Synthesis and Characterization of Nucleosides and Oligonucleotides with a Benzo[*a*]pyren-6-ylmethyl Adduct at Adenine N^6 or Guanine N^2

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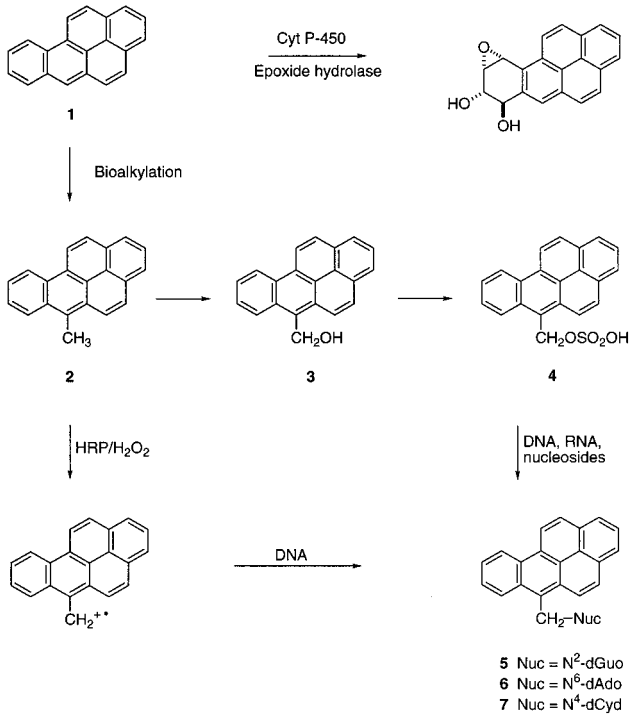
Benzo[*a*]pyrene (**1**) can be converted to reactive electrophilic species by a number of metabolic pathways, of which the route to the mutagenic and carcinogenic diol epoxide(s) is the best studied. An alternative and interesting pathway to a highly genotoxic electrophile is through alkylation at the 6 position to 6-methylbenzo[*a*]pyrene (**2**) followed by oxidation of the methyl group to give 6-hydroxymethylbenzo[*a*]pyrene (**3**). Esterification of **3**, especially to sulfate ester **4**, gives compounds which are both mutagenic and carcinogenic. The major DNA adduct identified from exposure of rats and mice to **4** is the guanine N^2 adduct [2′-deoxy-*N*^{2}-(benzo[*a*]pyren-6-ylmethyl)guanosine, **5**] which is also formed via activation of **2** to a radical cation species by horseradish peroxidase/H_2O_2 or iodine. To study the biological and structural properties of this adduct and the analogous adenine N^6 adduct (**6**), a nonbiomimetic synthesis of the adducted nucleosides **5** and **6** has been developed and has been extended to preparation of oligonucleotides containing **5** or **6** at a single site.

Introduction

Polycyclic aromatic hydrocarbons (PAHs)[1] are among the most ubiquitous and highly studied of the carcinogenic compounds found in the environment. Much effort has been expended correlating PAH structure with biological activity. This is a difficult task, given the complexities of metabolic activation, detoxification, reactivity of metabolites, and repair. It is clear, however, that the genotoxicity of PAHs arises from their metabolic conversion to electrophilic species capable of reacting with nucleophiles such as proteins and nucleic acids. There is strong evidence for the involvement of bay-region and fjord-region diol epoxides in the genotoxicity of PAHs such as benzo[*a*]pyrene (**1**, BP) and benzo[*c*]phenanthrene. Other pathways to genotoxic metabolites of PAHs have also been explored including one-electron oxidation to radical cation species by cytochrome P450 (*1*), oxidation to o-quinones (*2*) and hydroxylation of meso methyl groups followed by esterification.

One interesting PAH which appears to be activated in part by nondiol epoxide pathways is 6-methylbenzo[*a*]pyrene (**2**, 6-MeBP) (Scheme 1). An early study comparing the tumorigenicity of **1**, **2**, and 6-hydroxymethylbenzo[*a*]pyrene (**3**, 6-HOMeBP) when injected subcutaneously in rats showed that all of the compounds caused tumors at the injection site, and it was suggested that the 6-MeBP and hydroxylated derivative **3** could arise from BP by enzymatic alkylation at the most electrophilic meso position followed by oxidation; incubation of BP or 6-MeBP with rat liver homogenates yielded a number of

Scheme 1. Activation of Benzo[*a*]pyrene via Bioalkylation



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[1] Abbreviations: PAH, polycyclic aromatic hydrocarbon; BP, benzo[*a*]pyrene; 6-MeBP, 6-methylbenzo[*a*]pyrene; 6-HOMeBP, 6-hydroxymethylbenzo[*a*]pyrene; 6-SOMeBP, 6-sulfoxymethylbenzo[*a*]pyrene; 6-NH_2CH_2BP, 6-aminomethylbenzo[*a*]pyrene; N^2-6-MeBP dGuo, 2′-deoxy-*N*^{2}-(benzo[*a*]pyren-6-ylmethyl)guanosine; N^6-6-MeBP dAdo, 2′-deoxy-*N*^{6}-(benzo[*a*]pyren-6-ylmethyl)adenosine; t-PAC, 4-*tert*-butylphenoxyacetyl; TEAA, triethylammonium acetate; TMSE, trimethylsilylethyl; DIPEA, *N*,*N*-diisopropylethylamine; FAB, fast-atom bombardment mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; COSY, correlation spectroscopy.

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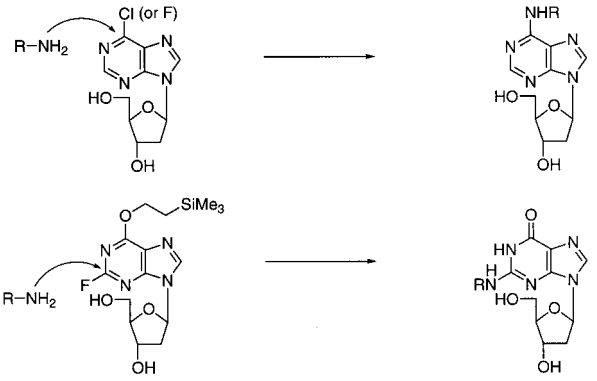
metabolites, one of which appeared to be 6-HOMeBP (*3*). The hypothesis was proposed that the 6-HOMeBP could be esterified in vivo and the esterified form would be a precursor of a stabilized carbonium ion able to react with cellular constituents. Esters (acetoxy and benzoyloxy) and halomethyl derivatives of 6-MeBP were prepared, and all were found to be tumorigenic upon injection (*4*). An extensive study by Cavalieri's group (*5*) on the carcinogenicity of 6-MeBP and related compounds gave results which were in general agreement with the earlier studies except that 6-MeBP was found to be more potent than 6-HOMeBP; this result was interpreted as meaning that there was a mechanism for activation of the 6-methyl compound which did not involve hydroxylation. They proposed that activation could occur by a one-electron oxidation process. Subsequently, they reported that 6-MeBP, when incubated with horseradish peroxidase, H_2O_2, and DNA gave adduct **5** bound through the 6-methyl group to the N^2 of deoxyguanosine (*6*); recently, this group has identified **5** among the products of iodine oxidation of 6-MeBP (*7*). Hydrolysates of DNA from the skin of mice treated with 6-MeBP also contained this adduct (*6*). The analogous guanosine adduct as well as adducts of adenosine and cytosine have been identified from the reaction of 6-(chloromethyl) benzo[*a*]pyrene with nucleosides (*8*). Support for the role of esterified derivatives in the activation of methylated PAHs also came from Cavalieri's group in studies which showed that the sulfate ester (**4**, 6-SOMeBP) of **3** was a potent carcinogen and that the sulfate and acetate esters were the most mutagenic of a group of 6-MeBP derivatives (esters, halides, etc) tested in *S. typhimurium* strains TA98 and TA100 (*9*).

