Arch Pharm Res Vol 23, No 2, 139-146, 2000

Medicinal Chemistry & Natural Products

Synthesis and Characterization of Oligonucleotides Containing Site-Specific Bulky N^2-Aralkylated Guanines and N^6-Aralkylated Adenines

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(Received December 10, 1999)

7- Bromomethylbenz[a]anthracene is a known mutagen and carcinogen. The two major DNA adducts produced by this carcinogen, i.e., N^2-(benz[a]anthracen-7-ylmethyl)-2′-deoxyguanosine (**2**, b[a]a^{2}G) and N^6-(benz[a]anthracen-7-ylmethyl)-2′-deoxyadenosine (**4**, b[a]a^{6}A), as well as the simpler benzylated analogs, N^2-benzyl-2′-deoxyguanosine (**1**, bn^{2}G) and N^6-benzyl-2′-deoxyadenosine (**3**, bn^{6}A), were prepared by direct aralkylation of 2′-deoxyguanosine and 2′-deoxyadenosine. To determine the site-specific mutagenicity of these bulky exocyclic amino-substituted adducts, the suitably protected nucleosides were incorporated into 16-base oligodeoxyribonucleotides in place of a normal guanine or adenine residues which respectively are part of the ATG initiation codon for the *lac Z′* α-complementation gene by using an *in situ* activation approach and automated phosphite triester synthetic methods. The base composition and the incorporation of the bulky adducts into synthetic oligonucleotides were characterized after purification of the modified oligonucleotides by enzymatic digestion and HPLC analysis.

Key words: 7-Bromomethylbenz[a]anthracene, DNA aralkylation, Carcinogen-DNA adducts, Oligodeoxyribonucleotide, Site-specific mutagenicity, Phosphite triester

INTRODUCTION

Carcinogen damage to cellular DNA is considered a key step in the initiation of chemical carcinogenesis. Carcinogens can react with a variety of sites on DNA bases and with several bases simultaneously. For this reason, it is difficult to evaluate how any one carcinogen adduct alters the structure or coding properties of the damaged DNA. The synthetic methods for the preparation of defined carcinogen-modified oligonucleotides bearing a single specific carcinogen-modified base at predetermined sequence locations have been developed to examine how individual carcinogen-DNA adducts disrupt the structure and stability of DNA, as well as mutagenic potency (Basu and Essigmann, 1988).

Bulky exocyclic amino-substituted adducts are produced by a wide variety of polycyclic aromatic hydrocarbon metabolites and carcinogenic aromatic amines (Dipple *et al.*, 1971; Dipple and Slade, 1970; Rayman and Dipple, 1973; Peck *et al.*, 1976; Natarajan and Flesher, 1973). Aralkyl halides (aralkylating agents) are halomethyl-substituted aromatic hydrocarbons and directly acting carcinogens which do not need any metabolic activations to modify nucleic acids (Moschel, 1994). Benzyl bromide is the simplest compound in this category. 7-Bromomethylbenz[a]anthracene (7-BrMeB[a]A), more complex aralkyl halide, is a known mutagen and carcinogen. Aralkylation of DNA by 7-BrMeB[a]A occurs predominantly on the amino groups of the bases in aqueous solution (Dipple *et al.*, 1971; Rayman and Dipple, 1973; Pei and Moschel, 1990). The exocyclic N^2-amino group on guanine and the N^6-amino group on adenine residues are the major site of aralkylation. N^2-(benz[a]anthracen-7-ylmethyl)-2′-deoxyguanosine (**2**, b[a]a^{2}G) and N^6-(benz[a]anthracen-7-ylmethyl)-2′-deoxyadenosine (**4**, b[a]a^{6}A) are the major modified deoxyribonucleosides produced in DNA by this carcinogen.

The primary objective of synthesis of oligonucleotides containing site-specific aralkylated modified bases is to examine the site-specific mutagenicity in *E. coli* and

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human cells of the major DNA adducts produced by 7-BrMeB[a]A, i.e., b[a]a^{2}G and b[a]a^{6}A when the modified base was incorporated into plasmid vectors. We then compare these data to those for the analogous but simpler benzylated derivatives of guanine and adenine, i.e., N^2-benzyl-2'-deoxyguanosine (**1**, bn^{2}G) and N^6-benzyl-2'-deoxyadenosine (**3**, bn^{6}A), to observe the effect of increasing size of the exocyclic amino group substituent on mutagenic potency and how substituted structure can influence the mutagenicity of bulky exocyclic amino-substituted adducts.

We now report preparative routes for the four modified nucleosides involving direct aralkylation of 2'-deoxyguanosine and 2'-deoxyadenosine and their efficient site-specific incorporation into synthetic oligonucleotides. Even though 7-BrMeB[a]A and benzyl bromide are known to be carcinogens, this is the first preparation and characterization of oligonucleotides containing b[a]a^{2}G, b[a]a^{6}A, bn^{2}G and bn^{6}A at defined sites using total synthetic method in order to evaluate the mutagenicity induced by site-specifically incorporated major adducts produced by 7-BrMeB[a]A as well as primary adducts produced by benzyl bromide.

