Effect of Benzo-Ring Hydroxyl Groups on Site-Specific Mutagenesis by Tetrahydrobenzo[*a*]pyrene Adducts at N^6 of Deoxyadenosine

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We have previously investigated the mutations induced on replication in *Escherichia coli* of the M13mp7L2 genome containing each of the eight possible adducts derived from the four optically active 7,8-diol 9,10-epoxide metabolites of benzo[*a*]pyrene (B[a]P) by alkylation of a specific deoxyadenosine (dAdo) residue at N^6. Observed mutational frequencies depended in part on the relative spatial orientations of the three hydroxyl groups in these adducts. To determine how the presence or absence of these hydroxyl groups affects mutational response, we have synthesized 16-mer oligonucleotides with the same sequence as one of those previously studied with the diol epoxide adducts, but containing B[a]P-dAdo adducts in which two or all three of the adduct hydroxyl groups were replaced by hydrogen. Transfection of the adducted M13 constructs into SOS-induced *Escherichia coli* consistently gave fewer infective centers than the control construct, with viabilities ranging from 8.4 to 44.9% relative to control. In general, decreasing the number of adduct hydroxyls decreased the total frequency of substitution mutations induced. For all but one of the present adducts, the total mutational frequency was lower than that for any of the previously reported diol epoxide adducts in the same sequence. Remarkably, this (9*S*,10*R*)-adduct with cis orientation of the dAdo residue and the 9-OH group gave the highest mutational frequency of all the B[a]P adducts studied in this sequence, including the diol epoxide adducts. With the present adducts, A → T transversions predominated, with smaller numbers of A → G transitions and even fewer A → C transversions.

Introduction

The occurrence of mutations, especially in genes that influence cell cycle regulation or proto-oncogene activation, is recognized as the initial event in the multistage process of carcinogenesis. Chemical carcinogens that alkylate DNA produce lesions in the form of covalent adducts (*1*). While most organisms are capable of repairing damaged DNA, some lesions either escape repair or are repaired inaccurately (*2*). Incorrect replication past such unrepaired lesions will result in mutations that could potentially lead to cell transformation.

The environmental pollutant benzo[*a*]pyrene (B[a]P)[1] is metabolized in mammals to two diastereomeric benzo[*a*]pyrene 7,8-diol-9,10-epoxides (B[a]P DE-1, in which the benzylic 7-hydroxyl group and the epoxide oxygen are cis, and B[a]P DE-2, in which these groups are trans) (*3*). Since each diastereomer exists as a pair of enantiomers, four DEs are metabolically formed. Each of these four DEs reacts with DNA via cis or trans opening of the epoxide ring by the exocyclic N^2 and N^6 amino groups of the purine nucleosides deoxyguanosine (dGuo) and deoxyadenosine (dAdo) (*4*, *5*); thus a total of eight isomeric adducts are possible from a given base. Our laboratory had previously investigated the effect of these eight isomeric B[a]P DE adducts at dAdo in two sequence contexts on the mutagenic outcome of translesion DNA replication in an *Escherichia coli*-M13 system (*6*, *7*). For all eight stereoisomers, base substitution mutations occurred at a high rate, with frequencies ranging from 5 to 68%. Observed mutational frequencies depended to some extent on the stereochemical relationships among the hydroxyl groups on C7, C8 and C9 of the DEs. However, chirality at the site of attachment of the amino group also had an influence on mutagenicity. This effect of chirality was much larger in the case of benzo[*c*]phenanthrene DE-dAdo adducts (*8*). In the present study we sought to determine the effect of the tetrahydro benzo-ring hydroxyl groups on the mutagenicity of B[a]P derivatives by the use of adduct-containing inserts **1**−**6** (Figure 1A) in which some or all of these hydroxyl groups in B[a]P DE adducts had been replaced by a hydrogen.

Experimental Procedures

T4 polynucleotide kinase, T4 DNA ligase, uracil DNA glycosylase, X-gal, and IPTG were obtained from USB Corp. (Cleve-

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[1] Abbreviations: B[a]P, benzo[*a*]pyrene; B[a]P DE, benzo[*a*]pyrene 7,8-diol-9,10-epoxides (B[a]P DE-1, in which the benzylic 7-hydroxyl group and the epoxide oxygen are cis, and B[a]P DE-2, in which these groups are trans); B[a]P H_4E, 7,8,9,10-tetrahydrobenzo[*a*]pyrene 9,-10-epoxide; 10-amino H_4 B[a]P, 10-amino-7,8,9,10-tetrahydrobenzo[*a*]-pyrene; 6-FP, 6-fluoro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)purine; UDG, uracil DNA glycosylase.

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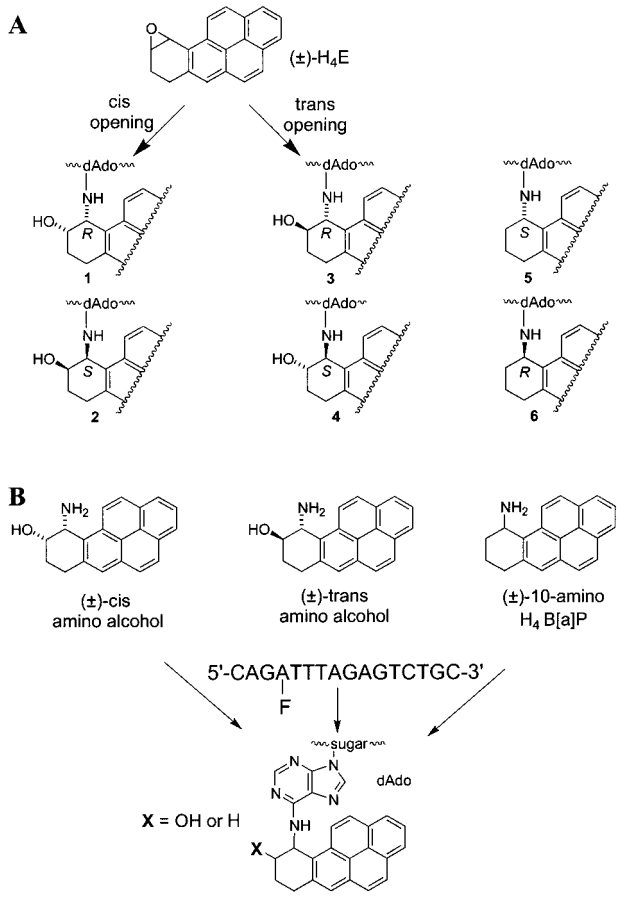


