Metabolism of 6-Substituted Benzo[a]pyrene Derivatives: O-Dealkylation and Regiospecificity in Aromatic Hydroxylation

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The carcinogen benzo[a]pyrene (BP) is metabolized by monooxygenase enzymes to phenols, dihydrodiols, and diones. The major product, 3-hydroxy-BP, is measured in the fluorescence assay for aryl hydrocarbon hydroxylase (AHH). In this assay, the BP phenols, which are readily oxidized to nonfluorescent diones, are not detected. Blocking the 6 position with various substituents prevents dione formation and stabilizes the phenolic metabolites. These substituted BP derivatives were used as possible alternative substrates to BP in the AHH assay and to investigate positional specificities of monooxygenase enzymes in aromatic hydroxylation, as well as O-dealkylation when the substrates were BP alkoxy derivatives. Phenolate anions at positions 1 and 12 could be distinguished from 3-phenolate anions of BP derivatives on the basis of the fluorescence excitation spectra. This permitted identification of phenolic metabolites of BP derivatives obtained after incubation with rat liver and lung microsomes. In some cases other metabolites were identified by high-pressure liquid chromatography. No worthy substitute for BP in the AHH assay was found, since the derivatives were hydroxylated at a lesser rate, hydroxylated at positions other than 3, metabolized by O-dealkylation, or yielded products less fluorescent than the 3-hydroxy-BP. AHH was found to have positional specificity for 3-hydroxylation of BP and some of its derivatives. The minor role of 1-hydroxylation is presumably due to a steric constraint of the enzyme. Three different enzyme activities were observed: (1) an uninduced 3-hydroxylase with some 1-hydroxylase activity; (2) a 3-methylcholanthrene-induced 3-hydroxylase, which has more 1-hydroxylase activity and is more sensitive to the structure of the substrate; (3) an uninducible O-dealkylase. Hydroxylase and O-dealkylase have the same regiospecificity: the hydroxylase can attack positions 1, 3, and 6, but not 12, while the O-dealkylase can dealkylate groups at positions 1, 3, and 6, but not 12. 6-Methoxy-BP was metabolized both by hydroxylation and O-dealkylation. Compounds metabolized mainly by hydroxylation were spectral type I. These included BP, 6-methoxy-BP, and 6-(methoxymethyl)-BP. Compounds metabolized by O-dealkylation, the 1,6- and 3,6-dialkoxy derivatives of BP, were spectral type II compounds, the first reported which do not contain a basic nitrogen atom.

Monooxygenase enzymes are responsible for the metabolism of many endogenous and foreign compounds, including steroids, drugs, carcinogens, and pesticides.[2-5] These enzymes are widely distributed in body tissues. Some of the reactions performed include aromatic hydroxylation, N- and O-dealkylation, and N-oxidation. Much or most of this activity is attributed to the cytochrome P-450 mediated monooxygenase enzyme.[2-7]

A commonly used measure of a tissue's monooxygenase activity is the determination of the aryl hydrocarbon hydroxylase (AHH).[8] For assaying this enzymatic activity, the tissue is incubated with the carcinogen benzo[a]pyrene (BP); the major metabolite, 30–60% of the total, is the highly fluorescent 3-phenol, 3-hydroxy-BP, which is extracted and measured. Other metabolites include the nonfluorescent 1,6-, 3,6-, and 6,12-diones, some dihydrodiols, and small amounts of the 1-, 7-, and 9-phenols.[9-11]

The AHH assay has several shortcomings. Not all of the phenolic metabolites are detected by the assay for two main reasons. Firstly, among the BP phenolic metabolites the AHH assay selects the 3- and 9-phenols because of their higher fluorescent yield in alkali when compared to the other BP phenols.[12] Secondly, some BP phenols readily oxidize in the presence of light, heat, and air to form diones,[13] as position 6 of BP is conjugated to positions 1, 3, and 12. Hydroxylation at position 6 reportedly accounts for most of the dione metabolites.[14,15] Also, a small amount of the 3-phenol formed is oxidized by chemical or further enzymatic activity to the 3,6-dione.[13,16,17]

Calculations of electron density within the BP molecule suggest that electrophilic substitution should occur most readily at positions 6, 1, 3, and 12, in decreasing order.[18] Experimentally, such is indeed the case with chemical electrophiles.[19-21] However, enzymatic hydroxylation occurs extensively only at positions 3 and 6. It is plausible to assume that enzymatic oxidation at position 6 occurs by an insertion mechanism[22] since the formation of a

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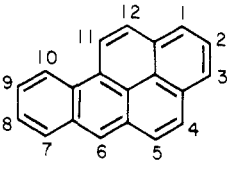
strained epoxide intermediate is unlikely. A similar mechanism might operate in the formation of 3-hydroxy-BP, as the yield of this metabolite is not decreased by the epoxide inhibitor 1,2-epoxy-3,3,3-trichloropropane,[23,24] thereby ruling out a 2,3-epoxide intermediate. The NIH shift mechanism observed in the formation of 3-hydroxy-BP[17] does not necessarily indicate, as suggested, the partial involvement of an epoxide intermediate. In fact, a possible alternative mechanism, i.e., an oxygen addition–rearrangement,[22] also entails an NIH shift. Very little 1-phenol[9] and no 12-phenol have been found among the metabolites of BP in various biological tissues. This could reflect a positional specificity of AHH due to a steric constraint in the active site. Alternatively, it could be due to the chemical propensity of some phenols, i.e., 6-hydroxy-BP,[14,15,23] some 3-hydroxy-BP,[17] and possibly most of 1-hydroxy-BP, to form diones. In such a case the AHH activity would not be detected in the assay.

The uncertainty associated with determination of the AHH activity could be resolved, and the assay possibly improved, by blocking position 6 of BP with an appropriate substituent. This would stabilize phenolic metabolites by preventing their oxidation to diones. Accordingly, a number of 6-substituted derivatives of BP were synthesized and tested as possible substrates in the assay. Some of these BP derivatives were synthetically hydroxylated to permit identification of the phenolic metabolites. The metabolism of 6-substituted BP derivatives was also used to investigate positional specificities of monooxygenase enzymes in aromatic hydroxylation as well as O-dealkylation when the substrates were BP alkoxy derivatives.



1, 6-methoxy
2, 1,6-dimethoxy
3, 3,6-dimethoxy
4, 6,12-dimethoxy
5, 1,6-diethoxy
6, 6-(methoxymethyl)
7, 1-acetoxy-6-methoxy
8, 3-acetoxy-6-methoxy
9, 12-acetoxy-6-methoxy
10, 1,3-diacetoxy-6-methoxy
11, 3-acetoxy-1,6-diethoxy
12, 12-hydroxy-6-methoxy
13, 1-hydroxy-6-methoxy
14, 3-hydroxy-6-methoxy
15, 1,6-diethoxy-3-hydroxy
16, 1,6-dihydroxy-6-methoxy

Experimental Section

Melting points are corrected. Infrared spectra were obtained on a Beckman IR 9 spectrometer and recorded in reciprocal centimeters. Ultraviolet spectra were obtained on a Cary 14 spectrometer and recorded in nanometers. NMR spectra were recorded in $CDCl_3$ solution at 100 MHz on a Varian HA-100 spectrometer. Chemical shifts are reported in parts per million downfield from an internal Me_4Si standard. Fluorescence spectra were obtained on an Aminco-Bowman spectrophotofluorometer 4-8202 SPF with slits of 5 (low resolution) and 0.1 mm (high resolution). The spectra reported are low resolution, unless otherwise specified. Routine fluorescence intensity measurements were made on a Turner 430 spectrofluorometer with excitation and emission slits which had half-power band-passes of 15 nm. Mass spectra at high and low resolution were obtained with an AEI MS-9 spectrometer operating at ionizing potentials of 50 and 70 eV, respectively. High-pressure LC was conducted on a Spectra Physics 3500B equipped with a 254-nm photometer to measure absorbance of the effluent. Fluorescence was monitored simultaneously with an Aminco-Bowman Fluoro-Monitor J4-7461. Two coupled 10-Partisil–ODS columns, each 25 × 0.43 cm, were used with a linear gradient of 35–85% methanol in water over 30 min, an inlet pressure of 1500 psi, and a flow rate of 1.5 mL/min. Microanalyses were performed by Midwest Microlab, LTD, Indianapolis, Ind.

