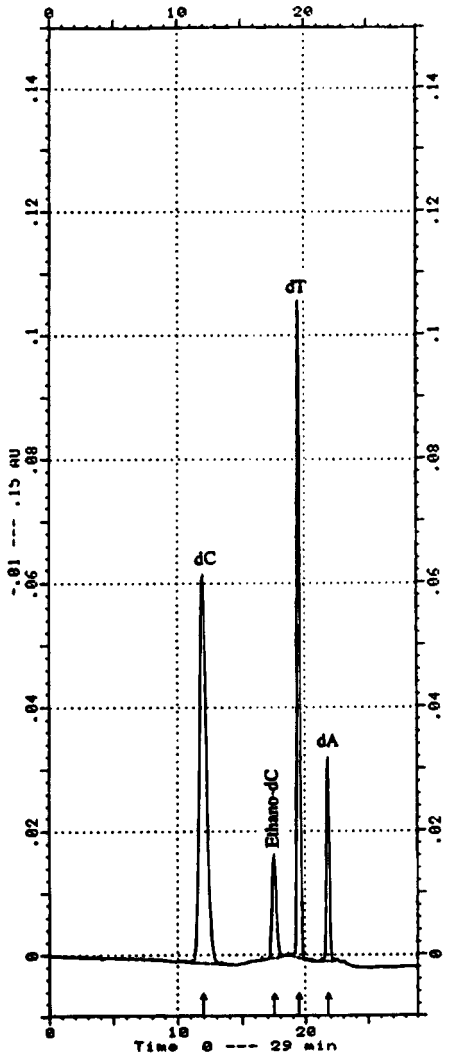


Figure 3. The HPLC profile of 2′-deoxynucleosides obtained as the result of enzymatic digestion of the oligodeoxynucleotide 5′-CCTTCXCTACTTTCCTCT (X = Ethano-dCyd, **20c**). HPLC conditions: elution with buffer (pH 6.85, Et_3N·HOAc, 0.05 M) and 0−30% acetonitrile (linear gradient) over 30 min at a flow rate of 1.0 mL/min.

Table 3. Nucleoside Content of the Oligomer 5′-d(CCTTCXCTACTTTCCTCT)-3′ Containing Ethano-dCyd[a]

nucleoside	nucleoside content (nmol)[b]	ratio[c]
dCyd	3.38 (4.08)	8.05 (8.0)
dThd	3.30 (4.08)	7.85 (8.0)
dAdo	0.42 (0.51)	1.00 (1.0)
Ethano-dCyd	0.43 (0.51)	1.07 (1.0)

[a] Ethano-dCyd 18-mer (3 μg) was used for the analysis of nucleoside content. [b] Nucleoside content was calculated by dividing the integrated HPLC peak area for each nucleoside under 260 nm with the values obtained from standard solutions. [c] Theoretical ratios are given in parentheses.

composition analysis, using the same conditions as were described for **19c**, and the individual nucleosides were found by enzymatic degradation to be present in the expected molecular ratios (Figure 3 and Table 3).

As discussed earlier, the syntheses of oligonucleotides containing 3HE-dCyd presents significant problems. Even if these were to be solved by careful synthesis and purification, the possibility of carrying out biological studies would be compromised by the rapid conversion of the residue to the 3HE-dUrd group. Because of the transient nature of the dCyd residue it seems likely that the lesion of importance is truly 3HE-dUrd, although

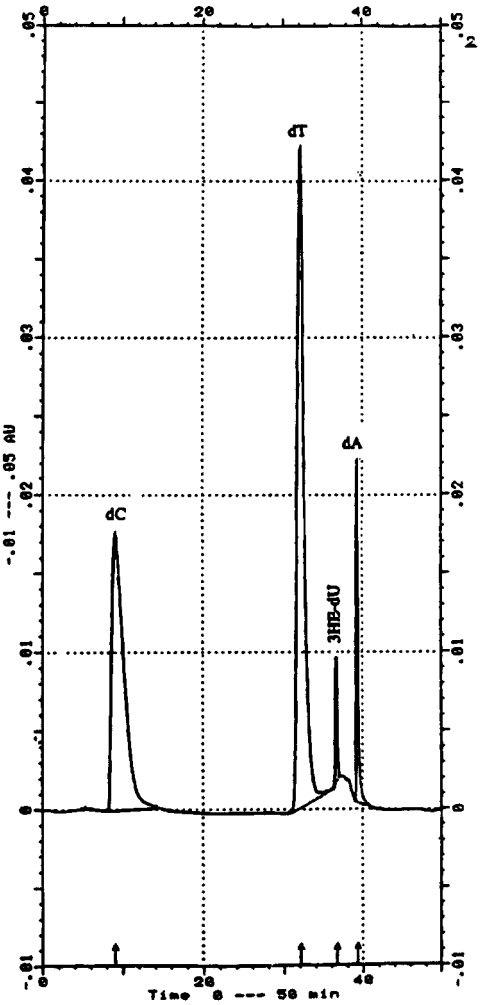


Figure 4. The HPLC profile of 2′-deoxynucleosides obtained as the result of enzymatic digestion of the oligodeoxynucleotide 5′-CCTTCXCTACTTTCCTCT (X = 3HE-dUrd, **21b**). The small bump under the 3HE-dU peak is the protein coeluted with the nucleoside. HPLC conditions: elution with water (pH 5.85) and 0−30% acetonitrile (linear gradient) over 60 min at a flow rate of 1.0 mL/min.

Table 4. Nucleoside Content of the Oligomer 5′-d(CCTTCXCTACTTTCCTCT)-3′ Containing 3HE-dUrd[a]

nucleoside	nucleoside content (nmol)[b]	ratio[c]
dCyd	2.38 (2.71)	7.18 (8.0)
dThd	2.40 (2.71)	8.27 (8.0)
dAdo	0.29 (0.39)	1.00 (1.0)
3HE-dUrd	0.26 (0.39)	0.90 (1.0)

[a] 3HE-dUrd 18-mer (2 μg) was used for the analysis of nucleoside content. [b] Nucleoside content was calculated by dividing the integrated HPLC peak area for each nucleoside under 260 nm with the values obtained from standard solutions. [c] Theoretical ratios are given in parentheses.

presently no direct biological evidence is available to support this suggestion. Nevertheless, the synthesis of oligodeoxynucleotides (**21a−c**) containing 3HE-dUrd rather than 3HE-dCyd was undertaken. In practice, we simply subjected all of the oligomers containing 3HE-dCyd to pH 8.0 and 40 °C in aqueous solution for more than 40 h. Electrospray mass spectroscopy confirmed the correct molecular mass of 5′-d(CCXAC) (**21a**) as 1453 daltons (calcd 1453). The oligomer **21b** then was selected for base composition analysis, using the same enzymatic degradation conditions as were described for **19c**, and the individual nucleosides were found to be present in the expected molecular ratios (Figure 4 and Table 4). The UV spectrum of 3HE-dUrd has λ_{max} 262 nm, and the

HPLC retention time was consistent with the 3HE-dUrd standard. We did not observe any peak corresponding to 3HE-dCyd in the HPLC profile (*28*).

