Identification of Disulfides from the Biodegradation of Dibenzothiophene

DAVID C. BRESSLER AND PHILLIP M. FEDORAK*

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Received 11 June 2001/Accepted 22 August 2001

Several investigations have identified benzothiophene-2,3-dione in the organic solvent extracts of acidified cultures degrading dibenzothiophene via the Kodama pathway. In solution at neutral pH, the 2,3-dione exists as 2-mercaptophenylglyoxylate, which cyclizes upon acidification and is extracted as the 2,3-dione. The fate of these compounds in microbial cultures has never been determined. This study investigated the abiotic reactions of 2-mercaptophenylglyoxylate incubated aerobically in mineral salts medium at neutral pH. Oxidation led to the formation of 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide, formed from two molecules of 2-mercaptophenylglyoxylate. Two sequential abiotic, net losses of both a carbon and an oxygen atom produced two additional disulfides, 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide and 2,2'-dithiosalicylic acid. The methods developed to extract and detect these three disulfides were then used for the analysis of a culture of *Pseudomonas* sp. strain BT1d grown on dibenzothiophene as its sole carbon and energy source. All three of the disulfides were detected, indicating that 2-mercaptophenylglyoxylate is an important, short-lived intermediate in the breakdown of dibenzothiophene via the Kodama pathway. The disulfides eluded previous investigations because of (i) their high polarity, being dicarboxylic acids; (ii) the need to lower the pH of the aqueous medium to <1 to extract them into an organic solvent such as dichloromethane; (iii) their poor solubility in organic solvents, (iv) their removal from organic extracts of cultures during filtration through the commonly used drying agent anhydrous sodium sulfate; and (v) their high molecular masses (362, 334, and 306 Da) compared to that of dibenzothiophene (184 Da).

Sulfur is the third most abundant element in crude oils (48), and sulfur heterocycles are common constituents of petroleum and liquids derived from coal. Condensed thiophenes such as benzothiophene, alkylbenzothiophenes, dibenzothiophene, and alkyldibenzothiophenes are among the most commonly found sulfur heterocycles. Based on data from Bence et al. (3), it is estimated that the *Exxon Valdez* spilled over 77 metric tons of dibenzothiophene and methyl-dibenzothiophenes into the environment. Although alkyl-condensed thiophenes are among the most recalcitrant compounds in petroleum-contaminated environments (3, 5), methyl- and dimethyldibenzothiophenes have been shown to be susceptible to biodegradation in laboratory studies (17, 18, 28) and by the examination of residues from oil-contaminated environments (2, 23). Bragg et al. (7) observed that during the biodegradation of oil released from the *Exxon Valdez*, the polar content of the biodegraded North Slope oil approached 60 to 70% of the total mass of residual oil, then biodegradation slowed substantially. Based on the Microtox assay, Zemanek et al. (54) demonstrated that the polar fraction of residual petroleum from oil-contaminated sites was often the most toxic fraction.

There are numerous reports on the microbial degradation of condensed thiophenes such as benzothiophene, dibenzothiophene, and their alkyl derivatives (2, 6, 14, 16, 17, 18, 23, 25, 26, 27, 28, 31, 35, 39, 44, 45, 50), and the three biodegradation pathways of dibenzothiophene and related compounds have been reviewed (8, 30). The biodesulfurization pathway is in-

hibited by the presence of sulfate, and this pathway is not considered a significant contributor to dibenzothiophene degradation in sulfate-containing environments (30). Likely the most common mode of biodegradation of dibenzothiophene in sulfate-containing environments is via the Kodama pathway, which can be presented as follows: dibenzothiophene $\rightarrow$ (+)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene $\rightarrow$ 1,2-dihydroxydibenzothiophene $\rightarrow$ *cis*-4-[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenic acid $\rightarrow$ *trans*-4-[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenic acid $\rightarrow$ 3-hydroxy-2-formylbenzothiophene (HFBT) (30). HFBT accumulates in pure cultures (26), but there are few studies on its fate. Mormile and Atlas (40) suggested that HFBT can be biodegraded further, but did not reveal anything about the fate of the carbon and sulfur atoms. Bressler and Fedorak (9) reported some chemical properties of purified HFBT and described the abiotic condensation of HFBT to form *cis*- and *trans*-thioindigo. They also showed that HFBT was mineralized by a mixed bacterial community, and they identified benzothiophene-2,3-dione in the extracts of these acidified cultures.