The possible biological importance of the sulfate ester was supported by the observation that rat and mouse liver cytosols were able to catalyze the reaction of 6-HOMeBP with guanosine and deoxyguanosine via a sulfotransferase enzyme (*10*). The formation of the adduct was completely dependent on the presence of the sulfo group donor, 3′-phosphoadenosine-5′-phosphosulfate. The major adduct isolated from treated DNA hydrolysates was the same N^2-deoxyguanosine adduct (**5**) identified by Cavalieri. Much smaller amounts of dA and dC adducts **6** and **7** were also detected. When the DNA from the livers of young rats which had been injected i.p. with 6-HOMeBP or sulfate ester **4** was examined, adduct **5** was the major compound identified; the dA and dC adducts were only found in animals injected directly with **4**. A subsequent study in $B6C3F_1$ mice showed a very high incidence of liver tumors in animals injected with one i.p. dose of 6-SOMeBP; 6-HOMeBP was only 10% as active (*11*).

The question of whether BP itself could actually give rise to aralkyl metabolites such as **2** and **3** in vivo was addressed by Stansbury et al. (*12*) in a ^{32}P-postlabeling study in which Sprague−Dawley weanling rats were injected subcutaneously with BP. Subcutaneous DNA was then examined for adducts arising by the diol epoxide pathway or by the benzylic ester route. The predominant pathway was via diol epoxides with adducts from this route being present at 15−50 times the level of the aralkyl products. However, the autoradiograms clearly showed the presence of adducts corresponding to those arising from injection of 6-MeBP, 6-HOMeBP, or 6-acetoxyMeBP indicating that BP could undergo in vivo activation by the alkylation, side-chain hydroxylation

Scheme 2. Nonbiomimetic Synthesis of Adducted Nucleosides and Oligonucleotides



route. Subsequently, it was shown that 6-HOMeBP and 6-SOMeBP could both act as complete carcinogens when injected subcutaneously (20 doses given three times a week); all animals developed sarcomas (*13*).

It appeared to us that DNA adducts arising from **2** and its metabolites would be excellent candidates for structural and biological studies because the parent hydrocarbon, whether activated through benzylic ester formation or one electron oxidation, appears to give only one major stable DNA adduct, the N^2 guanine adduct, with minor amounts of adenine and cytosine adducts also being detected. The question to be addressed is whether these adducts are involved in the genotoxicity of **2** and its metabolites. As an initial step toward answering this question, we undertook the synthesis of oligonucleotides containing an N^2 dGuo or N^6 dAdo adduct of **2** at a single site. We anticipated that the nonbiomimetic approach in which an amine equivalent of the electrophile is reacted with an oligonucleotide containing a halonucleoside (Scheme 2) would work well for these syntheses as it had for our previous work on site- and stereospecific adducts of PAH diol epoxides (*14*, *15*). This paper describes the synthesis and characterization of nucleosides and oligonucleotides containing adducts of 6-methylbenzo[*a*]pyrene on the exocyclic amino groups of adenine and guanine.

Experimental Section

Materials. Anhydrous dimethyl sulfoxide (Me_2SO) and diisopropylethylamine (DIPEA) were purchased from Aldrich in Sure/Seal bottles. THF was distilled from sodium/potassium alloy with benzophenone ketyl as indicator. Benzene was distilled from sodium. Other chemicals were used as purchased without further purification. Melting points are uncorrected. Unless otherwise noted, materials were obtained from commercial suppliers. All glassware, syringes, needles, and magnetic stirring bars used in moisture-sensitive reactions were oven-dried at 140 °C and stored in desiccators until used. All moisture-sensitive reactions were conducted under a nitrogen atmosphere.

Chromatography. Thin-layer chromatography was performed on silica gel precoated glass plates (EM Science) (Kieselgel 60 F254, precoated, 20 × 20 cm, 0.25 mm layer thickness). The chromatograms were visualized under UV (254 nm) or by staining with an anisaldehyde/sulfuric acid solution or 1% ninhydrin solution, followed by heating. Column chromatography was performed using silica gel 60, 70−230 mesh (EM Science). Oligonucleotides were desalted via a Sephadex G-25 column using a BioRad Biologic system. HPLC analyses and purifications were carried out on a gradient HPLC (Beckman Instruments; System Gold software) equipped with pump

module 125 and photodiode array detector module 168. Columns used were YMC-ODS-AQ (Waters, Milford, MA), 4.6 × 250 mm or 10 × 250 mm, or Luna C-8 or Phenyl-Hexyl (Phenomenex, Torrance, CA)), 4.6 × 250 mm or 10 × 250 mm. Centrifugal vacuum evaporation was performed using a RC10.22 evaporator equipped with a Titan Trap (Jouan, Winchester, VA).

Spectroscopy. Absorption spectra were obtained on a Hitachi U-3000 spectrophotometer. Fluorescence spectra were obtained on a PC1 Photon Counting Spectrofluorometer (ISS, Champaign, IL). Both types of spectra were obtained in phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, 1.0 M NaCl, 50 μM Na_2EDTA, pH 7.0) at ambient temperature. Samples were prepared at a concentration of 1.6 μM of modified strand (calculated ϵ at 260 nm, 12.65×10^4) and 1.7 μM complementary strand (calculated ϵ at 260 nm, 7.76×10^4).

^{1}H NMR and 2D spectra were recorded on a Bruker AC 300 or AM 400 NMR spectrometer with Me_2SO-d_6, CD_3CN, $CDCl_3$, or MeOH-d_4 as solvent. COSY spectra were obtained in the magnitude mode.

Mass Spectrometry. Low and high-resolution FAB mass spectra were obtained at the Mass Spectrometry Facility at the University of Notre Dame, Notre Dame, Indiana. Mass spectra of oligonucleotides were obtained using a Voyager Elite DE instrument (PerSeptive Biosystems). The system was operated in the negative ion mode using a matrix mixture of 2′,4′,6′-trihydroxyacetophenone monohydrate and ammonium hydrogen citrate.

Oligonucleotides. Oligodeoxynucleotides were synthesized on an Expedite 8909 DNA Synthesizer (PerSeptives Biosystems) on a 1-μmol scale using either the manufacturer's standard phosphoramidites or *tert*-butylphenoxyacetyl-protected (tBPA) phosphoramidites and standard synthesis protocol.