Experimental Section

Dimethylformamide (DMF), triethylamine, pyridine, and ethylene oxide were purchased from Fisher Scientific Co. (certified grade). HPLC-grade acetonitrile, choroform, ethyl acetate, methanol, benzene, and methylene chloride were purchased from Fisher Scientific Co. and were used as received, unless otherwise stated. DMF was dried over calcium hydride, decanted, and distilled prior to use. Pyridine was heated under reflux over calcium hydride and then distilled. Benzene and methylene chloride were boiled over P_2O_5 and then distilled. Triethylamine was heated with sodium wire for 6 h, decanted, and then distilled from calcium hydride.

2′-Deoxycytidine and (*N,N*-diisopropylamino)(2-cyanoethoxy)-chlorophosphine (>99% pure) were purchased from American Bionics Co. (Hayward, CA) and were used as supplied. Benzoyl chloride, 3-nitro-1,2,4-triazole, chloroacetaldehyde (50% aqueous solution; **Warning**: *carcinogenic substance*), 4,4′-dimethoxytrityl chloride, 4-chlorophenyl dichlorophosphate, tetrabutylammonium fluoride (1 M solution in THF), aluminum chloride, and *tert*-butyldimethylsilyl chloride (97% pure) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded in DMSO-d_6 on a General Electric QE-300 spectrometer unless otherwise noted. Chemical shifts are reported relative to an internal standard of tetramethylsilane. ^{31}P NMR spectra were recorded on a NT-300 spectrometer, and chemical shifts are reported relative to an external standard of $P(OCH_3)_3$. Fast atom bombardment (FAB) mass spectra were obtained on a Kratos MS-890/Ds 90 instrument. Glycerol or thiogylcerol was used as the matrix. Low-resolution mass spectra were obtained by using a Hewlett-Packard 5980A dodecapole mass spectrometer and a Hewlett-Packard 5988a mass spectrometer equipped with a particle beam LC/MS interface. Electrospray ionization mass spectra were acquired on a VG-TR10-2000 quadrupole mass spectrometer (Fisons Instrument, Davess, MA).

TLC was performed on EM 5539 silica gel 60 plates. All column chromatography was carried out with EM silica gel 60 (0.040−0.063 mm) with elution under the pressure. Melting points are uncorrected.

Oligonucleotides were synthesized on a DuPont CODER-300 automated DNA synthesizer using a controlled-pore glass (CPG) support with an (aminopropyl)succinate linker. Oligonucleotides were purified by reversed-phase HPLC (Waters 990 photodiode array detector) using a μ-Bondapak C_{18} (0.39 × 30 cm, Waters) column.

3,*N*^{4}-Etheno-2′-deoxycytidine (5,6-Dihydro-5-oxo-6-(β-D-2′-deoxyribofuranosyl)imidazo[1,2-*c*]pyrimidine (1). 2′-Deoxycytidine monohydrochloride (1.05 g, 4.6 mmol) and potassium phosphate (monobasic) (2 g) were dissolved in 40 mL of 25% chloroacetaldehyde aqueous solution. The solution was adjusted to pH 3.5 by means of 1 M KOH and then stirred at room temperature for 48 h. Thereafter, the aqueous solution was neutralized to pH 7 by adding 20% potassium carbonate solution, and sodium chloride was added to the saturation point. Extraction of the product was accomplished with ethyl acetate in a Salax continuous extraction apparatus over 2 h. The organic extract was dried over sodium sulfate and evaporated to dryness, and the residual material then was subjected to silica gel flash chromatography with EtOAc−MeOH (90:10) as the eluant. The product was crystallized from ethanol−hexane. Yield 81% (0.80 g); mp 137−138 °C; TLC R_f 0.41 (EtOAc−MeOH, 80:20). ^{1}H NMR: δ 7.79 (d, J = 2 Hz, 1H, H-2), 7.72 (d, J = 8 Hz, 1H, H-7), 7.37 (d, J= 2 Hz, 1H, H-3), 6.73 (d, J = 8 Hz, 1H, H-8), 6.40 (t, J = 6 Hz, 1H, H-1′), 5.33 (s, 1H, 3′-OH), 5.09 (s, 1H, 5′-OH), 4.29 (m, 1H, H-4′), 3.85 (m, 1H, H-3′), 3.50 (m, 2H, H-5′), 2.19 (m, 2H, H-2′); ^{13}C NMR: δ 145.42, 144.53, 132.53, 127.94, 112.66, 98.47, 87.75, 85.27, 70.38, 61.24, 40.10. FAB/MS (+ve ion, thioglycerol matrix): m/z 252 [(M + H)$^+$, 55], 136 [(heterocyclic base + 2H)$^+$, 100]. Anal. Calcd for $C_{11}H_{13}O_4N_3$: C, 52.59; H, 5.22; N,16.72. Found: C, 52.66; H, 5.19; N, 16.71. UV: λ_{max} = 272 nm, ε =12 000.