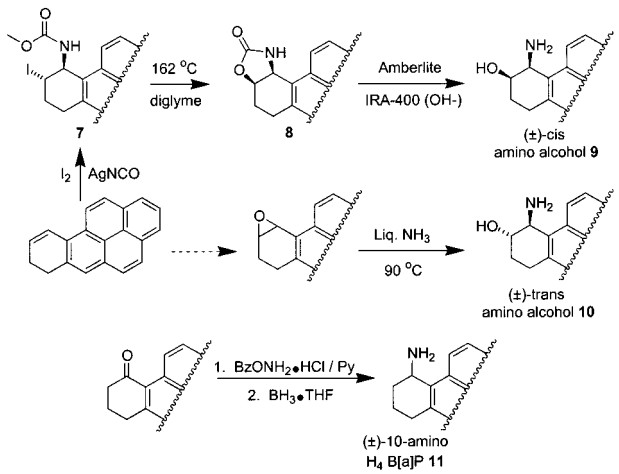
Figure 1. (A) Structures of the six optically active tetrahydro B[a]P dAdo adducts in the oligonucleotide 16-mers **1**−**6** prepared for the present study. (B) Reaction of the racemic H_4 B[a]P cis and trans amino alcohols and 10-amino H_4 B[a]P with the fluorinated oligonucleotide 16-mer. For graphical purposes the 6-FP residue is represented as a fluoro derivative of dAdo. Note that oligonucleotides **1** and **2** correspond to cis opening, and **3** and **4** to trans opening of the corresponding B[a]P tetrahydroepoxide (B[a]P H_4E).

land, OH). [γ-^{32}P]ATP and *Eco*RI restriction enzyme were from Amersham Corp. (Piscataway, NJ). *E. coli* strain SMH77 and the bacteriophage M13mp7L2 were generous gifts from Dr. C. W. Lawrence (University of Rochester, NY).

Synthetic Intermediates. Proton NMR spectra were obtained at 300 MHz. Chemical shifts (δ) are reported in parts per million, and coupling constants (J) are reported in hertz. Mass spectra were measured on a JEOL JMS-SX102 spectrometer with a direct exposure probe. Melting points are uncorrected. For structures and relative stereochemistry, see Scheme 1. The known precursor compounds (*9*), 9,10-dihydrobenzo[*a*]pyrene-7-(8*H*)-one and 7,8,9,10-tetrahydrobenzo[*a*]pyrene, were prepared using improved methods as described in the Supporting Information.

trans-9-Iodo-10-carbomethoxyamino-7,8,9,10-tetrahydrobenzo[a]pyrene (7). To a stirred mixture of 7,8-dihydrobenzo[*a*]pyrene (*10*) (1.7 g, 6.8 mmol), silver isocyanate (6 g, 40 mmol) and dry THF (40 mL) was added a solution of iodine (1.7 g, 6.8 mmol) in dry THF (10 mL) at room temperature over a period of 5 min in the dark. After being stirred for 30 min, the reaction mixture was treated with decolorizing charcoal (1 g) and filtered. The filtrate was evaporated to leave a reddish oil, which was dissolved in a mixture of THF (50 mL) and MeOH (350 mL) and refluxed for 2 h. After treatment with decolorizing charcoal (2 g) and filtration, the filtrate was concentrated to give a crystalline product (2.9 g, 94%). Recrystallization from MeOH gave colorless minute needles, mp 123 °C (decomp); ^{1}H NMR (acetone-d_6): δ 2.20−2.45 (m, 1H$_8$ and 1H$_{8'}$), 3.30−3.65 (m, 1H$_7$ and 1H$_{7'}$), 3.70 (s, MeCO), 5.10 (m, 1H$_9$), 6.05 (m, 1H$_{10}$), 7.32 (br d, 1NH, $J_{10,NH}$ = 6.9), 7.92−8.22 (m, 8 aromatic protons). HRMS (FAB$^+$, *m*/*z*) calcd for $C_{22}H_{18}INO_2$ (M^+): 455.0382. Found, 455.0384. Anal. calcd for $C_{22}H_{18}INO_2$: C, 58.04; H, 3.98. Found: C, 58.01; H, 3.89.

Scheme 1



Cyclic Carbamate of cis-9-Hydroxy-10-amino-7,8,9,10-tetrahydrobenzo[a]pyrene (8). A solution of iodocarbamate **7** (1.2 g, 2.6 mmol) in diglyme (12 mL) was refluxed under argon for 2.5 h. The reaction mixture was concentrated in vacuo to give a crystalline product (0.83 g, 99%). Recrystallization from THF-*n*-hexane gave colorless scales, mp 314−315 °C (decomp); ^{1}H NMR ($CDCl_3$): δ 2.08 (m, 1H$_{8'}$), 2.60 (m, 1H$_8$), 3.15 (dt, 1H$_7$, $J_{7,7'}$ = 16.0, $J_{7,8}$ = $J_{7,8'}$ = 4.0), 3.46 (m, 1H$_{7'}$), 5.33 (br s, 1NH), 5.45 (dt, 1H$_9$, $J_{9,10}$ = 8.0, $J_{9,8}$ = $J_{9,8'}$ = 4.0), 5.91 (d,1H$_{10}$, $J_{9,10}$ = 8.0), 8.00−8.27 (m, 8 aromatic protons). HRMS (FAB$^+$, *m*/*z*) calcd for $C_{21}H_{15}NO_2$ (M^+): 313.1103. Found: 313.1112. Anal. calcd for $C_{21}H_{15}NO_2$: C, 80.49; H, 4.82; N, 4.47. Found: C, 80.35; H, 4.85; N, 4.38.

