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Degradation of benz[*a*]anthracene by *Mycobacterium vanbaalenii* strain PYR-1

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Abstract

Cultures of *Mycobacterium vanbaalenii* strain PYR-1 grown in mineral salts medium and nutrients in the presence of benz[*a*]anthracene metabolized 15% of the added benz[*a*]anthracene after 12 days of incubation. Neutral and acidic ethyl acetate extractable metabolites were isolated and characterized by high performance liquid chromatography (HPLC) and uv–visible absorption, gas chromatography/mass (GC/MS) and nuclear magnetic resonance (NMR) spectral analysis. Trimethylsilylation of the metabolites followed by GC/MS analysis facilitated identification of metabolites. The characterization of metabolites indicated that *M. vanbaalenii* initiated attack of benz[*a*]anthracene at the C-1,2-, C-5,6-, C-7,12- and C-10,11-positions to form dihydroxylated and methoxylated intermediates. The major site of enzymatic attack was in the C-10, C-11 positions. Subsequent *ortho*- and *meta*-cleavage of each of the aromatic rings led to the accumulation of novel ring-fission metabolites in the medium. The major metabolites identified were 3-hydrobenzo[*f*]isobenzofuran-1-one (3.2%), 6-hydrofuran[3,4-*g*]chromene-2,8-dione (1.3%), benzo[*g*]chromene-2-one (1.7%), naphtho[2,1-*g*]chromen-10-one (48.1%), 10-hydroxy-11-methoxybenz[*a*]anthracene (9.3%), and 10,11-dimethoxybenz[*a*]anthracene (36.4%). Enzymatic attack at the C-7 and C-12 positions resulted in the formation of benz[*a*]anthracene-7,12-dione, 1-(2-hydroxybenzoyl)-2-naphthoic acid, and 1-benzoyl-2-naphthoic acid. A phenyl-naphthyl metabolite, 3-(2-carboxylphenyl)-2-naphthoic acid, was formed when *M. vanbaalenii* was incubated with benz[*a*]anthracene *cis*-5,6-dihydrodiol, indicating *ortho*-cleavage of 5,6-dihydroxybenz[*a*]anthracene. A minor amount of 5,6-dimethoxybenz[*a*]anthracene was also formed. The data extend and propose novel pathways for the bacterial metabolism of benz[*a*]anthracene.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) include pollutants of high human and ecotoxicological relevance due to their ubiquitous occurrence and potential toxic, mutagenic, and carcinogenic effects (Belkin et al. 1994; Blumer 1976; Harvey 1996).

Benz[*a*]anthracene is composed of four fused benzene rings. It is typically detected in concentrations of 14–312 mg kg^{-1} in soils from creosote waste sites, former wood treating operations, and manufactured gas plant sites (Sayles et al. 1999; Talley et al. 2002; Tiehm et al. 1997; Weissenfels et al. 1992).

Benz[*a*]anthracene is a suspected carcinogen that undergoes metabolic activation by microsomal cytochrome P450 monooxygenases and epoxide hydrolases to form biologically active products (Peltonen & Dipple 1995; Phillips & Grover 1994). The formation of an electrophilic bay-region dihydrodiol epoxide, benz[*a*]anthracene *trans*-3,4-dihydrodiol-1,2-epoxide, with subsequent formation of covalently linked DNA adducts has been implicated in the mutagenicity

and carcinogenicity of benz[*a*]anthracene (Braithwaite et al. 1998). Since microbial degradation of benz[*a*]anthracene has the potential to form reactive intermediates, the metabolites should be identified to see if potentially toxic intermediates are formed during the bioremediation of PAH-contaminated sites.

Whereas low-molecular weight PAHs (two or three rings) are usually readily degraded, high molecular weight PAHs, such as benz[*a*]anthracene, benzo[*a*]pyrene, chrysene, and dibenz[*a,h*]anthracene, resist extensive microbial degradation in soils and sediments (Cerniglia 1992; Kanaly & Harayama 2000; Mueller et al. 1996). Although there are many reasons why high molecular-weight PAHs are recalcitrant, a major explanation for lack of biodegradation is limited bioavailability due to the low water solubility and hydrophobic nature of PAHs (Talley et al. 2002).

Benz[*a*]anthracene is degraded to a limited extent by indigenous microflora in PAH-contaminated soils and sediments (Hughes et al. 1997; Tiehm et al. 1997). Pure cultures of *Pseudomonas saccharophila*, *P. fluorescens, Burkholderia cepacia, Sphingomonas paucimobilis*, *S. yanoikuyae,* and *Mycobacterium* spp. have the ability to degrade benz[*a*]anthracene (Caldini et al. 1995; Chen & Aitken 1999; Juhasz et al. 1996; Mahaffey et al. 1988; Schneider et al. 1996; Weissenfels et al. 1992; Ye et al. 1996). The rate of degradation of benz[*a*]anthracene in soil inoculated with a bacterial consortium or *Stenotrophomonas maltophilia* was low, but co-culturing with the fungus *Penicillium janthinellum* significantly increased metabolism (Boonchan et al. 2000). However, metabolites from benz[*a*]anthracene were not characterized in these studies.

Information on the isolation and identification of metabolites from the bacterial degradation of benz[*a*]anthracene is scarce. Benz[*a*]anthracene *cis*-1,2-, -8,9-, and 10,11-dihydrodiols were formed by a mutant strain B8/36 formerly thought to be a *Beijerinckia* sp. (Jerina et al. 1984), but now identified as *Sphingomonas yanoikuyae* (Gibson 1999; Khan et al. 1996). Further metabolism of those dihydrodiols by the wild-type strain B1 of this bacterium produced 1-hydroxy-2-anthroic acid, 3-hydroxy-2-phenanthroic acid, and 2-hydroxy-3-phenanthroic acid (Mahaffey et al. 1988). *Mycobacterium* sp. strain RJGII-135 grown in the presence of benz[*a*]anthracene formed five metabolites, two of which were characterized and identified as benz[*a*]anthracene *cis*-5,6- and *cis*-10,11-dihydrodiols (Schneider et al. 1996).

Mycobacterium sp. PYR-1, isolated from petrogenic chemical contaminated estuarine sediments, is capable of metabolizing low and high molecular weight PAHs (Heitkamp et al. 1988a, b; Khan et al. 2002). The biochemical pathways for the degradation of naphthalene, fluoranthene, anthracene, phenanthrene, pyrene, 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene by this strain have been described (Cerniglia 1992; Heitkamp et al. 1988a, b; Kelley & Cerniglia 1991; Kelley et al. 1990, 1991, 1993; Moody et al. 2001, 2003, 2004). In addition, *Mycobacterium vanbaalenii* PYR-1 has been used to degrade PAHs in soil–slurry microcosms and two-phase partitioning bioreactors (MacLeod & Daugulis 2003; Ramierz et al. 2001). In this paper, we report the detailed chemical structures of initial ring oxidation and novel ring-cleavage metabolites formed from benz[*a*]anthracene by *Mycobacterium vanbaalenii* strain PYR-1. Based upon this information, we extend and propose new pathways for the microbial degradation of benz[*a*]anthracene.