5′-*O*-(4,4′-Dimethoxytrityl)-3,*N*^{4}-etheno-2′-deoxycytidine (6). 3,*N*^{4}-Etheno-2′-deoxycytidine (1 g, 4 mmol) and 4,4′-dimethoxytrityl chloride (1.6 g, 1.2 equiv) were dissolved in 10 mL of pyridine. The reaction mixture (viscous yellow solution) was stirred at room temperature for 4 h (the reaction is almost complete in 1 h), then added to 50 mL of water, and extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were combined and back-washed with 30 mL of water and 5 mL of buffer solution (5% potassium phosphate, pH = 7), then dried over sodium sulfate and evaporated to dryness in vacuo to remove traces of pyridine. The desired product was separated by silica gel flash chromatography with EtOAc−hexane (50:50) followed by EtOAc as the eluant. The product was obtained as a white foam. Yield 91% (2 g); mp 95−101 °C; TLC: R_f 0.35 (EtOAc); ^{1}H NMR ($CDCl_3$): δ 7.22 (d, J = 2 Hz, 1H, H-2), 7.19 (d, J = 8 Hz, 1H, H-6), 6.96 (d, J = 2 Hz, 1H, H-3), 6.90−6.85 (m, 9H, ArH), 6.35 (d, J = 6 Hz, 4H, H ortho to OCH_3), 6.13 (t, J = 6 Hz, 1H, H-1′), 5.88 (d, J = 8 Hz, 1H, H-8), 4.16 (m, 1H, H-4′), 3.64 (m, 1H, H-3′), 3.31 (s, 6H, OCH_3), 3.03 (m, 2H, H-5′), 2.05 (m, H, H-2′β), 1.89 (m, 1H, H-2′α); ^{13}C NMR: δ 158.75, 145.72, 145.63, 144.39, 135.52, 132.49, 130.10, 128.18, 127.91, 127.27, 127.07, 113.21, 112.71, 99.02, 87.04, 86.27, 85.72, 71.37, 63.10, 55.21, 41.38. FAB/MS (+ve ion, thioglycerol matrix): m/z 554 [(M + H)$^+$, 18], 303 [(DMT + H)$^+$, 100)], 136 [(heterocyclic base + 2H)$^+$, 90]. Anal. Calcd for $C_{32}H_{31}O_6N_3$: C, 69.43; H, 5.64; N, 7.59. Found: C, 69.12; H, 5.81; N, 7.39.

3′-*O*-[(Diisopropylamino)(2-cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-3,*N*^{4}-etheno-2′-deoxycytidine (7). 5′-*O*-(4,4′-Dimethoxytrityl)-3,*N*^{4}-etheno-2′-deoxycytidine (0.2g) and 0.2 mL of Et_3N were dissolved in CH_2Cl_2 (2 mL). Under the protection of a nitrogen atmosphere, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.2 g, 1.5 equiv) was slowly added to the solution. The reaction mixture was stirred at room temperature for 1 h, the progress of the reaction being monitored by TLC (CH_2Cl_2−Et_3N, 95:5, as the mobile phase). After the reaction was complete, the mixture was evaporated to dryness and 30 mL of benzene was added. The benzene solution was filtered to remove insoluble material (Et_3N·HCl), and after removal of the benzene by evaporation, the desired product was obtained as a light yellow oil. Yield >99% (0.27 g); TLC: R_f 0.78 (CH_3Cl−Et_3N, 95:5); ^{1}H NMR ($CDCl_3$): δ 7.21 (d, J = 2 Hz, 1H, H-2), 7.17 (d, J = 8 Hz, 1H, H-6), 6.97 (d, J = 2 Hz, 1H, H-3), 6.90−6.84 (m, 9H, ArH), 6.32 (d, J = 6 Hz, 4H, H ortho to OCH_3), 6.12 (t, J = 6 Hz, 1H, H-1′), 5.86 (d, J = 8 Hz, 1H, H-8), 4.15 (m, 3H, H-4′, OCH_2), 3.61 (m, 1H, H-3′), 3.46 (m, 2H, 2CHN), 3.31 (s, 6H, OCH_3), 3.03 (m, 2H, H-5′), 2.76 (t , J = 6 Hz, CH_2CN), 2.07 (m, 1H, H-2′β), 1.90 (m, 1H, H-2′α), 1.25 (dd, 12H, $C(Pr^i)_2$); ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.56 and 147.41 (diastereomeric pair). This material was dried and without further purification was used in the automated DNA synthesizer. It showed incorporation efficiency of >85%.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2′-deoxyuridine (8). 2′-Deoxyuridine (4 g), *tert*-butyldimethylsilyl chloride (11.6 g, 4.4 equiv), and imidazole (10.5 g, 8.8 equiv) were dissolved in dry DMF (15 mL), and the mixture was stirred at room temperature overnight. The solution was then poured into 150 mL of water and extracted with CH_2Cl_2 (3 × 60 mL). The organic layer was evaporated to dryness under vacuum. The residue was further purified by silica gel flash chromatography with CH_2Cl_2−MeOH (99:1) as the eluant. A white foam was obtained as the final product. Yield 97% (7.8 g); mp 116−119 °C; TLC: R_f 0.30 (CH_2Cl_2−MeOH, 95:5); ^{1}H NMR ($CDCl_3$) δ 10.19 (s, 1H, NH), 7.78 (d, J = 8.0 Hz, 1H, H-6), 6.15 (t, J = 7.1 Hz, 1H, H-1′), 5.58 (d, J = 8.0 Hz, 1H, H-5), 4.28 (m, 1H, H-3′), 3.79 (m, 1H, H-5′), 3.63 (m, 1H, H-4′), 2.18 (m, 1H, H-2′β), 1.97 (m, 1H, H-2′α), 0.80 (s, 9H, $SiC(CH_3)_3$), 0.79 (s, 9H, $SiC(CH_3)_3$), 0.25 (s, 6H, $Si(CH_3)_2$), 0.23 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR: δ 164.16, 150.87, 140.35, 102.49, 87.99, 85.48, 71.40, 62.68, 42.11, 26.04,18.25, −4.35. Anal. Calcd for $C_{21}H_{40}N_2O_5Si_2$: C, 55.23; H, 8.84; N, 6.14. Found: C, 55.13; H, 8.72; N, 6.11.

4-(3-Nitro-1,2,4-triazolo)-1-[β-D-3,5-*O*-bis(*tert*-butyldimethylsilyl)-2-deoxyribofuranosyl]pyrimidin-2(1*H*)-one (9).

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2′-deoxyuridine (1 g) and 3-nitro-1,2,4-triazole (1 g, 4 equiv) were dissolved in pyridine (3 mL). The solution was cooled in an ice bath, and slowly there was added 4-chlorophenyl dichlorophosphate (1.07 g, 0.71 mL, 2 equiv). The reaction mixture was then stirred at room temperature for 3 days. Thereafter, the solvent was removed by evaporation on a rotary evaporator. The residue was dissolved in CH_2Cl_2 (60 mL) and washed with 5% of sodium bicarbonate solution (2 × 30 mL), and then with water once. The desired product was isolated by silica gel flash chromatography with CH_2Cl_2–MeOH (99:1) as the eluant. Yield 66% (0.80 g); mp 62–66 °C; TLC: R_f 0.5 (CH_2Cl_2–MeOH, 97:3). ^{1}H NMR ($CDCl_3$): δ 9.25 (s, 1H, triazole H), 8.70 (d, J = 8 Hz, 1H, H-6), 6.92 (d, J = 8 Hz, 1H, H-5), 6.15 (t, J = 7 Hz, 1H, H-1′), 4.31 (m, 1H, H-4′), 3.95 (m, 2H, H-5′), 3.72 (m, 1H, H-3′), 2.48 (m, 1H, H-2′), 2.11 (m, 1H, H-2′α), 0.76 (s, 9H, $SiC(CH_3)_3$), 0.75 (s, 9H, $SiC(CH_3)_3$), 0.09 (s, 6H, $Si(CH_3)_2$), 0.08 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR: δ 163.56, 158.14, 153.47, 148.47, 144.53, 93.32, 88.32, 88.01, 69.94, 61.66, 42.29, 5.60, 18.30, −5.68. Anal. Calcd for $C_{23}H_{40}N_6O_6Si_2$: C, 49.98; H, 7.29; N, 15.20. Found: C, 49.60; H, 7.41; N, 14.86.