MATERIALS AND METHODS

Ultraviolet absorption spectra were determined on a Milton Roy SLM-AMINCO 3000 diode array spectrophotometer. ^{1}H NMR spectra were recorded on a Varian VXR 500S spectrometer equipped with Sun 4/110 data stations or a Varian XL200 instrument interfaced to an Advanced data system. Samples were dissolved in dimethyl-d_6 sulfoxide and chemical shifts are reported as δ values (ppm) downfield from tetramethylsilane (TMS) as an internal standard. Positive ion (+ve) fast atom bombardment (FAB) and high-resolution electron impact (HREI) mass spectra (MS) were obtained with a reversed-geometry VG Micromass ZAB-2F spectrometer interfaced to a VG 2035 data system. A mixture of dithiothreitol and dithioerythritol (1:1) was used as the FAB matrix. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Thin-layer chromatography (TLC) was performed on Kodak silica gel (Eastman Kodak Co., Rochester, NY) adsorbent with fluorescent indicator (0.2 mm layer, 2.5 × 7.5 cm) and visualization was performed by UV illumination. Sephadex LH-20 was purchased from Pharmacia, Biotechnology AB, Uppsala, Sweden. Silica gel, 200-425 Mesh, 60Å (Aldrich Chemical Co., Milwaukee, WI), was used for flash chromatography. 2'-Deoxyguanosine, 2'-deoxyadenosine, snake venom phosphodiesterase (type VII from *Crotalus atrox* venom) and bacterial alkaline phosphatase (type III from *Escherichia coli*) were purchased from Sigma Chemical Co., St. Louis, MO. Most reagents and solvents were from Aldrich Chemical Co., Inc., Milwaukee, WI. 7-(Bromomethyl)benz[a]anthracene was prepared as described previously (Pei and Moschel, 1990). Standard nucleoside phosphoramidites were from Applied Biosystems, Inc. Oligomers were purified by HPLC on a semipreparative 10 mm × 25 cm Beckman Ultrasphere ODS column (5-mm particle size) using two Waters 6000A pumps, a Model 660 solvent programmer, a Model 450 variable-wavelength UV detector, and a Model U6K sample injector. Analytical samples were chromatographed on a 4.6 mm × 25 cm Beckman Ultrasphere ODS column (5-μm particle size) with a Hewlett-Packard Model 1090 Series II HPLC equipped with a diode array detector.

Synthesis

N^2-benzyl-2'-deoxyguanosine (1)

To a stirred suspension of 2'-deoxyguanosine (1.3 g, 4.87 mmol) in 25 mL of 2,2,2-trifluoroethanol was added 0.23 g of NaOH (5.75 mmol) at room temperature. When all NaOH and 2-deoxyguanosine had dissolved, benzyl bromide (0.55 mL, 4.63 mmol) was added dropwise over 3 min. The reaction was stirred at room temperature for 72 h. The resulting white solution was evaporated *in vacuo* to give a crude solid. The solid was dissolved in 150 mL of MeOH/H_2O/NH_4OH (10:90:3) and was loaded on a Sephadex LH-20 (2.8 × 78 cm) column eluted with MeOH/H_2O/NH_4OH (10:90:3) at 1 mL/min. UV absorp-tion was checked continuously at 280 nm and fractions (10 mL) were collected. N^2-benzyl-2'-deoxyguanosine (**1**) eluted in fractions 35~55 (780 mg, yield, 45%); R_f, 0.15 in MeOH/$CHCl_3$ (2:8); UV (pH 1) λ_{min} 234 nm (ε=0.468 × 10^4), λ_{max} 261 nm (ε=1.31 × 10^4), 281 nm (sh) (ε=0.825 × 10^4), (pH 6.9) λ_{min} 229 nm (ε=0.513 × 10^4), λ_{max} 256 nm (ε=1.315 × 10^4), 276 nm (sh) (ε=0.950 × 10^4), (pH 13) λ_{min} 239 nm (ε=0.673 × 10^4), λ_{max} 261 nm (ε=1.168 × 10^4); ^{1}H NMR δ 10.61 (1H, s, 1-NH, exchanges with D_2O), 7.90 (1H, s, 8-H), 7.20.-7.40 (5H, m, C_6H_5), 6.88 (1H, t, J_{CHNH}=5.52 Hz, $C_6H_5CH_2N$**H**, exchanges with D_2O), 6.14 (1H, t, J=6.94 Hz, H-1'), 5.26 (1H, br s, OH-3', exchanges with D_2O), 4.86 (1H, br s, OH-5', exchanges with D_2O), 4.50 (2H, d, J_{CHNH}= 5.67 Hz, C_6H_5C**H**$_2$NH, changes to a singlet on addition of D_2O), 4.33 (1H, m, H-3'), 3.79 (1H, m, H-4'), 3.50 (2H, m, H-5'), 2.57 (1H, m, H-2'b), 2.16 (1H, m, H-2'a); FAB$^+$ /*MSm/z* 358 ([$C_{17}H_{19}N_5O_4$+H]$^+$), 242 ([$C_{12}H_{11}N_5O$ +H]$^+$) ; Anal. Calcd for $C_{17}H_{19}N_5O_4$, C, H, N.

N^2-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine (2)

This compound was prepared essentially as described previously (Pei and Moschel, 1990). UV MeOH/H_2O/CH_3CN (70:25:5) λ 231 nm (ε=3.95 × 10^4), 258 nm (ε= 4.66 × 10^4), 270 nm (ε=5.0 × 10^4), 280 nm (ε=8.07 × 10^4), 291 nm (ε=9.66 × 10^4); Anal. Calcd for $C_{29}H_{25}N_5O_4.H_2O$, C, H, N.