cis-9-Hydroxy-10-amino-7,8,9,10-tetrahydrobenzo[a]pyrene (cis amino alcohol, 9). A mixture of cyclic carbamate **8** (370 mg, 1.2 mmol), Amberlite IRA-400 (OH^--form, 4 g), H_2O (30 mL), and THF (200 mL) was refluxed with stirring for 3 days. The reaction mixture was filtered, and the filtrate was evaporated to leave colorless crystals (320 mg, 94%). Recrystallization from THF-MeOH gave colorless needles, mp 227−228 °C (decomp); ^{1}H NMR (DMSO − D_2O): δ 1.85 (m, 1H$_{8'}$), 2.14 (m, 1H$_8$), 3.25 (m, 1H$_7$ and 1H$_{7'}$), 3.94 (dt, 1H$_9$, $J_{9,8'}$ = 12.4, $J_{9,10}$ = $J_{9,8}$ = 3.6), 4.73 (br d, 1H$_{10}$, $J_{9,10}$ = 3.6), 7.95−8.27 (m, 7 aromatic protons), 8.53 (d, 1H$_{11}$, $J_{11,12}$ = 9.3). HRMS (FAB$^+$, *m*/*z*) calcd for $C_{20}H_{18}NO$ (M^+ + 1): 288.1388. Found: 288.1392. Anal. calcd for $C_{20}H_{17}NO$: C, 88.52; H, 5.96; N, 4.87. Found: C, 88.45; H, 5.88; N, 4.90.

trans-9-Hydroxy-10-amino-7,8,9,10-tetrahydrobenzo[a]pyrene (trans amino alcohol, 10). A mixture of 9,10-epoxy-7,8,9,-10-tetrahydrobenzo[*a*]pyrene (*10*) (540 mg, 2.0 mmol) in liquid ammonia (∼50 mL) was heated at 85−95 °C for 18 h in a Parr high-pressure reactor. After cooling to dry ice temperature and evaporation of the ammonia under N_2, the crude product was washed with cold ether. Chromatography on a silica column packed in $CHCl_3$ and eluted with 7% MeOH in $CHCl_3$ provided 450 mg (85%) of off-white solid: mp 176−178 °C. ^{1}H NMR ($CDCl_3$): δ 2.25 (m, 1H$_{8'}$), 2.34 (m, 1H$_8$), 3.24 and 3.47 (each m, 1H$_7$ and 1H$_{7'}$), 4.32 (ddd, 1H$_9$, $J_{9,8'}$ = 5.5, $J_{9,10}$ =3.3, $J_{9,8}$ = 2.3), 4.88 (d, 1H$_{10}$, $J_{9,10}$ = 3.3), 7.50−8.04 (m, 4 aromatic protons), 8.17 (d, 1H$_{12,}$ $J_{11,12}$ = 9.1), 8.19 (br d, 1H$_3$) 8.21 (br d, 1H$_1$), 8.37 (d, 1H$_{11,}$ $J_{11,12}$ = 9.1). *m*/*z*: 288 (M^+ + 1). Treatment with Ac_2O/pyridine gave the 9-acetoxy 10-acetamido derivative (mp 258−260 °C). Anal. calcd for $C_{24}H_{21}NO_3$: C, 77.62; H, 5.66; N, 3.77. Found: C, 77.38; H, 5.77; N, 3.65.

10-Amino-7,8,9,10-tetrahydrobenzo[a]pyrene (10-amino H_4 B[a]P, 11). 7,8-dihydrobenzo[*a*]pyrene-10-(9*H*)-one (*11*) (1 g, 3.7 mmol) was converted to its *O*-benzyloxime by treatment with *O*-benzylhydroxylamine hydrochloride (2 g, 7.4 mmol) in

4 mL pyridine for 16 h at room temperature. The resultant product was directly reduced (*12*) to 10-amino-7,8,9,10-tetrahydrobenzo[*a*]pyrene **11** by treatment with borane-THF (26 mmol) in 50 mL THF for 1 h at 0 °C followed by 16 h at room temperature. After dilution with organic solvent and washing with NaOH, the organic layer was concentrated to give an oil (800 mg) that contained three major products (t_R = 1.8, 3.0 and 4.3 min) by HPLC on an Axxiom Sil column (9.5 × 250 mm) eluted at a flow rate of 10 mL/min with 20% EtOAc and 0.1% Et_3N in *n*-hexane (detection at 270 nm). The mixture was subjected to column chromatography on silica gel eluted with 20% EtOAc and 0.1% Et_3N in hexane to give three fractions, in order of elution, that were identified as 7,8,9,10-tetrahydro B[a]P (250 mg), the desired amine (**11**, 28 mg) and benzyl alcohol (420 mg). Although amine yields of 68−86% on reduction of other *O*-benzyloximes by the described procedure have been reported (*12*), the above reaction gave extremely poor yields of the desired amine as a result of over-reduction at the 10-position to give predominantly 7,8,9,10-tetrahydro B[a]P. The amine was further purified by HPLC as above to give a colorless powder (20 mg): ^{1}H NMR (acetone-d_6): δ 2.00 (m, 2H), 2.15 (m, 2H), 3.18 (m, $2H_7$), 4.80 (br s, NH_2 and $1H_{10}$), 7.80−8.27 (7 aromatic protons), 8.45 (d, $1H_{11}$, $J_{11,12}$ = 9.3). HRMS (EI, *m*/*z*) calcd for $C_{20}H_{17}N$ (M^+): 271.1361. Found: 271.1364. A portion was acetylated with Ac_2O and pyridine to give the acetamido derivative as a colorless powder. ^{1}H NMR ($CDCl_3$): δ 1.90−2.40 (m, 4H), 3.25 (m, $2H_7$), 2.00 (s, CH_3CO), 5.85 (br d, $1H_{10}$, $J_{10,NH}$ = 8.0), 6.09 (br d, 1NH), 7.90−8.26 (8 aromatic protons). HRMS (EI, *m*/*z*) calcd for $C_{22}H_{19}NO$ (M^+): 313.1466. Found: 313.1458.