3,*N*⁴-Ethano-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (10). Compound **14** (1 g), 2-chloroethylamine monohydrochloride (2 g), and triethylamine (4 mL) were dissolved in CH_3CN (4 mL). The reaction mixture was stirred at room temperature for 6 h and then evaporated to dryness in vacuo. The residue was dissolved in CH_2Cl_2 (30 mL) and washed with water three times. The final product was obtained from a silica gel column by elution with CH_2Cl_2–MeOH (97:3) as a colorless oil. Yield 86% (0.75 g); TLC: R_f 0.20 (CH_2Cl_2–MeOH, 97:3); ^{1}H NMR ($CDCl_3$) δ 7.23 (d, J = 8 Hz, 1H, H-6), 6.19 (t, J = 7 Hz, 1H, H-1′), 5.59 (d, J = 8 Hz, 1H, H-5), 4.28 (m, 1H, H-4′), 3.90–3.60 (m, 7H, H-5′, H-3′, NCH_2CH_2N), 2.05 (m, 1H, H-2′β), 1.95 (m, 1H, H-2′α), 0.79 (s, 9H, $SiC(CH_3)_3$), 0.78 (s, 9H, $SiC(CH_3)_3$), 0.07 (s, 6H, $Si(CH_3)_2$), 0.05 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR: δ 173.36, 148.69, 135.64, 96.99, 87.20, 84.8, 71.61, 62.64, 53.36, 43.92, 41.14, 25.69, 7.94, −4.70. FAB/MS (+ve ion, thioglycerol matrix): m/z 482 [(M + H)$^+$, 11], 138 [(heterocyclic base + 2H)$^+$, 100]. Anal. Calcd for $C_{23}H_{43}N_3O_4Si_2$: C, 57.35; H, 9.00; N, 8.73. Found: C, 57.08; H, 8.91; N, 8.67.

3,*N*⁴-Ethano-2′-deoxycytidine (3). 3,*N*⁴-Ethano-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (0.5 g) was dissolved in a solution of 1 M tetra-*n*-butylammonium fluoride in THF (1 mL) under ice bath cooling. The reaction solution was stirred at room temperature for 2 h, then the solvent was removed by evaporation. The product was separated by silica gel flash chromatography and was eluted by CH_2Cl_2–MeOH (80:20). The final product was crystallized from EtOAc/petroleum ether. Yield 95% (0.25 g); mp 169–171 °C; TLC: R_f 0.3 (CH_2Cl_2–MeOH, 80:20); ^{1}H NMR: δ 7.38 (d, J = 8 Hz, 1H, H-6), 6.13 (t, J = 7 Hz, 1H, H-1′), 5.72 (d, J = 8 Hz, 1H, H-5), 5.31 (s, 1H, 3′-OH), 5.07 (s, 1H, 5′-OH), 4.20 (m, 1H, H-4′), 3.80–3.70 (m, 5H, H-3′, NCH_2CH_2N), 3.52 (m, 2H, H-5′), 2.01 (m, 2H, H-2′); ^{13}C NMR: δ 15.42, 148.72, 136.58, 96.91, 87.43, 83.94, 71.11, 61.97, 53.30, 43.94, 39.91. FAB/MS (+ve ion, thioglycerol matrix): m/z 254 [(M + H)$^+$, 35], 138 [(heterocyclic base + 2H)$^+$, 100]. Anal. Calcd for $C_{11}H_{15}N_3O_4$: C, 52.17; H, 5.97; N, 16.59. Found: C, 52.07; H, 5.84; N, 16.51. UV, λ_{max} = 282 nm, ϵ = 8700.

3,*N*⁴-Ethano-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (11). 3,*N*⁴-Ethano-2′-deoxycytidine (0.25 g) and 4,4′-dimethoxytrityl chloride (0.4 g, 1.1 equiv) were dissolved in pyridine (2 mL), and the solution was stirred at room temperature overnight. The reaction mixture was evaporated to dryness in vacuo, and the residue was dissolved in CH_2Cl_2 and washed once with water and once with a buffer solution (5% potassium phosphate, pH 7). The final product was isolated by silica gel flash chromatography with EtOAc as the eluant. Yield 87% (0.47 g); mp 109–111 °C; TLC: R_f 0.38 (EtOAc–MeOH, 97:3); ^{1}H NMR ($CDCl_3$): δ 7.30–7.10 (m, ArH, H-6), 6.74 (d, J = 9 Hz, 4H, H ortho to OCH_3), 6.23 (t, J = 7 Hz, 1H, H-1′), 5.47 (d, J = 8 Hz, 1H, H-5), 4.23 (m, 1H, H-4′), 3.90–3.60 (m, 5H, H-3′, NCH_2CH_2N), 3.67 (s, 6H, OCH3), 3.52 (m, 2H, H-5′), 2.21 (m, 2H, H-2′β), 2.11 (m, 1H, H-2′α); ^{13}C NMR: δ 159.03, 155.02, 148.99, 144.83, 135.96, 135.55, 130.47, 128.56, 128.56, 127.35, 113.60, 97.00, 87.17, 86.30, 84.75, 71.79, 63.89, 55.58, 53.21, 44.31, 4.0.99. FAB/MS (+ve ion, thioglycerol matrix): m/z 556 [(M + H)$^+$, 10], 303 [(DMT + H)$^+$, 100], 138 [(heterocyclic base + 2H)$^+$, 87]. Anal. Calcd for $C_{32}H_{33}N_3O_6$: C, 69.17; H, 5.99; N, 7.56. Found: C, 69.09; H, 5.89; N, 7.45.