Bohonos et al. (6) found benzothiophene-2,3-dione in extracts of an acidified culture degrading dibenzothiophene and suggested it was an oxidized metabolite in the HFBT-producing pathway. This 2,3-dione has also been identified as a dibenzothiophene metabolite in HFBT-producing cultures (28) and it is thought to be formed from the acid-catalyzed cyclization of 2-mercaptophenylglyoxalate (14) (Fig. 1). Finkelstein et al. (19) have identified 2-mercaptobenzoic acid (thiosalicylic acid) as a further metabolite of dibenzothiophene degradation. To date, there is no proof that 2-mercaptobenzoic acid is formed directly from the 2,3-dione and not from a concurrent metabolic pathway. Finkelstein et al. (19) also identified 2,2'-di-

* Corresponding author. Mailing address: Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9. Phone: (780) 492-3670. Fax: (780) 492-9234. E-mail: phil.fedorak@ualberta.ca.

FIG. 1. pH-dependent equilibrium between benzothiophene-2,3-diones and 2-mercaptophenylglyoxalates.

thiosalicylic acid in dibenzothiophene-degrading culture and suggested that it was a product of 2-mercaptobenzoic acid oxidation forming the disulfide. Other than 2,2'-dithiosalicylic acid, no other disulfides have been reported as products of dibenzothiophene biodegradation.

Benzothiophene-2,3-dione, presumably originating from 2-mercaptophenylglyoxylate, is also a product of benzothiophene biodegradation. Eaton and Nitterauer (14) reported benzothiophene biotransformation by isopropylbenzene-degrading bacteria. The 2,3-dione was also observed in the extracts of acidified cultures incubated with methyl-substituted benzothiophenes containing the alkyl substituent on the benzene ring (16, 31, 44). Andersson and Bobinger (1) reported that when benzothiophene is subjected to photooxidation, it is converted though benzothiophene-2,3-dione to 2-sulfobenzoic acid, with an 85% yield of the acid. No investigations into microbial attack of the 2,3-dione have been reported.

The frequency with which 2,3-diones have been detected in acidified extracts of cultures degrading benzothiophenes and dibenzothiophenes prompted us to attempt to study the biodegradation of benzothiophene-2,3-dione. However, analytical difficulties were encountered even while monitoring the sterile controls, suggesting that abiotic reactions were occurring which complicated interpretation of the results. The observed abiotic reactions of HFBT in dibenzothiophene-degrading cultures (9), caused us to investigate whether 2-mercaptophenylglyoxylate could undergo abiotic reactions, leading to highly polar organic compounds such as 2,2'-dithiosalicylic acid. Development of analytical methods to detect these highly polar compounds allowed us to examine the culture supernatant of a dibenzothiophene-degrading bacterium for their presence. The understanding gained from studying the abiotic processes helped to explain the presence of two novel disulfides that are dicarboxylic acids similar to 2,2'-dithiosalicylic acid.

MATERIALS AND METHODS

Chemicals. Dibenzothiophene (>98%) was purchased from Fluka (Buch, Switzerland). Acetonitrile and dichloromethane (high-pressure liquid chromatography [HPLC] grade) were from Fisher Chemicals (Fairlawn, N.J.). Diazald and thianthrene were obtained from Aldrich (Milwaukee, Wis.). 2,2'-Dithiosalicylic acid (95% purity) was obtained from Lancaster Synthesis (Windham, N.H.). Diethylether was obtained from BDH Inc. (Toronto, Canada).

