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Benzo(*a*)pyrene-7,8-dihydrodiol 9,10-Oxide Adenosine and Deoxyadenosine Adducts: Structure and Stereochemistry

Abstract. *The structure and absolute stereoconfigurations of four adenosine adducts with (±)-7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (BPDE) and their deoxyadenosine analogs have been determined. They result from both* cis *and* trans *addition of the* N^6 *amino group of adenine to the 10 position of both enantiomers of BDPE. This was determined from studies of the nuclear magnetic resonance spectra, mass spectra, and circular dichroism spectra, as well as from their* pK_a *values and chemical reactivities.*

Considerable evidence has accumulated that the ultimate oncogenic metabolite of the ubiquitous environmental carcinogen benzo(*a*)pyrene (BP) is a 7,8-dihydrodiol 9,10-oxide (BPDE) (*1*). In previous studies from this laboratory (*2*) we demonstrated that the predominant RNA and DNA adduct present in human cells exposed to BP is formed by covalent linkage of the amino group at the 2 position of guanine (N^2) to the 10 position of a specific isomer: (+)-7β,8α dihydroxy-9α, 10α-epoxy7,8,-9,10-tetrahydrobenzo(*a*)pyrene (7*R*-BPDE). This adduct has also been detected in rodent cell cultures (*3*, *4*) and in mouse skin (*5*), and its complete stereochemistry has also been elucidated (*6*). Lesser amounts of N^2-substituted deoxyguanosine adducts derived from (±)-7β,8α-dihydroxy-9β,10β-expoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene have also been detected in vivo (*7*), and there is indirect evidence for the reaction of BPDE at the 7 position of guanine (*8*) and with phosphates of the DNA backbone (*5*).

In vitro studies with BPDE have demonstrated that under particular conditions there can also be significant reaction with adenine and to a lesser extent cytosine residues in synthetic homopolymers, RNA, and DNA (*9*, *10*). Small amounts of the DNA adducts have been tentatively identified in certain cell culture systems exposed to BP (*4*). There is also a report suggesting that the conformation of the DNA at sites of BPDE modification of adenine residues differs from that at sites of guanine modification (*11*). Because at present it is not known which of these types of nucleic acid modification are the most critical with respect to the carcinogenic process, we have elucidated the complete chemical structure and stereochemistry of certain BPDE-adenine adducts.

The quantities of deoxyadenosine derivatives that could be prepared were limited by the poor reactivity of deoxyadenosine monophosphate (dAMP), by the high cost of polydeoxyadenylate [poly(dA)], and by the difficulty experienced in hydrolyzing BPDE-modified poly(dA). Experiments were therefore directed toward elucidating the structures of the adenosine derivatives formed by modifying polyadenylate [poly(A)] with BPDE and then comparing these products with those formed with poly(dA) or dAMP. Poly(A) was reacted with (±)-BPDE and hydrolyzed to mononucleosides, and the modified residues were separated by LH-20 column chromatography and high-pressure liquid chromatography (HPLC). As was previously described (*10*), we obtained four major derivatives, which were shown to be BPDE-adenosine adducts by virtue of their pyrene-like ultraviolet absorption spectra and their circular dichroism (CD) spectra. The CD spectra of these four compounds (A-BPDE, **1** to **4**) were essentially identical in both shape and magnitude, except that the pairs **1** and **2** were opposite in sign from pairs **3** and **4** (Fig. 1). This result indicates that the spacial relationship between the pyrene and adenine chromophores is the same and thus the position of attachment of the two moieties is the