Chemicals. BP and 3-methylcholanthrene (3-MC) were purified by column chromatography on Brinkmann silica gel, using hexane–benzene (9:1), followed by recrystallization from benzene–methanol. Celite 545 was a gift of Johns Manville Co., and 3-hydroxy-BP was a gift of the National Cancer Institute. All other reagents were obtained commercially.

Benzo[*a*]pyrene-1,6-dione, -3,6-dione, and -6,12-dione. Oxidation of BP by the procedure of Antonello and Carlassare[25] yielded a mixture of the 1,6-, 3,6- and 6,12-diones in a 47:33:20 ratio, respectively. This mixture was completely resolved by chromatography on a column of a 2:1 (w/w) mixture of magnesia (USP heavy powder) and Celite 545. Unreacted BP eluted rapidly with benzene; the 6,12-dione and the 1,6-dione followed more slowly. The 3,6-dione was then eluted with chloroform. Physical properties of the three diones were as described by Antonello and Carlassare.[25]

6-Methoxybenzo[*a*]pyrene (1). This compound was prepared by following a published procedure:[19] NMR 9.03 (H_{10}, m), 8.95 (H_{11}, d), 8.61 (H_7, m), 8.31 (H_5, d), 8.20 (H_{12}, d), 8.16 (H_1, m), 7.98 (H_2, H_3, m), 7.90 (H_4, d), 7.80 (H_8, H_9, m), 4.18 ppm (CH_3O–, s).

1,6-Dimethoxybenzo[*a*]pyrene (2). To 40 mg (0.142 mmol) BP-1,6-dione dissolved in 25 mL of dioxane was added 20 mL of 5 N NaOH, and the mixture heated to reflux. Zinc dust (1.0 g) slurried in benzene was added to the stirred mixture, followed by dimethyl sulfate (5.0 mL, 25 mmol) in 1.0-mL aliquots over 10 min, alternated with 1.0 g of zinc dust. The reaction mixture was then partitioned between water and benzene. The organic layer was washed, dried ($MgSO_4$), and evaporated. The crude product was purified on an alumina column eluted with hexane–benzene (1:1). Recrystallization from benzene–hexane yielded 34 mg (79%) of light yellow crystals of 1,6-dimethoxy-BP, mp 187–187.5 °C (lit.[25] 185 °C). Spectral properties were as previously described.[25]

3,6-Dimethoxy- (3) and 6,12-Dimethoxybenzo[*a*]pyrene (4). These compounds were prepared from the corresponding diones as above. Physical properties were as previously described.[25]

1,6-Diethoxybenzo[*a*]pyrene (5). This compound was prepared from the 1,6-dione, as above, using diethyl sulfate in place of dimethyl sulfate. Prior to workup, excess alkyl sulfate was decomposed by adding phenol. The product was purified and recrystallized, as above, to yield 58% of 1,6-diethoxy-BP: mp 153–154 °C; MS *m/e* (high) 340.1450 (36.0, M^+), 311.1071 (79.6), 283.0758 (100.0), 226.0782 (45.4); UV λ_{max} (MeOH) 225 nm (ϵ 26 100), 257 (41 400), 267 (38 400), 289 (31 700), 301 (38 000), 384 (26 600), 393 (26 100), 405 (31 200), 419 (7800); NMR 8.99 (H_{10}, m), 8.91 (H_{11}, d), 8.59 (H_{12}, d), 8.56 (H_7, m), 8.10 (H_5, d), 7.88 (H_3, d), 7.75 (H_4, d), 7.75 (H_8, H_9, m), 7.33 (H_2, d), 4.32 [$-CH_2-$ (C_6), q], 4.30 [$-CH_2-$ (C_1), q], 1.65 [$-CH_3$ (C_6), t], 1.60 ppm [$-CH_3$ (C_1), t]. Anal. ($C_{24}H_{20}O_2$) C, H.

6-(Methoxymethyl)benzo[*a*]pyrene (6). 6-(Hydroxymethyl)-BP[26] was dissolved in dioxane. An equal volume of 5 N NaOH and an excess of dimethyl sulfate were added, and the mixture was heated on a steam bath for 2 h. Conversion to the ether was incomplete. The mixture was partitioned between benzene and water. The benzene layer was washed, dried ($MgSO_4$), and evaporated. The residue was chromatographed on an alumina column eluted with hexane–benzene (1:4). The forerunning fluorescent band was the product. Recrystallization from benzene–hexane gave light yellow crystals of 6-(methoxymethyl)-BP in 50% yield: mp 162–162.5 °C; MS *m/e* (high) 296.1200 (47.7, M^+), 266.1095 (30.6), 265.1017 (100.0), 263.0770 (19.6). Anal. ($C_{22}H_{16}O$) C, H.

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1-Acetoxy-6-methoxy- (7), 3-Acetoxy-6-methoxy- (8), 12-Acetoxy-6-methoxy- (9), and 1,3-Diacetoxy-6-methoxybenzo[a]pyrene (10). To 800 mg (2.84 mmol) 6-methoxy-BP in 400 mL of benzene was added dropwise, over 1 h, 2.488 g (5.6 mmol) of lead tetraacetate in 600 mL of glacial acetic acid. The mixture was then stirred at room temperature for 1.5 h and partitioned between water and benzene. The benzene layer was washed to neutrality, dried ($MgSO_4$), and evaporated. The crude product was chromatographed on a short silica gel column with hexane–benzene. Unreacted starting material eluted rapidly and was followed by the mixture of the three monoacetates (311 mg, 32%). 1,3-Diacetoxy-6-methoxy-BP was then eluted with benzene–$CHCl_3$ (9:1).

The diacetate fraction was further purified on a second silica gel column, with benzene–light petroleum ether (3:2) which eluted the monoacetate impurities. The diacetate was then eluted with benzene, dried, and recrystallized from chloroform–hexane. Golden-yellow crystals of the diacetate were formed: yield 45 mg (4%); mp 213–214 °C; UV λ_{max} (MeOH) 256 nm (ϵ 40 100), 260 (39 500), 267 (43 400), 289 (35 100), 301 (42 400), 368 (15 600), 380 (21 900), 387 (24 400), 391 (22 200), 393 (21 900), 399 (22 400), 413 (10 300), 433 (3300); NMR 8.97 (H_{10}, m), 8.92 (H_{11}, d), 8.57 (H_7, m), 8.29 (H_5, d), 8.17 (H_{12}, d), 7.87 (H_4, d), 7.79 (H_8, H_9, m), 7.66 (H_2, s), 4.17 (CH_3O–, s), 2.51 [CH_3CO– (C_1), s], 2.48 ppm [CH_3CO– (C_3), s].

The 1-, 3-, and 12-acetates were present in a 57:26:17 ratio, with methyl proton peaks at 2.52, 2.50, and 2.56 ppm, respectively. The monoacetate mixture was chromatographed in a silica gel column with benzene–light petroleum ether (4:1). The first few fractions contained pure 12-acetate, followed by fractions of mostly 1-acetate, and finally mixtures of the 1- and 3-acetates. Upon additional chromatography, the mixtures yielded pure samples of the 1- and 3-acetates. The three acetates were recrystallized from benzene–hexane.