Adducted Oligonucleotides. Oligonucleotides containing adducts corresponding to cis or trans opening of 9,10-epoxy-7,8,9,10-tetrahydro B[a]P by N^6 of dAdo (one hydroxyl group on the tetrahydro benzo-ring) or containing a N^6-10-(7,8,9,10-tetrahydrobenzo[*a*]pyrenyl) dAdo residue (no hydroxyl groups) were prepared by postoligomerization modification (*13*) of a support-bound oligonucleotide 16-mer, 5′-CAG(6-FP)TTTA-GAGTCTGC-3′ [Context II(A), corresponding to region 141−126 in the *supF* gene] containing a 6-fluoro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)purine (6-FP) residue at the modification site. The fluorinated oligonucleotide was prepared essentially as described (*14*) on a 15 micromole scale using 150 mg of 170 Å controlled pore glass (cpg) derivatized with N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine-3′-succinic acid (102 μmol/g), with the following modifications. 4,5-Dicyanoimidazole (0.5 M in CH_3CN, 250 μL) was used instead of 1*H*-tetrazole in the manual coupling with a 6-fold molar excess of 6-FP phosphoramidite. End capping following this step was omitted (*15*). Any failure to couple the fluorinated residue would eventualy result in a shorter oligonucleotide lacking the hydrocarbon, which is easily separable from the desired, adducted oligonucleotides on HPLC. The yield on manual coupling was essentially quantitative. The 5′-DMT group was removed from the support-bound oligonucleotide before use. Hydrocarbon adducted oligonucleotides were prepared on a 2 μmol scale. In a typical reaction, 20 mg of the above cpg-bound, fluorinated oligonucleotide, 12 mg of racemic, trans amino alcohol **10**, and 13 μL of triethylamine were heated at 40−45 °C for 65 h in 200 μL Me_2SO. Similar procedures were followed for postoligomerization synthesis with the racemic cis amino alcohol **9** and racemic 10-amino H_4 B[a]P **11**. Washing of the glass beads and cleavage and deprotection of the modified oligonucleotides were as described (*14*). The adducted oligonucleotides were purified by reversed-phase HPLC (Table 1). Isolated yields of each diastereomeric oligonucleotide were typically 15−25 A_{260}.

All oligodeoxynucleotides were further purified by electrophoresis on a denaturing 20% polyacrylamide gel. The oligonucleotides were detected by UV shadowing. The corresponding gel areas were cut out and crushed, incubated overnight in elution buffer [0.5 M NH_4OAc, 10 mM $Mg(OAc)_2$], and desalted on Waters Sep-Pak cartridges (Milford, MA) by elution with 60% methanol in water. The eluate was dried and then washed with 90% ethanol. Purity was confirmed by electrophoresis after

Table 1. HPLC Retention Times[a] and Configurational Assignments[b] for Oligonucleotides Containing Tetrahydro B[a]P-dAdo Adducts in Context II(A)

oligonucleotide[c]	configuration at C10	retention time (min)
1	*R*	21.7
2	*S*	23.7
3	*R*	21.3
4	*S*	22.7
5	*S*	24.2
6	*R*	26.9[d]

[a] On a 7 μm Hamilton PRP-1 column (10 × 250 mm) eluted at 3 mL/min with a linear gradient of acetonitrile in 0.1 M ammonium carbonate buffer, pH ~7.5, that increased the acetonitrile concentration from zero to 17.5% over 20 min, followed by a 5 min ramp to 50% acetonitrile and 5 min isocratic elution at this solvent composition. [b] Configurational assignments were based on CD spectra of the oligonucleotides (see text). [c] For structures see Figure 1. Note that for each pair of diastereomers the early and late eluting oligonucleotides always have the dAdo substituent in the α and β orientations, respectively, as shown in Figure 1A, although the Cahn−Ingold−Prelog nomenclature changes for **5** and **6** on application of the sequence rules. [d] An additional chromatographic step (Beckman Ultrasphere C_{18}, 10 × 250 mm, eluted at 3 mL/with the above solvent system) was required to separate this oligonucleotide (t_R 23.3 min) from ammonolysis product(s) of the organic protecting groups.

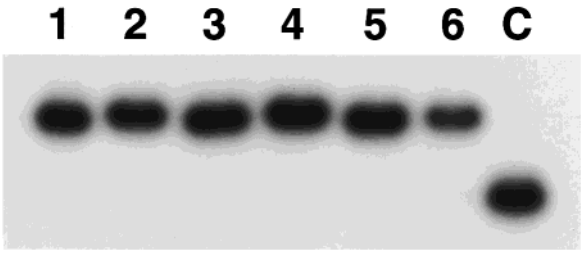


Figure 2. Polyacrylamide gel electrophoresis of the16-mer oligonucleotides **1**−**6** after end-labeling following their purification by HPLC and gel electrophoresis (see Experimental Procedures). Structures for **1**−**6** are shown in Figure 1. The lane labeled C corresponds to the unadducted 16-mer control.

[γ-^{32}P] ATP-labeling of aliquots (Figure 2).

All M13 minipreps were performed using QIAprep 8 M13 kits from Qiagen (Valencia, CA). DNA sequencing reactions were carried out using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit from PE Applied Biosystems (Foster City, CA). The sequencing runs were done on an ABI Prism 377 DNA Sequencer.

Construction of Site-Specifically Modified M13 Genomes. The construction of the M13 vectors was based on the procedure developed by Lawrence and co-workers (*16*, *17*) and is essentially the same as that used in previous studies in our laboratory (*6*, *7*). Briefly, bacteriophage M13mp7L2 (200 μg) was digested with *Eco*RI (800 units) in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, for 2.5 h at 30 °C. The enzyme was inactivated by heating at 65 °C for 15 min, and the solution was extracted with phenol/chloroform. The linearized DNA was then purified by ethanol precipitation. Each oligonucleotide 16-mer (50 pmol), with or without the adduct, was 5′-phosphorylated with kinase and annealed with a 56-mer uracil-containing scaffold (2 pmol) by heating them together to 50 °C for 5 min and slow cooling to room temperature overnight. The scaffold contained a middle sequence complementary to the 16-mer insert and 20-mer overhangs complementary to the ends of the linearized M13 DNA. The 16-mer/scaffold duplex was annealed with the linear M13 (1 pmol) by incubation at room temperature overnight. Ligation was done at 16 °C overnight upon addition of T4 DNA ligase (30 units). The ligation mixture was then incubated with uracil DNA glycosylase (UDG) (1 unit) to remove uracil residues in the scaffold. UDG produces abasic sites that lead to degradation of the scaffold by apurinic endonucleases

and exonucleases upon transfection into bacterial cells (*6*).

