Factors Determining Mutagenic Potential for Individual Cis and Trans Opened Benzo[*c*]phenanthrene Diol Epoxide-Deoxyadenosine Adducts†

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ABSTRACT: Four adducts that would result from trans opening at C-1 of benzo[*c*]phenanthrene 3,4-diol 1,2-epoxide (B[*c*]PhDE) isomers (i.e., DE-1 enantiomers, where the epoxide oxygen and benzylic hydroxyl group are cis, and DE-2 enantiomers, where they are trans) by the N^6-amino group of dAdo, together with the two cis opened N^6-dAdo adducts of B[*c*]PhDE-1, were incorporated into two oligonucleotides at the underlined site in 5′-TTTAGAGTCTGCTCCC [context I(A)] and 5′-CAGATTTAGAGTCTGC [context II(A)]. After ligation of these, and the corresponding unsubstituted oligonucleotides, into single-stranded M13mp7L2 bacteriophage and transfection into SOS-induced *Escherichia coli* SMH77, base substitution mutations induced by the different B[*c*]PhDE-dAdo adducts were determined. These findings were compared with data [Pontén et al. (1999) *Biochemistry 38*, 1144−1152] for cis opened B[*c*]PhDE-2-dAdo adducts in the same sequence contexts. In most cases, adducts with S absolute configuration at the site of attachment of the nucleoside to the hydrocarbon had higher mutation frequencies (1.9−56.5%) than the corresponding adducts with R configuration (0.05−5.6%). For adducts derived from B[*c*]PhDE-1, the predominant mutations were A→T transversions in context I(A) and A→G transitions for most of these adducts in context II(A). For adducts derived from B[*c*]PhDE-2, A→T base substitutions predominated for most of the trans adducts, but A→G mutations were favored by the cis adduct with S configuration in either context. Thus, the structural feature that most dramatically affected mutagenic activity was the configuration of the carbon at the attachment point, with S configuration mostly being associated with greater mutagenicity than the R configuration. However, other structural variations and sequence context also affected mutagenicity, indicating that a combination of structure and context effects define mutagenicity.

Polycyclic aromatic hydrocarbons (PAHs),[1] such as benzo[*c*]phenanthrene (B[*c*]Ph), are widespread environmental contaminants (*1*−*3*) that are known to require metabolic activation to bay- or fjord-region diol epoxides (DE) (*4*, *5*) for carcinogenic activity (*6*, *7*). For a given hydrocarbon, four configurationally isomeric diol epoxides can be generated (*8*). For B[*c*]PhDE, the DE-2 isomer is one of the most tumorigenic diol epoxides described to date (*6*).

Trans opened adducts are the major products of reaction of B[*c*]PhDE-2 isomers with DNA, and higher levels of dAdo than dGuo adducts are obtained (*9*−*13*). Cis opened adducts are minor products for the DE-2 isomers, but are more substantial in the case of the DE-1 isomers (*10*, *11*, *14*, *15*). Thus, a given hydrocarbon can form several adducts in DNA, and any one of these could be responsible for the mutations (*16*, *17*) in tumor suppressor genes or oncogenes required for tumorigenesis (*18*).

The development of site-specific mutation assays has allowed the mutagenic effects of a single adduct to be evaluated (reviewed in refs *19* and *20*). Both double-stranded and single-stranded DNA vectors have been used in such site-specific mutation studies for PAH diol epoxide−DNA adducts (*21*−*33*). The use of double-stranded vectors (*21*−*24*) has resulted in fairly low mutation frequencies, because of either strand-bias and/or the repair of the adduct. Use of single-stranded vectors eliminates strand bias and repair, and consequently, higher mutation frequencies are obtained. We have previously used the single-stranded vector (M13mp7L2) developed by Lawrence and colleagues (*34*, *35*) to study the mutagenic effects of cis and trans opened benzo[*a*]pyrene 7,8-diol 9,10-epoxide (B[*a*]PDE)-dAdo and -dGuo adducts (*30*, *33*) in several sequence contexts. To investigate the

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[1] Abbreviations: PAHs, polycyclic aromatic hydrocarbons; B[*c*]Ph, benzo[*c*]phenanthrene; B[*c*]PhDE-1, 3,4-diol 1,2-epoxide wherein the benzylic hydroxyl group and the epoxide oxygen are cis; B[*c*]PhDE-2, 3,4-diol 1,2-epoxide wherein the benzylic hydroxyl group and the epoxide oxygen are trans; B[*c*]PhDE-2/*R*, B[c]PhDE-dAdo adduct with R configuration at the adducted carbon (C_1); B[*c*]PhDE-2/*S*, dAdo adduct with S configuration at the adducted carbon (C_1); trans B[*c*]-PhDE-2/*R*, trans B[*c*]PhDE-2/*S*, etc., the structures are defined in Figure 1; DMT, dimethoxytrityl; B[*a*]P, benzo[*a*]pyrene; B[*a*]PDE, benzo[*a*]-pyrene 7,8-diol 9,10-epoxide.

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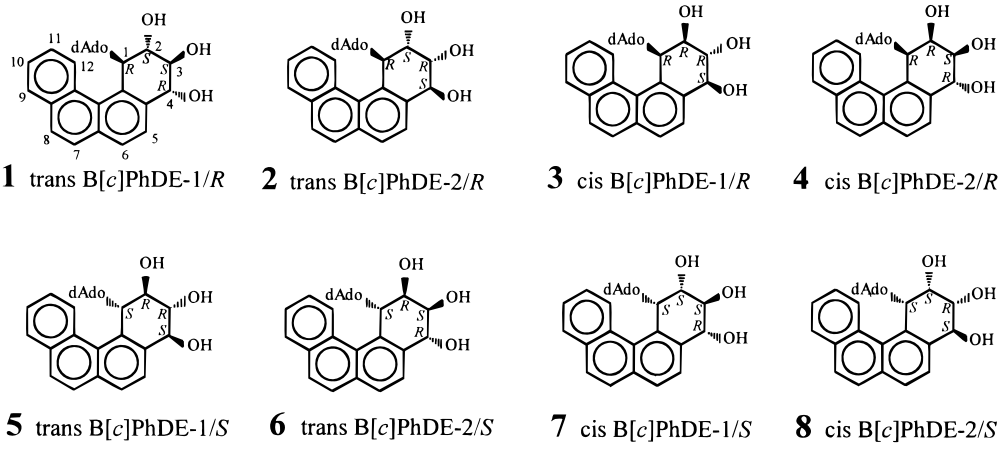

