Preparation of Oligodeoxynucleotides Containing a Diastereoisomer of α-(N^2-2′-Deoxyguanosinyl)tamoxifen by Phosphoramidite Chemical Synthesis

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Women treated with an antiestrogen tamoxifen (TAM) for endocrine therapy or prevention of breast cancer show an increased risk of developing endometrial cancer. TAM-DNA adducts have been detected in the liver of rodents treated with TAM and in the endometrium of women taking TAM. The major TAM adducts have been identified as diastereoisomers of trans- and *cis*-forms of α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-TAM) and α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen. In the study presented here, we prepared oligodeoxynucleotides containing a diastereoisomer of dG-N^2-TAM by phosphoramidite chemical synthesis. Initially, the *trans*- and *cis*-forms of α-aminotamoxifen (α-NH_2-TAM) were synthesized from α-hydroxytamoxifen using the Mitsunobu reaction followed by hydrolysis. Thereafter by coupling the *trans*- and *cis*-form of α-NH_2-TAM with the DMT-derivative of 2-fluoro-(O^6-2-(trimethylsilyl)ethyl)-2′-deoxyinosine, the *trans*- and *cis*-forms of DMT-dG-N^2-TAM, respectively, were prepared in high yield and used in the preparation of the phosphoramidite precursors. Large quantities of oligodeoxynucleotides containing a *trans*- or a *cis*-form of dG-N^2-TAM were prepared efficiently by automated DNA synthesizer. The incorporation of dG-N^2-TAM adduct into the oligodeoxynucleotides was confirmed using ^{32}P-postlabeling/polyacrylamide gel electrophoresis analysis. These site-specifically modified oligodeoxynucleotides will be used for exploring biological properties and three-dimensional structure of TAM−DNA adducts.

Introduction

Tamoxifen (TAM;[1] [*E*-1-[4-{2-(dimethylamino)-ethoxy}-phenyl]-1,2-diphenyl]-butene) is widely used in the endocrine therapy of breast cancer (*1*). This drug was recently approved as a prophylactic agent for women at high risk of developing this disease. Unfortunately, an increased incidence of endometrial cancer has been observed in breast cancer patients treated with TAM (*2*−*7*) and in women undergoing chemoprevention (*8*). TAM is a potent hepatocarcinogen in rats (*9*−*11*) and is listed as a human carcinogen by the International Agency of Research on Cancer (*12*).

TAM is metabolized in the liver of rodents and humans to α-hydroxytamoxifen (α-OHTAM), *N*-desmethyltamoxifen (*N*-desTAM), tamoxifen *N*-oxide (TAM *N*-oxide), and 4-hydroxytamoxifen (4-OHTAM) (*13*−*15*). Some *N*-desTAM is further metabolized to α-hydroxy-*N*-desmethyltamoxifen (α-OH-*N*-desTAM) (*14*, *16*). We found that α-OHTAM is sulfonated by rat and human hydroxysteroid sulfotransferases (HST) (*17*, *18*), after which they react with the exocyclic amino group of guanine in DNA via a short-lived carbocation intermediate (*19*). This results in the formation of two *trans* (fr-1 and fr-2) and two *cis* (fr-3 and fr-4) diastereoisomers of the α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-TAM, the structures in Figure 1) adducts (*20*, *21*). In fact, dG-N^2-TAM and α-(N^2-deoxyguanosinyl)-*N*-desmethyltamoxifen (dG-N^2-*N*-desTAM) adducts have been detected as major DNA adducts in the liver of rodents treated with TAM (*22*−*24*). dG-N^2-TAM adducts have also been detected in the endometrium of certain women taking TAM (*25*, *26*). dG-N^2-TAM adducts are miscoding and mutagenic lesions, generating primarily G → T transversions in mammalian cells (*27*, *28*). This adduct can be removed from DNA by nucleotide excision repair enzymes (*29*). TAM-DNA adducts, if not readily repaired, may contribute to the initiation of endometrial cancer.

dG-N^2-TAM-modified oligodeoxynucleotides were prepared previously in our laboratory by allowing an oligodeoxynucleotide containing a single dG to react with α-acetoxytamoxifen or TAM α-sulfate (*27*, *28*). However, such postsynthetic methods do not allow the preparation of oligodeoxynucleotides having a variety of sequence contexts nor can they be used to synthesize large quantities of such oligomers. To resolve these difficulties we synthesized the DMT-phosphoramidite derivative of dG-N^2-TAM and performed a synthesis of dG-N^2-TAM-modified oligodeoxynucleotides using an automated DNA synthesizer. Using this technique, dG-N^2-TAM adducts can be inserted into any sequence such as known mutational hot-spots, and used for mutagenesis, DNA repair, and 3D NMR structural studies.

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[1] Abbreviations: dG, 2′-deoxyguanosine; $dG_{3'p}$, 2′-deoxyguanosine 3′-monophosphate; TAM, tamoxifen; α-OHTAM, α-hydroxytamoxifen; α-NH_2-TAM, α-aminotamoxifen; dG-N^2-TAM, α-(N^2-deoxyguanosinyl)-tamoxifen; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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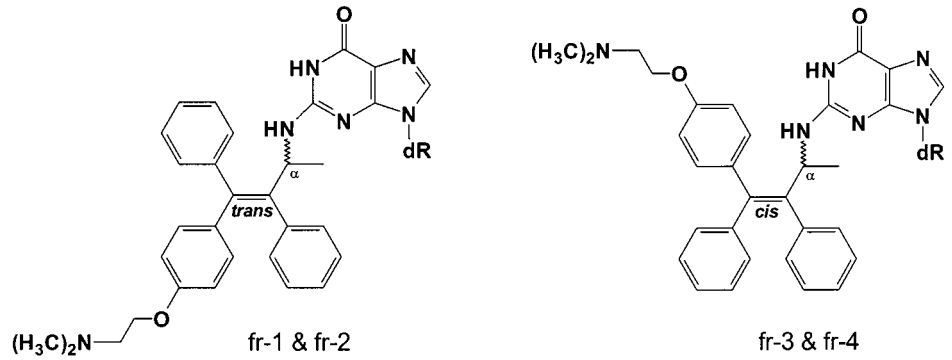


Figure 1. Structure of diastereoisomers of *trans*- and *cis*-dG-N^2-TAM.

Experimental Procedures

***Caution*:** TAM and its derivatives are genotoxic and should be handled with proper care. Waste materials must be discarded according to appropriate safety procedures.

Chemicals. Organic chemicals used for synthesis were supplied by Aldrich Chemical (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Whenever necessary, solvents were purified by standard procedures (*30*). Unless specified silica gel TLC plates were used. [γ-^{32}P]ATP (specific activity, 6000 Ci/mmol) was obtained from Amersham Corp (Arlington Heights, IL). Potato apyrase and alkaline phosphatase (type III) were purchased from Sigma (St. Louis, MO) and nuclease P1 was from Boehringer Mannheim (Indianapolis, IN). T4 polynucleotide kinase was purchased from Stratagene (La Jolla, CA).

Synthesis of Chemical Materials. The *trans*- and *cis*-forms of α-hydroxytamoxifen (α-OHTAM) were synthesized by the established protocol (*31*). Standard stereoisomers of *trans*- and *cis*-dG$_{3'p}$-N^2-TAM for ^{32}P-postlabeling analysis have been prepared previously (*18*). NMR spectroscopy was performed on Bruker AC 250 and Varian Gemini 300 MHz NMR instruments. FAB mass and the ESI negative ion mode were recorded on Quatro LC-micromass (Manchester) instrument. HPLC analyses and purifications were performed on a Waters 990 instrument, equipped with a photodiode array detector.