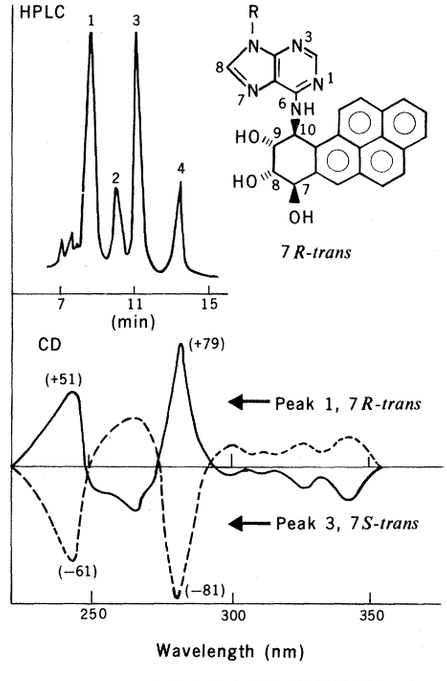

Fig. 1. High-pressure liquid chromatography profiles were obtained on a Du Pont 830 instrument with a Rainin C-18 column, 5-μm particle size, 250 by 2.1 mm (A-BPDE derivatives) or a Whatman ODS-2 column (dA-BPDE derivatives) operated with water-methanol mixtures at 50°C; the eluant was monitored at 280 nm. Preparative runs were performed on Rainin columns (250 by 6.4 mm). The CD spectra were measured in mixtures of water and methanol (1:1) on a JASCO J-40 instrument (*10*, *13*). All the A-BPDE and dA-BPDE derivatives, designated to **1–4** and **1′–4′**, respectively, by their HPLC elution order, had similar ultraviolet spectra which were dominated by the pyrene chromophore and were similar to that seen previously for the guanosine derivative (*2*). All the CD spectra were similar to each other except in sign. The molar ellipticities (Δε) at 244 and 279 nm, the absolute stereo configurations of the 7-hydroxyl group on addition of the adenine moities to the original BPDE were as follows: A-BPDE peak 1, +51, +79, 7*R-trans*; peak 2, +35, +60, 7*S-cis*; peak 3, −61, −81, 7*S-trans*; peak 4, −49, −81, 7*R-cis*; dA-BPDE peak 1′, +50, +68, 7*R-trans*; peak 2′, −49, −66, 7*S-trans*; peak 3′, +40, +61, 7*S-cis*; peak 4′, −36, −67, 7*R-cis*.

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same in all four compounds. To determine which adducts were derived from which of the two enantiomers of BPDE, poly(A) was reacted with the optically pure 7*R*-(+) enantiomer of BPDE (*6*). The modified polymer was digested, and the adenosine adducts were separated by HPLC. This sample contained only compounds A-BPDE **1** and A-BPDE **4**; thus, compounds A-BPDE **2** and **3** must be derived from the 7*S* enantiomer. The CD spectra were also used in *p*H titrations to determine the pK_a values of the adducts in 90 percent aqueous methanol (*12*). The values were in the range of 2.0 to 2.7, in good agreement with the values obtained when N^6-methyl- and N^6-(2-isopentenyl)adenosines (pK_a of 2.9 and 3.0, respectively) were measured in the same solvent. These results are consistent with attachment of the BPDE residue to N^6, rather than to the 1 or 7 positions of adenine (for additional evidence see below).

The nuclear magnetic resonance (NMR) spectra of compounds A-BPDE **3** and **4** were recorded in completely deuterated methanol (CD_3OD) (Fig. 2) and compared with the previously studied BPDE-guanosine derivatives (*13*). These spectra indicated that addition of the adenine was to the 10 position of the BPDE. Sterically it is most likely that the adenine residue is axial. From Dreiding models, we would then expect that the value of $J_{7,8}$ (J, spin-spin coupling constant) would be larger for *trans* than for *cis* addition. Thus compound **3**, with $J_{7,8}$ = 9 Hz, appears to be the *trans* adduct; whereas compound **4**, with $J_{7,8}$ = 3 Hz, is the *cis* adduct. In both cases the value of $J_{9,10}$ would be expected to be small; and this was the case (a singlet and 4 Hz, respectively). The downfield shift of 0.4 ppm of the hydrogen at position 10 of pyrene in compound **4**, when compared with compound **3**, is probably due to a slight difference in orientation with respect to the pyrene ring. The difference of about 0.3 ppm in the chemical shifts of the hydrogens at position 8 of adenine in compounds **3** and **4** may result from interactions with the hydroxyl group at the 9-*cis* position (in contrast to the *trans*) with the 7 position of adenine.

Compounds A-BPDE **3** and **4**, as their peracetates, had the same mass spectra with molecular ions at *m/e* (mass to charge) equal to 821. The high-resolution mass spectrum (*14*) of compound **4** showed a fragment of mass 444.1436 (65 percent relative abundance), which corresponded to $C_{26}H_{22}NO_6$ (444.1447). This fragment reflects transfer of the amino group from the 6 position of adenine (N^6) to the hydrocarbon moiety. A fragment of identical nominal mass 444, corresponding to losses of the acetylated ribose moiety and two acetic acids ($C_{27}H_{18}N_5O_2$ 444.1460), was not seen. This fragmentation pattern, which is similar to that previously seen with BPDE-guanosine adducts (*13*), confirms the point of attachment as being through the N^6 amino group (*14*). This assignment is further supported by the failure of the adducts to react with dimethylformamide dimethylacetal (*15*), while the model compounds adenosine, 1-methyladenosine, 2-methyladenine, and N^6-methyladenosine reacted with this reagent as predicted.