CGE. Capillary gel electrophoresis was performed on a Beckman P/ACE 5500 instrument using the manufacturer's ssDNA 100 gel capillary and Tris-borate-urea buffer. Samples were applied at −10 kV and run at −10 kV at 30 °C.

Enzyme Digestions. The oligonucleotides (0.2−0.6 A_{260} units), lyophilized in 1.5 mL microfuge tubes, were digested in a two-stage process. In the first step, buffer (20 μL, 0.01 M Tris-HCl, 0.01 M $MgCl_2$, pH 7.0) was added, followed by nuclease P1 (Sigma N-8630, 4 μL). After digesting for 3−6 h at 36 °C, Tris-HCl buffer (20 μL, 0.1 M, pH 9.0) was added, followed by snake venom phosphodiesterase (Sigma 5785, 0.04 units) and alkaline phosphatase (Sigma P-4282, 0.4 units). Digestion was continued at 37 °C for 3−6 h. H_2O-MeOH (1:1, 100 μL) was added to each sample and the digest was filtered (Ultrafree-MC, 0.45 μm, centrifugal filters, Millipore). The filters were rinsed with Me_2SO (50 μL) to elute adducted nucleosides **5** and **6** which are not very soluble in water. The digests were analyzed by HPLC on either a YMC-ODS-AQ or LUNA C-8 column (4.6 × 250 mm), with the following gradient: (A) 0.1 M ammonium formate, pH 6.4 (B), CH_3CN; 1 to 10% B over 15 min; 10 to 99% B over 20 min, at a flow rate of 1.5 or 1.0 mL/min.

Melting Studies. Adducted oligonucleotide and its complement were dissolved in melting buffer (10 mM Na_2HPO_4/NaH_2PO_4, 1.0 M NaCl, 50 μM Na_2EDTA, pH 7.0). The sample vials were heated to 100 °C, maintained at that temperature for 3 min, allowed to cool to room temperature and stored in the refrigerator overnight. Melting studies were performed using a Varian Cary 04E UV spectrophotometer. UV measurements were taken at one minute intervals with a 1 °C/min temperature gradient. The temperature was raised from 10 to 90 °C and cooled until a plateau was reached and no further hypochromicity was observed at the detection wavelength of 260 nm.

6-Formylbenzo[*a*]pyrene (8). A mixture of benzo[*a*]pyrene (0.50 g, 2 mmol), $POCl_3$ (330 μL, 3.6 mmol), *N*-methylformanilide (494 μL, 4.0 mmol), and 5 mL of *o*-dichlorobenzene was stirred in an oil bath at 95−100 °C for 2 h (*4*). The cooled reaction mixture was poured slowly into an aqueous solution (5 mL) containing 2.5 g of sodium acetate. The heterogeneous mixture was filtered. The filtrate was evaporated to dryness and the residue was dissolved in hot benzene (100 mL) and filtered. The benzene was evaporated under reduced pressure and the residue was purified by silica gel chromatography (10 and 20% MeOH/CH_2Cl_2 or benzene) to give compound **8** as a yellow powder (300 mg, 54% yield); mp 184 °C [lit. (*16*) mp 202−203 °C] ^{1}H NMR (300 MHz, Me_2SO-d_6,) δ 11.58 (s, 1H, CHO), 9.36−9.32 (m, 1H, H10), 9.28−9.25 (m, H11), 9.03 (d, 1H, H7, J = 9.6 Hz), 8.65 (d, 1H, H5, J = 9.2 Hz), 8.52 (d, 1H, H12, J = 7.8 Hz), 8.39 (d, 1H, H1, J = 7.5 Hz), 8.32 (d, 1H, H3, J = 9.6 Hz), 8.16 (t, 1H, H2, J = 7.5 Hz), 7.97−7.94 (m, 3H, H4, 8, 9).

6-Aminomethylbenzo[*a*]pyrene (10, 6-NH_2CH_2BP). 6-Formylbenzo[*a*]pyrene (**8**, 28 mg, 0.1 mmol) was suspended in 1 mL of anhydrous benzene. Sodium bis(trimethylsilyl)amide (0.1 mmol) was added dropwise over 3 min; the suspension was stirred for 20 min at room temperature as the solid went into solution. Chlorotrimethylsilane (0.1 mmol) was added dropwise over 2 min and stirring was continued for an additional 20 min. Silylimine **9** was reduced in situ without isolation. Borane-tetrahydrofuran complex (1 M solution in THF, 100 μL, 0.1 mmol) was added and the reaction was stirred for 30 min. After addition of 2 M HCl/MeOH (0.5 mL), the reaction was stirred at room temperature for 30 min, poured into 10 mL of 0.1 M NaOH, and extracted 4 times with ethyl acetate. The combined extracts were dried (Na_2SO_4) and concentrated in vacuo. The residual yellow oil was chromatographed on silica gel (40 g) (eluted with CH_2Cl_2:MeOH, 90:10) to give **10** (12 mg, 43%) as a yellow powder which migrated as one spot (with some tailing) on TLC. TLC R_f = 0.26, (CH_2Cl_2:MeOH, 9:1); mp 163−164 °C; ^{1}H NMR (400 MHz, Me_2SO-d_6) δ 9.28−9.26 (m, 1H, H10), 9.25 (d, 1H, H11, J = 9.1 Hz), 8.75−8.73 (m, 1H, H7), 8.48 (d, 1H, H5, J = 9.6 Hz), 8.41 (d, 1H, H12, J = 9.2 Hz), 8.34 (dd, 1H, H1, J_1 = 7.8, J_2 = 0.9 Hz), 8.20 (dd, 1H, H3, J_1 = 7.3, J_2 = 0.7 Hz), 8.09 (d, 1H, H2, J = 9.6 Hz), 8.04 (t, 1H, H4, J = 7.6 Hz), 7.90−7.87 (m, 2H, H8, 9), 4.83 (s, 2H, CH_2). HRMS (FAB) [MH^+] calcd for $C_{21}H_{16}N$: 281.1228, Found: 281.1204.