Context I(A): 5'-TTT**A**G**G**AGTCTGCTCCC

Context II(A): 5'-CA**G**G**A**TTTAGAGTCTGC

FIGURE 1: Structures of adducts formed from trans and cis opening of B[*c*]PhDE isomers by the exocyclic amino group of deoxyadenosine and the sequence contexts in which they were inserted. The adducts are labeled based on the diol epoxide from which they are derived. The configuration of the carbon at the attachment to dAdo is indicated (R or S).

effects of different PAH residues on mutation frequency and to establish a basis for determining general features that affect mutagenicity, we have extended this earlier work on B[*a*]PDE-dAdo adducts, derived from a parent compound with a planar structure, to studies with B[*c*]PhDE-dAdo adducts, derived from a parent compound with a nonplanar structure. The B[*c*]PhDEs, in contrast to B[*a*]PDE, react extensively with dAdo as well as with dGuo residues in DNA (*9*, *13*).

The present study reports the mutagenicities in each of two sequence contexts for six new DNA adducts formed from B[*c*]PhDEs through trans and cis opening of the epoxide ring at C_1 by the exocyclic amino group of deoxyadenosine. Comparable data for the other two related adducts was reported in an earlier study of sequence context effects (*32*). Each adduct was placed at the fourth nucleotide from the 5′-end in the two sequence contexts (see Figure 1 for structures and sequence contexts). The structural feature that most dramatically affected mutagenic activity was the configuration of the carbon at the point of attachment to the nucleoside residue. In most cases, the S configuration favored mutagenicity extensively over the R configuration.

MATERIALS AND METHODS

Oligonucleotide Synthesis. Twelve new oligonucleotides were prepared on a 2 μmol scale using 170 Å controlled-pore glass (CPG) loaded with N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine-3′-succinic acid (80−95 μmol/g). 5′-Dimethoxytrityl-3-diisopropylcyanoethylphosphoramidites corresponding to the cis and trans opened N^6-dAdo adducts of each B[*c*]PhDE, in which the three free hydroxyl groups on the hydrocarbon moiety were protected as their acetates, were chemically synthesized (*36*; H.Y., A.S.P., and D.M.J., in preparation). Each phosphoramidite was used as a diastereomeric mixture corresponding to either cis or trans ring opening of *racemic* DE-1 or DE-2. Support-bound oligonucleotides containing the appropriate 12-base sequence 3′ to the adduct were synthesized on an automated DNA synthesizer by standard methodology with modifications as described (*32*, *37*). The CPG beads bearing the oligonucleotide were removed from the column, and the modified dAdo residue was introduced manually by treatment with 7−9 μmol of a mixture of the appropriate phosphoramidite with 30−50 μL of 0.5 M 1H-tetrazole in acetonitrile for 16 h. Endcapping was omitted following the manual step. After returning the support-bound oligonucleotide to the column, the remaining three residues were added by reaction on the synthesizer, the oligonucleotide was cleaved from the support by the standard procedure, and protective groups were removed by ammonolysis (16 h, 58 °C). The diastereomeric, adducted oligonucleotides were separated by HPLC (*32*) after removal of the 5′-dimethoxytrityl protecting group. Yields on coupling of the modified phosphoramidites, estimated from the release of dimethoxytrityl alcohol on detritylation immediately following the manual coupling step, were variable. In the best case, a coupling yield of >80% gave a combined, isolated yield of 100 A_{260} units of the two diastereomeric, adducted oligonucleotides after HPLC. Retention times and HPLC conditions are given in Table 1. Homogeneity of the purified oligonucleotides was verified by HPLC analyses of the separated isomers.

For all the context I(A) oligonucleotides and the context II(A) oligonucleotide containing the trans B[*c*]PhDE-1 adducts **1** and **5**, the mutagenicity of the R adducts is extremely low relative to their diastereomeric S adducts, and even trace contamination with the S adduct could result in large errors in determining the distribution of specific mutations induced by the R adduct. In context I(A), the trans B[*c*]PhDE-2/*R* adduct, **2**, was found to be essentially nonmutagenic. The cis B[*c*]PhDE-2 R and S adducts, **4** and **8**, in context I(A) (Table 2 and ref *32*) produced very different distributions of mutations, an observation inconsistent with the possibility that the mutations observed with the cis B[*c*]PhDE-2/*R* adduct arose largely from the S adduct present as a contaminant. In contrast, the cis and trans B[*c*]PhDE-1 adducts in context I(A) and the trans B[*c*]PhDE-1 adduct in context II(A) *gave similar distributions of mutations from the R and S diastereomers*. In these cases, it was imperative to examine more closely the possibility of trace contamina-

Table 1: HPLC Retention Times[a] and Configurational Assignments for the Oligonucleotides

adduct[b]	retention time (min) context I(A)	retention time (min) context II(A)
1 trans B[*c*]PhDE-1/*R*	38.1[c]	14.8
5 trans B[*c*]PhDE-1/*S*	36.8[c]	14.0
2 trans B[*c*]PhDE-2/*R*	15.7	15.1
6 trans B[*c*]PhDE-2/*S*	17.1	13.6
3 cis B[*c*]PhDE-1/*R*	18.0	30.8[d]
7 cis B[*c*]PhDE-1/*S*	18.7	29.8[d]

[a] On a Hamilton PRP-1 column, 7 × 305 mm, eluted at 3.0 or 3.5 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, unless otherwise noted. [b]Configurational assignments of adducts at C_1 of the hydrocarbon are based on enzymatic hydrolysis of the early eluting member of each pair of diastereomeric oligonucleotides to the known, optically active nucleoside adducts (see text). [c]On the Hamilton column eluted at 3.0 mL/min with a linear gradient of methanol in 0.1 M ammonium carbonate buffer, pH ~7.5, that increased the methanol concentration from 0 to 50% over 50 min. [d]On a Hamilton column (7 μm, 10 × 250 mm) eluted at 3.0 mL/min with a linear gradient of methanol in 0.1 M ammonium carbonate buffer, pH ~7.5, that increased the methanol concentration from 0 to 50% over 40 min.

tion of the oligonucleotide containing the R adduct with its more mutagenic S diastereomer, to assess the reliability of the mutation distributions produced by the R adduct. We reexamined these R-adducted oligonucleotides by HPLC on a 5 μm, 4.1 × 150 mm Hamilton PRP-1 column eluted at 0.25 mL/min with a linear gradient that increased the proportion of acetonitrile in 0.1 M ammonium carbonate buffer from zero to 25% in 20 min followed by a ramp to 50% acetonitrile over the next 10 min [adducts **1** in context II(A) and **3** in context I(A), or a linear gradient of methanol in the same buffer that increased the proportion of methanol from zero to 100% in 50 min (adduct **1** in context I(A)]. Integration of the resulting chromatograms gave a limit of ≤0.1% S isomer in each sample. Chromatographic traces of these three oligonucleotides, showing the very low level of possible contamination by their S diastereomers, are given in the Supporting Information.

