seems unlikely that the protonation of a single acidic group is responsible for the observed intensity variations. As previously suggested by Burger and co-workers,[25] a conformational change triggered, at least in part, by the protonation of acidic groups alters the Trp environment enough to vary fluorescence, resonance Raman, and UV absorption properties.

Conclusions

The 222-nm resonance Raman data for bGH, pGH, and hGH at pH 8 show strong enhancement of the aromatic residues Phe, Trp, and Tyr. Pro is also enhanced. The pH-dependent variations of the Trp Raman vibrational cross sections in native bGH and pGH, compared to the analogous vibrations in native hGH, indicate the presence of a different Trp environment in native hGH. The pH dependence of Trp vibrational intensity changes in bGH and pGH correlates to fluorescence emission, suggesting the same protein group or molecular event is responsible for both effects.

Acknowledgment. We thank Scott Plaisted for supplying the carboxyl-terminal fragment from bGH and Paul Elzinga for performing the fluorescence lifetime experiments. We acknowledge Dr. Neil MacKenzie for helpful discussions.

Structures of Covalent Nucleoside Adducts Formed from Adenine, Guanine, and Cytosine Bases of DNA and the Optically Active Bay-Region 3,4-Diol 1,2-Epoxides of Dibenz[*a*,*j*]anthracene

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Abstract: Chemical structures of the principal adducts formed from DNA upon reaction in vitro with the four optically active 3,4-diol 1,2-epoxides derived from the *trans*-3,4-dihydrodiol enantiomers of dibenz[*a*,*j*]anthracene have been elucidated at the nucleoside level. In addition to the structures of deoxyadenosine (dA) and deoxyguanosine (dG) adducts, complete chemical characterization of a deoxycytidine (dC) adduct formed from a diol epoxide is reported for the first time. The site of covalent attachment is between the benzylic C-1 of the diol epoxide moiety and the exocyclic amino group of the base in all of these adducts, as deduced by chemical stability considerations and pH titration (pK_a = 2.1 and 9.3 of a dG adduct, pK_a = 2.2 of a dA adduct, and pK_a = 2.6 of a dC adduct). Upon acetylation, these adducts gave pentaacetates, as determined by their mass spectra. Since the purine or pyrimidine substituent must be pseudoaxial, conformational flexibility of the tetrahydro benzo ring is limited. Thus, coupling constants between methine protons on this ring of the adducts (as their pentaacetates), as well as effects of the purine on the chemical shift of the C-3 proton, when these two substituents are cis and pseudodiaxial, are diagnostic of the relative stereochemistry of the adducts (cis vs trans opening of the epoxide). All the dibenz[*a*,*j*]anthracene adducts which have *S* absolute configuration at the benzylic C-1 carbon of the tetrahydro aromatic moiety exhibit CD spectra with a positive band at 270–280 nm and a negative band at 290–300 nm. Thus, assignment of cis vs trans addition for minor adducts whose NMR spectra were unobtainable due to their low level of formation could be made on the basis of their CD spectra and the known absolute configurations of the parent diol epoxides.

Covalent modification of DNA by the chemically reactive bay-region diol epoxides, which are formed upon metabolic activation of polycyclic aromatic hydrocarbons,[1] presumably constitutes the first step in the tumorigenic and mutagenic processes initiated by these metabolites.[2] Covalent adduct formation upon reaction of nucleic acids with diol epoxides is well documented, particularly in the case of the benzo[*a*]pyrene (BaP) diol epoxides,[3] but definitive chemical characterization of many of these adducts (notably those derived from DNA) is unavailable. Recently, we reported complete spectroscopic characterization of 16 adducts formed upon cis and trans addition of the exocyclic nitrogen of purine residues in DNA to the benzylic C-1 position of the four optically active 3,4-diol 1,2-epoxides derived from benzo[*c*]-phenanthrene (BcPh).[4] In the present study, we describe the structural characterization of covalent nucleoside adducts[5] formed upon reaction of DNA with the four configurationally isomeric bay-region diol epoxides of dibenz[*a*,*j*]anthracene, whose structures are shown in Figure 1.

Dibenz[*a*,*j*]anthracene (DBA), one of the three possible isomeric dibenzanthracenes, is less carcinogenic than the dibenz[*a*,*h*]-anthracene isomer.[2] The bay-region theory[6] predicts that the

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(5) For brevity, the deoxyguanosine, deoxyadenosine, and deoxycytidine adducts are designated as dG, dA, and dC, respectively. The subscripts c and t are used to represent adducts derived from addition of the exocyclic amino group of DNA bases at the benzylic C-1 position by either cis or trans opening of the epoxide, respectively.

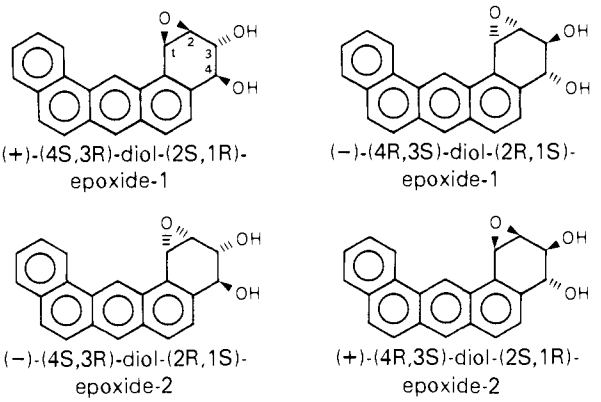

Figure 1. Enantiomers of the diastereomeric DBA diol epoxides. The diastereomers in which the benzylic C-4 hydroxyl group is cis to the epoxide oxygen are designated as diol epoxide-1, and the diastereomers in which these groups are trans are designated as diol epoxide-2. Isomers on the right half of the figure are derived from the metabolically predominant *trans*-(3*R*,4*R*)-dihydrodiol.

bay-region 3,4-diol 1,2-epoxides metabolically generated from dibenz[*a,j*]anthracene via the *trans*-3,4-dihydrodiol will be ultimate carcinogens. In the presence of calf thymus DNA in vitro, the optically active DNA diol epoxides undergo a combination of DNA-catalyzed hydration to tetraols (as previously observed with other diol epoxides[7,8]) and covalent addition to DNA.[9,10] The efficiency of covalent addition relative to hydrolysis is comparable to that observed with the BaP diol epoxides;[9,10] i.e., at a DNA concentration of ca. 1 mg/mL, 3–10% of each optically active diol epoxide is converted to adducts. A material balance[10] indicated that the preponderance of the UV absorption derived from the diol epoxides and not accounted for as adducts was recovered upon extraction of the solution of modified DNA with organic solvents prior to enzymatic hydrolysis: 88% in the case of racemic diol epoxide-1 and 90% in the case of racemic diol epoxide-2.[11] The (4*R*,3*S*)-diol (2*S*,1*R*)-epoxide-2 isomer of DBA is ca. 3 times more efficient in its binding to DNA than the other optically active isomers. In the cases of those diol epoxides for which tumor data are available, this is generally the most tumorigenic optically active isomer.[12] The DBA diol epoxides exhibit a preference (60–80% of total adducts) for adduct formation with deoxyguanosine (dG) residues. This pattern of reactivity resembles that observed for diol epoxides of BaP rather than for BcPh diol epoxides, which preferentially yield deoxyadenosine (dA) adducts.[4,13] The present study reports the structural characterization of the 16 possible cis and trans dA and dG adducts formed upon reaction of the exocyclic amino groups of these nucleoside residues in DNA with the four configurationally isomeric bay-region diol epoxides of dibenz[*a,j*]anthracene. Although adducts arising from deoxycytidine (dC) have been tentatively identified in the case of BaP[3a,b] and BcPh,[13] isolation and conclusive identification of a dC adduct from a diol epoxide was possible for the first time in the case of (−)-diol epoxide-2 of DBA.

