Bacterial Transformations of Naphthothiophenes

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Received 22 April 1997/Accepted 10 June 1997

Naphthothiophenes are minor components of fossil fuels, and they can enter the environment from oil spills. Naphtho[2,1-*b*]thiophene, naphtho[2,3-*b*]thiophene, and 1-methylnaphtho[2,1-*b*]thiophene were synthesized and used in biodegradation studies with 1-methylnaphthalene (1-MN)-degrading *Pseudomonas* strains W1, F, and BT1. Cultures were incubated with one of the naphthothiophenes with or without 1-MN, acidified, and extracted with CH₂Cl₂. The extracts were analyzed by gas chromatography with flame photometric and mass detectors to characterize sulfur-containing metabolites and with an atomic emission detector for quantification. Only strain W1 was able to grow on naphtho[2,1-*b*]thiophene, but strains F and BT1 cometabolized this compound if 1-MN was present. 1-MN was required by all three strains to metabolize naphtho[2,3-*b*]thiophene, which was more resistant to biodegradation than the [2,1-*b*] isomer. Two metabolites of naphtho[2,1-*b*]thiophene were purified, analyzed by ¹H nuclear magnetic resonance spectroscopy, and found to be 4-hydroxybenzothiophene-5-carboxylic acid (metabolite I) and 5-hydroxybenzothiophene-4-carboxylic acid (metabolite II). In cultures of strain W1 grown for 7 days on 52 μmol of naphtho[2,1-*b*]thiophene, >84% of the substrate was degraded and metabolites I and II accounted for 19 and 9%, respectively, of the original amount of naphtho[2,1-*b*]thiophene. When 1-MN was present, strain W1 degraded >97% of the naphtho[2,1-*b*]thiophene and similar amounts of metabolite II were produced, but metabolite I did not accumulate. 1-MN was shown to promote the further degradation of metabolite I, but not of metabolite II, by strain W1. Thus, 1-MN enhanced the biodegradation of naphtho[2,1-*b*]thiophene. Approximately 70% of the 1-methylnaphtho[2,1-*b*]thiophene added to cultures of strain W1 with 1-MN was recovered as 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid, the 3-methyl analog of metabolite I. The methyl substitution hindered further metabolism of 3-methyl-metabolite I even in the presence of 1-MN. Cometabolism of naphtho[2,3-*b*]thiophene yielded two products that were tentatively identified as 5-hydroxybenzothiophene-6-carboxylic and 6-hydroxybenzothiophene-5-carboxylic acids.

Sulfur is usually the most abundant hetero-element in crude oils, contributing from 0.04 to 5% of the mass of conventional crude oils (53). The types of organosulfur compounds identified in petroleum include thiols, sulfides, and thiophenes, with benzo- and dibenzothiophenes predominant in the heavier fractions, where sulfur content is the highest (20, 22). For example, in some Texas oils, as much as 70% of the organic sulfur is present as dibenzothiophene, and in some Middle East oils, the alkyl-substituted benzo- and dibenzothiophenes contribute up to 40% of the organic sulfur present (20). Thus, it is not surprising that most studies of the microbial metabolism of organosulfur compounds have focused on the condensed thiophenes that are found in the aromatic fraction of petroleum, especially those which are commercially available, namely, benzothiophene (5, 14, 16, 21, 35, 50), 3-methylbenzothiophene (16, 35), and dibenzothiophene (5, 9, 25–27, 29, 31, 32, 34, 38, 42, 43, 46, 54, 56). It is also not surprising that only a few studies have been done with alkyl-substituted benzothiophenes (35–37, 48) and dibenzothiophenes (33, 40, 45, 49), because these compounds are not commercially available and must be chemically synthesized before they can be used in biodegradation studies.

In addition to benzo- and dibenzothiophenes, other polycyclic aromatic sulfur heterocycles have been identified by gas chromatography (GC) analyses of solvent-refined coal liquids, coal tars, anthracene oils, shale oils, and higher-boiling-point petroleum fractions using a sulfur-selective flame photometric

detector (FPD) or an atomic emission detector (AED) and GC-mass spectrometry (GC-MS). The compounds identified include benzonaphthothiophenes, phenanthrothiophenes, dinaphthothiophenes, benzophenanthrothiophenes, and other compounds containing two to four benzene rings condensed with one or two thiophene rings (6, 7, 12, 39, 44, 59).

Using a very polar cyanopropyl stationary phase for GC analysis with an AED, Andersson and Schmid (1) detected naphtho[1,2-*b*]thiophene (C₁₂H₈S) in an Austrian shale oil and reported that this isomer coeluted with dibenzothiophene (C₁₂H₈S) during GC analysis with the nonpolar stationary phases typically used to analyze aromatic fractions. Naphtho[2,1-*b*]thiophene and naphtho[2,3-*b*]thiophene, the two other three-ring polycyclic aromatic sulfur heterocycles with the chemical formula C₁₂H₈S, were also detected. The coelution of dibenzothiophene with naphtho[1,2-*b*]thiophene is consistent with earlier reports of Burchill et al. (6, 7) that only three chromatographic peaks of the chemical formula C₁₂H₈S were detected by GC-MS analysis of coal tar and anthracene oil for the four possible isomers that contain a thiophene ring condensed with two benzene rings. Thus, naphthothiophenes are among the condensed thiophenes present in fossil fuels and their derivatives.

Previous studies using GC analyses with sulfur-selective detection to assess the biodegradation of crude oil in microbial cultures (17, 18, 23), in contaminated environments (15, 57), and within natural petroleum reservoirs (58) have demonstrated the loss of the peak corresponding to dibenzothiophene. This suggests that naphtho[1,2-*b*]thiophene, if it is present in the particular crude oils studied, is also susceptible to microbial degradation. However, none of the isomers of

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FIG. 1. Structures and ring-numbering conventions of naphtho[2,1-*b*]thiophene, naphtho[2,3-*b*]thiophene, and benzothiophene.

naphthothiophene are commercially available, and none of them have been previously subjected to biodegradation studies.

The objective of this work was to test the abilities of three 1-methylnaphthalene (1-MN)-degrading *Pseudomonas* strains, W1, BT1, and F, which have been previously shown to metabolize dibenzothiophene (34), to grow on and/or to cometabolize naphtho[2,1-*b*]thiophene and naphtho[2,3-*b*]thiophene. The structures and numbering of these chemically synthesized condensed thiophenes and benzothiophene are shown in Fig. 1. A small amount of 1-methylnaphtho[2,1-*b*]thiophene was also synthesized and used in these studies.

MATERIALS AND METHODS

Chemicals. 1-MN was purchased from Fluka (Buchs, Switzerland). Naphtho[2,1-*b*]thiophene (3), 1-methylnaphtho[2,1-*b*]thiophene (10), and naphtho[2,3-*b*]thiophene (8) were synthesized.

Bacterial cultures and culture methods. The isolation and characterization of the three 1-MN-degrading *Pseudomonas* strains, BT1, W1, and F, were described previously (16, 49). The 10-ml inocula used for biotransformation experiments with each of these isolates were obtained from 1-MN-grown maintenance cultures that were transferred weekly. These maintenance cultures were at least 4 days old when used to inoculate flasks for naphthothiophene biotransformation experiments. All cultures were grown in 500-ml Erlenmeyer flasks containing 200 ml of mineral medium supplemented with a trace metals solution (36), and they were incubated at 28°C on a rotary shaker.

