Articles

Synthesis and Duplex-Forming Properties of a Nonanucleotide Containing an N^6-Deoxyadenosine Adduct of a Bay-Region Diol Epoxide

Mahesh K. Lakshman, Jane M. Sayer, Haruhiko Yagi, and Donald M. Jerina*

Section on Oxidation Mechanisms, Laboratory of Bioorganic Chemistry, NIDDK, The National Institutes of Health, Bethesda, Maryland 20892

Received April 16, 1992

A protected derivative, corresponding to the adduct derived from trans opening of (±)-1α,2β-dihydroxy-3β,4β-epoxy-1,2,3,4-tetrahydrophenanthrene (in which the benzylic hydroxyl and epoxide groups are trans, phenanthrene diol epoxide-2) by the exocyclic amino group of 2′-deoxyadenosine (dA), has been synthesized by coupling (±)-1α,2β,3β-trihydroxy-4α-amino-1,2,3,4-tetrahydrophenanthrene with a disilyl derivative of 6-fluoro dA. The resulting pair of diastereomeric adducts was easily separated by HPLC, and the absolute configuration of each diastereomer was assigned from its CD spectrum by analogy to known tetrahydrophenanthrene analogs. The structures of these adducts were confirmed by comparison of the NMR spectra of the derived pentaacetates with those obtained for the corresponding compounds prepared from dAMP and phenanthrene diol epoxide-2. Acetylation of the free hydroxyl groups, desilylation of the sugar, 5′-*O*-(4,4′-dimethoxytrityl) protection, and generation of a 3′-*O*-[(*N*,*N*-diisopropylamino)(β-cyanoethoxy)phosphine] provided an intermediate that was incorporated, using modified solid-phase DNA synthesizer methodology, into the oligonucleotide d(GGT CA*C GAG) comprising codons 60–62 of the human K-*ras* b proto-oncogene sequence. The effect of an adduct on the T_m values for duplexes formed by this nonamer with complementary strands in which the residue opposite the modified dA is T or either of the "mismatched" purine nucleosides dA or dG was determined. Replacement of T with dG opposite the *modified* dA has little or no effect on T_m, whereas replacement of T with dA decreases the T_m of the modified duplex by 9 °C.

Bay-region diol epoxides, as exemplified by the 1,2-diol 3,4-epoxides of phenanthrene (see Scheme I) are oxidative metabolites of polycyclic aromatic hydrocarbons, many of which are mutagenic and/or carcinogenic.[1] These diol epoxides are thought to exert carcinogenic and other genotoxic effects predominantly through alkylation of DNA. Major products isolated from this reaction in vitro are adducts at the exocyclic amino groups of deoxyguanosine (dG) and deoxyadenosine (dA) residues, formed via cis and trans opening of the epoxide ring at the bay-region benzylic position.[2,3] In spite of a growing data base of structures of diol epoxide–mononucleoside adducts, the relationship of individual adducts to cell neoplasia is unknown. In order to understand better the biochemical events associated with cancer induction subsequent to adduct formation, we have undertaken a program to synthesize oligonucleotides in which a specific purine residue has been modified by diol epoxide adduct formation in a stereochemically defined manner. Since many if not most biologically significant oligonucleotide sequences contain multiple dA and/or dG residues, direct reaction of such oligonucleotides with diol epoxides would yield a mixture of products. Consequently, we have chosen a more general approach involving suitably protected nucleoside phosphoramidites into which the diol epoxide has been synthetically incorporated. We have recently reported the generation of oligonucleotides containing both cis[4] and trans[5] ring opened adducts of 3,4-epoxy-1,2,3,4-tetrahydrophenanthrene with dA, as well as of an analogous trans dG adduct.[6] The present report describes the first use of this methodology for the site specific incorporation of an adduct of a bay-region diol epoxide into an oligonucleotide.

Results and Discussion

Our general synthetic strategy for the generation of oligonucleotides specifically modified at dA residues by diol epoxide adduct formation has been to synthesize an activated dA adduct via coupling of an amino derivative of the hydrocarbon with an appropriately blocked 6-fluoro analog of dA, followed by steps for generating the blocked 3′-phosphoramidite. Reactions of fluoropurines with bay-region amino alcohols and amino esters derived from tetrahydrobenzo-ring derivatives of several polycyclic hydrocarbons have been well documented.[4-8] Trans ami-

(1) Thakker, D. R.; Yagi, H.; Levin, W.; Wood, A. W.; Conney, A. H.; Jerina, D. M. In *Bioactivation of Foreign Compounds*; Anders, M. W., Ed.; Academic Press: New York, 1985; pp 177–242. Jerina, D. M.; Cheh, A. M.; Chadha, A.; Yagi, H.; Sayer, J. M. In *Microsomes and Drug Oxidations: Proceedings of the 7th International Symposium*; Miners, J. O., Birkett, D. J., Drew, R., May, B. K., McManus, M. E., Eds.; Taylor and Francis: London, 1988; pp 354–362.

(2) Jerina, D. M.; Chadha, A.; Cheh, A. M.; Schurdak, M. E.; Wood, A. W.; Sayer, J. M. In *Biological Reactive Intermediates IV. Molecular and Cellular Effects and Their Impact on Human Health*; Witmer, C. M., Snyder, R., Jollow, D. J., Kalf, G. F., Kocsis, J. J., Sipes, I. G., Eds.; Plenum Press: New York, 1991; pp 533–553.

(3) (a) Agarwal, S. K.; Sayer, J. M.; Yeh, H. J. C.; Pannell, L. K.; Hilton, B. D.; Pigott, M. A.; Dipple, A.; Yagi, H.; Jerina, D. M. *J. Am. Chem. Soc.* **1987**, *109*, 2497–2504. (b) Cheng, S. C.; Prakash, A. S.; Pigott, M. A.; Hilton, B. D.; Roman, J. M.; Lee, H.; Harvey, R. G.; Dipple, A. *Chem. Res. Toxicol.* **1988**, *1*, 216–221. (c) Chadha, A.; Sayer, J. M.; Yeh, H. J. C.; Yagi, H.; Cheh, A. M.; Pannell, L. K.; Jerina, D. M. *J. Am. Chem. Soc.* **1989**, *111*, 5456–5463.

