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Microbially Mediated Formation of Benzonaphthothiophenes from Benzo[*b*]thiophenes

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Studies of the microbial metabolism of benzo[*b*]thiophene (molecular weight 134) by three *Pseudomonas* isolates showed the formation of benzothiophene sulfoxide, benzothiophene sulfone, and a sulfur-containing metabolite with a molecular weight of 234. Desulfurization of the high-molecular-weight product with nickel boride gave 1-phenylnaphthalene, indicating that the metabolite was benzo[*b*]naphtho[1,2-*d*]thiophene. Similarly, the isolates were capable of producing the analogous dimethyl-substituted benzonaphthothiophenes from methylbenzothiophenes that had the methyl substitution on the benzene ring. The formation of benzo[*b*]naphtho[1,2-*d*]thiophene was also observed when a petroleum-degrading mixed culture was incubated with benzothiophene-supplemented Prudhoe Bay crude oil. Investigations into the mechanism of formation of these high-molecular-weight compounds showed that they resulted from an abiotic, Diels-Alder-type condensation of two molecules of the sulfoxide, which were microbially produced from the respective benzothiophene, with the subsequent loss of two atoms of hydrogen and oxygen and one atom of sulfur. The condensation products also formed from the sulfoxides of benzothiophene and methylbenzothiophenes when the sulfoxides were enzymatically synthesized by oxidation of the benzothiophene with horse heart cytochrome *c* and H_2O_2.

The microbial removal of alkylbenzothiophenes and alkyldibenzothiophenes from the aromatic fraction of crude oil present in laboratory cultures (5, 16, 17) and from the aromatic fraction of crude oil spilled into the environment (3) or biodegraded within its natural reservoir (28, 29) has been demonstrated. Because of the complexity of the mixture of compounds present in crude oil, the metabolites of the biodegradation of these petroleum organosulfur compounds were not determined in those studies.

Various investigations with pure bacterial cultures and pure organosulfur compounds have demonstrated that benzothiophene (BT) and methylbenzothiophenes (methylBTs) are susceptible to microbial attack. For example, *Pseudomonas aeruginosa* PRG-1 oxidized BT dissolved in light oil but could not use this compound as a sole carbon source (25) and a dibenzothiophene-oxidizing isolate, *Pseudomonas alcaligenes* DBT2, oxidized BT to water-soluble products (18). However, the metabolites of BT oxidation were not identified in either of these studies.

In another investigation (6), enrichment cultures from several aquatic environments and from wastewater treatment plant effluents were able to oxidize BT when naphthalene was provided as a carbon source. Tentative identifications of the BT metabolites as BT sulfoxide, 2,3-dihydroBT-2,3-diol, and BT-2,3-dione were made on the basis of gas chromatography-mass spectrometry (GC-MS) analysis. Fedorak and Grbić-Galić (12) identified the products of biotransformation of BT and 3-methylBT by a 1-methylnaphthalene-utilizing bacterium, *Pseudomonas* sp. strain BT1, as BT-2,3-dione and 3-methylBT sulfoxide, respectively. A small amount of the sulfone of 3-methylBT was also produced.

These findings led to the prediction that if BT was substituted with a methyl group on the thiophene ring, the corresponding sulfoxide and sulfone would be formed, whereas if there was a methyl group on the benzene ring, the corresponding 2,3-dione would be formed (12). In a systematic study with *Pseudomonas* sp. strain BT1 and synthesized methylBTs, Saftić et al. (23) observed that this prediction held true for the metabolism of 2-, 3-, 4-, 5-, and 6-methylBTs and 2,3-dimethylBT. However, the metabolism of 7-methylBT yielded the 2,3-dione, sulfoxide, and sulfone in addition to several unidentified products.

A subsequent report (21) described the abilities of three other *Pseudomonas* strains to transform BT and each of the six isomers of methylBT. Sulfoxides and sulfones were frequently detected, and they were the most abundant products from 2- and 3-methylBTs. 2,3-Diones were observed as metabolites of BT and methylBTs with a methyl group on the benzene ring. However, these new strains also oxidized the sulfur atom of BT to give the sulfoxide and sulfone. Two of the isolates oxidized the methyl groups of the methylBTs, producing benzothiophenemethanols and benzothiophenecarboxylic acids. Isomers of tolyl methyl sulfoxide were also observed as thiophene ring cleavage products from 6- and 7-methylBTs. Recently, Eaton and Nitterauer (10a) identified several metabolites of BT produced by isopropylbenzene-degrading bacteria. These included 2-mercaptophenylglyoxalate and *trans*-4-[3-dihydroxy-2-thienyl]-2-oxo-but-3-enoate, which resulted from the cleavage of the thiophene ring and the benzene ring, respectively.

Kropp et al. (21) observed that some high-molecular-weight products were formed in cultures that were incubated in the presence of BT and those methylBTs that had the methyl group on the benzene ring. The molecular weight of the product from BT was 234, consistent with the chemical formula $C_{16}H_{10}S$. MethylBTs gave condensation products which were 28 mass units larger than the product from BT, consistent with the formula $C_{18}H_{14}S$. This paper describes the identification of these high-molecular-weight compounds and the mechanism of their formation.

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MATERIALS AND METHODS

Organosulfur compounds and other chemicals. Benzo[*b*]thiophene, benzo[*b*]naphtho[2,1-*d*]thiophene, and 1-phenylnaphthalene were purchased from Aldrich, Milwaukee, Wis. 3-Methylbenzo[*b*]thiophene was purchased from Lancaster Synthesis, Windham, N.H. 1-Methylnaphthalene and 2-phenylnaphthalene were purchased from Fluka, Buchs, Switzerland. The methods given by Andersson (1) were used for the syntheses of 2-, 4-, 5-, and 7-methylBTs and a mixture of 4- and 6-methylBTs. Sulfones of the BTs were synthesized by boiling the sulfur-containing compound with H_2O_2 in acetic acid for 15 min (7).

Crude preparations of the sulfoxides were prepared by oxidizing the BTs with horse heart cytochrome *c* (Sigma, St. Louis, Mo.) and H_2O_2 by scaling up the method of Vazquez-Duhalt et al. (26). Specifically, BT or 5-methylBT was dissolved in acetonitrile to 10 mM. This was diluted to 1 mM with 6 mM phosphate buffer (pH 6.1); then cytochrome *c* in the same buffer solution, and H_2O_2 were added to concentrations of 400 nM and 1 mM, respectively. The reaction mixture was stirred briefly and left to sit for 1 h at room temperature (20 to 22°C). Because H_2O_2 destroys the activity of cytochrome *c*, multiple hourly additions of the cytochrome and H_2O_2 were made over the course of 2 working days. The reaction mixture sat at room temperature between additions. On day 3, the reaction mixture was saturated with sodium chloride and extracted with diethyl ether to recover the products. In an attempt to produce enough BT sulfoxide to purify by column chromatography, 94 mg of BT was used for the reaction described above, with a total of 15 additions of the cytochrome *c* and H_2O_2 solutions.