1-Acetoxy-6-methoxy-BP was obtained as yellow, needle-like crystals: mp 142–144 °C; MS *m/e* (high) 340.1099 (29.5, M^+), 298.0993 (45.5), 283.0758 (100.0); UV λ_{max} (MeOH) 225 nm (ϵ 43 100), 255 (44 600), 267 (46 900), 279 (24 500), 290 (40 300), 302 (50 700), 360 (11 700), 376 (21 200), 384 (20 700), 389 (20 100), 397 (21 800), 412 (8800), 432 (2600); NMR 8.93 (H_{10}, m), 8.91 (H_{11}, d), 8.58 (H_7, m), 8.24 (H_5, d), 8.15 (H_{12}, d), 7.97 (H_3, d), 7.82 (H_4, d), 7.78 (H_8, H_9, m), 7.69 (H_2, d), 4.17 (CH_3O–, s), 2.52 ppm (CH_3CO–, s). Anal. ($C_{23}H_{16}O_3$) C, H.

3-Acetoxy-6-methoxy-BP was obtained as yellow-tan crystals: mp 205–208 °C; MS *m/e* (high) 340.1099 (29.5, M^+), 298.0993 (44.5), 283.0758 (100.0); UV λ_{max} (MeOH) 225 nm (ϵ 43 100), 255 (44 600), 267 (46 900), 279 (24 500), 290 (40 300), 302 (50 700), 360 (11 700), 376 (21 200), 384 (20 700), 389 (20 100), 397 (21 800), 412 (8800), 432 (2600); NMR 8.95 (H_{10}, m), 8.87 (H_{11}, d), 8.55 (H_7, m), 8.32 (H_5, d), 8.12 (H_1, d), 8.12 (H_{12}, d), 7.90 (H_4, d), 7.80 (H_8, H_9, m), 7.69 (H_2, d), 4.17 (CH_3O–, s), 2.50 ppm (CH_3CO–, s).

12-Acetoxy-6-methoxy-BP was obtained as yellow, needle-like crystals: mp 161–162 °C; MS *m/e* (high) 340.1099 (32.5, M^+), 298.0993 (35.4), 283.0758 (100.0); UV λ_{max} (MeOH) 227 nm (ϵ 37 100), 254 (42 500), 266 (45 300), 285 (43 500), 297 (57 600), 358 (12 200), 360 (12 200), 376 (23 500), 384 (18 400), 397 (25 900), 411 (7500); NMR 8.90 (H_{10}, m), 8.73 (H_{11}, s), 8.59 (H_7, m), 8.31 (H_5, d), 8.15 (H_1, m), 7.99 (H_2, H_3, m), 7.89 (H_4, d), 7.80 (H_8, H_9, m), 4.18 (CH_3O–, s), 2.56 ppm (CH_3CO–, s).

3-Acetoxy-1,6-diethoxybenzo[a]pyrene (11). To a stirred solution of 204 mg (0.60 mmol) 1,6-diethoxy-BP in 75 mL of benzene was added dropwise, over 50 min, 200 mg (0.45 mmol) of lead tetraacetate in 75 mL of glacial acetic acid. The reaction mixture was then partitioned between water and benzene. The benzene layer was washed to neutrality, dried ($MgSO_4$), and evaporated. The residue was chromatographed on a silica gel column eluted with benzene. Unreacted starting material eluted first, followed by the product. Recrystallization from benzene–hexane yielded 92 mg (38.5 or 51% if the yield is based on the limiting reagent lead tetraacetate) of golden-yellow crystals of 3-acetoxy-1,6-diethoxy-BP: mp 200.5–201.5 °C; MS *m/e* (high) 398.1491 (26.5, M^+), 328.1028 (23.6), 327.0996 (100.0), 299.0720 (47.9); UV λ_{max} (MeOH) 225 nm (ϵ 21 600), 258 (31 200), 269 (29 500), 292 (20 000), 303 (23 500), 384 (19 100), 394 (20 900), 405 (21 800), 421 (7200); NMR 8.95 (H_{10}, m), 8.85 (H_{11}, d), 8.53 (H_7, m), 8.52 (H_{12}, d), 8.11 (H_5, d), 7.74 (H_4, d), 7.74 (H_8, H_9, m), 7.15 (H_2, s), 4.29 (–CH_2–, q), 4.27 (–CH_2–, q), 2.48 (CH_3CO–, s), 1.64 (–CH_3, t), 1.60 (–CH_3, t). Anal. ($C_{26}H_{22}O_4$) C, H.

12-Hydroxy-6-methoxybenzo[a]pyrene (12). To a solution of 10 mg (0.029 mmol) of 12-acetoxy-6-methoxy-BP in 5 mL of ether was added 2 mL of ether saturated with $LiAlH_4$. After the addition of 3 mL of 10% HCl, the organic layer was washed to neutrality, dried (Na_2SO_4), and evaporated. The residue was recrystallized from $CHCl_3$–hexane to give 7.3 mg (83%) of lemon-yellow crystals of 12-hydroxy-6-methoxy-BP: mp (in vacuo) 256–257 °C; MS *m/e* (high) 298.0993 (37.8, M^+), 283.0758 (100.0); UV λ_{max} (MeOH) 219 nm (ϵ 26 200), 223 (26 500), 256 (33 900), 291 (37 200), 301 (40 200), 372 (10 100), 390 (18 500), 412 (18 800). Fluorescence λ_{max} (MeOH): excitation, 305, 405; emission, 442.

1-Hydroxy-6-methoxybenzo[a]pyrene (13). This compound was prepared as above. The phenol was purified on a short silica gel column with benzene–light petroleum ether (4:1) and recrystallized to yield 6.6 mg (75%) of light yellow crystals: mp (in vacuo) 230–232 °C; MS *m/e* (high) 298.0993 (43.4, M^+), 283.0758 (100.0); UV λ_{max} (MeOH) 257 nm (ϵ 43 500), 266 (40 500), 276 (27 400), 288 (35 500), 300 (40 800), 386 (22 000), 388 (22 100), 395 (22 900), 407 (24 600), 423 (7400). Fluorescence λ_{max} (MeOH): excitation, 309, 398; emission, 442. Anal. ($C_{21}H_{14}O_2$) C, H.

3-Hydroxy-6-methoxybenzo[a]pyrene (14). The phenol prepared as above did not crystallize readily, and evaporation of the solvent led to a darkening of the product. The small volume of ether solution was taken up in methylene chloride and evaporated slowly by a stream of dry nitrogen to produce tan crystals: MS *m/e* (high) 298.0993 (40.2, M^+), 283.0758 (100.0); UV λ_{max} (MeOH) 227 nm (ϵ 45 200), 257 (43 000), 261 (42 300), 269 (42 900), 297 (26 900), 311 (31 500), 351 (6100), 367 (14 900), 387 (25 800), 407 (14 900), 432 (13 800). Fluorescence λ_{max} (MeOH): excitation, 310, 377, 390; emission, 448.