Materials and methods

Chemicals

[5,6-^{14}C]Benz[*a*]anthracene (54.6 mCi mmol^{-1}) with a radiochemical purity of ≥98% was purchased from Chemsyn Science Laboratories (Lenexa, KS). Unlabeled benz[*a*]anthracene (99% pure) was purchased from Aldrich Chemical Company (Milwaukee, WI). Racemic benz[*a*]anthracene *cis*-5,6-dihydrodiol was synthesized by Dr Peter P. Fu at the National Center for Toxicological Research. Bacteriological media and reagents were purchased from BD Biosciences, Difco Laboratories (Detroit, MI). NMR solvents were purchased from Isotec, Inc. (Miamisburg, OH). Other solvents were purchased from J. T. Baker, Inc. (Phillipsburg, NJ), and were of the highest purity available.

Culture conditions

Cultures of *Mycobacterium vanbaalenii* PYR-1 were grown in 1-l Erlenmeyer flasks containing 500 ml of basal salts medium supplemented with 0.38 g l^{-1} each of peptone, yeast extract, and soluble starch (Heitkamp et al. 1988a,b). A 100 µl

aliquot of phenanthrene in *N,N*-dimethylformamide (DMF)(12 mg ml^{-1}) was added to each flask for enzyme induction. The cultures were grown for 4 days in the dark at 28 °C with shaking at 110 rpm. The cells (5 g wet wt.) from the flasks were harvested by centrifugation (7000 rpm for 10 min) and resuspended in 50 ml of the medium in 250 ml Erlenmeyer flasks. Benz[*a*]anthracene was dissolved in DMF and added to the cultures at a final concentration of 0.53 mM (6 mg in 50 ml medium). Flasks were harvested at 1, 3, 5, 10, 24, 48, 72, 96, 114, 144, 192, 264, and 288 h to determine the time of optimum metabolite production. A larger group of cultures was grown, treated with benz[*a*]anthracene in the same manner, and incubated for 114 h. Flasks containing only the culture and noninoculated flasks containing only benz[*a*]anthracene and medium were used as controls.

The contents of each flask were extracted as previously reported and dried (Moody et al. 2001). The pH of the remaining aqueous fraction was lowered to 2.5 with 6 M HCl and extracted again with three equal volumes of ethyl acetate to isolate the acidic metabolites. The residues were dissolved in 3 ml methanol and concentrated to approximately 100 µl, using a model SS21 Savant SpeedVac system (Savant Instruments, Holbrook, NY) for analysis by reversed-phase HPLC.

Radiolabel experiments

Mineralization experiments, as evidenced by CO_2 evolution, were carried out using a CO_2 trap containing 20 ml of 5 M NaOH. The phenanthrene-induced *Mycobacterium* cells (Moody et al. 2001) were added to 50 ml of basal salts medium supplemented with 0.38 g/ml each of peptone, yeast extract, and soluble starch and treated with 1.22 µCi ^{14}C-labeled benz[*a*]anthracene and 6 mg unlabeled PAH. A flask containing only the benz[*a*]anthracene and one with autoclaved *Mycobacterium* cells and ^{14}C-benz[*a*]anthracene were used as controls. Each flask was immediately sampled for CO_2 production by removing 1.0 ml of the trapping solution. This sample was added to 14 ml of Ultima Gold liquid scintillation fluid (Packard Instruments, Downers Grove, IL) and counted in a Packard Tri-Carb 2000A scintillation analyzer. Flasks were sampled and counted at 2, 3, 4, 5, 6, 24, 48, 96, 120, 144, 168, and 192 h.

Physical and chemical analysis

Benz[*a*]anthracene and its metabolites were separated by HPLC using a Hewlett-Packard model 1050 (Hewlett-Packard, Palo Alto, CA) chromatograph with a 4.6 × 250 mm C_{18} Inertsil ODS-3 5-µm column (MetaChem Technologies, Torrance, CA) at a flow rate of 1 ml min^{-1}. UV absorbance spectra were obtained on-line using a diode array (model 1040A) detector monitored at 254 nm. The compounds were eluted using a linear gradient of 40–95% methanol/water over 40 min. For collection of larger amounts of metabolites, a Beckman model 100A HPLC (Beckman Instruments, Fullerton, CA) with a 10 × 250 mm C_{18} Inertsil ODS-3 5-µm column (MetaChem) was used. The mobile phase and gradient were the same as above, but with a 5 ml min^{-1} flow rate. The concentration and relative amount of each metabolite was estimated from the peak areas of these compounds compared to internal standards.

Probe EI mass spectra were obtained on a TSQ 700 triple quadrupole mass spectrometer (ThermoFinnigan Corp., San Jose, CA) using a direct exposure probe and electron ionization (DEP/EI MS).

Gas chromatography/mass spectrometry (GC/MS) was performed on the same triple quadrupole mass spectrometer with separation of the metabolites using a J&W DB-5ms capillary column (30 m × 0.25 mm, 0.25 µm film thickness). GC/MS analyses were performed with a column temperature increase of 20 °C min^{-1} and a total analysis time of 30 min. Derivatization prior to GC/EI-MS analysis was performed by silylation with *N,O-bis*(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (Regis Technologies, Morton Grove, IL). The samples were dissolved in 250 µl of acetonitrile. One hundred microliters of dissolved sample and 150 µl of silylation reagent were mixed and allowed to react for 1 h at 60 °C. The injection volume was 0.5 µl.

All mass spectrometric measurements (GC/MS & DEP/MS) were at low resolution and no tandem mass spectrometry methods were employed. All fragmentation losses are reported as assumptions based on the proposed structures and available moieties. Observed mass losses and proposed composite loss species include: 1 (H), 15 (CH_3), 18 (H_2O), 28 (CO), 29 (HCO), 43 (CH_3 + CO), 44 (CH_3 + HCO), 44 (CO_2), 45 (CO_2H), 46

(H_2O + CO), 47 (H_2O + HCO), 57 (CO + HCO), 58 ($2CH_3$ + CO), 61 (CH_3 + CO + H_2O), 71 (CH_3 + 2CO), 72 (CH_3 + CO + HCO), and 72 (CO_2 + CO).