After inoculation, the cultures were given one of the naphthothiophenes either as a solid, for qualitative studies, or as a solution in dichloromethane (DCM) (50 to 150 μ l), for quantitative and time course studies which required reproducible amounts of naphthothiophene in replicate cultures. When it was used, the DCM quickly evaporated as the cultures incubated on the shaker. The amounts of naphthothiophenes given exceeded the aqueous solubilities of these compounds, so that they were present in cultures as a fine suspension or partitioned into the cosubstrate 1-MN, when it was present. In all studies with naphtho[2,1-*b*]thiophene and 1-methylnaphtho[2,1-*b*]thiophene, the amount of naphthothiophene given was approximately 10 mg per culture. In studies with naphtho[2,3-*b*]thiophene, this amount was given in only one experiment in which it was the sole carbon source. Subsequently, the amount of naphtho[2,3-*b*]thiophene was reduced to 5 mg per culture in cometabolism studies with 50 μ l of 1-MN present as the growth substrate. The same amount of 1-MN was used in cometabolism studies with naphtho[2,1-*b*]thiophene or 1-methylnaphtho[2,1-*b*]thiophene. For each biotransformation experiment, appropriate sterile controls were included to account for any abiotic loss and transformations. Although some evaporation occurred, none of the oxygenated products detected in the culture extracts were found in the sterile controls.

When the isolates from 1-MN-grown maintenance cultures were incubated with 10 mg of a naphthothiophene as the sole carbon source, they were always capable of oxidation of at least a small portion of the substrate, and formation of some metabolites was observed. However, the isolates were considered capable of growth on a naphthothiophene only if they survived 14 days of incubation of the culture and subsequent transfer (10 ml) into a second flask of naphthothiophene-containing medium and were still able to oxidize the naphthothiophene. Growth of isolate W1 on naphtho[2,1-*b*]thiophene after this transfer was demonstrated by monitoring the increase in CFU over the following 2-week incubation. At various intervals, 1.0 ml of the culture was removed and serially diluted in 9-ml volumes of sterile phosphate buffer (pH 7.2; 10 mM), and then 0.1 ml of these dilutions was plated onto Plate Count Agar (Difco, Detroit, Mich.). After 3 days of incubation of these plates at 28°C, the numbers of colonies present were determined.

Two metabolites of naphtho[2,1-*b*]thiophene were detected and were designated metabolites I and II. The following procedure was used to obtain a mixture of these two metabolites for use in subsequent studies to determine if they could be biodegraded. Five replicate cultures of isolate W1 were incubated for 5 days

with naphtho[2,1-*b*]thiophene as the sole carbon source. Then they were acidified, pooled, and extracted with DCM. Metabolites I and II were separated from unoxidized naphtho[2,1-*b*]thiophene and nonacidic products in the DCM extract by back extraction into aqueous 5% NaHCO₃. After acidification to a pH of <2 with concentrated H₂SO₄, the metabolites were extracted with DCM. The DCM extract was concentrated to dryness and redissolved in 5 ml of a solvent mixture of DCM and acetone (4:1, vol/vol). Then 1.0-ml portions were added to each of five flasks of sterile medium together with 50 μ l of 1-MN, and the mixtures were incubated as follows: one was acidified and extracted at time zero to determine the amounts of metabolites I and II added, another flask served as a sterile control that was incubated with the cultures, and the three remaining flasks were inoculated with strain W1 and incubated for 11, 31, or 98 h before extraction and analyses by GC-AED.

Solvent extraction. Cultures and sterile controls were acidified with 2 M H₂SO₄ to a pH of <2 and extracted with DCM (four times 20 ml) to recover substrates and products. The DCM extract was dried over anhydrous Na₂SO₄ and concentrated on a rotary evaporator. For quantitative experiments by GC-AED analysis, known amounts of the following compounds were added to acidified cultures as internal standards prior to extraction: benzo[*b*]naphtho[2,1-*d*]thiophene (99%; Aldrich, Milwaukee, Wis.) for cultures incubated with naphtho[2,1-*b*]thiophene, dibenzothiophene (98%; Fluka) for cultures incubated with 1-methylnaphtho[2,1-*b*]thiophene, and phenyl sulfoxide (97%; Aldrich) for cultures incubated with naphtho[2,3-*b*]thiophene. The internal standards used were chosen so that they would not coelute with substrates or products detected in preliminary experiments.

GC analyses. The methods used for GC-FPD (16) and routine GC-MS analyses (48, 49) have been described previously. To obtain high-resolution GC-MS data, analyses were done in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta (19). The operation of the GC-AED for quantitative analyses was outlined by Kropp et al. (33).

To facilitate GC-MS identification of some of the metabolites, trimethylsilyl (TMS) derivatives of compounds in culture extracts were made by silylation with *N,O*-bis(trimethylsilyl)acetamide (BSA) in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, Ill.; method 5). Also, some metabolites were methyl-esterified by treating culture extracts with an ethereal solution of diazomethane generated by using a Diazald kit (Aldrich).

¹H NMR spectroscopy. Metabolites were dissolved in deuterated DCM, and ¹H nuclear magnetic resonance (¹H-NMR) spectra were recorded on an AM 400 NMR spectrometer by personnel in the NMR Service Laboratory, Chemistry Department, University of Alberta. The sample isolation and purification procedure is described in more detail in Results.

Total sulfur analysis. DCM extracts were diluted to 1.0 ml with DCM in volumetric flasks, and 200- μ l portions were applied to filter paper (Whatman no. 42). After evaporation of the DCM, the filter paper samples were combusted in sealed Schöninger oxygen flasks and the SO₂ produced was trapped as H₂SO₄ in 10 ml of water to which 2 drops of 30% hydrogen peroxide had been added. After addition of 40 ml of 2-propanol, the amount of sulfate present was determined by microtitration with BaClO₄ (0.01077 M), using Thorin as the endpoint indicator (24).

Lyophilization and methyl-esterification. Cultures (200 ml) were made alkaline (pH > 12) by the addition of 0.5 g of NaOH before lyophilization. After lyophilization, the residue was transferred to a 100-ml round-bottom flask with 40 ml of methanol and 4 ml of concentrated H₂SO₄ and refluxed for 2 h. After cooling and the addition of 40 ml of water, the reaction mixture was extracted with DCM as described above.

RESULTS

The ability of the isolates to grow on the naphthothiophenes as the sole carbon source was tested. Only isolate W1 was capable of growth on naphtho[2,1-*b*]thiophene, and none of the isolates was capable of growth on naphtho[2,3-*b*]thiophene. The isolates were not tested for the ability to grow on 1-methylnaphtho[2,1-*b*]thiophene, because <80 mg of this compound was available. Thus, the results described below focus on the metabolism of naphtho[2,1-*b*]thiophene as the sole carbon source for isolate W1 and compare this with the cometabolism of naphtho[2,1-*b*]thiophene and 1-methylnaphtho[2,1-*b*]thiophene by 1-MN-grown cultures of this isolate. Subsequently, the cometabolism of naphtho[2,1-*b*]thiophene by isolates BT1 and F and of naphtho[2,3-*b*]thiophene by isolates W1, BT1, and F is briefly described.