1,6-Diethoxy-3-hydroxybenzo[a]pyrene (15). This compound could be prepared either by the above method or as follows. To a solution of 50 mg (0.12 mmol) of 3-acetoxy-1,6-diethoxy-BP in 5 mL of tetrahydrofuran under nitrogen was added 0.25 mmol of sodium methoxide in 1.0 mL of methanol. Starting material was hydrolyzed within 8 min. The base was then neutralized with dilute sulfuric acid. When 15 mL of water had been added, orange crystals formed. These were filtered out, washed with water, and dried: yield 34 mg (75%); mp (in vacuo) 192–195 °C, with a morphic change between 85 and 88 °C; MS *m/e* (low) 356 (47.0, M^+), 327 (100.0), 299 (66.5); UV λ_{max} (MeOH) 256 nm (ϵ 33 100), 262 (32 400), 271 (30 800), 297 (17 800), 311 (23 300), 378 (15 000), 397 (23 600), 417 (15 000), 443 (13 400). Fluorescence λ_{max} (MeOH): excitation, 311, 394, 399; emission, 466.

1,3-Dihydroxy-6-methoxybenzo[a]pyrene (16). This compound was prepared by $LiAlH_4$ hydrolysis of the 1,3-diacetate. However, it was not isolated because it was labile to oxidation by air. A methanolic solution of known concentration, based on total conversion of the diacetate to the dihydroxy derivative, showed the following UV spectrum with approximate extinction coefficients: UV λ_{max} (MeOH) 235 nm (ϵ 39 300), 279 (16 700), 312 (7640), 338 (7230), 354 (9530), 382 (13 800), 403 (14 700), 441 (6390).

Preparation of Microsomes. Microsomal preparations were obtained from 7- or 8-week-old male Wistar MRC rats (Eppley colony). Rats were injected ip with either 40 mg of 3-MC/kg of body weight in 2.5 mL of olive oil or 40 mg of phenobarbital/kg of body weight in 2.5 mL of saline twice daily for 3 days. They were fasted the final 24 h and sacrificed. Control rats received ip 2.5 mL of olive oil/kg of body weight and were fasted 24 h before sacrifice.

Livers or lungs from two rats were pooled and rinsed in 0.25 M sucrose solution containing 50 mM Tris buffer, 25 mM KCl, and 5 mM $MgCl_2$, pH 7.5 (sucrose–TKM). The organs were minced in 3 volumes of sucrose–TKM and homogenized in glass–glass (liver) of Teflon–glass (lung) systems. Microsomes were isolated according to Schenkman and Cinti[27] and resuspended in sucrose–TKM to a final protein concentration of 10 mg/mL for liver and 3–5 mg/mL for lungs. Protein concentrations were measured according to Lowry et al.[28] Fresh preparations

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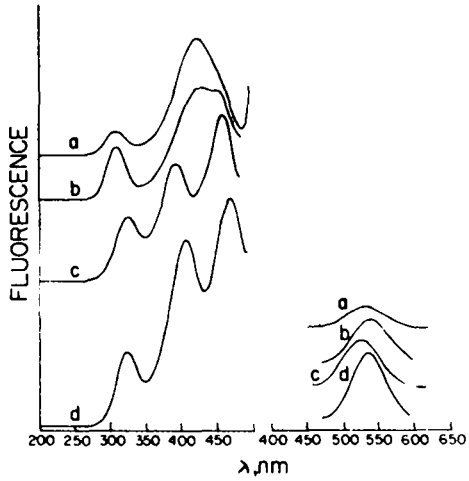

Figure 1. Fluorescence excitation (left) and emission (right) spectra of (a) 1-hydroxy-6-methoxy-BP, sodium phenolate, λ excitation 310 nm, λ emission 534 nm; (b) 12-hydroxy-6-methoxy-BP, sodium phenolate, λ excitation 310 nm, λ emission 540 nm; (c) 3-hydroxy-6-methoxy-BP, sodium phenolate, λ excitation 310 nm, λ emission 525 nm; and (d) 1,6-diethoxy-3-hydroxy-BP, sodium phenolate, λ excitation 320 nm, λ emission 538 nm.

of microsomes were used throughout.

Enzyme Assays. *p*-Nitroanisole *O*-demethylase activity was assayed by a modified method of Netter and Seidel[29] and AHH activity by a modified method of Nebert and Gelboin.[8] BP (80 nmol) was added to the incubation medium in 40 μL of acetone; all other substrates were added in 15 μL of Me_2SO. The incubation medium contained 0.5 mg of protein/mL (liver microsomes) or 1.0 mg of protein/mL (lung microsomes). Fluorescence spectra of basic solutions of the phenolic metabolites were obtained and the maxima for excitation and emission determined. Fluorescence excitation with the 1-phenols was at the maximum of the major peak and in the case of 3-phenols at the maximum of the central peak. Fluorescence emission was measured at the maximal wavelength for each compound. Phenolic metabolites were quantitated with basic solutions of 3-hydroxy-, 1-hydroxy-6-methoxy-, or 3-hydroxy-6-methoxy-BP. It was assumed that the 3-phenols of all monosubstituted 6-derivatives fluoresced with the same efficiency as 3-hydroxy-6-methoxy-BP. Enzyme activities were determined in duplicate and compared to a blank containing microsomes deactivated by heat (2 min at 100 °C) or prior addition of acetone. In either case, the blanks possessed less than 1% of the fluorescence of samples from active microsomes.

High-Performance Liquid Chromatography. Samples of metabolites were prepared for high-performance LC injection by extracting a 10-mL reaction mixture twice with an equal volume of ethyl acetate. The organic phases were combined and evaporated to dryness with nitrogen in the dark. The residue was dissolved in a standard volume of methanol for injection. Retention times and peak areas were calibrated with synthetic samples of substrates, diones, and phenols, both before and after chromatography of the metabolic products.

Substrate-Binding Difference Spectra. A procedure similar to that of Schenkman et al.[30] was used. One-milliliter samples contained 9.2 mg of protein and 27 nmol of substrate added in 13 μL of acetone (in the case of BP) or 5 μL of Me_2SO (with all other substrates). The medium was equal parts sucrose–TKM and 50 mM Tris buffer, pH 7.5.

Results

A. Fluorescence Spectra of Synthetic Phenols. Fluorescence excitation spectra permitted the phenolate anions of 1-hydroxy-6-methoxy- (Figure 1a) and 12-hydroxy-6-methoxy-BP (Figure 1b) to be distinguished from the phenolate anions of BP-3-phenols, including 3-hydroxy-6-methoxy- (Figure 1c), 1,6-diethoxy-3-hydroxy- (Figure 1d), and 3-hydroxy-BP.[8] In fact, the spectra of the 1- and 12-phenolates (Figure 1a,b) exhibited a minor peak around 308 nm and two poorly resolved major peaks between 400 and 475 nm, while the spectra of the 3-phenolates (Figure 1c,d) featured three well-resolved, evenly spaced peaks. The fluorescence intensities of the 12- and 1-phenolates were less than those of 3-phenolates (Table I) but were still considerable. The 12- and 1-phenols of 6-substituted BP derivatives are also reasonably stable. The metabolic products of BP derivatives may be studied by applying these spectral properties and the extent of enzymatic ring hydroxylation measured with authentic 1- and 3-phenols of BP derivatives as standards (see below).

Table I. Relative Fluorescence Intensity of Various BP Phenols[a]

compd	excitation, nm	emission, nm	rel fluorescence intensity
3-hydroxy-BP	394	520	100
3-hydroxy-6-methoxy-BP	401	525	79
1,6-diethoxy-3-hydroxy-BP	411	536	38
1-hydroxy-6-methoxy-BP	428	535	61
12-hydroxy-6-methoxy-BP	423	540	17

[a] Concentration 1.90×10^{-7} M in 1 N NaOH recorded with a Turner spectrofluorometer.