Preparation of dG-N^2-TAM-Modified Oligodeoxynucleotides by Chemical Synthesis. *2-[2-{(E)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-diphenyl-but-3-enyl}]isoindole-1,3-dione (**3a**).* *trans*-(*E*)-4-[4-(2-Dimethylamino-ethoxy)-phenyl]-3,4-diphenyl-but-3-en-2-ol (**2a**) was prepared by the procedure reported by Foster et al. (*31*). To a solution of **2a** (1.80 g, 4.65 mmol) in THF (60 mL) were added triphenylphosphine (1.25 g, 4.77 mmol) and phthalimide (0.71 g, 4.8 mmol) under an inert atmosphere, and stirring of the solution was continued at 0 °C for 5 min. Then diethylazodicarboxylate (0.84 g, 4.8 mmol) was added, and the mixture was stirred at 0 °C for 30 min. The reaction mixture was allowed to come to room temperature and stirred overnight. The solvent was evaporated and the residue was purified by flash chromatography using silica gel and as the eluent, ether:Et_3N, 9:1. Compound **3a** was obtained as pale yellow solids (1.14 g, 48%). R_f = 0.29 (eluent, ether:Et_3N, 9:1). The product was characterized as 2-[2-{(*E*)-4-(4-(2-dimethylaminoethoxy)phenyl)-3,4-diphenyl-but-3-enyl}]isoindole-1,3-dione (**3a**). FAB mass: *m/z* 517 ($M^+ + 1$). ^{1}H NMR ($CDCl_3$) δ ppm :1.56 (d, 3H, J = 7.2 Hz, HC−CH_3); 2.26 (s, 6H, N(CH_3)$_2$); 2.60 (t, 2H, J = 5.6 Hz, N−CH_2); 3.88 (t, 2H, J = 5.8 Hz, O−CH_2); 5.41 (q, 1H, J = 7.5 Hz, H−C(CH_3)); 6.51 (d, 2H, J = 9 Hz, H-3,5 of CC_6H_4O); 6.83 (d, 2H, J = 9 Hz, H-2,6 of CC_6H_4O); 7.05−7.71 (m, 14H, aromatic protons). ^{13}C NMR ($CDCl_3$) δ ppm: 17.66 (CH−CH_3), 45.05 (N(CH_3)$_2$), 49.69 (CH−CH_3), 57.36 (N−CH_2), 64.88 (O−CH_2), 112.67, 121.84, 125.90, 126.07, 127.69, 127.76, 127.88, 127.98, 130.02, 130.22, 131.17, 131.32, 132.68, 132.84, 133.88, 137.74, 138.43, 140.63, 141.18, 156.18, 166.99 (C═O).

*2-[2-{(Z)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-diphenyl-but-3-enyl}]isoindole-1,3-dione (**3b**).* The procedure employed was similar to that used for the *trans*-isomer (**3a**), in which *cis*-(Z)-4-[4-(2-dimethylamino-ethoxy)-phenyl]-3,4-diphenyl-but-3-en-2-ol (**2b**) (0.56 g, 1.44 mmol) was allowed to react with phthalimide (0.22 g, 1.49 mmol), triphenylphosphine (0.39 g, 1.49 mmol), and diethylazodicarboxylate (0.26 g, 1.49 mmol) in 20 mL of THF. This gave the desired product 2-[2-{(*Z*)-4-(4-(2-dimethylaminoethoxy)phenyl)-3,4-diphenyl-but-3-enyl}]isoindole-1,3-dione (**3b**), (0.4 g 53%), R_f = 0.14 (eluent, ether:Et_3N, 9:1). FAB mass: *m/z* 517 ($M^+ + 1$). ^{1}H NMR ($CDCl_3$) δ ppm: 1.61 (d, 3H, J = 7.5 Hz, HC−CH_3); 2.34 (s, 6H, N(CH_3)$_2$); 2.71 60 (t, 2H, J = 5.7 Hz, N−CH_2); 4.01 (dt, 2H, J = 6.0, 2.1 Hz, O−CH_2); 5.46 (q, 1H, J = 7.5 Hz, H−C(CH_3)); 6.82−7.70 (m, 18H, aromatic). ^{13}C NMR ($CDCl_3$) δ ppm: 18.21 (CH−CH_3), 45.82 (N(CH_3)$_2$), 50.54 (CH−CH_3), 58.20 (N−CH_2), 65.79 (O−CH_2), 114.46, 122.58, 125.82, 126.52, 127.20, 127.39, 128.33, 128.52, 129.61, 129.70, 130.60, 131.68, 131.93, 132.09, 133.48, 133.94, 138.84, 139.15, 141.49, 142.43, 157.68, 167.96 (C═O).

*[2-{(E)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-diphenyl but-3-enyl}]amine (**4a**).* A 33% solution of methylamine in absolute ethanol (36.4 mL) was added to a stirred solution of **3a** (1.13 g, 2.2 mmol) in 99.9% ethanol (18.3 mL) at room temperature. After 5 min, the reaction mixture was refluxed for 2.5 h. The mixture was then cooled to room temperature and the solvent was evaporated under vacuum. The residue was subjected flash chromatography on basic alumina, using as the eluent CH_2Cl_2:MeOH, 9:1. The title compound **4a** was obtained as a pale yellow solid (0.53 g, 63%), R_f = 0.42 on alumina TLC (eluent, CH_2Cl_2:MeOH, 9:1). FAB mass: *m/z* 387 ($M^+ + 1$). ^{1}H NMR ($CDCl_3$) δ ppm: 1.09 (d, 3H, J = 6.6 Hz, HC−CH_3); 2.27 (s, 6H, N(CH_3)$_2$); 2.62 (t, 2H, J = 5.7 Hz, N−CH_2); 3.89 (t, 2H, J = 5.7 Hz, O−CH_2); 4.05 (q, 1H, J = 6.6 Hz, $NH_2HC(CH_3)$); 6.53 (d, 2H, J = 6.6 Hz, H-3,5 of CC_6H_4O); 6.80 (d, 2H, J = 6.6 Hz, H-2,6 of CC_6H_4O); 7.16−7.39 (m, 10H, phenyls). ^{13}C NMR ($CDCl_3$) δ ppm: 22.41 (HC−CH_3); 45.34 (N(CH_3)$_2$); 48.28 ($NH_2HC(CH_3)$); 57.70 (N−CH_2); 65.12 (O−CH_2); 112.82, 125.81, 126.17, 127.05, 127.75, 128.79, 130.45, 130.59, 134.24, 138.09, 138.46, 141.91, 143.59, 156.26.

