Synthesis and Biochemical Properties of Cyanuric Acid Nucleoside-Containing DNA Oligomers

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1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid (cyanuric acid nucleoside, dY) (**1**) has been shown to be formed upon exposure of DNA components to ionizing radiation and excited photosensitizers. To investigate the biological and structural significance of dY residue in DNA, the latter modified 2′-deoxynucleoside was chemically prepared and then site-specifically incorporated into oligodeoxyribonucleotides (ODNs). This was achieved in good yields using the phosphoramidite approach. For this purpose, a convenient glycosylation method involving 3,5-protected 2-deoxyribofuranoside chloride and cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine) was devised. The anomeric mixture of modified 2′-deoxyribonucleosides (1/2 α/β) was resolved by silica gel purification of the 5′-*O*-dimethoxytritylated derivatives, and then, phosphitylation afforded the desired β-phosphoramidite monomer (**5**). After solid-phase condensation and final deprotection, the purity and the integrity of the modified synthetic DNA fragments were checked using different complementary techniques such as HPLC and polyacrylamide gel electrophoresis, together with electrospray ionization and MALDI-TOF mass spectrometry. The presence of cyanuric acid nucleoside in a 14-mer was found to have destabilizing effects on the double-stranded DNA fragment as inferred from melting temperature measurements. The piperidine test applied to dY-containing ODNs supported the high stability of cyanuric acid nucleoside inserted into the oligonucleotide chain. Several enzymatic experiments aimed at determining the biological features of such a DNA lesion were carried out. Thus, processing of dY by nuclease P_1, snake venom phosphodiesterase (SVPDE), calf spleen phosphodiesterase (CSPDE), and repair enzymes, including *Escherichia coli* endonuclease III (endo III) and Fapy glycosylase (Fpg), was investigated. Finally, a 22-mer ODN bearing a cyanuric acid residue was used as a template to study the in vitro nucleotide incorporation opposite the damage by the Klenow fragment of *E. coli* polymerase I.

Introduction

Several studies have established that 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo),[1] which may result from hydroxylation of the C8 position of 2′-deoxyguanosine (dGuo), is the major stable modified nucleoside present in oxidized DNA. The structural and biological features of 8-oxodGuo have been widely studied during the past decade (*1*−*5*). Interestingly, 8-oxodGuo is currently used as a biomarker of DNA oxidation induced by both endogenous (cellular metabolism, oxidative stress, etc.) and exogenous (ionizing radiation, photosensitizers, xenobiotics, etc.) agents (*6*, *7*). Several studies have shown that 8-oxodGuo, which exhibits a lower ionization potential than the normal DNA nucleosides, is highly reactive toward oxidizing reagents (*8*−*11*). This suggests that the latter nucleoside may also be sensitive to in vivo DNA oxidation. Recently, 8-oxodGuo has been found to be an efficient substrate of photosensitized reactions which give rise to a set of secondary products. Thus, at the nucleoside level, it was shown that the major product of singlet oxygen oxidation (type II mechanism) was the cyanuric acid nucleoside (**1**) (*12*, *13*). Although the latter model studies provided a large amount of information about the structure and the mechanism of formation of **1**, the presence of dY has not been established so far at the DNA level.

In this paper, we report the first synthesis of cyanuric acid nucleoside (**1**) and its site-specific insertion into several oligodeoxyribonucleotides using phosphoramidite chemistry. The modified DNA oligomers were used to determine the hybridization properties of **1** upon substitution of the guanine parent base. In addition, information about biochemical features, including enzymatic cleavage, repair, and mutagenicity, is provided.

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[1] Abbreviations: dY, 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)cyanuric acid; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ODN, oligodeoxyribonucleotide; DMTrCl, 4,4′-dimethoxytrityl chloride; TFA, trifluoroacetic acid; CSPDE, calf spleen phosphodiesterase; SVPDE, snake venom phosphodiesterase; FAB-MS, fast atom bombardment mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; endo III, endonuclease III; Fpg, formamidopyrimidine DNA glycosylase.

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Materials and Methods

General Procedures and Materials. 2,4,6-Trihydroxy-1,3,5-triazine (cyanuric acid) was from Fluka (Buchs, Switzerland). The silica gel (70−200 μm) used for the low-pressure column chromatography was purchased from SDS (Peypin, France). TLC was carried out on Merck DC Kieselgel 60 F-254 plastic sheets (Darmstadt, Germany). Acetonitrile and methanol (HPLC grade) were obtained from Carlo Erba (Milan, Italy). Buffers for high-performance liquid chromatography (HPLC) were prepared using water purified with a Milli-Q system (Milford, MA). The porous graphitized Hypercarb carbon column (98.5% carbon, particle size of 5 μm, porosity of 250 Å, 100 mm × 3 mm i.d.) was from Shandon (Runcorn, Cheshire, U.K.), whereas the Hypersil ODS column (5 μm, 4.6 mm × 250 mm i.d.) was purchased from Interchim (Montluçon, France). Unmodified deoxyribonucleoside phosphoramidites, protected with phenoxyacetyl for dAdo, isopropylphenoxyacetyl for dGuo, and acetyl for dCyd, were from Glen Research (Sterling, VA). Functionalized CPG supports, involving the phenoxyacetyl protective group for dAdo and dGuo and the isobutyryl protective group for dCyd, were purchased from Sigma (St. Louis, MO). 8-OxodGuo-containing oligonucleotide was prepared using a commercial phosphoramidite monomer from Glen Research.

Enzymes. Calf spleen phosphodiesterase (CSPDE) and snake venom phophodiesterase (SVPDE) were purchased from Boehringer Mannheim (Mannheim, Germany). Fpg and endonuclease III were kind gifts from S. Boiteux (CEA, Fontenay-aux-Roses, France). Nuclease P_1 (*Penicillium citrium*) and phosphatase alkaline were purchased from Sigma. T_4 polynucleotide kinase was obtained from Pharmacia Biotech (Uppsala, Sweden). Klenow fragment (exo$^-$) of *Escherichia coli* DNA polymerase I was from Amersham (Buckinghamshire, U.K.).

Mass Spectrometry Measurements. FAB (fast atom bombardment) mass spectrometry analyses were carried out on a VG ZAB 2-EQ apparatus (Manchester, U.K.). The samples were dissolved in either a glycerol or NBA matrix prior to be analyzed.