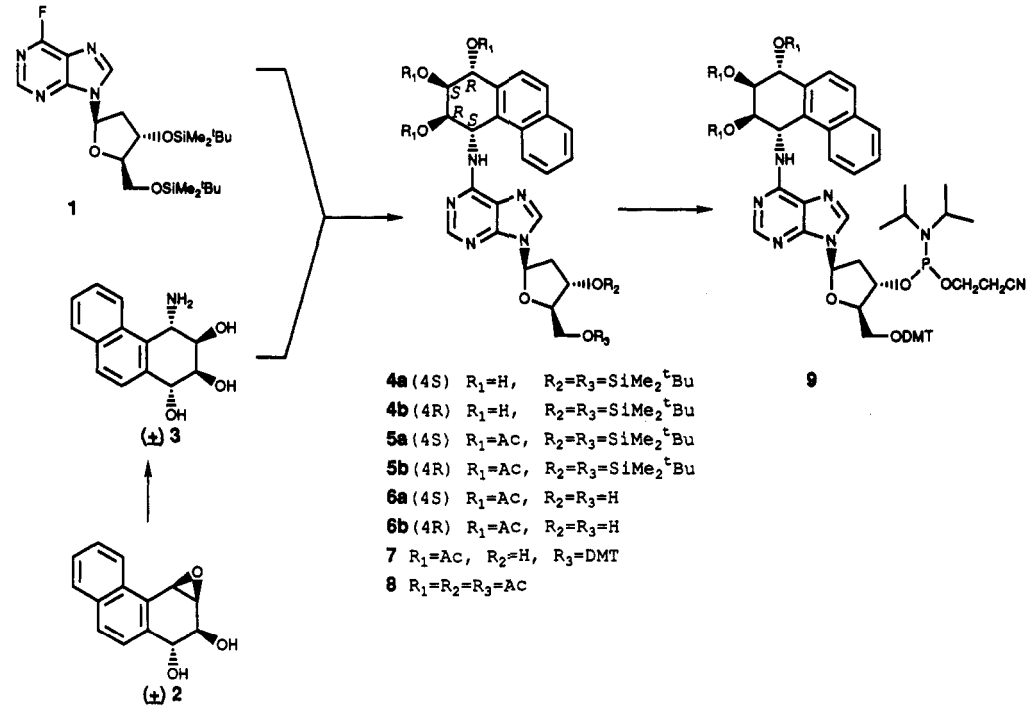
(4) Lakshman, M. K.; Sayer, J. M.; Jerina, D. M. *J. Org. Chem.* **1992**, *57*, 3438–3443.

(5) Lakshman, M. K.; Sayer, J. M.; Jerina, D. M. *J. Am. Chem. Soc.* **1991**, *113*, 6589–6594.

(6) Zajc, B.; Lakshman, M. K.; Sayer, J. M.; Jerina, D. M. *Tetrahedron Lett.* **1992**, *33*, 3409–3412.

(7) Lakshman, M.; Lehr, R. E. *Tetrahedron Lett.* **1990**, *31*, 1547–1550.

Scheme I



nolysis at C-4 of racemic phenanthrene 1α,2β-diol 3β,4β-epoxide **2** (series-2 diastereomer in which the epoxide group and the benzylic 4-hydroxyl group are trans) cleanly produces the amino triol **3**.[5,9] Thus, coupling of racemic amino triol **3** with the 3′,5′-bis-*O*-(*tert*-butyldimethylsilyl) derivative **1**[5] of 6-fluoro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)purine (6-FP)[10] at 90 °C (4 h) under the reaction conditions described[4] provided the pair of diastereomeric adducts **4a** and **4b** which were easily separated by HPLC on a Du Pont Golden Series SIL column, 6.2 × 80 mm, eluted at 3.0 mL/min with 3% MeOH–20% EtOAc–77% CH_2Cl_2; k' (**4a**) 4.9, k' (**4b**) 6.5.

Steric interference by the bay-region proton (H-5) in **3** restricts the amino group in the tetrahydrobenzo-ring to a pseudoaxial conformation. This diminishes the nucleophilicity of the amino group, and dramatic differences in the reactivities of pseudoequatorial vs pseudoaxial amines have been reported in prior syntheses of dA adducts from tetrahydro and diol epoxide derivatives of polycyclic aromatic hydrocarbons.[7,8] In amino triol **3**, the pseudoequatorial hydroxyl groups at C-1 and C-2 might compete with the pseudoaxial amino group in displacing the fluoride of **1** to give an ether rather than the desired secondary amine. This possibility was ruled out by the demonstration that our synthetic adducts were identical to authentic trans-opened diol epoxide–dA adducts prepared as described[3a,5] from racemic **2** and 2′-deoxyadenosine-5′-monophosphate (dAMP). In the adducts prepared by the latter route, the nitrogen source is the nucleoside, and thus ambiguity concerning the nature of the purine–hydrocarbon linkage is absent. The two pairs of nucleoside adducts (derived from cis and trans opening of the epoxide enantiomers) obtained after enzymatic cleavage of the 5′-phosphate group were easily separated by reversed-phase HPLC (Figure 1). Proton NMR spectra (Table I) of their pentaacetates were used to assign their relative (cis vs trans) stereochemistry of epoxide ring opening at C-4. In particular, for the diol epoxide-2 series, a large value of J for the vicinal pair of trans protons remote from the bay region is characteristic of adducts formed by trans opening of the epoxide ring.[2] Proof of structure of **4a** and **4b** was provided by the observation that the NMR spectra of the pentaacetates obtained upon desilylation and acetylation of these two compounds were identical with those of the pentaacetates of the trans adducts derived from dAMP (see Table I).

Table I. Partial ^{1}H NMR Data[a] for Selected Compounds (See Scheme I) and for the Acetylated Adducts Formed from Racemic 2 upon Reaction with dAMP

	methine hydrogens			
	H-4	H-3	H-2	H-1
synthesized compds				
4a	6.25	4.42	4.22	4.94
	($J_{1,2}$ = 8.4, $J_{2,3}$ = 2.2)			
4b	6.25	4.40	4.22	4.93
	($J_{1,2}$ = 8.4, $J_{2,3}$ = 2.2)			
5a	6.38	5.94	5.88	6.51
	($J_{1,2}$ = 8.4)			
5b	6.36	5.94	5.86	6.50
	($J_{1,2}$ = 9.2, $J_{2,3}$ = ~2.5)			
adducts from dAMP[b] (as acetates)				
cis-(1*S*,2*R*,3*S*,4*S*)[c]	6.85	5.76	5.62	6.30
	($J_{1,2}$ = 2.7, $J_{2,3}$ = 2.4, $J_{3,4}$ = 5.2)			
cis-(1*R*,2*S*,3*R*,4*R*)[c]	6.87	5.78	5.64	6.31
	($J_{1,2}$ = 2.9, $J_{2,3}$ = 2.3, $J_{3,4}$ = 5.3)			
trans-(1*R*,2*S*,3*R*,4*S*) (also from **4a**)	6.35	5.93	5.87	6.50
	($J_{1,2}$ = 8.7, $J_{2,3}$ = $J_{3,4}$ = ~2.0)			
trans-(1*S*,2*R*,3*S*,4*R*) (also from **4b**)	6.35	5.93	5.87	6.50
	($J_{1,2}$ = 9.0, $J_{2,3}$ = 2.5, $J_{3,4}$ = ~3.0)			

[a] In acetone-d_6 at 500 MHz. Line positions (δ) are in ppm, the acetone-d_6 peak (δ 2.04 ppm) being used as reference, and coupling constants (J) are in hertz. [b] Trans and cis indicate stereochemistry of epoxide ring opening at C-4. [c] The N^6-H proton signals for the cis-(4*S*) and cis-(4*R*) adducts appeared as doublets at δ 6.55 ppm with $J_{N\text{-}H,4}$ = 10.2 Hz and at δ 6.60 ppm with $J_{N\text{-}H,4}$ = 10.5 Hz, respectively.