Benzothiophene-2,3-dione was synthesized by the method of Hannoun et al. (22) from oxalyl chloride (Aldrich) and benzenethiol (Aldrich). After two recrystallizations from methanol, the large bright orange crystals had a melting point of 119 to 120°C, which agreed closely with literature value of 119 to 121°C (15). The crystals were found to have greater than 99% purity by HPLC and by gas chromatography (GC). 7-Methylbenzothiophene-2,3-dione was synthesized using the same method (22) from oxalyl chloride and 2-methylbenzenethiol

(Aldrich). The crystals had a melting point of 126°C, which agreed closely with the literature value of 126 to 127°C (12).

Abiotic reactions of benzothiophene-2,3-dione. Twenty milligrams of the dione was added to 200 ml of sulfate-free medium (pH 7) (10) in a 500-ml Erlenmeyer flask, and this was incubated in the dark at 28°C with shaking at 200 rpm. Samples (1 ml) were aseptically removed at various times for HPLC analysis. After 20 days of incubation, the solution was acidified with 5 ml of concentrated HCl, resulting in a pH of <1. The solution was then extracted three times with 50-ml portions of dichloromethane, and the pooled extracts were evaporated under reduced pressure to 1 ml. The concentrated extract was then transferred to a 1-dram (3.7 ml) vial and taken to dryness under a nitrogen stream. The resulting residue was then derivatized with an ethereal diazomethane solution (generated from Diazald according to the manufacturer's instructions). The derivatization was allowed to continue for 1 h, and then the sample was again evaporated to dryness with a nitrogen stream. The sample was redissolved in 100 μ l of dichloromethane and analyzed by gas chromatography-mass spectrometry (GC-MS).

In one experiment, benzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione were added to sterile medium (each at 100 mg/liter), incubated aerobically for 1 week, and extracted as described above.

Detection of disulfides in a dibenzothiophene-degrading culture. This study was done with a culture of *Pseudomonas* sp. strain BT1d that was used previously to produce HFBT, via the Kodama pathway, while growing on dibenzothiophene as its sole carbon and energy source (9). This isolate was inoculated into a 2-liter Erlenmeyer flask that contained 1.5 liters of mineral salts medium (31) and 2 g of dibenzothiophene (most of which remained as crystals). The culture was incubated at 28°C with shaking for 2 months, and 500 ml of this culture was first extracted with three 100-ml portions of dichloromethane to remove neutral compounds and then acidified with 10 ml of concentrated HCl and extracted with three 100-ml portions of dichloromethane to collect the acidic products. The latter three extracts were pooled, concentrated, and derivatized with diazomethane. The resulting derivatized mixture was then subjected to GC-MS analysis.

Analytical methods. Benzothiophene-2,3-dione, 2-mercaptophenylglyoxylate, and abiotically formed products in aqueous samples were analyzed by HPLC using a Hewlett Packard model 1050 chromatography system and a 5- μ m Li-Chrospher 100 RP-18 column (125 mm by 4 mm; Hewlett Packard) with a UV detector at 240 nm. An acidic (pH ~1) HPLC mobile phase consisting of (by volume) phosphoric acid (1.5%), KH_2PO_4 buffer (0.01 M, 64%), and acetonitrile (34.5%) was used at a flow rate of 2 ml/min.

To confirm the identity of the 2-mercaptophenylglyoxylate and to help characterize the unknown abiotic products that formed over time, the acidic mobile phase and RP-18 HPLC column were transferred to a Waters 2690 HPLC system which was equipped with a Waters 996 photodiode array detector allowing UV-visible scans for each eluted peak.

An Agilent Technologies 1100 mass selective detector was used for low-resolution HPLC-MS. Gradient elution using an acetonitrile-water mobile phase, from 0 to 50% acetonitrile, allowed the separation and identification of the primary abiotic product formed when the 2,3-dione was transformed in the sterile medium. To obtain a molecular formula of these products, a Micromass ZabSpec oaTOF instrument was used for direct loop injection electrospray high-resolution mass spectrometry under both positive and negative ionization conditions.