*[2-{(Z)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-diphenyl but-3-enyl}]amine (**4b**).* The procedure is similar to that used for the *trans*-isomer (**4a**). Compound **3b** (0.53 g, 1.02 mmol) in 8.5 mL of ethanol and 17 mL of a 33% solution of methylamine in ethanol gave **4b** as a pale orange colored solid (0.3 g, 76%). R_f = 0.25 (eluent, CH_2Cl_2:MeOH, 9:1). FAB mass: *m/z* 387 ($M^+ + 1$). ^{1}H NMR ($CDCl_3$) δ ppm: 1.11 (d, 3H, J = 6.6 Hz, HC−CH_3); 2.36 (s, 6H, N(CH_3)$_2$); 2.75 (t, 2H, J = 5.7 Hz, N−CH_2); 4.07−4.17 (m, 3H, O−CH_2 and $NH_2HC(CH_3)$); 6.89−7.20 (m, 14H, aromatic). ^{13}C NMR ($CDCl_3$) δ ppm: 22.90 (HC−CH_3); 45.85 (N(CH_3)$_2$); 48.58 ($NH_2HC(CH_3)$); 58.23 (N−CH_2); 65.88 (O−CH_2); 114.23, 125.66, 126.22, 127.15, 127.35, 129.87, 130.33, 130.92, 134.50, 138.41, 139.40, 142.5, 144.8, 157.5.

*N^2-[2-{(E)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-diphenyl but-3-enyl}]-2′-deoxyguano-sine (**6a**).* Compounds **4a** (84 mg, 0.22 mmol) and **5** (81 mg, 0.22 mmol) were dissolved in dry DMSO (5 mL), and the reaction mixture was stirred under an inert atmosphere at 75 °C for 3 days [until the disappearance of starting material was evident by TLC (eluent, CH_2Cl_2:MeOH, 7:3)]. The solvent was removed under high vacuum and 0.1% of acetic acid solution (2 mL) was added, and the mixture stirred

at room temperature for 2 h. The solvent was removed, and the dry residue was purified by flash chromatography on silica gel using as the eluent CH_2Cl_2:MeOH:Et_3N, 9:0.9:0.1. The title compound **6a** was obtained as a pale yellow solid (50 mg, 38%) $R_f = 0.20$ (TLC eluent CH_2Cl_2:MeOH, 7:3). FAB mass: *m/z* 637 ($M^+ + 1$). ^{1}H NMR (CD_3OD) δ ppm: 1.36 (d, 3H, $J = 7.2$ Hz, HC−CH_3); 2.30 (s, 6H, N(CH_3)$_2$); 2.70 (t, 2H, $J = 5.3$ Hz, N−CH_2); 3.92 (t, 2H, $J = 5.6$ Hz, O−CH_2), 5.09−5.17 (m, 1H, N−CH−CH_3), 6.55 and 6.65 (d, 2H, $J = 9$ Hz, H-3,5 of CC_6H_4O); 6.82 and 6.83 (d, 2H, $J = 9.3$ Hz, H-2,6 of CC_6H_4O); 7.09−7.51 (m, 10H, phenyls); 8.08, 8.06 (2s, 1H, H at C-8 of dG). Sugar moiety: 2.33−2.63 (m, 2H, 2′-CH_2); 3.69−3.81 (m, 2H, 5′CH_2−OH); 4.02−4.08 (m, 1H, 4′-CH); 4.50−4.56 (m, 1H, 3′-CH), 6.53−6.41 (m, 1H, 1′-CH). ^{13}C NMR (CD_3OD) δ ppm: 21.49 ($CHCH_3$), 41.66 and 42.45 (C2′ of sugar moiety), 45.76 (N(CH_3)$_2$); 50.44 and 50.65 (NHCHCH_3), 59.05 (N−CH_2), 63.31 and 63.37 (C5′ of sugar moiety), 66.22 and 66.26 (O−CH_2), 72.55 and 72.68 (C3′ of sugar moiety), 84.57 and 84.69 (C1′ of sugar moiety), 89.32 and 89.45 (C4′ of sugar moiety), 114.56 and 114.59, 117.56 and 117.57, 127.84, 128.30, 129.00, 129.72, 130.53 and 130.56, 131.95 and 132.03, 132.36 and 132.42, 132.47 and 132.52, 136.60 and 136.94, 137.34 and 137.59, 140.31 and 140.43, 141.75 and 141.89, 142.05 and 142.31, 143.65 and 143.72, 152.90 and 152.97, 153.14 and 153.18, 157.93 and 157.99.

*N^2-[2-{(Z)-4-(4-(2-Dimethylaminoethoxy)phenyl)-3,4-diphenyl but-3-enyl}]-2′-deoxyguano-sine (**6b**).* The procedure was similar to that used for the *trans*-isomer (**6a**). Compounds **4b** (100 mg, 0.26 mmol) and **5** (100 mg, 0.27 mmol) in 5 mL of dry DMSO after reaction were treated with 0.1% AcOH (5 mL) and gave the title compound **6b** as a pale yellow solid (63 mg, 38%), $R_f = 0.15$ (eluent CH_2Cl_2:MeOH, 7:3). FAB mass: *m/z* 637 ($M^+ + 1$). ^{1}H NMR (CD_3OD) δ ppm: 1.38 (d, 3H, $J = 7.2$ Hz, HC−CH_3); 2.35 (s, 6H, N(CH_3)$_2$); 2.78 (t, 2H, $J = 5.5$ Hz, N−CH_2); 4.01−4.09 (m, 2H, O−CH_2), 5.19 (q, 1H, $J = 7.1$ Hz, N−CH−CH_3), 6.89−7.37 (m, 14H, phenyls); 8.08, 8.05 (2s, 1H, H at C-8 of dG). Sugar moiety: 2.44−2.61 (m, 2H, 2′-CH_2); 4.06 (m, 2H, 5′CH_2−OH); 4.13 (m, 1H, 4′-CH); 4.52 (m, 1H, 3′-CH), 6.41 (t, 1H, $J = 6.9$ Hz, 1′-CH). ^{13}C NMR (CD_3OD) δ ppm: 21.42 ($CHCH_3$), 41.42 and 42.45 (C2′ of sugar moiety), 47.16 (N(CH_3)$_2$); 50.25 and 50.44 (NHCHCH_3), 59.25 and 59.30 (N−CH_2), 63.32 and 63.40 (C5′ of sugar moiety), 66.69 and 66.74 (O−CH_2), 72.59 and 72.73 (C3′ of sugar moiety), 84.56 and 84.75 (C1′ of sugar moiety), 89.35 and 89.47 (C4′ of sugar moiety), 115.65, 117.41 and 117.62, 126.93, 127.70, 128.37 and 128.40, 128.80, 130.98 and 131.12, 131.60 and 131.66, 131.89 and 131.94, 135.82, 137.26, 137.58, 140.06, 140.22, 141.90, 142.10, 142.33, 142.60, 144.10, 152.80, 152.92, 153.10, 153.14,157.35.