Experimental Section

Calf thymus DNA (Sigma Chemical Co.) was dialyzed for 16 h against 10 mM Tris-HCl buffer (pH 7.0–7.4) before use. 2′-Deoxyguanosine 5′-monophosphate, 2′-deoxyadenosine 5′-monophosphate, and 2′-deoxycytidine 5′-monophosphate were obtained as sodium salts from Sigma Chemical Co. and used as such. *Escherichia coli* alkaline phosphatase, type III, was obtained from Sigma as a suspension in 2.5 M ammonium sulfate. Optically active and racemic dibenz[*a,j*]anthracene 3,4-diol 1,2-epoxides[14] were prepared as described.

Proton NMR spectra were measured at 300 MHz in acetone-d_6 (Varian XL-300). Chemical ionization (NH_3 gas) mass spectra were measured on a Finnigan MAT 4500 quadrupole mass spectrometer with a direct exposure probe. Circular dichroism (CD) spectra were measured on a JASCO Model J500A spectropolarimeter equipped with a data processing system for signal averaging. CD spectra of the adducts in methanol were normalized to 1.0 absorbance unit at 290 nm. UV spectra of chromatographic eluates were monitored on-line by use of a liquid chromatograph equipped with a Hewlett-Packard 1040A diode array detector. Titration curves[15] for unacetylated adducts (−)-DE1/dG_c, (−)-DE2/dA_t, and (−)-DE2/dC_t were determined at 25 °C by measurement of the CD spectra upon addition of aliquots of a stock solution of each adduct to solutions whose pHs were maintained with either HCl (1–100 mM), NaOH (10 mM), or buffers (5 mM). The final composition of each mixture was 1:9 methanol/water. Reversibility of the spectral changes observed at the pH extrema (1.1 and 11.8) was verified by adjusting the pH of these solutions to 5–8 and redetermining the CD spectra. These spectra were found to be identical with those measured directly in buffer solutions near neutrality. The pK_a values were determined from plots of θ_{obs} vs pH at 279 nm for (−)-DE1/dG_c and 293 nm for (−)-DE2/dA_t and (−)-DE2/dC_t.

Analytical HPLC of products (as previously described[10]) was carried out on a Du Pont Zorbax ODS column, 4.6 × 250 mm, eluted at 1.0 mL/min as follows: (A) for diol epoxide-1 adducts, a linear gradient of acetonitrile/methanol/water from 25:5:70 (at time zero) to 30:6:64 (at 15 min) followed by a linear gradient up to 50:15:35 acetonitrile/methanol/water (at 30 min); (B) for diol epoxide-2 adducts, a linear gradient of methanol/water from 60:40 to 80:20 in 20 min. Chromatographic eluates were monitored at 290 nm.

Quantities of adducts suitable for structural studies were prepared via reaction of the racemic diol epoxides (4–5 mg in 1 mL of a 10–20% solution of DMSO in THF) and the appropriate deoxynucleoside 5′-monophosphate (20–50 mg/mL in 20–100 mL of Tris-HCl buffer, pH 7), except for the diol epoxide-2 adducts of deoxyguanosine, which were prepared from the diol epoxide (5 mg) and DNA (39 mg) in 50 mL of the above buffer containing 5 mL of acetone. Details of the experimental procedures, including binding to calf thymus DNA, enzymatic hydrolyses to the nucleoside level, and the HPLC conditions used to isolate individual adducts are described elsewhere.[10]

The dC adduct of (−)-diol epoxide-2 was isolated from the reaction of 1 g of 2′-deoxycytidine 5′-monophosphate (disodium salt) dissolved in 10 mL Tris-HCl buffer (50 mM, pH 7) and 1.1 mg of (−)-diol epoxide-2 dissolved in 75 μL of freshly distilled THF containing 25 μL of DMSO. After incubation at 37 °C overnight, the reaction mixture was extracted five times with ethyl acetate (10 mL) and three times with diethyl ether (10 mL). The aqueous portion was freed from organic solvent by purging with nitrogen. All hydrocarbon-containing products were then adsorbed on a C_{18} Sep-Pak (Waters Associates). The Sep-Pak was eluted with methanol, and the methanol solution was evaporated to dryness, after which 10 mL of Tris-HCl buffer (50 mM, pH 9) and 20 units of *E. coli* alkaline phosphatase were added to the residue. Enzymatic digestion was allowed to proceed for 24 h at 37 °C. Following this, the reaction mixture was passed through a C_{18} Sep-Pak, and the adducts were finally eluted with methanol. The methanol extract was subjected to chromatography on a Du Pont Zorbax phenyl column (4.6 × 250 mm), eluted with 20:30:50 acetonitrile/methanol/water at 1 mL/min. The major

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Table I. Covalent Binding Efficiency, Adduct Distribution, and Retention Times of the dA, dG, and dC Adducts of Bay-Region Diol Epoxides of Dibenz[*a*,*j*]anthracene[a]

diol epoxide enantiomer[b]	% bound	adduct distribution (%) (rt)[c]				
		dG		dC	dA	
		cis	trans	trans	cis	trans
(−)-(*R*,*S*,*R*,*S*)-DE1	3–4	30–33 (16.8)	32–33 (14.3)		31–34 (24.2)	4–5 (22.9)
(+)-(*S*,*R*,*S*,*R*)-DE1	3–4	45–52 (15.4)	18–24 (16.1)		5–10 (24.9)	21–25 (22.2)
(−)-(*S*,*R*,*R*,*S*)-DE2	~3	4–5 (11.1)	57–64 (9.0)	12–14 (10.4)	5–6 (16.9)	15–18 (15.5)
(+)-(*R*,*S*,*S*,*R*)-DE2	9–10	16–23 (9.7)	58–64 (10.9)		<1 (17.8)	19–20 (14.8)

[a] Binding of the diol epoxides to calf thymus DNA was carried out at 37 °C in 1:10 tetrahydrofuran/Tris-HCl buffer, pH 7.0, or 1:10 acetonitrile/buffer, pH 7.4 (cf. ref 10). No significant differences in results were observed under these two sets of reaction conditions. [b] Absolute configurations at the tetrahydro benzo ring carbon atoms are designated in sequence starting with the carbon bearing the benzylic hydroxyl group. [c] Retention time in minutes of the adducts under analytical HPLC conditions as described in the text.

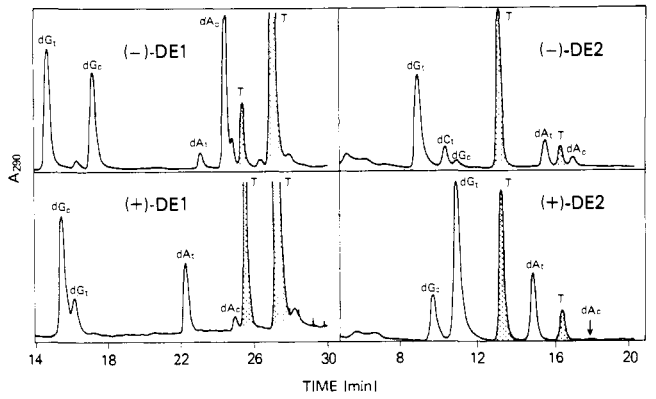

Figure 2. Reverse-phase HPLC profiles of the nucleoside adducts derived from the four optically isomeric 3,4-diol 1,2-epoxides of DBA upon reaction with DNA. For chromatographic conditions, see text. Tetraol peaks (T) are shaded. Tetraols that are derived by trans opening of the epoxide at C-1 emerge first from the column.