N^6-benzyl-2'-deoxyadenosine (3)

To a stirred solution of 2'-deoxyadenosine (5 g, 19.9 mmol) in 200 mL of water containing 9 g of Na_2CO_3 was added benzyl bromide (6 mL, 50.57 mmol). The reaction mixture was stirred at 55°C for 24 h. After cooling to room temperature, the resulting pale yellow solution was extracted with $CHCl_3$ (3 × 200 mL). The combined organic layer was dried over anhydrouse Na_2SO_4 and filtered. The filtrate was evaporated *in vacuo* to produce a pale yellow semi-solid which was solidified with anhydrous ether to give N^6-benzyl-2'-deoxyadenosine (**3**) as a white solid (800 mg, yield, 10.3%). To a stirred remaining aqueous phase was added benzyl bromide (5 mL, 42.14 mmol) and NaOH (4 g), respectively. The reaction was stirred additionally at 55°C for 24 h. The workup procedures were carried out as described above to obtain **3** (600 mg, yield, 8.8 %); R_f=0.31 in MeOH/$CHCl_3$ (5:95); UV (pH 1) λ_{max} 266 nm ($\varepsilon = 1.906 \times 10^4$), (pH 6.9) λ_{max} 270 nm ($\varepsilon = 1.920 \times 10^4$), (pH 13) λ_{max} 270 nm ($\varepsilon = 1.972 \times 10^4$); ^{1}H NMR δ 8.41 (1H, br s, N^6-$C_6H_5CH_2N$**H**, exchanges with D_2O), 8.36 (1H, s, 8-H), 8.19 (1H, s, 2-H), 7.18-7.35 (5H, m, C_6H_5), 6.36 (1H, t, J=6.2 Hz, H-1'), 5.30 (1H, m, OH-3', exchanges with D_2O), 5.20 (1H, m, OH-5', exchanges with D_2O), 4.71 (2H, br s, N^6-C_6H_5C**H**$_2$), 4.41 (1H, m, H-3'), 3.88 (1H, m, H-4'), 3.57 (2H, m, H-5'), 2.73 (1H, m, H-2'b), 2.26 (1H, m, H-2'a); FAB$^+$/MS *m/z* 342 ([C_{17} H_{19} N_5O_3 +H]$^+$), 226 ([$C_{12}H_{11}N_5$+H]$^+$); Anal. Calcd for C_{17} $H_{19}N_5O_3$, C, H, N.

N^6-(benz[*a*]anthracen-7-ylmethyl)-2'-deoxyadenosine (4)

To a stirred solution of 2'-deoxyadenosine (5 g, 19.9 mmol) in 20 mL of *N,N*-dimethylformamide was added 7-bromomethylbenz[a]anthracene (1.2 g, 3.74 mmol). The reaction mixture was stirred at 37°C in the dark for 24 h. The resulting yellow color solution was poured into the 5% aqueous sodium bromide (500 mL) with rapid stirring. The white precepitate formed was filtered and dried to give a crude 1-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine. This solid was dissolved in 500 mL of MeOH and warmed to 37°C, and sodium hydroxide (1 N, 100 mL) was added. The floppy solid formed upon finishing addition of sodium hydroxide was filtered off after brought the reaction solution into room temperature to obtain a clear pale yellow solution. The clear reaction solution was allowed to proceed in the dark at 37°C overnight. The resultant white solid was collected by filtration, washed with water, and dried *in vacuo* to give **4** as a white solid (1.1 g, yield, 60%); UV MeOH/H_2O/CH_3CN (70:25:5) λ 260 nm ($\varepsilon = 4.55 \times 10^4$), 271 nm ($\varepsilon = 5.82 \times 10^4$), 280 nm ($\varepsilon = 9.06 \times 10^4$), 291 nm ($\varepsilon = 0.39 \times 10^4$); ^{1}H NMR δ 7.60-9.52 (14H, m, ArH + H-8 + H-2 + N^6-ArCH_2N**H**, intergrates for 13 protons on addition of D_2O), 6.38 (1H, t, H-1'), 5.74 (2H, br s, N^6-ArC**H**$_2$), 5.33 (1H, m, OH-3', exchanges with D_2O), 5.22 (1H, m, OH-5', exchanges with D_2O), 4.43 (1H, m, H-3'), 3.90 (1H, m, H-4'), 3.59 (2H, m, H-5'), 2.74 (1H, m, H-2'b), 2.29 (1H, m, H-2'a); FAB$^+$/MS *m/z* 492 ([$C_{29}H_{25}N_5O_3$ + H]$^+$), 376 ([$C_{24}H_{17}N_5$+H]$^+$); Anal. Calcd for $C_{29}H_{25}N_5O_3$, C, H, N.

