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IDENTIFICATION OF THE MAJOR ADDUCTS FORMED BY REACTION OF BENZO[A]PYRENE DIOL EPOXIDE WITH DNA *IN VITRO*

Key terms: chemical carcinogens, activation, microsomes, high resolution mass spectrometry, high pressure liquid chromatography

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Abbreviations: BaP, benzo[a]pyrene;

BaP diol epoxide, (±)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene;

HRMS, high resolution mass spectrometry;

DMSO, dimethylsulfoxide;

HPLC, high pressure liquid chromatography.

TMS, trimethylsilyl

ABSTRACT

Covalent binding of the benzo[a]pyrene metabolite (±)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene to calf thymus DNA was investigated. Enzymatic hydrolysis of the carcinogen modified DNA and subsequent separation via reversed-phase HPLC resulted in the detection and isolation of 7 distinct products. High resolution mass spectrometry indicates that these products are covalent adducts of deoxyguanosine, deoxyadenosine, and deoxycytidine. The deoxyguanosine and deoxyadenosine adducts involve binding between the activated hydrocarbon (BaP diol epoxide) and exocyclic amino groups of the respective purines.

The potent carcinogen benzo[a]pyrene (BaP) is known to undergo metabolism in vivo to a chemically reactive intermediate, which can then react with cellular RNA, DNA and protein (1,2). It is widely accepted that these covalent interactions with cellular macromolecules, particularly DNA, are an essential initial step in the process of carcinogenesis (3,4). Recent work has implicated 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BaP diol epoxide, Structure I) as the molecular species responsible for binding to nucleic acids in vivo (5-7). Weinstein and co-workers have shown that the major RNA adduct found in cell culture is identical to the product obtained by reacting BaP diol epoxide with poly(G), and involves a covalent bond between C-10 of the hydrocarbon and the N^2 exocyclic amino group of guanine (8,9). A similar structure has been reported for reaction of the isomeric diol epoxide, 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, with poly(G) (10). The structures of the products obtained in the reaction between BaP diol epoxide and DNA have not been reported, primarily because only microgram quantities of such adducts can be readily isolated from the analogous reaction with DNA.

We have isolated 7 distinct products from the reaction of BaP diol epoxide with calf thymus DNA, and by the use of high sensitivity, high resolution mass spectrometry (HRMS) the structures of these products were determined. The results show that adducts are formed between BaP diol epoxide and the bases guanine, adenine, and cytosine, and involve reactions with the exocyclic amino group of guanine and adenine.

MATERIALS AND METHODS

Adduct Formation and Isolation. Formation of microsomal enzyme-activated BaP-DNA adducts has been described (11). Racemic BaP diol epoxide was synthesized according to the procedure of McCaustland and Engel (12). Diol epoxide (12 nmol per mg DNA) in 100 µl DMSO was added to a solution of 100 mg calf thymus DNA (Sigma Chemical Co.) in 100 ml 0.01 M phosphate buffer, pH 7.2 at 37°C. After 24 hr, unbound BaP diol epoxide and its hydrolysis products were removed by ethyl acetate extraction. The DNA was then precipitated with ethanol and the precipitate heated to remove intercalated material. The DNA was enzymatically hydrolyzed with deoxyribonuclease II, spleen phosphodiesterase, and alkaline phosphatase (Sigma Chemical Co.), and the modified nucleosides isolated by Sephadex LH-20 chromatography. This fraction was then further separated by high pressure liquid chromatography (HPLC).

High Pressure Liquid Chromatography. HPLC was carried out on a Varian Model 8500 chromatograph equipped with Valco injector and two Waters Associates µ-Bondapak C_{18} columns (4.1 mm x 30 cm) connected in series. Fifty percent water-methanol was used as the eluting solvent, at a flow rate of 1 ml/min. A fluorescence detector (Schoeffel Instrument Co.) was used to monitor column effluent; excitation at 248 nm and emission above 390 nm was used to detect the characteristic 7,8,9,10-tetrahydrobenzo[a]pyrene chromophore.

Derivatization Procedure. Each HPLC peak (containing 0.1-1.0 µg of adduct) was collected, taken to dryness, and methylated according to the procedure of Hakamori (13).

Trimethylsilyl ether derivatives were made by reacting the dried sample with an excess of trimethylsilylimidazole (Pierce Chemical Co.)

for 2-3 hr at 100°C, according to the procedure of Axelson and Sjövall (14).