All modified and unmodified oligonucleotides were characterized by electrospray ionization mass spectrometry measurements (ESI-MS) on a Micromass Platform model 3000 spectrophotometer (Manchester, U.K.). Typically, 0.1 OD of the sample was dissolved in a solution of acetonitrile and water (50/50, v/v) containing 1% triethylamine prior to be analyzed in the negative mode. The modified nucleoside dY and its 5′-DMTr-protected derivative were analyzed by ESI-MS in both the negative and positive modes. For the positive mode analysis, the sample was dissolved in a solution of acetonitrile and water (50/50, v/v) that contained 0.5% formic acid.

MALDI mass spectra were obtained with a commercially available time-of-flight mass spectrometer (Voyager-DE, Perseptive Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser and a pulsed delay source extraction. Spectra were recorded from 256 laser shots with an accelerating voltage of 25 kV in the linear and positive modes. For the matrix, a mixture of 3-hydroxypicolinic acid and picolinic acid in a 4/1 (w/w) ratio was dissolved in an aqueous acetonitrile solution (50%) that contained 0.1% TFA and a small amount of Dowex-50W 50X8-200 cation-exchange resin (Sigma). Typically, 1 μL of the sample was added to 1 μL of the matrix, and the resulting solution was stirred. The resulting sample was then placed on the top of the target plate and allowed to dry by itself. The spectra were calibrated with a 1 pmol/μL solution of myoglobin (*m*/*z* 16 952), using the same conditions that were described for the analysis of oligonucleotides.

Labeling of Oligonucleotides. Oligonucleotides (10 pmol) were labeled at the 5′-terminus with 10 μCi of [γ-^{32}P]ATP (2 pmol, 10 mCi/mL) from Amersham upon incubation with T_4 polynucleotide kinase (9.5 units) in 10 μL of supplied buffer at 37 °C for 30 min. The reaction was quenched by addition of 1 μL of a 0.5 M EDTA solution (pH 8). Unincorporated [γ-^{32}P]-ATP was removed by purification of the oligonucleotide on a MicroSpin column (Pharmacia Biotech).

Synthetic Procedures. (1) 1-(2-Deoxy-α,β-D-*erythro*-pentofuranosyl)cyanuric Acid (1 and 2). A mixture of 2,4,6-trihydroxy-1,3,5-triazine (330 mg, 2 mmol), 2-deoxy-3,5-di-*O*-*p*-toluoyl-D-*erythro*-pentofuranosyl chloride (*14*) (800 mg, 2 mmol), potassium nonafluorobutanesulfonate (1625 g, 2.4 equiv), hexamethyldisilazane (0.295 mL, 0.8 equiv), and trimethylsilyl chloride (0.785 mL, 3.1 equiv) was refluxed in 20 mL of anhydrous acetonitrile for 16 h, under conditions of moisture exclusion. The course of the reaction was followed by TLC ($CHCl_3$/MeOH, 90/10, v/v). After evaporation of the solvents to dryness, the residue was taken up in $CHCl_3$ and washed successively with saturated $NaHCO_3$ and water. The major products corresponding to the mixture of α and β anomers of the 3′,5′-diprotected nucleoside (R_f = 0.48) were not isolated, but directly submitted to alkaline deprotection. Thus, the glycosylation mixture was placed in a 1% NaOH methanol solution (50 mL) which was stirred for 1 h at room temperature. Solvents were removed by evaporation, and the resulting residue was dissolved in water (50 mL), prior to being washed with diethyl ether (50 mL) and chloroform (50 mL). The two anomers **1** and **2** were then purified on a short silica gel column that was eluted with a step gradient of MeOH in $CHCl_3$ (from 0 to 20%): yield of 270 mg (55%); 1/2 α/β (from integration of the HPLC and NMR peaks area of the anomeric mixture); R_f = 0.53 (70/30 $CHCl_3$/MeOH); λ_{max} (H_2O, pH 7) 220 nm (shoulder); ESI-MS (negative mode) *m*/*z* 244.22 [M − H]$^-$ (calcd mass 245.19).

For β anomer **1**: ^{1}H NMR (400.13 MHz, D_2O) δ 11.39 (s, 2H, NH), 6.64 (dd, J = 5.3 and 8.6 Hz, 1H, H-1′), 4.63 (m, 1H, H-3′), 4.02 (m, 1H, H-4′), 3.93 (m, 1H, H-5′), 3.85 (m, 1H, H-5″), 2.96 (m, 1H, H-2′), 2.35 (m, 1H, H-2″); ^{13}C NMR (100.61 MHz, DMSO-d_6) δ 149.50 (2C, C-2 and C-6), 148.70 (1C, C-4), 87.59 (1C, C-4′), 82.04 (1C, C-1′), 71.09 (1C, C-3′), 62.46 (1C, C-5′), 36.85 (1C, C-2′).

For α anomer **2**: ^{1}H NMR (400.13 MHz, D_2O) δ 11.39 (s, 2H, NH), 6.55 (pseudo-t, J = 7.6 and 7.5 Hz, 1H, H-1′), 4.43 (m, 1H, H-3′), 4.40 (m, 1H, H-4′), 3.91 (m, 1H, H-5′), 3.77 (m, 1H, H-5″), 2.77 (m, 1H, H-2′), 2.72 (m, 1H, H-2″); ^{13}C NMR (100.61 MHz, DMSO-d_6) δ 149.69 (2C, C-2 and C-6), 148.70 (1C, C-4), 85.63 (1C, C-4′), 81.74 (1C, C-1′), 70.40 (1C, C-3′), 61.50 (1C, C-5′), 36.32 (1C, C-2′).

(2) 1-[2-Deoxy-5-(4,4′-dimethoxytrityl)-α,β-D-*erythro*-pentofuranosyl]cyanuric Acid (3 and 4). The anomeric mixture of compounds **1** and **2** (0.41 mmol, 100 mg) was dried by repeated coevaporation with anhydrous pyridine and then dissolved in pyridine (10 mL). The solution was cooled in an ice/water bath and kept under an argon atmosphere. Then, 4,4′-dimethoxytrityl chloride (DMTrCl, 195 mg, 1.4 equiv) was added under stirring. After 1 h, the cooling bath was removed and the reaction was continued at room temperature overnight. Subsequently, 1 mL of methanol was added and the resulting solvent mixture was evaporated. The oily residue was dissolved in ethyl acetate (50 mL) and washed with a saturated $NaHCO_3$ aqueous solution (50 mL) and water (50 mL). The organic phase that contained the two 5′-protected anomers **3** and **4** was dried over Na_2SO_4 and evaporated under reduced pressure. The mixture of α and β anomers was resolved by silica gel column chromatography (0 to 5% step gradient of methanol in $CHCl_3$ in the presence of 1% triethylamine) to afford the title compounds **3** and **4** in a yield of 40.5 and 19%, respectively (1/2 α/β). One-dimensional TLC analysis was performed using $CHCl_3$/MeOH (90/10, v/v) with 1% TEA as the solvent. The R_f values were 0.63 for the α-anomer **4** and 0.52 for the β-anomer **3**: ESI-MS (negative mode) *m*/*z* 546.09 [M − H]$^-$ (calcd mass 547).