*5′-O-(4,4′-Dimethoxytrityl)-N^2-[2-{(E)-4-(4-(2-dimethylaminoethoxy)phenyl)-3,4-diphenyl-but-3-enyl}]-2′-deoxyguanosine (**8a**).* Compounds **4a** (100 mg, 0.26 mmol) and **9** (175 mg, 0.26 mmol) were dissolved in anhydrous DMSO (4 mL), and triethylamine (0.036 mL, 0.29 mmol) was added, and the reaction mixture was stirred at 75 °C for 5 days (i.e., until the disappearance of the starting materials as indicated by TLC (eluent, CH_2Cl_2:CH_3-OH:Et_3N, 95:4:1). The solvent was removed under vacuum and the residue was purified by flash chromatography on silica gel using CH_2Cl_2:CH_3OH:Et_3N, 95:4:1, as eluent. The product **8a** was obtained as a pale yellow solid (164 mg, 67%), $R_f = 0.39$ (eluent CH_2Cl_2:CH_3OH:Et_3N, 95:4:1). FAB mass: *m/z* 939 ($M^+ + 1$). ^{1}H NMR (CD_3OD) δ ppm: 1.37 (d, 3H, $J = 7.1$ Hz, HC−CH_3); 2.21, 2.26 (2s, 6H, N(CH_3)$_2$); 2.57 (t, 2H, $J = 5.7$ Hz, N−CH_2); 3.91 (t, 2H, $J = 5.4$ Hz, O−CH_2), 5.04−5.21 (m,1H, N−CH−CH_3), 6.36−7.51 (m, 29H, aromatic and C1′-H of sugar moiety); 7.85 (s, 1H, H at C-8 of dG). Sugar moiety: 2.32−2.54 (m, 1H, 2′-CH_2); 2.81−2.98 (m, 1H, 2″CH_2); 3.27−3.24 (m, 1H, 5′CH_2−OH); 3.51−3.59 (m, 1H, 5″CH_2−OH); 3.71 (s, 3H, OCH_3); 3.75 (s, 3H, OCH_3); 4.14−4.18 (m, 1H, 4′-CH); 4.49−4.56 (m, 1H, 3′-CH).

*5′-O-(4,4′-Dimethoxytrityl)-N^2-[2-{(E)-4-(4-(2-dimethylaminoethoxy)phenyl)-3,4-diphenyl-but-3-enyl}]-2′-deoxyguanosine (**8b**).* The procedure was similar to that used for the *trans*-isomer **8a**. Compounds **4b** (126 mg, 0.33 mmol) and **9** (200 mg, 0.33 mmol) were dissolved in dry DMSO (5 mL) and triethylamine (0.046 mL, 0.36 mmol) was added. Reaction and workup were conducted as before to give the title compound **8b**. Both of the diastereoisomers could be separated and isolated by flash chromatography on silica gel using CH_2Cl_2:CH_3OH:Et_3N, 95:4:1, as the eluent. Fraction **1** (71 mg) $R_f = 0.39$, and fraction **2** (67 mg) $R_f = 0.29$ (eluent CH_2Cl_2:MeOH:Et_3N, 95:4:1). Overall yield = 47%.

*Fraction **8b-1**.* FAB mass: *m/z* 939 ($M^+ + 1$). ^{1}H NMR (CD_3-OD) δ ppm: 1.36 (d, 3H, $J = 6.9$ Hz, HC−CH_3); 2.26 (s, 6H, N(CH_3)$_2$); 2.69 (t, 2H, $J = 5.4$ Hz, N−CH_2); 4.06 (t, 2H, $J = 5.4$ Hz, O−CH_2), 5.26 (q,1H, $J = 6.9$ Hz, N−CH−CH_3), 6.76−7.41 (m, 28H, aromatic); 7.85 (s, 1H, H at C-8 of dG). Sugar moiety: 2.50−2.58 (m, 2H, 2′-CH_2); 3.69 (broad base singlet, 8H, OCH_3 and 5′CH_2−OH); 4.12−4.21 (m, 1H, 4′-CH); 4.51−4.61 (m, 1H, 3′-CH); 6.40 (t, 1H, $J = 6.5$ Hz, 1′CH).

*Fraction **8b-2**:* FAB mass: *m/z* 939 ($M^+ + 1$). ^{1}H NMR (CD_3-OD) δ ppm: 1.34 (d, 3H, $J = 6.9$ Hz, HC−CH_3); 2.30 (s, 6H, N(CH_3)$_2$); 2.73 (t, 2H, $J = 5.1$ Hz, N−CH_2); 4.09 (t, 2H, $J = 5.1$ Hz, O−CH_2), 5.17 (q, 1H, $J = 6.9$ Hz, N−CH−CH_3), 6.72−7.41 (m, 28H, aromatic); 7.83 (s, 1H, H at C-8 of dG). Sugar moiety: 2.28−2.54 (m, 2H, 2′-CH_2); 3.68 (singlet, 6H, OCH_3); 3.58−3.74 (m, 2H. 5′CH_2−OH); 4.14−4.21 (m, 1H, 4′-CH); 4.47−4.58 (m, 1H, 3′-CH); 6.41 (t, 1H, $J = 6.5$ Hz, 1′CH).

*5′-O-(4,4′-Dimethoxytrityl)-N^2-[2-{(E)-4-(4-(2-dimethylaminoethoxy)phenyl)-3,4-diphenyl but-3-enyl}]-2′-deoxyguanosine 3′-O-(2-cyanoethoxy, N,N-diisopropyl) phosphoramidite (**10a**).* Compound **8a** (0.20 g, 0.21 mmol) was dried by azeotropic treatment with anhydrous pyridine (2 × 10 mL) and placed under vacuum for 3 h. Anhydrous 1-H-tetrazole (17 mg, 0.25 mmol) was added to a flame dried flask and kept under a nitrogen atmosphere. A solution of **8a** in anhydrous CH_2Cl_2 (4 mL) was injected into the reaction flask followed by 2-cyanoethyl N,N,N,N-tetraisopropyl phosphoramidite (100 mg, 0.31 mmol). The reaction mixture was stirred at room temperature for 3 h and then added to a saturated solution of sodium bicarbonate (27 mL) and extracted with CH_2CL_2 (5 × 30 mL). The organic layer was separated and dried ($MgSO_4$) then evaporated. The residue was purified by flash column chromatography using silica gel (CH_2-Cl_2:MeOH:Et_3N, 95:4:1) to give **10a** as a pale yellow solid (225 mg, 95%) $R_f = 0.61$ (eluent, CH_2Cl_2:CH_3OH:Et_3N, 95:4:1). FAB mass: *m/z* 1156 ($M^+ + H_2O$). ^{1}H NMR (CD_3OD) δ ppm: 0.99−1.22 (m, 12H, 2[(CH_3)$_2$CH]); 1.34 (d, 3H, $J = 6.6$ Hz, $CHCH_3$); 2.18, 2.23 (2s, 6H, N(CH_3)$_2$); 2.34−2.79 (m, 6H, 2′CH_2 of sugar moiety, NCH_2, CH_2CN); 3.31−3.68 (m, 6H, 5′CH_2 of sugar moiety, P−O−CH_2, 2NCH(CH_3)$_2$); 3.69 (s, 3H, OCH_3); 3.72 (s, 3H. OCH_3); 3.89 (t, 2H, $J = 5.3$ Hz, OCH_2); 4.22−4.36(m, 1H, 4′CH of sugar moiety); 4.65−4.76 (m, 1H, 3′CH of sugar moiety); 5.05−5.19 (m,1H, HNCHCH$_3$); 6.38−7.50 (m, 29H, aromatic and 1′CH); 7.88−7.91 (m, 1H, H at C8 of dG). ^{31}P NMR (CD_3OD) δ ppm: 154.66, 154.09, 153.88, 153.84.