5'-*O*-(4,4'-Dimethoxytrityl)-N^2-benzyl-2'-deoxyguano-sine (5)

To a stirred solution of N^2-benzyl-2'-deoxy-guanosine (357 mg, 1 mmol) in 10 mL of anhydrous pyridine were added 4,4-dimethoxytrityl chloride (508.25 mg, 1.5 mmol), triethylamine (0.21 mL, 1.5 mmol) and DMAP (6.11 mg, 0.05 mmol), successively. This reaction mixture was stirred for 3 h under dry nitrogen gas at room temperature. The resulting dark yellow solution was poured into 7% aqueous $NaHCO_3$ (250 mL) and extracted with $CHCl_3$ (250 mL× 3), the combined organic layer was dried over anhydrous Na_2SO_4 and filtered off. The filtrate evaporated to a crude dark yellow oil was coevaporated with toluene (2 × 5 mL) to remove pyridine. The dark yellow semi-solid was dissolved in minium volume of $CHCl_3$ and was loaded on a silica gel (200-425 mesh) column (3 × 15 cm) eluted with EtOH/$CHCl_3$ (10:90) to give **5** as a thin yellow solid (544 mg, yield, 78%); R_f=0.16 in MeOH/$CHCl_3$ (5:95); ^{1}H NMR δ 10.63 (1H, s, 1-NH, exchanges with D_2O), 7.80 (1H, s, 8-H), 7.17-7.36 (14H, m, C_6H_5 + DMT), 6.84 (1H, br s, $C_6H_5CH_2N$**H**, exchanges with D_2O), 6.80 (4H, m, H ortho to OCH_3), 6.18 (1H, t, J= 6.6 Hz, H-1'), 5.30 (1H, d, J = 4.6 Hz, OH-3', exchanges with D_2O), 4.35 (3H, m, C_6H_5C**H**$_2$NH+H-3'), 3.93 (1H, m, H-4'), 3.71 (6H, s, OCH_3), 3.22 and 3.08 (2H, m, H-5'), 2.68 (1H, m, H-2'b), 2.20 (1H, m, H-2'a); FAB$^+$/MS *m/z* 682 ([$C_{38}H_{37}N_5O_6Na$+H]$^+$), 304 ([$C_{21}H_{19}O_2$+H]$^+$), 242 ([$C_{12}H_{11}N_5O$+H]$^+$); HRMS (FAB$^+$, PNBA/LiI) calcd for [$C_{38}H_{37}N_5O_6Li^+$] 666.2903, found 666.2915.

5'-*O*-(4,4-Dimethoxytrityl)-N^2-(benz[*a*]anthracen-7-ylmethyl)-2-deoxyguanosine (6)

To a stirred solution of N^2-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine (507.55 mg, 1 mmol) in 20 mL of anhydrous pyridine were added 4,4'-dimethoxytrityl chloride (508.25 mg, 1.5 mmol), triethylamine (0.21 mL, 1.5 mmol) and DMAP (6.11 mg, 0.05 mmol), successively. The reaction mixture was stirred in the dark for 3.5 h under dry nitrogen gas at room temperature. The resulting dark yellow solution was poured into the 7% aqueous $NaHCO_3$ (250 mL) with vigorus stirring and extracted with $CHCl_3$ (3 × 250 mL), the combined organic layer was dried over anhydrous Na_2SO_4 and filtered off. The filtrate evaporated to a crude dark yellow oil was coevaporated with toluene (2 × 5 mL) to remove pyridine. The dark yellow semi-solid was dissolved in minium volume of $CHCl_3$ and was loaded to a silica gel column (grade 633, 200~425 mesh, 60Å, 3 × 15 cm) eluted with EtOAc/$CHCl_3$ (30:70). Fractions (50 × 20 mL) were collected to remove fast moving products and impurities. The eluting solvent

mixture was changed to isopropyl alcohol/chloroform (10 :90). Fractions (25 × 20 mL) were pooled and evaporated *in vacuo* to give **6** as a dark yellow solid (570 mg, yield, 70%): ^{1}H NMR δ 10.15 (1H, s, 1-NH, exchanges with D_2O), 9.57-6.74 (25H, m, ArH+H-8+DMT), 6.92 (1H, m, $ArCH_2N\mathbf{H}$, exchanges with D_2O), 6.43 (1H, t, H-1), 5.43 (2H, m, $ArC\mathbf{H}_2$), 5.23 (1H, m, OH-3, exchanges with D_2O), 4.55 (1H, m, H-3), 4.02 (1H, m, H-4), 3.73 (2H, m, H-5), 3.65 (6H, s, OCH_3), 2.88 (1H, m, H-2b), 2.42 (1H, m, H-2a); FAB$^+$/MS *m/z* 833 ($[C_{50}H_{43}N_5O_6Na+H]^+$), 304 ($[C_{21}H_{19}O_2+H]^+$); HRMS (FAB$^+$, PNBA/NaI) calcd for $C_{50}H_{43}N_5O_6Na$ 832.3111, found 832.3097.

5'-*O*-(4,4'-Dimethoxytrityl)-*N*^{6}-benzyl-2'-deoxyadenosine (7)

5'-*O*-(4,4'-Dimethoxytrityl)-*N*^{6}-benzyl-2'-deoxyadenosine was prepared on a 1-mmol scale (483 mg, yield, 75%) as described for **5**; R_f=0.69 in MeOH/$CHCl_3$ (5:95); ^{1}H NMR δ 8.37 (1H, br s, N^6-$C_6H_5CH_2N\mathbf{H}$, exchanges with D_2O), 8.24 (1H, s, 8-H), 8.04 (1H, s, 2-H), 7.16-7.36 (14H, m, C_6H_5 + DMT), 6.81 (4H, m, ortho to OCH_3), 6.34 (1H, t, H-1), 5.34 (1H, m, OH-3, exchanges with D_2O), 4.69 (2H, br s, N^6-$C_6H_5C\mathbf{H}_2$), 4.40 (1H, m, H-3), 3.98 (1H, m, H-4), 3.72 (6H, s, OCH_3), 3.19 (2H, m, H-5), 2.87 (1H, m, H-2b), 2.31 (1H, m, H-2a); FAB$^+$/MS *m/z* 644 ($[C_{38}H_{37}N_5O_5+H]^+$), 340 ($[C_{38}H_{37}N_5O_5$-DMT+H$]^+$); HRMS (FAB$^+$) calcd for $C_{38}H_{37}N_5O_5$ 643.2794, found 643.2784.