Organic extracts containing benzothiophene-2,3-dione or other sulfur heterocycles were analyzed by GC with a sulfur-selective detector (10). Most extracts were analyzed by GC-MS using a Hewlett Packard 5890 series II GC with a 5970 series mass selective detector and a 30-m DB-5 capillary column (J&W Scientific,

Folsom, Calif.). The GC temperature program used for all of these analyses was 90°C for 1 min and then 5°C/min to 280°C for 21 min.

RESULTS

Abiotic transformation products of benzothiophene-2,3-dione. Benzothiophene-2,3-dione dissolved in sterile mineral salts medium (pH 7) was incubated aerobically, and samples were removed for HPLC analyses. Over the 20 days of the incubation, five peaks (designated A, B, C, D, and E) were detected. Within <1 h of incubation, most of the 2,3-dione (peak A; retention time, 3.3 min) was gone, and two new peaks (B and C; retention times, 1.4 and 1.8 min, respectively) appeared in the HPLC chromatogram. Based on the absorbance at 240 nm, the area of peak B was about 10 times that of peak C, which was about 10 times that of peak A. The UV-visible spectrum of peak A had absorption maxima at 222, 255, and 309 nm, which closely matched the maxima of benzothiophene-2,3-dione at 222, 256, and 310 nm reported by Eaton and Nitterauer (14). Peak B was identified as 2-mercaptophenylglyoxylate based on its UV-visible spectrum that had maxima at 231, 266, and 336 nm, which corresponded to previously reported maxima of 232, 266, and 334 nm (14).

After 24 h of incubation, peaks A and B had all but disappeared, with the formation of peak C and the appearance of peak D (retention time, 2.9 min). After 7 days of incubation, peak E (retention time, 4.9 min) appeared in the HPLC chromatogram.

HPLC-MS, operated under both positive and negative ionization conditions, was used to help identify compound C. The low-resolution, positive ionization mass spectrum showed an $(M + 1)^+$ ion of m/z 401, indicating the presence of a compound with a molecular weight of 400, and other ions corresponding to the addition of a potassium ion (m/z 438.9) and another potassium ion (m/z 476.9) to this compound. Analysis of the negative ionization mass spectrum revealed that the actual mass of compound C was 362, and the addition of one potassium ion corresponded to the ion at m/z 400. The data from the negative ionization mass spectrum suggested that the observed ion at m/z 401 in the positive spectrum already contained one potassium ion. High-resolution mass spectrometry revealed the molecular formula of compound C to be $C_{16}H_9O_6S_2K_1$, with an exact mass of 400.955516. Other potassium adducts were identified with the formulae $C_{16}H_8O_6S_2K_2$ (mass = 438.911678) and $C_{16}H_8O_6S_2K_3$ (mass = 476.868088). The potassium adducts were present because the mineral salts medium used for the experiment was buffered with potassium hydrogen phosphate. This molecular formula was consistent with the oxidation of 2-mercaptophenylglyoxylate, resulting in the formation of a disulfide (Fig. 2).

Initial attempts using organic extractions and GC-MS to detect compounds C, D, and E originating from benzothiophene-2,3-dione incubated in sterile medium were unsuccessful. Using the procedures routinely employed in this laboratory (16, 28, 29, 33, 44, 45), which included acidifying the medium to approximately pH 2, extracting with dichloromethane, drying the extract with anhydrous sodium sulfate, concentrating the extract, derivatizing with diazomethane, and analyzing by GC-MS with upper oven temperature of 250°C, failed to show any peaks other than benzothiophene-2,3-dione.

2-Mercaptophenylglyoxylate

A disulfide dicarboxylic acid

FIG. 2. Oxidation of 2-mercaptophenylglyoxylate to a disulfide. The potassium adduct was detected by HPLC-MS under positive ionization conditions.