Metabolites from naphtho[2,1-*b*]thiophene. When a 13-day-old culture of isolate W1 incubated with naphtho[2,1-*b*]thiophene as the sole carbon source was extracted and the extract was concentrated to 1 ml for GC analysis, a precipitate was observed to fall out of solution. GC-FPD analysis of the satu-

FIG. 2. From GC-MS analysis, the mass spectrum observed for the two abundant sulfur-containing products detected during analysis of underivatized extracts of cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene. These products were identified as 4- and 5-hydroxybenzothiophene, which formed by thermal decarboxylation in the GC injection port liner of the metabolites 4-hydroxybenzothiophene-5-carboxylic acid and 5-hydroxybenzothiophene-4-carboxylic acid.

rated solution showed the presence of two abundant sulfur-containing compounds, eluting at 19.4 and 19.8 min, respectively, and 10 minor sulfur-containing peaks. GC-MS analysis showed that the mass spectra of the two abundant compounds were virtually identical, so only one is shown (Fig. 2). The molecular ion at m/z 150 was consistent with the compound being a hydroxybenzothiophene. The m/z 121 ion results from the loss of CHO ($M-29$)⁺, which is characteristic of phenols (52).

When a culture extract was treated with BSA to prepare the TMS ethers of the suspected hydroxybenzothiophenes, the precipitate that had been present in the extract was dissolved, and GC analysis revealed that the two abundant peaks at 19.4 and 19.8 min were gone from the chromatogram and two abundant sulfur-containing derivatives with retention times of 33.0 and 33.2 min were present. The mass spectra from GC-MS analysis of the two derivatives were virtually identical, so only one is shown in Fig. 3. The derivatives were not the expected

FIG. 3. From GC-MS analysis, the mass spectrum observed for the two abundant sulfur-containing metabolites detected in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene after treatment with BSA. These metabolites were identified as 4-hydroxybenzothiophene-5-carboxylic acid and 5-hydroxybenzothiophene-4-carboxylic acid.

TMS ethers of the hydroxybenzothiophenes. The ion at m/z 147 is indicative of the incorporation of two TMS moieties into the molecule (47). TMS derivatives typically lose a methyl group to give a strong ($M-15$)⁺ ion, which was likely the m/z 323 ion in Fig. 3. If this were true, then the molecular ion would be at m/z 338, which is not visible in Fig. 3. This molecular weight was consistent with benzothiophene substituted with a carboxylic group and a hydroxyl group, both of which had reacted with a TMS group.

This led to the hypothesis that the metabolism of naphtho[2,1-*b*]thiophene by isolate W1 led to the formation of two isomers of hydroxybenzothiophene carboxylic acid which were present in the culture extract and would react with BSA to give the TMS derivatives observed (Fig. 3). When underivatized, these metabolites were too polar to be chromatographed under the conditions used, but they underwent a thermal decarboxylation in the heated GC injection port liner to give the two isomers of hydroxybenzothiophene observed by GC analysis of underivatized extracts (Fig. 2). This thermal decomposition is discussed below.

Because it seemed likely that the precipitate in the concentrated DCM extract was the highly polar hydroxybenzothiophene carboxylic acids, a concentrated extract was filtered through glass wool in a Pasteur pipette to remove the precipitate. After the glass wool was rinsed with 0.5 ml of DCM to remove compounds readily soluble in this solvent, it was rinsed with methanol which was collected in a separate vial. After concentration under a stream of nitrogen, only one isomer of hydroxybenzothiophene, with a retention time of 19.4 min, was detected by GC analysis of this methanol rinse. Thus, by this method, the isomer of hydroxybenzothiophene carboxylic acid that was the least soluble in DCM was purified.

After evaporation to dryness, the purified residue was analyzed by high-resolution mass spectrometry with the sample introduced into the source of the instrument on a room temperature probe. The mass spectra were recorded as the probe was heated. The first 25 scans showed the presence of a single abundant compound with a molecular ion at m/z 194 and a chemical formula of $C_9H_6SO_3$ (data not shown), consistent with the structure of underivatized hydroxybenzothiophene carboxylic acid. The mass spectrum also showed the presence of the base peak at m/z 176 resulting from loss of H_2O ($M-18$)⁺ from the molecular ion. No abundant ion at m/z 150 (Fig. 2) was detected during these scans, so no hydroxybenzothiophene was present. However, as the probe was heated, the ion at m/z 194 disappeared and ions at m/z 150 and 121 (Fig. 2) came to predominate the mass spectra that were observed. The chemical formula for the molecular ion (m/z 150) was C_8H_6SO , consistent with hydroxybenzothiophene. This compound was the result of thermal decomposition of the hydroxybenzothiophene carboxylic acid, because if a hydroxybenzothiophene was present as a contaminant in the sample introduced into the mass spectrometer, the hydroxybenzothiophene would vaporize from the probe first and predominate the earlier scans, since it would be more volatile than hydroxybenzothiophene carboxylic acid. Thus, the hydroxybenzothiophenes (Fig. 2) were not metabolites from naphtho[2,1-*b*]thiophene but were artifacts produced by thermal decarboxylation in the GC injection port liner of the two major metabolites produced during the growth of isolate W1. These metabolites were hydroxybenzothiophene carboxylic acids.

The identification of these two major metabolites in culture extracts and all subsequent quantitation by GC-AED analysis were further aided by the use of diazomethane derivatization. Reports in the literature (e.g., reference 41) suggest that diazomethane is capable of reacting with carboxylic acids and

FIG. 4. From GC-MS analysis, the mass spectrum observed for the two abundant sulfur-containing metabolites detected in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene after treatment with diazomethane. These metabolites were identified as 4-hydroxybenzothiophene-5-carboxylic acid and 5-hydroxybenzothiophene-4-carboxylic acid.

phenols to give methyl esters and methyl ethers, respectively. Although we have been able to demonstrate methyl-esterification with authentic standards of 1-naphthoic, 3-methyl-2-thiophenecarboxylic, and 5-methyl-2-thiophenecarboxylic acids, we have not been able to form the methyl ethers of 2-naphthol, *m*-cresol, and 1,2-dihydroxynaphthalene by treatment with diazomethane. This is consistent with our observations from treatment of the two major metabolites of naphtho[2,1-*b*]thiophene with diazomethane that only the more acidic carboxyl group of these metabolites was reactive. The mass spectra of the two methyl-esterified hydroxybenzothiophene carboxylic acids were virtually identical, so only one is shown in Fig. 4. The molecular ion at m/z 208 was consistent with the methyl esters of hydroxybenzothiophene carboxylic acids, as was the base peak at m/z 176 which results from loss of CH_3OH ($M-32$)⁺ from the molecular ion. The abundant ion observed at m/z 148 results from the loss of $\text{C}_2\text{H}_4\text{O}_2$ ($M-60$)⁺ from the molecular ion, and a subsequent loss of CO (28 atomic mass units [amu]) would give the ion at m/z 120. These fragmentations are consistent with the structural assignments in Fig. 4.