(8) Kim, S. J.; Harris, C. M.; Jung, K.-Y.; Koreeda, M.; Harris, T. M. *Tetrahedron Lett.* **1991**, *32*, 6073–6076.
(9) For a synthesis of phenanthrene 3,4-diol 1,2-epoxides, see: Whalen, D. L.; Ross, A. M.; Yagi, H.; Karle, J. M.; Jerina, D. M. *J. Am. Chem. Soc.* **1978**, *100*, 5218–5221.
(10) Robins, M. J.; Basom, G. L. *Can. J. Chem.* **1973**, *51*, 3161–3169.

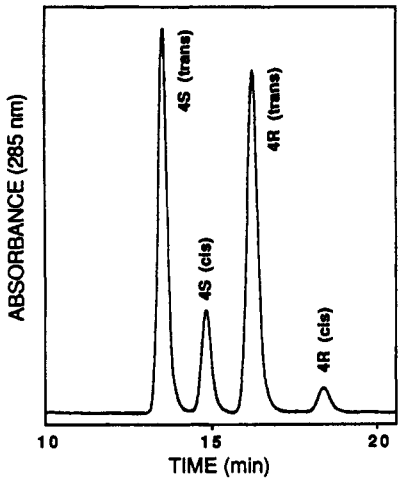


Figure 1. HPLC trace of the mixture of nucleoside adducts formed via reaction of racemic **2** with 2′-deoxyadenosine 5′-monophosphate. For chromatographic conditions, see Experimental Section. Absolute configurations at the N-substituted benzylic carbon atom are indicated. Since trans opening of the epoxide ring results in inversion of configuration at this carbon, the first- and last-eluting peaks are derived from the (1*R*,2*S*)-diol (3*S*,4*R*)-epoxide, whereas the two central peaks are derived from its enantiomer, the (1*S*,2*R*)-diol (3*R*,4*S*)-epoxide.

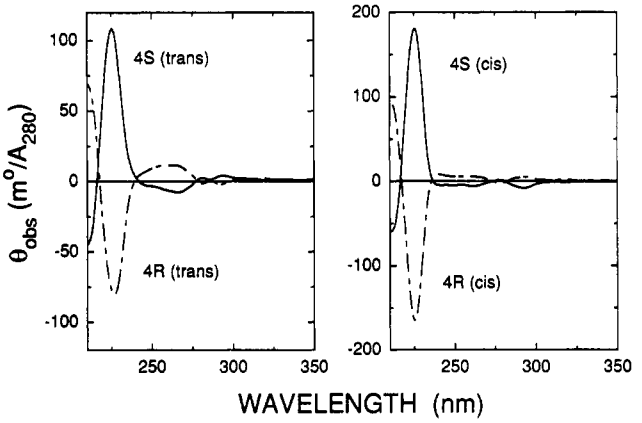


Figure 2. Circular dichroism spectra in methanol, normalized to 1.0 absorbance unit at 280 nm, for the nucleoside adducts formed via reaction of racemic **2** with 2′-deoxyadenosine 5′-monophosphate. The CD spectra of the adducts obtained upon desilylation of **4a** and **4b** were essentially identical to those shown in the left panel for the 4*S* (trans) and 4*R* (trans) adducts, respectively.

Assignment of absolute configurations to the pairs of cis and trans opened adducts obtained from dAMP and the enantiomers of **2** was made on the basis of their CD spectra (Figure 2), which exhibit strong exciton coupling bands between the hydrocarbon and purine moieties. An empirical relationship[11] exists between the shape of the CD curves for diol epoxide–purine nucleoside adducts and their absolute configurations at the N-substituted benzylic carbon. Consistent with this relationship, the intense short wavelength band (225 nm) in the CD spectra of dA adducts derived from optically active tetrahydrophenanthrene 3,4-epoxides[4,5] is negative when the absolute configuration at C-4 is *R* and positive when this absolute configuration is *S*. Since the carbinol centers contribute little to the observed CD spectra, absolute configurations were assigned to the diol epoxide adducts by comparison of their CD spectra (Figure 2) with those of the already assigned tetrahydroepoxide adducts.[5] Thus, a strong positive CD band

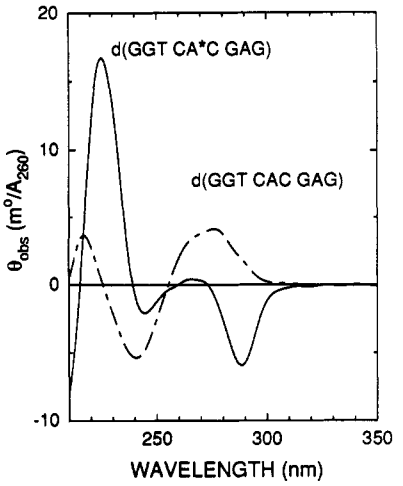


Figure 3. Circular dichroism spectra, normalized to 1.0 absorbance unit at 260 nm, of the normal d(GGT CAC GAG) and adducted d(GGT CA*C GAG) nonanucleotides in 20 mM sodium phosphate buffer (pH 7.0, ionic strength 100 mM maintained with NaCl). The adducted strand corresponds to that which would form by trans opening of the (1*R*,2*S*,3*S*,4*R*) diol epoxide-2 stereoisomer to form the N^6-adduct with (1*R*,2*S*,3*R*,4*S*)-absolute configuration.

at 225 nm requires (4*S*)-configuration, whereas a nearly identical band of opposite sign requires (4*R*)-configuration. CD spectra of the products obtained upon desilylation of **4a** and **4b** were essentially identical to those of the 4*S* (trans) and 4*R* (trans) adducts, respectively, derived from the nucleoside 5′-phosphate (Figure 2), thus establishing the absolute configurations of these synthesized adducts.