2′-Deoxy-N^6-(benzo[*a*]pyren-6-ylmethyl)adenosine (6, N^6-6MeBP dAdo). 6-Chloropurine-2′-deoxyriboside (**11**) (*17*, *18*) (6 mg, 0.02 mmol) and compound **10** (3 mg, 0.01 mmol) were dissolved in 400 μL of anhydrous Me_2SO and 19 μL (0.11 mmol) of *N*,*N*-diisopropylethylamine (DIPEA) was added to the solution. The mixture was stirred for 4 h at 55 °C. The product was purified directly from the reaction mixture by HPLC (YMC-ODS-AQ column, 10 × 250 mm) with the following gradient: (A) H_2O, (B) CH_3CN, 10% to 20% B over 5 min, hold for 3 min, 20% to 99% B over 10 min, hold for 5 min; flow rate 4 mL/min. Adduct **6** eluted at 20.8 min. The adduct was recovered almost quantitatively (98%). ^{1}H NMR (400 MHz, Me_2SO-d_6,) δ 9.28 (d, 2H, H10, H11, J = 9.0 Hz), 8.77 (d, 1H, H7, J = 8.4 Hz), 8.63 (d, 1H, H5, J = 9.4 Hz), 8.45 (d, 1H, H12, J = 9.1 Hz), 8.36 (d, 1H, H1, J = 7.7 Hz), 8.30 (s, br, 1H, H2 adenine), 8.21 (d, 1H, H3, J = 7.3 Hz), 8.08 (t, 1H, H2, J_1 = 9.3, J_2 = 7.2 Hz), 8.05 (s, 1H, H8 adenine), 8.04 (d, 1H, H4, J = 7.6 Hz), 7.86 (m, 2H, H8,9), 6.36 (t, 1H, H1′), 5.88 (br, 2H, CH_2), 5.31 (d, 1H, OH, J = 4.0 Hz), 5.20 (br, 1H, OH), 4.39 (br, 1H, H3′), 3.87 (d, 1H, H4′, J = 2.5 Hz), 3.59−3.50 (m, 2H, H5′, H5″), 2.72 (m, 1H, H2″), 2.27 (m, 1H, H2′). HRMS (FAB) [MH^+] calcd for $C_{31}H_{26}N_5O_3$: 516.2036. Found: 516.2045.

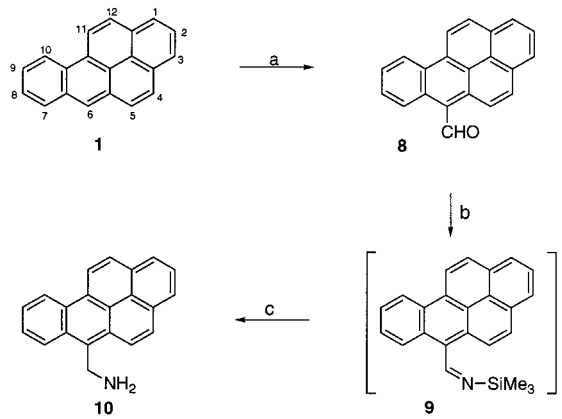
2′-Deoxy-N^2-(benzo[*a*]pyren-6-ylmethyl)guanosine (5, N^2-6-MeBP dGuo). 2-Fluoro-(O^6-trimethylsilylethyl)-2′-deoxyinosine (**12**) (*19*) (10 mg, 0.03 mmol) and **10** (3 mg, 0.01 mmol) were dissolved in 600 μL of anhydrous Me_2SO followed by the addition of 19 μL (0.11 mmol) of DIPEA. The reaction mixture was stirred for 5 h at 45 °C. The products were separated directly from the reaction mixture by HPLC (YMC-ODS-AQ column, 10 × 250 mm) with the following gradient: (A) H_2O, (B) CH_3CN, 50 to 99% B over 10 min, hold for 5 min, 99 to 40% B over 5 min; 40 to 50% B over 5 min; flow rate of 4.0 mL/min. Products eluted at 6.4 min (compound **5**) and 17.2 min (**13**, O^6-trimethylsilylethyl-protected **5**). Compound **5** (0.6 mg, 10%) and compound **13** (1.9 mg, 27%) were collected. Adduct **13** (1.9 mg) was dissolved in 1 mL of H_2O-MeOH (1:2). Hydrochloric acid (0.05 M, 80 μL) was added and the mixture was stirred for 5 h at room temperature and 3 h at 50 °C to ensure complete

removal of the O^6-trimethylsilylethyl protecting group. Adduct **5** was purified using the HPLC conditions described above for **13** to yield 1.4 mg (88% yield for deprotection step). Therefore, total recovered yield of **5** was 2.0 mg (overall 34%). ^{1}H NMR (400 MHz, Me_2SO-d_6/D_2O) δ 9.32−9.27 (m, 1H, H10, H11), 8.75−8.73 (m, 1H, H7), 8.60 (d, 1H, H5, J = 9.6 Hz), 8.48 (d, 1H, H12, J = 9.2 Hz), 8.38 (d, 1H, H1, J = 7.7 Hz), 8.24 (d, 1H, H3, J = 7.2 Hz), 8.19 (d, 1H, H2, J = 9.6 Hz), 8.07, (t, 1H, H4, J = 7.6 Hz), 7.99 (s, 1H, H8 guanine), 7.95−7.92 (m, 2H, H8, H9), 6.43 (t, 1H, H1′, J_1 = 7.2, J_2 = 6.6 Hz), 5.64 (s, 2H, CH_2), 4.47−4.46 (m, 1H, H3′), 3.92 (m, 1H, H4′), 3.63−3.60 (m, 2H, H5′, H5″), 2.75 (m, 1H, H2″), 2.38 (m, 1H, H2′). HRMS (FAB) [MH^+] calcd for $C_{31}H_{26}N_5O_4$: 532.1985. Found: 532.1982.

5′-G-GCA*-GGT-GGT-G-3′ (14, *ras*11,3A*, A* = N^6-6MeBP dAdo). The modified oligonucleotide (2 × 1-μmol), 5′-G-GCA*-GGT-GGT-G-3′ where A* is 6-fluoropurine-2′-deoxyriboside, was prepared using automated solid-phase synthesis with the phosphoramidite of 6-fluoropurine-2′-deoxyriboside (*15*, *20*). The final detritylation was carried out on the synthesizer, but the oligonucleotide was not further deprotected or released from the solid support. 6-Aminomethylbenzo[*a*]pyrene (**10**, 7 mg, 0.02 mmol) was placed in a 3-dram vial containing the immobilized oligonucleotide suspended in anhydrous Me_2SO (400 μL) and DIPEA (70 μL). The vial was capped and the mixture was heated for 3 days at 48 °C under N_2. After cooling to room temperature, the supernatant was removed, and the residual beads were washed with CH_3OH (3 × 1 mL). The beads were transferred to a 10-mL vial and concentrated NH_4OH (7 mL) was added. The tightly closed vial was heated for 8 h at 60 °C for cleavage from the solid support and deblocking. After cooling, the vial was allowed to stand until the excess NH_4OH had evaporated. The beads were separated by centrifugation and washed with water (2 × 1 mL). The combined solution was filtered (0.22 μm, Millipore), transferred into a conical tube (50 mL), and lyophilized. The dry material was dissolved in H_2O-MeOH (1:1, 1 mL), and purified on a YMC-ODS-AQ column (10 × 250 mm) using the following gradient: (A) 0.1 M ammonium formate, pH 6.45 (B) CH_3CN, 1 to 10% B over 5 min; 10 to 20% B over 5 min; hold for 5 min; 20 to 80% B over 5 min, flow rate 4.0 mL/min. The product (**14**) eluted at 13.4 min and was collected (110 A_{260} units). After removal of solvent by vacuum centrifugation the fraction was desalted on a Sephadex G-25 (Bio-Rad Laboratories) column (1.5 × 40 cm) with elution by H_2O to give the pure oligonucleotide (40 A_{260} units after a second HPLC purification). MS (MALDI-TOF) m/z calcd for [M − H]$^-$ 3715.6, observed 3715.3.