The isomer of hydroxybenzothiophene carboxylic acid that was purified as the insoluble compound in the concentrated DCM solution was methyl-esterified and analyzed by GC. This methyl ester was the first of the two methyl esters to elute; thus, it was designated the methyl ester of metabolite I. The ^1H NMR spectrum of this methyl ester was recorded. The ^1H NMR spectrum of the methyl ester of metabolite II was obtained by NMR analysis of a mixture of the two isomers isolated from a culture extract by back extraction into 5% NaHCO_3 prior to diazomethane treatment. The signals due to metabolite II could be deduced because the NMR spectrum of pure metabolite I was obtained previously and because metabolite II was present in approximately one-third the amount of metabolite I.

Figure 5a and b show the aromatic region of the NMR spectra of metabolites I and II. Metabolites I and II also showed the phenolic proton as a singlet at 11.60 and 11.65 ppm, respectively, and the carboxymethyl group as a singlet at 4.0 and 4.1 ppm, respectively, which are not shown in Fig. 5. The aromatic region (7 to 8 ppm) shows the aromatic benzene

FIG. 5. ^1H NMR (400 MHz) spectra of methyl esters of metabolite I (a) and a mixture of metabolites I and II (b) detected in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene and of the only abundant metabolite (c) detected in cultures of isolate W1 grown on 1-MN in the presence of 1-methylnaphtho[2,1-*b*]thiophene. These metabolites were derivatized with diazomethane prior to analysis. The shaded signals in panel b are from metabolite II.

protons H-6 and H-7 with a coupling constant of approximately 9 Hz and the aromatic thiophene protons H-2 and H-3 with a coupling constant of approximately 5 Hz (52). The chemical shifts of the thiophenic protons are not useful in deducing the positions of the hydroxy and carboxymethyl functional groups in metabolites I and II, because we cannot predict the effects that these substituents on the benzene ring will have on the chemical shifts of the thiophenic protons. However, it is simple enough to distinguish the thiophenic protons from the benzene protons on the basis of their smaller *ortho* coupling constants. Then, by using only the chemical shifts of protons H-6 and H-7, it is possible to deduce which isomer of hydroxybenzothiophene carboxylic acid corresponds to each of metabolites I and II.

The ^1H NMR spectrum of benzothiophene in benzene, acetone, and carbon tetrachloride was reported by Balkau and Heffernan (2), with unambiguous assignments for all six protons. In all three solvents, the chemical shift of H-6 was significantly upfield from that of H-7 (−0.52, −0.59, and −0.54 ppm, respectively). A carboxymethyl group on a benzene ring is strongly deshielding (+0.74 ppm) to protons at positions *ortho* to it but has relatively little effect on the chemical shift of the *meta* (+0.07 ppm) or *para* (+0.2 ppm) protons (28). A hydroxy group on a benzene ring is strongly shielding to protons *ortho* (−0.50 ppm) and *para* (−0.40 ppm) to it but has relatively little effect on the *meta* proton (−0.14 ppm) (28). Thus, for the methyl ester of 4-hydroxybenzothiophene-5-carboxylic acid, the carboxymethyl group at position 5 would be

FIG. 6. From GC-AED analyses, concentration profile during the oxidation of naphtho[2,1-*b*]thiophene to metabolites I and II and other minor, unidentified sulfur-containing products in cultures of isolate W1 incubated with naphtho[2,1-*b*]thiophene as the sole carbon source.

expected to deshield the *ortho* proton H-6, which is further upfield in benzothiophene. The hydroxy group at position 4 would be expected to shield the *para* proton H-7, which is further downfield in benzothiophene. Thus, the upfield proton H-6 of benzothiophene would become the downfield proton, and the downfield proton H-7 of benzothiophene would become the upfield proton, in the methyl ester of 4-hydroxybenzothiophene-5-carboxylic acid. Thus, a hydroxy group at position 4 and a carboxymethyl group at position 5 of benzothiophene would decrease the difference in chemical shift of protons H-6 and H-7 from approximately 0.55 ppm (2) to approximately 0.33 ppm. This is consistent with the difference of 0.37 ppm observed for protons H-6 and H-7 in metabolite I (Fig. 5a). Thus, metabolite I was identified as 4-hydroxybenzothiophene-5-carboxylic acid.

By similar logic, it can be seen that the ^1H NMR spectrum of metabolite II is consistent with the structure of the methyl ester of 5-hydroxybenzothiophene-4-carboxylic acid. The shielding effect of the hydroxy group on the *ortho* proton H-6 of benzothiophene, which is already upfield, would be expected to cause it to resonate even further upfield. The deshielding effect of the carboxymethyl group on the *para* proton H-7 of benzothiophene, which is already downfield, would be expected to cause it to resonate even further downfield. Thus, a hydroxy group at position 5 and a carboxymethyl group at position 4 of benzothiophene would increase the difference in chemical shift of protons H-6 and H-7 from approximately 0.55 ppm (2) to approximately 1.1 ppm. This is what is observed in the ^1H NMR spectrum of metabolite II (Fig. 5b), which has the benzene protons H-6 and H-7 separated by 0.9 ppm. Thus, metabolite II was identified as 5-hydroxybenzothiophene-4-carboxylic acid.

Quantitative studies of naphtho[2,1-*b*]thiophene metabolism by strain W1. Plate counts showed that the density of a culture of isolate W1 increased from 2.3×10^4 to 1.5×10^7 CFU/ml after 7 days of incubation in medium containing 52 μmol of naphtho[2,1-*b*]thiophene as the sole carbon source. Replicate cultures were prepared for a time course experiment, and Fig. 6 shows the depletion of naphtho[2,1-*b*]thio-

phene over 14 days in cultures of isolate W1 grown on this substrate as the sole carbon source. Each time point in Fig. 6 represents the results from GC-AED analysis of a single culture extract. Figure 6 shows the concomitant production of metabolites I and II and other minor sulfur-containing peaks which were detected but not identified. At most, only 8.2% of the total sulfur from naphtho[2,1-*b*]thiophene was present in these 7 to 13 minor products. The results indicate that after naphtho[2,1-*b*]thiophene was depleted from the culture at 7 days, there was no significant degradation of metabolites I and II (Fig. 6).

The recovery of metabolites and unaltered substrate, as determined by GC-AED analysis of extracts of cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene for 7 days, is summarized in Table 1 (lines 1 and 2). In one trial, the naphtho[2,1-*b*]thiophene was essentially gone in 7 days (line 1), whereas the second trial showed that 8.4 of the initial 52 μmol (16%) of the substrate remained (line 2). This difference was responsible for the better total recovery in the second trial of 46% (line 2) compared to the first trial (36%; line 1), because the percentages of the sulfur from naphtho[2,1-*b*]thiophene that was detected as metabolites I, II, and the unidentified products were similar in the two trials.

Total sulfur analysis of DCM extracts gave a slightly higher recovery of sulfur from naphtho[2,1-*b*]thiophene than was observed by GC-AED analysis (Table 1, lines 1 and 2), suggesting that there may be a small quantity of sulfur-containing metabolites that are extracted from cultures with DCM but are not amenable to GC analysis. The recovery from the corresponding sterile control (75%) suggests that 25% of the initial substrate was lost due to evaporation. This indicates that 25% (line 1) and 10% (line 2) of the sulfur from naphtho[2,1-*b*]thiophene is not recovered by DCM extraction of cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene.

Cometabolism of naphtho[2,1-*b*]thiophene by strain W1.