Bacterial cultures used. The isolation and characterization of *Pseudomonas* strains W1 and F (21, 24) and *Pseudomonas pseudoalcaligenes* SB(G) (21) have been described previously. A mixed culture of petroleum-degrading bacteria, designated SLPB (13), was also used.

General culture methods and media. Biotransformation studies with strain SB(G) were done with cell suspensions as described by Kropp et al. (21). In short, the culture was grown on plate count agar (Difco, Detroit, Mich.) and the cells were suspended in sterile 3 mM phosphate buffer (pH 7.2). This suspension was added to 200 ml of mineral medium (16) to give an optical density at 600 nm of between 0.5 and 0.8. After the addition of 4 mg of BT or 4 to 6 mg of methylBT, this cell suspension was incubated on a rotary shaker for 18 h prior to acidification and extraction to recover the metabolites.

The mineral medium used for growing cultures of isolates W1 and F in biotransformation studies was modified from that of Fedorak and Westlake (16) by increasing the phosphate concentration eightfold to provide greater buffer capacity at pH 7.0. The modified medium contained (per 900 ml) NH_4Cl, 1.0 g; Na_2SO_4, 2.0 g; KNO_3, 2.0 g; $FeSO_4 \cdot 7H_2O$, trace; trace metal solution (12), 1 ml. To this was added 100 ml of a buffer prepared by adding a solution of KH_2PO_4 (4 g/100 ml) into a solution of K_2HPO_4 (4 g/100 ml) until the pH was 7.0. The buffered medium was then sterilized by autoclaving. Prior to inoculation, 1.0 ml of a separately sterilized solution of $MgSO_4 \cdot 7H_2O$ (4 g/100 ml) was added to each 200-ml portion of medium.

The BTs studied in biotransformation experiments with strains W1 and F would not support the growth of these isolates. Therefore, 1-methylnaphthalene or glucose was used as the growth substrate and the biotransformations of the BTs were observed. The growth substrates were sterilized separately, and 50 mg was added per 200 ml of modified medium. Each 200-ml portion of medium also received 2 to 5 mg of BT or a methylBT, and the culture was incubated for 7 days (unless otherwise stated) prior to extraction. Occasionally, the amount of BT added to cultures was increased to as much as 45 mg/200-ml culture. For each biotransformation experiment, appropriate sterile controls were incubated to account for any abiotic transformations. All cultures and controls were incubated at 28°C with shaking at 200 rpm.

In experiments to determine the amount of high-molecular-weight compound produced from BT by strains W1 and F, cultures of these isolates were given daily additions of 20 μl of 1-methylnaphthalene into which 4.12 mg (31 μmol) of BT had been dissolved. These cultures were incubated in screw-cap flasks to minimize evaporative loss of the BT and were opened daily to replenish oxygen in the culture headspace. After 10 days of incubation, when each culture had received a total of 41.2 mg (310 μmol) of BT, each culture received 4 mg of benzo[*b*]naphtho[2,1-*d*]thiophene to serve as a surrogate standard to quantify the amount of high-molecular-weight product that had formed.

A mixed culture, designated SLPB (13), was used in experiments to determine if the high-molecular-weight product would be formed in the presence of crude oil. This culture was grown in 200 ml of mineral medium (16) with 0.2 ml of Prudhoe Bay crude oil supplemented with 8 mg of BT.

General analytical methods. After incubation, the cultures were acidified with sulfuric acid to pH < 2 and extracted with dichloromethane (4 times 20 ml) to recover substrates and products. To screen for the presence of sulfur-containing products, the extracts were analyzed by GC with a 30-m DB-5 capillary column in an instrument equipped with a flame ionization detector (FID) and a sulfur-selective flame photometric detector (FPD) (12).

Routine GC-MS analyses were done with a model 5890 gas chromatograph (Hewlett-Packard) fitted with a 30-m DB-1 or DB-5 capillary column and coupled to a 5970 series mass-selective detector. In some instances, GC-MS was done by the personnel in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta. The instrument and conditions have been described previously (16).

Column chromatography procedure. To purify the polar products from the cytochrome *c*-mediated oxidation of BT, the silica gel column chromatography method of Fedorak and Andersson (11) was used with a few modifications. Specifically, the prepared column was developed with 5 ml of *n*-pentane, 5 ml of dichloromethane–*n*-pentane (20:80), and 25 ml of dichloromethane–*n*-pentane (50:50) as previously reported (11), but the volume of methanol-benzene (50:50) used as the final solvent was increased from 30 to 45 ml. The first 50 ml to elute from the column was collected as the aromatic fraction. The next 5-ml fraction was collected, concentrated to 100 μl, and analyzed by GC to ensure that no thiophenes or polar compounds were present, confirming a clean separation of these compounds. The final 20 ml was collected to contain the polar compounds, namely the sulfoxide and sulfone of BT.

Nickel boride desulfurization procedure. To help determine the structure of some sulfur-containing, high-molecular-weight products, the nickel boride desulfurization reaction of Back et al. (4) was used. The procedure was verified by using dibenzothiophene and benzo[*b*]naphtho[2,1-*d*]thiophene, which yielded biphenyl and 2-phenylnaphthalene, respectively.

RESULTS

Identification of the high-molecular-weight compound from BT. Dichloromethane extracts of the BT-containing medium that had been incubated with any one of the three *Pseudomo-*

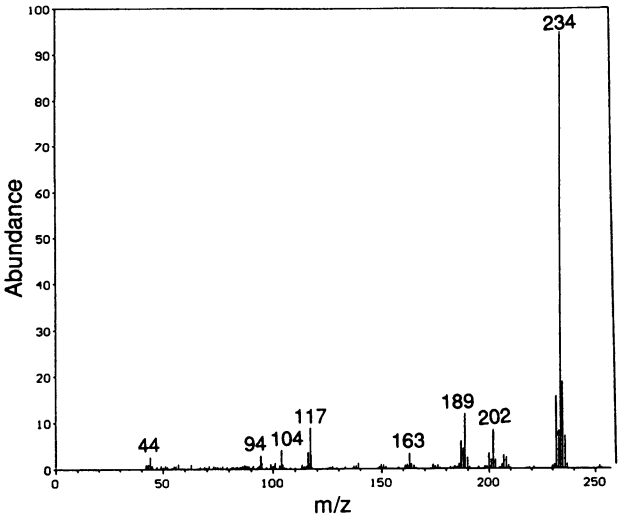

FIG. 1. Mass spectrum obtained from GC-MS analysis of the high-molecular-weight compound found in the extract of a cell suspension of isolate SB(G) incubated with BT.

nas isolates showed the presence of a high-molecular-weight, sulfur-containing compound that eluted from the GC column at a late retention time. GC-MS analysis gave a mass spectrum for the compound with a strong molecular ion at *m/z* 234 (Fig. 1), corresponding to the empirical formula $C_{16}H_{10}S$. Figure 2 shows four products with this chemical formula that could arise from a condensation reaction between two molecules of BT with the loss of two atoms of H and one atom of S. These include the three isomers with a central thiophene ring (the benzonaphthothiophenes) and one example of a condensation product with a peripheral thiophene ring (phenanthro[2,1-*b*]-thiophene). The mass spectrum of the compound found in culture extracts was very similar to that of benzo[*b*]naphtho-[1,2-*d*]thiophene and the other two benzonaphthothiophenes (20).