High Resolution Mass Spectrometry. Electron impact high resolution mass spectra were determined by direct probe using a modified Kratos-AEI MS-902 mass spectrometer operated on-line at M/ΔM 10,000 in real-time employing a Xerox Sigma 7/LOGOS-II computer system (15,16).

RESULTS

Modification of DNA and Adduct Isolation. The covalent binding of BaP to DNA *in vitro* results in a level of DNA base modification equal to one hydrocarbon adduct per 40,000 nucleotides (2). The microsome-mediated binding conditions used in this work resulted in a similar level of binding, and BaP diol epoxide concentrations used (12 nmol/mg DNA) give a level approximately 5-fold higher. The concentrations of carcinogens and the pH used in these experiments approximate those that could occur *in vivo*. Figure 1 shows an HPLC profile obtained by co-injecting the modified nucleosides obtained from BaP diol epoxide-DNA and microsome-activated (G-^{3}H)BaP-DNA. Seven distinct peaks are evident in the fluorescence trace and are labeled P1-P7. A minor component eluting between P5 and P6 was sometimes found but not investigated further in this study. HPLC of the BaP diol epoxide-DNA adducts alone results in a fluorescence trace identical to Figure 1, but chromatography of the microsome-activated BaP-DNA sample alone resulted in peaks 1, 4, 5 and 7 only. The reaction of BaP diol epoxide with DNA thus gives 3 additional products (P2, P3, P6) not seen in the reaction with microsome-activated BaP plus DNA. All products exhibit the characteristic fluorescence and absorbance spectrum of 7,8,9,10-tetrahydrobenzo[a]pyrene. Enzymatic hydrolysis of microsome-activated (G-^{3}H)BaP-DNA results in about 40% of the total bound radioactivity eluting from Sephadex

LH-20 as BaP-mononucleosides. The bulk of the remaining unhydrolyzed material exhibits the characteristic fluorescence spectrum of 7,8,9,10-tetrahydrobenzo[a]pyrene.

Structure Analysis. The structures of P1-P7 were determined by HRMS of their permethyl or persilyl derivatives. Verification of elemental compositions for P1, P3 and P5 was obtained by perdeuteriomethylation with CD_3I. The molecular weight of the main component, P5, was also obtained on the underivatized adduct using low resolution field desorption mass spectrometry.

P1 had a retention time on HPLC identical to that of the hydrolysis product of BaP diol epoxide, BaP tetraol (7,8,9,10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene). HRMS of the permethyl and perdeuteriomethyl derivative of P1 gave molecular ions at m/q 376 ($C_{24}H_{24}O_4$) and m/q 388 ($C_{24}H_{12}D_{12}O_4$), respectively. The fragmentation pattern in both cases was identical to that of authentic permethylated BaP tetraol, obtained by hydrolyzing BaP diol epoxide in water. Yang et al. (17) and Yagi et al. (18) have shown that hydrolysis of BaP diol epoxide gives two isomers, corresponding to cis and trans addition of water at C-10 of the BaP diol epoxide. The other BaP tetraol isomer has a retention time similar to that of P4, and is unresolved from that component.

Figure 2 shows composite nominal mass plots for the HRMS of permethyl derivatives of P7, P2, and P5. The mass spectrum of permethyl-P6 was found to be identical to that of permethyl-P7; permethyl-P3 was identical to that of permethyl-P5; and permethyl-P4 was identical to permethyl-P2. All six spectra exhibit ions due to fragmentation of the hydrocarbon moiety corresponding to cleavage of the hydrocarbon-nucleoside bond (Table 1), generating the trimethoxy-tetrahydrobenzo[a]pyrene moiety at m/q 345 (composition

$C_{23}H_{21}O_3$, ion "a" in Figure 3). Other ions are then generated by successive loss of the elements of CH_3OH, leading to the aromatized monohydroxybenzo[a]-pyrene species at m/q 267 ($C_{20}H_{11}O$). This fragment can then lose CO to give the major ion at m/q 239 ($C_{19}H_{11}$). These fragmentation processes are outlined schematically in Figure 3.