3′-*O*-[(Diisopropylamino)(2-cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-3,*N*⁴-ethano-2′-deoxycytidine (12). 5′-*O*-(4,4′-Dimethoxytrityl)-3,*N*⁴-ethano-2′-deoxycytidine (0.2 g) and Et_3N (0.2 mL) were dissolved in CH_2Cl_2 (2 mL). Under the protection of a nitrogen atmosphere, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.2 g, 1.5 equiv) was slowly added to the solution dropwise. The mixture was stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC using CH_2Cl_2–Et_3N (95:5) as the mobile phase. After the reaction was complete (1 h), the mixture was evaporated to dryness, and benzene (30 mL) was then added. The solution was filtered to remove the insoluble salt (Et_3N·HCl), and the benzene was evaporated to give the product as a light yellow oil. Yield >99% (0.27 g); TLC: R_f 0.75 (CH_3Cl–Et_3N, 95:5); ^{1}H NMR ($CDCl_3$): δ 7.31–7.11 (m, 10H, ArH, H-6), 6.72 (d, J = 9 Hz, 4H, H ortho to OCH_3), 6.21 (t, J = 7 Hz, 1H, H-1′), 5.45 (d, J = 8 Hz, 1H, H-5), 4.21 (m, 3H, H-4′, OCH_2), 3.90–3.50 (m, 7H, H-3′, NCH_2CH_2N, 2CHN), 3.65 (s, 6H, OCH_3), 3.53 (m, 2H, H-5′), 2.73 (t, J = 6.0 Hz, 2H, CH_2CN), 2.20 (m, 1H, H-2′β), 2.10 (m, 1H, H-2′α), 1.25 (dd, 12 H, $C(Pr^i)_2$); ^{31}P NMR (121 Hz, $CDCl_3$) δ 147.5 and 147.3 (diastereomeric pair). This material was used in an automated DNA synthesizer directly (incorporation efficiency >85%).

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (13). 2′-Deoxycytidine monohydrochloride (1.052 g, 4 mmol), *tert*-butyldimethylsilyl chloride (2.64 g, 17.6 mmol), and imidazole (2.40 g, 35.2 mmol) were dissolved in dry DMF (4 mL). The solution was stirred at room temperature for 24 h and then poured into 60 mL of water. The aqueous solution was extracted with 3 × 30 mL of CH_2Cl_2. The combined organic extracts were washed with 20 mL of water and dried over sodium sulfate, and the residue, obtained after the evaporation of the solvents, was crystallized from CH_3CN. Yield 93% (1.6 g); mp 184–186 °C; TLC: R_f 0.51 (CH_2Cl_2–MeOH, 80:10); ^{1}H NMR ($CDCl_3$) δ 7.89 (d, J = 8 Hz, 1H, H-6), 6.18 (t, J = 7 Hz, 1H, H-1′), 5.62 (d, J = 8 Hz, 1H, H-5), 4.29 (m, 1H, H-4′), 3.82 (m, 2H, H-5′), 3.70 (m, 1H, H-3′), 2.32 (m, 1H, H-2′β), 2.01 (m, 1H, H-2′α), 0.84 (s, 9H, $SiC(CH_3)_3$), 0.83 (s, 9H, $SiC(CH_3)_3$), 0.44 (s, 6H, $Si(CH_3)_2$), 0.42 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR: δ 166.02, 155.88, 140.37, 94.45, 87.17, 85.66, 70.77, 62.19, 41.95, 25.60, 17.75, −5.67. Anal. Calcd for $C_{21}H_{40}N_2O_5Si_2$: C, 55.23; H, 8.83; N, 6.13. Found: C, 55.58; H, 9.12; N, 5.96.

3-(2-Hydroxyethyl)-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (14). 3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (2 g), aluminum chloride (0.2 g, exposed to air for 3 h before usage), and CH_3CN (12 mL) were mixed together in a high pressure reaction container kept at ~−70 °C by means of a dry ice. Ethylene oxide (14 mL) was then quickly poured in. The container was sealed and stirred at 55 °C for 8 days. The reaction vessel was cooled in dry ice before opening, and the contents were then dissolved in CH_2Cl_2 (100 mL) and washed with 5% ammonium chloride solution (2 × 30 mL) and then water (2 × 30 mL). The organic layer was dried over sodium sulfate and evaporated to dryness. The residual material was purified by using silica gel flash chromatography twice. The first column was washed with 1% Et_3N in $CHCl_3$, whereas the second column was eluted with EtOAc–hexane (50:50) followed by EtOAc (gradient mobile phase). The purified material finally was crystallized from EtOAc at the temperature of dry ice. Yield 69% (1.5 g); mp 66–70 °C; TLC: R_f 0.38 (CH_3Cl–Et_3N, 95:5). ^{1}H NMR ($CDCl_3$): δ 7.21 (d, J = 8 Hz, 1H, H-6), 6.13 (t, J = 7 Hz, 1H, H-1′), 5.45 (d, J = 8 Hz, 1H, H-5), 4.25 (m, 1H, H-4′), 4.15 (t, J = 5Hz, 2H, NCH_2-), 3.62 (m, 4H, H-5′, $HOCH_2$-), 3.59 (m, 1H, H-3′), 2.07 (m, 1H, H-2′β), 1.90 (m, 1H, H-2′α), 0.78 (s, 9H, $SiC(CH_3)_3$), 0.77 (s, 9H, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$), 0.01 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR: δ 160.4, 150.60, 131.90, 101.31, 87.31, 85.30, 71.34, 62.56, 62.53, 45.46, 41.28, 25.62, 18.25, −5.58. FAB/MS: m/z 500 [(M + H)$^+$, 24.6], 182 (13.7), 156 (19.4), 112 (8.3), 93 (22.9). Anal. Calcd for

$C_{23}H_{45}N_3O_5Si_2$: C, 55.27; H, 9.08; N, 8.41. Found: C, 55.51; H, 9.25; N, 8.27.

3-(2-Hydroxyethyl)-2′-deoxycytidine (4). Compound **14** (50 mg) was dissolved in a THF solution (0.1 mL) of 1 M TBAF with cooling in an ice bath. The reaction solution was stirred at room temperature for 30 min, after which the THF was removed by evaporation. The residue was purified by silica gel flash chromatography using MeOH–CH_2Cl_2 (10:90) as the mobile phase. The final product was crystallized from ethanol. Yield 95% (25 mg); ^{1}H NMR: δ 7.08 (d, J = 8 Hz, 1H, H-6), 6.14 (t, J = 7 Hz, 1H, H-1′), 5.77 (d, J = 8 Hz, 1H, H-5), 5.23 (s, 1H, 3′-OH), 4.98 (s, 1H, 5′-OH), 4.32 (m, 1H, H-4′), 3.98 (t, J = 5 Hz, 2H, NCH_2-), 3.74 (m, 4H, H-5′, -OCH_2-), 3.51 (m, 2H, H-3′, HOCH), 2.05 (m, 2H, H-2′). FAB/MS: m/z 272 [$(M + H)^+$, 25.2], 156 [(heterocyclic base + 2H)$^+$, 100). UV: λ_{max} = 278 nm, ϵ = 11 000.