NMR spectra were recorded at 500.13 MHz on a Bruker AM500 spectrometer (Bruker Instruments, Billerica, MA). The metabolites were dissolved in 0.5 ml 99.96% acetone-d_6. $^{-1}$H chemical shifts are reported on the δ scale (ppm) by assigning the residual solvent peak to 2.04 ppm. Typical $^{-1}$H data acquisition parameters were: data size, 32 K; sweep width, 7042 Hz, filter width,

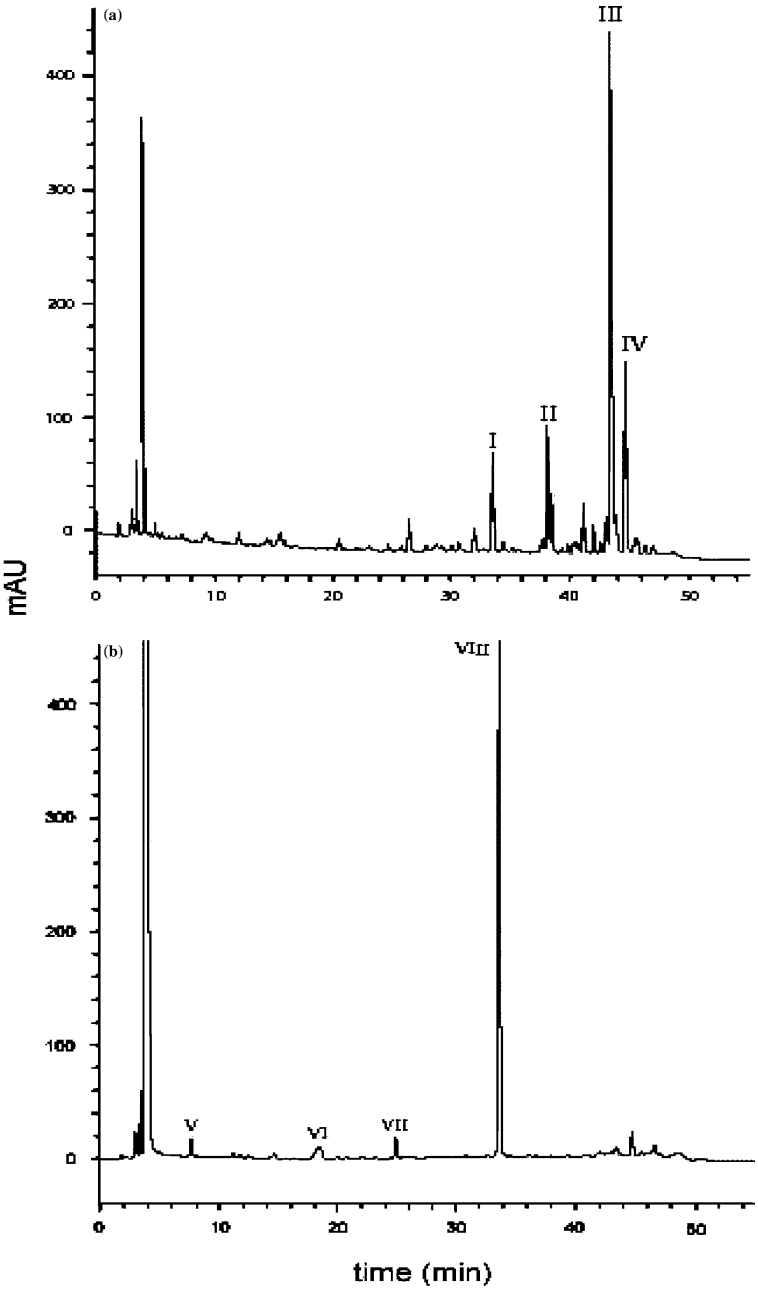

Figure 1. Reversed-phase HPLC chromatograms showing benz[*a*]anthracene and the metabolites formed by *M. vanbaalenii* PYR-1. (a) Neutral extract. (b) Acidic extract.

Table 1. HPLC, mass, and proton nuclear magnetic resonance spectral data for benz[*a*]anthracene and its metabolites produced from *Mycobacterium* sp. PYR-1

Compound name	Retention time (min)	Molecular weight	Significant ions produced by electron ionization [*m*/*z* (% relative intensity)]	NMR chemical shifts (ppm)[a]	Coupling constants (Hz)[b]
Naphtho[2,1-*g*]chromen-10-one (I and VIII)	34.2	246	247(21), 246(100), 218(33), 189(41), 163(6), 123(5), 109(4), 94.6(21), 93.6(5)	6.52(H9), 7.72(H3), 7.76(H2), 7.83(H5), 7.90(H6), 8.00(H4), 8.17(H8), 8.30(H7), 8.67(H12), 8.89(H1)	$J_{1,2}$ = 8.6, $J_{1,3}$ = 1.1, $J_{2,3}$ = 7.7, $J_{2,4}$ = 1.5, $J_{3,4}$ = 7.7, $J_{5,6}$ = 8.8, $J_{8,9}$ = 9.5
10-Hydroxy-11-methoxy-benz[*a*]anthracene (II)	38.8	274	275(22), 274(100), 259(91), 231(29), 203(12), 202(42), 137(12), 115(19), 101(23)	4.09(H11), 7.34(H5), 7.63(H3), 7.65(H9), 7.70(H2), 7.82(H6), 7.83(H8), 7.90(H4), 8.42(H7), 8.95(H1), 9.34(H12)	$J_{1,2}$ = 8.0, $J_{1,3}$ = 1.3, $J_{2,3}$ = 7.7, $J_{2,4}$ = 1.3, $J_{3,4}$ = 7.7, $J_{5,6}$ = 8.8, $J_{8,9}$ = 9.0
10,11-Dimethoxy-benz[*a*]anthracene (III)	44.2	288	289(23), 288(100), 273(33), 261(17), 245(16), 230(29), 227(14), 202(23), 200(10), 144(12), 101(11)	4.05(H10), 4.12(H11), 7.57(H9), 7.64(H3), 7.66(H5), 7.71(H2), 7.83(H6), 7.88(H4), 7.90(H8), 8.44(H7), 8.94(H1), 9.48(H12)	$J_{1,2}$ = 8.2, $J_{1,3}$ = 1.3, $J_{2,3}$ = 7.5, $J_{3,4}$ = 7.5, $J_{5,6}$ = 8.8, $J_{8,9}$ = 9.0
6-Hydrofurano[3,4-*g*]chromene-2,8-dione (V)	7.3	202	202(67), 174(11), 173(100), 145(30), 117(8), 89(12), 63(5)	5.47(H6), 6.61(H3), 7.72(H9), 7.98(H5), 8.13(H4)	$J_{3,4}$ = 9.7, $J_{5,6}$ = 0.9
3-Hydrobenzo[*f*]isobenzofuran-1-one (VI)	19.8	184	184(75), 183(11), 156(13), 155(100), 127(58), 126(18), 101(3), 77(5), 63(5)	5.55(H3), 7.65(H7), 7.71(H6), 8.08(H5), 8.13(H4), 8.20(H8), 8.51(H9)	$J_{3,4}$ = 1.1, $J_{5,6}$ = 8.4, $J_{5,7}$ = 1.3, $J_{6,7}$ = 7.5
Benzo[*g*]chromen-2-one (VII)	24.9	196	197 (14), 196 (100), 168 (30), 140(16), 139 (27), 70 (7)	6.47 (H3), 7.54 (H6), 7.62 (H7), 7.79 (H9), 8.00 (H8), 8.03 (H5), 8.12 (H4), 8.27 (H10)	$J_{3,4}$ = 9.7, $J_{5,6}$ = 8.4, $J_{6,7}$ = 7.7, $J_{6,8}$ = 1.3, $J_{7,8}$ = 8.6,$J_{7,9}$ = 1.3
Benz[*a*]anthracene (IV)	44.5	228	229(19), 228(100), 226(23), 224(5), 114(12), 113(10), 101(5),	7.58(H9,10), 7.64(H3), 7.71(H2), 7.72(H5), 7.88(H6), 7.91(H4), 8.11(H8), 8.23(H11), 8.49(H7), 8.96(H1), 9.38(H12)	$J_{1,2}$ = 8.2, $J_{2,3}$ = 7.5, $J_{2,4}$ = 1.5, $J_{3,4}$ = 7.7, $J_{5,6}$ = 8.8

[a]Dissolved in deuterated acetone.
[b]First order only.