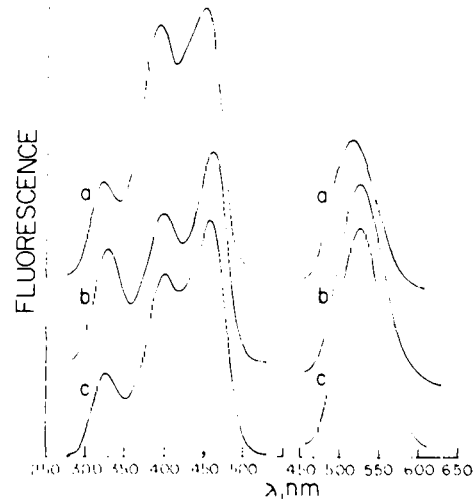

Figure 2. Fluorescence excitation (left) and emission (right) spectra of base-extractable metabolites obtained from 3-MC-induced liver microsomes: (a) BP; (b) 6,12-dimethoxy-BP; (c) 6-methoxy-BP.

B. Metabolic Hydroxylation of Benzo[*a*]pyrene and Some of Its Derivatives. The various substrates were incubated with liver and/or lung microsomes. As observed by others,[31-33] the small amount of organic solvent used to dissolve the substrates affected AHH activity. BP derivatives were metabolized most rapidly when added in

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Table II. Rates of 3-Hydroxylation of BP and Its Derivatives by Liver and Lung Microsomes from Uninduced or Induced Rats[a]

substrate	control	PB	3-MC	3-MC/control
		A. Liver		
BP	2950 ± 14 (2)	1160 ± 15 (2)	5810 ± 240 (5)	2.0
6-methyl-BP	200 (1)	ND[c]	ND	ND
6-(hydroxymethyl)-BP	885 ± 90 (2)	ND	1510 ± 290 (2)	1.7
6,12-dimethoxy-BP	~2900[b] (1)	ND	~5700 (1)	2
6-methoxy-BP	3120 ± 490 (5)	1980 ± 420 (2)	9010 ± 225 (3)	2.9
6-(methoxymethyl)-BP	1450 ± 215	ND	7040 ± 1160 (2)	4.9
		B. Lung		
BP	84 ± 19	49 ± 4 (2)	618 ± 47 (3)	7.4
6-(hydroxymethyl)-BP	36 ± 12 (2)	ND	182 ± 38 (2)	5.1
6-methoxy-BP	79 ± 14 (5)	69 ± 9 (2)	576 ± 57 (3)	7.3
6-(methoxymethyl)-BP	48 ± 2 (2)	ND	654 ± 1 (2)	13.6

[a] Rates are expressed in units of pmol of product 30 min^{-1} (mg of protein)$^{-1}$. Figures are the mean values plus or minus standard error, obtained from duplicate determinations with microsomes from the number of individual rats in parentheses. [b] The rates of hydroxylation from fluorescence intensities could not be quantitated due to the lack of an authentic sample of 3-hydroxy-6,12-dimethoxy-BP. The approximate values were deduced as described under Results. [c] ND = not determined.

Table III. Metabolites of BP Derivatives Exhibiting Fluorescence Spectra of BP Phenols

compd[a]	λ_{max}, excitation (rel intensity)[b]	λ_{max}, emission
3-hydroxy-6-methoxy-BP	325 (0.39), 394 (0.72), 458 (1.0)	525
3-phenols of		
BP (H, I)[c]	325 (0.35), 396 (0.93), 452 (1.0)	522
6-(hydroxymethyl)-BP (H, C)	338 (0.20), 404 (0.46), 466 (1.0)	530
6,12-dimethoxy-BP (H, I)	328 (0.53), 401 (0.71), 460 (1.0)	526
6-methoxy-BP (H, C)	326 (0.35), 401 (0.78), 457 (1.0)	526
6-methoxy-BP (H, I)	326 (0.34), 401 (0.77), 457 (1.0)	528
6-methoxy-BP (L, C)	325 (0.29), 401 (0.79), 458 (1.0)	524
6-methoxy-BP (L, I)	326 (0.35), 401 (0.76), 458 (1.0)	525
3,6-dimethoxy-BP (H, C)	326 (0.35), 400 (0.71), 458 (1.0)	525
3,6-dimethoxy-BP (H, I)	326 (0.35), 400 (0.77), 458 (1.0)	525
3,6-dimethoxy-BP (L, I)	325 (0.35), 400 (0.76), 460 (1.0)	525
1-hydroxy-6-methoxy-BP	308 (0.20), 420 (1.0), 450 sh[d] (0.75)	534
1-phenols of		
1,6-dimethoxy-BP (H, C)	325 (0.81), 428 (1.0), 455 sh (0.86)	534
1,6-diethoxy-BP (H, C)	318 (0.17), 427 (1.0), 450 sh (0.81)	534
1,6-diethoxy-BP (H, I)	318 (0.15), 427 (1.0), 450 sh (0.81)	534
1,6-diethoxy-BP (L, I)	316 (0.18), 424 (1.0), 450 sh (0.85)	534
1,6-dipropoxy-BP (H, C)	316 (0.21), 426 (1.0), 450 sh (0.90)	534
1-phenols and 3-phenols of		
6-(methoxymethyl)-BP (H, C)	326 (0.30), 392 sh (0.52), 403 (0.55), 4.60 (1.0)	530
6-(methoxymethyl)-BP (H, I)	318 (0.25), 425 sh (0.78), 455 (1.0)	534
6-(methoxymethyl)-BP (L, I)	318 (0.25), 425 sh (0.78), 455 (1.0)	534

[a] Fluorescence spectra of authentic phenols and metabolites of some BP derivatives in 1 N NaOH. [b] Peak intensities are expressed relative to the peak of greatest intensity. [c] Abbreviations: H = hepatic, L = lung, C = control, I = 3-MC induced. [d] sh = shoulder.

Me_2SO or polyols. Me_2SO was chosen as the solvent for these compounds because of its lesser viscosity and greater solubilizing properties compared to polyols. Anomalously, BP was hydroxylated most rapidly when added in acetone, which was retained as its solvent. The base-extractable metabolites were isolated and their fluorescence intensities calibrated with a phenolic authentic sample. In the case of 6-substituted derivatives, it was assumed that their corresponding 3-phenols fluoresced with the efficiency of the 3-hydroxy-6-methoxy-BP. The metabolic profile of some BP derivatives was studied by high-performance LC.

Benzo[*a*]pyrene. The base-extractable metabolites of BP (Figure 2a) exhibited a fluorescence excitation spectrum resembling that of 3-hydroxy-BP, although, as reported elsewhere,[8,34] the shallow troughs indicated the presence of small amounts of other phenols. Both liver and lung microsomes gave the same product. The 3-hydroxylase activity was inducible in both organs by 3-MC but was decreased in this strain of rat by treatment with PB (Table II), as observed by others.[35,36] The difference spectrum of microsomes incubated with BP exhibited a maximum at 388 nm and a minimum at 414 nm, a type I pattern.

6-Methyl- and 6-(Hydroxymethyl)benzo[*a*]pyrene. These two substrates were also mainly hydroxylated at position 3, as evidenced by their fluorescence excitation spectra. 6-Methyl-BP was hydroxylated by liver microsomes at about one-fifteenth the rate of BP (Table II, section A). 6-(Hydroxymethyl)-BP was hydroxylated by liver and lung microsomes at rates appreciably less than those of BP. Induction patterns with 3-MC were similar to those of BP.