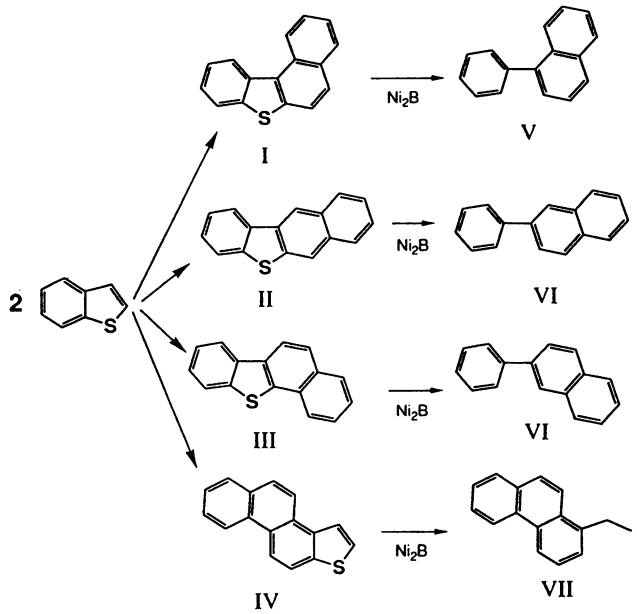

FIG. 2. Possible structures of the sulfur-containing metabolite with molecular weight 234, and the compounds that would be produced after desulfurization with nickel boride. I, Benzo[*b*]naphtho[1,2-*d*]thiophene; II, benzo[*b*]naphtho[2,3-*d*]thiophene; III, benzo[*b*]naphtho[2,1-*d*]thiophene; IV, phenanthro[2,1-*b*]thiophene; V, 1-phenylnaphthalene; VI, 2-phenylnaphthalene; VII, 1-ethylphenanthrene.

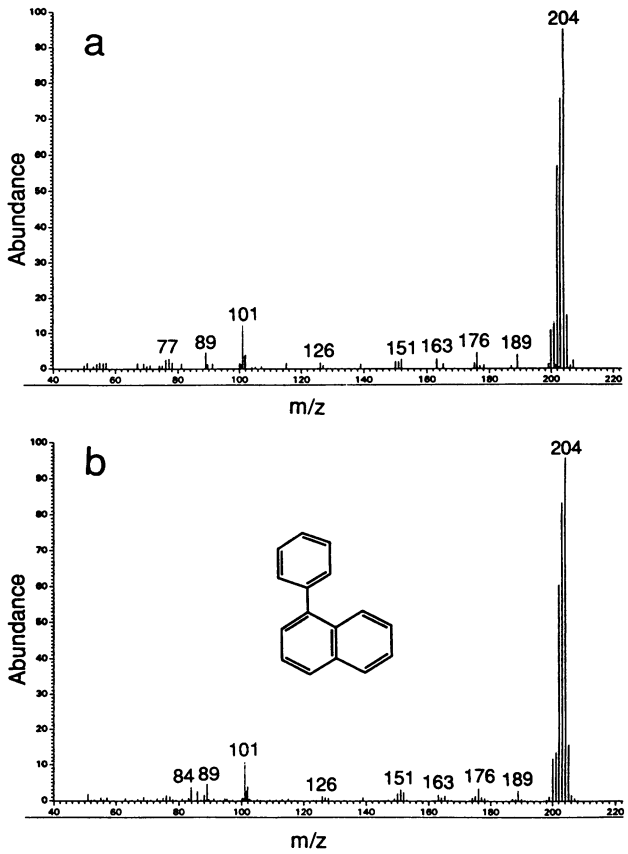

FIG. 3. (a) Mass spectrum obtained from GC-MS analysis of the desulfurized metabolite from isolate SB(G) incubated in the presence of BT. (b) Mass spectrum of authentic 1-phenylnaphthalene.

The GC retention time of the high-molecular-weight metabolite did not match that of an authentic standard of benzo[*b*]-naphtho[2,1-*d*]thiophene; therefore, it was not this isomer. No authentic standards of the other possible sulfur-containing compounds shown in Fig. 2 were available, so the nickel boride desulfurization reaction was used to identify the high-molecular-weight compound. This reaction distinguishes between benzo[*b*]naphtho[1,2-*d*]thiophene and benzo[*b*]naphtho[2,3-*d*]thiophene because the former gives 1-phenylnaphthalene and the latter gives 2-phenylnaphthalene upon desulfurization. These two isomers of phenylnaphthalene were readily separated by the GC method used.

Desulfurization of the high-molecular-weight metabolite yielded a product with the same GC retention time as 1-phenylnaphthalene (compound V, Fig. 2). The mass spectrum of the desulfurized metabolite is shown in Fig. 3a and it matches that of authentic 1-phenylnaphthalene shown in Fig. 3b. Thus, the high-molecular-weight product was benzo[*b*]naphtho[1,2-*d*]thiophene (compound I, Fig. 2).

Verification that the formation of benzo[*b*]naphtho[1,2-*d*]-thiophene was microbially mediated. Evidence suggested that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene in cell sus-

pensions of isolate SB(G) and in 1-methylnaphthalene- or glucose-grown cultures of isolates W1 and F was microbially mediated. For example, the high-molecular-weight compound never formed in the sterile controls that contained medium and BT. Furthermore, when 100 μg of chloramphenicol per ml was added to the cell suspension of isolate SB(G), the high-molecular-weight compound was not formed from BT.

Haines et al. (19) and Andersson and Bobinger (2) identified benzo[*b*]naphtho[2,1-*d*]thiophene as a photooxidation product of BT. To verify that the condensation product was not the result of a photochemical reaction, duplicate cultures of isolate W1 were grown on 1-methylnaphthalene in the presence of BT and duplicate sterile controls were incubated with these cultures. Two flasks, one with the inoculated culture and one with the sterile control, were wrapped in aluminum foil to prevent light from reaching the BT-containing medium, and the remaining two flasks were left uncovered. After 7 days of incubation, the contents of the flasks were extracted and analyzed by GC-FPD. The sterile controls had no sulfur-containing compounds other than the BT originally added. On the other hand, GC analyses of the extracts from both of the cultures of isolate W1 showed the presence of the high-molecular-weight product regardless of whether light had been excluded from the incubation. These results clearly indicate that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene in cultures containing BT was microbially mediated and was not the result of a photochemical reaction of BT or of a photochemical reaction of the microbial metabolites of BT oxidation.