5'-*O*-(4,4-Dimethoxytrityl)-*N*^{6}-(benz[*a*]anthracen-7-ylmethyl)-2-deoxyadenosine (8)

5'-*O*-(4,4'-Dimethoxy-trityl)-*N*^{6}-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine was prepared on a 1-mmol scale as described for **6** to give **8** as a pale yellow solid (660 mg, yield, 83.1%); ^{1}H NMR δ 7.11-9.52 (23H, m, ArH+H-8+H-2+N^6-$ArCH_2N\mathbf{H}$ +DMT), 6.80 (4H, m, H ortho to OCH_3), 6.39 (1H, t, H-1'), 5.72 (2H, br s, $ArC\mathbf{H}_2$), 5.38 (1H, m, OH-3', exchanges with D_2O), 4.48 (1H, m, H-3'), 3.99 (1H, m, H-4'), 3.69 (6H, s, OCH_3), 3.18 (2H, m, H-5'), 2.87 (1H, m, H-2'b), 2.34 (1H, m, H-2'a); FAB$^+$/MS *m/z* 817 ($[C_{50}H_{43}N_5O_5Na+H]^+$), 513 ($[C_{50}H_{43}N_5O_5Na$-DMT+H$]^+$); HRMS (FAB$^+$, PNBA/NaI) calcd for $C_{50}H_{43}N_5O_5Na$ 816.3158, found 816.3144.

3'-[*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite derivatives of 5 and 7

Phosphoramidite derivatives fo **5** and **7** were prepared essentially as described previously (Pauly *et al.*, 1988). To a slow stirred anhydrous 0.1 M solution of 1H-tetrazole in acetonitrile (1 mL) was added 2-cyanoethyl *N,N,N',N'*-tetraisopropyl phosphorodiamidite (0.03788 g, 40 μL, 1.2×10^{-4} mol) under argon atmosphere at room temperature. 5'-*O*-(4,4'-Dimethoxytrityl)-*N*^{2}-benzyl-2'-deoxyguanosine(**5**) (66 mg, 1.00×10^{-4} mol) or 5'-*O*-(4,4'-Dimethoxytrityl)-*N*^{6}-benzyl-2'-deoxyadenosine (**7**) (65 mg, 1.00×10^{-4} mol) was added. The reaction mixture was stirred under argon gas at room temperature for 3~4 h. The progress of reaction was monitored by thin-layer chromatography [(TLC, MeOH/$CHCl_3$ (5:95)] [R_f values for the **5** and **7** were 0.16 and 0.69] and the appearance of the corresponding 3-[*O*-(2-cyanoethyl) *N,N*-diisopropylphosphoramidites] were 0.36 and 0.91, respectively.

3'-[*O*-(2-cyanoethyl) *N,N*-diisopropyl phosphorami-dite derivatives of 6 and 8

Phosphoramidite derivatives of **6** and **8** were prepared as same scale as described in derivatives of **5** and **7** expect that reaction solvent was $CH_3CN/CHCl_3$. R_f values for the **6** and **8** were 0.24 and 0.77. The appearance of the corresponding 3-[*O*-(2-cyanoethyl) *N,N*-diisopropyl-phosphoramidites] were 0.41 and 0.98. When the conversion was judged complete by TLC, the reactions were filtered to remove the precipitated diisopropyl ammonium tetra-zolide, and the filtrate was used immediately for DNA synthesis.

Synthesis and Purification of Oligonucleotides

Oligodeoxyribonucleotides were synthesized on a 10-mm scale by using an Applied Biosystems, Inc., Model 380B DNA synthesizer as previously described (Pauly *et al.*, 1988), except that N^2-substituted 2'-deoxyguanosines and N^6-substituted 2'-deoxyadenosines were allowed to couple for 60 min. The stepwise coupling yield for the modified phosphoramidites was 95% as determined by trityl cation release. The terminal 5'-*O*-(4,4'-dimethoxytrityl) group bearing oligomers were chromatographed on a 10 mm × 25 cm Ultrasphere ODS column (5-mm particle size) using a linear gradient of 10~40% acetonitrile in 0.1 M TEAA, pH 7 over 60 min at a flow rate of 2 mL/min. The retention time for all the oligonucleotides bearing a 5'-*O*-(4,4'-dimethoxytrityl) group was in the range of 45~52 min. The recovered oligonucleotides were lyophilized and detritylated by treatment with 80% acetic acid at room temperature for 15 min followed by coevaporated with ethanol. All samples were redissolved in 0.1 M TEAA, pH 7 and chromatographed on a 10 mm × 25 cm column using a gradient of 5~40% acetonitrile in 0.1 M TEAA over 60 min at a flow rate of 2 mL/min. Under these chromatographic conditions, elution times for the nontritylated *N*^{2}-benzyl-2'-deoxyguanosine, *N*^{2}-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine, *N*^{6}-benzyl-2'-deoxyadenosine, and *N*^{6}-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine containing oligonucleotides were 23.58, 27.55, 28.58, and 39.50 min, respectively. Portions of all these nontritylated oligomers (~50 OD_{260} units) were further purified by preparative polyacrylamide gel electrophoresis using gels that were 20% acrylamide/*N,N'*-methylenebisacrylamide (19:1) and 7 M urea, run in 0.089 M Tris-borate/0.089 M boric acid/0.001 M EDTA, pH 8, extracted at 55°C overnight. The