For β anomer **3** (91 mg): ^{1}H NMR (400.13 MHz, acetone-d_6) δ 7.62−6.85 (m, 12H, aromatic H of DMTr), 6.56 (dd, J = 4.6 and 8.9 Hz, 1H, H-1′), 4.49 (m, 1H, H-3′), 3.98 (m, 1H, H-4′), 3.81 (s, 6H, OCH_3), 3.39 (m, 1H, H-5′), 3.23 (m, 1H, H-5″), 2.76 (m, 1H, H-2′), 2.21 (m, 1H, H-2″).

For α anomer **4** (43 mg): ^{1}H NMR (400.13 MHz, acetone-d_6) δ 7.60−6.78 (m, 12H, aromatic H of DMTr), 6.49 (pseudo-t, 1H, H-1′), 4.44 (m, 1H, H-3′), 4.42 (m, 1H, H-4′), 3.80 (s, 6H, OCH_3),

3.36 (m, 1H, H-5′), 3.21 (m, 1H, H-5″), 2.63 (m, 1H, H-2′), 2.57 (m, 1H, H-2″).

(3) 1-{2-Deoxy-3-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-5-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl}cyanuric Acid (5). Compound **3** (0.145 mmol, 80 mg) was coevaporated twice with dry pyridine and subsequently dissolved in anhydrous CH_2Cl_2 (2 mL) under an argon atmosphere. Diisopropylammonium tetrazolate (13 mg, 0.075 mmol) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyldiamidite (50 μL, 0.16 mmol) were added to the solution under stirring. The reaction was monitored by TLC (95/5/1 CH_2Cl_2/MeOH/TEA). After 2 h at room temperature, the mixture was diluted with ethyl acetate (25 mL) and washed with a saturated $NaHCO_3$ aqueous solution (30 mL). The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The resulting residue was purified by flash chromatography on a silica gel column with a step gradient of methanol (0 to 3%) in $CHCl_3$/TEA (99/1, v/v) as the mobile phase. Then, the collected fractions corresponding to the two diastereomers were dried to afford compound **5** as a white foam (yield of 68%, 0.098 mmol, or 73 mg): $R_f = 0.25$ (95/5/1 CH_2Cl_2/MeOH/TEA); ^{31}P NMR (101.21 MHz, CD_3COCD_3) δ 149.53 and 149.25 (two diastereoisomers); FAB-MS (negative mode) *m*/*z* 745.9 $[M - H]^-$.

Stability Studies of 1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)cyanuric Acid (1) under the Alkaline Conditions Used for Chemical Synthesis of ODNs. Aqueous ammonia (32%, 500 μL) was added to 0.5 AU_{230nm} of compound **1** (the same experiment was performed with the α-isomer **2**) in sealed tubes. The solutions were kept at either room temperature or 55 °C. Then, the reactions were stopped at increasing time intervals (0, 1, 2, 4, 8, 16, and 24 h) by freezing the samples in liquid nitrogen and subsequent lyophilization. Samples were analyzed by HPLC using a Hypercarb column. The elution was achieved with a 0 to 10% linear gradient of acetonitrile in 25 mM ammonium formiate buffer over the course of 30 min (flow rate of 0.5 mL/min; UV detection at 230 nm).

Stability Studies of 1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)cyanuric Acid (1) under the Acid Conditions Used for Chemical Synthesis of ODNs. A similar procedure, as described above for the alkali stability assay, was used. This involved incubation of compound **1** in an 80% acetic acid aqueous solution for 0, 1, 2, 4, 8, 16, and 24 h at room temperature.

Stability Studies of the 1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)cyanuric Acid (1) under Oxidizing Conditions Used for Chemical Synthesis of ODNs. Similarly, compound **1** was incubated in a 0.1 M oxidizing solution of iodine for 0, 1, 2, 4, 8, 16, and 24 h at room temperature.

Solid-Phase Synthesis of Oligodeoxyribonucleotides. The synthesis of cyanuric acid-containing oligodeoxyribonucleotides was performed at 1 μmol scale using an Applied Biosystems Inc. 392 DNA synthesizer, with retention of the 5′-terminal DMTr group (trityl-on mode). The standard 1 μmol DNA cycle was used with a slight modification. This consisted in increasing the duration of condensation by a factor of 4 for the modified nucleoside phosphoramidite **5** (120 s instead of 30 s for normal nucleoside phosphoramidites). Under these conditions, a coupling efficiency of more than 90% for the modified monomer **5** was achieved.

Deprotection and Purification of Oligodeoxyribonucleotides. Upon completion of the synthesis, the alkali-labile protecting groups of the oligodeoxyribonucleotides were removed by treatment with concentrated aqueous ammonia (32%) at room temperature for 4 h. Solvents were removed by evaporation under vacuum. Then, the crude 5′-DMTr oligomers were purified and deprotected on-line by reverse-phase HPLC using a polymeric support, as previously described (*15*). The modified 22-mer ODN **10**, used in repair and replication studies, was further purified by preparative polyacrylamide gel electrophoresis and, then, was desalted using a NAP-25 Sephadex column (Pharmacia).

Piperidine Treatment of the Cyanuric Acid-Containing Oligodeoxyribonucleotides. Oligonucleotides were treated with a freshly made 1 M piperidine aqueous solution at 90 °C for 30 min. The reactions were carried out on 0.01 OD of 5′-^{32}P-labeled modified oligodeoxyribonucleotides in 100 μL of the piperidine solution in sealed tubes. After cooling, the samples were coevaporated twice with water and then loaded onto a 20% polyacrylamide denaturing gel. The electrophoresis was carried out at 1300 V for 3 h, and subsequently, the gel was exposed to X-ray films.

Digestion of Modified ODNs by Nuclease P_1 and Alkaline Phosphatase. The modified trinucleotide d(TYT) (**6**) that contained a cyanuric acid residue at the central position (1 AU_{260nm}) was digested into nucleotides upon incubation for 2 h at 37 °C with 5 EU (enzyme units) of nuclease P_1 in a 30 mM NaOAc and 0.1 mM $ZnSO_4$ aqueous solution (pH 5.5) in a total volume of 50 μL. Then, 10 EU of calf intestinal alkaline phosphatase in 500 mM Tris and 1 mM EDTA (pH 8.5, 5 μL) was added, and the resulting mixture was further incubated for 1 h. The digestion mixture of 2′-deoxyribonucleosides was then analyzed by HPLC onto a Hypercarb column. The elution was performed with a 0 to 30% linear gradient of CH_3CN in 0.25 mM ammonium formiate buffer over 40 min at a flow rate of 0.5 mL/min (UV detection at 230 nm). The different collected products were analyzed by ESI-MS in the negative mode.