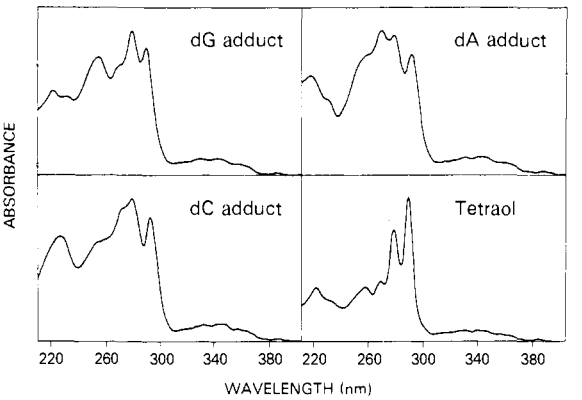

Figure 3. Typical UV spectra of adducts and tetraols formed from 3,4-diol 1,2-epoxides of DBA. Spectra were obtained by on-line monitoring of chromatographic eluates with a diode array detector. The compounds are (+)-DE2/dG_t, (+)-DE2/dA_t, (−)-DE2/dC_t, and the trans tetraol from (+)-diol epoxide-2.

product, which had the same retention time (8.7 min) on HPLC as the (−)-DE2/dC_t adduct obtained from DNA, was isolated for NMR and CD studies.

Adducts were acetylated with acetic anhydride in pyridine. The acetylated adducts from deoxyadenosine with diol epoxide-2 were purified on a Du Pont Golden Series SIL column (6.2 × 80 mm) eluted with methylene chloride/ethyl acetate/methanol (95:3:2) at 1 mL/min. All other acetylated adducts from diol epoxides-1 and -2 with deoxyadenosine and deoxyguanosine were purified on a Perkin-Elmer HS-3 C_{18} column (4.6 × 100 mm) eluted with acetonitrile/water (50:50) at 2 mL/min. In all cases, absorbance was monitored at 290 nm; retention times ranged from 2 to 5 min.

Results and Discussion

Treatment[10] of calf thymus DNA with each optically active isomer of the DBA 3,4-diol 1,2-epoxides, followed by a three-step enzymatic hydrolysis to the nucleoside level, resulted in multiple altered nucleosides which were separable by HPLC (Figure 2 and Table I). The origin of individual products was established by HPLC and UV spectral comparison with adducts obtained from the reactions of the diol epoxides with the separate nucleotides, followed by enzymatic hydrolysis of the phosphate group. Assignments of structure (discussed in detail in following sections) to the peaks observed on reverse-phase HPLC are indicated[5] in the figure. In our chromatographic systems, the adducts derived from deoxyadenosine by trans addition to the oxirane ring of both diol epoxides-1 and -2 are eluted earlier from C_{18} columns than their cis-adduct counterparts. No generalizations can be made with respect to the elution order of the dG adducts. Furthermore, the elution order for several of these adducts was solvent dependent: The cis adduct of (+)-diol epoxide-1 with deoxyguanosine was eluted earlier than the corresponding trans adduct in an aqueous solvent system modified with acetonitrile and methanol, but this order was reversed when the organic modifier was methanol alone.

The dG and dA adducts of DBA exhibit characteristic UV spectra (Figure 3), distinct from each other and from the tetraols, which served to identify the nucleoside component of each adduct.[9] Even though the ultraviolet spectra of the adducts of deoxyguanosine and deoxyadenosine in methanol show a λ_{max} of 280–281 nm, the relative intensities (cf. Figure 3) of the bands at lower wavelengths (ca. 257 and 270 nm) distinguish these adducts from each other and from the tetraols, which have a λ_{max} of 290 nm and much weaker absorption at 257 and 270 nm. The dC adduct from (−)-diol epoxide-2 shows a λ_{max} of 280 nm with weak absorption at 257 nm and a shoulder at 270 nm. Thus, on the basis of UV spectra, it was possible to distinguish dG, dA, and dC adducts and the tetraols.

Mass spectra (CI, NH_3) of the acetylated adducts[16] exhibited $(M + 1)^+$ peaks at m/z 790 (dA adducts) or 806 (dG adducts), corresponding to the pentaacetates. The dG adducts exhibited a base peak at m/z 746 due to the loss of an acetate, whereas in the case of the dA and dC adducts, the $(M + 1)^+$ peak was the base peak. The mass spectrum of the acetylated deoxycytidine adduct confirmed that it was a pentaacetate (m/z 766). The mass spectra of selected unacetylated deoxyguanosine adducts [(−)-DE2/dG_t and (−)-DE2/dG_c] exhibited a base peak (m/z 480) due to loss of the sugar, whereas the free deoxycytidine adduct showed a $(M + 1)^+$ peak at m/z 556 and a peak at m/z 440 due to loss of the sugar. Mass spectrometry of unacetylated adducts is thus useful in distinguishing between dG and dC adducts, which elute from ODS columns in the same region.

Site of Substitution. As in the case of the benzo[*c*]phenanthrene adducts, the acid–base behavior of selected purine adducts from

(16) Mass spectra were obtained for all of the dA and dG adducts (as their acetates) except for (+)-DE2/dA_t, for which the NMR sample in deuterated chloroform had decomposed.

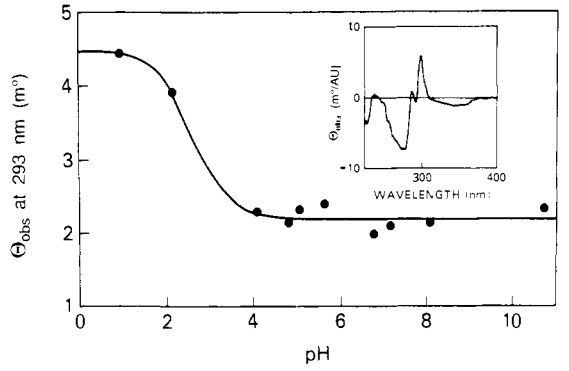

Figure 4. Titration curve of (−)-DE2/dC_t measured by CD spectroscopy at 293 nm in 1:9 methanol/water. The inset shows the CD spectrum of (−)-DE2/dC_t measured in methanol.