Assignment of the specific purine or pyrimidine in each adduct was deduced from the fragmentation patterns of their respective permethylated derivatives. Von Minden and McCloskey have shown that a major fragmentation pathway of permethylated nucleosides involves cleavage of the C-N glycosidic bond to give a "base + H" ion (19). Table II shows the results obtained for permethyl-P2 through P7; in all cases fragments due to the heterocyclic base are of high relative intensity. This allows the assignment of each adduct to a particular nucleoside. Permethyl-P7 (and permethyl-P6) has an intense ion at m/q 149 ($C_6H_7N_5$), corresponding to a monomethyl adenine. Another ion at m/q 119 ($C_5H_3N_4$) corresponds to loss of the elements of CH_3N from the intact base. Similarly, permethyl-P5 (and permethyl-P3) has an ion at m/q 179 ($C_7H_9ON_5$), indicative of dimethylguanine. This elemental composition was confirmed by perdeuteriomethylation, which gave m/q 185 ($C_7H_3D_6ON_5$). Permethyl-P2 (and permethyl-P4) has a low intensity ion at m/q 125 ($C_5H_7ON_3$) corresponding to monomethyl cytosine, with an intense (100% relative intensity) ion at m/q 111 ($C_4H_5ON_3$).

The overall fragmentation for each adduct is dominated by the stability of the aromatic hydrocarbon and purine or pyrimidine moieties. The results obtained for permethyl-P7 serve to illustrate this point. A molecular ion is observed at m/q 637 ($C_{36}H_{39}O_6N_5$). Successive losses of the elements of methanol give m/q 605 ($C_{35}H_{35}O_5N_5$) and 574 ($C_{34}H_{32}O_4N_5$), and loss of the elements of both CH_3OH and the deoxyribose gives m/q 461 ($C_{28}H_{23}O_2N_5$) and

430 ($C_{27}H_{20}ON_5$). Cleavage of this hydrocarbon-purine bond results in the hydrocarbon and base ions discussed above. Permethyl-P2 (and permethyl-P4) gives similar results, except that a molecular ion (expected at m/q 613) is not seen. The highest mass ion, m/q 469 ($C_{28}H_{27}O_4N_3$), corresponds to loss of the deoxyribose from a cytosine-BaP diol epoxide adduct. Successive losses of the elements of CH_3OH result in m/q 437 ($C_{27}H_{23}O_3N_3$) and 406 ($C_{26}H_{20}O_2N_3$). Cleavage of the hydrocarbon-pyrimidine bond then results in the hydrocarbon and base series of ions.

Von Minden and McCloskey have presented extensive data on the fragmentation patterns of permethylated nucleosides (19). A characteristic ion for permethyl deoxyguanosine, permethyl deoxyadenosine, and permethyl deoxycytidine involves the "base + H" ion (i.e., loss of deoxyribose) which includes a dimethylamino function. This species can then lose methyleneimine (CH_2NH) or the entire dimethylamino function. In the spectra reported here, dimethyladenine or dimethylcytosine ions are not found; only monomethyl species are present. For the adenosine adduct (permethyl-P7), this suggests that direct linkage to the purine (e.g., C-8) does not occur. The occurrence of a relatively low abundance (1.9% relative intensity) nitrogen-containing hydrocarbon fragment at m/q 279 ($C_{21}H_{13}N$) indicates bonding through the N^6 exocyclic amino group. The apparent high stability associated with the benzylic carbonium ion formed by cleavage of the hydrocarbon-nucleoside bond (ion "a", Figure 3) explains the relatively low intensity of these nitrogen-containing hydrocarbon fragments. For permethyl-P2 (and permethyl-P4), no such ion can be found, leaving open the possibility of alkylation <u>via</u> the O^2, N-3, or N^4 positions of cytosine.

The major component, permethyl-P5, contains guanine but otherwise follows the same general fragmentation scheme observed for permethyl-P7 and permethyl-P2.

High mass ions include m/q 523 ($C_{30}H_{29}O_4N_5$, $M^{+\cdot}$ - deoxy-ribose), followed by successive losses of the elements of CH_3OH to give m/q 490 ($C_{29}H_{24}O_3N_5$), 459 ($C_{28}H_{21}O_2N_5$), and the major ion 428 ($C_{27}H_{18}ON_5$, 19.3% relative intensity). Elemental compositions of major ions were confirmed by perdeuteriomethylation with CD_3I. A molecular ion is not observed in the electron-impact spectrum; the highest mass ion that occurs is at m/q 604 ($C_{35}H_{34}O_5N_5$, $M^{+\cdot}$ - $C_2H_7O_2$). The molecular weight for P5 was therefore obtained by field desorption mass spectrometry. The field desorption spectrum of underivatized P5 was found to consist of a single peak at m/q 592, corresponding to 569 + Na. The electron impact fragmentation pattern of the base-containing ions does not permit assignment of the alkylation site on guanine. In particular, no nitrogen-containing hydrocarbon ion could be found, making it difficult to distinguish between N^2 or O^6 alkylation. Alkylation at C-8 or other ring positions of the purine would be expected to generate a trimethylguanine species, which was not found. The absence of a BaP moiety containing nitrogen is again presumably due to the high stability associated with ion "a" (Figure 3). A persilyl ether derivative of P5 was, therefore, prepared in order to obtain additional structural information. The composite nominal mass spectrum of pertrimethylsilyl-P5 is shown in Figure 4. Table 3 lists the major ions observed for P5-TMS. The overall pattern is similar to that of the permethylated derivatives: loss of the deoxyribose followed by successive losses of trimethylsilanol [$(CH_3)_3SiOH$] to give m/q 561 ($C_{31}H_{31}O_2N_5Si_2$). This is then followed by cleavage of the base-hydrocarbon bond to give the base (m/q 223, $C_8H_{13}ON_5Si$) or hydrocarbon series of ions. In this case, however, a relatively low intensity ion corresponding to a nitrogen-containing BaP species is seen at m/q 355 ($C_{23}H_{21}ONSi$, 0.2% relative intensity). This is consistent with alkylation on the N^2 exocyclic amino group, as was found for alkylation of poly(G) by BaP diol epoxide (9).