5′-G-GCA-GG*T-GGT-G-3′ (15, *ras*12,2G*, G* = N^2-6MeBP dGuo). The modified oligonucleotide (2 × 1 μmol), 5′-G-GCA-GG*T-GGT-G-3′, where G* is 2-fluoro-O^6-(trimethylsilylethyl)-2′-deoxyinosine, was prepared using the phosphoramidite of the 2-fluoronucleoside (*19*) and *t*-PAC phosphoramidites for the other bases. The final detritylation was carried out on the synthesizer, but the oligonucleotide was not further deprotected or released from the solid support. 6-Aminomethylbenzo[*a*]pyrene (**10**, 8 mg, 0.03 mmol) was placed in a 3-dram vial containing the immobilized oligonucleotide suspended in anhydrous Me_2SO (400 μL) and DIPEA (80 μL). The vial was capped and the mixture was heated for 5 days at 60 °C under N_2. After cooling to room temperature, the supernatant was removed, and the residual beads were washed with CH_3OH (3 × 1 mL). The beads were transferred to a 10-mL vial and concentrated NH_4OH (7 mL) was added. The tightly closed vial was heated for 1 h at 60 °C for cleavage from the solid support and deblocking. After cooling, the vial was allowed to stand until the excess NH_4OH had evaporated. The beads were separated by centrifugation and washed with MeOH:H_2O (1:1, 2 × 1 mL). The combined solution was filtered, transferred into a conical tube (50 mL), and lyophilized. The dry material was dissolved in MeOH:H_2O (1 mL), and purified on a LUNA Phenyl-Hexyl column (Phenomenex, 4.5 × 250 mm) using the following gradient: (A) 0.2 M triethylammonium acetate (TEAA), pH 7.02 (B) CH_3OH, 0 to 20% B over 10 min, hold for 5 min, 20 to 90% B over 12 min, hold for 8 min; flow rate 1.0 mL/min. The material (silylated at the O^6 position) eluting at 29.8 min was collected and lyophilized. A second HPLC purification was performed using 0.1 M TEAA and CH_3CN with the same gradient. Oligonucleotide **15**, which had desilylated at the O^6 positon during the first purification and lyophilization, eluted at 14.30 min. After removal of solvent by vacuum centrifugation the fraction was desalted on a Sephadex G-25 (Bio-Rad Laboratories) column (1.5 × 40 cm) with elution by H_2O to give purified oligonucleotide **15** (13 A_{260} units). The adducted oligonucleotide was analyzed by enzyme hydrolysis and the homogeneity was checked by capillary gel electrophoresis. MS (MALDI-TOF) m/z calcd for [M−H]$^-$ 3715.6, observed 3715.9.

Scheme 3. Synthesis of 6-Aminomethylbenzo[*a*]pyrene (10)



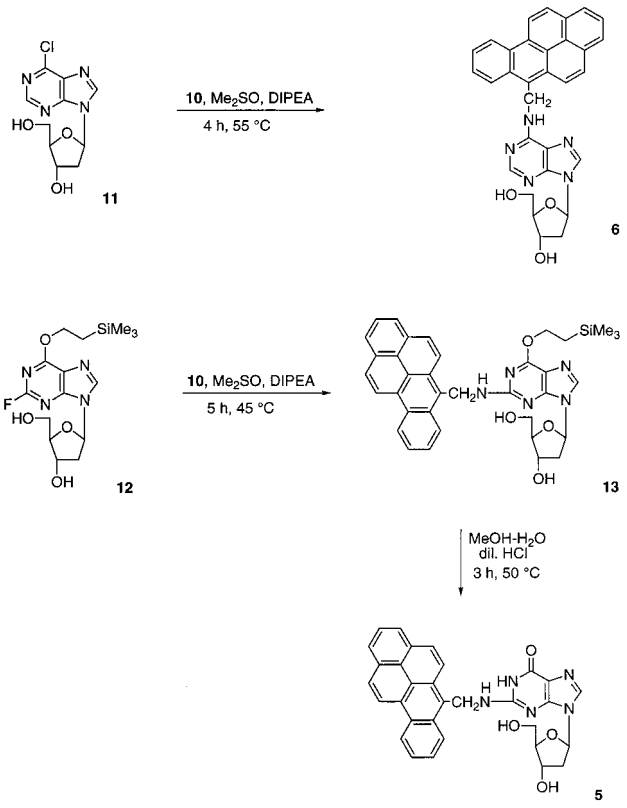
(a) $POCl_3$, *N*-methylformanilide, *o*-dichlorobenzene. (b) NaN($SiMe_3$)$_2$, Me_3SiCl, benzene. (c) BH_3-THF.

Results and Discussion

Two options were considered for the synthesis of oligonucleotides containing adducts of 6-MeBP. The first and apparently simplest was to synthesize the adducted nucleosides by published procedures, using the sulfate ester of 6-HOMeBP or a haloderivative such as 6-chloromethyl benzo[*a*]pyrene, followed by conversion of the adducted nucleosides to phosphoramidites. However, we were concerned that solubility problems might be encountered with the modified amidite and that coupling, especially of the dGuo amidite might be inefficient. Others have obtained relatively modest coupling yields, even after extended coupling times, with dGuo amidites modified at N^2 with bulky groups such as benzo[*a*]pyrene (*21*, *22*). We decided, therefore, to investigate the post-oligomerization approach to these adducts.

The implementation of the strategy depicted in Scheme 2 required the synthesis of 6-aminomethylbenzo[*a*]pyrene (**10**). Dewhurst and Kitchen (*16*) had attempted to prepare **10** via either catalytic hydrogenation of the 6-nitrile or lithium aluminum hydride reduction of the 6-carboxamide but were not able to obtain pure material. We chose to start with 6-formyl derivative **8** which was commercially available at the time we initiated this project; we have also synthesized it by the route shown in Scheme 3 (*16*, *23*). Our original plan was to synthesize the amine by reduction of the oxime, a route used previously for the preparation of pyrenylmethylamine (*24*). The oxime of **8** was obtained in high yield but neither Zn/acetic acid nor lithium aluminum hydride reduction gave satisfactory yields of pure material, in part because of the insolubility of the oxime. The next route chosen was through imine reduction; the one shown

Scheme 4. Synthesis of N^6 Ado and N^2 Guo Adducts of 6-Methylbenzo[*a*]pyrene (6 and 5)



in Scheme 3 was the most successful. The silylimine was generated in situ and reduced with borane in THF to give **10** in 43% yield.