3-(2-Hydroxyethyl)-2′-deoxyuridine (5). Compound **4** (1mg) was dissolved in Tris-HCl buffer (0.1 mL, 0.05 M, pH 8) and incubated at 37 °C for 10 h. The reaction mixture then was injected onto a reversed-phase HPLC using a μ-Bondapak C_{18} (0.39 × 30 cm, Waters) column. The mobile phase was water and CH_3CN (0–30% of organic gradient in 30 min). FAB/MS: m/z 273 [$(M + H)^+$, 31.4], 157 [(heterocyclic base + 2H)$^+$, 100). UV: λ_{max} = 262 nm, ϵ = 9 500.

3-[2-(Benzoyloxy)ethyl]-*N*^{4}-benzoyl-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (15). 3-(2-Hydroxyethyl)-3′,5′-bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (0.5 g) was dissolved in pyridine (3 mL). The solution was cooled in an ice bath, and there was slowly added 0.5 mL of benzoyl chloride. The solution was warmed slowly to room temperature and stirred for 1 h, then cooled in an ice bath to 5 °C. Methylene chloride (20 mL) was added together with 5 mL of concentrated NH_3. The solution was stirred for a further 15 min, the solvents were evaporated to dryness, and the residue was then passed through a silica gel column using hexane–EtOAc (90:10) as the mobile phase. The final product was a water-white oil. Yield 94% (0.65 g); TLC: R_f 0.61 (EtOAc); ^{1}H NMR ($CDCl_3$): δ 8.05–7.30 (m, 10H, ArH), 7.64 (d, J = 8 Hz, 1H, H-6), 6.52 (d, J = 8 Hz, 1H, H-5), 6.19 (t, J = 7 Hz, 1H, H-1′), 4.61 (m, 4H, NCH_2-CH_2O), 4.31 (m, 1H, H-4′), 3.70 (m, 3H, H-5′, H-3′), 2.08 (m, 1H, H-2′β), 1.95 (m, 1H, H-2′α), 0.82 (s, 9H, $SiC(CH_3)_3$), 0.80 (s, 9H, $SiC(CH_3)_3$), 0.02 (s, 6H, $Si(CH_3)_2$), 0.01 (s, 6H, $Si(CH_3)_2$); ^{13}C NMR: δ 176.94, 166.33, 156.62, 150.03, 136.39, 136.25, 132.77, 131.15, 130.00, 129.67, 129.58, 128.16, 128.05, 98.19, 87.73, 85.95, 71.05, 62.28, 61.62, 41.79, 41.65, 25.68, 17.88, −5.58.

3-(2-Benzoyloxyethyl)-*N*^{4}-benzoyl-2′-deoxycytidine (16). 3-[2-(Benzoyloxy)ethyl]-*N*^{4}-benzoyl-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxycytidine (0.5 g) was dissolved in a THF solution (0.5 mL) of 1 M tetrabutylammonium fluoride, with cooling in an ice bath. The reaction solution was stirred at room temperature for about 2 h, after which the THF was removed by evaporation. The residue was purified by the silica gel flash chromatography with EtOAc as the eluant, and the crude product was crystallized from EtOAc/petroleum ether. Yield 94% (0.31 g); mp 114–120 °C; TLC: R_f 0.30 (EtOAc); ^{1}H NMR $CDCl_3$): δ 8.07–7.32 (m, 10H, ArH), 7.63 (d, J = 8 Hz, 1H, H-6), 6.55 (d, J = 8 Hz, 1H, H-5), 6.17 (t, J = 7 Hz, 1H, H-1′), 4.60 (m, 4H, NCH_2CH_2O), 4.31 (m, 1H, H-4′), 3.73 (m, 3H, H-5′, H-3′), 2.10 (m, 1H, H-2′β), 1.95 (m, 1H, H-2′α); ^{13}C NMR: δ 176.96, 166.44, 156.59, 150.07, 136.42, 136.29, 132.81, 131.19, 130.04, 129.71, 129.62, 128.20, 128.09, 98.23, 87.77, 85.98, 71.11, 62.33, 61.66, 41.81, 41.68. Anal. Calcd for $C_{25}H_{25}N_3O_7$: C, 62.62; H, 5.26; N, 8.76. Found: C, 62.39; H, 5.54; N, 8.74.

3-[2-(Benzoyloxy)ethyl]-*N*^{4}-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (17). 3-[2-(Benzoyloxy)-ethyl]-*N*^{4}-benzoyl-2′-deoxycytidine (1 g, 4 mmol) and 4,4′-dimethoxytrityl chloride (1.8 g, 1.2 equiv) were dissolved in pyridine (10 mL). The reaction mixture (a thick yellow solution) was stirred at room temperature for 4 h (the reaction was almost complete after 1 h; TLC analysis). There was then added 50 mL of water, and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, washed with water (30 mL) and buffer solution (10 mL, 5% potassium phosphate, pH 7), dried over sodium sulfate, and evaporated to dryness under reduced pressure. The residue was purified by silica gel flash chromatography using EtOAc–hexane (15:85) as the eluant followed by EtOAc–hexane (95:5). The desired product was obtained as a noncrystalline white foam. Yield 92% (1.5 g); mp 85–91 °C; TLC: R_f 0.45 (EtOAc); ^{1}H NMR $CDCl_3$): δ 8.10–7.30 (m, 20H, ArH, H-6), 6.80 (d, J = 6 Hz, 4H, H ortho to OCH_3), 6.29 (d, J = 8 Hz, 1H, H-5), 6.17 (t, J = 7 Hz, 1H, H-1′), 4.61 (m, 4H, NCH_2CH_2O), 4.37 (m, 1H, H-4′), 3.75 (m, 3H, H-5′, H-3′), 3.70 (s, 6H, OCH_3), 2.15 (m, 1H, H-2′β), 1.95 (m, 1H, H-2′α); ^{13}C NMR: δ 177.13, 165.51, 158.78, 156.10, 150.05, 144.17, 137.71, 136.14, 136.01, 135.46, 132.87, 130.05, 129.72, 129.50, 129.14, 128.63, 128.16, 127.36, 127.16, 127.10, 113.36, 98.30, 87.36, 87.06, 85.88, 70.97, 62.71, 61.63, 55.31, 41.77, 47.17. Anal. Calcd for $C_{46}H_{43}N_3O_9$: C, 70.67; H, 5.54; N, 5.37. Found: C, 70.50; H, 5.66; N, 5.33.