*5′-O-(4,4′-Dimethoxytrityl)-N^2-[2-{(Z)-4-(4-(2-dimethylaminoethoxy)phenyl)-3,4-diphenyl but-3-enyl}]-2′-deoxyguanosine 3′-O-(2-Cyanoethoxy,N,N-diisopropyl) Phosphoramidite (**10b**).* The title compound was prepared by the procedure similar to that used for the *trans*-isomer **10a**. The phosphoramidite's derivative of both the diastereoisomers was prepared separately. **8b-1** (71 mg, 0.08 mmol) in 1.5 mL of CH_2Cl_2 was added to 1-H-tetrazole (6.1 mg, 0.09 mmol) and 2-cyanoethyl N,N,N,N-tetraisopropyl phosphoramidite (36.6 mg, 0.09 mmol) to give **10b-1** (60 mg, 70%) $R_f = 0.35$ (eluent, CH_2Cl_2:CH_3OH:Et_3N, 95:4:1). ^{1}H NMR (CD_3OD) δ ppm: 0.99−1.14 (m, 12H, 2[(CH_3)$_2$CH]); 1.38 (d, 3H, $J = 6.9$ Hz, $CHCH_3$); 2.27 (2s, 6H, N(CH_3)$_2$); 2.27−2.37 (m, 6H, 2′CH_2 of sugar moiety, NCH_2, CH_2CN); 3.31−3.57 (m, 4H, P−O−CH_2, 2NCH(CH_3)$_2$); 3.73 (broad base singlet, 8H, 2OCH_3, 5′CH_2 of sugar moiety); 4.08 (t, 2H, $J = 5.2$ Hz, OCH_2); 4.23−4.39 (m, 1H, 4′CH of sugar moiety); 4.63−4.79 (m, 1H, 3′CH of sugar moiety); 5.15−5.25 (m, 1H, HNCHCH$_3$); 6.35−6.51 (m, 1H, 1′CH of sugar moiety); 6.79−7.48 (m, 28H, aromatic); 7.922, 7.91 (2s, 1H, C8−H of dG). ^{31}P NMR (CD_3OD) δ ppm: 153.77, 153.86. FAB mass: *m/z* 1156 ($M^+ + H_2O$). **8b-2** (67 mg, 0.07 mmol) in 1.5 mL of CH_2Cl_2, was added to 1-H-tetrazole (5.8 mg,

0.08 mmol), 2-cyanoethyl N,N,N,N-tetraisopropylphosphoramidite (34.3 mg, 0.08 mmol) to give **10b-2** (50 mg, 61%) R_f = 0.31 (eluent, CH_2Cl_2:CH_3OH:Et_3N, 95:4:1). FAB mass: *m/z* 1156 ($M^+ + H_2O$). ^1H NMR (CD_3OD) δ ppm: 0.99−1.21 (m, 12H, 2[$(CH_3)_2CH$]); 1.34(d, 3H, J = 4.8 Hz, $CHCH_3$); 2.32 (s, 6H, $N(CH_3)_2$); 2.39 (t, 1H, J = 5.7 Hz, 1H of CH_2CN); 2.52−2.94 (m, 5H, 2′CH_2 of sugar moiety, NCH_2, 1H of CH_2CN); 3.28−3.35 (m, 2H, P−O−CH_2); 3.53−3.73 (m, 4H, 2$NCH(CH_3)_2$, 5′CH_2 of sugar moiety); 3.70 (s, 6H, 2OCH_3,); 4.10 (t, 2H, J = 5.2 Hz, OCH_2); 4.18−4.32 (m, 1H, 4′CH of sugar moiety); 4.72−4.85 (m, 1H, 3′CH of sugar moiety); 5.12−5.21 (m,1H, $HNCHCH_3$); 6.39−6.52 (m, 1H, 1′CH of sugar moiety); 6.72−7.41(m, 28H, aromatic); 7.89 (s, 1H, C8−H of dG). ^{31}P NMR (CD_3OD) δ ppm: 154.39, 153.60.

Synthesis of Oligodeoxynucleotides. dG-N^2-TAM-modified oligodeoxynucleotides were synthesized on an Applied Biosystems DNA synthesizer at the 0.25, 1.0, or 10 μmol, as described previously (*32*). Upon completion of the synthesis, the beads from the cassette were incubated at 55 °C for 16 h in concentrated ammonium hydroxide. The supernatant liquid was decanted and the residual beads were washed twice with water (2 × 500 μL). The combined aqueous fractions were lyophilized and dissolved in 0.5 mL of 0.1 M TEAA buffer at pH 6.8, filtered, and subjected to HPLC for isolation of the DMT-protected oligodeoxynucleotides. HPLC separation was performed on PRP-1 column (0.46 × 25 cm) at room temperature using a linear gradient of (A) 0.1 M TEAA, pH 6.8, containing 16−40% of acetonitrile over a period of 30 min, with a flow rate of 1.0 mL/min. The fractions containing the oligomer were collected and lyophilized. To remove the DMT protection, these samples were treated with 80% acetic acid (1 mL) for 30 min, neutralized with 0.1 M TEAA buffer pH 6.8 (2 mL), and lyophilized. The DMT-deprotected oligodeoxynucleotides were purified on μBondapak C_{18} (0.78 × 30 cm, Waters) using 0.05 M TEAA, pH 7.0, containing 10−20% acetonitrile, eluted over 60 min at a flow rate 2.0 mL/min.

^{32}P-Postlabeling/Polyacrylamide Gel Electrophoresis Analysis. An unmodified or dG-N^2-TAM-modified oligodeoxynucleotide (250 pg) was digested at 37 °C for 2 h in 30 μL of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM $CaCl_2$, using micrococcal nuclease (15 units) and spleen phosphodiesterase (0.15 units). The reaction mixture was incubated at 37 °C for another 1 h with nuclease P1 (1.0 unit). After the incubation, 100 μL of water was added. The reaction samples were extracted twice with 200 μL of butanol. The butanol fractions were combined, back-extracted with 50 μL of distilled water, evaporated to dryness, and then used for ^{32}P-postlabeling analysis. Approximately 95% of TAM adducts were recovered by butanol extraction. The concentration of the oligodeoxynucleotide was estimated as 30 μg = O.D.$_{260nm}$ 1.0.

The DNA digests were incubated at 37 °C for 30 min with 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) and 1 μL of 3′-phosphatase-free T4 polynucleotide kinase (10 units/μL) (*33*) and then incubated with apyrase (50 milliunits) for another 30 min, as described previously (*18*). A part of the ^{32}P-labeled sample was electrophoresed for 5 h on a nondenaturing 30% polyacrylamide gel (35 × 42 × 0.04 cm) with 1400−1600 V/20−40 mA. A 30% polyacrylamide gel was prepared from 40% polyacrylamide solution (60 mL), 10× TBE buffer, pH 7.0 (10 mL), distilled water (10 mL), 10% ammonium persulfate (0.6 mL), and TEMED (35 μL). A 10× TBE buffer (pH 7.0) was prepared from 1 M tris-base, 2.24 M boric acid, and 25.5 mM EDTA. Standard stereoisomers of *trans*- and *cis*-forms of $dG_{3'p}$-N^2-TAM (*18*) were also labeled with ^{32}P and subjected to PAGE. The position of ^{32}P-labeled adducts was established by a β-phosphorimager analysis (Molecular Dynamics Inc.).

Isolation of dG-N^2-TAM. To isolate dG-N^2-TAM, the pooled butanol extracts were subjected to liquid chromatography on a reverse-phase μBondapak C_{18} column (0.78 × 30 cm, Waters), eluted over 45 min at a flow rate of 2.0 mL/min with a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10 to 70% acetonitrile. Isocratic HPLC conditions using 65% of 0.05 M triethylammonium acetate, pH 7.0, and 35% acetonitrile were also used for the purification. HPLC analysis was performed on a Waters 990 HPLC instrument, equipped with a photodiode array detector.