8900 Hz; acquisition time, 2.33 s; flip angle, 90°; relaxation delay, 0 s; temperature, 301 K. For spectra recorded under quantitative conditions, a 10- to 20-s relaxation delay was used. For measurement of coupling constants, the free induction decay (FID) was zero-filled to 64 K, resulting in a final data point resolution of 0.215 Hz per point. Coupling constants reported are first order. Those that are non-first order and those of overlapping resonances are omitted. Assignments were made

from homonuclear decoupling experiments, nuclear Overhauser effect (NOE) experiments, integration, and analysis of substituent effects.

Metabolism of benz[a]anthracene cis-5,6-dihydrodiol

Cultures of *M. vanbaalenii* PYR-1 were prepared as described above and incubated with 2 mg of synthetic benz[*a*]anthracene *cis*-5,6-dihydrodiol. GC/MS analyses of trimethylsilyl derivatized extracts from 114 h incubations were performed as described above for benz[*a*]anthracene metabolism.

Results

Metabolism of benz[a]anthracene

Mycobacterium vanbaalenii PYR-1 does not grow well with benz[*a*]anthracene as the sole carbon and energy source. Therefore, we grew *M. vanbaalenii* PYR-1 in a mineral salts medium containing nutrients and phenanthrene as described previously (Heitkamp 1988a,b; Moody et al. 2001). Washed cell suspensions of phenanthrene induced cultures incubated in the presence of benz[*a*]anthracene were able to metabolize the PAH.

Mycobacterium vanbaalenii PYR-1 degraded about 5% of the added [^{14}C-]benz[*a*]anthracene to numerous metabolites after 114 h of incubation. Trace levels of $^{14}CO_2$ were formed over the same time period. Approximately 15% of the benz[*a*]anthracene was metabolized after 12 days of incubation. In order to obtain sufficient amounts of metabolites for structure elucidation, large-scale biotransformation studies were conducted. Neutral and acidic ethyl acetate extractable metabolites were analyzed by HPLC and GC/MS.

Identification of metabolites

Three metabolites and benz[*a*]anthracene were observed in the neutral extract HPLC chromatogram (Figure 1a). The first metabolite I, which accounted for 48.1% of the total metabolites, eluted at 34.2 min and produced an EI mass spectrum (Table 1) with a base peak at *m*/*z* 246 $M^{+\bullet}$ and significant fragment ions at *m*/*z* 218 $[M–CO]^+$, 189 $[M–CO–HCO]^+$, and 94.6 $[189]^{++}$. The losses of 28 (CO) and 29 (HCO) are associated with carbonyl moieties and hydroxylated PAHs. The NMR spectrum consisted of six doublets, two triplets, and two singlets that, after homonuclear decoupling and NOE experiments, showed substitution at C-10 and C-11. Two possible structures were considered, one with a carbonyl at C-10 and an oxygen atom replacing C-11 and the other with an oxygen replacing C-10 and a carbonyl at C-11. The former was determined to be correct, since it would result in an upfield chemical shift for H9 (Table 1). The structure of metabolite I, analogous to one formed by *M. vanbaalenii* PYR-1 from anthracene (Moody et al. 2001), was identified as naphtho[2,1-*g*]chromen-10-one. Furthermore, our study provides evidence for the unidentified metabolite BAA IV formed from benz[*a*]anthracene by *Mycobacterium* sp. strain RJGII-135 (Schneider et al. 1996).

The second neutral extract peak, II, eluted at 38.8 min accounted for 9.3% of the total metabolites. The EI mass spectrum (Table 1) of the compound showed a base peak at *m*/*z* 274 $[M]^{+\bullet}$ and fragment ions at *m*/*z* 259 $[M–CH_3]^+$, 231 $[M–CH_3–CO]^+$, 202 $[M–CH_3–CO–HCO]^+$, 137 $[274]^{++}$, and 101 $[202]^{++}$. The $^{-1}$H NMR spectrum (Table 1) showed ten aromatic proton resonances and a singlet at 4.09 ppm that integrated as three, indicating substitution at two carbons with one of the substitutions either a methyl or a methoxy group. Further, the coupling patterns showed that the substitutions were either on the ring with H1 through H4 or the ring with H8 through H11. NOE and homonuclear decoupling experiments identified H5 and H6 at 7.34 and 7.82 ppm, respectively. Selective saturation of the singlet resonance at 8.42 ppm, H7, produced an NOE at two resonances, 7.82 ppm (H6) and 7.83 ppm (H8). Homonuclear decoupling experiments showed that H8 and H9 were coupled only to each other. Selective saturation of the singlet resonance at 4.09 ppm (representing a methyl group) produced an NOE to the aromatic singlet at 9.34 ppm, H12. That showed the aromatic singlet to be spin-coupled to H11. The substitution at C10 showed no protons in deuterated methanol and was initially assumed to be a hydroxyl. The mass spectral data showed the addition of mass weight equivalent to two oxygen atoms and one methylene group. The losses of 15 (CH_3), 28 (CO) and 29 (HCO) are consistent with the identifica-

tion of metabolite II as 10-hydroxy-11-methoxybenz[*a*]anthracene.

Neutral extract peak III eluted at 44.2 min and produced an EI mass spectrum with a base peak at *m*/*z* 288 $M^{+\bullet}$ and fragment ions at *m*/*z* 273 $[M–CH_3]^+$, 245 $[M–CH_3–CO]^+$, 230 $[M–CH_3–CO–CH_3]^+$, and 202 $[M–CH_3–CO–CH_3–CO]^+$. The $^{-1}$H NMR spectrum consisted of ten aromatic proton resonances and two singlets, both integrating as three, at 4.05 and 4.12 ppm. Based on the identification of metabolite II, it was assumed that III was the dimethoxy-substituted compound and homonuclear decoupling experiments showed that the substitution was at C11 and C12. Selective saturation of the singlet at 4.12 ppm (H11) produced an NOE to the aromatic singlet at 9.48 ppm (H12); saturation of the singlet at 4.05 ppm (H10) produced an NOE to the doublet at 7.57 ppm (H9). The mass spectrum indicated the addition of the mass weight equivalent of two oxygen atoms and two methylene groups. The mass spectrum is consistent with the loss of two methoxy groups. Metabolite III was identified as 10,11-dimethoxybenz[*a*]anthracene. The dimethoxylated derivative accounted for 36.4% of the total metabolites.