Enzymatic Digestion of ODNs by 3′- or 5′-Exonuclease followed by MALDI-TOF Mass Spectrometry Analysis. ODNs were precipitated twice in a 0.3 M ammonium acetate/ethanol (1/3, v/v) solution prior to the enzymatic digestions.

(1) Digestion by Calf Spleen Phosphodiesterase (5′-Exo). ODNs (0.1 AU_{260nm}) were incubated at 37 °C with 10^{-3} EU of calf spleen phosphodiesterase (2 units/mL) in 0.02 M ammonium citrate (pH 5) in a total volume of 30 μL. Aliquots (1.5 μL) were withdrawn after increasing periods of time, and the reactions were quenched by addition of 50 μL of water and subsequent freezing of the samples in liquid nitrogen. Then, the samples were lyophilized and analyzed by MALDI-TOF spectrometry following the conditions described above.

(2) Digestion by Snake Venom Phosphodiesterase (3′-Exo). ODNs (0.1 AU_{260nm}) and 3×10^{-4} EU of snake venom phosphodiesterase (3 units/mL) were added to 30 μL of 0.02 M ammonium citrate (pH 9). The resulting solution was incubated at 37 °C, and aliquots (1.5 μL) were withdrawn after increasing periods of time. The reactions were quenched by addition of 50 μL of water and the samples frozen in liquid nitrogen. The samples were lyophilized before being analyzed by MALDI-TOF spectrometry following the conditions described above.

Thermal Denaturation Studies. The melting experiments were carried out using 1 nmol of ODN duplex. Thus, the oligonucleotides [5′-d(ATC GT**X** ACT GAT CT)-3′] where **X** is G or Y (0.125 and 0.118 AU_{260nm}, respectively) and their complementary sequence [5′-d(AGA TCA GTC ACG AT)-3′] (0.125 AU_{260nm}) were mixed in 20 μL of a buffer that contained 0.01 M sodium phosphate, 0.1 M NaCl, and 1 mM EDTA (pH 7). The DNA fragments were annealed by heating at 90 °C for 3 min followed by slow cooling to 4 °C over the course of 3 h. The hybridization solutions were diluted with 400 μL of buffer. Then, UV absorbance measurements were taken in a 0.8 mL quartz cell (0.2 cm path length) with a UV/vis spectrophotometer equipped with a Peltier temperature controller. The absorbance of the sample was monitored at 260 nm from 15 to 85 °C at a heating rate of 1 °C/min. The reported data are the average of three melting curves per ODN duplex.

DNA Repair Studies of Cyanuric Acid by Fpg and Endonuclease III. The modified ODN **10** (10 pmol) was 5′-end-labeled using [γ-^{32}P]ATP and then purified using MicroSpin G-25 columns. Nonlabeled modified ODN **10** (95 pmol) and the complementary strand [5′-d(AGA TCA GTC ACG ATC CGA AGT G)-3′] (150 pmol) were added to afford a double-stranded DNA fragment that contained the lesion. The hybridization was performed in 10 μL of buffer, consisting of 20 mM Tris-HCl, 1 mM EDTA, and 100 mM KCl (pH 7.5). This was achieved by heating the resulting solution for 5 min at 80 °C and cooling slowly to 4 °C. Water (40 μL) was added, and the solution was

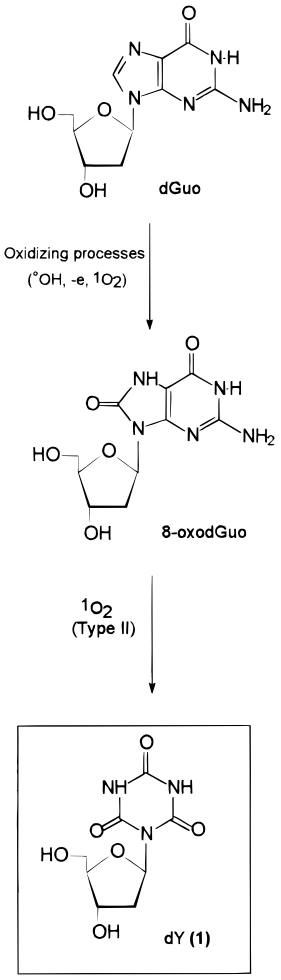
aliquoted (5 μL per reaction). Then, 5 μL of enzyme (Fpg or endo III) at a final concentration of 2−100 ng/μL in a 2× buffer was added to the aliquoted solutions of double-stranded DNA. Reactions using increasing amounts of enzyme (2, 5, 10, 15, 25, 50, 75, and 100 ng/μL) were performed at 37 °C for 30 min and stopped by addition of formamide (15 μL). Samples were denatured by heating at 85 °C for 5 min and then electrophoresed on a 20% polyacrylamide−7 M urea gel at 1300 V for 30 min in TBE buffer [50 mM Tris, 50 mM boric acid, and 50 mM EDTA (pH 8)]. The reaction products were visualized by autoradiography after exposure of the gel to X-ray films.

Modified ODN Replication Assays. Typically, 10 pmol of modified DNA template **10** was annealed with 10 pmol of the complementary 5′-^{32}P-end-labeled 10-mer primer [5′-d(AGA TCA GTC A)-3′] in 10 μL of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 0.2 mg of BSA buffer. The primer−template solution was incubated at 80 °C for 5 min, and then cooled slowly to 4 °C, at least, for 2 h. Subsequently, primer elongation by the Klenow fragment was carried out at 25 °C in the same buffer over the course of 1 h. Each sample contained 0.5 pmol of the labeled primer−template duplex, 1 unit of polymerase, and the four dNTPs (each at 100 μM) in a final volume of 25 μL. The reactions were stopped by addition of formamide (15 μL). Samples were denatured by heating at 85 °C for 5 min and then electrophoresed on a 20% polyacrylamide−7 M urea gel at 1300 V for 30 min in TBE buffer. The reaction products were visualized by exposing the gel to X-ray films. A similar experiment using the unmodified 22-mer template [5′-d(CAC TTC GGA TCG TGA CTG ATC T)-3′] was performed as a control.