The configuration of the adduct in each oligonucleotide was established by enzymatic hydrolysis to nucleosides of the early eluting member of each diastereomeric pair of oligomers (*32*, *38*). After isolation of the monomeric adducts by HPLC (*32*), their absolute configurations were determined by comparison of their CD spectra with those of the known dAdo adducts from B[*c*]PhDE [positive at ~250 nm and negative at ~280 nm for the S adducts (*13*)].

Aliquots of the unsubstituted and the adducted oligonucleotides were further purified by electrophoresis on a denaturing 20% polyacrylamide gel (*30*, *32*). After detection by UV-light, gel slices containing the oligonucleotides were cut out. The oligonucleotides were extracted with an ammonium acetate and magnesium acetate solution (0.5 M and 10 mM, respectively) overnight and then adsorbed to reversed-phase Sep-Pak columns and recovered by elution with methanol in water (60%). After drying, the eluate was dissolved in water and the purity of the oligonucleotides determined on ^{32}P-end-labeled aliquots.

Construction of Modified Vector DNA and Mutagenesis Assay. Construction of modified vector DNA was as described (*30*, *32*). In brief, bacteriophage M13mp7L2 was linearized with *Eco*RI (2 units/μg of DNA at 30 °C for 2.5 h) and then purified by phenol extraction. Ligation of oligonucleotide sequences was principally as described by Lawrence and co-workers (*34*). However, a uracil-containing scaffold, in which the terminal sequences were complementary to the M13 ends, and the middle sequence to the oligonucleotide to be inserted, was used (*30*, *32*). In some cases, the scaffold (2 pmol) was annealed with the M13 (1 pmol), followed by annealing with the phosphorylated oligonucleotide (100 pmol). Most constructions, however, followed an alternative protocol where the scaffold was annealed with the phosphorylated oligonucleotide (50 pmol) followed by annealing with the M13. After ligation with T4 DNA ligase (30 units), the constructs were treated with uracil-DNA-glycosylase (1 unit) to generate abasic sites in the scaffold, thereby making it susceptible to cleavage by cellular endonucleases. The presence of adducts in the modified vectors was verified by primer extension reactions (*32*). Thereafter, control- or adduct-containing vector was transfected into competent ($CaCl_2$), SOS-induced (40 J/m^2 UV light) (*35*) *Escherichia coli* SMH77 cells (20 ng of DNA/

Table 2: Base Substitutions for B[*c*]PhDE Deoxyadenosine Adducts and Mutation Frequencies in Contexts I(A) and II(A)[a]

	sequence context							
	I(A) TTT**A**G**A**GTCTGCTCCC				II(A) CA**G**A**T**TTAGAGTCTGC			
	mutation no.				mutation no.			
adduct	A→T	A→G	A→C	MF_{tot}[b] (%)	A→T	A→G	A→C	MF_{tot}[b] (%)
1 trans B[*c*]PhDE-1/*R*	10	1	0	0.22 ± 0.13	20	32	4	0.38 ± 0.10
2 trans B[*c*]PhDE-2/*R*	3	2	0	0.05 ± 0.04	33	4	0	1.4 ± 0.46
3 cis B[*c*]PhDE-1/*R*	18	3	1	0.43 ± 0.18	29	22	0	8.2 ± 2.2
4 cis B[*c*]PhDE-2/*R*[c]	38	15	4	0.62 ± 0.16	39	41	2	5.6 ± 1.2
5 trans B[*c*]PhDE-1/*S*	137	11	6	24.0 ± 3.4	35	41	1	9.3 ± 2.0
6 trans B[*c*]PhDE-2/*S*	73	19	1	14.9 ± 2.8	31	11	1	1.9 ± 0.57
7 cis B[*c*]PhDE-1/*S*	88	15	4	9.8 ± 1.8	24	46	1	5.2 ± 1.2
8 cis B[*c*]PhDE-2/*S*[c]	39	67	2	31.9 ± 5.1	29	146	0	56.5 ± 5.6

[a] In the context I(A) control sequence 1 A→T mutation was found in 16 539 plaques screened and in the context II(A), 2 A→G mutations in 11 109 plaques. The background mutation frequency was thus less than 0.02%. The total numbers of plaques screened for context I(A): trans B[*c*]PhDE-1/*R* 5084; trans B[*c*]PhDE-2/*R* 9505; cis B[*c*]PhDE-1/*R* 5083; cis B[*c*]PhDE-2/*R* 9140; trans B[*c*]PhDE-1/*S* 643; trans B[*c*]PhDE-2/*S* 625; cis B[*c*]PhDE-1/*S* 1093; and cis B[*c*]PhDE-2/*S* 339 plaques. Total numbers of plaques screened for context II(A): trans B[*c*]PhDE-1/*R* 14 742; trans B[*c*]PhDE-2/*R* 2570; cis B[*c*]PhDE-1/*R* 620; cis B[*c*]PhDE-2/*R* 1466; trans B[*c*]PhDE-1/*S* 824; trans B[*c*]PhDE-2/*S* 2297; cis B[*c*]PhDE-1/*S* 1361; and cis B[*c*]PhDE-2/*S* 310 plaques. [b]MF_{tot} is total mutation frequency in percent ±2 × SE (for a 95% confidence interval) (*39*). [c]Data from ref *32*.