When cultures of isolate W1 were incubated with naphtho[2,1-*b*]thiophene in the presence of 1-MN as a cosubstrate, the naphtho[2,1-*b*]thiophene was depleted from the cultures within 7 days in both of the quantitative trials (Table 1, lines 3 and 4). Also, amounts of metabolite II (lines 3 and 4) which were comparable to those observed in the incubations with naphtho[2,1-*b*]thiophene as the sole carbon source (lines 1 and 2) were produced. However, when 1-MN was included in the cultures (lines 3 and 4), metabolite I was not observed to accumulate as it had when 1-MN was excluded (lines 1 and 2), although it was still detected. The fact that metabolite I did not accumulate in cultures containing 1-MN contributed to the lower total recovery observed by GC-AED analysis of DCM extracts (12 and 13% in two trials). Total sulfur analysis of DCM extracts showed that there was only a small amount of organic sulfur in the extracts that was not amenable to GC analysis. After the evaporative loss observed in the sterile control was accounted for, the data showed that 59% (Table 1, line 3) and 56% (line 4) of the sulfur from naphtho[2,1-*b*]thiophene were not recovered by DCM extraction.

To begin characterization of this fraction of sulfur from naphtho[2,1-*b*]thiophene that was not recovered by DCM extraction of acidified cultures, a culture was lyophilized and treated with a methyl-esterification procedure prior to DCM extraction (Table 2, line 1). Total sulfur analysis of the extract obtained by this procedure showed an increased recovery of 17.1 μmol of organosulfur (32% of the 54 μmol naphtho[2,1-*b*]thiophene given). This increase does not include the 5.8 μmol of sulfur observed by total sulfur analysis of a control that did not receive naphtho[2,1-*b*]thiophene. This control was established to account for any sulfur recovered by this method that

TABLE 1. Quantification of naphtho[2,1-*b*]thiophene and 1-methylnaphtho[2,1-*b*]thiophene and their sulfur-containing metabolites as determined by GC-AED analyses of DCM extracts of acidified cultures of *Pseudomonas*^a

Strain	Substrate(s)	μmol of sulfur from NT or 1-MeNT recovered as:				Total recovery		Recovery by total sulfur analysis (%)	Recovery in sterile control (%)
		Unaltered thiophenic substrate	Metabolite I ^b	Metabolite II ^c	Other sulfur-containing metabolites ^d	(δmol)	(%)		
W1 ^e	NT ^f	0.2	11	4.6	3.1 [11]	18.9	36	50	75
	NT ^f	8.4	8.9	4.3	2.2 [8]	23.8	46	65	75
	1-MN and NT ^g	1.4	0.5	4.5	0.3 [11]	6.7	12	20	79
	1-MN and NT ^f	0.2	0.1	5.1	1.4 [5]	6.8	13	23	79
	1-MN and 1-MeNT ^h	2.5	34 ⁱ	0 ^j	3.3 [6]	39.8	81	ND ^k	ND
	1-MN and 1-MeNT ^f	0.8	35 ⁱ	0 ^j	3.8 [7]	39.6	76	ND	ND
BT1	1-MN and NT ^g	0.1	0	0	1.0 [13]	1.1	2	15	79
F	1-MN and NT ^g	25	0.1	0.8	0.3 [6]	26.2	49	49	79

^a The three *Pseudomonas* strains were incubated for 7 days. The total recovery by GC-AED analysis is also compared with the recovery by total sulfur analysis and with the recovery in the corresponding sterile controls as determined by GC-AED. NT, naphtho[2,1-*b*]thiophene; 1-MeNT, 1-methylnaphtho[2,1-*b*]thiophene.

^b 4-Hydroxybenzothiophene-5-carboxylic acid.

^c 5-Hydroxybenzothiophene-4-carboxylic acid.

^d Micromoles of organosulfur present in other minor sulfur peaks which were not identified and the number of peaks (in brackets) which are represented by this total.

^e With isolate W1, the results of two separate trials are shown for each set of substrates.

^f A total of 52 μmol of naphtho[2,1-*b*]thiophene or 1-methylnaphtho[2,1-*b*]thiophene was initially in the culture.

^g A total of 54 μmol of naphtho[2,1-*b*]thiophene was initially in the culture.

^h A total of 49 μmol of 1-methylnaphtho[2,1-*b*]thiophene was initially in the culture.

ⁱ The 3-methyl analog of metabolite I.

^j The 3-methyl analog of metabolite II.

^k ND, not determined.

resulted from microbial incorporation of sulfate provided in the medium into organic compounds. The observed increase in total sulfur recovery (Table 2) suggests that there was a significant portion of sulfur from naphtho[2,1-*b*]thiophene that was present in cultures as organic metabolites that were too polar to be recovered by DCM extraction of acidified cultures.

Some of the metabolites that were too polar to be recovered by DCM extraction, leading to the low recoveries of total sulfur observed above, may result from the further biodegradation of metabolite I, which appeared to be promoted by the presence of 1-MN in the cultures of isolate W1. This possibility was tested by adding a mixture of metabolites I and II to four replicate cultures of isolate W1 and a sterile control. One culture was extracted immediately and analyzed by GC-AED, showing that 3.9 and 2.8 μmol of metabolites I and II, respectively, had been added to each flask. The remaining three cultures were incubated for 11, 31, and 98 h and then extracted and analyzed by GC-AED. Analysis of the sterile control that

was incubated for 98 h detected 4.0 μmol (103%) and 2.7 μmol (96%) of metabolites I and II, respectively. No depletion of metabolite II was observed in the cultures of isolate W1 (2.8 μmol, 100% recovered after 98 h). However, after 11 h of incubation, 3.4 μmol (87%) of metabolite I remained, whereas after 31 h of incubation, only 0.04 μmol (1%) of metabolite I was present in the culture extract. Thus, the presence of 1-MN in cultures of isolate W1 clearly stimulated the degradation of metabolite I, which accumulated in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene as the sole carbon source. Metabolite II accumulated in cultures of isolate W1 regardless of whether 1-MN was present.

Cometabolism of 1-methylnaphtho[2,1-*b*]thiophene by strain W1. Cultures of isolate W1 grown on 1-MN in the presence of 1-methylnaphtho[2,1-*b*]thiophene accumulated a single abundant, sulfur-containing product. GC-MS analysis of underivatized extracts gave a mass spectrum with a molecular ion at *m/z* 164 and an abundant fragment (M-29)⁺ at *m/z* 135 from loss of CHO (data not shown), which are consistent with the structure of hydroxy-substituted methylbenzothiophene. However, GC-MS analysis after treatment of the extract with diazomethane suggested that this hydroxymethylbenzothiophene resulted from decarboxylation in the GC injection port liner of a hydroxymethylbenzothiophene carboxylic acid. The mass spectrum of the methyl-esterified hydroxymethylbenzothiophene carboxylic acid metabolite is shown in Fig. 7. The molecular ion at *m/z* 222 is consistent with the structure shown, as are the fragments at *m/z* 190 (M-32)⁺ and *m/z* 162 (M-60)⁺ which result from loss of CH₃OH and C₂H₄O₂, respectively. Again, only the carboxy group of the metabolite was observed to react with diazomethane to form a methyl ester. However, when the methyl-esterified metabolite was treated with BSA, the hydroxy group was observed to react with 1 eq of BSA (72 amu) to form the TMS ether with a molecular ion at *m/z* 294 and an abundant fragment (M-15)⁺ at *m/z* 279 (data not shown).