DISCUSSION

The results described in this study are consistent with independent labeling studies which identified P3 and P5 as being guanine adducts, P6 and P7 as adenine adducts, and P4 as probably being a cytosine adduct (11). Interpretation of the mass spectral fragmentation data is most consistent with binding via the exocyclic amino group of adenine and guanine, while cytosine may involve O^2, N-3, or N^4 alkylation. Bonding is presumed to occur at C-10 of the hydrocarbon, since studies have shown that this occurs in the reactions of a wide variety of nucleophiles with BaP diol epoxide (18,20). The structures for P2 (P4), P5 (P3), and P7 (P6) are shown below as IV, II and III, respectively.

The BaP diol epoxide used in this study was racemic. Enzymatic formation of I is known to be highly stereoselective for a single enantiomer (17,21,22) and explains the fact that P2, P3, and P6 were not seen in the adducts obtained from DNA modified by microsome-activated BaP. P2, P3, and P6 would then correspond to diastereomers of P4, P5, and P7 (one enantiomer of BaP diol epoxide reacted with the enantiomeric deoxyribonucleoside) and each diastereomeric pair could be expected to give identical electron impact spectra. This explanation is supported by circular dichroism spectra of the adducts (unpublished data, this laboratory). The alternative explanation is that multiple adducts can be formed by cis or trans addition at C-10 of the hydrocarbon, but model studies suggest that trans addition predominates for BaP diol epoxide (17,18). It should be noted that several minor peaks were occasionally observed in the HPLC chromatogram of these DNA adducts (usually <1% of the reported species) and could be due to products formed by cis addition at C-10 or possibly other alkylation sites on the nucleoside bases.

In carrying out analogous experiments with the other geometric isomer of BaP diol epoxide, 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, we have found that the major products do not co-chromatograph with the microsome-activated BaP-DNA adducts represented in Figure 1. The structures of these products are currently under investigation.

These results provide the first unambiguous determination of the overall molecular structures of the products obtained in the alkylation of DNA by BaP diol epoxide, and extend the available information on alkylation of DNA by polynuclear aromatic carcinogens. Essigman et al. have recently reported that the 2,3-oxide of Aflatoxin B1 forms covalent adducts with the N-7 of guanine residues (23). 2-Acetylaminofluorene is known to bind to both C-8 and N^2 of guanine (24-26), and both BaP diol epoxide and synthetic 7,12-dimethylbenz[a]anthracene-5,6-oxide bind principally to the N^2 of guanine in poly(G) (27). Adducts with adenine or cytosine in DNA with these polynuclear carcinogens have not been reported. Little is known about the effects of these modifications on DNA structure, or how they might alter the functional properties of nucleic acids <u>in vivo</u>. BaP diol epoxide is also capable of catalyzing strand scission in both DNA and RNA, but the physiological significance of this process is not known (28). The results of these binding studies, together with those reported for simple alkylating agents (29), suggest that a multiplicity of sites may be involved in the biological activity of chemical carcinogens.