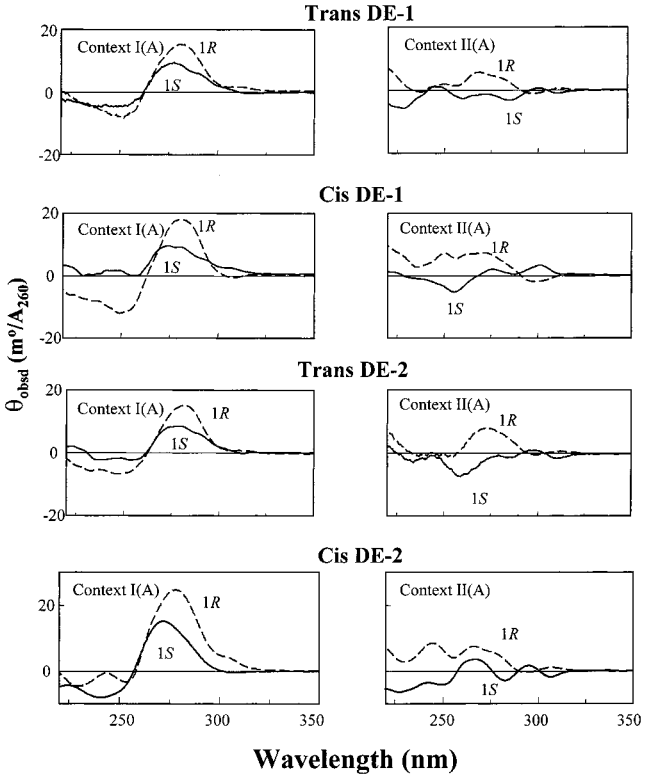

FIGURE 2: Circular dichroism spectra of the synthesized oligonucleotides containing cis and trans opened B[*c*]PhDE adducts in 0.02 M phosphate buffer, pH 7, with ionic strength adjusted to 0.1 M with NaCl. Spectra are normalized to 1.0 absorbance unit at 260 nm. Absolute configurations were assigned by enzymatic hydrolysis to the known nucleoside adducts (see text). Previously published spectra of the cis opened DE-2 adducts (*32*) are shown for comparison.

50 μL or 20 ng of DNA/100 μL), and the progeny were screened for mutations by differential hybridization of nitrocellulose filters containing the progeny M13 DNA, or by sequencing, all as described earlier (*30*, *32*).

RESULTS

Synthesis of the four oligonucleotides [context I(A) and context II(A), each containing a cis B[*c*]PhDE-2/*S* or cis B[*c*]-PhDE-2/*R*-dAdo adduct] and the determination of their mutagenicity in the system used here have been described (*32*). In the present study, we have prepared the remaining 12 oligonucleotides corresponding to the two diastereomeric trans B[*c*]PhDE-2 adducts as well as the cis and trans dAdo adducts derived from B[*c*]PhDE-1 in the same two sequence contexts (for structures, see Figure 1). The syntheses utilized standard phosphoramidite methodology with diastereomeric phosphoramidite mixtures, each corresponding to either cis or trans opening of either (+/−)-DE-1 or (+/−)-DE-2, to give (for a given sequence) a total of four pairs of diastereomeric oligonucleotides that were quite readily separated by HPLC (cf. Table 1).

Circular dichroism spectra of the complete set of oligonucleotides, along with the configurational assignment for each one, determined by enzymatic hydrolysis to nucleosides of the early eluting member of each diastereomer pair, are shown in Figure 2. Context I(A) oligonucleotides containing B[*c*]PhDE adducts with R configuration at the point of attachment of the base to the hydrocarbon consistently

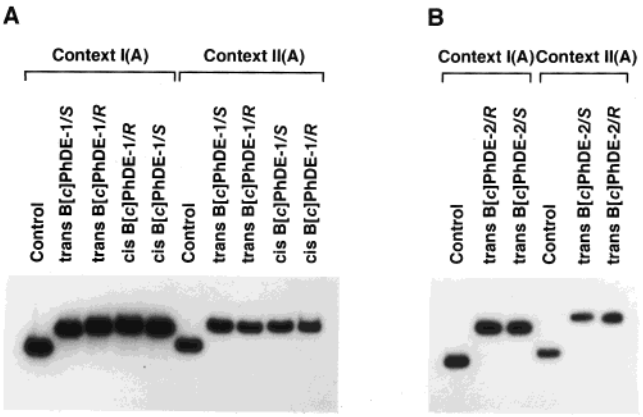

FIGURE 3: Polyacrylamide gel electrophoresis of end-labeled oligonucleotides after purification by HPLC and gel electrophoresis. (A) Trans and cis B[*c*]PhDE-1 adducts in contexts I(A) and II(A), as shown; (B) trans B[*c*]PhDE-2 adduct in contexts I(A) and II-(A), as shown. Controls are unsubstituted 16-mers.

exhibited a positive CD band at ~280 nm that was stronger and shifted slightly to longer wavelength relative to the corresponding bands of the S adducts. Although the CD signals for the adducted context II(A) oligonucleotides were quite weak throughout the wavelength range, context II(A) oligonucleotides with R adducts also showed positive CD signals in the 250−280 nm region, whereas the corresponding S adducts had much weaker and/or negative CD bands in this region. CD spectra are sensitive to changes in secondary structure of nucleic acids so the spectral differences between an adduct in context I(A) and the same adduct in context II(A) may reflect conformational differences that may also affect mutagenesis.

Upon HPLC analysis of the separated R- and S-adducted oligonucleotides under essentially the same conditions employed for their preparative separation, each oligonucleotide was chromatographically homogeneous. In cases where extremely low levels of contamination by the S diastereomer could significantly affect the results for the R diastereomer, rechromatography of the R-adducted oligonucleotides indicated that ≤0.1% of the S isomer was present.

The adducts were further purified by gel electrophoresis to remove any shorter, modified sequences that could result from failure of chain extension after coupling of the hydrocarbon adduct. After this step, the 12 new adducted oligonucleotides were shown to be free of such contaminating shorter sequences as well as unmodified oligonucleotide by gel electrophoresis of radiolabeled aliquots (Figure 3). The electrophoretic mobility of the adducted oligonucleotides was less than that of the unmodified control oligonucleotides, as expected.

The efficiency of ligation of oligonucleotides into the M13 bacteriophage vector ranged from 22 to 44% (estimated from the fraction of total DNA in the ligation reaction that was circular). The ligation efficiency for adducted oligonucleotides ranged from 73 to 88% of that for the corresponding unsubstituted controls. Very minor differences of ligation efficiencies were seen between the R and S adducted constructs (see Supporting Information for ligation efficiencies in each construct).