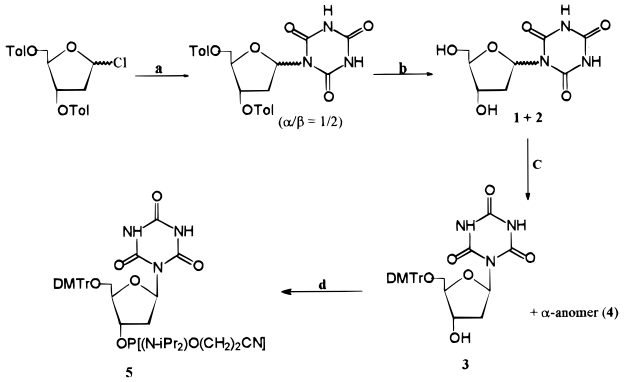
Results and Discussion

Synthesis of the Modified Phosphoramidite Building Block and Its Insertion into Defined Sequence Oligonucleotides. (1) The Cyanuric Acid Nucleoside. Synthesis of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-cyanuric acid **1** (βdY) and its chemical incorporation into ODNs have not been reported so far in the literature. Our synthetic pathway (Scheme 2) for affording dY involves the direct glycosylation of silylated cyanuric acid with the 3,5-protected 2-deoxyribofuranoside chloride. After removal of the toluoyl groups from the sugar moiety and subsequent silica gel purification, an unresolvable mixture of anomeric nucleosides **1** and **2** was obtained in a 55% yield. The structure of **1** and **2** was assigned on the basis of UV, mass, and ^{1}H and ^{13}C NMR measurements of the anomeric nucleoside mixture. This received further confirmation by comparing the latter results with the previously reported data for the β anomer prepared by photosensitized oxidation (*12*). Thus, the UV spectrum of dY (Figure 1) exhibits a lack of absorption at 260 nm and a maximum, as a shoulder, at 220 nm. The mass measurement, in the electrospray ionization mode, is indicative of an empirical formula of $C_8H_{11}O_6N_3$. The two anomers **1** and **2** show a major difference in the ^{1}H NMR features. This concerns the multiplicity of H-1′, which appears as a doublet of doublets for the β-anomer **1** and a triplet for the α-anomer **2**. The results are in agrement with the values already reported for modified pyrimidine 2′-deoxyribonucleosides exhibiting two carbonyl groups in ortho positions with respect to the *N*-glycosidic bond (*16*, *17*). This mimics for both anomers a syn conformation since one of the two carbonyl group lies over the sugar moiety. As a result, in both cases there was a shift in the dynamic equilibrum between the C2′-endo and C3′-endo furanose puckered forms toward the latter one. This leads to a decrease in the trans $J_{1',2'}$ coupling of the β anomer, whereas an increase in the trans $J_{1',2''}$ coupling of the α anomer is noted. Finally, NMR and reverse-phase HPLC analyses revealed that the β anomer was obtained as the major product (1/2 α/β).

Scheme 1. Formation of Cyanuric Acid Nucleoside (1) from Irradiated and Photosensitized DNA Constituents



Scheme 2[a]



[a] (a) 2,4,6-Trihydroxy-1,3,5-triazine (1 equiv), $C_4F_9SO_3K$ (2.4 equiv), HMDS (0.8 equiv), TMSCl (3.1 equiv), dry CH_3CN, reflux, 16 h; (b) 1% NaOH, MeOH, 1 h, 55% **1** and **2**; (c) DMTrCl (1.4 equiv), pyridine, 16 h, 40.5% **3**; (d) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyldiamidite (1.1 equiv), *N*,*N*′-diisopropylammonium tetrazolate (0.5 equiv), CH_2Cl_2, 2 h, 68% **5**.

(2) Preparation of Modified Phosphoramidite Building Blocks. The anomeric mixture of nucleosides was then submitted to 5′-dimethoxytritylation and easily resolved at this step by silica gel chromatography to afford **3** (β anomer) as a pure product. The structure of

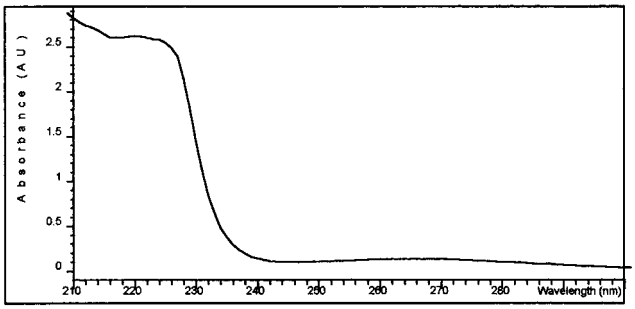


Figure 1. UV spectrum, in water (pH 7), of dY.

Table 1. Sequences and Molecular Masses of the Modified Oligonucleotides Synthesized and Used in This Study[a]

sequence (5′−3′)	length	mass calcd (Da)	mass found (Da)
6 T(**Y**)T	3	853	852.31
7 ATC T(**Y**)T ACT	9	2667	2665.60
8 ATC T(**8-oxoGua**)T ACT	9	2704	2703.27
9 ATC GT(**Y**) ACT GAT CT	14	4232	4230.73
10 CAC TTC GGA TC(**Y**) TGA CTG ATC T	22	6679	6676.93

[a] Y is cyanuric acid; 8-oxoGua is 8-oxo-7,8-dihydroguanine. All the oligonucleotide masses have been obtained by ESI-MS measurements in the negative mode.

both the 5′-DMTr-protected anomers (**3** and **4**) was confirmed by ^{1}H NMR analyses and ESI-MS measurements. Then, the dimethoxytritylated β anomer **3** was treated with the 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyldiamidite phosphitylating reagent, affording the expected phosphoramidite building block **5** in a good yield (68%) after silica gel column chromatography.

It should be noted that the stability of βdY under the usual conditions of synthesis and deprotection of oligodeoxyribonucleotides on solid support was demonstrated. The ability to use **1** in standard automated ODN synthesis was assessed by investigating its stability under the various acid, alkali, and oxidizing conditions used during the chemical preparation of DNA fragments. Thus, βdY **1** was shown to be stable under the latter conditions. Further studies performed on the α-anomer **2** confirmed the good stability of the cyanuric nucleoside toward chemical degradation and anomerization.

(3) Synthesis and Characterization of the Oligonucleotides. Several oligodeoxyribonucleotides (Table 1) bearing a βdY residue were synthesized on solid support using the phosphoramidite chemistry, with the modifications previously described in Materials and Methods. This allowed a coupling efficiency of more than 90% for the modified monomer. After ammonia deprotection at room temperature for 4 h, the crude 5′-tritylated ODNs were purified by RP-HPLC on polymeric support using an on-line detritylation/purification procedure (*15*). The purity and the homogeneity of the material were assessed by several approaches, including analytical HPLC, polyacrylamide gel electrophoresis of 5′-^{32}P-labeled fragments, and mass spectrometry measurements. The molecular masses of the oligonucleotides were inferred from electrospray ionization mass spectrometry measurement in the negative mode (Table 1). These results confirmed the incorporation and the integrity of βdY **1** into the oligomers.