Phosphoramidite **9** [(1*R*,2*S*,3*R*,4*S*)-N^6-[4-(1,2,3-triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(*N*,*N*-diisopropylamino)(β-cyanoethoxy)-phosphinyl]-2′-deoxyadenosine, derived from **4a**] was incorporated[4] into the oligonucleotide comprising codons 60–62 of the human K-*ras* b proto-oncogene sequence,[12] d(GGT CA*C GAG), where A* represents the modified residue. Since this adducted oligonucleotide is longer than the modified pentamers reported by us in previous studies,[4,5] its CD spectrum might have been expected to be dominated by interactions among the bases. This is not the case, as indicated by the marked differences between the CD spectra of the normal and modified nonamers (Figure 3). In particular, the strong positive band at 225 nm is ascribed to an interaction between the oligonucleotide and the naphthalene chromophore[5] (^1B_b transition, parallel to the long axis) of the hydrocarbon (4*S*-absolute configuration). The moderately strong negative band at 289 nm, which results in a reversal of sign relative to the unmodified oligonucleotide, presumably involves the longer wavelength transition (^1L_a, parallel to the short axis) of the naphthalene moiety.

Both the normal and adducted nonamers form duplexes upon annealing with the complementary DNA strand, d(CTC GTG ACC), as demonstrated by the melting curves shown in Figure 4. At a total strand concentration of approximately 10 μM, the observed T_m of 43 °C for the normal duplex at 100 mM ionic strength (NaCl) is the same as that calculated[13] (in 1 M NaCl) for this sequence at the same oligonucleotide concentration. Phenanthrene diol epoxide adduct formation at the central dA residue

(11) Sayer, J. M.; Chadha, A.; Agarwal, S. K.; Yeh, H. J. C.; Yagi, H.; Jerina, D. M. *J. Org. Chem.* **1991**, *56*, 20–29.

(12) McCoy, M.; Bargmann, C.; Weinberg, R. *Molec. Cell Biol.* **1984**, *4*, 1577–1582.

(13) Breslauer, K. J.; Frank, R.; Blöcker, H.; Marky, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3746–3750. Marky, L. A.; Breslauer, K. J. *Biopolymers* **1987**, *26*, 1601–1620.

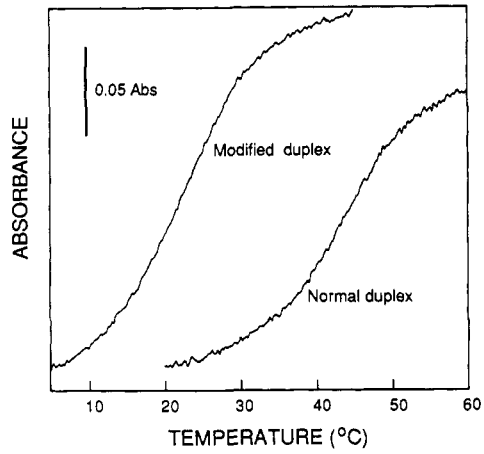


Figure 4. Melting curves for the normal duplex d(GGT CAC GAG)·d(CTC GTG ACC) and the modified duplex containing a phenanthrene diol epoxide adduct at the central dA residue. Measurements were made in the buffer solution described in the legend of Figure 3 at a concentration of each strand of approximately 5 μM (total strand concentration 10 μM).

reduces the T_m to 23 °C. The presence of a phenanthrene diol epoxide adduct has an effect on the T_m of the present nonamer duplex that is similar to that of a 7-methylene-12-methylbenz[*a*]anthracene adduct[14] at N^6 of the central adenine in the duplex between the nonamer d(GTG CAA TCC) and its complementary strand (T_m of this duplex decreased by 11–12 °C in 1.5 M NaCl and by an estimated 20–25 °C in 150 mM salt).

An attractive mechanism for mutagenesis mediated by the presence of an adduct involves the formation of base mismatches opposite the modified base. For example, covalent modification at C-8 of dG in DNA by an aminofluorene moiety leads predominantly to G → T transversions, and the duplex formed between an undecanucleotide thus modified at a dG residue and the complementary strand containing a dA mismatch opposite the dG adduct has a stable structure that has been determined by NMR.[15] Similarly, NMR studies have elucidated the structure of a duplex formed by a nonanucleotide containing a 1,N^6-ethenodeoxyadenosine residue with its complementary strand containing dG opposite the modified dA.[16] The presence of such modified dA residues (formed by reaction of DNA with chloroacetaldehyde) can result in the misincorporation of dG opposite the modified dA during DNA replication in vitro,[17] as well as in A → C transversions in vivo.[16] In the case of a hydrocarbon adduct, alkylation of dA by a benzo[*c*]phenanthrene diol epoxide gives rise to a preponderance of A → T transversions,[18] an observation that suggested the possible stabilization of a dA mismatch opposite a diol epoxide-modified adenine base. In contrast, dA adduct formation by a benzo[*a*]pyrene diol epoxide led to approximately equal numbers of A → T, A → C, and A → G substitutions.[19] To explore the possibility that specific base mismatches may be favored opposite a diol epoxide-modified base, we determined T_m values for the duplexes formed from the modified nonamer and the mismatch-containing strands, d(CTC GAG ACC) and d(CTC GGG ACC) (Table II). The presence of an adduct decreased the selectivity for the base in the complementary position, although some selectivity remained. Thus, duplexes of approximately equal thermal stability were formed with either T or dG opposite the phenanthrene diol epoxide–dA adduct, whereas a *less stable* duplex was formed with dA in this position. Further studies are in progress to ascertain whether preference for a particular base mismatch in the position complementary to a diol epoxide adduct depends on the parent hydrocarbon. It will also be of interest to determine whether a preference for specific mismatches in oligonucleotide duplexes as a result of diol epoxide adduct formation is a good predictor of nucleotide misincorporation during replication of DNA thus modified.

Table II. Melting Temperatures of Normal d(GGT CAC GAG) and Adducted Oligonucleotide Duplexes with the Fully or Partially Complementary Strands d(CTC GXG ACC) as a Function of the Base, X, Opposite the Central Deoxyadenosine Residue[a]

X	T_m (normal duplex)	T_m (adducted duplex)
T	43	23
G	36	24
A	25	14[b]

[a] For conditions see text. [b] No change was observed in the T_m upon decreasing the pH to 5.8.

Experimental Section

Proton NMR data were obtained at 500 MHz in acetone-d_6. Chemical shifts (δ) are reported in ppm, and coupling constants (*J*) are reported in Hz. For the adduct derivatives, the conventional phenanthrene numbering has been used for the hydrocarbon, singly primed numbers (1′–5′) are used for the deoxyribose unit, and doubly primed numbers (2″ and 8″ for the protons) are used for the purine.