The presence of adduct in an M13 construct consistently resulted in lower yields of M13 progeny upon transfection into SOS-induced *E. coli* than found with unadducted

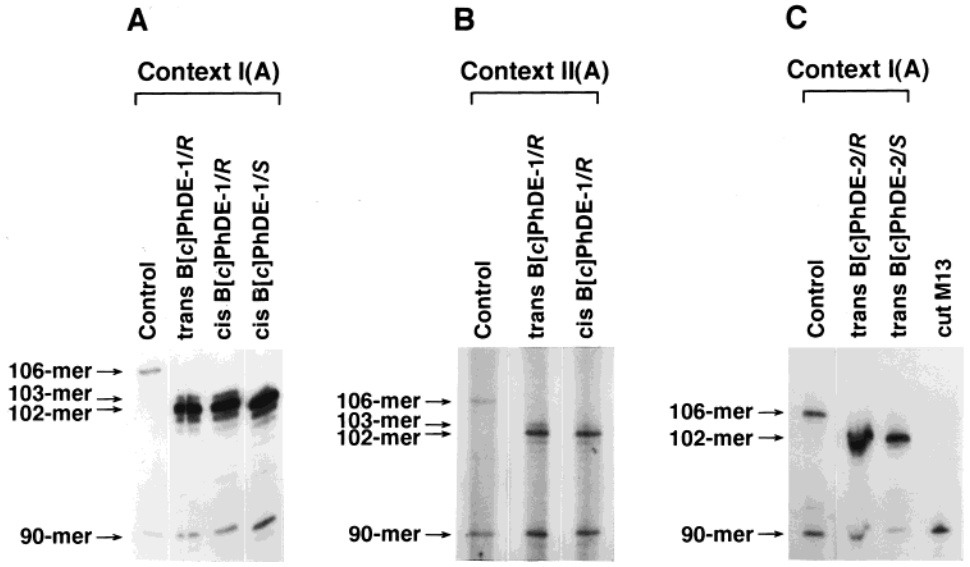

FIGURE 4: Examples of polyacrylamide gel electrophoresis of [α-^{32}P]dATP-labeled primer extension products from reaction with M13 constructs and Sequenase. (A and B) Constructs with the trans and cis B[*c*]PhDE-1 adducts in contexts I(A) and II(A), as shown. (C) Constructs with the trans B[*c*]PhDE-2 adducts in context I(A) and cut M13 (90-mer control). Controls are constructs with unsubstituted insert oligonucleotides. The cis B[*c*]PhDE-1/*S* in panel A, cis B[*c*]PhDE-1/*R* in panel B, and trans B[*c*]PhDE-2/*S* in panel C are from a constructs giving a high mutation frequency (8−15%) shown as positive controls.

oligonucleotides. Progeny yields varied for different adducts and ranged from 11 to 50.5% of the plaque yields found with the corresponding control constructs (see Supporting Information for survival data for each experiment). In general, low plaque yields coincided with high mutation frequencies and vice versa.

Table 2 summarizes the mutation data collected for the 12 new oligonucleotide constructs and includes, for comparison, data obtained earlier for the cis B[*c*]PhDE-2/*S* and B[*c*]PhDE-2/*R* adducts (see Supporting Information for the number of mutants and the mutation frequencies in each experiment). For several constructs, mutation frequencies were low (<1%) whereas for several others, very substantial frequencies were obtained. Although it is reasonable to expect that mutation frequencies could vary substantially with specific adduct structure and sequence context, it was important to establish that adduct was truly present in the constructs that gave particularly low mutation frequencies.

For this reason, most of the constructs were used as templates in a primer extension assay using Sequenase 2.0 in the presence of [α-^{32}P]dATP (*32*). The primer used was such that its extension on the cut M13 template yielded a 90-mer product. In constructs derived from unsubstituted control oligonucleotides, both the 90-mer and a 106-mer primer extension product were obtained as a result of some ligation failure at each end of the oligonucleotide insert (Figure 4). In adduct-containing constructs, the primer extension products were 90-mer and 102-mer (elongation to one base before the adduct site) oligonucleotides. In some cases, insertion of a base opposite the adduct site was also noted (103-mer, Figure 4). However, in concert with earlier studies (*32*, *40*), the polymerase was unable to extend the primer beyond the adduct site. The presence of products resulting from termination by the adduct together with the absence of 106-mers in all the adducted constructs indicated that adduct was present in all the constructs, even in those that gave low mutation frequencies.

Overall Mutation Frequencies. In most cases, the overall mutation frequencies for adducts with S configuration (see Figure 1 for structures) were greater, sometimes dramatically so, than those for adducts with R configuration. For adducts with S configuration, mutation frequency ranged from 1.9 to 56.5% (Table 2), whereas the corresponding adducts with R configuration exhibited frequencies of 0.05−8.2%. In the case of the trans B[*c*]PhDE-2 adducts in context I(A), the adduct with S configuration was ∼300-fold more mutagenic than the corresponding R adduct. In only one case did the R adduct give a higher mutation frequency than its corresponding S adduct [in context II(A), cis B[*c*]PhDE-1/*R*, 8.2%, and cis B[*c*]PhDE-1/*S*, 5.2%, Table 2]. These findings indicate that, for the B[*c*]Ph-dAdo adducts, the configuration at the C_1 carbon substituted by dAdo is a major determinant of mutagenic activity.

The majority of mutants recovered came from replication of the constructs that contained adducts with S configuration. Within this set of constructs, cis opened DE-2 adducts were substantially more mutagenic than trans opened DE-2 adducts, particularly in context II(A) (∼30-fold). However, cis opened DE-1 adducts were somewhat less active than trans opened DE-1 adducts. Cis opened adducts derived from DE-2 gave much higher mutation frequencies than cis opened adducts derived from DE-1, whereas for trans opened adducts, DE-1 adducts were somewhat more mutagenic than DE-2 adducts.

Sequence context effects on overall mutagenicity of the B[*c*]PhDE adducts (cf. ref *32*) were also observed. For most adducts with S configuration, mutation frequency was higher in context I(A) than in context II(A) (0.6−8-fold), whereas the mutation frequencies found for adducts with R configuration suggested that the converse applied to them [i.e., ∼2−28-fold greater activity in II(A) than in I(A)].