Piperidine Stability of Cyanuric Acid Inserted into Oligodeoxyribonucleotides. The preparation of

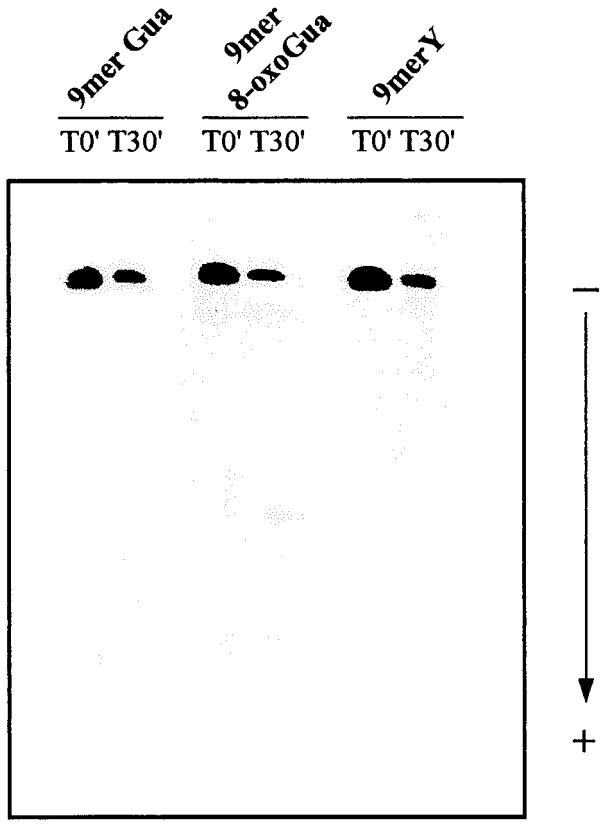


Figure 2. PAGE analysis of the 5′-end-labeled 9-mer 5′-(ATC T**X**T ACT)-3′ with **X** being Gua, 8-oxoGua, or Y, after treatment with a 1 M piperidine aqueous solution at 90 °C for 0 and 30 min.

modified oligonucleotides that contained the cyanuric acid lesion allowed the determination of the stability of **1** under the piperidine conditions used to reveal alkali-labile DNA modifications in oxidized oligonucleotides. The stability experiment was extended to normal dGuo and the 8-oxodGuo lesion incorporated into DNA. Then, the comparative study of the stability was performed by treating the 5′-^{32}P-labeled oligonucleotides 5′-d(ATC T**X**T ACT)-3′ where **X** is Gua, 8-oxoGua, or Y with piperidine at 90 °C for 30 min. In a subsequent step, the resulting DNA fragments were analyzed by denaturing polyacrylamide gel electrophoresis (Figure 2). It was found that the cyanuric acid-containing oligonucleotide **7** was stable under piperidine treatment. Thus, cyanuric acid cannot be considered an alkali-labile site, as well as its oxidized 8-oxodGuo precursor which has been recently re-evaluted as a piperidine-stable modification (*11*, *18*−*20*).

Thermal Denaturation Studies. To determine the structural effect of the incorporation of cyanuric acid into DNA, the thermal stability of the dY·dC base pair was evaluated. Thus, ODN **9**, which contained a ***β***dY lesion at the central position, was annealed with its complementary DNA strand 5′-d(AGA TCA GTC ACG AT)-3′. The melting temperature (T_m) of the duplex was determined by UV measurements at 260 nm. Interestingly, the substitution of a dGuo residue with dY in the middle of the sequence led to an important decrease in the T_m of 15 °C (the T_m of the duplex that contained cyanuric acid = 40 ± 1 vs 55 ± 1 °C for the unmodified duplex). The noticeable observed decrease in the melting temperature suggests that the insertion of cyanuric acid significantly induces a local destabilization of the hybrid

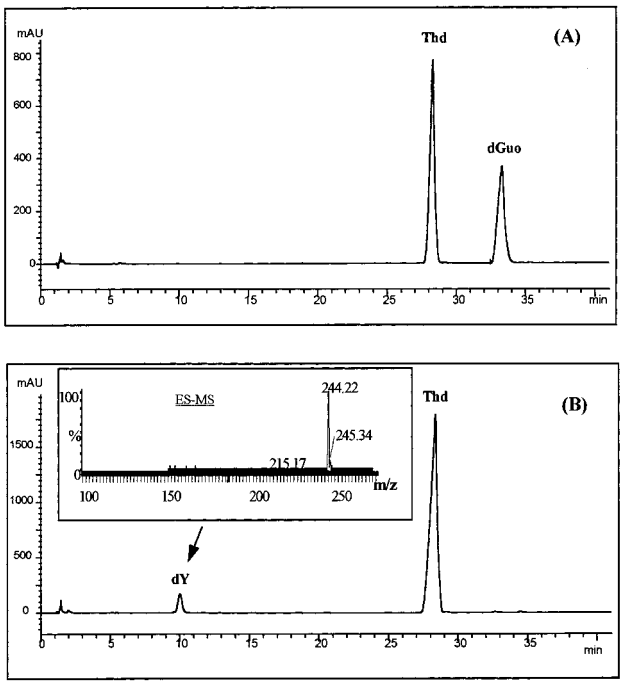


Figure 3. RP-HPLC analysis of the enzymatic digestion mixture of d(TGT) (A) and d(TYT) (B) treated with 5 EU of nuclease P_1 at 37 °C for 2 h and then with 10 EU of alkaline phosphatase for 1 h (the chromatographic conditions are reported in Materials and Methods). The detection of dY, which exhibits a lack of absorption at 260 nm, was achieved at 230 nm ($A_{230/260nm}$ = 11) upon injection of a large amount of nucleosides arising from the digestion of 1 AU_{260nm} of trinucleotide d(TYT). The inset shows the electrospray mass spectrum, in the negative mode, of the collected dY residue.