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TABLE 1. Fragments derived from hydrocarbon moiety of permethylated adducts (P2-P7)

m/q	Elemental composition
345	$C_{23}H_{21}O_3$
344	$C_{23}H_{20}O_3$
313	$C_{22}H_{17}O_2$
312	$C_{22}H_{16}O_2$
298	$C_{21}H_{14}O_2$
297	$C_{21}H_{13}O_2$
282	$C_{21}H_{14}O$
267	$C_{20}H_{11}O$
239	$C_{19}H_{11}$
226	$C_{18}H_{10}$
215	$C_{17}H_{11}$

TABLE 2. Fragments derived from the nucleoside base moiety of permethylated adducts (P2-P7)

Adduct	m/q	Elemental composition
P-2 (P-4)	125	$C_5H_7N_3O$
	112	$C_4H_6N_3O$
	111	$C_4H_5N_3O$
P-5 (P-3)	179	$C_7H_9ON_5$
	150	$C_6H_6ON_4$
P-8 (P-7)	149	$C_6H_7N_5$
	120	$C_5H_4N_4$

TABLE 3. Major fragments found in pertrimethyl-silyl-P5

m/q	Elemental composition
732	$C_{30}H_{19}O_4N_5(TMS)_3$
651	$C_{25}H_{14}O_3N_5(TMS)_3$
561	$C_{25}H_{13}O_2N_5(TMS)_2$
518	$C_{20}H_{11}O_3(TMS)_3$
472	$C_{25}H_{13}ON_5$ TMS
428	$C_{20}H_{10}O_2(TMS)_2$
356	$C_{20}H_{11}O_2$ TMS
355	$C_{20}H_{12}ON$ TMS
223	$C_5H_4ON_5$ TMS

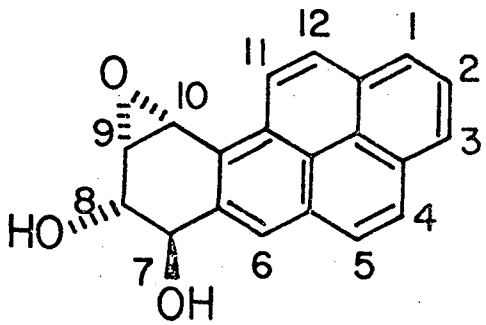
FIGURE CAPTIONS

Fig. 1. HPLC profile of coinjection of diol epoxide DNA adducts plus adducts obtained from microsome-activated (G-^{3}H) BaP and DNA. The 7 major products are labelled P1-P7. Fluorescence units are arbitrary. Full scale for fluorescence represents approximately 50 ng of material.

Fig. 2. Composite nominal mass plots (abscissa, % relative intensity vs. ordinate, m/q) for permethyl-P2 (P2-CH_3, top), permethyl-P5 (P5-CH_3, center), and permethyl-P7 (P7-CH_3, bottom).

Fig. 3. Schematic fragmentation pathway of the hydrocarbon moiety from a diol epoxide-DNA adduct. "B" refers to a DNA base.

Fig. 4. Composite nominal mass plot (abscissa, % relative intensity vs. ordinate, m/q) for pertrimethylsilyl ether of P5.