Frequencies of Individual Base Substitutions. A summary of the frequencies for individual base substitutions is presented in Figure 5. These data again illustrate the differences in mutagenic activity for adducts with S and R

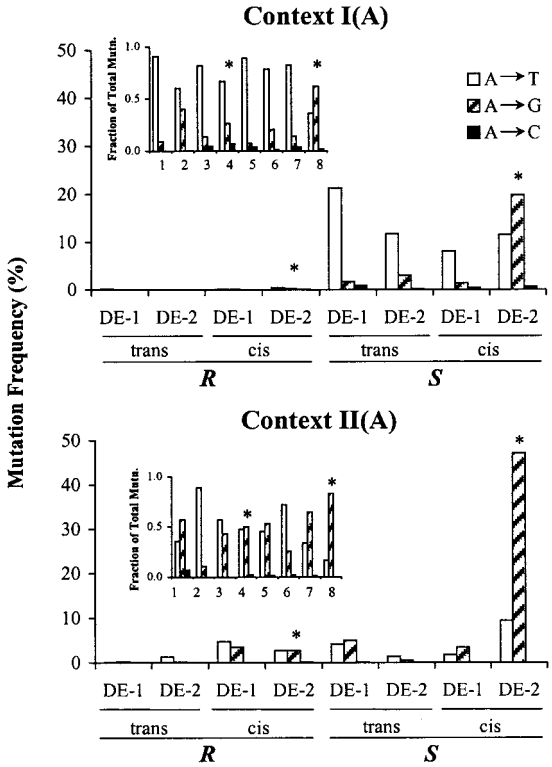

FIGURE 5: Frequencies of base substitution mutations in contexts I(A) and II(A) for dAdo adducts resulting from trans or cis opening of B[*c*]PhDE-1 and B[*c*]PhDE-2. Inserts in these figures show the distribution of each type of base substitution as a fraction of total mutations. B[*c*]PhDE-1 and B[*c*]PhDE-2 dAdo adduct structures (**1**−**8**) are shown in Figure 1. (*) Data taken from Pontén et al. (*32*).

configurations and show that they are particularly dramatic in the context I(A) environment. Furthermore, this figure shows that sequence context has a major influence on the type of base substitution mutation that is preferred. Generally, context I(A) seems to favor A→T mutation whereas context II(A) favors A→G mutation. However, in certain cases, adduct structure overrides this effect. Thus, the constructs containing the trans B[*c*]PhDE-2/*S* adduct and the cis B[*c*]-PhDE-2/*S* adduct preferentially give A→T and A→G mutations, respectively, regardless of sequence context.

In summary, the interplay of several factors is involved in determining the mutagenic properties of the carcinogen−deoxyribonucleoside adducts examined in these investigations. Configuration at the site of carcinogen−nucleoside bonding had a substantial effect on frequencies of mutation, and this was more apparent in context I(A) than in context II(A). Another major observation concerns the effect of sequence context on the types of base substitution mutation recovered. The two contexts studied differ in that context II(A) favors A→G mutations whereas context I(A) favors A→T mutations even though structural effects override this tendency in some cases. Other effects on mutation frequency depend on structural features such as whether the adduct has arisen by cis or trans opening of the epoxide ring or whether it is derived from DE-1 or DE-2.

DISCUSSION

The present report describes the mutagenic activities in the single-stranded M13mp7L2 system (*34*) of six B[*c*]PhDE-dAdo adducts in the two sequence environments, context I(A) and context II(A) (Figure 1), and complements our previous report on two cis opened dAdo adducts derived from B[*c*]-PhDE-2 (*32*). We have previously determined the mutagenic activities in the same system for the eight dAdo adducts derived from both cis and trans opening of the four isomeric B[*a*]PDEs [(+)- and (−)-DE-1 and DE-2] in the same two sequences (*30*, *33*) (Table 3). Together, these reports allow comparisons of the mutagenic activities of all known diol epoxide-dAdo adducts from both B[*c*]Ph and B[*a*]P in two sequence contexts. In their reactions with DNA, the diol epoxides derived from these two hydrocarbons represent opposite ends of the range of observed base selectivities (*15*). For example, the *R*,*S*-diol *S*,*R*-epoxide of B[*a*]P reacts almost exclusively (95%) with guanine in DNA (*41*,*42*), whereas the corresponding B[*c*]Ph metabolite reacts more extensively (66%) with adenine (*9*, *10*, *13*), and its extent of DNA adduct formation relative to hydrolysis to tetraols is much greater than that of the corresponding B[*a*]PDE. Although the combined data set for the two hydrocarbons in two sequence contexts is substantial, it nevertheless represents a relatively narrow sampling of all possible variations in structure and sequence context. However, within this data set, some significant observations relating to the effects of sequence context, hydrocarbon residue, and adduct stereochemistry on both total and individual base substitution mutation frequencies have emerged.

Table 3: Total Mutation Frequency (%) for B[*a*]PDE-dAdo Adducts[a]

adduct	context I(A)	context II(A)
1 trans B[*a*]PDE-1/10*R*	37	8.0
2 trans B[*a*]PDE-2/10*R*	58	19.5
3 cis B[*a*]PDE-1/10*R*	68.2	24.2
4 cis B[*a*]PDE-2/10*R*	49.3	9.5
5 trans B[*a*]PDE-1/10*S*	44.4	10.7
6 trans B[*a*]PDE-2/10*S*	50.7	5.6
7 cis B[*a*]PDE-1/10*S*	28.1	13.0
8 cis B[*a*]PDE-2/10*S*	26.5	17.0

[a] Data published previously (*30*, *33*).

Effects of Sequence Context. For each of the eight B[*a*]P-derived adducts (*30*, *33*), total mutation frequency was higher in context I(A) than in context II(A). For the B[*c*]PhDE adducts, all four adducts with R configuration were particularly poor mutagens in context I(A), and consequently, for five of the eight adducts studied, mutation frequencies were greater in context II(A) than in I(A). For the B[*a*]P adducts, A→T transversion frequencies were higher in context I(A) (∼19−62% of total progeny; 71−92% of observed mutations) than in context II(A) (∼3−19% of total progeny; 31−78% of observed mutations), and A→G transition frequencies for each adduct were higher in context II(A) (∼2−8.5% of total progeny; 13−65% of observed mutations) than in context I(A) (∼1−3.5% of total progeny; 2−8% of observed mutations). Sequence context effects were similar for the B[*c*]-PhDE-derived adducts to the extent that for most (seven of eight) of these adducts, A→G transition frequencies were greater in context II(A) (∼0.1−47% of total progeny; 10−83% of observed mutations) than in context I(A) (∼0.02−20% of total progeny; 5−62% of observed mutations). Thus, the most consistent sequence effect observed is that context II(A) seems to favor A→G mutation more than context I(A). A more limited observation is that for all adducts with S

configuration at the site of nucleoside attachment, A→T mutation frequencies are greater in context I(A) than in context II(A).