N^6-[4-(1,2,3-Trihydroxy-1,2,3,4-tetrahydrophenanthrenyl)]-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (4a and 4b). 6-Fluoro-9-(2-deoxy-3,5-bis-*O*-(*tert*-butyldimethylsilyl)-β-D-*erythro*-pentofuranosyl)purine (1) (70 mg, 0.15 mmol) and (±)-aminotriol **3** (18 mg, 73.5 μmol) were heated in a mixture of DMF (1 mL), 2,6-lutidine (17 μL, 0.15 mmol), and HMDS (0.78 mL, 3.7 mmol) at 90 °C. The reaction was monitored by reversed-phase HPLC (Beckman Ultrasphere ODS, 5 μm, 4.6 × 250 mm, eluted at 1.5 mL/min with a gradient that was ramped from 40% MeOH in 10 mM NH_4OAc (pH 7) to 100% MeOH over 30 min. After 4 h the reaction was complete as evidenced by the disappearance of the amino triol (t_R 3.5 min) and the appearance of the adducts (t_R 33.4 min). Evaporation of the cooled reaction mixture under reduced pressure was followed by addition of toluene, evaporation, and drying under vacuum. Addition of MeOH and evaporation gave an off white-gray solid, which was subjected to HPLC separation on a Du Pont Golden Series SIL column (6.2 × 80 mm) eluted at 3 mL/min with 3% MeOH–20% EtOAc–77% CH_2Cl_2. The *early*-eluting **4a** (t_R 4.2 min, k' 4.9) and *late*-eluting **4b** (t_R 5.4 min, k' 6.5) diastereomers were obtained in yields of 23 and 25 mg, respectively, for a combined yield of 90%: ^{1}H NMR **4a** δ 8.46, 8.14 (2s, 2 $H_{2'',8''}$); 7.78–7.92 (m, 4 $H_{5,8,9,10}$); 7.42 (t, 1 H_6, J = 7.0); 7.35 (t, 1 H_7, J = 7.0); 6.45 (t, 1 $H_{1'}$, J = 6.6); 6.25 (br s, 1 H_4); 4.94 (d, 1 H_1, J = 8.4); 4.77 (m, 1 $H_{3'}$); 4.42 (br s, 1 H_3); 4.22 (dd, 1 $H_{2'}$, J = 2.2, 8.4); 3.98 (m, 1 $H_{4'}$); 3.91 (dd, 1 $H_{5'}$, J = 5.5, 11.0); 3.78 (dd, 1 $H_{5'}$, J = 3.7, 11.0); 2.98 (m, 1 $H_{2'}$); 2.45 (ddd, 1 $H_{2'}$, J = 4.0, 6.2, 13.2); 0.88–0.90 (18 H, *t*-Bu); 0.07–0.20 (12 H, Me); ^{1}H NMR **4b** δ 8.46, 8.10 (2s, 2 $H_{2'',8''}$); 7.76–7.94 (m, 4 $H_{5,8,9,10}$); 7.42 (t, 1 H_6, J = 7.0); 7.35 (t, 1 H_7, J = 7.0); 6.44 (t, 1 $H_{1'}$, J = 6.2); 6.25 (br s, 1 H_4); 4.93 (d, 1 H_1, J = 8.4); 4.78 (m, 1 $H_{3'}$); 4.40 (br s, 1 H_3); 4.22 (dd, 1 H_2, J = 2.2, 8.4); 3.98 (m, 1 $H_{4'}$); 3.92 (dd, 1 $H_{5'}$, J = 5.5, 11.0); 3.80 (dd, 1 $H_{5'}$, J = 4.0, 11.0); 2.98 (m, 1 $H_{2'}$); 2.45 (ddd, 1 $H_{2'}$, J = 4.0, 6.2, 13.2); 0.88–0.90 (18 H, *t*-Bu); 0.07–0.20 (12 H, Me); HRMS calcd for $C_{36}H_{54}N_5O_6Si_2$

(14) Stezowski, J. J.; Joos-Guba, G.; Schönwälder, K.-H.; Straub, A.; Glusker, J. P. *J. Biomol. Struct. Dyn.* **1987**, *5*, 615–637.
(15) Norman, D.; Abuaf, P.; Hingerty, B. E.; Live, D.; Grunberger, D.; Broyde, S.; Patel, D. J. *Biochemistry* **1989**, *28*, 7462–7476.
(16) de los Santos, C.; Kouchakdjian, M.; Yarema, K.; Basu, A.; Essigman, J.; Patel, D. J. *Biochemistry* **1991**, *30*, 1828–1835.
(17) Singer, B.; Spengler, S. J. In *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*; Singer, B., Bartsch, H., Eds.; IARC Scientific Publications 70; IARC: Lyon, 1986; pp 359–371.
(18) Bigger, C. A. H.; Strandberg, J.; Yagi, H.; Jerina, D. M.; Dipple, A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2291–2295.
(19) Wei, S.-J. C.; Chang, R. L.; Wong, C.-Q.; Bhachech, N.; Cui, X. X.; Hennig, E.; Yagi, H.; Sayer, J. M.; Jerina, D. M.; Preston, B. D.; Conney, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11227–11230.

(M + 1) 708.3613, found (**4a**, *early*) 708.3646 and (**4b**, *late*) 708.3666.

(1*R*,2*S*,3*R*,4*S*)-*N*^{6}-[4-(1,2,3-Triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (5a). The *early*-eluting diastereomer **4a** (21 mg, 30 μmol) was stirred in 1:1 Ac_2O/pyridine (1 mL) overnight at rt. The mixture was evaporated, and the residue was repeatedly treated with PhH which was evaporated. After being dried under vacuum, **5a** (24 mg, quant) gave a single peak (t_R 2.4 min) upon analytical HPLC on a Du Pont Golden Series SIL column (6.2 × 80 mm) eluted at 3 mL/min with 1% MeOH in CH_2Cl_2 and was used without further purification in the subsequent step: ^{1}H NMR δ 8.46, 8.10 (2s, 2 $H_{2'',8''}$); 7.95 (m, 2 $H_{8,9}$); 7.88 (d, 1 H_5, J = 8.1); 7.78 (br, N*H*); 7.51 (t, 1 H_6, J = 7.0); 7.42 (t, 1 H_7, J = 7.0); 7.36 (d, 1 H_{10}, J = 8.8); 6.51 (d, 1 H_1, J = 8.4); 6.47 (t, 1 $H_{1'}$, J = 6.6); 6.38 (br, 1 H_4); 5.94 (narrow m, 1 H_3); 5.88 (br d, 1 H_2, J = 8.4); 4.78 (br s, 1 $H_{3'}$); 3.98 (m, 1 $H_{4'}$); 3.92 (dd, 1 $H_{5'}$, J = 5.5, 11.0); 3.80 (dd, 1 $H_{5'}$, J = 3.7, 11.0); 3.00 (quint, 1 $H_{2'}$, J_{app} = 6.2); 2.46 (ddd, 1 $H_{2'}$, J = 3.7, 5.9, 13.2); 1.90–2.20 (9 H, OAc); 0.80–0.90 (18 H, *t*-Bu); 0.07–0.20 (12 H, Me); HRMS calcd for $C_{42}H_{60}N_5O_9Si_2$ (M + 1) 834.3930, found 834.3959.