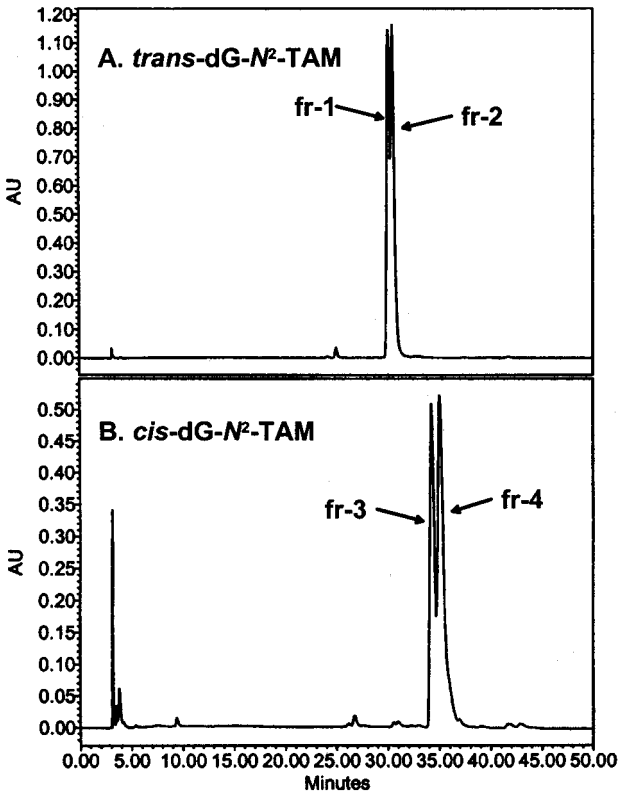
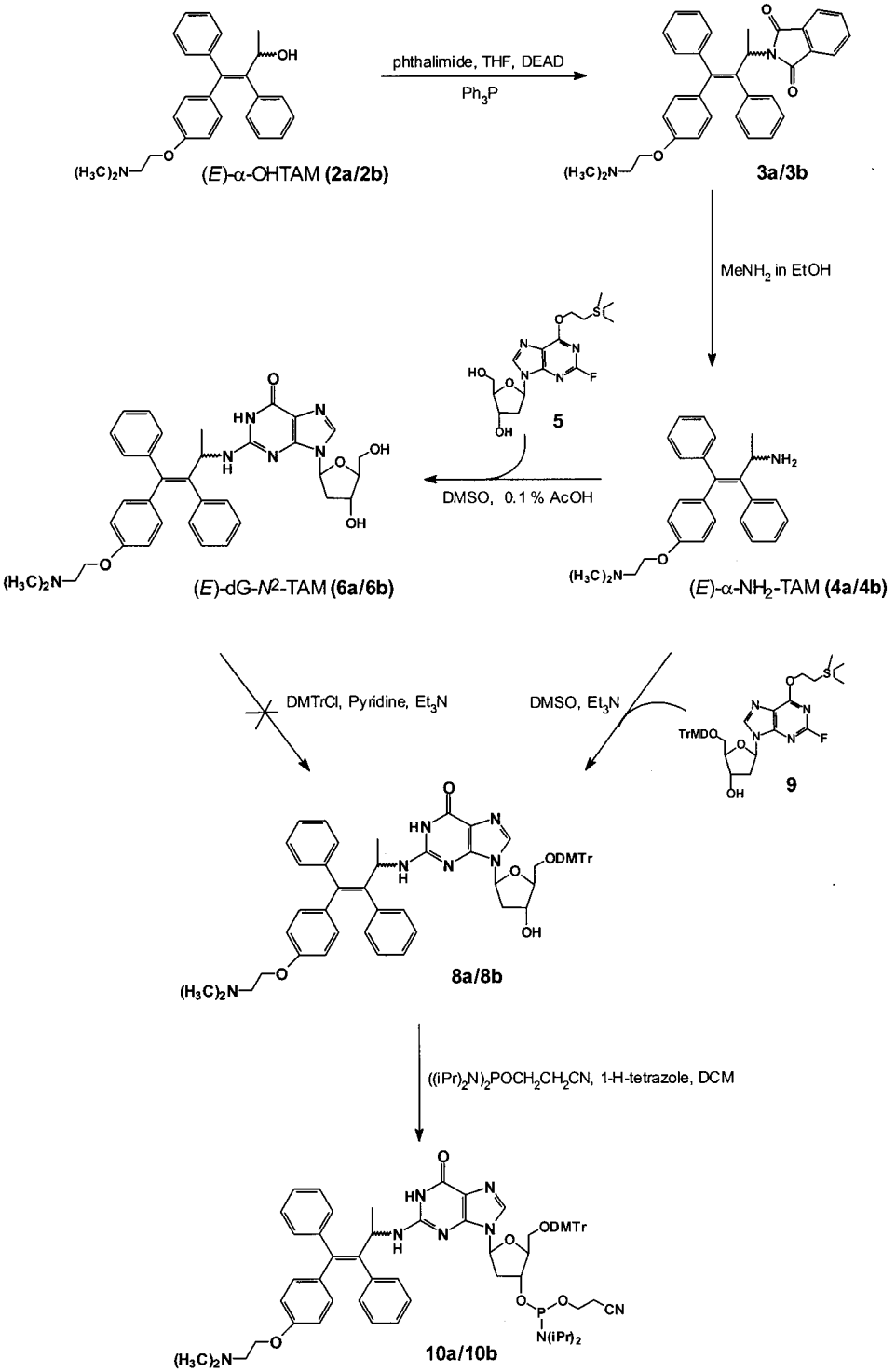


Figure 2. HPLC separation of *trans*- and *cis*-dG-N^2-TAM. *trans*-isoforms (A) or *cis*-isoforms (B) of dG-N^2-TAM were isolated on a μBondapak C_{18} (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate (pH 7.0), containing 10 to 70% acetonitrile with an elution time of 45 min and a flow rate of 1.0 mL/min.

Results and Discussion

Preparation of *trans*- and *cis*-dG-N^2-TAM. (*E*)-α-NH_2-TAM (**4a**) and (*Z*)-α-NH_2-TAM (**4b**) were synthesized from (*E*)-α-OHTAM (**2a**) and (*Z*)-α-OHTAM (**2b**), respectively, using the Mitsunobu reaction (*34*) followed by hydrolysis (*35*), as shown in Scheme 1. The detailed synthetic conditions, and purification of products are described in the Materials and Methods. 2-Fluoro-(O^6-trimethylsilylethyl)-2′-deoxyinosine (**5**) was coupled with (*E*)-α-NH_2-TAM (**4a**) or (*Z*)-α-NH_2-TAM (**4b**) in anhydrous DMSO. The products were treated with a low concentration of acetic acid to remove the O^6-silyl group and then purified by column chromatography to give the *trans*- (**6a**) or *cis*-forms (**6b**) of dG-N^2-TAM in approximately 38% yield. When the *trans*-form of dG-N^2-TAM (**6a**) was subjected to HPLC, two diastereoisomers (fr-1 and fr-2) were detected at 30.1 and 30.4 min (Figure 2A) although the separation was not well as reported previously (*20*). With the *cis*-form of dG-N^2-TAM (**6b**), two diastereoisomers (fr-3 and fr-4) were detected at 34.2 and 35.0 min (Figure 2B). The ^1H NMR, FAB mass and the UV spectra of **6a** and **6b** were consistent with those of the products obtained by reacting dG with TAM α-sulfate or α-acetoxytamoxifen (*20*). Since the yield of dG-N^2-TAM was much higher than that obtained from the postsynthetic method (*20*), ^{13}C NMR spectra of **6a** and **6b** were determined and described in the Materials and Methods.