Effects of Hydrocarbon Residue. In either sequence context, the mutagenic activity of the cis B[*c*]PhDE-2/*S* adduct far exceeded that of any other B[*c*]PhDE adduct. This particular adduct was the only B[*c*]PhDE adduct that exhibited greater mutagenic activity than the corresponding B[*a*]PDE adduct. All of the other B[*a*]PDE-derived adducts were more active than their B[*c*]PhDE-derived counterparts, particularly in context I(A). In this sequence, all four B[*c*]-PhDE adducts with 1R configuration were extremely weak mutagens; in several cases, >100-fold less active than the corresponding 10R B[*a*]PDE adducts.

Of the three possible base substitutions, A→C transversions were relatively rare and were the least frequently observed substitution mutations for B[*c*]PhDE adducts in context I(A), as well as for adducts from both hydrocarbons in context II(A). The B[*c*]PhDE adducts generally gave a higher percentage of A→G transitions than their B[*a*]PDE counterparts in both sequence contexts. As previously noted, A→G mutations are also favored by context II(A) in comparison with context I(A). As a result of these two factors, the B[*c*]PhDE adducts in context II(A) generally gave the highest overall percentage of A→G transitions (as high as 83% of total mutations in the case of cis B[*c*]PhDE-2/*S*), whereas the B[*a*]PDE adducts in context I(A) gave the lowest percentage of these mutations.

In summary, the mutagenicity of the B[*c*]PhDE adducts in the present system tends to be lower than that of the B[*a*]-PDE adducts and is much more sensitive to the stereochemistry of the adduct. Most notably, the huge difference in frequencies for R versus S adducts in context I(A) for the B[*c*]PhDE adducts described herein was not observed for the B[*a*]PDE adducts.

Adduct Structure: Effect of Configuration at the Carbon Bonded to the Purine. Within this series of DE adducts, it is possible to analyze the effects of inverting the configuration at C_1 of the B[*c*]Ph moiety or C_{10} of the B[*a*]P moiety, either with or without concomitant inversion at one or more of the other three chiral carbon atoms in the cyclohexene ring.

The mutagenicities of adducts that *differ only in configuration at the carbon bonded to the purine*, i.e., C_1 for B[*c*]-PhDE adducts and C_{10} for B[*a*]PDE adducts, can be compared by relating the activities of cis DE-1/*S* (**7**) with trans DE-1/*R* (**1**), of cis DE-2/*S* (**8**) with trans DE-2/*R* (**2**), of trans DE-1/*S* (**5**) with cis DE-1/*R* (**3**), and of trans DE-2/*S* (**6**) with cis DE-2/*R* (**4**) (see Figure 1). In such comparisons, B[*a*]PDE adducts differ at most by a factor of ~2, with the R configuration generally being more active than the S configuration. For B[*c*]PhDE adducts, because of the low mutagenic activities of the R adducts, very large differences in activity (up to ~600-fold) are observed in context I(A), with the mutagenic activity being markedly lower for adducts with the R configuration. Cis opened adducts often display higher mutagenicity than trans opened adducts (see below). However, in context I(A), the trans 1S adducts **5** and **6** are much more mutagenic than the cis 1R adducts **3** and **4**. Thus, in these cases, S configuration at the site of attachment of the purine rather than cis orientation of the purine and its vicinal hydroxyl group seems to be the dominant influence on the mutagenicity.

The effect of *inversion of configuration at all four chiral carbon atoms* is examined by comparison of the mutagenic activity of a given S adduct with that of the corresponding R adduct [e.g., trans DE-1/*S* (**5**) versus trans DE-1/*R* (**1**), etc.; cf. Figure 1]. For B[*a*]PDE adducts, relatively minor (~3-fold) differences result from this inversion of configuration, whereas for B[*c*]PhDE adducts, huge differences (up to ~300-fold) are observed in context I(A), and largely reflect the low mutagenicity of the 1R relative to the 1S adducts. Some substantial differences for B[*c*]PhDE adducts are also found in the context II(A) environment, with adducts with S configuration at C_1 being more mutagenic than those with R configuration.

Somewhat similar conclusions arise if the effect of *inversion at both the carbon atom bonded to the nucleoside (C_1 or C_{10}) and its vicinal carbon atom (C_2 or C_9)* is examined by comparing activities for trans DE-2/*S* (**6**) with trans DE-1/*R* (**1**), etc. Again, the B[*a*]PDE adducts were not particularly sensitive to these structural changes, whereas most B[*c*]PhDE/*R* adducts were much less mutagenic than their B[*c*]PhDE/*S* counterparts in both sequence contexts. *In summary, comparisons of structures that include changes in configuration at the point of attachment of the hydrocarbon to the purine are largely dominated by the effects at this chiral center.*

Adduct Structure: Effects of Configuration at Carbons Not Bonded to the Purine. Other effects of adduct structure can be probed by pairwise comparisons of adducts with the same configuration at the site of attachment to the purine and inversions of configuration at other chiral centers. For example, a given (cis or trans) DE-2/*S* adduct differs from the corresponding DE-1/*S* adduct (or DE-2/*R* from DE-1/*R*) only in that the two hydroxyl groups of the parent trans dihydrodiol are inverted. Thus, the effect on mutagenicity of the presence of 3*S*,4*R*-hydroxyl groups versus 3*R*,4*S*-hydroxyl groups can be assessed by comparing data obtained for **4** with **3**, **7** with **8**, **1** with **2**, as well as **6** with **5** (see Figure 1 for structures). Comparison of such adduct pairs indicates that the configuration of these hydroxyl groups has only a modest effect on mutagenicity for most such B[*c*]-PhDE and B[*a*]PDE adduct pairs. The effects were generally greater in context II(A) than in context I(A).

Pairs of structures that differ only in the configuration of the hydroxyl group vicinal to the purine substituent are **3** and **2**, **6** and **7**, **4** and **1**, and **5** and **8** (Figure 1). For B[*a*]-PDE adducts, the configuration of this hydroxyl group at C_9 had little effect on mutagenicity. For B[*c*]PhDE adducts, effects were more pronounced. For adducts with R configuration at C_1, the R configuration at C_2 (cis) increased the mutation frequencies relative to the S configuration at C_2 (trans) by ~5−14-fold. For the 1S adducts, the effects of configuration at C_2 were somewhat smaller. Since each pair of adducts compared consists of one cis and one trans opened adduct, these observed effects could result either from the absolute configuration of the vicinal hydroxyl group or from the cis versus trans orientation of this hydroxyl group relative to the purine.