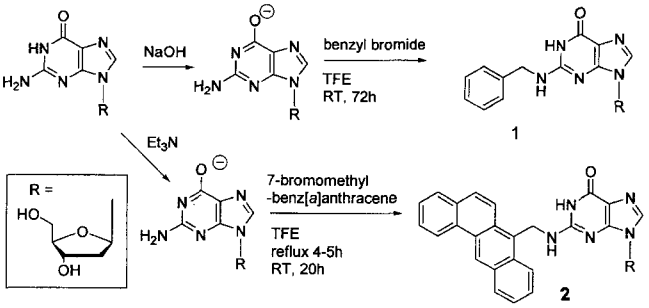
extracted oligonucleotides were applied to a Sep-Pak C_{18} (Water Millipore Cartridge) preflushed with MeOH and water, respectively. Salts were removed by eluting water. The oligonucleotides were eluted from the Cartridge with 5 mL of TEAA/MeOH (1:1). All oligonucleotides were chromatographed again under the same chromatographic conditions used in the purification of nontritylated oligomers. All these homogeneous oligonucleotides were then lyophilized and stored at -20°C.

Nucleoside Composition Analysis

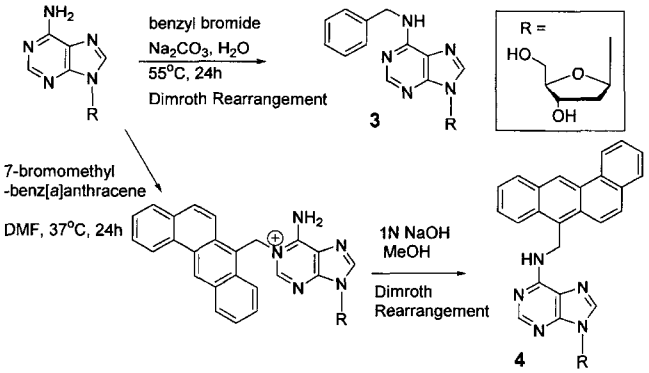
Oligonucleotides (0.6~0.7 OD_{260} units) were treated with 0.1 unit of phosphodiesterase and 2.5 units of alkaline phosphatase in a total volume of 0.91 mL of 0.05 M Tris-HCl and 0.01 M $MgCl_2$, pH 8.0, for 3 h at 37°C with occasional shaking. The oligomer containing N^6-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine was incubated for 20 h at 37°C. To a all incubation mixture were then added 0.04 mL of a 10 nM solution of 2'-deoxyuridine as a chromatographic internal standard and 0.05 mL of acetonitrile to make a 5% of acetonitrile solution, respectively. The resulting mixture (250 μL) was directly injected and analyzed by reversed-phase HPLC (4.6 mm × 25 cm Beckman Ultrasphere ODS column) on a Hewlett-Packard Model 1090 Series II HPLC equipped with a diode array detector. The column was eluted with for 10 min at 5% of acetonitrile (CH_3CN) in 0.1 M TEAA (pH 7), then a gradient of 5 to 10% CH_3CN in TEAA from 10~20 min, 10 to 30% CH_3CN from 20~40 min, then 30 to 70% from 40~60 min at a flow rate of 1 mL/min. All these HPLC analysis was carried out at 23°C. The retention times under these chromatographic conditions: dC, 3.7 min. dU, 4.5 min. dG, 5.9 min. dT, 7.6 min. dA 11.0 min. bn^2G, 34.2 min. bn^6A, 39.9 min. $b[a]a^2G$, 51.3 min. $b[a]a^6A$, 56.8 min. Peak areas were converted to nanomoles of nucleoside by comparison with calibration curves generated with authentic standards.

RESULTS AND DISCUSSION

We developed preparative routes for the four modified nucleosides involving direct aralkylation of 2'-deoxyguanosine and 2'-deoxyadenosine based on the solvent effect site-selectivity study (Dipple *et al.*, 1982; Dipple *et al.*, 1984) (Scheme I, II). These studies showed that product distributions were markedly dependent on the reaction solvent. Briefly, in dipolar aprotic solvents [e.g., *N,N*-dimethylacetate (DMA) or *N,N*-dimethylformamide (DMF)] reaction occurred primarily on ring nitrogen sites (i.e., the 7-position on guanine nucleosides and the 1-position in adenine nucleosides). In solvents of greater ionizing power (e.g., aqueous organic solvent mixtures or water) reaction at exocyclic sites (i.e., the N^2- and O^6-position on guanine nucleosides and the N^6-position on