Sterile medium containing 100 mg of benzothiophene-2,3-dione per liter was incubated for 20 days, and HPLC analysis showed that compounds C, D, and E formed during this time. Thianthrene was added to the sterile medium as an internal standard, and the pH of the medium was adjusted to less than 1 with HCl, yielding a faint yellow color that was extracted into the dichloromethane. After removing residual water by filtering the extract through anhydrous sodium sulfate and derivatizing with diazomethane, the sample was analyzed by GC-MS with the temperature program modified to have a final oven temperature of 280°C. Thianthrene eluted after 25.4 min, and three new peaks (retention times, 38.3, 40.9, and 45.1 min) were detected. As described later, these were determined to be compounds E, D, and C, respectively.

During the preparation of this sample, it was observed that a considerable amount of material appeared to precipitate during the acidification prior to extraction and that some material collected on the drying agent while the extract was filtered through the anhydrous sodium sulfate. To recover the latter material, the drying agent was dissolved in a 0.1 M HCl solution, which was then extracted with dichloromethane and concentrated but not dried with sodium sulfate, before diazomethane derivatization and GC-MS analysis. The resulting chromatogram showed a small amount of thianthrene, the in-

ternal standard, and large quantities of these three new peaks, indicating that they were retained by the drying agent. These studies demonstrated that these three products were quite insoluble in acidic aqueous medium and in dichloromethane. Tetrahydrofuran was found to be a superior solvent for these three compounds.

The mass spectra of the methylesters of these three abiotic products are shown in Fig. 3. The product with the longest retention time (45.1 min) had a weak molecular ion at m/z 390 and a base peak of m/z 195. This molecular weight corresponded to the methyl ester of the disulfide formed from 2-mercaptophenylglyoxylate, which was detected as a potassium adduct by HPLC-MS (Fig. 2). Cleavage of the relatively weak S-S bond would yield the base peak of m/z 195 (Fig. 3). The molecular ion of compound D was m/z 362 (Fig. 3), which is 28 mass units less than that of compound C, and molecular ion of compound E was m/z 334 (Fig. 3), which is 28 mass units less than that of compound D. Stepwise net losses of carbon and oxygen atoms (see Discussion), as illustrated in the lower portion of Fig. 4, would account for these differences.

Two sequential net losses of carbon and oxygen atoms from compound C would yield 2,2'-dithiosalicylic acid, which is commercially available. Derivatization and GC-MS analysis of the authentic standard gave the same retention time (38.3 min) and mass spectrum as that shown for compound E in Fig. 3. In this case, the cleavage of the S-S bond yielded the base peak of m/z 167. Authentic 2,2'-dithiosalicylic acid was also subjected to HPLC analysis with a diode array detector. It had the same retention time and absorption maxima (218, 249, and 310 nm) as compound E in the mixture of abiotic products from benzothiophene-2,3-dione analyzed in the same manner. Thus, compound E was 2,2'-dithiosalicylic acid. From this HPLC analysis, the absorption maxima for compound C were 234, 268, 338, and 315 nm, and those for compound D were 221 and 320 nm.

The fragmentation pattern of compound D (Fig. 3) is consistent with a net loss of a carbon and an oxygen atom of compound C. Cleavage of the S-S bond yields the base peak at m/z 195, which is from the moiety observed in compound D, and the major ion at m/z 167 (28 mass units less than the base peak), which is from a moiety that arose after the net loss of a carbon and an oxygen atom.

Detection of disulfides in a dibenzothiophene-degrading culture. After the three disulfides were observed as abiotic oxidation products of 2-mercaptophenylglyoxylate, it was hypothesized that these disulfides should be observed in a dibenzothiophene-degrading bacterial culture that is known to yield benzothiophene-2,3-dione in organic extracts from acidified medium. To test this hypothesis, *Pseudomonas* BT1d was grown on dibenzothiophene as the sole carbon and energy source, and the culture was extracted under acidic conditions (pH < 1). The extract was subjected to diazomethane derivatization and analyzed by GC-MS. As shown in Fig. 5, benzothiophene-2,3-dione (retention time, 15 min) was also detected in the chromatogram, indicating the presence of 2-mercaptophenylglyoxylate, which should lead to the abiotic formation of the disulfides. Indeed, all three disulfides (compounds C, D, and E) were abundant in this extract. Also observed in Fig. 5 were HFBT and a CH₂ insertion product from treatment with diazomethane (21) (retention times, 16.6 and 17.9 min, respec-

tively), residual dibenzothiophene, and thioindigo (retention time, 45.7 min).