(1*S*,2*R*,3*S*,4*R*)-*N*^{6}-[4-(1,2,3-Triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyadenosine (5b). The *late*-eluting diastereomer **4b** (22 mg, 31 μmol) was acetylated as described for the *early*-eluting diastereomer. The product **5b** from this reaction (24 mg, quant) also gave a single peak (t_R 3 min) on HPLC as above: ^{1}H NMR δ 8.46, 8.04 (2s, 2 $H_{2'',8''}$); 7.95 (m, 2 $H_{8,9}$); 7.88 (d, 1 H_5, J = 8.0); 7.80 (br, N*H*); 7.51 (t, 1 H_6, J = 7.0); 7.43 (t, 1 H_7, J = 7.0); 7.36 (d, 1 H_{10}, J = 8.8); 6.50 (d, 1 H_1, J = 9.2); 6.44 (br, 1 $H_{1'}$); 6.36 (br, 1 H_4); 5.94 (t, 1 H_3, J_{app} = 2.6); 5.86 (br d, 1 H_2, J = 9.2); 4.80 (m, 1 $H_{3'}$); 3.98 (m, 1 $H_{4'}$); 3.93 (dd, 1 $H_{5'}$, J = 5.5, 11.0); 3.80 (dd, 1 $H_{5'}$, J = 3.7, 11.0); 3.02 (quint, 1 $H_{2'}$, J_{app} = 6.2); 2.45 (m, 1 $H_{2'}$); 1.90–2.20 (9 H, OAc); 0.80–0.90 (18 H, *t*-Bu); 0.07–0.20 (12 H, Me); HRMS calcd for $C_{42}H_{60}N_5O_9Si_2$ (M + 1) 834.3930, found 834.3956. Anal. Calcd for $C_{42}H_{59}N_5O_9Si_2$: C, 60.48; H, 7.13. Found: C, 60.67; H, 6.75.

(1*R*,2*S*,3*R*,4*S*)-*N*^{6}-[4-(1,2,3-Triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-2′-deoxyadenosine (6a). To a stirred solution of disilyl triacetate **5a** (22 mg, 26 μmol) in anhydrous THF was added n-$Bu_4N^+F^-$ in THF (58 μL of a 1 M solution, 2.2 equiv), and the mixture was stirred at rt for 1.5 h. Evaporation of the reaction mixture followed by chromatography of the crude product on a 500-μm, 20- × 20-cm silica preparative TLC plate developed with 10% MeOH in CH_2Cl_2 gave **6a** (11 mg, 69%) as a clear glass: ^{1}H NMR δ 10.80 (s, N*H*); 8.45, 8.07 (2s, 2 $H_{2'',8''}$); 7.98 (d, 1 H_9, J = 8.8); 7.95 (d, 1 H_8, J = 8.1); 7.88 (d, 1 H_5, J = 8.1); 7.51 (t, 1 H_6, J = 8.1); 7.44 (t, 1 H_7, J = 8.4); 7.36 (d, 1 H_{10}, J = 8.8); 6.50 (d, 1 H_1, J = 9.2); 6.43 (br t, 1 $H_{1'}$, J = 7.0); 6.35 (br, 1 H_4); 5.93 (br m, 1 H_3); 5.84 (d, 1 H_2, J = 9.2); 4.63 (d, 1 $H_{3'}$, J = 5.1); 4.08 (br, 1 $H_{4'}$); 3.80 (m, 1 $H_{5'}$); 3.68 (dd, 1 $H_{5'}$, J = 2.9, 12.5); 2.92 (m, 1 $H_{2'}$); 2.37 (m, 1 $H_{2'}$); 1.90–2.20 (9 H, OAc); HRMS calcd for $C_{30}H_{32}N_5O_9$ (M + 1) 606.2200, found 606.2175.

(1*S*,2*R*,3*S*,4*R*)-*N*^{6}-[4-(1,2,3-Triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-2′-deoxyadenosine (6b). Disilyl triacetate **5b** from the *late* diastereomer was desilylated as above using 61 μL of n-$Bu_4N^+F^-$ in THF. Chromatography as described gave the desilylated compound **6b** (11.6 mg, 69%) as a pale yellow solid: ^{1}H NMR δ 10.80 (s, N*H*); 8.45, 8.10 (2s, 2 $H_{2'',8''}$); 7.95 (m, 2 $H_{8,9}$); 7.86 (d, 1 H_5, J = 8.1); 7.51 (t, 1 H_6, J = 7.3); 7.46 (t, 1 H_7, J = 8.1); 7.36 (d, 1 H_{10}, J = 8.4); 6.49 (d, 1 H_1, J = 9.2); 6.42 (br t, 1 $H_{1'}$, J = 7.0); 6.37 (br, 1 H_4); 5.93 (t, 1 H_3, J = 2.2); 5.86 (d, 1 H_2, J = 9.2); 4.64 (d, 1 $H_{3'}$, J = 5.5); 4.08 (m, 1 $H_{4'}$); 3.82 (dd, 1 $H_{5'}$, J = 2.6; 12.5); 3.70 (br d, 1 $H_{5'}$, J = 12.5); 2.94 (septet, 1 $H_{2'}$, J_{app} = 5.1); 2.36 (dd, 1 $H_{2'}$, J = 5.1, 12.8), 1.90–2.20 (9 H, OAc); HRMS calcd for $C_{30}H_{32}N_5O_9$ (M + 1) 606.2200, found 606.2198.

(1*R*,2*S*,3*R*,4*S*)-*N*^{6}-[4-(1,2,3-Triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine (7). Nucleoside **6a** (25 mg, 41 μmol), Li_2CO_3 (6.1 mg, 82 μmol), and $DMT^+BF_4^-$ (41 μmol) were dried under vacuum for 15 min. 2,6-Lutidine (0.1 mL) was added, and the mixture was stirred under argon at rt for 1.5 h. Since TLC showed the presence of starting nucleoside another portion of $DMT^+BF_4^-$ (41 μmol) was added, and the mixture was stirred for an additional 1.5 h. The mixture was cooled to 0 °C, and another equivalent of $DMT^+BF_4^-$ (41 μmol) was added. The mixture was allowed to warm to rt and stirred for 10 min. Cooling, addition of the DMT reagent, and stirring for 10 min at rt were repeated an additional time, after which reaction was essentially complete. The mixture was diluted with CH_2Cl_2/aq $NaHCO_3$. The aqueous layer was extracted once with CH_2Cl_2, and the combined organic layers were dried (Na_2SO_4) and evaporated. Chromatography of the crude mixture on a silica column packed in 1% Et_3N in CH_2Cl_2 and eluted with 1% Et_3N–2% MeOH–97% CH_2Cl_2 gave **7** (26 mg, 69%) as a pale yellow foam: ^{1}H NMR δ 8.40, 8.03 (2s, 2 $H_{2'',8''}$); 7.95 (d, 1 H_9, J = 8.4); 7.93 (d, 1 H_8, J = 8.1); 7.86 (d, 1 H_5, J = 7.7); 7.74 (s, N*H*); 6.80–7.50 (aromatic, 16 H); 6.50 (m, 2 $H_{1,1'}$); 6.36 (br s, 1 H_4); 5.94 (t, 1 H_3, J = 2.6); 5.88 (d, 1 H_2, J = 8.8); 4.69 (m, 1 H_3); 4.14 (m, 1 H_4); 3.72 (s, 6 H, OMe); 3.35 (m, 2 $H_{5'}$); 2.97 (m, 1 $H_{2'}$); 2.48 (m, 1 $H_{2'}$); 1.90–2.20 (9 H, OAc).