Some laboratory methods were evaluated to verify that the benzo[*b*]naphtho[1,2-*d*]thiophene was not formed during culture extraction or preparation for GC analysis. To ensure that the formation of the high-molecular-weight condensation product was not caused by the acidification to pH < 2 prior to extraction, duplicate cultures of isolate W1, grown for 7 days on 1-methylnaphthalene in the presence of BT, were extracted and analyzed. One of these cultures was acidified to pH < 2 prior to extraction, and the other culture was extracted at neutral pH. GC analyses showed that both of the culture extracts contained benzo[*b*]naphtho[1,2-*d*]thiophene.

To ensure that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene was not an artifact of some other aspect of the liquid-liquid extraction procedure which was routinely used, another method to recover the high-molecular-weight product from aqueous cultures was used. In this case, a 200-ml culture of isolate F, grown on 1-methylnaphthalene in the presence of 25 mg of BT for 7 days, was freeze-dried overnight. The residue was washed with acetonitrile, and after concentration under nitrogen, the wash was analyzed by GC, which confirmed the presence of benzo[*b*]naphtho[1,2-*d*]thiophene. A 200-ml sterile control containing 1-methylnaphthalene and 25 mg of BT was carried through the same procedure, and no condensation product was detected in the acetonitrile wash of the freeze-dried material. These experiments confirmed that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene was not caused by the conditions of the liquid-liquid extraction procedure that was routinely used.

Dimethylbenzonaphthothiophenes from methylBTs. In addition to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene from BT, the formation of high-molecular-weight products from 4-, 5-, 6-, and 7-methylBTs in cultures of isolates SB(G), W1, and F was observed (21). All of these products gave very similar mass spectra with an abundant molecular ion at *m/z* 262 and few fragmentations. This molecular weight is consistent with the empirical formula $C_{18}H_{14}S$, which is 28 mass units greater than benzo[*b*]naphtho[1,2-*d*]thiophene, suggesting dimethyl-substituted products. The mass spectrum shown in Fig. 4a is that of the product which was formed from 5-methylBT in a 1-methylnaphthalene-grown culture of isolate F. The proposed structure shown in Fig. 4a has the methyl groups substituted at positions 3 and 10 of the benzo[*b*]naphtho[1,2-*d*]-thiophene nucleus, which is consistent with the condensation mechanism discussed below. If 5-methylBT condenses by the same mechanism which gives benzo[*b*]naphtho[1,2-*d*]thiophene from BT, then 3,10-dimethylbenzo[*b*]naphtho[1,2-*d*]-thiophene is postulated to be the condensation product from 5-methylBT. This high-molecular-weight product was desulfurized with nickel boride to give a compound with a molecular weight of 232 (Fig. 4b). This is consistent with a dimethyl-substituted 1-phenylnaphthalene. The fragments at *m/z* 217 and 202 represent the loss of one and two methyl groups, respectively. However, no authentic standard was available to positively identify this compound.

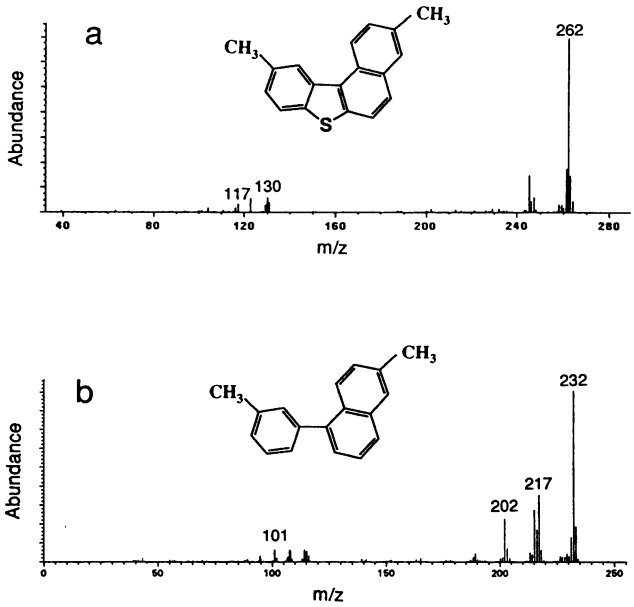

FIG. 4. Mass spectra obtained from GC-MS analysis of a sulfur-containing, high-molecular-weight compound found in the extract of a culture of isolate F grown on 1-methylnaphthalene in the presence of 5-methylBT (a) and of the product after nickel boride desulfurization of the high-molecular-weight compound (b).

Methyl-substituted benzonaphthothiophenes from a mixture of BT and 5-methylBT. When isolate F was incubated with 1-methylnaphthalene in the presence of both BT and 5-methylBT, four high-molecular-weight products were detected. Two of these had the same retention times and mass spectra as benzo[*b*]naphtho[1,2-*d*]thiophene and 3,10-dimethylbenzo[*b*]-naphtho[1,2-*d*]thiophene, which were previously identified as products from BT and 5-methylBT, respectively. The other two products eluted between the unsubstituted and dimethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes during GC analysis. These both gave very similar mass spectra, so only one is shown (Fig. 5), with a strong molecular ion at *m/z* 248. This molecular weight is consistent with the two possible monomethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes which could form by the condensation of a molecule of BT with a molecule of 5-methylBT.

Mechanism of formation of benzo[*b*]naphtho[1,2-*d*]thiophene. Figure 6 shows six possible schemes that might lead to an initial Diels-Alder condensation reaction, the product of

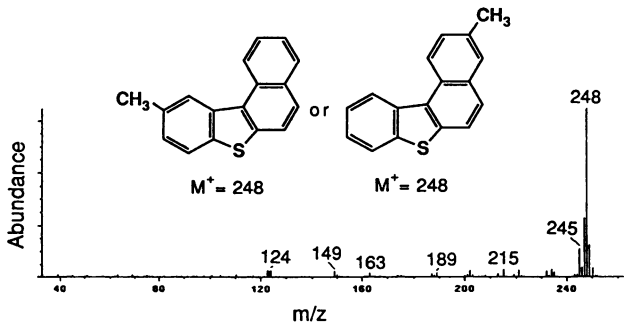

FIG. 5. Mass spectrum obtained from GC-MS analysis of one of the high-molecular-weight sulfur-containing metabolites detected in a culture of isolate F grown on 1-methylnaphthalene in the presence of a mixture of BT and 5-methylBT.

which could undergo subsequent reactions leading to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene. The goal of this work was to determine which of these schemes was the most likely mechanism for the initial condensation step. Benzo[*b*]naphtho[1,2-*d*]thiophene was never formed in sterile controls containing BT, eliminating scheme A and indicating the important role of the bacterial isolates in mediating the formation of these condensation products. It was postulated that after microbial oxidation of BT to the sulfoxide or sulfone, an abiotic condensation reaction occurred, leading to the formation of the high-molecular-weight product (schemes B to F). Extracts of BT-containing cultures of isolates W1, SB(G), and F, in which benzo[*b*]naphtho[1,2-*d*]thiophene had been first identified, always contained BT and its sulfoxide and sulfone.