Peak IV had the same UV characteristics, retention time, mass spectrum (consisting of ions at *m*/z 228 $M^{+\bullet}$, 226 $[M\text{-}H_2]^+$, 114 $[228]^{++}$, and 113 $[226]^{++}$) and NMR spectrum as authentic benz[*a*]anthracene (Table 1).

HPLC analysis of the acid extract yielded four metabolites (Figure 1b). The first one, V, eluted at 7.3 min and was approximately 1.3% of the total metabolites. Its EI mass spectrum showed a molecular ion at *m*/*z* 202 $M^{+\bullet}$ with fragment ions at *m*/*z* 173 $[M–HCO]^+$, 145 $[M–HCO–CO]^+$, 117 $[M–HCO–2CO]^+$, and 89 $[M–HCO–3CO]^+$, indicating a probable cleavage of one of the benz[*a*]anthracene rings. The paucity of NMR resonances also indicated that considerable degradation had taken place; the spectrum consisted of two aromatic singlets and two aromatic doublet resonances that each integrated as one, and a singlet resonance at 5.47 ppm that integrated as two. Homonuclear decoupling and other NOE experiments showed that the aromatic doublet resonances were coupled to one another (H3 and H4) and were, therefore, on the same ring. The experiments also showed that the singlet at 5.47 ppm (H6) had a long-range coupling ($J_{5,6} = 0.9$ Hz) to the singlet at 7.98 ppm (H5). Irradiation of the singlet at 5.47 also produced an NOE to the singlet at 7.98 ppm. These data showed that the protons were on adjacent rings. Irradiation of the singlet at 7.98 ppm produced an NOE to the doublet resonance at 8.13 ppm (H4). The singlet at 7.72 ppm showed no connections to any of the other resonances. The mass spectral data showed several 28-Da losses, indicating probable carbonyl losses. The proposed structure for metabolite V based on the NMR and MS data was 6-hydrofuran[3,4-*g*]chromene-2,8-dione.

Peak VI (19.8 min) which was approximately 3.2% of the total metabolites had EI mass spectral ions at *m*/*z* 184 $[M]^+$, 155 $[M–HCO]^+$, and 127 $[M–HCO–CO]^+$. The $^{-1}$H NMR spectrum had the same number and type of resonances as metabolite V, as well as two triplets that each integrated as two. Homonuclear decoupling and NOE experiments showed that the molecule had the same hydrofuran ring as metabolite V, but that the ring at the other end of the molecule had no substitutions at its carbons. Metabolite VI was identified as 3-hydrobenzo[*f*]isobenzofuran-1-one.

Peak VII eluted at 24.9 min and produced $^{-1}$H NMR and EI mass spectra identical to those of benzo[*g*]chromene-2-one, an anthracene metabolite formed by *M. vanbaalenii* PYR-1 (Moody et al. 2001). This ring-fission product accounted for approximately 1.7% of the total metabolites.

Peak VIII had the same UV, mass and NMR spectra as metabolite I. Although the retention times were slightly different, 33.9 min for VIII compared to 34.2 for I, VIII was identified as naphtho[2,1-*g*]chromen-10-one.

Since some of the compounds were formed in limited amounts, NMR analysis could not be conducted to rigorously identify the metabolites. Therefore, we conducted GC/MS analysis of neutral and acidic ethyl acetate extracts from 144 h incubations before and after trimethysilylation of the samples (Tables 2 and 3). In addition to the identification of the minor metabolites, all of the major metabolites (Peaks I–VIII in Figures 1a, b) were confirmed (Table 2).

The neutral ethyl acetate extract before derivatization contained one metabolite with a retention time of 14.4 min (ions at *m*/*z* 244 and 215). The trimethylsilylated metabolite (10.8 min) gave a molecular ion at *m*/*z* 316 (244 + 72) and fragment ions at *m*/*z* 301 $[M–CH_3]^+$, 225, and 197 (Table 3). Both the underivatized and the deriva-

Table 2. GC/MS spectral properties of benz[*a*]anthracene and the metabolites formed by *M. vanbaalenii* PYR-1[a]

Compound name	GC retention time (min)	Molecular weight	Significant ions [*m*/*z* (% relative intensity)]
1,2-Dihydroxyanthracene or 2,3-dihydroxyphenanthrene	8.1	210	210(5), 182(12), 181(100), 153(5), 139(3), 130(3), 125(3), 111(5), 97(14), 83(17), 69(11), 57(8), 55(23)
1,2-Dihydroxyanthracene or 2,3-dihydroxyphenanthrene	8.7	210	210(47), 182(14), 181(100), 166(18), 165(19), 152(10), 151(19), 107(40), 85(5), 83(4), 77(8)
3-Hydrobenzo[*f*]isobenzo-furan-1-one (VI)	10.1	184	184(82), 183(11), 156(12), 155(100), 139(7), 128(7), 127(53), 126(16), 101(3), 87(2), 77(4), 63(4)
Benzo[*g*]chromen-2-one (VII)	10.7	196	197(10), 196(100), 168(34), 140(19), 139(38), 113(3), 70(4)
6-Hydrofuran-[3,4-*g*]chromene-2,8-dione (V)	11.6	202	203(8), 202(70), 174(11), 173(100), 145(29), 117(8), 102(3), 89(12), 63(4)
Benz[*a*]anthracene (IV)	13.4	228	228(100), 226(23), 114(8), 113(6)
Benz[*a*]anthracene-7,12-dione	14.2	258	259(19), 258(100), 257(22), 230(28), 202(39), 201(22), 200(22), 101(12), 100(8), 88(5)
Benz[*a*]anthracene dione	14.9	258	259(18), 258(100), 257(5), 230(22), 202(35), 201(12), 200(16), 101(8), 100(6), 88(4)
Naphtho[2,1-*g*]chromen-10-one (I and VIII)	15.6	246	247(17), 246(100), 218(34), 189(37), 163(4), 123(3), 109(3), 94.6(15), 81.5(3)
Hydroxymethoxybenz[*a*]anthracene	15.9	274	275(20), 274(100), 260(12), 259(68), 241(5), 231(16), 203(15), 202(29), 200(5), 137(4), 129(4), 101(16)
Dimethoxybenz[*a*]anthracene	16.3	288	289(22), 288(100), 273(31), 255(7), 231(6), 230(32), 227(6), 202(12), 101(5)
10-hydroxy-11-methoxybenz[*a*]anthracene (II)	16.7	274	275(19), 274(100), 260(18), 259(99), 231(33), 215(4), 213(4), 203(10), 202(37), 201(6), 200(7), 176(4), 137(7), 129.6(6), 115.6(14), 101(14), 88(4)
10,11-Dimethoxybenz[*a*]anthracene (III)	16.8	288	289(20), 288(100), 273(30), 245(15), 230(23), 227(16), 217(7), 202(15), 144(8), 113(4), 106.6(4)
Dimethoxybenz[*a*]anthracene	17.3	288	289(21), 288(100), 273(37), 245(19), 230(26), 227(10), 217(7), 215(6), 202(17), 200(6), 144(7), 106.6(5)
Hydroxymethoxybenz[*a*]anthracene	18.8	274	275(18), 274(100), 260(13), 259(68), 231(14), 213(3) 203(15), 202(28), 200(6), 137(8), 129.4(5), 115.8(3), 101(15), 100 (5), 88(3)