(1*R*,2*S*,3*R*,4*S*)-*N*^{6}-[4-(1,2,3-Triacetoxy-1,2,3,4-tetrahydrophenanthrenyl)]-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-[(*N*,*N*-diisopropylamino)(β-cyanoethoxy)phosphinyl]-2′-deoxyadenosine (9). The 5′-DMT blocked nucleoside **7** (26 mg, 28 μmol) was stirred in anhydrous CH_2Cl_2 (0.5 mL). *N*,*N*-Diisopropylethylamine (25 μL, 0.14 mmol) was added to the mixture, and the reaction vial was transferred to a glove bag filled with dry argon. 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (13 μL, 56 μmol) was added. The vial was capped under argon, and the reaction mixture was stirred at rt for 50 min. The mixture was diluted with CH_2Cl_2 and washed twice with brine. After drying (Na_2SO_4) and evaporation of the organic layer, the crude product was chromatographed on a silica column packed in 1% Et_3N in CH_2Cl_2 and eluted with 1% Et_3N–3% MeOH–96% CH_2Cl_2. Phosphoramidite **9** was obtained as a white solid (28 mg, 90%). The mixture of diastereomers **9** was of high purity as judged by its ^{31}P NMR (121.4 MHz in acetone, 0.1 M phosphoric acid as an external standard): 149.86, 149.81 ppm.

Oligonucleotide Synthesis. The modified oligonucleotide d(GGT CA*C GAG) was prepared following the method described[4] using a 2-μmol controlled pore glass (CPG) column on an Applied Biosystems Model 292 synthesizer, with an anhydrous solution of *tert*-butyl hydroperoxide as the oxidant to minimize aqueous contamination of the support-bound oligonucleotide. A manual step (1.8 h) was used for the coupling of the modified nucleoside phosphoramidite **9** (6.4 mg, 5.4 μmol) to the growing chain. A yield for this step of ~15% relative to the CPG-bound oligonucleotide was obtained, based on the yields of DMT cation released immediately prior and subsequent to the coupling. The adducted 5′-DMT nonamer, after cleavage from the column and standard deblocking, was purified by HPLC on a Hamilton PRP-1 column (10 μ, 7 × 305 mm) eluted at 2.5 mL/min with a gradient of 20% B to 60% B over 15 min, followed by a ramp to 100% B over the next 5 min, where solvent A is 0.1 M $(NH_4)_2CO_3$, pH 7.5, and solvent B is a 1:1 mixture of A with CH_3CN, adjusted after mixing to pH 7.5; t_R 16.3 min; yield 14.6 A_{260} units. Following detritylation (80% AcOH in H_2O) and extraction of the DMT alcohol with ethyl acetate, 12.5 A_{260} units of the product d(GGT CA*C GAG) were obtained, corresponding to ~16% of the amount of the unmodified oligonucleotide obtained by synthesis on the same scale using a fully automated but otherwise identical procedure. The adducted oligonucleotide, which contained only trace impurities by reversed-phase HPLC, was subjected to a final purification on the Hamilton column, eluted at 2.5 mL/min with a gradient of 0–35% B over 20 min, followed by a ramp to 100% B over 10 min; t_R 19.7 min, yield 8.8 A_{260} units. The resultant material was homogeneous (≥95%) by capillary zone electrophoresis (elution at 13.9 min) under the conditions described[4] as well as by ion-exchange chromatography (Dionex NucleoPac PA-100, 4 × 250 mm, eluted at 1.5 mL/min with a linear gradient from 0.15 M to 1.5 M ammonium acetate, pH 6.0, in 10% acetonitrile over 18 min); t_R 12 min. The sample was dissolved in 5% aqueous ammonium hydroxide and analyzed by negative-ion electrospray MS, 2991.9; calcd 2992.1. Unmodified oligonucleotides were prepared by automated synthesis (1 or 2 μmol scale) and purified as their 5′-DMT derivatives on the Hamilton column, eluted at 2.5 mL/min with a gradient of 20–100% B in 15 min. Electrospray MS, d(GGT CAC GAG), 2764.0; d(CTC GTG ACC), 2674.1; d(CTC GAG ACC), 2682.5; d(CTC GGG ACC), 2699.7, were as expected.

Determination of T_m. Melting curves of oligonucleotide duplexes were measured in 20 mM sodium phosphate buffer, pH

7.0 ± 0.1 (Na_2HPO_4/NaH_2PO_4 (6:4)), containing sufficient NaCl to give a total ionic strength of 100 mM. The concentration of each single-stranded oligonucleotide was estimated from its absorbance at 260 nm (H_2O) and published extinction coefficients[20] for the mononucleotides, without compensation for hypochromicity. A sufficient quantity of each strand was introduced into the buffer to give a ~5 μM solution of that strand. (Thus, the duplex concentration would be ~5 μM whereas the total concentration of both single strands would be ~10 μM.) The strands were annealed by heating at least 10 °C above the T_m, followed by cooling (1 °C/min) at least 14 °C below the T_m. Melting curves were recorded at 260 nm [d(GGT CAC GAG)·d(CTC GTG ACC)] or 273 nm (all others) in a stirred 3-mL cuvette using a spectrophotometer equipped with a temperature-programmable cell holder and a temperature probe. Initial temperatures were selected so that the T_m fell near the midpoint of the 40° temperature gradient used. The temperature was ramped up at a rate of 0.5 °C/min over a period of 80 min. The absorbance was recorded as a function of time, which was converted to temperature, and the transition temperature (T_m) was determined from the midpoint of the resultant sigmoid curve of absorbance vs temperature. In several cases repeated determinations on the same sample verified the reproducibility of the measurements and the reversibility of the thermal transition.