Subsequently, a sample of the nonacidified supernatant of another culture of strain BT1d grown on dibenzothiophene was analyzed by HPLC. Initially, none of the disulfides were detected in this supernatant, although several UV-absorbing compounds were present in the chromatogram. However, after extracting the neutral supernatant with dichloromethane, disulfides C and D were detected by HPLC. Other extractable metabolites had masked these disulfides in the HPLC analysis of the unextracted supernatant. Based on the detector response at 240 nm, the amount of disulfide C was about 10 times greater than the amount of disulfide D. The concentration of disulfide E was too low to detect by HPLC, but trace amounts of disulfide E were detected in the same sample after acidic (pH < 1) extraction, derivatization, and analysis by GC-MS. Thus, the more abundant disulfides could be detected by HPLC analysis of nonacidified supernatant after an extraction to remove other dibenzothiophene metabolites.

Abiotic formation of disulfides in a mixture of benzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione. A mixture of benzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione was prepared in sterile medium, and after 8 days of aerobic incubation the organic compounds were extracted from the acidified medium and analyzed by GC-MS. Figure 6 shows a portion of the chromatogram in which seven disulfides were detected. The most abundant disulfide (retention time, 44.2 min) was a homodimer formed from two molecules of 2-mercaptophenylglyoxylate derived from benzothiophene-2,3-dione (i.e., compound C). Another abundant disulfide (retention time, 41.7 min) had a molecular weight of 418 and a base peak of m/z 209, consistent with a homodimer formed from two molecules of 3-methyl-2-mercaptophenylglyoxylate derived from 7-methylbenzothiophene-2,3-dione (Fig. 1). The peak at 42 min (Fig. 6) had a molecular weight of 404, with a base peak of m/z 195 and another abundant ion at m/z 209, suggesting that this compound was a heterodimer formed from 2-mercaptophenylglyoxylate and 3-methyl-2-mercaptophenylglyoxylate. The peak at 40.8 min (Fig. 6) was compound D, from the net loss of a carbon and an oxygen atom of compound C. The peak at 38.9 min had a molecular weight of 390, with a base peak of m/z 209, suggesting that it was product of the homodimer formed from 3-methyl-2-mercaptophenylglyoxylate, which subsequently underwent a net loss of a carbon and an oxygen atom. The peak at 39.2 min had a molecular weight of 376 with a base peak of m/z 209 and a major ion of m/z 167, suggesting that it was a product of the heterodimer after the net loss of a carbon and an oxygen atom. Finally, the peak at 38.2 min was the least abundant of these disulfides, and it corresponded to compound E.

DISCUSSION

This investigation indicates that 2-mercaptophenylglyoxylate is likely an important intermediate in dibenzothiophene biodegradation through the Kodama pathway, because considerable amounts of the three disulfides were detected in extracts of *Pseudomonas* BT1d grown on dibenzothiophene (Fig. 5). The observation of the abiotic oxidation of 2-mercaptophenylglyoxylate to give compound C and the subsequent net loss of

FIG. 3. Mass spectra of the three disulfides (C, D, and E) detected by GC-MS analysis. Compound C is the dimethyl ester of 2-oxo-2-(2-thiophenyl)ethanoic acid disulfide, D is the dimethyl ester of 2-oxo-2-(2-thiophenyl)ethanoic acid 2-benzoic acid disulfide, and E is the dimethyl ester of 2,2'-dithiosalicylic acid (or 2,2'-dithiobenzoic acid).