The possibility that BT sulfone condensed with either BT (scheme C) or another molecule of BT sulfone (scheme F) to give benzo[*b*]naphtho[1,2-*d*]thiophene was easily tested by using synthesized BT sulfone. Benzo[*b*]naphtho[1,2-*d*]thiophene was not detected in extracts of cultures of isolates W1 or F grown on either 1-methylnaphthalene or glucose in the presence of BT sulfone nor in sterile controls containing glucose or 1-methylnaphthalene and a mixture of BT sulfone and BT. Thus, neither scheme C nor F leads to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene.

Thus, it appeared that the condensation reaction to give benzo[*b*]naphtho[1,2-*d*]thiophene involved BT sulfoxide (scheme B, D, or E), which could not be synthesized by chemical means. Thus, the method of Vazquez-Duhalt et al. (26), using cytochrome *c* and H_2O_2, was scaled up to synthesize a modest amount of sulfoxide from 94 mg of BT. However GC-FPD analysis of ether extracts of the reaction mixture showed that little of the BT had been oxidized and that under these reaction conditions both the sulfoxide and sulfone of BT, along with several other sulfur-containing compounds, were formed. Interestingly, GC-MS analysis also showed that benzo[*b*]naphtho[1,2-*d*]thiophene was present in the extract of the reaction mixture. GC-FID analysis, with commercially available benzo[*b*]naphtho[2,1-*d*]thiophene as a quantitative standard, showed that 1.4 mg of the high-molecular-weight product had formed in this reaction. This observation demonstrated that active microbial growth was not required for the formation of the condensation product. The cytochrome *c*-catalyzed oxidation of BT was sufficient to promote the formation of benzo[*b*]naphtho[1,2-*d*]thiophene.

The polar compounds BT sulfoxide and BT sulfone were separated from the excess BT and the condensation product by silica gel column chromatography. GC analysis revealed that the first 50 ml of solvent collected from the column contained the BT and benzo[*b*]naphtho[1,2-*d*]thiophene that had been present in the extract of the cytochrome *c* reaction mixture. The next 5 ml of solvent to elute from the column was concentrated approximately 50-fold, and GC analysis of the concentrate showed no sulfur-containing compounds. This confirmed that all the BT and benzo[*b*]naphtho[1,2-*d*]thiophene had eluted in the first 50 ml of eluent and that none of the oxidized products had eluted. GC-MS analysis showed that the last 20 ml of methanol-benzene to elute contained BT sulfoxide and sulfone. Furthermore, GC-MS analysis showed benzo[*b*]naphtho[1,2-*d*]thiophene in this polar fraction. This suggested that the high-molecular-weight product was formed by abiotic condensation of the compounds present in the polar fraction, thereby ruling out scheme B. The benzo[*b*]naphtho[1,2-*d*]thiophene formation during silica gel column chromatography is consistent with the enhancement of the rate of Diels-Alder reactions by adsorption onto silica gel, as previously reported (27).

The cytochrome *c*-catalyzed oxidation procedure was also done with a mixture of BT and 5-methylBT. GC-MS analysis of the ether extract of the reaction mixture showed that it contained BT, 5-methylBT, the corresponding sulfones, BT sulfoxide, benzo[*b*]naphtho[1,2-*d*]thiophene, two isomers of monomethyl benzo[*b*]naphtho[1,2-*d*]thiophene, and a dimethylbenzo[*b*]naphtho[1,2-*d*]thiophene. Interestingly, no 5-methylBT sulfoxide was detected in the extract, presumably because it readily reacted to form the condensation products or was readily oxidized further to the respective sulfone. Alternatively, all of this sulfoxide may have decomposed in the GC injection port liner (11).

The observations from the cytochrome *c* oxidation of the mixture of BT and 5-methylBT provided a simple means to

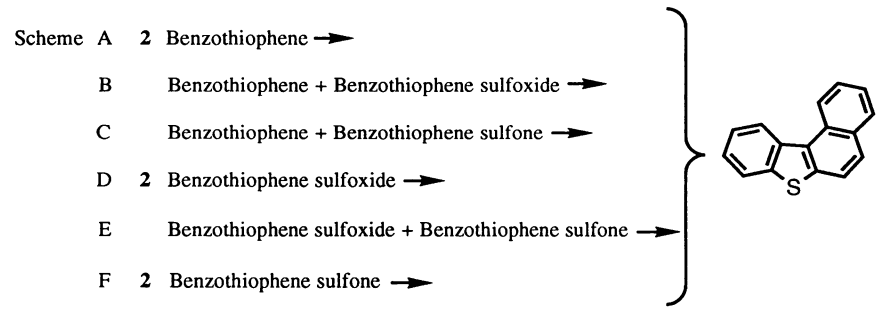

FIG. 6. Six possible schemes that might lead to a Diels-Alder condensation resulting in the formation of benzo[*b*]naphtho[1,2-*d*]thiophene.

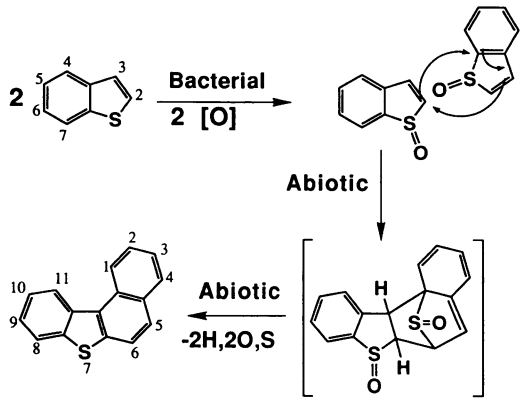

FIG. 7. Proposed mechanism for the formation of benzo[*b*]naphtho[1,2-*d*]thiophene by abiotic condensation of microbially produced BT sulfoxide.

determine whether the sulfones played a role in the condensation mechanism (scheme E). BT (14 mg) and 3 mg of chemically synthesized 5-methylBT sulfone were added to a reaction mixture containing cytochrome *c* and H_2O_2. At the end of the reaction time, the mixture was extracted and analyzed by GC-MS, which showed that the only high-molecular-weight product that formed was benzo[*b*]naphtho[1,2-*d*]-thiophene. This indicated that the condensation did not involve the sulfone, because, if it had, the 5-methylBT sulfone would have reacted with the BT sulfoxide (formed by the cytochrome *c* oxidation) to give a monomethyl benzo[*b*]naphtho[1,2-*d*]thiophene. To further verify this, the cytochrome *c* oxidation procedure was done with 5 mg of 5-methylBT and 13 mg of chemically synthesized BT sulfone. If the sulfone was involved in the condensation mechanism, a monomethylated condensation product would have been detected. However, the only condensation product present was a dimethylbenzo[*b*]-naphtho[1,2-*d*]thiophene, which formed through the condensation of two molecules of 5-methylBT sulfoxide, produced by the cytochrome *c* oxidation. Thus, scheme D describes the formation of benzo[*b*]naphtho[1,2-*d*]thiophene from BT sulfoxide.