3′-*O*-[(Diisopropylamino)(2-cyanoethoxy)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-3-[2-(benzoyloxy)ethyl]-*N*^{4}-benzoyl-2′-deoxycytidine (18). 3-(2-Benzoyloxyethyl)-*N*^{4}-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine (150 mg) and 0.2 mL of Et_3N were dissolved in CH_2Cl_2 (2 mL). Under the protection of a nitrogen atmosphere, 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite reagent (0.2 g, 1.5 equiv) was slowly added to the solution, and the reaction mixture was stirred at room temperature for 1 h. The progress of the reaction was monitored by TLC (CH_2Cl_2 + 5% Et_3N as the mobile phase). After the reaction was complete, the mixture was evaporated to dryness, and 30 mL of benzene was then added. The benzene solution was filtered to remove the insoluble salt (Et_3N·HCl) and then evaporated, and the desired phosphoramidite product was obtained as a light yellow oil. Yield >99% (195 mg); TLC: R_f 0.95 (CH_3Cl–Et_3N, 95:5); ^{1}H NMR ($CDCl_3$): δ 8.11–7.31 (m, 20H, (m, 20H, ArH, H-6), 6.81 (d, J = 6 Hz, 4H, H ortho to OCH_3), 6.28 (d, J = 8 Hz, 1H, H-5), 6.15 (t, J = 7 Hz, 1H, H-1′), 4.61 (m, 4H, NCH_2CH_2O), 4.20 (m, 3H, H-4′, OCH_2), 3.75 (m, 3H, H-5′, H-3′), 3.70 (s, 6H, OCH_3), 3.44 (m, 2H, 2CHN), 2.76 (t, J = 6.0 Hz, 2H, CH_2CN), 2.09 (m, 1H, H-2′β), 1.88 (m, 1H, H-2′α), 1.23 (dd, 12H, $C(Pr^i)_2$); ^{31}P NMR (121 MHz, $CDCl_3$): δ 147.4 and 147.2 (diastereomeric pair). This material was used in the DNA automated synthesizer directly (incorporation efficiency >85%).

Synthesis of Oligodeoxynucleotides on a Solid Support. The syntheses of the oligodeoxynucleotides containing one of the modified nucleosides **1, 2**, or **3**, located at a predesignated position, were performed on a CPG support using a fully automated DNA synthesizer and commercially available, conventionally-protected deoxynucleoside 2-cyanoethyl phosphoramidites. The syntheses of all these oligonucleotides were carried out a 1 μmol scale. The synthetic cycle consisted essentially of detritylation (2% dichloroacetic acid in CH_2Cl_2), condensation (phosphoramidite derivative and tetrazole in CH_3CN), capping (Ac_2O lutidine and *N*-methylimidazole), and oxidation (I_2 and H_2O in THF) stages. The coupling efficiency of the modified DMT phosphoramidite in the syntheses of oligodeoxynucleotides was measured by the absorbance of the effluent DMT cation solution in CH_2Cl_2. Upon completion of the synthesis, all base-labile protective groups on the oligodeoxynucleotide were removed by heating with concentrated ammonia at 55 °C for 15 h. This led to the 5′-terminal-O-DMT oligodeoxynucleotides which were then purified by reversed-phase HPLC. Thereafter, the terminal 5′-DMT group was removed by treatment with 80% acetic acid for 30 min at room temperature. The oligomers were further purified first by electrophoresis on 20% polyacrylamide gel in the presence of 7 M urea and then by a second HPLC purification. The oligomers at this stage were found to be homogeneous as judged by gel electrophoresis.

The oligomers containing 3HE-dUrd were further hydrolyzed in the aqueous solution at pH 8.0, 37 °C, for 30 h before the purification procedures. This allowed the complete conversion of all 3HE-dCyd residues to the corresponding 3HE-dUrd residues (*12, 26*). The structures and sequences of the oligomers were then further confirmed by enzymatic degradation and electrospray mass spectroscopy. These analyses are discussed in detail in the discussion sections above dealing with 3HE-dCyd and 3HE-dUrd.

Base Composition Analysis. The base compositions of the oligodeoxynucleotides with respect to their individual nucleosides were checked by enzymatic hydrolysis using the following procedure (*27*). To the oligodeoxynucleotide (3.0 μg) in 100 μL of sodium acetate buffer (0.03 M, pH 5.3) containing 2-mercaptoethanol (10 mM) were added 5 μL of zinc sulfate solution (20 mM) and 2 units of nuclease P1 (Boehringer, W. Germany). The mixture was incubated for 2 h at 37 °C, and thereafter the pH was adjusted to 8.5 by the addition of 20 μL of Tris-HCl (0.5 M) The sample then was reincubated at 37 °C with 3 units of bacterial alkaline phosphatase (Sigma, St. Louis, MO) for an additional 2 h. Samples thus obtained were heated in boiling water for 3 min, evaporated in a vacuum desiccator, then extracted with MeOH (2 × 350 μL), and dried. For analysis, the residue was dissolved in 30 μL of water and then injected directly onto an HPLC column (μ-Bondapak C_{18} 0.39 × 30 cm, Waters).

Acknowledgment. This work was supported by NIH Grant CA4799504 awarded by the National Cancer Institute. We are grateful to Ms. Peilin Yu and Dr. V. Bodapudi for their experimental assistance. We also thank Drs. A. P. Grollman and S. Shibutani for their interest in this program.

References

(1) Bartsch, H., and Montesano, R. (1975) Mutagenic and Carcinogenic Effects of Vinyl Chloride. *Nucleic Acids Res.* **3,** 93–113.

(2) Creech, J. L., and Johnson, M. N. (1974) Angiosarcoma of Liver in the Manufacture of Polyvinyl Chloride. *J. Occup. Med.* **16,** 150–151.

(3) Huberman, E., Bartsch, H., and Sachs, L. (1975) Mutation Induction in Chinese Hamster V79 Cells by Two Vinyl Chloride Metabolites, Chloroethylene Oxide and 2-Chloroacetaldehyde. *Int. J. Cancer* **16,** 639–644.

(4) Oesch, F., and Doerjer, G. (1982) Detection of N^2,3-Ethenoguanine in DNA after Treatment with Chloroacetaldehyde in vitro. *Carcinogenesis* **3,** 663–665.

(5) Malaveille, C., Bartsch, H., Montesano, R., Barbin, A., and Camus, A. M. (1975) Mutagenicity of Vinyl Chloride, Chloroethylene Oxide, Chloroacetaldehyde and Chloroethanol. *Biochem. Biophys. Res. Commun.* **63,** 363–370.