6,12-Dimethoxybenzo[*a*]pyrene. 6,12-Dimethoxy-BP was apparently hydroxylated exclusively at position 3 by liver microsomes, since the base-extractable metabolites

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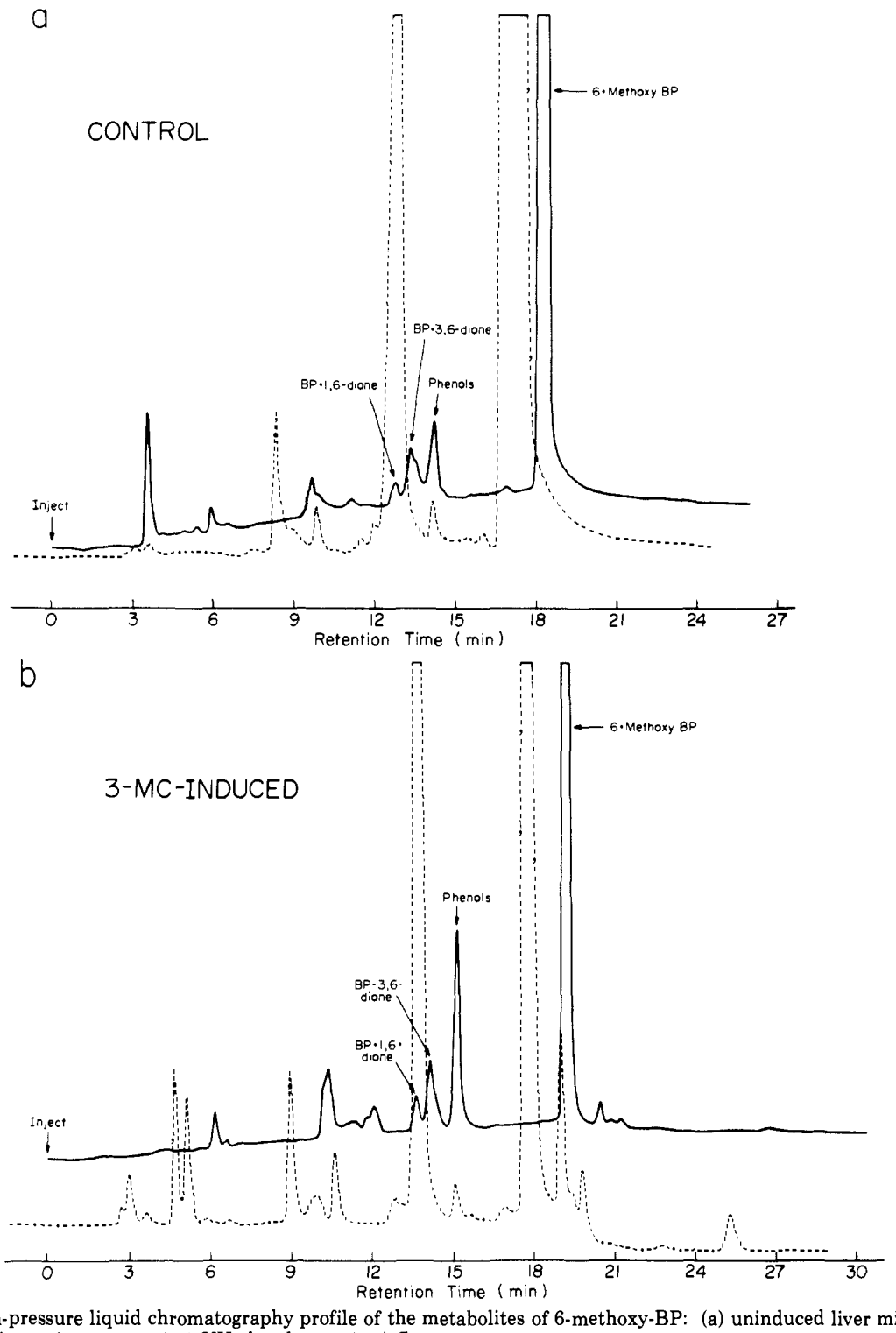

Figure 3. High-pressure liquid chromatography profile of the metabolites of 6-methoxy-BP: (a) uninduced liver microsomes; (b) 3-MC-induced liver microsomes. (—) UV absorbance, (- - -) fluorescence.

exhibited the fluorescence spectrum of a pure 3-phenol (Figure 2b and Table III). This activity was inducible by 3-MC (Table II, section A). The lack of a sample of 3-hydroxy-6,12-dimethoxy-BP precluded quantitation of the rate of hydroxylation. 3-Hydroxy-6-methoxy-BP could not be used to calibrate this activity, as the fluorescence intensity of the disubstituted phenol would be less than that of the monosubstituted one. However, the fluorescence intensities of the phenolic metabolites of 6,12-dimethoxy-BP were approximately 60% of those of BP metabolites in uninduced and 3-MC-induced liver in microsomes, suggesting that the hydroxylation occurred at a comparable rate.

6-Methoxybenzo[*a*]pyrene. As previously reported,[37] this compound was hydroxylated at position 3 (Figure 2c and Table III) by liver and lung microsomes. The spectra suggest the presence of small amounts of other phenols, since the troughs are shallower than in the case of the pure 3-phenol (Figure 1c). The hydroxylation rate with liver microsomes was slightly higher than that with BP (Table II, section A), while the induction patterns were similar. Since the 6-substitution decreased the fluorescence of the corresponding 3-phenol, the absolute fluorescence of the

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Table IV. Rates of O-Dealkylation of Several BP Derivatives and of *p*-Nitroanisole by Liver and Lung Microsomes from Uninduced or Induced Rats[a]

substrate	control	PB	3-MC	PB/ control	3-MC/ control
		A. Liver			
BP[b]	2 950 ± 14 (2)	1 115 ± 15 (2)	5 810 ± 241 (5)	0.39	2.0
1,6-dimethoxy-BP	5 740 ± 500 (2)	ND	ND	ND	ND
1,6-diethoxy-BP	6 040 ± 369 (3)	2 850 ± 379 (2)	3 360 ± 389 (2)	0.47	0.56
1,6-dipropoxy-BP	620 (1)	ND	818 (1)	ND	1.3
3,6-dimethoxy-BP	2 190 ± 260 (2)	ND	1 253 (1)	ND	0.57
p-nitroanisole	20 550 ± 0 (2)	102 600 ± 0 (2)	229 400 ± 25 800 (3)	5.0	11.2
		B. Lung			
BP	84 ± 19 (5)	49 ± 4 (2)	618 ± 47 (3)	0.58	7.4
1,6-diethoxy-BP	56 ± 3 (3)	48 ± 11 (2)	156 ± 21 (2)	0.86	2.8
3,6-dimethoxy-BP	42 (1)	ND	ND	ND	ND

[a] Rates are expressed in units of pmol of product 30 min^{-1} (mg of protein)$^{-1}$. Figures are the mean values plus or minus standard error, obtained from duplicate determinations on microsomes from the number of individual rats in parentheses. [b] The rate of 3-hydroxylation of BP is included for comparison.

metabolites of 6-methoxy-BP was not appreciably enhanced compared to the metabolites of BP.

When resolved by high-performance LC (Figure 3a), the major metabolites coeluted with and exhibited the fluorescence and nonfluorescence characteristics of 3-hydroxy-6-methoxy-BP and BP-diones, i.e., BP-1,6-dione and -3,6-dione, respectively. The 1,6- and 3,6-diones, in the approximate ratio 1:2, were formed in a slightly smaller quantity than the phenol. This indicates that 6-methoxy-BP is O-demethylated at a significant rate. The initial product of demethylation, i.e., 6-hydroxy-BP, would rapidly oxidize to diones. When 6-methoxy-BP was metabolized by the same quantity of 3-MC-induced microsomal liver protein, the area of the high-performance LC phenolic peak increased by a factor of 2.7 (Figure 3b), which compares well with the fluorimetric factor of 2.9 (Table II, section A). No increase was noted in the areas of the dione peaks, which indicates that the *O*-demethylase activity was not inducible by 3-MC. The difference spectrum of microsomes incubated with 6-methoxy-BP was that of a type I compound, with a maximum at 384 nm and a minimum at 414 nm.