DBA is consistent with substitution on the exocyclic nitrogen.[10] Values of the pK_a for representative unacetylated adducts were determined by measurement[15] of the pH-dependent changes in their CD spectra at 279 nm [(−)-DE1/dG_c] or 293 nm [(−)-DE2/dA_t]. For (−)-DE1/dG_c, the pK_a values[10] were 2.1 and 9.3, which are very similar to those observed for analogous dG adducts from BcPh and BaP diol epoxides.[3c,4,15] The pK_a of 9.3 indicates an ionizable hydrogen at N-1 and excludes alkyl substitution at N-1 or O-6.[17] Since alkylation at N-3 gives a cation which could readily lose this hydrogen to form a neutral species, the presence of a weakly acidic proton at N-1 would also appear inconsistent with alkylation at N-3; moreover, such alkylation products may be unstable as the nucleosides.[18a] A representative deoxyadenosine adduct, (−)-DE2/dA_t, gave a single pK_a of 2.2,[10] which is similar to those measured for adducts of dA with BcPh[4] and BaP[3d] diol epoxides. Under the conditions employed in their formation and enzymatic release from DNA, these adducts are stable. This stability excludes substitution at N-3 or N-7 of deoxyadenosine or deoxyguanosine, since adducts at these positions should undergo facile ring opening or deglycosylation reactions.[18] Although only one adduct of each base was titrated, similarities among the UV, NMR, and CD spectra of the DBA adducts are consistent with the conclusion that all the purine adducts characterized from DBA, like their BcPh analogues, result from substitution at the exocyclic amino group.

For (−)-DE2/dC_t (Figure 4), the observed pK_a of ca. 2.6 is appreciably lower than the value of 4.2 previously measured[19a] for N^4-ethyldeoxycytidine in water. Nevertheless, it is most consistent with substitution at this position and not with substitution at N-3 or O-2, since pK_a values for adducts at these positions are expected to be >8.[19] Doublets corresponding to the cytosine protons at C-5 and C-6 (at δ 5.85 and 7.76 ppm, respectively) in the ^{1}H NMR spectrum of this adduct as its acetate were identified by decoupling. These chemical shifts are consistent with those observed for the corresponding protons of 2′-deoxycytidine and its adducts with 10-azabenzo[*a*]pyrene 4,5-oxide.[20b] Thus, alkyl substitution at either C-5 or C-6 is ruled out. All of the above data point to the exocyclic amino nitrogen as the site of substitution in the dC adduct, as is seen in the adducts derived from the purine bases.

NMR Spectra: Stereochemistry of Addition. Of the 16 possible cis and trans addition products from dA and dG with the four optically isomeric diol epoxides, all but two were obtained in sufficient quantities for NMR spectral study. ^{1}H NMR spectra were measured after acetylation of the adducts since we had previously observed in the case of BcPh[4] that such acetylation products give excellent quality spectra in acetone. Table II lists the chemical shifts and coupling constants for the four methine protons of the tetrahydro benzo ring in the acetylated purine and pyrimidine nucleoside adducts from DBA diol epoxides. Partial spectra of typical adducts are shown in Figure 5. That these acetylation products were pentaacetates had been shown by their mass spectra. Although the signal for the N-H proton of the exocyclic amino group was not identified in any of these acetylated purine nucleoside adducts, a combination of NMR and mass spectral evidence indicated that this nitrogen had not been acetylated, since the chemical shifts for the methine protons of the tetrahydro benzo ring and the sugar were consistent with acetylation of all five hydroxyl groups. A similar conclusion had previously been reached in the case of the analogous BcPh adducts.[4]

In the case of the acetylated deoxyadenosine adducts derived from both diol epoxides-1 and -2, signals corresponding to protons at C-2 and C-8 of adenine (listed in the supplementary material) were identified at ca. δ 8.1 and 8.8 ppm, respectively. The signals for these protons were very broad in the acetates of the early-eluting (more polar) isomer of each pair of dA adducts (cf. Figure 5). These isomers were shown to have the stereochemistry resulting from trans addition of the purine to the epoxide (see below). This selective broadening was seen in different solvents (benzene-d_6, THF-d_8), but the peaks did sharpen somewhat in benzene-d_6 and acetone-d_6 at 40 °C, suggestive of a conformational effect. The purine protons at C-8 of acetylated deoxyguanosine adducts appear between δ 8.05 and 8.13 ppm, with no apparent pattern in the variations among their chemical shifts.

Distinction between adducts derived from cis or trans addition to the oxirane ring was possible upon examination of the NMR spectra and was most conveniently accomplished by comparison with the corresponding tetraacetates of the isomeric tetraols, 1,2,3,4-tetrahydroxy-1,2,3,4-tetrahydrodibenz[*a*,*j*]anthracenes (Figure 6), whose relative stereochemistry had previously been determined.[14] As described in detail elsewhere,[14] the tetraols were produced upon cis and trans hydration of the dibenz[*a*,*j*]anthracene 3,4-diol 1,2-epoxides, with the exception of the cis-2 tetraol, which was prepared by oxidation of *trans*-3,4-dihydroxy-3,4-dihydrodibenz[*a*,*j*]anthracene with osmium tetroxide. The signals corresponding to the methine protons on the benzo ring of the adducts (Table II) were assigned on the basis of direct decoupling experiments as well as by comparison with the tetraol tetraacetate spectra.

As is the case for analogous adducts derived from BcPh,[4] adducts resulting from cis vs trans addition of the purine amino group to C-1 of the diol epoxide-1 isomers could be distinguished by analysis of coupling constants. In both the cis and trans adducts, the bulky purine substituent at C-1, constrained by steric hindrance in the bay region, is forced into a pseudoaxial orientation. This conformational constraint causes the benzo ring to adopt a half-chair conformation in which the methine hydrogens at C-2, C-3, and C-4 of the cis adducts from diol epoxide-1 are all pseudoaxial. The large couplings $J_{2,3}$ (11–12 Hz) and $J_{3,4}$ (8.0–8.2 Hz) observed for the cis adducts are consistent with this conformation. The cis dA adducts from diol epoxide-1 differ markedly from the corresponding cis dG adducts in the chemical shift of H-3 ($\Delta\delta$ ca. 0.3 ppm downfield for dA relative to dG adducts), but not of H-4 ($\Delta\delta$ 0.04 ppm). This observation also supports the conclusion that in these dA adducts cis addition to the epoxide has occurred, since in such adducts the methine hydrogen at C-3 is axial and on the same side of the benzo ring as the purine substituent, and is thus susceptible to its magnetic effect. In the trans adducts from diol epoxide-1, H-3 is on the opposite side of the ring from the purine, and consequently the chemical shift for this proton is insensitive to the identity of the purine base.

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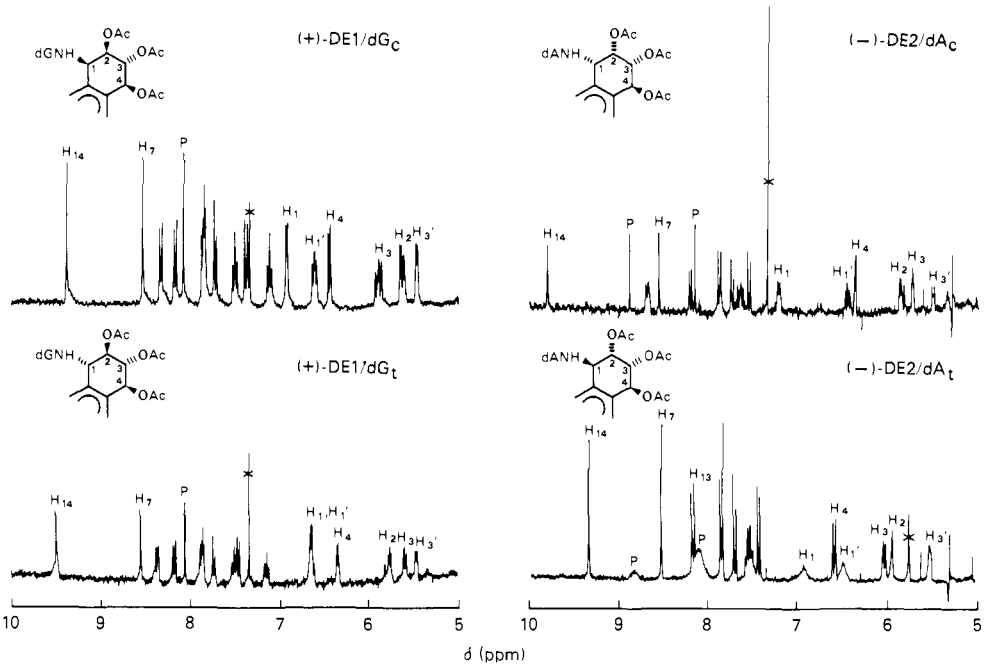

Figure 5. Partial NMR spectra (300 MHz, acetone-d_6) showing the benzo ring methine proton resonances of typical acetylated adducts: deoxyguanosine (dG) with (+)-diol epoxide-1 and deoxyadenosine (dA) with (−)-diol epoxide-2. Protons derived from the sugar are designated with a prime symbol. Purine protons are indicated with the letter P.