Comparisons of mutation data for adducts **5** with **7**, **6** with **8**, **1** with **3**, or **2** with **4** (Figure 1) illustrate the effect of inverting the configurations of all three hydroxyl groups. For B[*a*]PDE adducts, the effects of such transformations on mutagenicity were modest (up to 3-fold). For B[*c*]PhDE

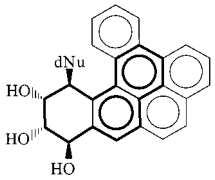

FIGURE 6: Differences in adduct structure between B[*a*]PDE and B[*c*]PhDE adducts. Common structural features are bold. The B[*a*]-PDE adducts additionally contain the naphthalene ring system (indicated in lighter type) on the right side of the figure. The B[*c*]-PhDE adducts consist of the bold structure together with the benzene ring system shown (in lighter type) at the top of the figure.

adducts, some more substantial effects were noted (up to ~30-fold), and configurations that corresponded to cis opened adducts gave the greatest activity.

Overall, variations in configuration at the chiral carbons of the hydrocarbon moiety that are not involved in bonding to the nucleoside result in changes in mutagenicity that are larger for the B[*c*]PhDE adducts than for the B[*a*]PDE adducts. Furthermore, the largest differences in activity observed for B[*c*]PhDE adducts coincide with configurational changes that switch between trans and cis opened adducts, with higher mutagenicity generally being associated with the cis opened adducts. Earlier studies of trans and cis B[*a*]PDE adducts (*25*, *31*) have also reported greater mutagenic activities for cis adducts. Interestingly, studies of DNA repair of B[*a*]PDE adducts have indicated that cis adducts are more readily repaired than the corresponding trans adducts (*43*, *44*).

Our eventual goal is to understand the structural basis for the pronounced effect on mutagenicity of configuration at the carbon atom bonded to the nucleoside that we have observed here for the first time with B[*c*]PhDE (but not B[*a*]-PDE) adducts. As shown in Figure 6, both of these hydrocarbons share some common structural features (heavy lines). In addition to this structural overlay, the B[*a*]PDE adducts contain a naphthalene ring system well removed from the bond joining the hydrocarbon residue to the purine ring, whereas the B[*c*]PhDE adducts contain a benzo ring that is close to this bond. This ring forms a hindered fjord region which results in conformational differences between the cyclohexene rings of the B[*c*]Ph and B[*a*]P adducts. For example, the four cis and trans opened, DE-1 and DE-2 dAdo adducts (as the nucleoside pentaacetates) derived from B[*c*]-Ph prefer more boatlike conformations than their B[*a*]P counterparts, as indicated by NMR coupling constants (*13*, *45*). In double-stranded DNA, NMR solution structures (*46*) for trans opened *R*-dAdo adducts derived from B[*a*]P (*47*, *48*) and B[*c*]Ph (*49*) diol epoxides are similar in overall characteristics; namely, the aromatic portion of the hydrocarbon is intercalated toward the 5′-end of the adducted strand, the glycosidic torsion angle for the adducted dAdo is anti, and nearly normal hydrogen bonding is maintained between the modified adenine and its complementary thymine. On the other hand, *S*-dAdo adducts derived from the two hydrocarbons differ significantly from each other. For example, it has been possible to determine solution structures for oligonucleotides containing both trans opened 1*S* (*50*)- and 1*R* (*49*)-dAdo adducts of B[*c*]PhDE-2 with a normal, complementary T. In contrast, oligonucleotides with the corresponding trans opened 10*S*-dAdo adducts from B[*a*]-PDE-2 have not been amenable to NMR structure determination because of conformational heterogeneity (*51*). Notably also, the T_m values of duplex oligonucleotides containing 10*S*-dAdo adducts of B[*a*]PDEs are markedly depressed relative to their 10R counterparts (*47*, *51*), whereas oligonucleotides containing 1*R*- and 1*S*-dAdo adducts of B[*c*]-PhDE-2 exhibit identical melting points (*52*). These observations all suggest that the 10*S*-B[*a*]P-dAdo adducts exhibit a poor fit into duplex DNA, with resultant structural disorder at the adduct site. Although it is difficult to extrapolate data from double-stranded DNA to the structure at a replication fork, our most reasonable prediction a priori was that B[*a*]-PDE/*S*-dAdo adducts might show corresponding abnormalities in their mutagenicity (either very high or very low) when compared to the B[*a*]PDE/*R*-dAdo adducts and/or to both configurations of the B[*c*]PhDE-dAdo adducts. Instead, the adducts that are apparently anomalous because of their extremely low mutagenicity are B[*c*]PhDE/*R* adducts. On the basis of the above criteria, these adducts were not expected to behave atypically. An unusual conformation with a pseudoequatorial purine substituent has been reported (*49*) for the cyclohexene ring of the trans opened, B[*c*]PhDE-2/*R* dAdo adduct in an oligonucleotide duplex (sequence environment ~C<u>A</u>C~). This conformation differs from that of the B[*c*]PhDE-2/*S* counterpart in the same sequence (*50*). The generality of this unusual conformation, as well as its possible relevance to biological activity, remains to be established for other B[*c*]PhDE/*R* adducts and other sequence contexts.

In summary, the mutagenic activity of B[*c*]PhDE adducts is much more dependent on structural variables than the mutagenic activity of B[*a*]PDE adducts. Consequently, the spread of mutation frequencies among the adducts derived from the eight isomeric B[*a*]PDEs was less than a factor of 5 in both sequence contexts, whereas large differences among these isomers were observed for the B[*c*]PhDE adducts. For the B[*c*]PhDE adducts, the configuration at the site of nucleoside attachment has profound effects on mutagenic activity. The B[*a*]PDE adducts tend to be more mutagenic than B[*c*]PhDE adducts, particularly in context I(A). The B[*c*]-PhDE adducts induce a larger fraction of A→G transitions than the B[*a*]PDE adducts. For both sets of hydrocarbon adducts, A→T base substitutions are more favored in context I(A) than in context II(A), whereas A→G mutations are more favored in context II(A). Although some trends are emerging from these investigations, it will be necessary to resolve fully the effects of individual variables, such as sequence context, before the complex effects of the structural variables can be understood.

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SUPPORTING INFORMATION AVAILABLE

Typical HPLC traces of purified, adducted oligonucleotides, as well as tables listing mutations, survival and ligation efficiencies in the various experiments. This material

is available free of charge via the Internet at http://pubs.acs.org.

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