6-(Methoxymethyl)benzo[*a*]pyrene. This compound was metabolized at approximately one-half the rate of BP by liver and lung microsomes (Table II). The fluorescence excitation spectrum of the base-extractable metabolites exhibited the three-peak pattern of a 3-phenol (Figure 4a). However, 3-MC induced the rate of metabolism of 6-(methoxymethyl)-BP markedly more than the rate of metabolism of BP or the other derivatives (Table II). This induction was accompanied by a change in the fluorescence spectrum of the metabolites. The trough between the second and third excitation peaks vanished (Figure 4b and Table III), indicating the presence of a phenol with an excitation peak in the region 410–430 nm. The emission maximum was also shifted a few nanometers to the red. Similar changes were obtained with 3-MC-induced lung microsomes (Figure 4c and Table III). The high-resolution fluorescence spectrum of the liver 3-MC-induced metabolites (Figure 5a) resembles the superimposed spectra of the 1- (Figure 5b) and 3-phenolate (Figure 5c), with the 425-nm peak of the 1-phenolate visible. Assuming that the spectral characteristics and the relative fluorescence intensities (Table I) of the 1- and 3-phenolates of 6-(methoxymethyl)-BP do not differ significantly from those of the corresponding phenolates of 6-methoxy-BP, the results suggest that 3-MC-induced microsomes produce the 1- and 3-phenols of 6-(methoxymethyl)-BP in approximately equal quantities. When resolved by high-performance LC, most of the metabolites from both uninduced and 3-MC-induced microsomes eluted in a monophenol peak. The difference spectra with both control and induced microsomes were type I, with maxima at 387 nm and minima at 408 nm.

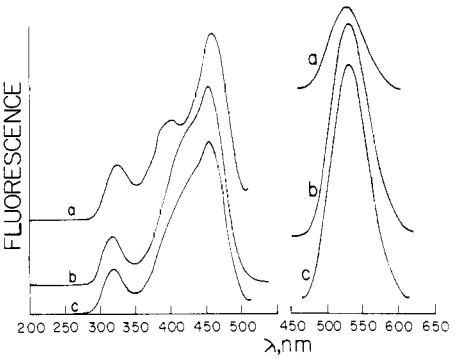

Figure 4. Fluorescence excitation (left) and emission (right) spectra of base-extractable metabolites of 6-(methoxymethyl)-BP: (a) uninduced liver microsomes; (b) 3-MC-induced liver microsomes; (c) 3-MC-induced lung microsomes.

C. O-Dealkylation of Benzo[*a*]pyrene Derivatives. BP 1,6- and 3,6-dialkoxy derivatives were metabolized by O-dealkylation. Thus, 1,6-dimethoxy-, 1,6-diethoxy-, and 1,6-dipropoxy-BP were dealkylated by liver and lung microsomes to yield the corresponding 1-phenols, while 3,6-dimethoxy-BP was metabolized to 3-hydroxy-6-methoxy-BP (Table III). The rate of O-dealkylation in liver microsomes was about the same for 1,6-dimethoxy- and 1,6-diethoxy-BP, while it was much slower for 1,6-dipropoxy-BP (Table IV, section A). Induction by PB or 3-MC decreased this activity, in contrast to the effect on *p*-nitroanisole *O*-demethylase (Table IV, section A). Metabolic activity in the lungs was low (Table IV, section A). Lung microsomes from 3-MC-treated rats metabolized 1,6-diethoxy-BP about three times faster than controls. The fluorescence spectrum was that of the 1-phenol (Table III) and not that of the 1,6-diethoxy-3-hydroxy-BP (Figure 1d), indicating that *O*-dealkylase activity had been induced in the lung.

The difference spectra of microsomes incubated with these compounds were type II, with maxima at 432 (1,6-dimethoxy- and 1,6-diethoxy-BP) and 441 nm (3,6-dimethoxy-BP), and minima at 410 (1,6- and 3,6-dimethoxy-BP) and 411 nm (1,6-diethoxy-BP).

Discussion

AHH clearly has a positional specificity for 3-hydroxylation of BP and some of its derivatives. The minor conversion to 1-phenol[9] and the lack of 12-phenol

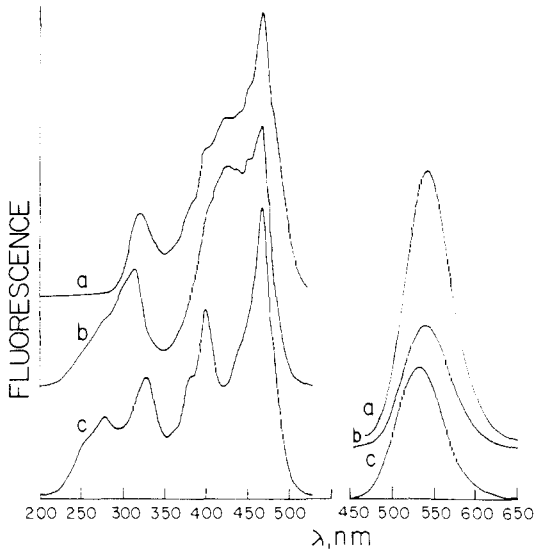

Figure 5. High-resolution fluorescence excitation (left) and emission (right) spectra of (a) base-extractable metabolites of 6-(methoxymethyl)-BP obtained from 3-MC-induced liver microsomes; (b) 1-hydroxy-6-methoxy-BP, sodium phenolate; (c) 3-hydroxy-6-methoxy-BP, sodium phenolate.

in BP metabolism are presumably due to a steric constraint of the enzyme and not to the instability of the phenolic products. When hydroxylation of position 6 was blocked, no suitable substitute for BP in the AHH assay was produced. Substrates which were 3-hydroxylated at comparable rates to BP, i.e., 6-methoxy- and 6,12-dimethoxy-BP (Table II, section A), did not improve the sensitivity of the assay, as the higher degree of substitution of the products decreased their fluorescence yields. Other substrates were 3-hydroxylated at a much lower rate; 6-methyl- and 6-(hydroxymethyl)-BP (Table II, section A) were hydroxylated at positions other than 3, i.e., 6-(methoxymethyl)-BP (Figure 4b,c and Table III), or metabolized by O-dealkylation, i.e., 1,6- and 3,6-dialkoxy ethers (Table III). Therefore, we cannot recommend any substitute for BP in the measurement of 3-hydroxylase activity.

6,12-Dimethoxy-BP is presumably hydroxylated solely at position 3 (Figure 2b). Other derivatives, however, are probably hydroxylated at more than one position, since their base-extractable metabolites exhibited fluorescence excitation spectra with slightly shallower troughs between the two most prominent peaks than was the case with pure 3-phenols. Peak maxima were also shifted several nanometers to the red for both excitation and emission (Table III). Upon induction by 3-MC, the troughs became even shallower with the metabolites of BP and 6-methoxy-BP (Figure 2a,c), while the trough disappeared altogether with the metabolites of 6-(methoxymethyl)-BP (Figure 4b). This was accompanied by a further red shift in maxima. These observations suggest a change in positional specificity upon induction. The presence of either the 1- or 9-phenol of BP could account for the shallow troughs, but 9-phenols would shift the emission maxima to the blue,[12] while 1-phenols would shift them to the red. Therefore, AHH probably has some specificity for position 1 of BP derivatives. Although the 12-phenol shows a fluorescence excitation spectrum similar to the 1-phenol (Figure 1a,b), it is assumed that hydroxylation of BP derivatives occurs at position 1, as no 12-phenol has ever been identified among the BP metabolites.[9,12] The specificity for the 1 position is preferentially induced by 3-MC, indicating that the induced AHH has a set of steric constraints different from those of the uninduced AHH. The positional specificity is affected by the location of substituents in the substrate. Thus, a 12-methoxy group prevents 1-hydroxylation, even by 3-MC-induced microsomes (Figure 2b). On the other hand, a methoxymethyl group at position 6 causes induced microsomes to hydroxylate positions 1 and 3 (Figure 4b,c and 5a) at roughly equal rates. The long side chain probably causes the substrate to fit into the active site at an unusual angle. This particular fit increases the rate of attack on both positions 1 and 3, with preferential increase at position 1.