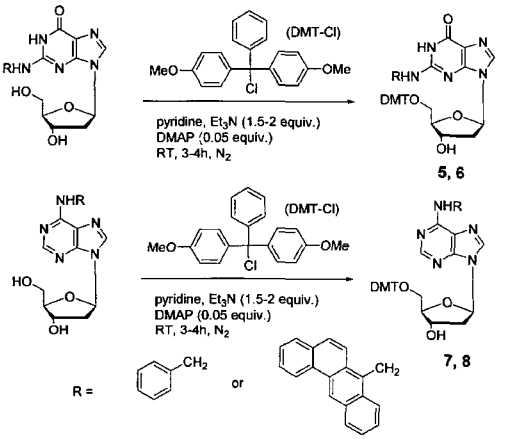
Scheme I. Preparation of N^2-Aralkylated 2'-deoxyguanosines



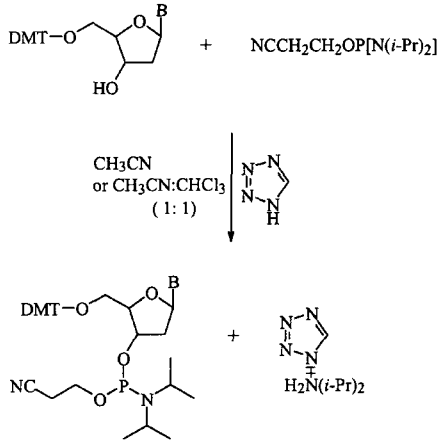
Scheme II. Preparation of N^6-Aralkylated 2'-deoxyadenosines

adenine nucleosides) occurred in addition to reaction at ring nitrogen sites. N^2-benzyl-2'-deoxyguanosine (**1**) was prepared by treatment of the anionic 2'-deoxyguanosine with benzyl bromide in trifluoroethanol (TFE) and was purified by Sephadex LH-20 gel chromatography. Benzylation of the anionic of 2'-deoxyguanosine ($dGuo^-$) induced by NaOH in TFE produced **1** in 45% yield (Moon and Moschel, 1998). This is one step direct modification and the reaction condition is very unique for the preparation of substantial amount of N^2-amino group benzylated 2'-deoxyguanosine with high selectivity. N^2-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine (**2**) was essentially prepared as published procedure (Pei and Moschel, 1990). The reaction of 7-BrMeB[a]A with $dGuo^-$ induced by NaOH in TFE produced **2** in 6 % yield which is ½ N^2-amino group aralkylation than that of $dGuo^-$ induced by triethylamine (12% yield) (Pei and Moschel, 1990). This observation can be explained that 7-BrMeB[a]A reacted with NaOH to produce 7-hydroxymethylB[a]A which results in the deactivation of 7-BrMeB[a]A due to the 7-BrMeB[a]A is more reactive than benzyl bromide. Aralkylation at the N^2-position on guanine nucleosides was shown to increase substantially in aqueous reactions involving the nucleoside anion compared to the neutral nucleoside even though the exocyclic amino group is not formally charged in the anion (Pei and Moschel, 1990; Moschel *et al.*, 1984; Moschel *et al.*, 1986). However, the total yield of the N^2-substituted products was fairly

low due to the high nucleophilicity of the aqueous solvents which led to preferential hydrolysis of the benzylating agents rather than to reaction with the nucleoside. The weakly nucleophilic but protic polar solvent TFE and the iminolate tautomeric form of the 2'-deoxyguanosine anion appear to be essential for benzylation at the exocyclic N^2-position (Moon and Moschel, 1998). The quantitative amount of N^6-benzyl-2'-deoxyadenosine (**3**) was prepared in an alkali aqueous solvent system (~20% yield). We expect that benzylation of 2'-deoxyadenosine in dipolar aprotic solvent (i.e., DMA or DMF) produces 1-benzyl-2'-deoxyadenosine, followed by induction of the Dimroth rearrangement to produce **3**. However, this reaction conditions produced depurinated product, viz., N^6-benzyladenine (Brookes *et al.*, 1968). Therefore, the alternative route for the preparation of **3** was the benzylation of 2'-deoxyadenosine in an aqueous solvent system. Benzylation predominantly occurred at exocyclic N^6-amino group of 2'-deoxyadenosine in an aqueous solvent to produce **3** which resulted in the direct benzylation of 2'-deoxyadenosine, even if there is a formation of 1-benzyl-2'-deoxyadenosine that undergoes Dimroth rearrangement in an alkali condition to produce **3**. N^6-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine (**4**) was prepared by a modification of the published procedure (Dipple *et al.*, 1971). The reaction of 2'-deoxyadenosine with 7-BrMeB[a]A in DMF produced 1-(benz[a] anthracen-7-ylmethyl)-2'-deoxyadenosine, followed by Dimroth rearrangement in methanolic alkali condition to obtain **4**. However, as shown in the preparation of **3**, the reaction of 2'-deoxyadenosine with benzyl bromide in DMF, followed by treatment of methanolic alkali produced N^6-benzyladenine. The reason for the depurination is as yet not clear and remains to be elucidated.