To estimate the apparent ligation efficiency (defined as the ratio of circular DNA to total DNA), the components of the vector construction mixture (~100 ng of DNA) were separated by agarose gel electrophoresis and transferred to nitrocellulose by Southern blotting. A [γ-^{32}P]ATP-labeled probe complementary to a sequence in the M13 construct, 5′-GGCGAAAGGGGGATGTGC (1 ng/mL hybridization solution), was used to detect closed circular and linear DNA, the amounts of which were quantified by PhosphorImager analysis (Storm 860, Molecular Dynamics, Inc.).

Mutagenesis Assay. *E. coli* strain SMH77 cells were SOS-induced by UV irradiation (254 nm) at 40 J/m^2 for 40 s (*18*) before they were made competent by $CaCl_2$ treatment. For each transfection, an aliquot of the ligation mixture (20 ng) was added to 100 μL of the competent cell suspension, heat shocked at 42 °C for 90 s, cooled on ice for 120 s and incubated at room temperature for 10 min. The DNA/cell suspension was then mixed with top agarose containing X-gal and IPTG, poured onto 2XYT agar plates, and grown at 37 °C overnight. Survival was estimated by comparing the number of blue plaques (containing the insert) obtained from equal amounts of total DNA with and without adduct (with the survival of the control taken as 100%). Either three or four separate ligations were performed followed by transfection, and the survival results were averaged. Mutagenicity data were generally obtained from three ligation-transfection experiments, except for the two most mutagenic adducted sequences **1** and **3,** which gave 595 and 697 total plaques, respectively, and good agreement between the data from two replicate experiments.

Mutation analysis of progeny phage was done by differential oligonucleotide hybridization as described (*6*). The DNA from the plaques produced on each plate was transferred to four Protran nitrocellulose membranes (Schleicher & Schuell). After baking the membranes for 2 h at 80 °C in a vacuum oven, they were washed with 3× SSC containing 0.1% SDS for 2 h at 37 °C. To eliminate nonspecific binding, they were then incubated in 2× Prehybridization/Hybridization solution (Gibco/BRL) for 1.5 h at 37 °C. One of four [γ-^{32}P]ATP-labeled 13-mers designed to be complementary to either side of the adduct site, 5′-TCTAAA**X**CTGCAC, where the adduct is opposite X (X=A, C, G, or T), was added to each transfer membrane at 37 °C. The solutions were cooled slowly to room temperature and incubated overnight with constant agitation. The membranes were then washed with 6× SSC at room temperature (4 × 30 min) and finally at the stringent temperature 35.5 °C for another 30 min. The membranes were subsequently exposed to X-ray film with an intensifying screen at −70 °C overnight. The autoradiographs were compared to the actual plates to identify single-base mutations at the target site. The DNA from any plaque that did not correspond to any signal in the autoradiograph was extracted and sequenced.

Results

Adducted Oligonucleotides. The required oligonucleotides were constructed by postoligomerization modification (*13*) of a 16-mer containing a 6-fluoropurine base (Figure 1B), utilizing racemic 10-amino tetrahydro B[a]P **11** as well as the racemic cis and trans amino alcohols **9** and **10**. The cis and trans amino alcohols are easily distinguished from each other by the NMR coupling constants between the two H8 protons and H9. Thus, for the trans amino alcohol (diaxial amino and hydroxyl substituents), H9 is equatorial, and $J_{9eq,8eq}$ and $J_{9eq,8ax}$ are both relatively small (2.3 and 5.5 Hz). In contrast, for the cis amino alcohol (axial amino and equatorial hydroxyl group), H9 is axial, and $J_{9ax,8ax}$ is 12.4 Hz. The trans amino alcohol was easily prepared (85%) from the corresponding 9,10-epoxide of 7,8,9,10-tetrahydro B[a]P (Scheme 1) by ammonolysis in liquid ammonia under pressure as previously described for the corresponding amino triols from B[a]P DEs (*19*). The corresponding cis amino alcohol was prepared by the general method of Hassner et al. (*20*) from 7,8-dihydro B[a]P in three steps (Scheme 1), each of which proceeded with ≥94% yield. In contrast, preparation of 10-amino H_4 B[a]P via reduction of the *O*-benzyloxime proved quite difficult, as the major reaction pathway resulted in loss of the 10-amino group to give 7,8,9,10-tetrahydro B[a]P.

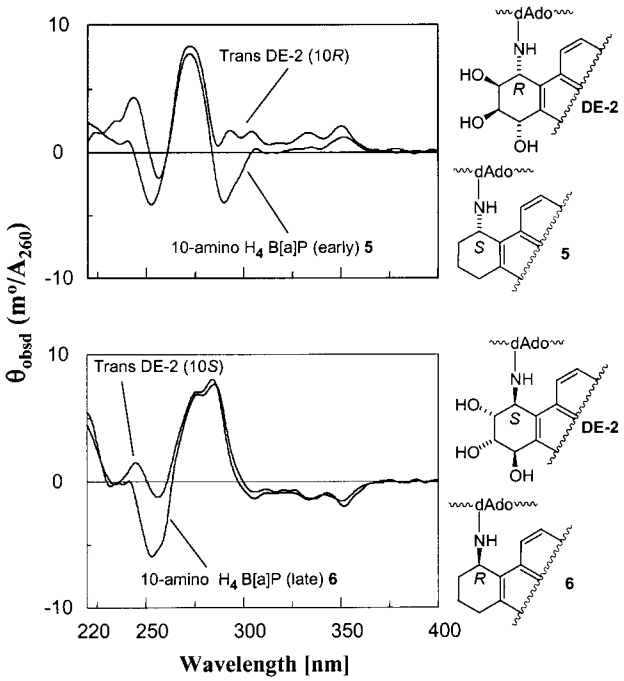


Figure 3. Circular dichroism spectra of oligonucleotides in 0.02 M phosphate buffer, pH 7, with ionic strength adjusted to 0.1 M with NaCl. Spectra are normalized to 1.0 absorbance unit at 260 nm. Note that the early (**5**) and late (**6**) eluting adducted oligonucleotides (Table 1) with no hydroxyl groups on the saturated ring (10-amino H_4 B[a]P adduct; partial structures as indicated) exhibit major CD bands that are very similar to those for the oligonucleotides with the same sequence (*14*) containing B[a]P DE adducts (3 hydroxyl groups). Corresponding similarities are observed with oligonucleotides containing the cis opened DE adducts (*7*) as well as the cis and trans opened B[a]P H_4E adducts in oligonucleotides **1**−**4** (not shown).