[a] Neutral extracts after 144 h incubation of benz[*a*]anthracene with *M. vanbaalenii* PYR-1.

tized metabolite MS data are consistent with a hydroxybenz[*a*]anthracene structure (Yao et al. 1998). The position of hydroxylation could not be determined. The trimethylsilylated acid neutral extract contained four possible trimethylsilylated metabolites at 12.4, 12.5, 12.6 and 13.3 min that

Table 3. GC/MS spectral properties of minor metabolites formed by *M. vanbaalenii* PYR-1 from benz[*a*]anthracene and benz[*a*]anthracene *cis*-5,6-dihydrodiol[a,b]

Compound name	GC retention time (min)	Molecular weight	Significant ions [*m*/*z* (% relative intensity)]
Hydroxy-BA (TMSi)	10.8	316	316(82), 301(27), 225(100), 197(78)
BA-dihydrodiol (diTMSi)	12.4	406	406(100), 391(18), 316(25), 147(15), 73(17), 197(78)
BA-dihydrodiol (diTMSi)	12.5	406	406(100), 316(25), 228(16), 147(10), 73(22), 391(28)
BA *cis*-5,6-dihydrodiol (diTMSi)	12.6	406	406(100), 391(33), 375(16), 316(54), 228(15), 115(17), 147(69), 73(23)
3-(2-carboxylphenyl)-2-naphthoic acid (diTMSi)[b]	12.8	436	436(17), 421(6), 377(3), 319(100), 303(4), 147(21), 101(4), 73(17)
BA *cis*-10,11-dihydrodiol (diTMSi)	13.3	406	406(100), 391(12), 318(14), 317(56), 316(83), 228(16), 147(10), 114(26), 73(29)
BA-7,12-dione	14.2	258	258(100), 257(22), 230(28), 202(39), 201(22), 200(22), 101(12)
Hydroxy-BA	14.4	244	245(20), 244(100), 215(50)
5,6-dimethoxy BA[b]	14.8	288	288(100), 273(47), 255(11), 245(55), 230(54), 202(11), 200(12), 101(12), 75(16)
BA-dione	15.0	258	258(100), 257(5), 230(22), 202(36), 201(12), 200(16), 101(9)
5,6-dihydroxy BA (diTMSi)[b]	15.2	404	404(100), 346(4), 316(30), 286(9), 73(7)
1-benzoyl-2-naphthoic acid (TMSi)[c]	18.6	348	348(83), 347(13), 333(15), 261(4), 231(100), 215(8), 202(24), 167(14), 73(28)
1-(2-hydroxybenzoyl)-2-naphthoic acid (diTMSi)[c]	20.6	436	436(8), 421(3), 377(1), 319(100), 303(5), 147(9), 73(18)

[a]Neutral and acidic extracts, which were analyzed as trimethylsilylated (TMSi) derivatives.
[b]Metabolites formed from benz[*a*]anthracene *cis*-5,6-dihydrodiol incubations.
[c]Tentative identification since there can be positional differences in the carboxylic acid and hydroxyl moieties.

gave molecular ions at *m*/*z* 406 and fragment ions at *m*/*z* 391 $[M–CH_3]^+$ and 316 $[M–(CH_3)_3–SiOH]^+$ that are consistent with trimethylsilylated benz[*a*]anthracene dihydrodiols (Schneider et al. 1996) (Table 3). The compound at 12.6 min had a retention time and mass spectral fragmentation pattern and uv–visible absorption spectrum identical to that of authentic *cis*-5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene (benz[*a*]anthracene *cis*-5,6-dihydrodiol). The compound at 13.3 min was the predominant dihydrodiol isomer which accounted for approximately 60.3% of the total dihydrodiols formed by *M. vanbaalenii* PYR-1. It had uv–visible and mass spectral data consistent with those of benz[*a*]anthracene *cis*-10,11-dihydrodiol (Jerina et al. 1984; Schneider et al. 1996). The positions of substitution and whether the compounds at 12.4 and 12.5 min were *cis* or *trans* isomers could not be determined due to limited material and the lack of authentic standards. However, based on the identification of benzo[*g*]chromen-2-one, one of the dihydrodiols is likely to be benz[*a*]anthracene *cis*-1,2-dihydrodiol.

A compound in the neutral extract with a GC/MS retention time of 14.2 min gave a molecular ion at *m*/*z* 258 (100) and fragment ions at *m*/*z* 257 $[M–H]^+$, 230 $[M–CO]^+$, 202 $[M–2CO]^+$ and 101 $[202]^{++}$ similar to those reported (Jang & McDow