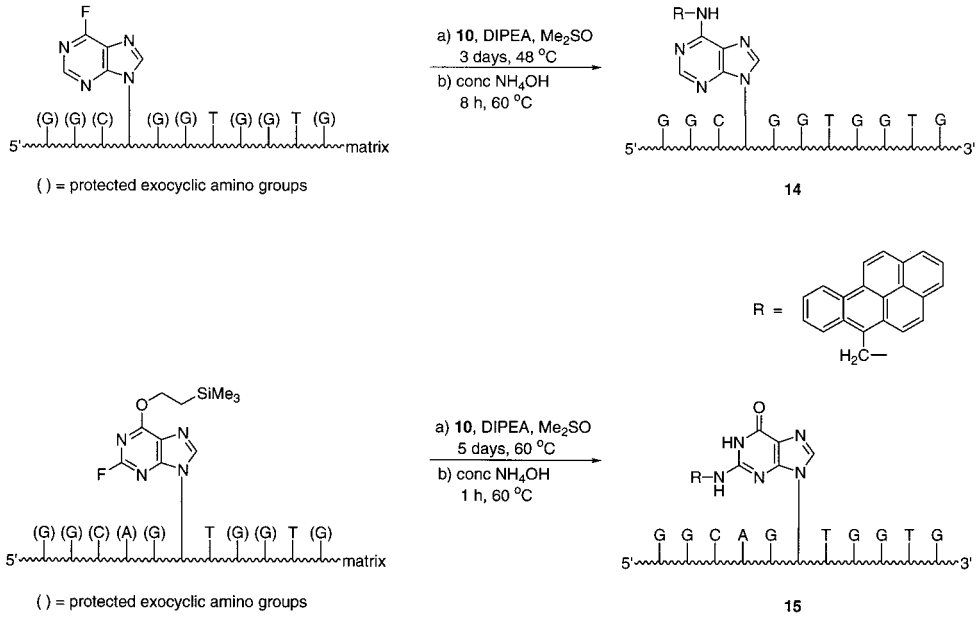
Adducted nucleosides **5** and **6** were prepared by reaction of the appropriate halonucleoside with amine **10** (Scheme 4). 6-Chloropurine 2′-deoxyriboside (**11**) reacted quantitatively with **10** to yield deoxyadenosine adduct **6**. The analogous adenosine adduct had been identified by Royer et al. (*8*) from the reaction of 6-(chloromethyl)-benzo[*a*]pyrene with the riboside, although the product was not fully characterized. 2′-Deoxyguanosine adduct **5** was obtained in a similar fashion by reaction of **10** with fluoronucleoside **12**. The initial isolation gave both O^6-protected product **13** and final product **5** which had undergone deprotection probably due to fluoride ion generated during the reaction. Isolated **13** was deprotected by heating with dilute HCl in MeOH-H_2O. The lower yield of the guanine adduct reflects not only the two step procedure but also the poor solubility of this adduct. The ^{1}H NMR spectrum of **5,** assigned by a combination of COSY and NOE difference spectroscopy, was in agreement with those published previously (*6*, *10*).

With nucleoside standards in hand, synthesis of adducted oligonucleotides was undertaken. In the interest of comparing an adenine N^6 and a guanine N^2 adduct in the same sequence, we chose to put both adducts in the 11-mer sequence, 5′-GGC**A**G**G**TGGTC-3′ (Scheme 5) (in separate syntheses, not with both adducts in the same oligonucleotide). This sequence is part of the N-*ras* protooncogene which we have used in several of our previous studies (*19*, *25*). The adenine adduct was introduced at the 11,3 position to give oligonucleotide **14** and the guanine adduct at position 12,2 to yield **15**.

Although the 6-chloropurine nucleoside was used for preparation of the N^6 adenine nucleoside adduct, the 6-fluoropurine nucleoside was chosen as the halo constituent for the oligonucleotide synthesis because of its increased reactivity (*15*, *26*). The matrix-bound oligonucleotide was reacted with amine **10** in Me_2SO at 48 °C for 3 days. The reaction proceeded well as seen in the HPLC trace of the crude oligonucleotide after removal from the beads and deprotection (Figure 1).

Synthesis and purification of guanine-adducted oligonucleotide **15** proved to be more difficult. The phosphoramidite of 2-fluoro-*O*^{6}-(trimethylsilylethyl) 2′-deoxyinosine was used for the synthesis of the oligonucleotide (Scheme 5) (*19*). The reaction with amine **10** was carried out with matrix-bound fully protected oligonucleotide. The HPLC of the crude oligonucleotide (with the O^6-

Scheme 5. Synthesis of Oligonucleotides Bearing Adenine N^6 and Guanine N^2 Adducts of 6-Methylbenzo[*a*]pyrene (6 and 5)[a]



[a] Protected exocyclic amino groups appear in parentheses.

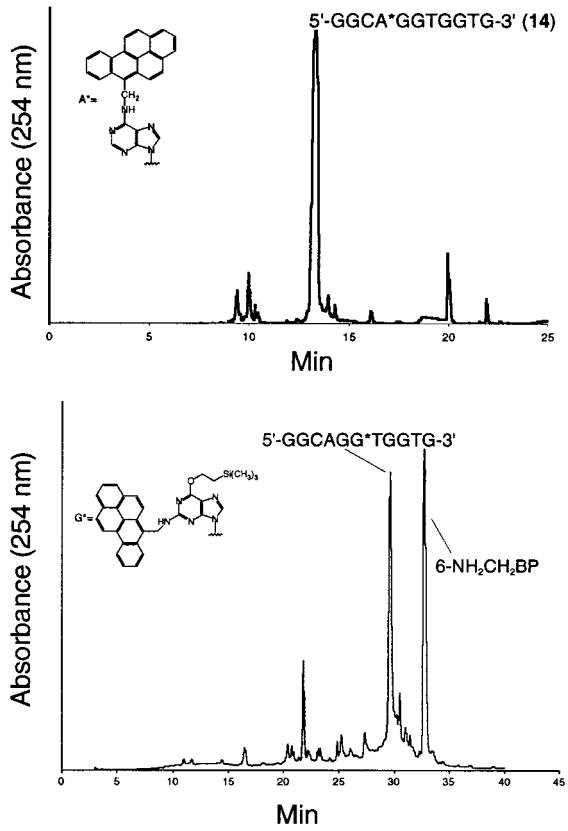


Figure 1. HPLC chromatograms of crude adducted oligonucleotide reactions. Upper trace: 5′-GGC**A***GGTGGTG-3′ (**14**, *ras* 11,3A* where A* = N⁶-6-MeBP dAdo). Lower trace: 5′-GGCAG**G***TGGTG-3′ (**15**, *ras* 12,2G* where G* = N²-6-MeBP dGuo with O⁶ protection).

TMSE group intact) after removal from the beads and deprotection with NH_4OH is shown in Figure 1. Considerable difficulty was encountered with the chromatography of oligonucleotide **15** and its *O*⁶-TMSE precursor and the chromatogram shown was only obtained after experimentation with several columns and solvent conditions. Chromatography on YMC-ODS-AQ columns with ammonium formate buffers under conditions which had been quite satisfactory for separations of this particular sequence bearing other adducts at 12,2 gave broad unresolvable peaks. Changing to triethylammonium acetate (TEAA) buffers helped, but the best separations were obtained on the newly available Luna Phenyl-Hexyl column with 0.1 or 0.2 M TEAA, pH 7.01/acetonitrile. The problems probably arose from interaction of the very hydrophobic aromatic N² adduct with the multiple guanines in this particular sequence. Oligonucleotide **14**, with an adenine adduct in the same sequence, did not show this anomalous chromatographic behavior. We have had similar problems with the chromatography of N² guanine adducts of benzo[*a*]pyrene diol epoxide in this sequence.[2]

The purified oligonucleotides were characterized by denaturing capillary gel electrophoresis (Figure 2), mass spectroscopy (MALDI-TOF), and enzymatic digestion with nuclease P1 followed by snake venom phosphodiesterase and alkaline phosphatase. Chromatograms of the enzymatic digests are shown in Figure 3; the identity of the adducted nucleosides (**6** from oligonucleotide **14** and **5** from **15**) was confirmed by comparison with the independently synthesized nucleoside adducts. One problem that arose in the enzyme digestions, especially with the guanine adduct, is the poor solubility of the adducts.