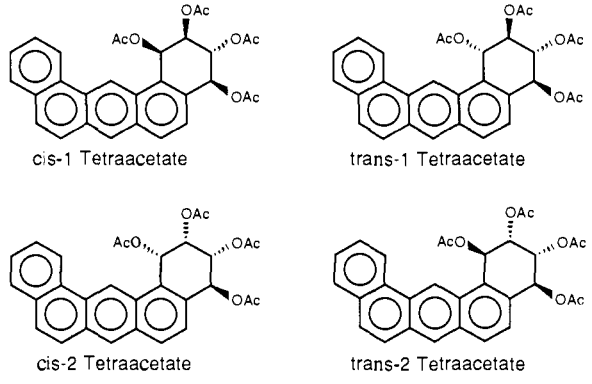

Figure 6. Structures of the tetraol tetraacetates derived from the DBA diol epoxides. The designations 1 and 2 identify the diastereomeric diol epoxide precursors.

For the trans adducts derived from diol epoxide-1, the pseudoaxial purine moiety at C-1 forces the benzo ring to adopt a conformation in which all the methine hydrogens are pseudoequatorial. Thus, values of $J_{2,3}$ (6.6–7.1 Hz) and $J_{3,4}$ (4.8–5.5 Hz) for these isomers are substantially smaller than those in the case of the cis adducts. The small values of $J_{3,4}$ confirm that the trans adducts derived from DBA diol epoxide-1 favor a chair conformation rather than the more boat-like conformation favored by the corresponding BcPh derivatives,[4] in which the hydrogens at C-3 and C-4 are more axial ($J_{3,4}$ ca. 8 Hz). Analogously, in the cis adducts from diol epoxide-1, the relatively large value (ca. 8 Hz) for $J_{3,4}$ in the DBA derivatives, as opposed to 2–5 Hz for the BcPh derivatives,[4] is also consistent with a chair conformation for the DBA and a boat conformation for the BcPh adducts. As in the case of acetylated nucleoside adducts of BcPh diol epoxide-1[4] as well as tetraol tetraacetates derived from diol epoxide-1 isomers,[4,21] the signal for the bay-region benzylic H-1 exhibits a downfield shift in the cis-1, relative to the trans-1, derivatives.

Unlike the adducts formed from diol epoxide-2 in the BcPh series,[4] adducts resulting from cis vs trans addition of purines to diol epoxide-2 in the DBA series were easily distinguishable from each other by their coupling constants, as well as by the effect of the base moiety on the chemical shifts for the protons at C-3. As in the case of the diol epoxide-1 adducts, a chair conformation for these diol epoxide-2 adducts is preferred, with the C-1 substituent pseudoaxial. In the cis adducts from diol epoxide-2 this preferred conformation has the hydrogens at C-3 and C-4 pseudoequatorial, such that $J_{3,4}$ is small (ca. 3 Hz). In the trans adducts, these hydrogens are pseudoaxial, and $J_{3,4}$ is ca. 9 Hz. The chemical shift of the methine hydrogen at C-3 is dependent on the identity of the base, such that the signal for this proton in the trans dA adducts from diol epoxide-2 appears ca. 0.25 ppm farther downfield than the corresponding proton signal in the trans dG adducts. This observation is consistent with a conformation for the trans adducts in which this methine hydrogen and the purine moiety are cis and diaxial. The conformations of these acetylated diol epoxide-2 adducts as shown by their coupling constants closely resemble those of the corresponding DBA tetraol tetraacetates, as well as analogous derivatives from other hydrocarbons, such as the tetraol tetraacetates and several nucleophilic addition products from benzo[*a*]pyrene diol epoxide-2.[21] The cis-2 tetraol tetraacetate, as well as the two acetylated cis adducts from diol epoxide-2 that were obtained in sufficient quantities for NMR spectroscopy, exhibits a small downfield shift (ca. 0.3 ppm) for the proton at C-1 relative to the trans adducts. This is analogous to previous observations in the BcPh series,[4] although the magnitude of the difference for the DBA derivatives is much smaller.

For both cis and trans adducts from a given diol epoxide isomer, the signal for the benzylic H-1 always appears downfield (ca. 0.3–0.5 ppm) in the dA adducts relative to the corresponding dG adducts, and conversely, the signal for H-1′ of the sugar generally appears downfield in the dG adducts relative to the corresponding dA adducts.

In general, each pair of diastereomers, having the same relative stereochemistry for the tetrahydro benzo ring but derived from enantiomeric diol epoxides, exhibited identical chemical shifts and coupling constants for the four methine hydrogens on this ring. Exceptions were noted in the case of (+)- and (−)-DE1/dG$_c$, in which the C-1 proton of the adduct derived from the (−)-diol epoxide exhibited a small (0.11 ppm) downfield shift relative to this proton in the adduct derived from the (+)-diol epoxide, and in the case of (+)- and (−)-DE2/dG$_t$, in which the signal for H-2 in the (−)-diol epoxide-2 adduct appeared slightly upfield (0.08 ppm) relative to that for H-2 in the (+)-diol epoxide-2 adduct.

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Table II. ^{1}H NMR Data[a] for the Methine Protons of the Acetylated Deoxyribonucleoside Adducts and of the Tetraol Tetraacetates from Dibenz[*a,j*]anthracene Diol Epoxides-1 and -2