The BPDE deoxyadenosine derivatives were much more difficult to prepare in large quantities, and therefore their structures were deduced from the adenosine analogs. Four modified deoxyadenosines (**1′**, **2′**, **3′**, **4′**) were obtained from the modification of poly(dA) with (±)-BPDE. Only compounds **2′** and **3′** were obtained from modification reactions with the purified 7*S*- (−) enantiomer of BPDE. Analysis of their CD spectra and comparison with the CD spectra of the A-BPDE compounds (Fig. 1) indicated that the dA-BPDE compounds **1′**, **2′**, **3′**, and **4′**, corresponded to compounds **1**, **3**, **2**, and **4** in the ribose series, respectively.

Modification of calf thymus DNA with (±)-BPDE gives mainly the 7*R*-BPDE-dG (deoxyguanylate) adduct (*16*). The same is true with plasmid DNA, but with plasmid DNA appreciable amounts (8 percent) of dA adducts are also formed (*17*). Analysis by HPLC of these adducts on a Whatman ODS-2, rather than a Waters C-18 column, which does not resolve compounds dA-BPDE **2′** and **3′**, indicates that the major dA adduct in DNA is compound **2′**, the ratios of compounds **1′** to **4′** being 10, 80, 0, and 10 percent, respectively. Thus, although modification of the N^2 amino group of deoxyguanosine residues occurs preferentially with the 7*R*-BPDE, the N^6 amino group of deoxyadenosine reacts preferentially with the 7*S* enantiomer.

Our results are consistent with previous mass spectra studies of a BPDE deoxyadenosine adduct that also provided evidence for substitution at the N^6 position of adenine (*9*). Our results provide more complete information on such

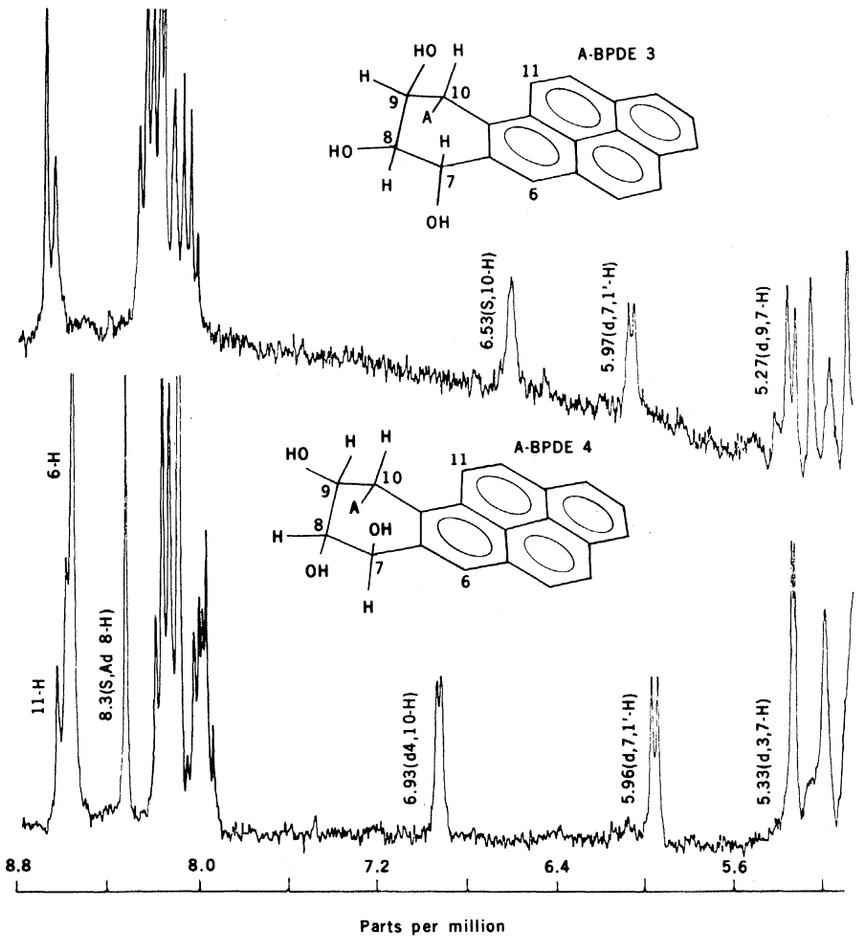

Fig. 2. The NMR spectra were obtained in CD_3OD at 250 MHz (*18*). In the structures *A* represents adenosine linked through the N^6 amino group.

structures, including their stereochemistry. It is of interest that the N^6 position of adenine is in the major groove of the DNA double helix, whereas the N^2 position of guanine is in the minor groove. The significance of attack by BPDE at these two different sites, with respect to stereochemical aspects, interference with base pairing, recognition by DNA repair enzymes, and the carcinogenic process, remains to be determined.