The diastereomeric adducted oligonucleotides derived from reaction of the support-bound, fluorinated 16-mer with each racemic amino alcohol or with racemic 10-amino H_4 B[a]P were well separated on HPLC (Table 1). Absolute configurations at C10, the point of attachment of N^6 of dAdo to the hydrocarbon, were assigned on the basis of the circular dichroism (CD) spectra of the purified oligonucleotides (Figure 3). In the *monomeric nucleoside adducts*, the hydroxyl groups have a negligible effect on the appearance of the CD spectra, as shown by comparison of the spectra of B[a]P H_4E adducts (*21*, *22*) at N^6 of dAdo with the corresponding DE adducts (*23*, *24*). Similarly, the present B[a]P adducted *oligonucleotides* lacking two or three of the benzo-ring hydroxyl groups exhibited CD spectra whose long-wavelength bands were very similar to those reported (*7*, *14*) for the corresponding DE-adducted oligonucleotides. For example, the CD spectra for the H_4 B[a]P-modified oligonucleotides **5** and **6** (prepared from 10-amino H_4 B[a]P in this study) are compared with those for the previously assigned trans DE-2 adducted analogues in Figure 3. Similar results were obtained with the adducted oligonucleotides **1**−**4**

(not shown). On the basis of their CD spectra, all three early-eluting oligonucleotides (**1**, **3**, and **5**) had the same spatial orientation at C10 of the hydrocarbon relative to the dAdo substituent (see Figures 1 and 2), and all three late-eluting eluting oligonucleotides (**2**, **4**, and **6**) had the opposite orientation. Note that early- and late-eluting adducts correspond to 10*R* and 10*S*, respectively, for the amino alcohol derivatives, but the nomenclature (although *not* the relative spatial orientation of the dAdo substituent and the hydrocarbon) reverses for the 10-amino B[a]P derivatives because of the application of the sequence rule.

Construction of Site-Specifically Modified M13 Genomes. The M13 vectors were constructed based on the method of Lawrence and co-workers (*16*, *17*) with minor modifications (*6*). After digestion with *Eco*RI, the linear M13 DNA was recircularized by annealing and ligating a duplex consisting of a 56-mer scaffold and a 16-mer oligonucleotide insert with or without the adduct. Ligation efficiencies, defined as the amount of circular DNA as a fraction of the sum of linear and circular DNA, were determined by running an aliquot of the constructs on an agarose gel, transferring the DNA onto a membrane by Southern blotting and radioactive probing. Ligation of the unadducted 16-mer into the M13 vector occurred with an apparent efficiency of 36%, whereas the oligonucleotides **1**, **2**, **3**, **4**, **5**, and **6** containing the dAdo adducts were ligated with somewhat lower efficiencies of 26, 26, 20, 29, 27, and 28%, respectively, that were quite similar to each other. These apparent ligation efficiencies for the adducted 16-mers are comparable to those observed wirh cis opened B[a]P DE-dAdo adducts in the same sequence (*7*), and may result from distortion of the DNA by the adducts.

When transfected into SOS-induced *E. coli*, the adducted M13 constructs gave fewer plaques than the control M13 construct. The values for survival were determined from the ratio of infective centers containing the insert (blue plaques) obtained from a given amount of adducted relative to control DNA. These values were 13, 23, 13, 19, 33, and 46%, for the constructs with dAdo adducts **1**, **2**, **3**, **4**, **5**, and **6**, respectively. In the presence of adducts, low apparent survival values reflect contributions from two factors: (1) a smaller fraction of the total DNA that is circular and contains the insert, and (2) reduced efficiency of DNA polymerases to replicate past the adducts. Consequently, the survival data by themselves would overestimate the contribution of the latter factor (*25*). In the present study, apparent ligation efficiencies for all six adducted oligonucleotides were quite large (~60−70% of control). Observed survival values (13−46%) that are much less than this suggest that the present H_4 B[a]P dAdo adducts are inefficiently bypassed. Interestingly, the recoveries of progeny in the present experiments are generally higher than those (<10%) previously observed (*6*, *7*) with the B[a]P DE adducted oligonucleotides relative to the control.

Mutational Frequencies. Mutation data for the six adducted oligonucleotide inserts are summarized in Table 2. Overall mutational frequencies ranged from 0.2 to 29.2%. Oligonucleotides **1** (10*R*), **3** (10*R*), and **5** (10*S*), having the same spatial arrangement of the pyrene rings with respect to dAdo, gave higher mutational frequencies than their counterparts, **2** (10*S*), **4** (10*S*), and **6** (10*R*). For the H_4E adducts, those with the hydroxyl group and the dAdo in a cis arrangement (**1** and **2**) showed higher mutational frequencies than their trans counterparts (**3** and **4**). With respect to the hydroxyl groups on the tetrahydro benzo-ring, **5** and **6** (no hydroxyl groups) had lower mutational frequencies than **1**, **2**, **3**, and **4** (one hydroxyl group).

Table 2. Frequencies of Individual Replication Events and Total Base Substitution Mutations Resulting from Replication Past the B[a]P Adducts in Inserts 1−6 in SOS-Induced Cells[a]

	no. of A → A	no. of A → T	no. of A → G	no. of A → C	survival (%)	MF_{tot}[b] (%)
1[c]	421	165	5	4	13	29.2
2[d]	1918	28	16	2	23	2.3
3[e]	649	43	5	0	13	6.9
4[f]	2110	12	3	1	19	0.8
5[g]	2380	43	4	1	33	2.0
6[h]	3556	4	1	1	46	0.2