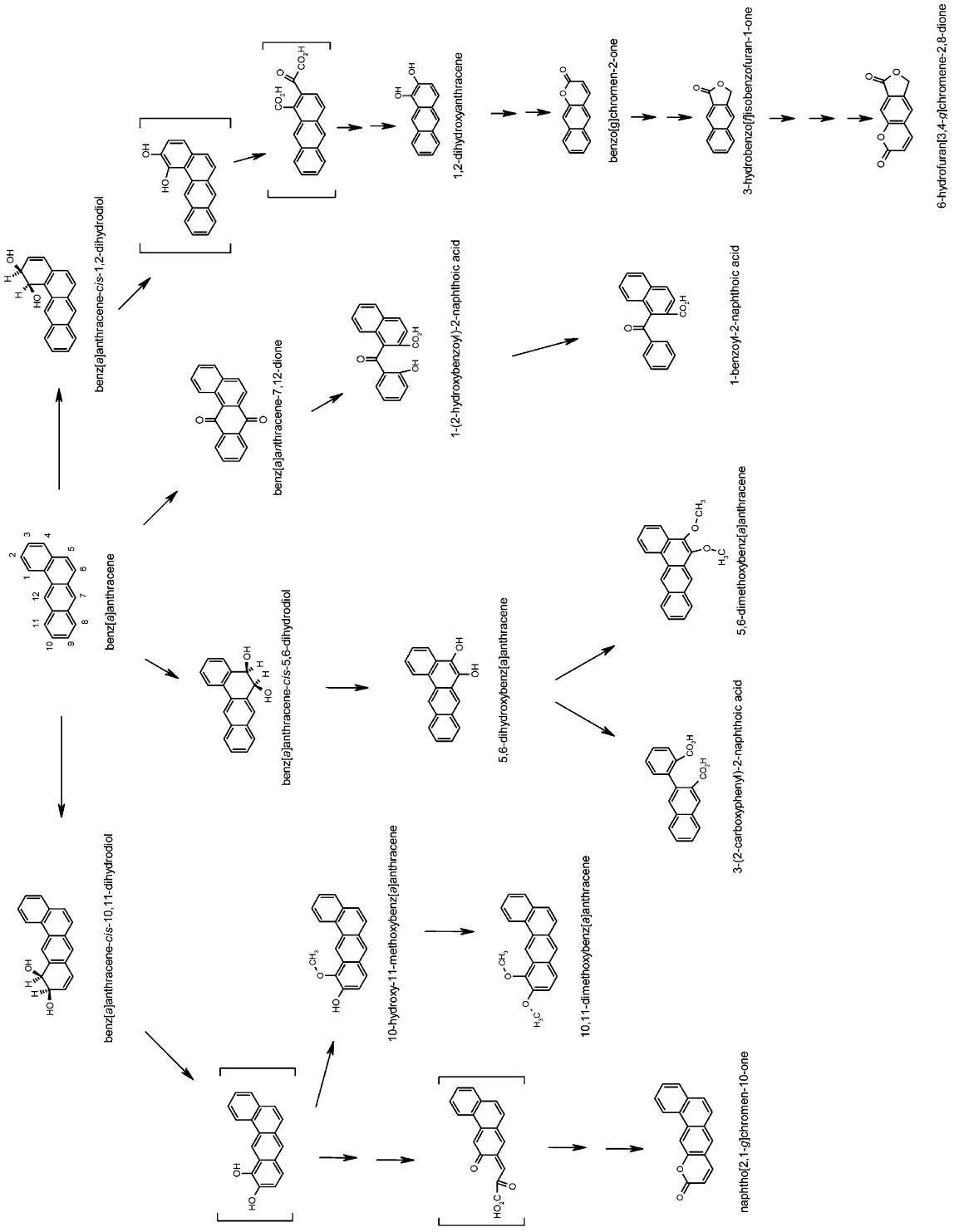

Figure 2. Pathway proposed for the degradation of benz[*a*]anthracene by *M. vanbaalenii* PYR-1. The compounds in brackets have not been isolated.

1997; Yao et al. 1998) for benz[*a*]anthracene-7,12-dione. A compound at 15.0 min also gave a molecular ion at *m*/*z* 258 and a fragmentation pattern similar to a benz[*a*]anthracene dione standard.

GC/MS analysis of three trimethylsilylated metabolites from acidic ethyl acetate extracts had retention times of 12.8, 18.6, and 20.6 min (Table 3).

The mass spectrum of the component at 20.6 min gave a molecular ion at *m*/*z* 436 with fragment ions at *m*/*z* 421 $[M–CH_3]^+$, 377, 319 $[M–(CH_3)_3–Si–CO)]^+$, 303, 147 $[(CH_3)_3–Si–Si–(CH_3)_3+H]^+$, and 73 $[(CH_3)_3–Si]^+$. The component at 18.6 min produced a molecular ion at *m*/*z* 348 with fragment ions at *m*/*z* 333 $[M–CH_3]^+$, 261, 231 $[M–(CH_3)_3–Si–CO)]^+$, 202, and 73 $[(CH_3)_3–Si]^+$. The mass spectral data were similar to those reported by Yao et al. (1998) for 1-(2-hydroxybenzoyl)-2-naphthoic acid and 1-benzoyl-2-naphthoic acid, respectively. The compound at 12.8 min had a molecular ion at *m*/*z* 436 which was determined to be a phenyl naphthyl metabolite (see below).

Metabolism of benz[a]anthracene cis-*5,6-dihydrodiol*

An experiment was conducted to determine whether benz[*a*]anthracene *cis*-5,6-dihydrodiol was metabolized by *M. vanbaalenii* PYR-1. GC/MS analysis of trimethylsilyl derivatized acid extracts (Table 3) after 144 h of incubation indicated a major compound at 12.8 min that had an apparent molecular weight of 436. The mass spectrum consisted of ions at *m*/*z* 436 $M^{+\bullet}$, 421 $[M–CH_3]^+$, 319 $[M–(CH_3)_3–Si–CO)]^+$, 303, 147 $[(CH_3)_3–Si–Si–(CH_3)_3 + H]^+$, and 73 $[(CH_3)_3–Si]^+$. The $[M]^{+\bullet}$ at *m*/*z* 436 could be the weight of a ring-opened *ortho*-cleavage diacid structure (292 + 144 Da). The mass spectral data were consistent with those of ditrimethylsilyl-3-(2-carboxylphenyl)-2-naphthoic acid (Yao et al. 1998). Approximately 91% of the benz[*a*]anthracene *cis*-5,6-dihydrodiol was converted to the phenyl naphthyl dicarboxylic acid. An additional compound at 15.2 min had an apparent molecular weight of 404. The mass spectrum suggests a dihydroxylated derivatized intermediate, such as 5,6-dihydroxybenz[*a*]anthracene. The mass spectrum of another metabolite at 14.8 min gave an $[M]^{+\bullet}$ at *m*/*z* 288 and major fragments at *m*/*z* 273 $[M–CH_3]^+$, 245 $[M–CH_3–CO]^+$, 230 $[M–CH_3–CO–CH_3]^+$, 202 $[M–CH_3–CO–CH_3–CO]^+$, and 200, consistent with 5,6-dimethoxybenz[*a*]anthracene (Yao et al. 1998).

Discussion

Benz[*a*]anthracene was metabolized by *M. vanbaalenii* PYR-1 *via* at least four degradation pathways. *M. vanbaalenii* initially attacked benz[*a*]anthracene in the 1,2-, 5,6-, 10,11- and 7,12- positions (Figure 2). The predominant site of metabolism was at the C-10,11-positions. In contrast to previous studies on naphthalene, pyrene, phenanthrene, anthracene, benzo[*a*]pyrene, and 7,12-dimethylbenz[*a*]anthracene degradation (Heitkamp et al. 1988a, b; Kelley et al. 1990; Moody et al. 2001, 2003, 2004), the isomeric *cis*-dihydrodiols were transient metabolites formed in low yield and identified by comparison of UV spectra and GC/MS data of the trimethylsilylated derivatives to those in the literature (Schneider et al. 1996). The formation of *cis*-1,2-, 5,6- and 10,11-dihydrodiols is consistent with similar structures proposed for naphthalene, anthracene, phenanthrene, pyrene and benzo[*a*]pyrene degradation by *M. vanbaalenii* PYR-1 (Heitkamp et al. 1988a,b; Kelley et al. 1990; Moody et al. 2001, 2003, 2004). The relative ratio of the *cis*-1,2-, 5,6-, and 10,11-benz[*a*]anthracene dihydrodiols was approximately 5:1:9, respectively. Schneider et al. (1996) reported that *Mycobacterium* sp. RJG11-135 formed the benz[*a*]anthracene *cis*-5,6- and 10,11-dihydrodiols. However, no evidence was provided for enzymatic attack in the C-1,2- or C-7,12- positions nor were ring-fission products identified. Jerina et al. (1984) showed that *Sphingomonas yanoikuyae*, formerly identified as *Beijerinckia* sp. B-836, oxidized benz[*a*]anthracene preferentially in the 1,2- positions with minor metabolism in the 8,9- and 10,11- positions. In addition to the novel metabolites identified in the present study, the data reported in this investigation extend and propose novel pathways for the bacterial metabolism of benz[*a*]anthracene.