[2] Unpublished results.

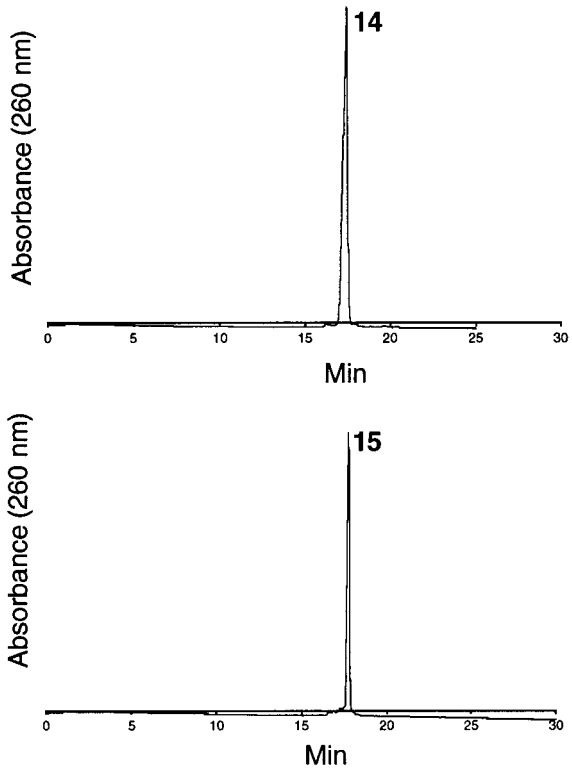


Figure 2. Capillary gel electropherograms of purified adducted oligonucleotides **14** and **15**. Upper trace: *ras* 11,3A* (**14**). Lower trace: *ras* 12,2G* (**15**).

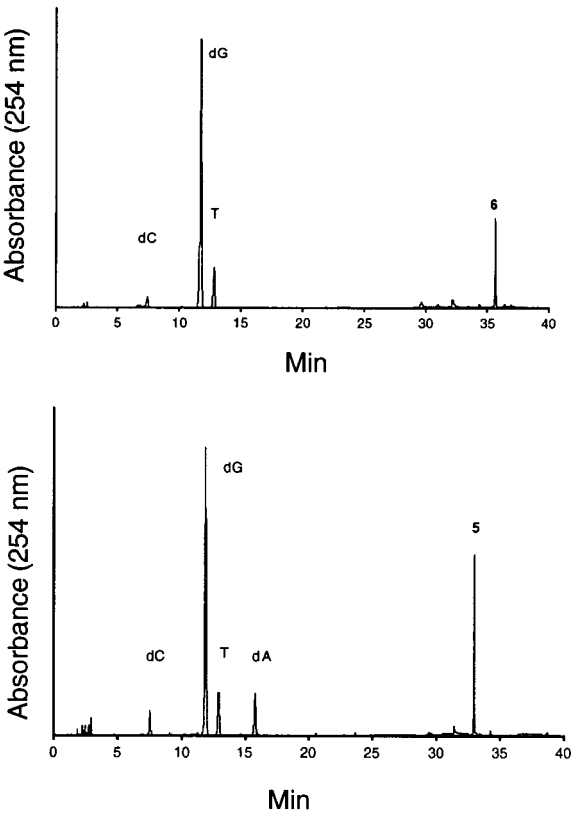


Figure 3. HPLC chromatograms of enzymatic digests of oligonucleotides **14** (upper trace) and **15** (lower trace). The peak labeled **6** is N⁶-6-MeBP dAdo and **5** is N²-6-MeBP dGuo.

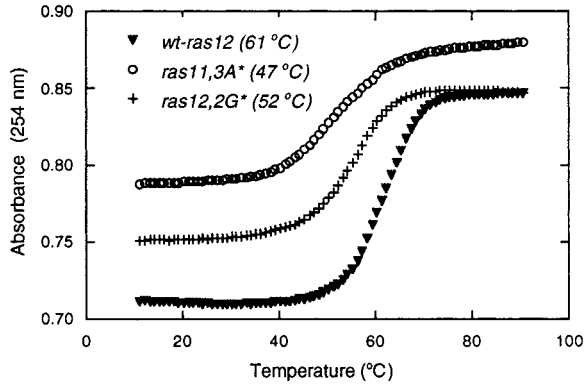


Figure 4. Thermal stability of oligonucleotide duplexes (10 mM Na_2HPO_4/NaH_2PO_4, 1.0 M NaCl, 50 mM Na_2EDTA, pH 7.0) (▼) unmodified: 5′-GGCAGGTGGTG-3′ (○) *ras* 11,3A* (**14**), (+) *ras* 12,2G* (**15**).

In our routine procedure, the enzyme digest is diluted with water and filtered through a 0.45 μm filter before HPLC analysis. It was necessary to rinse the filter with Me_2SO to recover the adducts.

Melting Profiles. The thermal stability of duplexes formed by oligonucleotides **14** and **15** with complementary strand, 5′-CACCACCTGCC-3′, was examined and the results are shown in Figure 4. Both the adenine and the guanine adduct destabilized the duplex, the former by 14 °C and the latter by 9 °C. These numbers are similar to those seen in studies of the thermal stability of oligonucleotides bearing N^2 dGuo (*27*−*29*) or N^6 dAdo (*30*) trans adducts of *anti*-benzo[*a*]pyrene diol epoxide, keeping in mind, however, that sequence context can affect thermal stability (*27*).

Absorption and Emission Spectra. Both absorption and fluorescence emission spectroscopy have been extensively used for studying interactions between DNA and PAHs (*21*, *27*, *28*, *31*−*33*). Changes in absorption and emission maxima and intensities have been interpreted in terms of orientation of PAH adducts, particularly in double-stranded DNA. Orientations of PAH adducts in DNA are divided into two general types termed site I and site II. In site I, conformations with considerable base-stacking or intercalative character, there is a pronounced red shift in the emission maxima and a marked diminution in fluorescence intensity upon duplex formation, whereas in site II, or external, groove-bound conformers, there is relatively little red shift and only moderate quenching, indicating that the adduct is still relatively available to solvent in the duplex.