[a] For the control sequence, 3668 plaques were screened and no mutation at the targeted site was detected (background MF < 0.027%). [b] The percentage of total progeny that had undergone base substitution mutations at the adduct site. [c] Nontargeted mutations for the insert sequence 5′−$C_1A_2G_3A_4T_5T_6T_7A_8G_9A_{10}$-$G_{11}T_{12}C_{13}T_{14}G_{15}C_{16}$, where the adduct is at A_4, were as follows through footnote h: 1 C_1 → T; 4 A_2 → G; 1 A_2 → C; 1 G_3 → T; 1 T_5 → A; 1 A_8 → G; 2 G_9 → A; 2 A_{10} → C; 1 G insert between T_{14} and G_{15}; 3 C_{16} deletions. [d] 1 C_1 → G; 3 A_2 → C, 2 G_3 → C; 1 T_5 → A; 1 A_8 → C; 3 G_9 → A; 3 G_9 → C; 4 A_{10} → C; 1 A_{10} → T; 1 G insert between A_{10} and G_{11}; 1 C_{16} deletion. [e] 1 G_3 → T; 1 G_9 → A; 4 C_{16} deletions. [f] 3 C_1 → T; 4 A_2 → T/G_3 → T/T_5 → G; 2 G_3 → C; 4 G_9 → A; 1 A_{10} → C; 1 A_{10} → T; 1 G_{11} → A; 1 C_{16} deletion; 1 deletion of first 6 nucleotides. [g] 1 T_5 → A; 1 T_6 → A; 2 A_8 → G; 1 G_9 → A; 1 G_9 → T; 1 G insert between A_{10} and G_{11}; 6 C_{16} deletions. [h] 1 A_2 → C; 1 G_3 → T; 1 T_7 → A; 1 A_8 → G; 3 G_9 → A; 2 A_{10} → C; 1 A_{10} → T; 1 G_{11} → A; 4 C_{16} deletions.

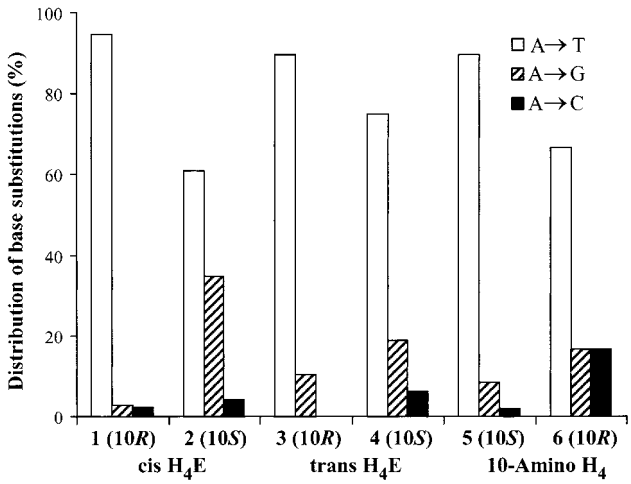


Figure 4. Distribution of individual base substitution mutations as a fraction of total mutations induced by B[a]P-dAdo adducts in the 16-mer inserts **1**−**6**. Structures are shown in Figure 1.

The distribution of mutations is given in Figure 4. For all six adducts investigated, the major base substitution mutations observed were A → T transversions, with frequencies ranging from 60.9 to 94.8% of total mutations. The frequencies for A → G transitions were higher than for A → C transversions in all cases except for **6**, where these two mutational events occurred with the same frequency.

Discussion

An objective of our ongoing studies on site-specific mutations induced by polycyclic aromatic hydrocarbon

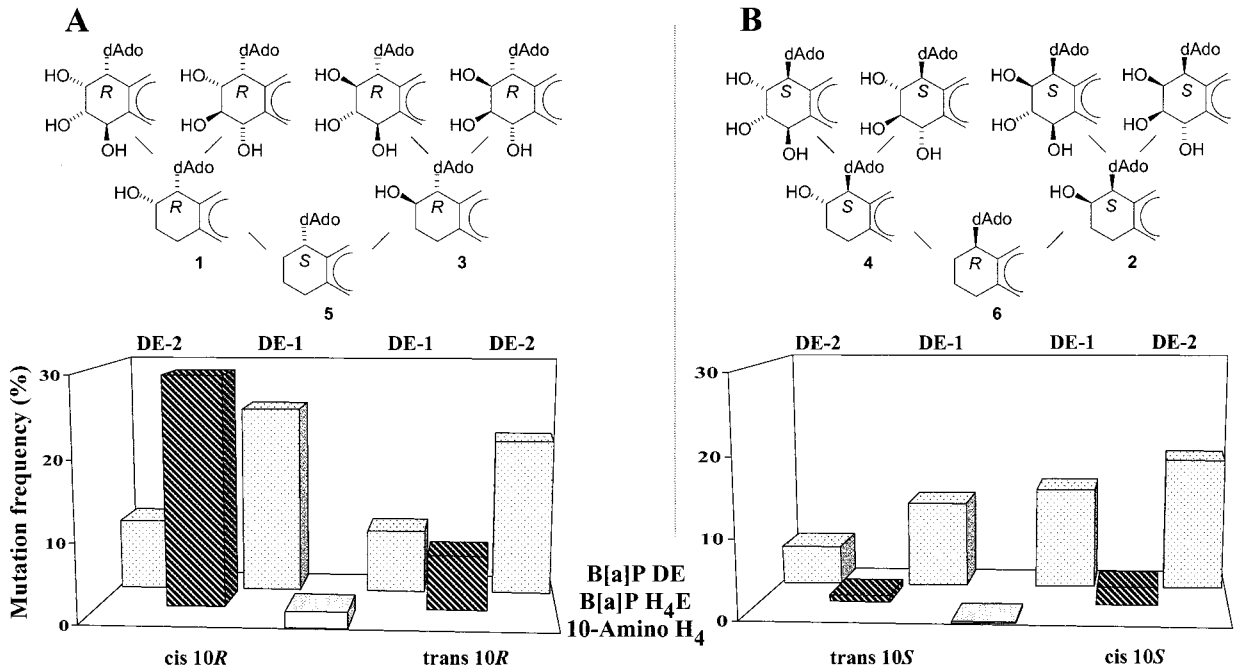


Figure 5. Total frequencies of base substitution mutations induced by the B[a]P-dAdo adducts in 16-mers **1**−**6** in comparison with those induced by the related B[a]P diol epoxide (DE-1 and DE-2) dAdo adducts in the same sequence context. Data for the DE adducts are from Page et al. (*6*, *7*). The top row of structures (DE adducts) correspond to the rear row of bars in each bar graph. All of the adducts on side A have the same spatial orientation at C10, and the adducts on side B have the opposite orientation. The structures are arranged such that those on the right-hand edge of side A and those along the left-hand edge of side B, etc., are mirror images of each other.