On the basis of these observations, we propose a mechanism, shown in Fig. 7, whereby two molecules of BT sulfoxide, produced by bacterial or cytochrome *c* oxidation of BT, condense by a Diels-Alder-type mechanism with the subsequent loss of one atom of sulfur, two atoms of hydrogen, and two atoms of oxygen, resulting in the formation of benzo[*b*]-naphtho[1,2-*d*]thiophene. The sulfoxide of BT functions as both diene and dienophile in this reaction. The last two steps in the mechanism are considered to be abiotic because of the observed formation of the condensation product in the polar fraction obtained by silica gel chromatography.

The methyl- and dimethyl-substituted benzo[*b*]naphtho [1,2-*d*]thiophenes observed in cultures of isolates SB(G), W1, and F are believed to form by the same mechanism. Although the sulfoxides of 2-methylBT and 3-methylBT have been observed in other studies (12, 21, 23), condensation products from these isomers were never detected (21). This is consistent with the mechanism shown in Fig. 7, because the condensation would be hindered by a methyl group on the thiophene ring.

Table 1 summarizes the high-molecular-weight products found in various cultures incubated with methylBTs. Assuming that the mechanism given in Fig. 7 applies to all these condensation reactions, the postulated identities of the methyl- and dimethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes are given in Table 1. However, no authentic standards were available to unequivocally identify these compounds. Although it is possible that four isomers of dimethyl-substituted benzo-[*b*]naphtho[1,2-*d*]thiophene could be formed from the mixture of 4- and 6-methylBT, only two peaks on the gas chromatogram were detected. However, these peaks may have contained coeluting isomers.

TABLE 1. Postulated identities of methyl- and dimethyl-substituted benzonaphthothiophenes produced in cultures incubated with various methylBTs

Substrate	No. of HMW[a] products detected by GC-MS	Postulated identities of products[b]	Bacterial strains studied[c]
4-MethylBT	1	4,11-DimethylBNT	F
5-MethylBT	1	3,10-DimethylBNT	SB(G), F
7-MethylBT	1	1,8-DimethylBNT	W1, F
4- and 6-MethylBTs[d]	2	4,9-DimethylBNT 4,11-DimethylBNT 2,9-DimethylBNT 2,11-DimethylBNT	W1, SB(G), F
BT and 5-methylBT	4[e]	3-MethylBNT 10-MethylBNT 3,10-DimethylBNT	F

[a] HMW, high molecular weight.
[b] Presuming that the mechanism shown in Fig. 7 applies; BNT, benzo[*b*]naphtho[1,2-*d*]thiophene.
[c] Not all strains were tested on each substrate.
[d] The synthesis for 6-methylBT yielded a mixture of this compound and 4-methylBT.
[e] Unsubstituted benzo[*b*]naphtho[1,2-*d*]thiophene was also detected in these culture extracts.

Quantification of the amount of benzo[*b*]naphtho[1,2-*d*]-thiophene produced by isolates W1 and F. Cultures of isolates W1 and F that had been incubated in screw-cap flasks and had received 1-methylnaphthalene and BT daily for 10 days were used to determine the amount of benzo[*b*]naphtho[1,2-*d*]thiophene produced. Peak areas from the GC-FID were compared with those of a known amount of the commercially available benzo[*b*]naphtho[2,1-*d*]thiophene added to the culture before extraction. These results showed that the 200-ml cultures of isolates W1 and F produced 2.7 mg (11.4 μmol) and 2.5 mg (10.7 μmol) of benzo[*b*]naphtho[1,2-*d*]thiophene, respectively, from 41.2 mg (310 μmol) of BT. Because two molecules of BT are required to condense to give one molecule of the condensation product, the maximum amount of benzo[*b*]naphtho[1,2-*d*]thiophene that could have formed was 155 μmol. Thus, the amounts of benzo[*b*]naphtho[1,2-*d*]thiophene produced by isolates W1 and F corresponded to yields of 7.4 and 6.9%, respectively.

Formation of benzo[*b*]naphtho[1,2-*d*]thiophene by a mixed culture incubated with Prudhoe Bay crude oil. The dichloromethane extract from a 14-day-old culture of an oil-degrading mixed culture, SLPB, grown on BT-supplemented crude oil was analyzed by GC-FPD, and the extract from a sterile control was analyzed in the same manner (Fig. 8). GC-MS analysis of the extract from the viable culture confirmed the presence of the sulfoxide and sulfone of BT and the presence of benzo[*b*]naphtho[1,2-*d*]thiophene. GC-FID analyses, with benzo[*b*]naphtho[2,1-*d*]thiophene as a quantitative standard, showed that 0.04 mg of the condensation product was formed from the 8 mg of BT added to the oil. This amount of BT was approximately 5% of the weight of the oil added. In another experiment, 1 mg of BT was added, and the benzo[*b*]naph-