The O-dealkylase activity which metabolizes BP 1,6-ethers and 3,6-ethers is decreased upon treatment of the rat with PB or 3-MC (Table IV). Apparently, the induced forms of cytochrome P-450 have lesser activity and dilute the uninduced forms. The *O*-dealkylase attacks the alkyl groups at the 1, 3, and 6 positions, but not at the 12 position, as in the case of 6,12-dimethoxy-BP which was preferentially hydroxylated at position 3 [Figure 2b, Tables II (section A) and III]. 6-Methoxy-BP was both 3-hydroxylated and O-demethylated (Figure 3). The hydroxylase activity was inducible but the *O*-demethylase activity was not, suggesting it is similar to the activity discussed above. Since a BP derivative can be O-dealkylated at positions 1, 3, and 6, but not at 12, then a constraint is set upon any hypothetical model of the active site involved.

The 1,6- and 3,6-ethers of BP are type II compounds. This is anomalous in two respects. First, except for puromycin, the difference spectra of all O-dealkylated compounds thus far determined are type I, including *p*-nitroanisole,[38] 7-ethoxycoumarin,[36] acetophenetidin,[32] and codeine.[39] Secondly, these are the first type II compounds reported which do not contain a basic nitrogen atom. This challenges the hypothesis that the type II ligation to the heme group of P-450 requires such a basic nitrogen.[30,40] Apparently, the oxygen of the BP ethers can serve in the same capacity, although the data do not suffice to determine which of the groups is involved in the ligation. The O-demethylation of 6-methoxy-BP suggests ligation to the side chain being oxidized, as does the N-demethylation of the type II compound *p*-chloro-*N*-methylaniline.[32]

The metabolism of the BP ethers resembles that of puromycin and may be effected by the same enzyme. Puromycin, a type II compound,[41,42] is metabolized by both N- and O-demethylation.[43] The rate of O-demethylation, approximately 2 nmol 30 min^{-1} (mg of microsomal protein)$^{-1}$, is comparable to the rate of O-dealkylation of BP ethers. Neither *O*-dealkylase is induced in the liver by 3-MC. Puromycin is also a competitive inhibitor for the type I site.[41] It is possible N-demethylation occurs at the type I site and the O-demethylation at the type II site, since sterically hindered amines bind to type I and not type II sites.[40] The rate of N-demethylation is over 10 times more than that of O-demethylation, but this would not necessarily determine the binding spectrum, since there is no direct relationship between the kinetics of binding and the rate of metabolism of type II compounds.[30] Similarly, 6-methoxy-BP may be metabolized at two different

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sites. The inducible 3-hydroxylation occurs at a type I site, while the noninducible O-demethylation presumably occurs at a type II site.

In summary, the metabolism of BP and its derivatives involves at least three different enzyme activities: (1) a 3-hydroxylase in uninduced microsomes which has some 1-hydroxylase activity, (2) a 3-hydroxylase in 3-MC-induced microsomes which has more 1-hydroxylase activity and which is more sensitive to the structure of the substrate, and (3) an O-dealkylase which is not inducible. This last activity may or may not involve the uninduced 3-hydroxylase. Both have the same positional specificity: the hydroxylase can attack positions 1, 3, and 6, but not 12, while the O-dealkylase can O-dealkylate groups at positions 1, 3, and 6, but not 12. However, the hydroxylation occurs at a type I site and the O-dealkylation at a type II site, and, accordingly, different active sites are involved. The various activities may be due to different forms of cytochrome P-450. Both uninduced and induced microsomes contain a number of forms of these enzyme systems, all of which vary somewhat in substrate specificity,[6,7,44,45] positional specificity,[34,46] and stereospecificity.[46] For example, warfarin[46] can be hydroxylated at five different positions. This is reported to involve at least four different monooxygenases, one of which is not inducible by PB or 3-MC.

Detoxification and elimination of aromatic hydrocarbons generally proceeds through an initial oxidation of the molecule. There has been considerable study of the effects of enzyme induction on the rate of hydrocarbon hydroxylation. However, this study indicates that induction may also affect the position of the initial oxidation. This factor would modify the metabolic pathways, which could conceivably affect the biological activity and disposition of the compound, as well as the change in the rate of oxidation. The biological activity of aromatic hydrocarbons in different tissues and species presumably reflects both the disparate inducibilities and positional specificities of enzymes in different tissues. This important problem warrants further comparison of the products of initial metabolic oxidation of polycyclic aromatic hydrocarbons in uninduced and induced tissues.

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Studies on Cardiovascular Agents. 6. Synthesis and Coronary Vasodilating and Antihypertensive Activities of 1,2,4-Triazolo[1,5-*a*]pyrimidines Fused to Heterocyclic Systems

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The synthesis and coronary vasodilating and antihypertensive activities of 1,2,4-triazolo[1,5-*a*]pyrimidines fused to pyrrole, thiophene, pyran, pyridine, and pyridazine are described. Among these compounds, 8-*tert*-butyl-7,8-dihydro-5-methyl-6*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*a*]pyrimidine (**23**) was found to be the most promising potential cardiovascular agent, having been shown to be more potent in coronary vasodilating activity than trapidil [7-(diethylamino)-5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidine] and approximately equipotent to guanethidine sulfate in antihypertensive activity.

Some 1,2,4-triazolo[1,5-*a*]pyrimidine derivatives are known to possess potent pharmacological activities. For example, trapidil [7-(diethylamino)-5-methyl-1,2,4-triazolo[1,5-*a*]pyrimidine] is used as a coronary vasodilator[1] and 2-amino-4,5-dihydro-6-methyl-5-oxo-4-propyl-1,2,4-triazolo[1,5-*a*]pyrimidine has shown significant activity against bronchospasm.[2] Therefore, in attempts to prepare new types of 1,2,4-triazolo[1,5-*a*]pyrimidine derivatives superior to trapidil, we have synthesized a number of compounds which have a pyrrolotriazolopyrimidine nucleus resulting from the fixation of an ethylamino chain of trapidil to the triazolopyrimidine nucleus in a continuing search for cardiovascular agents.[3] Furthermore, triazolopyrimidines fused to thiophene, pyran, pyridine, and pyridazine were prepared and screened for coronary vasodilating and antihypertensive activities. Among these compounds, 8-*tert*-butyl-7,8-dihydro-5-methyl-6*H*-pyrrolo[3,2-*e*][1,2,4]triazolo[1,5-*a*]pyrimidine (**23**) was found to be the most promising potential cardiovascular agent. This paper deals with the synthesis and biological activity of the title compounds.

Chemistry. The synthetic routes to the 7,8-dihydro-6*H*-pyrrolotriazolopyrimidines **5–28** and **30–102** are outlined in Scheme I. Condensation of the 3-aminotriazoles (1; R^1 = H, CH_3, C_6H_5, and 4-CH_3O-C_6H_4) with the appropriate α-acetyl-γ-butyrolactones in the presence of boron trifluoride led to the 3-[[1-(tetrahydro-2-oxo-3-furyl)ethylidene]amino]triazoles **2a–f** (Table I), which are

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