DNA adducts has been to evaluate the effect of adduct structure on mutagenicity. Previously, we reported data for dAdo adducts derived from the cis and trans opening of B[a]P DEs (*6*, *7*) and benzo[*c*]phenanthrene DEs (*8*) in two sequence contexts. In those studies, we observed a dependence of the mutational frequencies on the stereochemical relationships among the hydroxyl groups as well as on the configuration at the site of attachment of the DEs to the adenine base. The aim of the present study was to investigate systematically the effect of the hydroxyl groups on mutational response by examining adducts in which some or all of these hydroxyl groups were absent. The bay-region tetrahydroepoxide, 9,10-epoxy-7,8,9,10-tetrahydro B[a]P (B[a]P H_4E) (see Figure 1), which lacks hydroxyl groups at C7 and C8, has been shown to form adducts with both dAdo and dGuo in DNA in vitro (*21*). This tetrahydroepoxide is formed on mammalian metabolism of 7,8-dihydro B[a]P (*26*−*28*), with the (+)-(9*S*,10*R*)-enantiomer predominating upon cytochrome P450-mediated metabolism. This stereoselectivity is analogous to that for the formation of the B[a]P DEs (*3*). Racemic B[a]P H_4E is generally more mutagenic than the corresponding B[a]P DEs in several strains of *Salmonella typhimurium*, but is somewhat less mutagenic than the B[a]P DEs in Chinese hamster V79 cells (*29*). However, the optically active (+)-B[a]P H_4E is about three times more mutagenic in V79 cells than the corresponding (+)-B[a]P DE-2 (R. L. Chang, personal communication). In contrast, racemic B[a]P H_4E was not tumorigenic on mouse skin (*30*), presumably as a consequence of its high chemical reactivity (*31*) and its sensitivity to epoxide hydrolase (*28*) which likely result in its rapid hydrolysis to inactive diols.

On the basis of the above considerations, we anticipated that the BaP H_4E adducts would be *at least as mutagenic and might well be more mutagenic* than the corresponding DE adducts. Thus, we chose context II(A) for the present experiments, since the relatively low mutational frequency observed with DE adducts in this sequence would potentially allow a better assessment of *increases* in mutational frequency than context I(A), whose mutational frequency with the DE adducts was already quite high (*6*, *7*). Surprisingly, however, only one of the H_4E adducts (the cis 10*R* adduct in **1**; see below) was more mutagenic than the DEs, and all the other adducts were much less so.

There is no clear effect of adding hydroxyl groups on the mutational distribution (Figure 4). In all cases A → T transversions predominated (≥60%). The set of adducts configurationally related to **5** (same orientation of dAdo relative to the hydrocarbon) exhibited a higher preference (≥90%) for A → T mutations than those (**2**, **4**, and **6**) with the opposite configuration at C10**.** Comparison with our previous data (*6*, *7*) for B[a]P DE adducts in this sequence context indicated that the 10*R* DE adducts also exhibited a higher preference for this mutation relative to their 10*S* counterparts. Concomitant with lower proportions of A → T mutations, **2**, **4**, and **6** gave relatively higher percentages (17−35%) of A → G transitions. The highest percentage of A → G transitions (35%) in the present series of compounds was observed with the cis 10*S* B[a]P H_4E adduct in **2**. Notably, in the same sequence II(A) (*7*), the cis 10*S* B[a]P DE-1 adduct also gave the highest percentage (65%) of this mutation. In all cases but one (insert **6**), A → C mutations, which were also relatively infrequent with the B[a]P DE adducts, were uncommon.

Total mutational frequencies for adducts lacking two or three of the hydroxyl groups, as well as those previously reported for the DE adducts, are summarized in Figure 5. For this purpose, we divided the adducts into two families, one formally derived from the *S* adducted oligonucleotide **5** and the other from the *R* adducted

oligonucleotide **6** by successive additions of hydroxyl groups on the tetrahydro ring. The figure is arranged so that the left- and right-hand families of structures, **A** and **B**, are mirror images of each other, e.g., enantiomeric pairs of trans opened adducts are on the inside edges of the graphs **A** (*R*) and **B** (*S*), and enantiomeric pairs of cis adducts are on their outside edges. Pairs of adducts occupying equivalent left-to-right positions in graphs **A** and **B** differ only in their absolute configuration at C10. In general, the 10-amino H_4 derivative **5** and its related 10*R* H_4E adducts **1** and **3** (**A**), as well as the related DE adducts, gave higher mutational frequencies than the corresponding adducts with the opposite spatial orientation at C10 (**B**). This trend is opposite to that observed with the benzo[*c*]phenanthrene DE-dAdo aducts (*8*). Interestingly, the cis 10*R* adduct in **1** was the most mutagenic of all the adducts studied. With this one outstanding exception, the mutational frequency decreased within each family as the number of hydroxyl groups was decreased from three to one to none. Thus, contrary to our original expectation, decreasing the number of hydroxyl groups generally decreased the mutagenicity of these B[a]P adducts.

Within a set of adducts (**A** or **B**) with the same spatial orientation at C10, the H_4E adducts in oligonucleotides **1** and **2**, in which the dAdo substituent and the hydroxyl group are cis, gave higher mutation frequencies than their trans counterparts **3** and **4**. For the 10*S* DE adducts (**B**), a similar general pattern of higher mutagenicity for adducts with cis orientation of the dAdo and 9-OH substituents is similar, but this pattern is less pronounced with the 10*R* DE adducts (**A**). It has previously been observed that cis-opened B[a]P DE adducts in an N-*ras* sequence (*32*) were severalfold more mutagenic than the corresponding trans-opened adducts. In the present work, the high mutagenicity of the cis 10*R* H_4E adduct **1** is particularly striking. Since dAdo adducts are formed from racemic B[a]P H_4E on reaction with DNA in vitro (*21*), it is tempting to speculate that a cis dAdo adduct or adducts may be in part responsible for the high mutagenicity observed in previous studies with this tetrahydroepoxide.

Supporting Information Available: Improved methods for the preparation of 9,10-dihydrobenzo[*a*]pyrene-7-(8*H*)-one and 7,8,9,10-tetrahydrobenzo[*a*]pyrene. This material is available free of charge via the Internet at http://pubs.acs.org.

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