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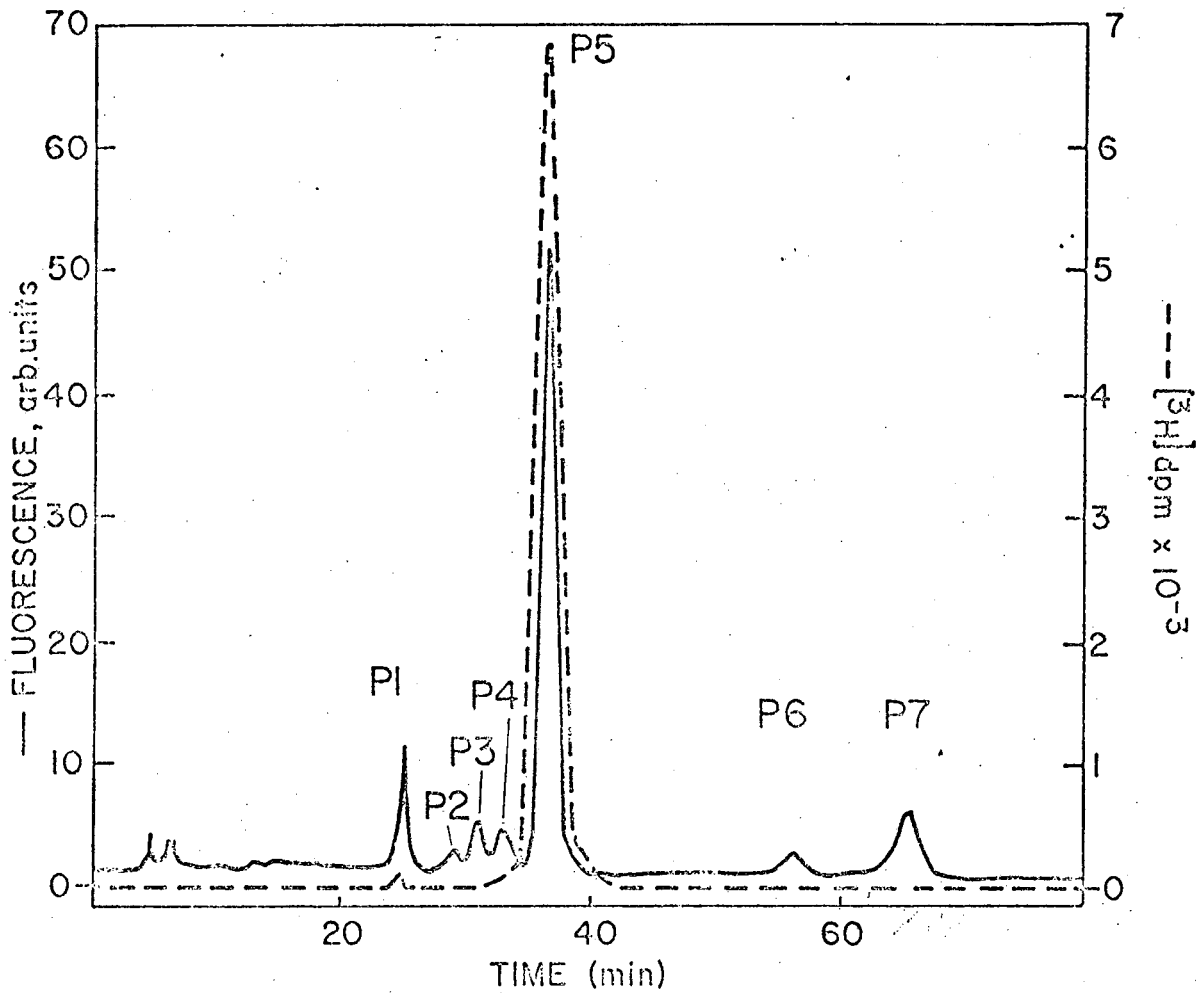
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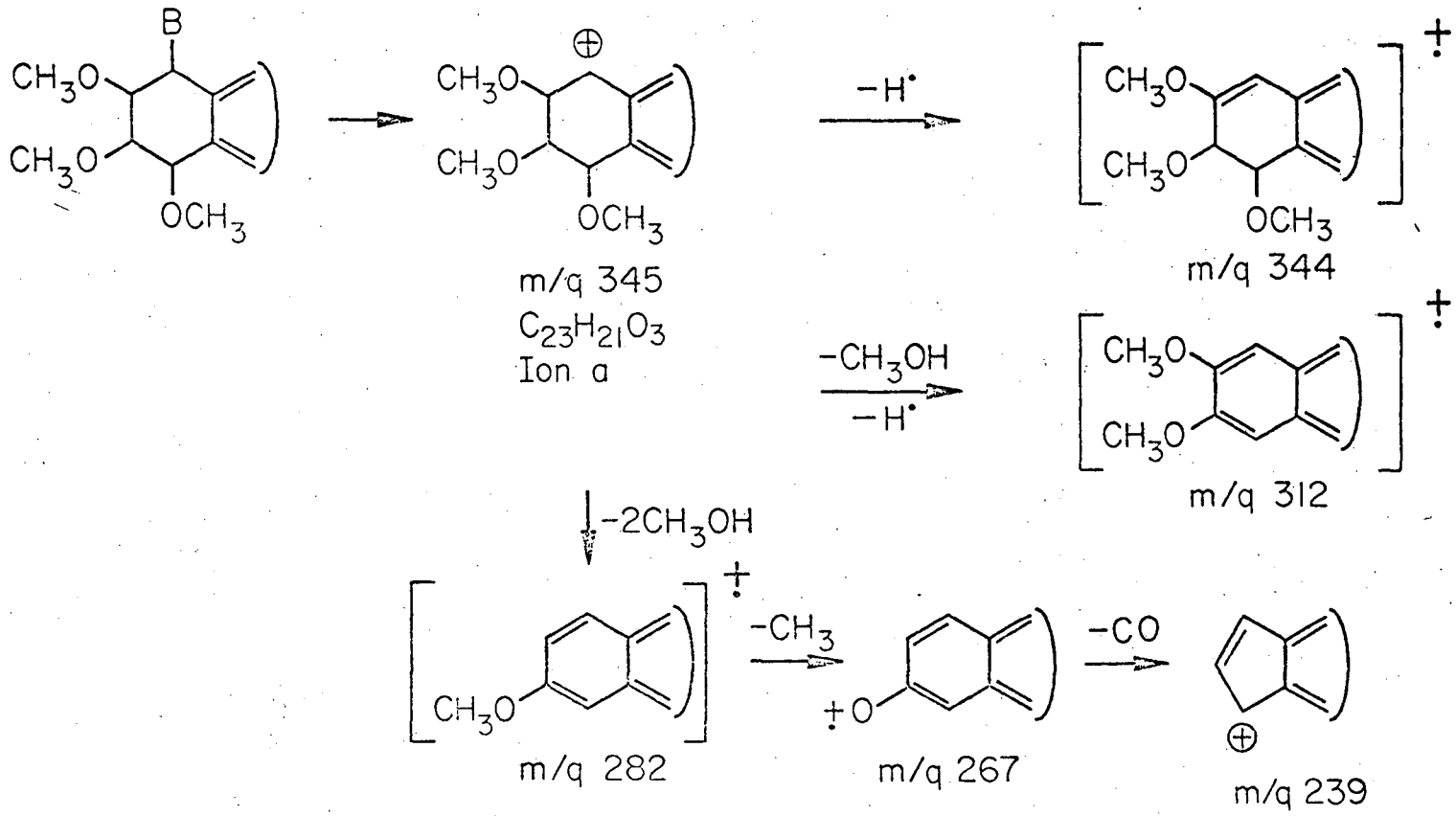