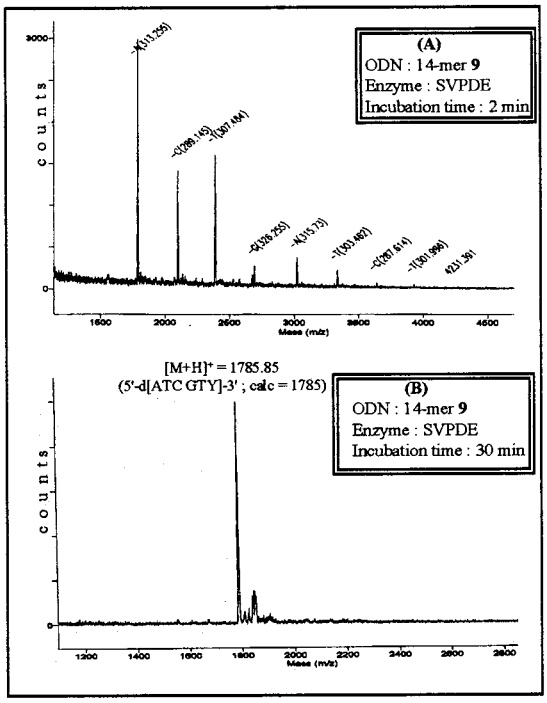


Figure 4. MALDI mass spectra of the products resulting from the digestion of modified oligonucleotide **9** by the 3′-exonuclease after incubation for 2 (A) and 30 min (B).

DNA structure, which may be mostly ascribed to a lack of the hydrogen bonding with the complementary cytosine base.

Nuclease-Mediated Digestions of Modified Oligonucleotides That Contained Cyanuric Acid. The ability of several nucleases to cleave the phosphodiester linkages between dY and the normal 2′-deoxyribonucleosides was assessed using cyanuric acid nucleoside-containing oligodeoxyribonucleotides.

First, the trimer d(TYT) was submitted to the action of nuclease P_1, followed by bacterial alkaline phosphatase. In addition, the unmodified trinucleotide d(TGT) which was used as a reference was treated under the same conditions. The resulting mixture of 2′-deoxyribonucleosides arising from the digestion of d(TYT) was then analyzed by reverse-phase HPLC (Figure 3B). Two HPLC peaks, including those of thymidine (retention time of 28 min) and of an additional product (retention time of 10 min), were observed. The latter compound was collected and then assigned as **1** by both HPLC co-injection with an authentic sample of βdY and ESI-MS analysis. This clearly shows the ability of nuclease P_1 to cleave cyanuric acid nucleoside from DNA. The latter observation provides additional support for the presence and the integrity of **1** in the synthetic oligomers.

Similar enzymatic digestion experiments were performed with the trinucleotide d(TYT) using exonucleases, including snake venom phosphodiesterase (SVPDE, 3′-exo) and calf spleen phosphodiesterase (CSPDE, 5′-exo), in combination with bacterial akaline phosphatase. Surprisingly, no peak corresponding to cyanuric acid nucleoside **1** was detected during the reverse-phase HPLC analysis of both the digestion hydrolysates. This indicated that the latter exonucleases failed to release the damaged nucleoside from the oligonucleotidic chain (data not shown). To confirm these results, enzymatic digestions by SVPDE and CSPDE of the 14-mer modified ODN **9** were performed. Thus, the course of the hydrolysis of the DNA strand by the nucleases was followed by withdrawing aliquots from the digestion mixtures after increasing periods of time. The DNA fragments were then analyzed by MALDI-TOF mass spectrometry (*21*, *22*). Using the latter powerful technique, the different molecular ions that were observed correspond to the digested DNA fragments which differ in mass by successive loss of nucleotides. The difference in mass between two adjacent peaks allows us to identify the released nucleotide and then to determine the overall sequence of the oligomer.

The unmodified 14-mer oligodeoxyribonucleotide 5′-d(ATC GTG ACT GAT CT), used first as a control, was totally hydrolyzed by both 5′- and 3′-exonucleases in less than 15 min, allowing the determination of the complete sequence (data not shown). In contrast, the presence of nucleoside **1** in **9** induces a total resistance to digestion by both SVPDE (3′-exo) and CSPDE (5′-exo). The mass spectra after 2 and 30 min of hydrolysis by SVPDE are shown in Figure 4. It was found that the enzyme induced the release of the first height nucleotides at the 3′-end of the oligonucleotide sequence (Figure 4A) but failed to cleave the phosphodiester linkage between **1** and thymidine, even after a prolonged treatment. This was inferred from the observation of a single peak at *m*/*z* 1785.85 Da (Figure 4B) that corresponds to the 6-mer [5′-d(ACT GTY)-3′] (calcd mass of 1785 Da). On the other hand, the digestion of ODN **9** by CSPDE for 30 min (Figure 5) provided the 9-mer [5′-d(YAC TGA TCT)-3′] (calcd mass of 2692 Da; found mass of 2691.82 Da). This shows that the enzyme is able to digest sequentially the ODN from the 5′-end until it reaches the cyanuric acid nucleoside

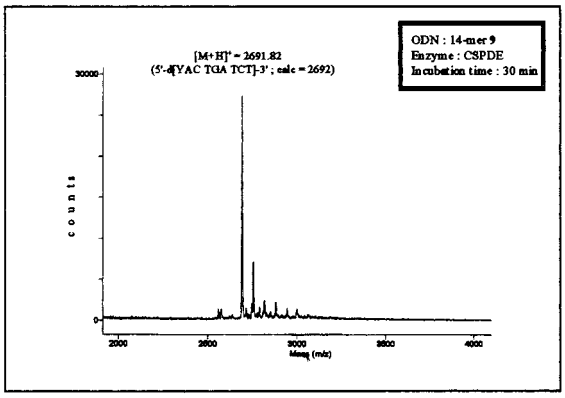


Figure 5. MALDI mass spectrum of the products resulting from the digestion of modified oligonucleotide **9** by the 5′-exonuclease after incubation for 30 min.

whose phosphodiester bond was found to resist to further cleavage.

Repair Assays of Cyanuric Acid-Containing Oligonucleotides with Fpg and Endonuclease III Proteins. To investigate the biological significance of the cyanuric acid base in oxidized DNA, attempts were made to asses whether **1** may be a substrate for two base excision repair enzymes, including formamidopyrimidine DNA *N*-glycosylase (Fpg) and endonuclease III (endo III) proteins. The *E. coli* Fpg protein is a well-known repair enzyme which is able to excise several modified purine bases from DNA duplexes, through both glycosylase and AP-endonuclease activities (*23*, *24*). Substrates recognized and excised by Fpg include 8-oxo-7,8-dihydroguanine (8-oxoG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-guanine), 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (Me-Fapy-guanine), and 4,6-diamino-5-formamidopyridine (Fapy-adenine) (*2*, *4*, *25*−*28*). It was also shown that 5-hydroxycytosine (5-OHC), 5,6-dihydrothymine (DHT), and *N*-3-(2-hydroxyisobutyric acid)urea, three modified pyrimidine bases, are also recognized and excised by Fpg (*29*−*31*).