Scheme 1. Synthetic Protocol for dG-N^2-TAM-Modified Oligodeoxynucleotides



[a] The synthetic steps are described only for the *trans*-isomers (**a**), but the same procedure was employed for the *cis*-isomers (**b**).

Preparation of dG-N^2-TAM-Modified Oligodeoxynucleotides by Phosphoramidite Automated Synthesis. To incorporate the dG-N^2-TAM adduct into an oligodeoxynucleotide by automated DNA synthesis, the preparation of the DMT-phosphoramidite derivative is required. On the basis of the efficient production of dG-N^2-TAM by allowing 2-fluoro-(O^6-trimethylsilylethyl)-2′-deoxyinosine (**5**) to react with α-NH_2-TAM, we attempted to synthesize the DMT derivatives of both *trans*- and *cis*-dG-N^2-TAM using DMTrCl (Scheme 1). However, this reaction failed to give the 5′-O-tritylated product. The reason may be due to the steric interference of the bulky TAM moiety on the N^2-position of the base hindering the formation of the DMT derivative. Therefore, the DMT-derivative of 2-fluoro-(O^6-trimethylsilylethyl)-2′-deoxyinosine (**9**) was prepared according to a reported method (*36*) and coupled separately with the *trans*- and *cis*-forms of α-NH_2-TAM (**4a** and **4b**) (Scheme 1). This procedure successfully accomplished the synthesis of the required DMT derivatives (**8a** and **8b**).

The phosphoramidite derivative of *trans*-dG-N^2-TAM (**10a**) was prepared from **8a** (Scheme 1). Since DMT

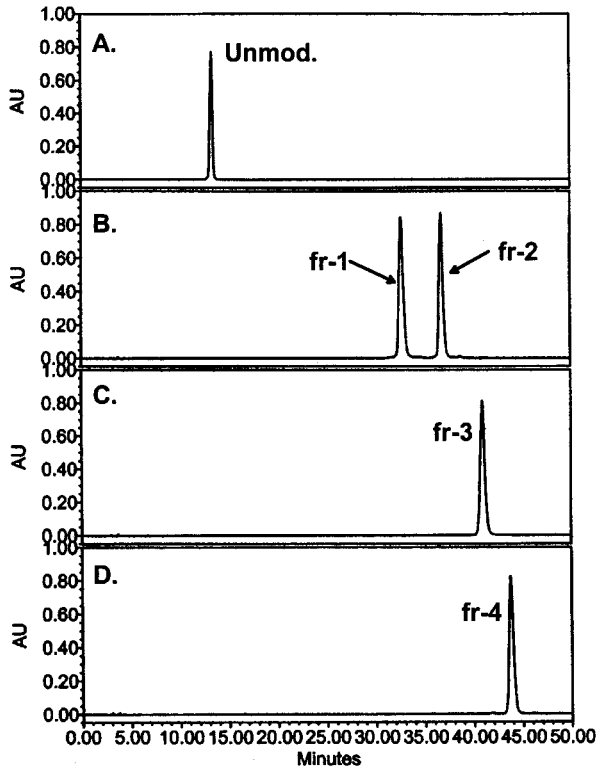


Figure 3. HPLC separation of 15-mer oligomers containing a single *trans*- or *cis*-dG-N^2-TAM. A mixture of 15-mer oligodeoxynucleotides (5′GAG GTG CXT GTT TGT, where X is dG-N^2-TAM) containing fr-1 and fr-2 of *trans*-dG-N^2-TAM (B) or 15-mer containing fr-3 (C) or fr-4 (D) of the *cis*-dG-N^2-TAM was isolated by HPLC. Separation of the unmodified 15-mer is shown in panel A. These oligomers were isolated on a μBondapak C_{18}, using a linear gradient of 0.05 M triethylammonium acetate (pH 7.0), containing 10 to 30% acetonitrile with an elution time of 60 min and a flow rate of 1.0 mL/min.

derivative of *cis*-dG-N^2-TAM (**8b**) (but not the *trans*-form) was resolved into the two diastereoisomers (**8b-1**, fr-3 and **8b-2**, fr-4) on the TLC plate, the phosphoramidite derivatives of *cis*-dG-N^2-TAM (**10b-1** and **10b-2**) were prepared from each of the separated DMT derivatives, **8b-1** and **8b-2**. Using the phosphoramidite precursors (**10a**, **10b-1**, and **10b-2**), several oligodeoxynucleotides containing a dG-N^2-TAM were prepared by solid-phase synthesis and purified by HPLC as described in the Materials and Methods. The coupling efficiency for 0.25−10 μmol scale synthesis was >98% and resulted in good yields of the DNA oligomers.

For example, the oligodeoxynucleotides 5′GAGGTGCX-TGTTTGT, (where X is dG-N^2-TAM as a single diastereoisomer) were prepared by automated chemical synthesis. The sequence of the 15-mer oligomer was selected from codons 271−275 of *P53* mutational hot-spots. When the *trans*-form of dG-N^2-TAM phosphoramidite (**10a**) was used, two oligomers containing fr-1 or fr-2 of *trans*-dG-N^2-TAM were separated by HPLC at 32.7 and 36.8 min, respectively (Figure 3B). Oligodeoxynucleotides containing fr-3 or fr-4 of *cis*-dG-N^2-TAM were prepared separately from the DMT-*cis*-dG-N^2-TAM phosphoramidite (**10b-1** or **10b-2**). The retention times of the oligomers containing fr-3 or fr-4 were 41.0 and 43.8 min, respectively (Figure 3, panels C and D). These modified oligomers can be separated from the corresponding unmodified oligomer (Figure 3A). The UV spectra of the *trans*- and *cis*-dG-N^2-TAM-modified oligomers were similar to that of the corresponding unmodified oligomer (data not shown). Since monomeric dG-N^2-TAM shows weak UV absorbance above 300 nm as noted previously (*20*), dG-N^2-TAM-modified oligomers also have weak UV absorbance in this region. Several other 15-mer, 19-mer, and 24-mer *trans*-dG-N^2-TAM-modified oligomers were synthesized, and again these modified oligomers were resolved into the two diastereoisomeric oligomers by HPLC (data not shown). The molecular weight of dG-N^2-TAM-modified oligomers was measured by FAB mass spectroscopy in negative ion mode. For example, the spectra