Scheme III. Preparation of N^2-Aralkylated 5-*O*-(4,4-Dimethoxytrityl)-2'-deoxyguanosines and N^6-Aralkylated 5-*O*-(4,4-Dimethoxytrityl)-2'-deoxyadenosines



Scheme IV. Preparation of N^2-Aralkylated 5'-*O*-(4,4-DMT)-2'-deoxyguanosine and N^6-Aralkylated 5'-*O*-(4,4-DMT)-2'-deoxyadenosine Phosphoramidite Derivatives

Site-specifically carcinogen modified oligonucleotides were prepared by total synthesis approach. Even the total synthesis approach is labor extensive, this method efficiently lead to the incorporation of carcinogen-modified base into a defined DNA sequence context. The modified four nucleosides were converted to 5'-*O*-4,4-dimethoxytrityl derivatives according to standard method (Scheme III) and subsequently to 3'-*O*-(2-cyanoethyl)diisopropylphosphoramidite derivatives using *in situ* activation approach as described (Scheme IV) (Pauly *et al.*, 1988). The latter were immediately incorporated into DNA using automated DNA synthetic method. The stepwise coupling efficiency for the standard and modified phosphoramidites were>97% and 95% as indicated by release of trityl cation, respectively. Aralkylated bases have been incorporated into DNA segments by using fully protected nucleoside precursors (Stezowski *et al.*, 1987; Casale and McLaughlin, 1990; Lee *et al.*, 1990; Lee *et al.*, 1995; Morningstar *et al.*, 1997) or less efficiently by reaction of an aralkyl halide with synthetically prepared DNA under aqueous/organic solvent conditions (Reardon *et al.*, 1990; Hruszkewycz *et al.*, 1991; Hruszkewycz and Dipple, 1991). The useful amounts of several specific arylamine-modified oligodeoxyribonucleotides have been prepared by direct reactions (Sharma and Box, 1985; Lasko *et al.*, 1987; Page *et al.*, 1996) between aromatic amine derivatives and preformed DNA segments. This in situ activation method enabled us to conserve nucleoside starting materials and

5'-d (A A C A G C C A T A T G G C C C)-3'

5'-d (A A C A G C C A T A T G*G C C C)-3'

5'-d (A A C A G C C A T A*T G G C C C)-3'

The asterisk marks the position of incorporation of the modified guanine and adenine derivatives.

Fig. 1. Synthetic DNA Sequences Containing N^2-Aralkylated Guanine and N^6-Aralkylated Adenine Nucleosides

Table I. Nucleoside Composition of Synthetic Oligodeoxyribonucleotides

	nucleoside composition found[a] (nucleoside composition expected)								
Oligonucleotide	dCyd	dGuo	dThd	dAdo	bn^2G	bn^6A	$b[a]a_2G$	$b[a]a^6A$	e $260(\times10^{-5})^c$
G*=G, A*=A	6.00	2.98	2.03	4.98	NP[b]	NP	NP	NP	1.29
	(6)	(3)	(2)	(5)					
G*=bn^2G	5.89	2.07	1.98	5.06	1.00	NP	NP	NP	1.35
	(6)	(2)	(2)	(5)	(1)				
A*=bn^6A	5.93	3.09	1.94	3.96	NP	1.08	NP	NP	1.39
	(6)	(3)	(2)	(4)		(1)			
G*=$b[a]a^2G$	5.80	2.09	2.01	5.16	NP	NP	0.94	NP	1.50
	(6)	(2)	(2)	(5)			(1)		
A*=$b[a]a^6A$	5.80	3.11	1.99	4.07	NP	NP	NP	1.03	1.49
	(6)	(3)	(2)	(4)				(1)	

[a] Results are the average of at least three determinations
[b] NP, not present
[c] Calculated from the total number of nanomoles of nucleosides liberated in a digest of a known OD_{260} value

eliminated the need to isolate and store larger amounts of cyanoethylphosphine intermediates of the carcinogen-modified nucleosides.

All the synthetic oligonucleotides (Fig. 1) were purified by reversed-phase HPLC (see Materials and Methods) to afford between 190 and 250 OD_{260} units of chromatographically homogeneous oligodeoxyribonucleotide from a single 10-mmol-scale synthesis. If necessary, portions of these oligomers were further purified by polyacrylamide gel electrophoresis (PAGE) and high pressure liquid chromatography (HPLC), successively. These oligomers were characterized by enzymatic digestion and followed by chromatographic analyses (see Materials and Methods) as described previously (Pauly *et al.*, 1988). It is interesting to describe that in the case of $b[a]a^6A$ containing oligomer, this oligomer needs much longer incubation time (20 h) to be completely digested to their component nucleosides than that of bn^2G, bn^6A, and $b[a]a^2G$ containing oligomers (3 h). This result indicates that aralkylation of N^6-amino group of 2'-deoxyadenosine, *i.e.*, $b[a]a^6A$ is much more resist for phosphordiesterase digestion than that of N^2-amino group of 2'-deoxyguanosine, *i.e.*, $b[a]a^2G$. After characterizing the samples by digestion to their component 2'-deoxyribonucleosides with snake venom phosphodiesterase and bacterial alkaline phosphatase, we quantified the liberated nucleosides. Table I shows excellent agreement between the expected and experimentally determined nucleoside compositions, conforming that the synthetic oligonucleotides were free of significant contamination by incompletely deprotected bases or possible base contaminants produced during synthesis and deprotection steps (Borowy-Borowski and Chambers, 1987). These oligonucleotides containing the aralkyl modified adducts at defined sites are being evaluated for their effect on site-specific mutagenicity in both *E. coli* and human cells.

ACKNOWLEDGEMENTS

This research was supported by the Brain Korea 21 project.

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