(6) Scherer, E., Van Der Laker, C. J., Gwinner, L. M., and Laib, R. J. (1981) Modification of Deoxyguanosine by Chloroethylene Oxide. *Carcinogenesis* **2,** 671–677.

(7) Kohn, K. W., and Gibson, N. W. (1986) DNA Cross-link by Chloroethylating Agents. In *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Singer, B., and Bartsch, H., Eds.) pp 155-162, IARC Scientific Publications, Lyon, France.

(8) Ludlum, D. B. (1986) Formation of Cyclic Adducts in Nucleic Acids by the Haloethylnitrosoureas. In *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis* (Singer, B., and Bartsch, H., Eds.) pp 137-146, IARC Scientific Publications, Lyon, France.

(9) Parker, S., Kirk, M. C., Ludlum, D. B., Koganty, R. R., and Lown, J. W. (1986) Reaction of 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) with Guanosine: Evidence for a New Mechanism of DNA Modification. *Biochem. Biophys. Res. Commun.* **139,** 31–36.

(10) Montgomery, J. A. (1981) The Chemistry of the Nitrosoureas. In *Nitrosoureas in Cancer Treatment* (Serrou, B., Schein, P.S., and Imbach, J.-L., Eds.) pp 13-20, Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands.

(11) Tong, W. P., Kohn, K. W., and Ludlum, D. B. (1982) Modifications of DNA by Different Haloethylnitrosoureas. *Cancer Res.* **42,** 4460–4464.

(12) Solomon, J. J., and Segal, A. (1989) DNA Adducts of Propylene Oxide and Acrylonitrile Epoxide: Hydrolytic Deamination of 3-Alkyl-dCyd to 3-Alkyl-dUrd. *Environ. Health Perspect.* **81,** 19–22.

(13) Solomon, J. J., Li, F., Mukai, F., and Segal, A. (1991) Characterization and Quantitation of in vitro DNA Adducts of Ethylene Oxide, Hydroxyethylation at N-3 of Cytosine Results in a Rapid Hydrolytic Deamination to a Uracil Adduct. *Environ. Mol. Mutagen.*, Abstract 246.17.

(14) Solomon J. J., Decker-Samuelian, K., Li, F., Mukai, F., and Segal, A. (1990) On the Rapid Hydrolytic Deamination of Epoxide-Generated 3-Hydroxyalkyl Cytosine to 3-Hydroxyalkyl Uracil. Proceedings of the 38th ASMS Conference on Mass Spectrometry and Allied Topics Tucson, AZ.

(15) Barrio, J., Secrist, J., III, and Leonard, N. (1972) Fluorescent Adenosine and Cytidine Derivatives. *Biochem. Biophys. Res. Commun.* **46,** 597–604.

(16) Caruthers, M. H. (1987) DNA synthesis for Nonchemists: The Phosphoramidite Method on Silica Support. In *Synthesis and Applications of DNA and RNA* (Narang, S. A., Eds.) pp 47-89, Academic Press, Orlando, FL.

(17) Schaller, H., Lerch, G. W., and Khorana, H. G. (1963) Studies on Polynucleotides. XXIV. The Stepwise Synthesis of Specific Deoxyribopolynucleotides (4). Protected Derivatives of Deoxyribonucleosides and New Synthesis of Deoxyribonucleoside-3′ Phosphates. *J. Am. Chem. Soc.* **85,** 3821–3827.

(18) Barone, A. D., Tang, J., and Caruthers, M. H. (1984) In situ Activation of Bis-Dialkylaminophosphines—A New Method Synthesizing Deoxyoligonucleotides on Polymer Support. *Nucleic Acids Res.* **12,** 4051–4061.

(19) Beaucage, S. L., and Caruthers, M. H. (1981) Deoxynucleoside Phosphoramidites—A New Class of Key Intermediates for Deoxypolynucleotide Synthesis. *Tetrahedron Lett.* **22,** 1859–1862.

(20) Dosanjh, M. K., Chenna, A., Kim, E., Fraenker-Conrat, H., Samson, L., and Singer, B. (1994) All Four Known Cyclic Adducts Formed in DNA by the Vinyl Chloride Metabolite Chloroacetaldehyde are Released by a Humna DNA Glycosylase. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 1024–1028.

(21) Gombar, C. T., Tong, W. P., and Ludlum, D. B. (1980) Mechanism of Action of the Nitrosoureas-IV, Reaction of Bis-chloroethyl Nitrosourea and Chloroethyl Nitrosourea with Deoxyribonucleic Acid. *Biochem. Pharmacol.* **29,** 2639–2643.

(22) Marinelli, E. R., Johnson, F., Iden, C. R., and Yu, P. (1990) Synthesis of 1,N^2-(1,3-Propano)-2′-deoxyguanosine and Incorporation into Oligodeoxynucleotides: A Model for Exocyclic Acrolein–DNA Adducts. *Chem. Res. Toxicol.* **3,** 49–58.

(23) Maggio, A. F., Lucas, M., Barascut, J. L., Pompon, A., and Imbach, J. L. (1986) Synthese Regiospecifique de Dinucleosides Pontes: Contribution a L'etude du Mecanisme D'action des Nitrosourees (Regioselective synthesis of linked dinucleosides: reaction mechanism of nitrosoureas). *Nouv. J. Chim.* **10,** 643–650.

(24) Sung, W. (1981) Chemical Conversion of Thymidine into 5-Methyl-2′-deoxycytidine. *J. Chem. Soc., Chem. Commun.* 1089.

(25) Lin, T., Gao, Y., and Mancini, R. (1983) Synthesis and Biological Activity of Various 3′-Azido and 3′-Amino Analogues of 5-Substituted Pyrimidine Deoxyribonucleosides. *J. Med. Chem.* **26,** 1691–1696.

(26) Li, F., Segal, A., and Solomon, J. J. (1992) In Vitro Reaction of Ethylene Oxide with DNA and Characterization of DNA Adducts. *Chem.-Biol. Interact.* **83,** 35–54.

(27) Sowers, L., Sedwick, W. D., and Shaw, B. (1989) Hydrolysis of N^3-methyl-2′-deoxycytidine: Model compound for reactivity of protonated cytosine residues in DNA. *Mutat. Res.* **215,** 131–138.

(28) Gehrke, C. W., McCune, R. A., Gama-Sosa, M., Ehrlich, M., and Kuo, K. C. (1984) Quantitative Reversed-phase High Performance Liquid Chromatography of Major and Modified Nucleosides in DNA. *J. Chromatogr.* **301,** 199–219.

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