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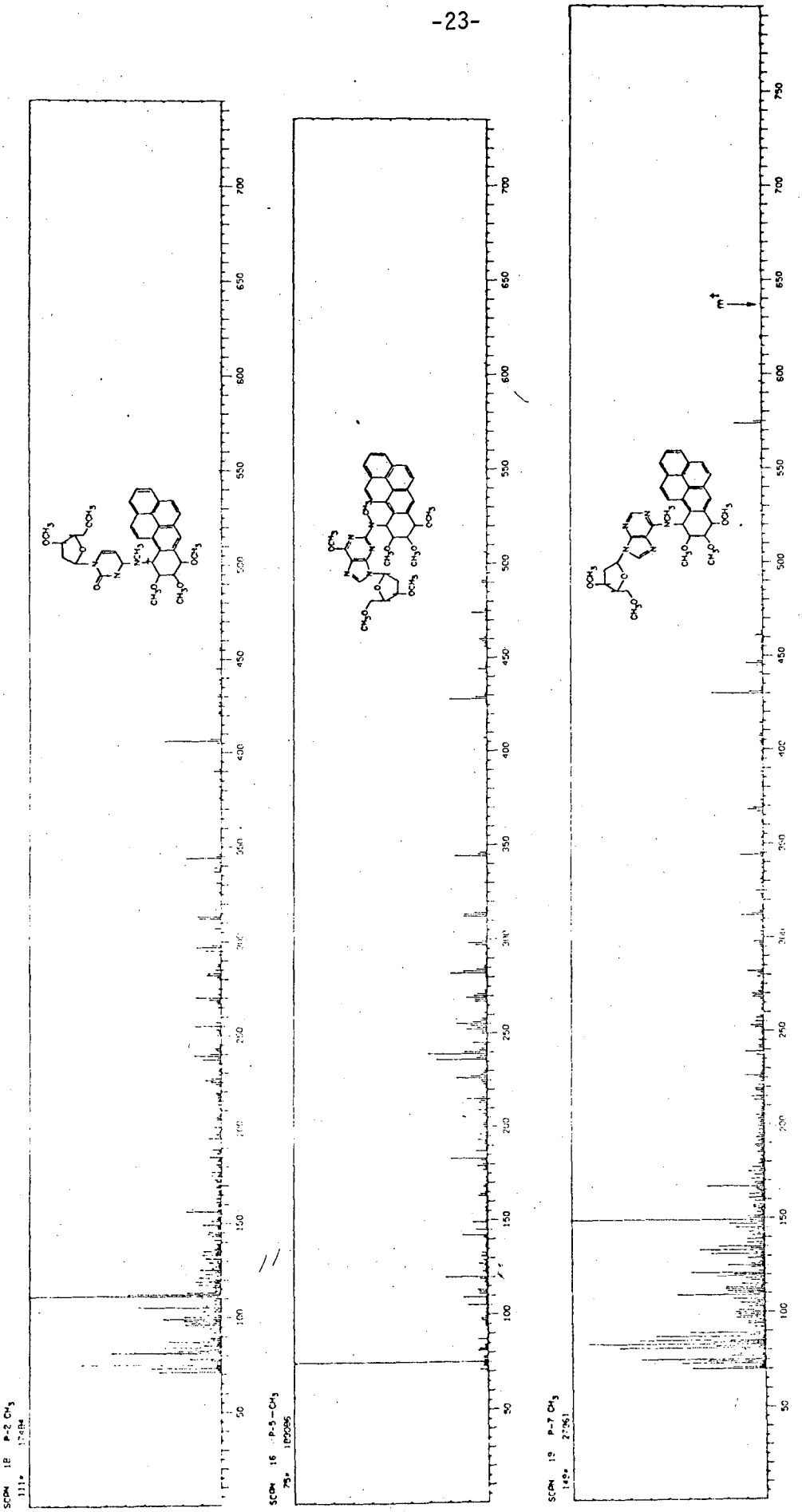
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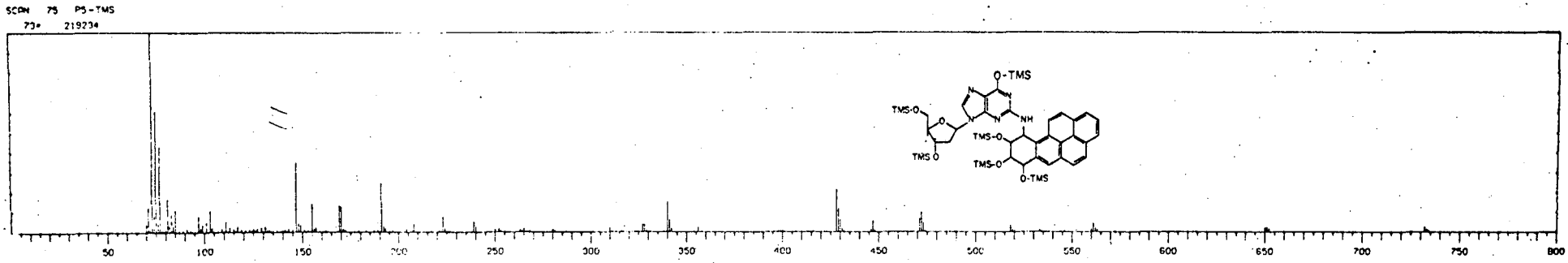
SCAN 16 P-5-CH3
SCAN 19 P-7 CH3
m+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TMS-O
TMS O
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TMS-O
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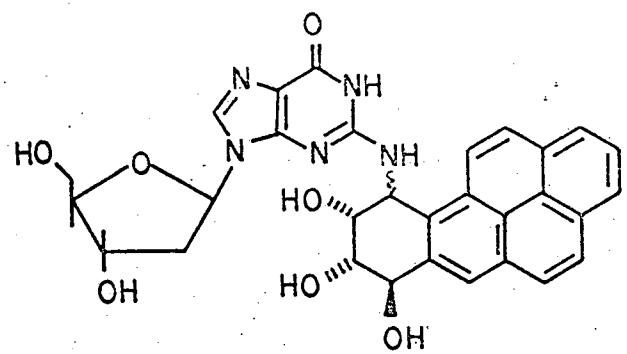
800