Formation of N^6-Adducts from 1,2,3,4-Tetrahydrophenanthrene-1,2-diol 3,4-Epoxide (2) and 2'-Deoxyadenosine 5'-Monophosphate and Comparison with Adducts Derived from the Coupling of 1 and 3. The adducts were prepared as described[3a,5] from 2'-deoxyadenosine 5'-monophosphate (250 mg in 12.5 mL of H_2O, pH 7.25) and the racemic diol epoxide **2** (5 mg, 20 μmol in 1 mL of acetone). The nucleoside adducts obtained upon enzymatic hydrolysis (*E. coli* alkaline phosphatase, 19 units) were separated by HPLC on a Beckman Ultrasphere ODS column (5 μm, 10 × 250 mm), eluted at 3 mL/min with 11% CH_3CN–30% MeOH–59% H_2O (Figure 1). Absolute configurations were assigned by comparison of the CD spectra of the adducts in MeOH (Figure 2) with those of the previously characterized adducts derived from tetrahydrophenanthrene 3,4-oxide.[5] For these adducts, a strong positive CD band at 225 nm corresponds to (4*S*)-absolute configuration at the N-substituted benzylic carbon atom, and a band at the same wavelength with approximately the same magnitude but opposite sign corresponds to (4*R*)-absolute configuration at this center.

Proton NMR spectra (see Table I) were measured for the pentaacetate derivatives (72 h at rt with pyridine/Ac_2O). These acetates were purified by HPLC on a Du Pont Golden Series SIL column (6.2 × 80 mm) eluted at 2.5 mL/min with 1.5% MeOH–4.9% EtOAc–93.6% CH_2Cl_2: t_R (min) (trans-4*S*-adduct), 3.8; (trans-4*R*-adduct), 3.7; (cis-4*S*-adduct), 3.2; cis-4*R*-adduct), 2.9.

For comparison with the adducts prepared from dAMP, a mixture of **4a** and **4b** was prepared as described from **1** (20 mg) and amino triol **3** (10 mg) in a mixture of DMF (0.7 mL), pyridine (0.03 mL), and HMDS (0.87 mL) at 90 °C for about 18 h. The adducts were directly desilylated with 0.5 mL of n-$Bu_4N^+F^-$ (1 M solution in THF) over 2 h and evaporated to dryness. The entire reaction mixture containing the diastereomeric *early*- and *late*-eluting adducts was subjected to preparative HPLC as described above for the adducts prepared from dAMP. The separated adducts were acetylated with pyridine (100 μL) and Ac_2O (50 μL) in the presence of DMAP (1 mg) at 50 °C overnight. After evaporation of the pyridine, chromatography of the products on a 250-μm silica plate developed with 5% MeOH in CH_2Cl_2 gave 2 mg each of **8** (4*S*) and the corresponding (4*R*)-pentaacetate. The ^1H NMR spectra of both pentaacetates were identical with those of the pentaacetates of the trans adducts derived from the reaction of racemic phenanthrene diol epoxide-2 with dAMP.

Supplementary Material Available: Proton NMR spectra of compounds **4a,b–9** (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(20) Dawson, R. M. C.; Elliott, D. C.; Elliott, W. H.; Jones, K. M. *Data for Biochemical Research*, 3rd Ed.; Clarendon Press: Oxford, 1986; pp 103–114.

Partial Synthesis of 9,10-Syn Diterpenes via Tosylhydrazone Reduction: (−)-(9β)-Pimara-7,15-diene and (−)-(9β)-Isopimaradiene[1]

Min Chu and Robert M. Coates*

Department of Chemistry, University of Illinois, 1209 West California Street, Urbana, Illinois 61801

Received March 9, 1992

(9β)-Pimara-7,15-diene (**3**), a proposed intermediate in the biosynthesis of the momilactone phytoalexins (**1** and **2**) from rice, and its C-13 epimer, (9β)-isopimara-7,15-diene (**4**), were synthesized from methyl pimara- and isopimara-8,15-dien-18-oates (**8b** and **8a**, respectively). Allylic oxidation of **8a** and **8b** as well as the derived diterpene hydrocarbons **15a** and **15b** with chromium trioxide–dipyridine complex afforded 8,15-dien-7-ones **9a**, **9b**, **16a**, and **16b** (35–54%). Lithium–ammonia reduction of **9a**, **16a**, and **16b** gave predominantly *trans,anti,trans*-isopimara- and -pimara-15-en-7-ones **10**, **17a**, and **17b**. In contrast, catecholborane reduction of the tosylhydrazones of **9a** and **9b** provided methyl (9β)-isopimara- and (9β)-pimara-7,15-dien-20-oates (**23a** and **23b**) having the 9,10-syn stereochemistry. The parent diterpenes, **3** and **4**, were obtained by carboxyl-to-methyl conversions. In a collaborative investigation **3** was tentatively identified as one of five diterpene hydrocarbons produced upon incubation of (*E,E,E*)-geranylgeranyl pyrophosphate with a crude enzyme extract from UV-treated rice plants.

The momilactones A (**1**), B (**2**), and C comprise a small group of oxygenated diterpenes isolated from rice husk.[2] Characterized originally as germination inhibitors,[2a] momilactones A and B were subsequently identified[3] as phytoalexins[4] of the rice plant, *Oryza sativa*. The most notable structural feature of these pimara-7,15-diene[5]

(1) Portions of this research were presented at the following meetings of the American Chemical Society: National Convention, Atlanta, GA, April 17, 1991; Great Lakes Regional Meeting, Dekalb, IL, May 31, 1990.

(2) (a) Kato, T.; Tsunakawa, M.; Sasaki, N.; Aizawa, H.; Fujita, K.; Kitahara, Y.; Takahashi, N. *Phytochem.* **1977**, *16*, 45–58. (b) Kato, T.; Aizawa, H.; Tsunakawa, M.; Sasaki, N.; Kitahara, Y.; Takahashi, N. *J. Chem. Soc., Perkin Trans. 1* **1977**, 250–254. (c) Tsunakawa, M.; Ohba, A.; Sasaki, N.; Kabuto, C.; Kato, T.; Kitahara, Y.; Takahashi, N. *Chem. Lett.* **1976**, 1157–1158.

(3) (a) Cartwright, D. W.; Langcake, P.; Pryce, R. J.; Leworthy, D. P.; Ride, J. P. *Nature* **1977**, *267*, 511–513. (b) Cartwright, D. W.; Langcake, P.; Ride J. P. *Physiol. Plant Pathol.* **1980**, *17*, 259–267. (c) Cartwright, D. W.; Langcake, P.; Pryce, R. J.; Leworthy, D. P.; Ride, J. P. *Phytochem.* **1981**, *20*, 535–537.

(4) (a) Bailey, J. A.; Mansfield, J. W. *Phytoalexins*; J. Wiley: New York, 1982. (b) West, C. A. *Naturwiss.* **1981**, *68*, 447–457.

0022-3263/92/1957-4590$03.00/0