Absorption and emission spectra for single- and double-stranded oligonucleotides **14** and **15** are shown in Figure 5. The absorption spectrum of single-stranded oligo-

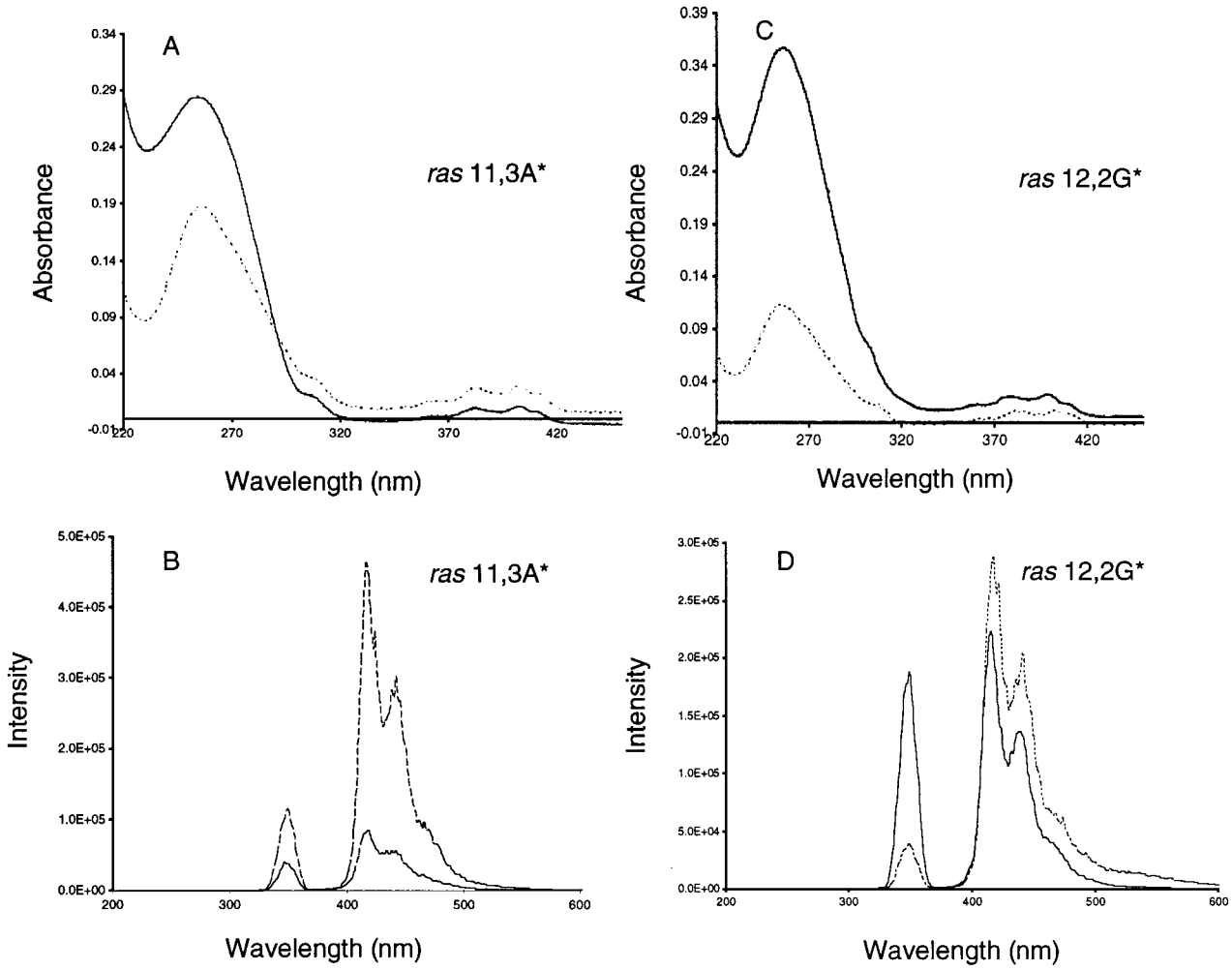


Figure 5. Absorption and fluorescence emission spectra for single (- - -) and double (—)-stranded oligonucleotides **14** and **15**. Emission intensity is in arbitrary units. (A) absorption and (B) emission spectra for *ras* 11,3A* (**14**); (C) absorption and (D) emission spectra for *ras* 12,2G* (**15**). Spectra were obtained in phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, 1.0 M NaCl, 50 μM Na_2EDTA, pH 7.0) at ambient temperature. Samples were prepared at a concentration of 1.6 μM modified strand and 1.7 μM complementary strand.

Table 1. UV Absorption and Fluorescence Emission Characteristics of Oligonucleotides Containing 6-Methylbenzo[*a*]pyrene Adducts on dAdo N⁶ and dGuo N² [a]

sample	UV wavelength, λ_{max}	fluorescence excitation, λ	fluorescence emission, λ_{max}
6-NH_2CH_2BP (**10**)	395, 373, 355	350	436, 412
*ras*11,3−6-MeBP-dAdo (**14**)			
single-strand	402, 381, 363	350	445, 420
double-strand	402, 381, 362	350	442, 419
ras 12,2−6-MeBP-dGuo (**15**)			
single-strand	402, 381, 363	350	443, 420
double-strand	397, 377, 358	350	441, 417

[a] Samples were run at ambient temperature in buffer containing 10 mM Na_2HPO_4/NaH_2PO_4, 1 M NaCl, 50 μM Na_2EDTA, pH 7.0.

nucleotide **14** containing the N⁶ dAdo adduct showed long-wavelength maxima at 402, 381, and 363 nm (Table 1); upon formation of duplex the maxima did not change but there was a significant decrease in intensity in the long wavelength region. Similarly the fluorescence emission was significantly decreased upon duplex formation. Both of these effects indicate that in the duplex the polycyclic adduct is involved in considerable base-stacking, but the large decrease in thermal stability (T_m 47 °C versus 61 °C for unmodified duplex) argues against a fully intercalated orientation.

In contrast, absorption and emission spectra for oligonucleotide **15** containing the N² dGuo adduct showed relatively little difference in intensity in the long wavelength part of the spectrum upon duplex formation. The shift to shorter wavelengths in the absorption spectrum upon duplex formation may reflect base-stacking of the adduct with other bases in the single-stranded purine-rich oligonucleotide which is decreased upon duplex formation. These data are in accord with orientation of the dGuo adduct in the minor groove with considerable exposure to solvent. A similar conclusion was reached by Casale and McLaughlin (*21*) in an earlier study on an oligonucleotide bearing an N² dGuo adduct of 9-methylanthracene. In accordance with earlier observations on other PAH adducts (*34*, *28*, *35*) the intensity of the fluorescence emission of the guanine adduct is much less than that of the adenine. This effect has been attributed to fluorescence quenching by photoinduced electron transfer from guanine. The question of whether the adducts of 6-MeBP exist in one predominant conformation awaits further examination by high-field NMR or X-ray crystallography; low-temperature fluorescence studies of these adducts would also be interesting (*33*).

In addition to their possible role in genotoxicity, these very hydrophobic adducts with their high intrinsic fluorescence should be useful tools for studying DNA−protein interactions, as, for example, the thermodynamics of the interaction of BPDE-adducted oligonucleotides with the *Escherichia coli* repair protein UvrA was examined by fluorescence spectroscopy (*36*).

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