On the other hand, previous studies have shown that endonuclease III recognizes modified thymine and cytosine bases, including 5,6-dihydrothymine (DHT), 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol), 5-hydroxy-5,6-dihydrothymine, 5,6-dihydrouracil (DHU), 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol), 5-hydroxy-5,6-dihydrouracil, 5-hydroxyuracil (5-OHU), 6-hydroxy-5,6-dihydrocytosine, 5-hydroxycytosine (5-OHC), urea, methyltartronyl-*N*-urea, 5-hydroxy-5-methylhydantoin, and alloxan (*31*−*35*).

Thus, to obtain further information about the substrate specificity of both Fpg and endo III proteins, the modified 22-mer ODN **10** that contained dY at a central position was incubated with the repair enzyme. This involved 5′-^{32}P-end labeling and subsequent hydridization of modified ODN **10** with its complementary sequence 5′-d(AGA TCA GTC ACG ATC CGA AGT G)-3′, which contains a cytosine in front of the cyanuric acid lesion. Then, the damaged base excision by the repair enzymes was determined by searching for the strand breakage of the ODN using polyacrylamide gel electrophoresis. Using the latter technique, it was shown that neither the Fpg enzyme nor the endonuclease III protein is able to cleave the modified DNA duplex at the site of **1** (data not shown). Investigations are currently in progress to determine the possible implication of bases other than

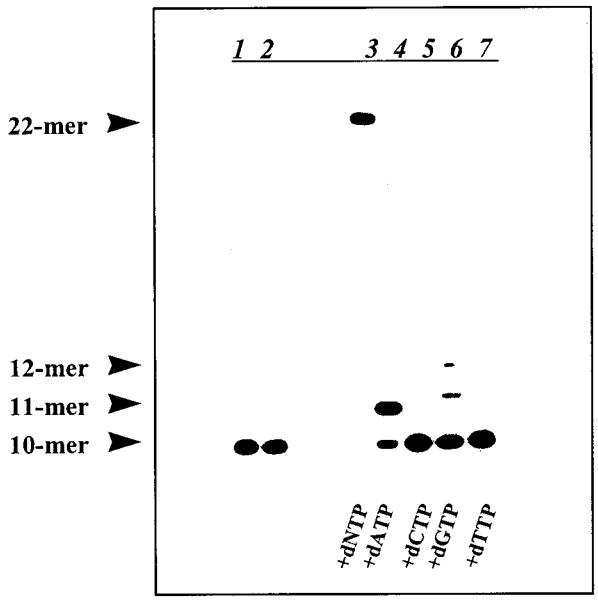


Figure 6. Primer extension reactions catalyzed by the Klenow fragments. Using the modified 22-mer template [5′-d(CAC TTC GGA TC**Y** TGA CTG ATC T)-3′] primed with a ^{32}P-labeled 10-mer [5′-d(AGA TCA GTC A)-3′] (lane 1), primer extension reactions were carried out by adding 1 unit of Klenow fragment polymerase (lane 2, reference without nucleoside triphosphates) in the presence of dNTP (lane 3), dATP (lane 4), dCTP (lane 5), dGTP (lane 6), and dTTP (lane 7), as described in Materials and Methods. Then, the reaction mixtures were subjected to denaturing 20% PAGE, and the extended products were visualized by exposing the gel to X-ray film.

cytosine in front of dY during the repair reaction and also the excision of this lesion by other oxidized purine-specific repair enzymes, namely, yeast and human Ogg1 proteins (*36*−*40*).

In Vitro Replication Experiments with DNA Polymerase. The ability of the Klenow fragment to extend a primer annealed with a template bearing a cyanuric acid nucleoside was investigated. The primer was ^{32}P-labeled at its 5′-end so that extension by nucleotide incorporation could be observed by sequencing polyacrylamide gel electrophoresis (PAGE), the intensity of each band being proportional to the number of molecules that terminates the synthesis at a given position of the template. Figure 6 shows the denaturing PAGE bands obtained by elongation of the 10-mer primer 5′-d(AGA TCA GTC A)-3′ in the presence of 5′-d(CAC TTC GGA TC**Y** TGA CTG ATC T)-3′ (template that contained a cyanuric acid residue in the central position). Thus, when an equimolar concentration of the four natural deoxyribonucleoside triphosphates was used in the reaction mixture (lane 3), it was found that no chain termination opposite cyanuric acid occurred during the extension of the primer. Indeed, readthroughs giving rise to a full-length 22-mer product were observed. This indicated that the cyanuric acid residue does not block in vitro DNA synthesis.

To determine the nature of the base incorporated opposite cyanuric acid, the same primer extension experiments with Klenow polymerase were performed by the "one-nucleotide extension assay". This was achieved by adding a single 2′-deoxyribonucleoside triphosphate to each reaction mixture. The autoradiography (Figure 6, lanes 4−7) shows that dAMP was predominantly incorporated opposite cyanuric acid (lane 4), dGMP being also inserted to a lower extent (lane 6). These results show that the cyanuric acid base exhibits an important

miscoding potential during the replication step, leading to the mutagenic G > T and G > C transversions.

Conclusion and Perspectives

The synthesis of cyanuric acid nucleoside (**1**) and its incorporation into several oligonucleotides by the phosphoramidite approach were carried out for the first time. The synthetic oligomers were isolated in good yields and characterized by complementary techniques, showing the integrity of the incorporated modified nucleoside. The piperidine stability experiment performed with the modified oligonucleotides supported the high stability of the cyanuric acid lesion inserted into DNA strands. A significant decrease in the melting temperature of a double-stranded 14-mer DNA fragment that contained a Y·C base pair was observed. This suggests that the presence of cyanuric acid induces a local destabilization of the duplex DNA structure. The processing of **1** by different nucleases was studied. Thus, it was shown that nuclease P_1 is able to quantitatively cleave the cyanuric acid residue from the oligonucleotides while both SVPDE and CSPDE failed to release the latter modification from the DNA fragment. On the other hand, the ability of two repair enzymes, namely, the Fpg and the endonuclease III proteins, to excise cyanuric acid was investigated. A series of experiments was performed which clearly indicated that cyanuric acid was not the substrate for either repair enzyme. The biochemical study was then extended to the evaluation of the mutagenic properties of the cyanuric acid lesion. This involved the determination of the base-specific incorporation directed by this residue during in vitro replication using the Klenow fragment. Thus, **1** does not block the polymerase which incorporates adenine opposite the damage, guanine being also slighty inserted.

The cyanuric acid nucleoside and the different modified ODNs prepared in this work are actually used as tools for investigations aimed at determining the mechanism and the level of formation of **1** in single- and double-stranded oxidized DNA.

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