Only one isomer of hydroxymethylbenzothiophene carboxylic acid, which could be the 3-methyl-substituted isomer of either metabolite I or II, was detected by GC analysis. To determine which isomer was present, the ¹H NMR spectrum of

TABLE 2. Comparison of the recovery of organosulfur from naphtho[2,1-*b*]thiophene by DCM extraction of acidified cultures and by lyophilization^a

Strain	μmol of organosulfur recovered by:		Additional organosulfur found with lyophilization ^c (μmol)
	DCM extraction	Lyophilization ^b	
W1	9.1	32 (5.8)	17.1
BT1	11	34 (3.5)	19.5
F	17	34 (3.9)	13.1

^a The three *Pseudomonas* strains were incubated with 1-MN and 54 μmol of naphtho[2,1-*b*]thiophene for 7 days. Recovery was determined by total sulfur analysis of extracts obtained by the two methods.

^b Numbers in parentheses are micromoles of organosulfur that were also observed by total sulfur analysis of controls that did not receive naphtho[2,1-*b*]thiophene.

^c Additional sulfur found = amount found by lyophilization - (amount found in control + amount found in DCM extract).

FIG. 7. From GC-MS analysis, the mass spectrum of the only abundant metabolite detected in cultures of isolate W1 grown on 1-MN in the presence of 1-methylnaphtho[2,1-*b*]thiophene after treatment with diazomethane. The metabolite was identified as 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid.

the methyl-esterified metabolite, which was purified from a culture extract by back extraction into 5% NaHCO₃ prior to diazomethane derivatization, was recorded, and part of the spectrum is shown in Fig. 5c. The NMR spectrum showed the hydroxy proton as a singlet at 11.8 ppm, the protons on the carboxymethyl group as a singlet at 3.9 ppm, and the methyl protons as a singlet at 2.7 ppm (data not shown). The thiophenic proton H-2 at 6.95 ppm is readily distinguished from the benzene protons H-6 and H-7 because the adjacent carbon atom does not bear any protons, so there is no strong coupling with proton H-2 and it appears as a singlet. The chemical shifts of protons H-6 and H-7 are very similar to those of metabolite I (Fig. 5a), suggesting that the metabolite formed from 1-methylnaphtho[2,1-*b*]thiophene in cultures of isolate W1 incubated with 1-MN is 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid (i.e., 3-methyl-metabolite I).

The recovery of this metabolite after 7 days of incubation of the W1 culture is summarized in Table 1 (lines 5 and 6). In two separate trials, the 1-methylnaphtho[2,1-*b*]thiophene was almost completely degraded, with only 2.5 μ mol (5.1% [Table 1, line 5]) and 0.8 μ mol (1.5% [line 6]) remaining after 7 days of incubation. A small amount of the sulfur from the depleted substrate was present as six or seven unidentified minor products, but the vast majority of the organosulfur (34 and 35 μ mol, representing 69 and 67%, respectively) was present as 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid in the two trials. The total recoveries by GC-AED analysis of 81 and 76% in the two trials agree very closely with the recovery of naphtho[2,1-*b*]thiophene in the sterile control (Table 1, lines 3 and 4) of 79%. A quantitative sterile control with approximately 50 μ mol of 1-methylnaphtho[2,1-*b*]thiophene was not used because of the small amount of this substrate that was available. However, it is likely that its losses due to volatility would be comparable to those of naphtho[2,1-*b*]thiophene (Table 1, lines 3 and 4). A qualitative sterile control with 5 mg of 1-methylnaphtho[2,1-*b*]thiophene was incubated, and the results verified that an active culture of isolate W1 was necessary for the formation of 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid.

Cometabolism of naphtho[2,1-*b*]thiophene by strains BT1 and F. Neither isolate BT1 nor isolate F was capable of growth on naphtho[2,1-*b*]thiophene, but both isolates were able to oxidize this compound when provided with 1-MN as a growth substrate. After 7 days of incubation with isolate BT1, the 54 μ mol of naphtho[2,1-*b*]thiophene was essentially consumed (0.1 μ mol remained), and only trace amounts of 13 sulfur-containing metabolites, none of which were consistent with metabolites I and II, were detected (Table 1, line 7). This led

to the very low total recovery by GC-AED analysis of 2%. Total sulfur analysis revealed that there was only a small portion of sulfur from naphtho[2,1-*b*]thiophene present in the DCM extract that was not amenable to GC analysis. Only 15% recovery of the sulfur from naphtho[2,1-*b*]thiophene was observed by total sulfur analysis of the extract (Table 1, line 7). Thus, most of the sulfur from naphtho[2,1-*b*]thiophene was left in the aqueous phase after DCM extraction.

Isolate F degraded naphtho[2,1-*b*]thiophene to a lesser extent over the 7-day incubation, with 25 of the original 54 μ mol (46%) remaining. However, of the depleted substrate sulfur, only 0.1 and 0.8 μ mol were detected as metabolites I and II, respectively, and there was only a small amount (0.3 μ mol) of six minor unidentified products (Table 1, line 8). In the case of isolate F, total sulfur analysis of DCM extracts gave the same recovery as had been observed by GC-AED (49% [Table 1, line 8]). Thus, the cometabolism of naphtho[2,1-*b*]thiophene by 1-MN-grown cultures of isolates BT1 and F did not result in the accumulation of metabolites I or II or other sulfur-containing metabolites that are amenable to DCM extraction. As a result, there was a large fraction of the sulfur from naphtho[2,1-*b*]thiophene that was not accounted for by the methods used (64% for isolate BT1 and 30% for isolate F).

In a separate experiment, the recovery of total sulfur by DCM extraction of cultures that had been lyophilized and methyl-esterified prior to extraction was compared with the recovery of total sulfur by DCM extraction of acidified cultures (Table 2, lines 2 and 3). With both isolates, there was an increase in the amount of organosulfur detected in cultures that were lyophilized (19.5 and 13.1 μ mol for isolates BT1 and F, respectively) over that observed in cultures that were acidified and extracted. This suggests that a significant portion of the sulfur from the depleted substrate still existed in the form of organic metabolites that were too polar to be extracted from acidified cultures with DCM.

Cometabolism of naphtho[2,3-*b*]thiophene by strains W1, BT1, and F. None of the three isolates was capable of growth on naphtho[2,3-*b*]thiophene, but all were able to oxidize this compound when 1-MN was provided as a growth substrate. In cultures that were incubated for 14 days with 25 μ mol of naphtho[2,3-*b*]thiophene, the observed depletion of the substrate in cultures of isolates W1, BT1, and F was 32, 64, and 30%, respectively. The recovery from the corresponding sterile control was 95%. No sulfur-containing metabolites or other products, such as the hydroxybenzothiophenes observed to form by decarboxylation of the hydroxybenzothiophene carboxylic acid metabolites in the GC injection port liner, were detected by GC analysis of underivatized extracts. However, after derivatization with diazomethane or BSA, two derivatized metabolites with the same mass spectra as in Fig. 3 (after BSA treatment) or in Fig. 4 (after diazomethane treatment) were detected in extracts of cultures of isolates W1 and F. By analogy with the identifications of metabolites I and II, the metabolites from naphtho[2,3-*b*]thiophene were presumed to be 5-hydroxybenzothiophene-6-carboxylic and 6-hydroxybenzothiophene-5-carboxylic acids. However, neither isomer was purified and analyzed by NMR to determine which peak from GC analysis corresponds to which isomer. The total amounts of these two metabolites detected in cultures of isolates W1 and F, respectively, correspond to 8.1 and 7.7% of the naphtho[2,3-*b*]thiophene given. These two products were not detected in derivatized extracts of BT1 cultures, in which no metabolites were observed to accumulate over the 14-day incubation.