compound (as the acetate)	methine hydrogens			
	H-1	H-2	H-3	H-4
cis-1 tetraol	7.46	5.65	5.91	6.54
	($J_{1,2}$ = 3.7, $J_{2,3}$ = 11.5, $J_{3,4}$ = 8.1)			
(+)-DE1/dG$_c$	6.92	5.65	5.92	6.45
	($J_{1,2}$ = 4.2, $J_{2,3}$ = 11.8, $J_{3,4}$ = 8.2)			
(−)-DE1/dG$_c$	7.03	5.63	5.93	6.45
	($J_{1,2}$ = 4.5, $J_{2,3}$ = 11.9, $J_{3,4}$ = 8.2)			
(+)-DE1/dA$_c$	7.35	5.74	6.25	6.41
	($J_{1,2}$ = 4.5, $J_{2,3}$ = 10.9, $J_{3,4}$ = 8.0)			
(−)-DE1/dA$_c$	7.30	5.74	6.24	6.41
	($J_{1,2}$ = 4.7, $J_{2,3}$ = 11.5, $J_{3,4}$ = 8.1)			
trans-1 tetraol	7.19	5.71	5.49	6.47
	($J_{1,2}$ = 4.5, $J_{2,3}$ = 8.1, $J_{3,4}$ = 7.2)			
(+)-DE1/dG$_t$[b]	6.64	5.78	5.60	6.35
	($J_{1,2}$ = 4.3, $J_{2,3}$ = 6.7, $J_{3,4}$ = 4.8)			
(−)-DE1/dG$_t$[b]	6.60	5.80	5.60	6.32
	($J_{1,2}$ = 4.1, $J_{2,3}$ = 6.6, $J_{3,4}$ = 4.9)			
(+)-DE1/dA$_t$	7.05	5.80	5.62	6.49
	($J_{1,2}$ = 4.2, $J_{2,3}$ = 7.1, $J_{3,4}$ = 5.4)			
(−)-DE1/dA$_t$	7.00	5.80	5.61	6.49
	($J_{1,2}$ = 4.2, $J_{2,3}$ = 7.1, $J_{3,4}$ = 5.5)			
cis-2 tetraol	7.38	5.81	5.63	6.31
	($J_{1,2}$ = 4.8, $J_{2,3}$ = 2.6, $J_{3,4}$ = 3.5)			
(+)-DE2/dG$_c$	6.86	5.90	5.68	6.31
	($J_{1,2}$ = 5.4, $J_{2,3}$ = 2.7, $J_{3,4}$ = 2.8)			
(−)-DE2/dG$_c$[c]				
(+)-DE2/dA$_c$[c]				
(−)-DE2/dA$_c$	7.23	5.87	5.72	6.37
	($J_{1,2}$ = 5.2, $J_{2,3}$ = 2.6, $J_{3,4}$ = 2.9)			
trans-2 tetraol	7.10	5.81	5.66	6.57
	($J_{1,2}$ = 3.7, $J_{2,3}$ = 2.5, $J_{3,4}$ = 8.7)			
(+)-DE2/dG$_t$	6.52	5.92	5.75	6.58
	($J_{1,2}$ = 3.4, $J_{2,3}$ = 2.3, $J_{3,4}$ = 8.9)			
(−)-DE2/dG$_t$	6.47	5.84	5.78	6.60
	($J_{1,2}$ = 2.9, $J_{2,3}$ = 2.8, $J_{3,4}$ = 8.8)			
(+)-DE2/dA$_t$	6.90	5.92	6.03	6.59
	($J_{1,2}$ = ~3, $J_{2,3}$ = 2.0, $J_{3,4}$ = 9.0)			
(−)-DE2/dA$_t$	6.95	5.95	6.03	6.59
	($J_{1,2}$ = ~3, $J_{2,3}$ = 2.5, $J_{3,4}$ = 9.0)			
(−)-DE2/dC$_t$	6.69	5.79	5.69	6.55
	($J_{1,2}$ = 2.8, $J_{2,3}$ = 2.2, $J_{3,4}$ = 8.9)			

[a] Spectra were measured in acetone-d_6 at 300 MHz. Line positions are reported in ppm, the acetone-d_6 peak (δ 2.04 ppm) being used as reference, and coupling constants (J) are given in hertz. [b] Assignments are based on the assumption that H-1 exhibits the furthest downfield shift. Because of the similarity in coupling constants $J_{1,2}$ and $J_{3,4}$, the alternative interpretation (H-4 furthest downfield, with reversed assignments of chemical shifts for H-1 and H-4 and for H-2 and H-3) would lead to identical structural conclusions. [c] Insufficient material for satisfactory NMR spectra.

A partial ^{1}H NMR spectrum of the observed adduct from (−)-diol epoxide-2 and deoxycytidine, (−)-DE2/dC$_t$ as the pentaacetate, is shown in Figure 7. By analogy with the tetraol tetraacetates and the acetylated dA and dG adducts, the observed large coupling of 8.9 Hz for H-3 and H-4 on the tetrahydro benzo ring of the DBA moiety (Table II) indicates that this dC adduct resulted from trans addition of deoxycytidine to the oxirane of (−)-diol epoxide-2.

Listings of the chemical shifts for H-7, H-13 and H-14 of the hydrocarbon moiety, as well as for selected sugar protons, are given in the supplementary material. A pronounced upfield shift (0.32–0.64 ppm) of H-13 is observed in all of the acetylated dA and dG adducts when compared to the tetraol tetraacetates; however, in the case of (−)-DE2/dC$_t$, this proton signal was shifted downfield by ca. 0.1 ppm. The protons at C-7 and C-14 of the hydrocarbon appear as clearly distinguishable singlets in the spectra of the acetylated adducts as well as those of the tetraol tetraacetates. Adduct formation has virtually no effect on the chemical shift of H-7, whereas the signal for H-14 of several of

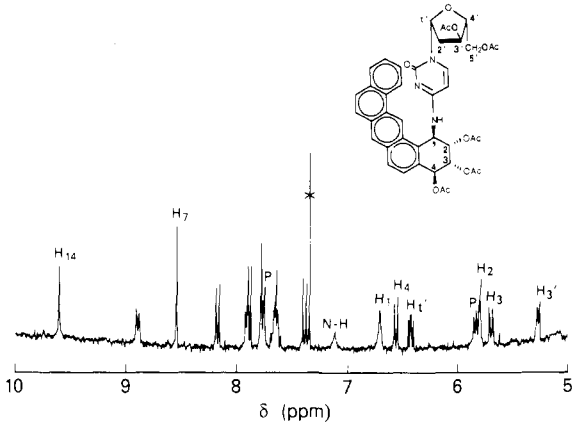

Figure 7. Partial NMR spectrum of the acetylated deoxycytidine adduct of (−)-diol epoxide-2 (300 MHz, acetone-d_6). The four methine protons of the benzo ring are shown. The pyrimidine protons (P) H-6 and H-5 appear at δ 7.76 and 5.85 ppm, respectively. Protons designated with a prime symbol are derived from the sugar.

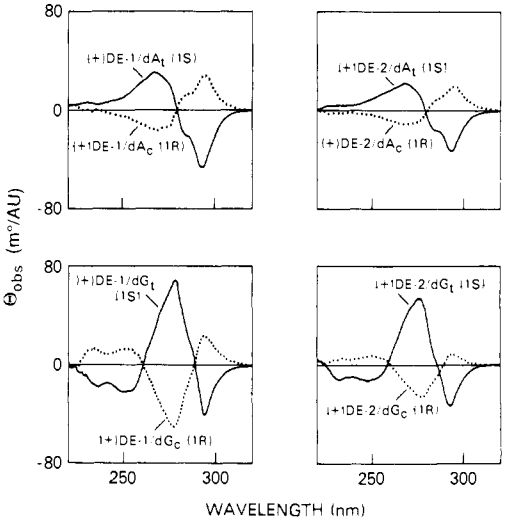

Figure 8. Circular dichroism spectra in methanol of the adducts derived from deoxyguanosine and deoxyadenosine with (+)-dibenz[*a,j*]-anthracene (4*S*,3*R*)-diol (2*S*,1*R*)-epoxide-1 and (+)-dibenz[*a,j*]-anthracene (4*R*,3*S*)-diol (2*S*,1*R*)-epoxide-2. The spectra have been normalized to 1 absorbance unit at 290 nm. Corresponding derivatives of the enantiomers of these diol epoxides exhibited CD spectra that were essentially mirror images of those shown (see supplementary material).

the dA adducts appears 0.1–0.4 ppm farther downfield relative to the corresponding dG adducts or tetraol tetraacetates. Notably, the dA adducts in which this proton is deshielded (trans-1 and cis-2 adducts) all have the adenine substituent and an acetoxy group in 1,3-diaxial orientation. We speculate that steric interaction between these two groups may cause the purine to occupy (on average) a position in which it is rotated away from the tetrahydro benzo ring and is close to H-14, such that edge deshielding of this proton by the aromatic rings of adenine is possible.