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14. The high-resolution spectrum was measured on a JEOL-01SG-2 instrument at 8 kV, 4.4 A; the probe temperature was 250°C. Further losses of 43 (CH_3CO) and 60 (CH_3COOH) mass units occurred from the *m/e* 444 fragment to give ions at 401 ($C_{24}H_{19}NO_5$, 50 percent) and 384 ($C_{24}H_{18}NO_4$, 100 percent). Consistent with the proposed structure were the ions corresponding to losses from the molecular ion of (i) CH_3COOH to give fragment 761 ($C_{40}H_{35}N_5O_{11}$, 20 percent), followed by losses of CH_3COO to 702 (20 percent) and CH_3COOH to 642 ($C_{36}H_{28}N_5O_7$, 85 percent); (ii) CH_3COO and the benzo(*a*)pyrene moiety to give an ion at *m/e* 334 ($C_{14}N_{16}N_5O_5$, 10 percent); and (iii) the ribose moiety and CH_3COO to give an ion at *m/e* 503 ($C_{28}H_{21}N_5O_4$, 10 percent), followed by a loss of CH_3CO to 460 ($C_{27}H_{18}N_5O_3$, 5 percent).
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Generation of Unidirectionally Propagated Action Potentials in a Peripheral Nerve by Brief Stimuli

Abstract. *Single, unidirectionally propagated action potentials can be elicited in peripheral nerves by electrical stimuli of short duration. Propagation in one direction is blocked anodically by means of a quasi-trapezoidal stimulus wave form and a modified tripolar electrode configuration. Propagation in the other direction proceeds unhindered. This technique may be applicable to collision blocking of motor nerves for neural prostheses.*

Electrical stimulation of a peripheral nerve ordinarily elicits two action potentials, which propagate in opposite directions from the stimulus site. We report a technique in which a single unidirectionally propagated action potential is generated by brief current pulses. This mode of stimulation could be used to effect motor-nerve blockage by introducing antidromic impulses in the peripheral nerve. The block would arise from the head-on collision (and subsequent mutual annihilation) of the natural efferent impulses and the artificially generated antidromic impulses. Directionally controlled stimulation is essential in producing antidromic impulses in order that the muscle receive no artificially generated neural activity.

We demonstrated the technique successfully in 15 cats by using the preparation shown in Fig. 1A. Regulated current pulses were delivered to the sciatic nerve through an asymmetrical tripolar cuff electrode (Fig. 1B). Of the current returning to the cathode, 10 to 30 percent originated from the proximal anode; the remainder flowed from the closer distal anode. Orthodromic propagation was blocked by the potential gradient arising between the cathode and distal anode, which interfered with the flow of excitatory action currents. Spurious orthodromic excitation that occurred distal to the block as a result of stimulus current spread was suppressed by the tripolar electrode configuration, which tended to contain current flow within the insulator. The stimulus wave form consisted of a square leading edge and a 350-μsec plateau phase followed by an exponential falling phase (*1*). Electromyogram (EMG) tracings were obtained by recording distally (R1) from the medial gastrocnemius with intramuscular electrodes of fine stainless steel wire. Compound sciatic neurogram tracings were obtained by recording proximally (R2) with 45-μm straight wires inserted into the nerve trunk with hypodermic needles (29 gauge).

Figure 2A shows a typical sequence of responses recorded at R1 and R2 with increasing stimulus amplitude. A maximal EMG response was elicited at an amplitude of 0.5 mA; as the amplitude was increased to 6 mA, the response disappeared. Simultaneously, the neurographic response grew to a maximum as smaller fibers were recruited. This demonstrates the feasibility of preventing orthodromic propagation from the stimulus site to the muscle. The persistence of the antidromic response is implied by the maximal sciatic discharge at R2. However, an additional experiment was required to demonstrate conclusively that the particular axons innervating the medial gastrocnemius were among those discharging antidromically. This was accomplished by establishing that, after the blocking stimulus, these axons were refractory on the proximal side of the blocking cuff.

Stimulating hook electrodes (S2) were placed proximal to the cuff and their stimulus amplitude was adjusted to produce a maximal EMG response with a 100-μsec pulse. Neurogram recording sensitivity was adjusted such that the compound action potential (recorded at R2) elicited by a maximal test stimulus at S1 to the medial gastrocnemius branch was clearly visible (trace a in Fig. 2B). Stimuli were then delivered at S2 immediately after the blocking pulse. The blocking pulse itself elicited a strong neural discharge at R2 (traces b to h). Stimulation at S2 failed to elicit any further neural activity when delayed from the onset of the blocking pulse by 1.75 msec or less (traces b and c). This indicates that all axons normally excited by stimulation at S2, including those innervating the medial gastrocnemius, were refractory in this time period, hav-

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