FIG. 8. Proposed pathways for the formation of metabolites I and II. Hypothesized intermediates in the formation of metabolites I and II that were not detected (structures in brackets) and the proposed locations of *meta* cleavage of dihydroxy-substituted naphtho[2,1-*b*]thiophenes (dashed line) are indicated.

DISCUSSION

These results indicate that naphtho[2,3-*b*]thiophene is more resistant to microbial attack than is naphtho[2,1-*b*]thiophene. In cultures of isolates W1, BT1, and F incubated for 14 days with 1-MN and 25 μmol of naphtho[2,3-*b*]thiophene, the observed depletion of the naphthothiophene was 32, 64, and 30%, respectively. However, after only 7 days of incubation with 1-MN and 54 μmol of naphtho[2,1-*b*]thiophene, 97, 99, and 53% losses were observed in cultures of isolates W1, BT1, and F, respectively (Table 1). These results, taken together with the fact that only naphtho[2,1-*b*]thiophene supported the growth of any of the isolates, suggest that naphtho[2,1-*b*]thiophene is more susceptible to bacterial degradation.

Cultures of isolate W1 that were grown with naphtho[2,1-*b*]thiophene as the sole carbon source accumulated 4-hydroxybenzothiophene-5-carboxylic acid (metabolite I) and 5-hydroxybenzothiophene-4-carboxylic acid (metabolite II) as abundant products after 7 days of incubation, with little, if any, further degradation of these metabolites occurring (Fig. 6). These metabolites presumably result from dioxygenase attack at positions 8 and 9 and at positions 6 and 7 of naphtho[2,1-*b*]thiophene, respectively, to give the corresponding dihydrodiols. Subsequent dehydrogenation to form the diols, followed by *meta* cleavage, loss of pyruvate, and oxidation of the resulting hydroxyformylbenzothiophenes, yielded these two isomers of hydroxybenzothiophene carboxylic acid. Figure 8 shows this proposed pathway. This is analogous to the upper pathway for naphthalene catabolism which converts naphthalene to salicylic acid (13) and which has also been shown to be both

biochemically and genetically similar to the pathway responsible for the oxidation of dibenzothiophene to 3-hydroxy-2-formylbenzothiophene (11), phenanthrene to 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid (11, 30, 51), and anthracene to 2-hydroxy-3-naphthoic acid (30, 51).

The formation of two isomers of hydroxybenzothiophene carboxylic acid from naphtho[2,1-*b*]thiophene requires a suite of broad-specificity enzymes which can attack either at positions 8 and 9 or at positions 6 and 7 of naphtho[2,1-*b*]thiophene and carry out the subsequent oxidation reactions. The greater amount of metabolite I produced suggests that positions 8 and 9 are favored for initial dioxygenation. This is consistent with the observation of Kiyohara et al. (30) for *Pseudomonas putida* AC10 carrying plasmid IP7, which encodes the enzymes for oxidation of naphthalene, phenanthrene, and anthracene, that phenanthrene was preferentially oxidized at positions 3 and 4, yielding 1-hydroxy-2-naphthoic acid, but 2-hydroxy-1-naphthoic acid was formed as a minor product. This minor product results from initial dioxygenation of phenanthrene at positions 1 and 2. These sites of dioxygenase attack of phenanthrene to give the major and minor products are analogous to those of naphtho[2,1-*b*]thiophene oxidized by isolate W1 to give metabolites I and II, respectively.

The accumulation of hydroxybenzothiophene carboxylic acids from growth of isolate W1 on naphtho[2,1-*b*]thiophene over a 7-day incubation period resulted in moderate total recoveries of sulfur from naphtho[2,1-*b*]thiophene by GC-AED analysis of 36 and 46%, in two trials (Table 1). Total sulfur analysis of the DCM extracts gave slightly higher recoveries, suggesting that small amounts of DCM-extractable organic sulfur that were not detected by GC-AED analysis may be present. After accounting for evaporative losses in the sterile control incubated with these cultures, there was only 25 and 10% of the sulfur from naphtho[2,1-*b*]thiophene that was not detected by DCM extraction and total sulfur analysis in the two trials. The sulfur from the depleted substrate that was not recovered by DCM extraction, was not amenable to GC analysis, or was present in numerous minor sulfur-containing peaks detected in the GC-AED chromatogram, but not identified, could potentially exist as intermediates in the formation of metabolites I and II or as metabolites in alternative pathways to those which cleave the external homocyclic ring of naphtho[2,1-*b*]thiophene.

Cultures of isolate W1 that were grown with 1-MN and naphtho[2,1-*b*]thiophene accumulated metabolite II to levels similar to those observed without 1-MN, but metabolite I was not observed to accumulate (Table 1). This resulted in lower total recovery by GC-AED analysis and lower recovery by total sulfur analysis of DCM extracts. The portion of sulfur that was not present in DCM extracts appears to exist, at least in part, in the form of organic metabolites that are too polar to be extracted with DCM, but were recovered by the lyophilization and methyl-esterification procedure (Table 2).

Compared to isolate W1 grown on naphtho[2,1-*b*]thiophene as the sole carbon source, the increase in the fraction of the sulfur from naphtho[2,1-*b*]thiophene that is not recovered by DCM extraction is correlated with the decrease in the amount of metabolite I that was observed to accumulate. The decreased accumulation of metabolite I may be due to the stimulation of its further degradation by the presence of the co-substrate 1-MN. Alternatively, the effect of 1-MN may be to promote the oxidation of naphtho[2,1-*b*]thiophene by some other pathway so that less substrate is oxidized by the dioxygenase at positions 8 and 9. However, this would also be expected to decrease the amount of naphtho[2,1-*b*]thiophene

entering the pathway of dioxygenase attack at positions 6 and 7 that leads to formation of metabolite II. Because the amount of metabolite II accumulated is nearly the same as when 1-MN was excluded, this is not likely the effect of 1-MN. Indeed, 1-MN was shown to promote the degradation of metabolite I, but not metabolite II, when these metabolites were separated from extracts of cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene and fed to cultures of isolate W1 incubated in the presence of 1-MN.

Cultures of isolate W1 incubated with 1-MN and 1-methylnaphtho[2,1-*b*]thiophene accumulated the 3-methyl-substituted isomer of metabolite I in 67 and 69% yields over 7 days of incubation. Total recovery of sulfur as 3-methyl-metabolite I, unoxidized substrate, and several minor sulfur-containing products as determined by GC-AED analysis (76 and 81% [Table 1, lines 5 and 6]) was approximately the same as was observed with the sterile control containing 1-MN and naphtho[2,1-*b*]thiophene (75 to 79% [Table 1]). Assuming that the evaporative losses of 1-methylnaphtho[2,1-*b*]thiophene were essentially the same as for naphtho[2,1-*b*]thiophene, there was nearly a stoichiometric recovery of sulfur from the cultures of isolate W1 grown on 1-MN in the presence of 1-methylnaphtho[2,1-*b*]thiophene.