The presence of the sugar moiety in all of the adducts (as shown by the mass and NMR spectra of their pentaacetates), along with the absence of a downfield shift[22] for H-8 of the purine, confirms the lack of substitution at N-7. Signals corresponding to H-1′ and H-3′ of the sugar were identified at δ 6.4–6.7 ppm and ca. 5.5 ppm, respectively, in all of the dA and dG adducts whose NMR spectra were measured. In several of the adducts, the two nonequivalent hydrogens at C-2′ (δ ca. 2.6 and 3.2–3.5 ppm) as well as H-4′ and H-5′ (δ 4.2–4.5 ppm) were also identifiable, although detailed analyses of their coupling constants were not undertaken. Since all of these derivatives are pentaacetates, their

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conformations as reflected by NMR spectra are not necessarily the same as those of the *unacetylated* adducts as monomers or in oligo- or polynucleotides.

Circular Dichroism Spectra. Figure 8 shows circular dichroism (CD) spectra in methanol of the unacetylated adducts derived from deoxyguanosine and deoxyadenosine with (+)-DBA (4*S*,3*R*)-diol (2*S*,1*R*)-epoxide-1 and (+)-DBA (4*R*,3*S*)-diol (2*S*,1*R*)-epoxide-2. Each of the corresponding eight spectra (supplementary material) for the adducts from their enantiomers, (−)-DBA (4*R*,3*S*)-diol (2*R*,1*S*)-epoxide-1 and (−)-DBA (4*S*,3*R*)-diol (2*R*,1*S*)-epoxide-2, is essentially the mirror image of the spectrum of the adduct derived from the (+) enantiomer shown. The strong CD spectra observed for the purine adducts result from the exciton interaction of the aromatic systems of the bases with the benz[*a*]anthracene chromophore in the hydrocarbon moiety. The sign of the CD bands is dependent on the spatial relationship between the hydrocarbon and base chromophores and has been used to propose absolute configurations for hydrocarbon adducts in some cases,[23,24] although in the absence of independent corroboration such assignments are only tentative because of uncertainty regarding the directions of the electric dipole transition moments[25] of the bases. The skew sense between the electric transition moments of the purine and hydrocarbon chromophores depends on the orientation of the bond between nitrogen and the benzylic C-1, and hence on the absolute configuration at C-1 in these adducts. Thus, the sign of the CD bands is determined by the absolute configuration at C-1. Although this sign cannot be derived a priori from theoretical considerations, given the information currently available, assignment of absolute configuration to most of the present adducts was possible on the basis of independent evidence (known absolute configurations of the starting epoxides and relative configurations of the adducts from NMR spectroscopy). Thus, it was possible to develop an *empirical* relationship, analogous to that previously reported for the BcPh adducts,[4] between the signs of the CD bands and the absolute configurations of the adducts. For the DBA derivatives, a positive CD band at 270–280 nm and a negative band at 290–300 nm were observed for compounds whose absolute configuration was unambiguously assigned to be 1*S* from their relative configurations and the known absolute configurations of the parent epoxides. Conversely, CD spectra of comparable intensity but opposite in sign were observed for DBA adducts whose absolute configuration was 1*R*. The CD spectra of the BcPh adducts[4] are analogous to those observed for the DBA adducts, both in general appearance and in the relationship between sign and absolute configuration at C-1; for example, the 1*S* adducts in the BcPh series exhibit a positive band at ca. 260 nm and a negative band at ca. 280–290 nm.

This empirical relationship provides confirmatory evidence for the structures of the two minor DBA adducts, (−)-DE2/dG_c and (+)-DE2/dA_c, which had similar UV spectra and were known by mass spectrometry to be isomeric with the other dG and dA adducts, respectively, but were not obtained in sufficient quantities for NMR spectroscopy. Specifically, (−)-DE2/dG_c has a positive CD band at 275 and a negative band at ca. 290 nm and thus has 1*S* absolute configuration. Since its parent diol epoxide, (−)-DBA diol epoxide-2, had 1*S* absolute configuration, addition to the epoxide must have involved retention of configuration at this center, and hence, the stereochemistry of addition must be cis. Similarly, (+)-DE2/dA_c is also a cis adduct since its CD spectrum (Figure 8) corresponds to 1*R* absolute configuration of the adduct and the parent diol epoxide had 1*R* absolute configuration.

The CD spectrum of the unacetylated deoxycytidine adduct, (−)-DE2/dC_t, is shown in Figure 4 (inset). As in the case of trans adducts of the purines, dA and dG, with (−)-diol epoxide-2, there is a negative band at 260–280 nm and a positive band at 290–300 nm. Although these CD bands for (−)-DE2/dC_t are about 8-fold less intense than the corresponding bands for the dA or dG adducts, their signs are consistent with *R* absolute configuration at C-1, as required by trans addition (vide NMR) of cytosine to the (−)-epoxide, which has *S* absolute configuration at this carbon. Thus, it appears that the relationship between the sign of the CD bands and the absolute configuration at C-1 observed for purine nucleoside adducts applies to adducts of deoxycytidine as well.

Summary and Conclusions

The present work represents a continuation of our investigation of the relationship between structure and DNA-binding properties of the diol epoxides derived metabolically from polycyclic aromatic hydrocarbons and provides a major step in the development of a structural and spectroscopic data base of the covalent nucleoside adducts thus formed. The results of this study, as well as data for diol epoxides derived from benzo[*a*]pyrene,[3] benzo[*c*]-phenanthrene,[4] and 7,12-dimethylbenz[*a*]anthracene,[26] strongly indicate that for these diol epoxides the major sites of modification of DNA are at the exocyclic amino groups of the purine and pyrimidine bases, although, in the case of a benzo[*a*]pyrene diol epoxide, O-6 and N-7 derivatives[27] of guanine have been suggested as possible minor adducts. Similarly, the polycyclic aromatic hydrocarbon derivatives 9,10-epoxy-7,8,9,10-tetrahydrobenzo-[*a*]pyrene and 9,10-epoxy-7,8,9,10-tetrahydrobenzo[*e*]pyrene yield adducts at the N-2 position of guanine.[28] The K-region arene oxide 7,12-dimethylbenz[*a*]anthracene 5,6-oxide also gives mainly N-2-substituted adducts upon reaction with polyguanylic acid,[23] although C_2-OH[24] and C-8[29] adducts have been characterized upon reaction of this arene oxide with guanosine at alkaline pH and are apparently formed as minor adducts from the hydrocarbon with RNA in cell culture.[30] The K-region 4,5-oxide derived from 10-azabenzo[*a*]pyrene gives cis and trans N-4 adducts of deoxycytidine upon reaction with calf thymus DNA.[20b] In contrast to the polycyclic aromatic epoxides, a number of other epoxides react predominantly at the ring nitrogen positions of guanine or adenine. The simple aliphatic derivative propylene oxide[31] forms adducts at N-7 of guanine and N-3 of adenine. An adduct at N-3 of adenine has been reported from the reaction of 9-anthryloxirane with poly(dA-dT) or with DNA.[32] Upon reaction with DNA, styrene oxide yields predominant adducts at N-7 of guanine, along with minor quantities of N-2 and O-6 adducts.[33] The carcinogens aflatoxin B_1[34] and vinyl chloride,[35] which are thought to be metabolized via epoxide intermediates, also give rise to DNA adducts resulting from modification at N-7 of guanine. Moschel, Hudgins, and Dipple have suggested[36] that electrophiles that react via transition states with substantial delocalized carbocation character may form adducts preferentially at the exocyclic amino groups of nucleoside residues. Thus, these electronic factors as well as steric requirements of the aromatic moieties could account for the selectivity toward exocyclic amino groups shown by epoxide

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derivatives of polycyclic aromatic hydrocarbons as compared to other typical epoxides.