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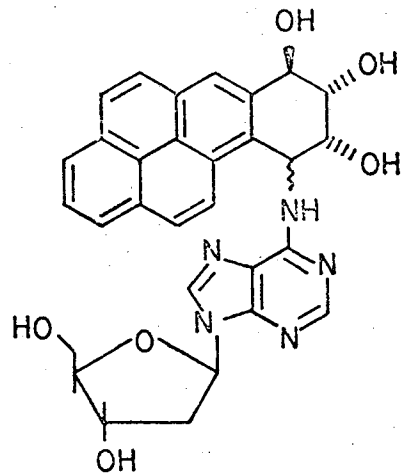
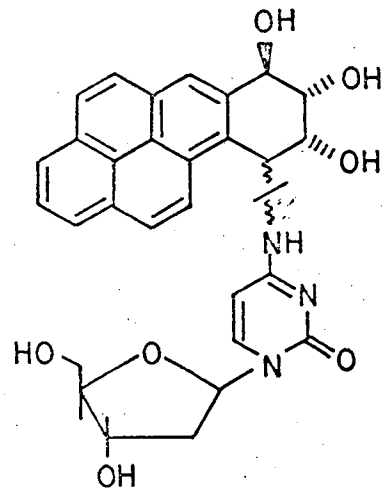


III



IV

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