The K-region metabolite, benz[*a*]anthracene *cis*-5,6-dihydrodiol, was transformed by *M. vanbaalenii* PYR-1 to 3-(2-carboxyphenyl)-2-naphthoic acid, indicating 5,6-dehydrogenation to form 5,6-dihydroxybenz[*a*]anthracene, followed by *ortho*-cleavage to form the phenyl naphthyl dicarboxylic acid. The detection of 5,6-dimethoxy benz[*a*]anthracene provided further evidence for the dihydroxylated benz[*a*]anthracene intermedi-

ate. PAH K-region dioxygenation to give *cis*-dihydrodiols with subsequent *ortho*-cleavage to form dicarboxylic acids is a unique finding in the metabolism of PAHs by *Mycobacterium* spp. (Dean-Ross & Cerniglia 1996; Heitkamp et al. 1988a, b; Moody et al. 2001, 2003, 2004; Schneider et al. 1996; Vila et al. 2001).

M. vanbaalenii PYR-1 was also able to degrade benz[*a*]anthracene to benz[*a*]anthracene- 7,12-dione with further oxidation to form 1-(2-hydroxybenzoyl)-2-naphthoic acid and 1-benzoyl-2-naphthoic acid. Data in this pathway have not been described for the bacterial degradation of benz[*a*]anthracene; however, a similar metabolic pathway was reported for the oxidation of anthracene by the ligninolytic fungus *Irpex lacteus* (Cajthaml et al. 2002). These compounds are also found as products of benz[*a*]anthracene ozonation reactions (Yao et al. 1998).

Ozonation and Fenton oxidation are primary chemical treatment methods to enhance the bioavailability of PAHs by forming oxidized reaction products that are more susceptible to microbial degradation (Lee & Hosomi 2001; Yao et al. 1998). Fenton oxidation of benz[*a*]anthracene occurs at the C-7 and C-12 positions to form benz[*a*]anthracene-7,12-dione as the major oxidation product (Lee & Hosomi 2001). Fenton oxidation followed by microbial treatment of contaminated soil is effective in degrading benz[*a*]anthracene-7,12-dione (Lee & Hosomi 2001). Our investigation could provide a metabolism profile data base for the identification of intermediates using combined treatment technologies. Ozonation of benz[*a*]anthracene occurs initially in the C-7,12- and C-5,6- positions to form quinones and ring-cleavage products that could act as substrates for further microbial degradation (Yao et al. 1998). Interestingly, *M. vanbaalenii* PYR-1 carried out a free radical attack similar to singlet oxygen reactions.

M. vanbaalenii PYR-1 oxidized benz[*a*]anthracene predominantly in the 10,11- positions to dihydroxylated intermediates that underwent *O*-methylation and ring cleavage reactions. The identification of 10-hydroxy-11-methoxybenz[*a*]anthracene and 10,11-dimethoxybenz[*a*]anthracene shows a metabolism similar to that previously found in benzo[*a*]pyrene degradation (Moody et al. 2004). We recently determined that *M. vanbaalenii* PYR-1 has a constitutive catechol methyltransferase that is involved in the detoxification of PAHs (Kim et al. 2004). The identification of naphtho[2,1-*g*]chromen-10-one represents a ring-closure product most likely formed by *meta*-cleavage of 10,11-dihydroxybenz[*a*]anthracene. This reaction is analogous to our previous studies in the degradation of anthracene and benzo[*a*]pyrene by *M. vanbaalenii* PYR-1 (Moody et al. 2001, 2004).

The formation of acid-extractable metabolites, 6-hydrofuran[3,4-*g*]chromene-2,8-dione and benzo[*g*]chromen-2-one, involved the same ring opening and closing reactions in the C-10 and C-11 positions as for naphtho[2,1-*g*]chromen-10-one. However, further breakdown of the compound resulted in the ring containing C-1 through C-4 being removed in the case of 6-hydrofuran[3,4-*g*] chromene-2,8-dione, 3-hydrobenzo[*f*]isobenzofuran-1-one, and benzo[*g*]chro-men-2-one. The rings containing C-5 and C-6 of 6-hydrofuran [3,4-*g*]chromene-2,8-dione and 3-hydrobenzo[*f*] isobenzofuran-1-one were also attacked.

The novel benzocoumarin-type ring closure metabolites identified from acid extracts of culture broths of *M. vanbaalenii* PYR-1 suggested a fourth possible degradation pathway (Figure 2). The formation of benzo[*g*]chromen-2-one indicates that dioxygenation occurred in the C-1 and C-2 positions of benz[*a*]anthracene, yielding the benz[*a*]anthracene *cis*-1,2-dihydrodiol. Dehydrogenation of the dihydrodiol to 1,2-dihydroxybenz[*a*]anthracene with subsequent ring cleavage and aromatic ring closure could lead to the formation of benzo[*g*]chromen-2-one. This ring fission product could act as a substrate for further oxidation and ring cleavage pathways.

This investigation advances our knowledge of the bacterial degradation of benz[*a*]anthracene. We have shown that *M. vanbaalenii* PYR-1 metabolizes benz[*a*]anthracene by at least four pathways, which suggests that the organism uses multiple dioxygenases and monooxygenases. We previously identified and characterized genes for the large and small subunits of a pyrene dioxygenase, *nid*A and *nid*B, respectively (Khan et al. 2001). In addition, we have recently reported on the genes responsible for the degradation of phenanthrene *via* the phthalic acid pathway in *Mycobacterium* species (Stingley et al. 2004a, b). The versatility of *M. vanbaalenii* to enzymatically attack multiple sites on the aromatic rings of high molecular weight PAHs to produce dihydroxylated intermediates and ring-cleavage products will

be extremely useful in the remediation of potentially genotoxic compounds from the environment. Since we have recently reported that the PAH-degrading genes from *M. vanbaalenii* PYR-1 are also found in other *Mycobacterium* spp. (Brezna et al. 2003), this adds further support of the importance of Gram-positive nocardioform bacteria in environmental degradation of PAHs.

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