Unlike the BcPh diol epoxides, the DBA diol epoxides exhibit low efficiency of covalent binding to DNA,[9,10] relative to hydrolysis. In general the DBA diol epoxides show some preference (cf. Table I) for dG adduct formation (60–80% of total adducts), whereas this preference is reversed in the case of the BcPh diol epoxides, such that 50–90% of the adducts formed from the BcPh diol epoxides involve dA residues. Despite these differences, some notable similarities do emerge in the patterns of adduct formation from the individual optically active isomers in the BcPh and the DBA series.

(i) For dA adduct formation, the ratio of cis to trans addition to diol epoxide-1 is highly dependent on the absolute configuration of the diol epoxide. For example, the ratio of cis to trans dA adducts from both DBA and BcPh (4*S*,3*R*)-diol (2*S*,1*R*)-epoxides-1 is ca. 1:3, whereas the ratio of cis to trans dA adducts from the enantiomeric (4*R*,3*S*)-diol (2*R*,1*S*)-epoxides-1 from both hydrocarbons is ca. 7:1. In the case of diol epoxide-2, trans dA adduct formation is preferred by a factor of 3:1 for the (4*S*,3*R*)-diol (2*R*,1*S*)-epoxide-2 from DBA and by a factor of 2:1 for this diol epoxide isomer from BcPh. A much greater preference for trans dA adduct formation is exhibited by the (4*R*,3*S*)-diol (2*S*,1*R*)-epoxide-2 enantiomers: >20:1 for DBA and >40:1 for BcPh.

(ii) Trans-adduct formation from dG and the (4*S*,3*R*)-diol (2*R*,1*S*)-epoxide-2 isomers is substantially favored over cis-adduct formation, by >10:1 for DBA and >20:1 for BcPh. With the (4*R*,3*S*)-diol (2*S*,1*R*)-epoxide-2 enantiomers, trans dG adduct formation is also preferred, by a factor of ca. 3 for DBA and of ca. 10 for BcPh.

(iii) Only the (4*S*,3*R*)-diol (2*R*,1*S*)-epoxide-2 isomer gives appreciable quantities of a dC adduct, in the case of both DBA and BcPh. Deoxycytidine adducts have also been reported from BaP diol epoxide-2 enantiomers,[3a,b] but they have not been well characterized, and it is not clear whether only one or both enantiomers of the diol epoxide give rise to these adducts.

From these results it is apparent that chiral interactions between the diol epoxides and the DNA molecule are highly important in determining the preferred sites and orientations of adduct formation. Further investigations using oligonucleotides of defined structure and sequence will be required to determine the nature of these interactions, as well as their possible implications for the differential mutagenicities and tumorigenicities of the optically active isomeric diol epoxides.

Supplementary Material Available: Listing of ^{1}H NMR chemical shifts for the base and sugar hydrogens of the acetylated deoxyribonucleoside adducts, as well as selected hydrogens of the hydrocarbon moieties in these adducts, and CD spectral traces of the adducts derived from (−)-dibenz[*a*,*j*]anthracene (4*R*,3*S*)-diol (2*R*,1*S*)-epoxide-1 and (−)-(4*S*,3*R*)-diol (2*R*,1*S*)-epoxide-2 (4 pages). Ordering information is given on any current masthead page.

Communications to the Editor

The Absolute Configuration and Synthesis of Natural (−)-Dolastatin 10[1]

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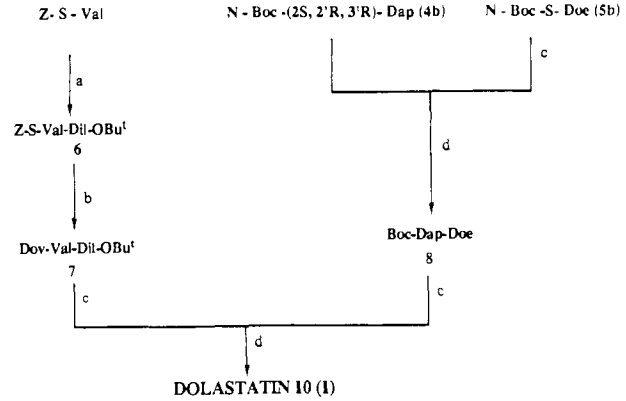
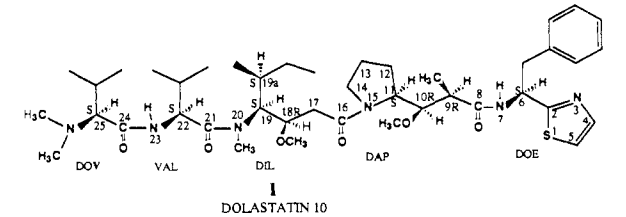
The fascinating sea hare *Dolabella auricularia* was known to certain ancient Greeks and Romans for various medical or nefarious[2,3] purposes. As early as 200 BC the Greek Nicandros recommended extracts of this opisthobranch (Subclass, Mollusca phylum) for treatment of certain diseases.[3] Over the past 16 years we have vigorously pursued the powerful cytostatic and antineoplastic constituents of *D. auricularia* collected in the Western Indian Ocean. In 1987 we reported the isolation and structure of dolastatin 10 (**1**), the most potent (i.e., lowest in vivo dose) antineoplastic substance known to date.[4] Due to the few milligrams of amorphous dolastatin 10 available for structure determination combined with the chiral complexity (9 asymmetric centers), the absolute configuration was not ascertained.

The most attractive solution to both the dolastatin 10 stereochemical and preclinical supply problems resided in an effective synthesis of the natural isomer from among 512 possible corresponding to the one-dimensional structure. We herein report that the absolute configuration of natural (−)-dolastatin 10 corresponds to structure **1** and we give its total synthesis. The genesis of a practical solution to these challenging problems arose from our

Scheme I[a]



[a] a. $(CH_3)_3COCl$, NMM, (3*R*,4*S*,5*S*)-Dil-OBu^t·HCl, $CHCl_3$. b. Dov-OPfp, 10% Pd/C, H_2, dioxane. c. CF_3CO_2H, CH_2Cl_2. d. Diethyl phosphorocyanidate (DEPC), TEA, DME, 1 h at 0 °C, 2 h at room temperature.



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DOLASTATIN 10

(1) Dedicated to Professor Carl Djerassi's 65th birthday. Contribution 189 in the series Antineoplastic Agents. For Part 188 see: Singh, S. B.; Pettit, G. R.; Herald, D. L. *J. Am. Chem. Soc.*, in preparation.

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