Other than the work by Kropp et al. (34), the majority of studies that have attempted quantitative analysis of the microbial metabolism of condensed thiophenes are those that addressed the biodesulfurization of model compounds in which the cleavage of C—C bonds is undesirable (27, 40, 45, 46, 55, 56). In studies with the same three *Pseudomonas* strains (34), between 2.8 and 38% of the sulfur from dibenzothiophene was recovered as DCM-extractable metabolites that were amenable to GC-AED analysis. Comparable results appear in Table 1, which shows that 2 to 49% of the sulfur from naphtho[2,1-*b*]thiophene was recovered and detected in the same manner.

The methyl group in 1-methylnaphtho[2,1-*b*]thiophene appears to direct the attack of the dioxygenase at carbons 8 and 9, yielding the 3-methyl analog of metabolite I as the major product. In fact, the 3-methyl analog of metabolite II was not detected (Table 1, lines 5 and 6), so the dioxygenase attack at positions 6 and 7 was not significant. Also, with 1-methylnaphtho[2,1-*b*]thiophene there was not a significant fraction of sulfur from the substrate that was present in cultures as organosulfur-containing metabolites that were too polar to be extracted with DCM, as had been observed with unsubstituted naphtho[2,1-*b*]thiophene. Thus, this highly polar fraction from naphtho[2,1-*b*]thiophene likely results from oxidation by an alternative pathway to those which cleave the external homocyclic ring to give metabolites I and II. This alternative pathway was likely blocked by the methyl group at position 1, with the result that essentially all the substrate was oxidized at positions 8 and 9, leading to such high recoveries of 3-methyl-metabolite I, a metabolite which is amenable to the extraction and analytical methods used.

1-MN was shown to promote the further degradation of metabolite I, but the methyl group of 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid (3-methyl-metabolite I) prevents further degradation of the latter compound. This suggests that the further degradation of metabolite I in cultures of isolate W1 incubated with 1-MN occurs by a pathway that is blocked by the methyl group at position 3 of 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid.

The bacterial metabolism of benzothiophene and of methyl- and dimethyl-substituted benzothiophenes that do not bear methyl groups on the thiophene ring has been studied (14, 16, 35, 37, 48). Those investigations showed that the thiophene ring is oxidized and cleaved to give 2-mercaptophenylglyox-

alates, which are often detected as the benzothiophene-2,3-diones formed by acid-catalyzed dehydration during culture extraction (14). Also, Eaton and Nitterauer (14) showed that both the homocyclic and the heterocyclic rings of benzothiophene were cleaved by their isopropylbenzene-degrading bacteria. Thus, it is possible that similar oxidations of the thiophene ring of naphtho[2,1-*b*]thiophene compete with dioxygenation at positions 6 and 7 and at positions 8 and 9. However, no naphtho[2,1-*b*]thiophene-1,2-dione was found in any of our culture extracts, although it may have been one of the minor compounds that was not identified. Furthermore, it is possible that oxidation at positions 2 and 3 of metabolite I are responsible for its subsequent degradation in cultures of isolate W1 grown on 1-MN and naphtho[2,1-*b*]thiophene. This is consistent with the observation that a methyl group located on the thiophene ring of metabolite I (i.e., 3-methyl-metabolite I) prevented its further degradation.

Isolate W1 is known to oxidize the methyl groups of some methyl- and dimethylbenzothiophenes to methanols and carboxylic acids (35, 37) and of some methylbenzothiophenes to methanols (49). However, in this study, there was no evidence of oxidation of the methyl group of 1-methylnaphtho[2,1-*b*]thiophene, although some of the minor sulfur-containing metabolites that were not identified may have been a methanol or carboxylic acid. Nonetheless, only 3.3 and 3.8% of the sulfur from 1-methylnaphtho[2,1-*b*]thiophene was recovered as other sulfur-containing metabolites (Table 1), so if the methanol and/or carboxylic acid was formed, it was a minor product.

The cometabolic oxidation of naphtho[2,1-*b*]thiophene by cultures of isolates BT1 and F resulted in extensive degradation of the substrate without accumulation of metabolites detected by GC analysis of culture extracts. Metabolites I and II were present in trace amounts in extracts of cultures of isolate F (Table 1, line 8) but were not detected in extracts of BT1 cultures (Table 1, line 7). Increased recovery by lyophilization and methyl-esterification procedures suggested that a significant portion of the sulfur from the depleted substrate was still present as organic metabolites that were not detected by the DCM extraction and GC analysis methods used (Table 2).

The cometabolism of naphtho[2,3-*b*]thiophene by cultures of the three isolates grown on 1-MN also resulted in extensive depletion of the parent compound. For isolate BT1, no metabolites were observed to accumulate despite the fact that 64% of the substrate was degraded. With isolates W1 and F, approximately 25% of the sulfur from the depleted substrate was present as 5-hydroxybenzothiophene-6-carboxylic acid and 6-hydroxybenzothiophene-5-carboxylic acid. These metabolites would result from dioxygenase attack at positions 5 and 6 and positions 7 and 8, respectively, of naphtho[2,3-*b*]thiophene. Thus, the broad specificity of the dioxygenase that allows isolates W1, BT1, and F to grow on 1-MN also allows the cometabolic oxidation of both naphtho[2,1-*b*]thiophene and naphtho[2,3-*b*]thiophene.

Two interesting observations of this study are (i) the effect of 1-MN on the further degradation of 4-hydroxybenzothiophene-5-carboxylic acid (metabolite I) in cultures of isolate W1 and (ii) the effect of the methyl group of 1-methylnaphtho[2,1-*b*]thiophene on its metabolism. The effect of the 1-MN on subsequent degradation of metabolite I from bacterial metabolism of naphtho[2,1-*b*]thiophene suggests that cocontaminants in the environment will influence the fate of condensed thiophenes. This is important because condensed thiophenes are found in environments contaminated with petroleum and other fossil fuel derivatives in complex mixtures of saturated and aromatic hydrocarbons. Thus, many other compounds would be present to serve as cosubstrates that could influence

the fate of the condensed thiophenes. The methyl group of 1-methylnaphtho[2,1-*b*]thiophene was shown to influence the location of dioxygenase attack of the naphtho[2,1-*b*]thiophene nucleus, and the 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid that was formed was resistant to further degradation. As has been observed before (4, 33, 35), the methyl substitution pattern of condensed thiophenes can also influence the microbial metabolism of these compounds. Thus, studies with 1-methylnaphtho[2,1-*b*]thiophene provided some clues to the mechanism for further degradation of metabolite I, but these have not been elucidated.

ACKNOWLEDGMENTS

Funding for these studies was provided by Environment Canada, through the Groundwater and Soil Remediation Program, contract KA168-2-2191; by the Canadian Petroleum Products Institute; and by the Natural Sciences and Engineering Research Council of Canada. Financial support for the syntheses of the naphthothiophenes was given by the Fonds der Chemischen Industrie. An award from the NATO Collaborative Research Grants Programme is also gratefully acknowledged. K. G. Kropp was supported by a Queen Elizabeth II Doctoral Fellowship in Environmental Studies.

We thank J. Hoyle for technical assistance and L. Harrower for the use of his GC-MS system.

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