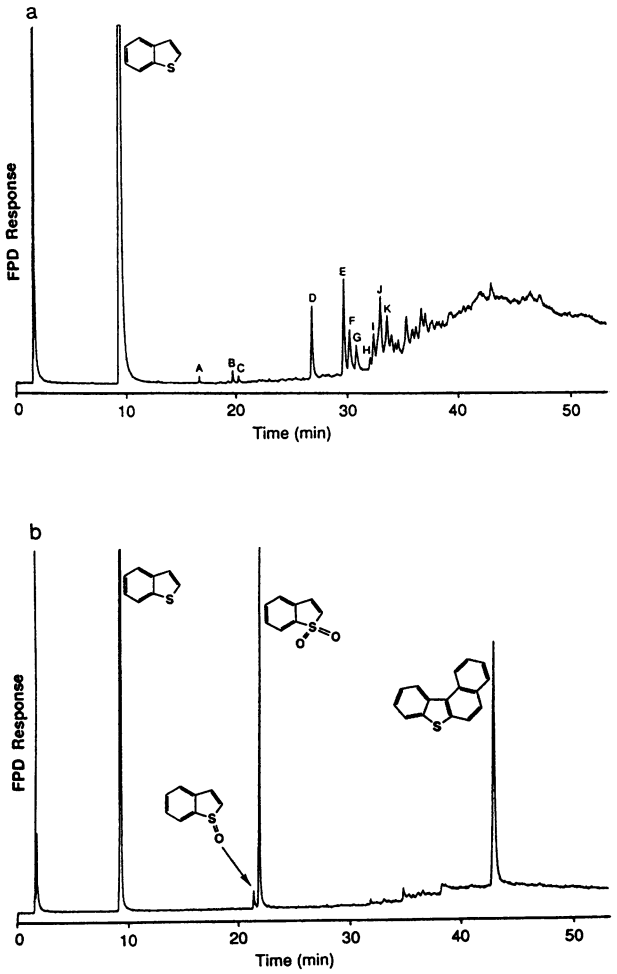

FIG. 8. GC-FPD analysis of BT-supplemented Prudhoe Bay crude oil extracted from a sterile control (a) and from a culture of the oil-degrading mixed culture SLPB after 14 days of incubation (b). Peak designations: A, C_2-BT; B and C, C_3-BTs; D, dibenzothiophene; E to G, C_1-dibenzothiophenes; H to K, C_2-dibenzothiophenes.

tho[1,2-*d*]thiophene peak was just detectable in the culture extract.

DISCUSSION

The oxidation of some of the BTs to their respective sulfoxides by the activity of the three *Pseudomonas* isolates SB(G), W1, and F or by the cytochrome *c*-catalyzed oxidation reaction resulted in the formation of benzo[*b*]naphtho[1,2-*d*]-thiophenes. Proving the involvement of sulfoxides in the condensation mechanism was complicated by the difficulty experienced in synthesizing and isolating pure BT sulfoxide. Chemical methods of oxidizing the sulfur atom do not stop at the sulfoxide but proceed to the sulfone. Vazquez-Duhalt et al. (26) reported the oxidation of a number of organosulfur compounds, including BT, to their respective sulfoxides by horse heart cytochrome *c* in small-scale reactions. Sulfones were not detected by GC-MS and high-pressure liquid chromatography analyses in their study. In the current study, the synthesis of about 100 mg of BT sulfoxide was attempted by using a scaled-up version of their method. However, despite multiple additions of cytochrome *c* and H_2O_2, quantitative oxidization of the BT was not achieved. As well, the scaled-up method yielded the sulfoxide and sulfone as determined by GC-MS analyses. The isolation of pure BT sulfoxide was further complicated by the formation of the condensation product, benzo[*b*]naphtho[1,2-*d*]thiophene, from the BT sulfoxide in the reaction mixture and on the silica gel column. Although the attempted synthesis of pure BT sulfoxide was unsuccessful, the formation of the condensation product in the cytochrome *c*-mediated oxidation reaction made it possible to determine that only the sulfoxides (scheme C, Fig. 6) were involved in the condensation reaction.

GC analyses of some of the extracts from cultures of isolates SB(G), W1, and F incubated in the presence of methylBTs failed to detect the corresponding sulfoxide intermediates, although the dimethylbenzonaphthothiophenes were detected (21). It is likely that the sulfoxide had condensed to form the high-molecular-weight product or that it had been oxidized by the bacterial culture to the sulfone. However, it is possible that the undetected sulfoxides decomposed in the GC injection port liner (11).

There are a few reports on methods for the chemical synthesis of benzo[*b*]naphtho[1,2-*d*]thiophene. For example, Davies et al. (9) observed its formation as a minor product when the reaction mixture from the oxidation of BT to BT sulfone by H_2O_2 and acetic acid (7) was extracted with chloroform, dried, and distilled under reduced pressure (boiling point, 280°C at 15 mm Hg). Minor amounts of the sulfone of benzo[*b*]naphtho[1,2-*d*]thiophene were also produced. In other studies (8, 9), 6a,11b-dihydrobenzo[*b*]naphtho[1,2-*d*]-thiophene sulfone was obtained in 80 to 90% yield from the controlled pyrolysis of BT sulfone (180 to 200°C in tetralin). The aromatization of this compound by sequential bromination and treatment with alcoholic potassium hydroxide followed by reduction of the sulfone with $LiAlH_4$ has been used for the synthesis of benzo[*b*]naphtho[1,2-*d*]thiophene (9). This synthesis requires reaction conditions that are much more severe than those that lead to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene from the microbially produced BT sulfoxide reported here. Indeed, the cytochrome *c*-catalyzed oxidation of BTs to their sulfoxides, which spontaneously condense, should serve as a simple method for the synthesis of small amounts of benzo[*b*]naphtho[1,2-*d*]thiophenes, although isolation of the condensation product from other sulfur-containing products will be required.

The microbially mediated formation of benzonaphthothiophenes from BTs will produce compounds that are less volatile, less water soluble, and less mobile than the parent compounds. Whether this occurs in petroleum- or creosote-contaminated environments has yet to be determined. However, our laboratory tests with Prudhoe Bay crude oil supplemented with BT to a concentration that was sufficient to allow detection of the metabolites showed that a mixed culture produced BT sulfoxide, BT sulfone, and benzo[*b*]naphtho-[1,2-*d*]thiophene. Kropp et al. (21) demonstrated that the microbial population in a river water sample also produced BT sulfoxide from BT added to crude oil. The presence of the microbially produced sulfoxide may lead to the abiotic formation of the benzonaphthothiophene. These transformations would probably increase the recalcitrance of the residual petroleum or creosote and may affect its toxicity.

As a general rule, the larger the number of aromatic rings in a polycyclic aromatic molecule, the more recalcitrant it is to biodegradation. The condensation described in this paper transforms a two-ring sulfur heterocycle into a four-ring sulfur heterocycle, which is presumably quite resistant to microbial attack. For example, although the commercially available

benzo[*b*]naphtho[2,1-*d*]thiophene has been oxidized to its sulfoxide by cytochrome *c* (26), repeated attempts to demonstrate microbial transformation of the former compound have failed (22a). In addition, the greater the number of alkyl carbons on an aromatic ring system, the more recalcitrant the molecule. For instance, naphthalene is more susceptible to biodegradation than are the C_1-naphthalenes, which are more susceptible to biodegradation than are the C_2-naphthalenes (14). Similarly, dibenzothiophene is more susceptible to biodegradation than are the C_1-dibenzothiophenes, which are more susceptible to biodegradation than are the C_2-dibenzothiophenes (5, 15, 16). The condensation reactions involving methylBTs, which were demonstrated in the current study, yielded C_1- and C_2-benzo[*b*]naphtho[1,2-*d*]thiophenes, which are probably more resistant to microbial degradation than benzo[*b*]naphtho[1,2-*d*]-thiophene is.