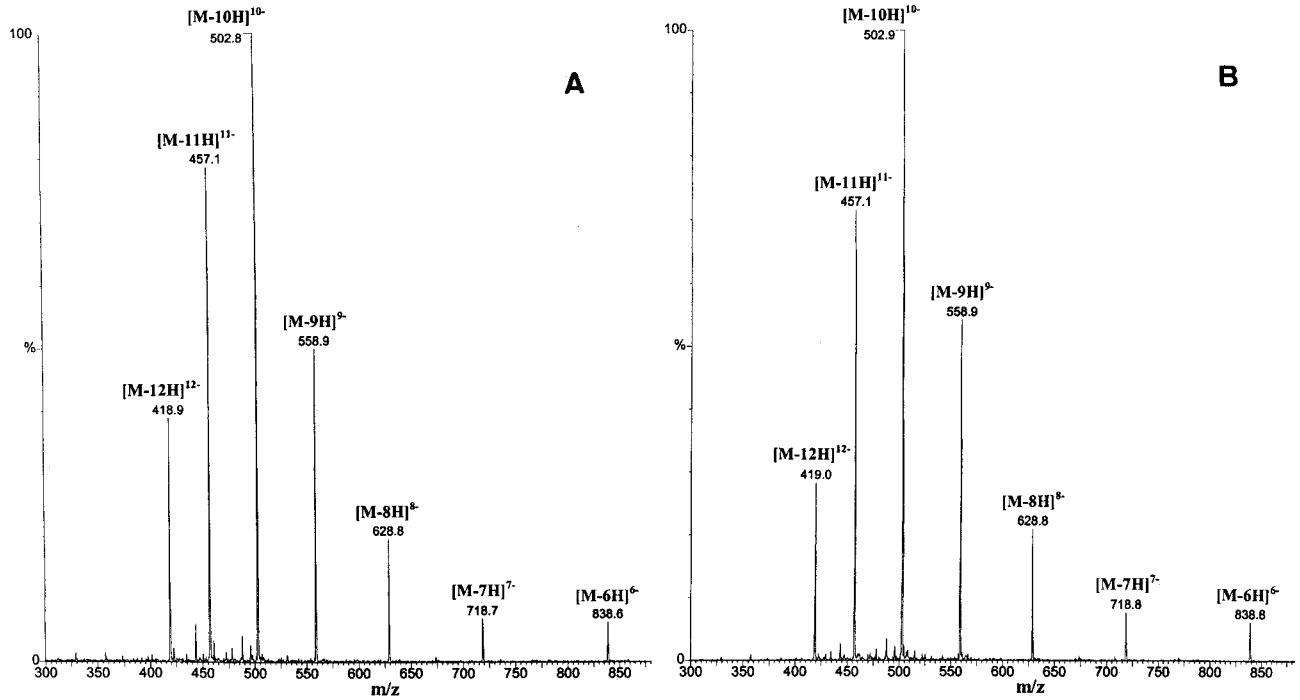


Figure 4. LC/MS/MS analysis of dG-N^2-TAM-modified oligodeoxynucleotides. A 15-mer oligodeoxynucleotide (5′GAG GTG CXT GTT TGT, where X is dG-N^2-TAM, 5040 Da) containing a *trans*-isoform (fr-2, A) or a *cis*-isoform (fr-4, B) of dG-N^2-TAM. The instrument was operated in negative ion mode using electrospray ionization, as described in Materials and Methods.

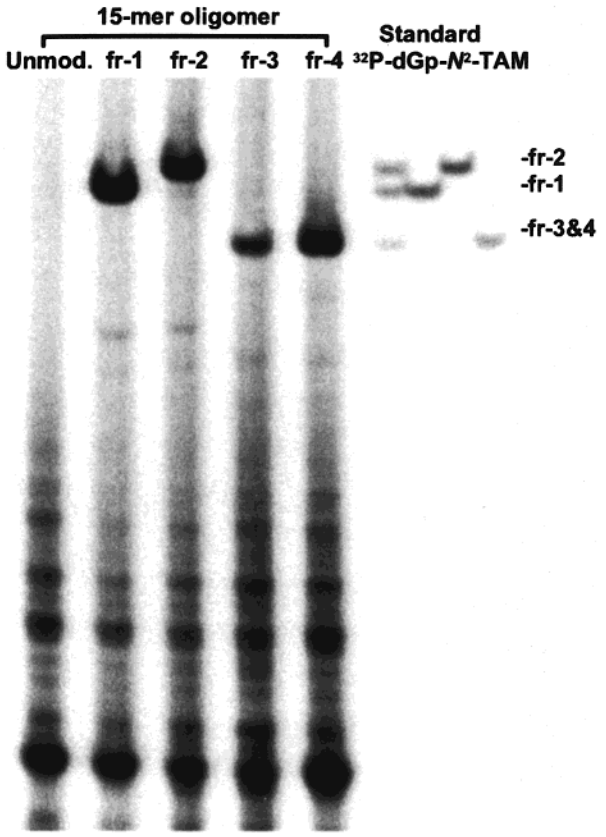


Figure 5. ^{32}P-Postlabeling analysis of dG-N^2-TAM-modified oligodeoxynucleotides. 15-mer oligodeoxynucleotides (250 pg, 5′-GAGGTGCXTGTTTGT, where X is dG or dG-N^2-TAM) containing a single dG or single diastereoisomer of *trans*-isoforms (fr-1 and fr-2) or *cis*-isoforms (fr-3 and fr-4) of dG-N^2-TAM were digested enzymatically, and labeled with ^{32}P as described in Material and Methods. ^{32}P-labeled samples were subjected for 5 h to a nondenaturing 30% polyacrylamide gel (35 × 42 × 0.04 cm). Standard of *trans*- and *cis*-forms of $dG_{3'p}$-N^2-TAM were also labeled with ^{32}P and subjected on PAGE. The position of ^{32}P-labeled adducts was established using a β-phosporimager.

of 15-mer oligomers containing a *trans* (fr-2)- or *cis* (fr-4)-dG-N^2-TAM (5′GAGGTGCXTGTTTGT, where X is dG-N^2-TAM) exhibited an ion at *m*/*z* 5039, identifying the molecular mass as 5040 Da (Figure 4, panels A and B).

To confirm the incorporation of the adduct into the oligomer, the modified oligomer is generally digested enzymatically and the resulting adducted nucleoside is isolated by HPLC and identified using a standard (*27*). Instead of this approach, we demonstrate that ^{32}P-postlabeling/polyacrylamide gel electrophoresis (^{32}P-postlabeling/PAGE) developed recently in our laboratory can be used to confirm the incorporation of dG-N^2-TAM adducts into oligomers. When dG-N^2-TAM-modified 15-mer oligodeoxynucleotides were analyzed (Figure 5), the *trans*-dG-N^2-TAM adducts migrated slower than the *cis* adducts. The migration of each adduct was consistent with that of standard *trans*-diastereoisomers (fr-1 and fr-2) or *cis*-diastereoisomers (a mixture of fr-3 and fr-4). The two *trans*-diastereoisomers were resolved on the gel while the two *cis*-diastereoisomers were not. No adducts were observed in the unmodified oligomer (Figure 5). Thus, the oligomers referred as fr-1, fr-2, fr-3, and fr-4 were confirmed to contain the expected diastereoisomer of dG-N^2-TAM adduct.

Thus, phosphoramidite chemical synthesis permits the preparation of substantial quantities of oligomers containing dG-N^2-TAM lesion(s) in virtually any sequence context. These dG-N^2-TAM-modified oligomers will be used for mutagenesis, DNA repair, and 3D NMR structural studies. In addition, such modified oligomers can be used as standards for ^{32}P-postlabeling analysis to quantify TAM−DNA adducts in animal and human DNA samples.

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