Eastmond et al. (10) tested a variety of polycyclic aromatic hydrocarbons and structurally similar polycyclic aromatic sulfur heterocycles for their toxicity to the zooplankton *Daphnia magna*. They observed 50% lethal concentrations of 0.22 mg/liter for benzo[*b*]naphtho[1,2-*d*]thiophene and 63.7 mg/liter for BT. Thus, the product of the microbially mediated condensation is more toxic than BT. However, benzo[*b*]naphtho[1,2-*d*]thiophene showed no mutagenicity in the standard Ames or preincubation Ames test (22).

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REFERENCES

1. **Andersson, J. T.** 1986. Gas chromatographic retention indices for all C_1- and C_2-alkylated benzothiophenes and their dioxides on three different stationary phases. J. Chromatogr. **354**:83–98.
2. **Andersson, J. T., and S. Bobinger.** 1992. Polycyclic aromatic sulfur heterocycles. II. Photochemical oxidation of benzo[*b*]thiophene in aqueous solution. Chemosphere **24**:383–388.
3. **Atlas, R. M., P. D. Boehm, and J. A. Calder.** 1981. Chemical and biological weathering of oil, from the Amoco Cadiz spillage, within the littoral zone. Estuarine Coastal Shelf Sci. **12**:589–608.
4. **Back, T. G., K. Yang, and H. R. Krouse.** 1992. Desulfurization of benzo- and dibenzothiophenes with nickel boride. J. Org. Chem. **57**:1986–1990.
5. **Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald.** 1986. Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures. J. Environ. Anal. Chem. **23**:289–303.
6. **Bohonos, N., T.-W. Chou, and R. J. Spanggord.** 1977. Some observations on biodegradation of pollutants in aquatic systems. Jpn. J. Antibiot. **30**(Suppl.):275–285.
7. **Bordwell, F. G., B. B. Lampert, and W. H. McKellin.** 1949. Thianaphthene chemistry. III. The reaction of 2,3-dibromo-2,3-dihydro- and 2-bromo-thianaphthene-1-dioxide with synthesis secondary amines. J. Am. Chem. Soc. **71**:1702–1705.
8. **Bordwell, F. G., W. H. McKellin, and D. Babcock.** 1951. Benzothiophene chemistry. V. The pyrolysis of benzothiophene 1-dioxide. J. Am. Chem. Soc. **73**:5566–5568.
9. **Davies, W., N. W. Gamble, and W. E. Savige.** 1952. The direct conversion of thionaphthen into derivatives of 9-thiafluorene. J. Chem. Soc. **1952**:4678–4683.
10. **Eastmond, D. A., G. M. Booth, and M. L. Lee.** 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles in *Daphnia magna*. Arch. Environ. Contam. Toxicol. **13**:105–111.

10a. **Eaton, R. W., and J. D. Nitterauer.** 1994. Biotransformation of benzothiophene by isopropylbenzene-degrading bacteria. J. Bacteriol. **176**:3992–4002.

11. **Fedorak, P. M., and J. T. Andersson.** 1992. Decomposition of two methylbenzothiophene sulphoxides in a commercial GC injection port liner. J. Chromatogr. **591**:362–366.
12. **Fedorak, P. M., and D. Grbić-Galić.** 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. **57**:932–940.
13. **Fedorak, P. M., and T. M. Peakman.** 1992. Aerobic microbial metabolism of some alkyl thiophenes found in petroleum. Biodegradation **2**:223–236.
14. **Fedorak, P. M., and D. W. S. Westlake.** 1981. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can. J. Microbiol. **27**:432–443.
15. **Fedorak, P. M., and D. W. S. Westlake.** 1983. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. **29**:291–296.
16. **Fedorak, P. M., and D. W. S. Westlake.** 1984. Degradation of sulfur heterocycles in Prudhoe Bay crude oil by soil enrichments. Water Air Soil Pollut. **21**:225–230.
17. **Fedorak, P. M., and D. W. S. Westlake.** 1986. Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. **51**:435–437.
18. **Finnerty, W. R., K. Shockley, and H. Attaway.** 1983. Microbial desulfurization and denitrification of hydrocarbons, p. 83–91. *In* J. E. Zajic, D. C. Cooper, T. R. Jack, and N. Kosaric (ed.), Microbial enhanced oil recovery. PennWell Publishing Co., Tulsa, Okla.
19. **Haines, W. E., R. V. Helm, G. L. Cook, and J. S. Ball.** 1956. Purification and properties of ten organic sulfur compounds-second series. J. Phys. Chem. **60**:549–555.
20. **Karcher, W., R. J. Fordham, J. J. Dubois, P. G. J. M. Glaude, and J. A. M. Ligthart.** 1985. Spectral atlas of polycyclic aromatic compounds. Dordrecht Reidel Publishing Co., Boston.
21. **Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak.** 1994. Bacterial transformations of benzothiophene and methylbenzothiophenes. Environ. Sci. Technol. **28**:1348–1356.
22. **Pelroy, R. A., D. L. Stewart, Y. Tominaga, M. Iwao, R. N. Castle, and M. L. Lee.** 1983. Microbial mutagenicity of 3- and 4-ring polycyclic aromatic sulfur heterocycles. Mutat. Res. **117**:31–40.

22a. **Saftić, S., and P. M. Fedorak.** Unpublished data.

23. **Saftić, S., P. M. Fedorak, and J. T. Andersson.** 1992. Diones, sulfoxides, and sulfones from the aerobic cometabolism of methylbenzothiophenes by *Pseudomonas* strain BT1. Environ. Sci. Technol. **26**:1759–1764.
24. **Saftić, S., P. M. Fedorak, and J. T. Andersson.** 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. **27**:2577–2584.
25. **Sagardía, F., J. J. Rigau, A. Martínez-Lahoz, F. Fuentes, C. López, and W. Flores.** 1975. Degradation of benzothiophene and related compounds by a soil *Pseudomonas* in an oil-aqueous environment. Appl. Microbiol. **29**:722–725.
26. **Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak.** 1993. Cytochrome *c* as a biocatalyst for the oxidation of thiophenes and organosulfides. Enzyme Microb. Technol. **15**:494–499.
27. **Veselovsky, V. V., A. S. Gybin, A. V. Lozanova, A. M. Moiseenkov, and W. A. Smit.** 1988. Dramatic acceleration of the Diels-Alder reaction by adsorption on chromatography adsorbents. Tetrahedron Lett. **29**:175–178.
28. **Westlake, D. W. S.** 1983. Microbial activities and changes in the chemical and physical properties of oil, p. 102–111. *In* E. C. Donaldson and J. B. Clark (ed.), Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Bartlesville Energy Technology Centre, Bartlesville, Okla.
29. **Williams, J. A., M. Bjorøy, D. L. Dolcater, and J. C. Winters.** 1986. Biodegradation of South Texas Eocene oils-effects on aromatics and biomarkers. Org. Geochem. **10**:451–461.