FIG. 4. Summary of biotic and abiotic reactions that would yield the compounds detected in the extract of the dibenzothiophene-degrading culture shown in Fig. 5. These include the dimerization of 2-mercaptophenylglyoxalate to compound C and the subsequent net loss of carbon and oxygen atoms to yield compounds D and E.

a carbon and an oxygen atom to give compound D is the first report of these two disulfides being formed in a dibenzothiophene-degrading culture. Finkel'stein et al. (19) observed 2,2'-dithiosalicylic acid (compound E) as a product of dibenzothiophene bacterial biodegradation, but their report implied that the disulfide was formed through the oxidation of 2-mercaptobenzoic acid. Indeed, 2-mercaptobenzoic acid is very sensitive to air, and it is oxidized by molecular oxygen to 2,2'-

dithiosalicylic acid (4). Although it is possible that a small amount of the 2-mercaptobenzoate was formed from 2-mercaptophenylglyoxalate before being oxidized to form the disulfide (compound E), our HPLC analyses of abiotic controls indicate that the vast majority of the 2-mercaptophenylglyoxalate was rapidly oxidized to form compound C before the net loss of a carbon and an oxygen atom occurred. Subsequently, 2,2'-dithiosalicylic acid was observed after a few days of incubation of the sterile controls.

Munday (41) studied the autoxidation of several thiophenols (aromatic thiols) to disulfides at neutral pH and observed that addition of copper, iron, or cobalt salts catalyzed their dimerization. These trace metals were present in the media that we used, and thus, they would hasten the dimerization of 2-mercaptophenylglyoxalate to form compound C, shown in Fig. 2. Munday (41) also showed that this autoxidation generated active oxygen species, such as O_2^- and H_2O_2 , and that this reaction was inhibited by superoxide dismutase and catalase. These disulfides generated H_2O_2 in erythrocytes in vitro and induced oxidative damage in these cells (42). However, aerobic microbial cells that produce superoxide dismutase and catalase should not be severely affected by these active oxygen species.

The phenylglyoxyl moiety ($-C_6H_4COCO_2H$) is part of compounds C and D (Fig. 4). Containing an α -keto acid, this moiety is susceptible to decarbonylation and decarboxylation, and there are numerous reports of these reactions of phenylglyoxylic acid (11, 34, 46, 47, 53). Chen et al. (11) calculated that the activation energy for decarbonylation is lower than that for decarboxylation. Decarbonylation of phenylglyoxylic acid results in the loss of a carbon and an oxygen atom, yielding CO and benzoic acid (11, 53). The proposed mechanism of this decarbonylation (11) involves the migration of the OH from the carboxyl carbon to the carbonyl carbon and subsequent loss of the carboxyl carbon as CO. In contrast, the oxidative decarboxylation of phenylglyoxylic acid results in the loss of CO_2 , but still yields benzoic acid (46, 47). This reaction is catalyzed by iron (a component in our medium) in the presence of H_2O_2 (that could be produced by the dimerization of 2-mercaptophenylglyoxalate, an aromatic thiol) as reported by Munday (41).

The result of either of these abiotic reactions is the net loss of a carbon and an oxygen atom from the parent α -keto acid. Although it is not known which mechanism occurred in our sterile medium containing benzothiophene-2,3-dione or in the dibenzothiophene-degrading culture, either a decarbonylation or an oxidative decarboxylation would give the observed net losses of carbon and oxygen atoms shown in Fig. 4.

March (38) noted that α -keto acids and α -keto esters can be decarbonylated by heating. However, the detection of compounds D and E in our work was not likely an artifact of the exposure of compound C to heating during GC-MS analyses, because compounds D and E were both detected by HPLC analyses in which the column was at room temperature.

Many of the products of microbial degradation of dibenzothiophene have been known since the studies of Kodama and coworkers in the early 1970s (26, 27), and these products have been observed by other workers (6, 28, 35). However, with the exception of Finkel'stein et al. (19), no other studies have detected a disulfide in the extracts of dibenzothiophene-degrading cultures. The disulfides eluded other investigators be-

FIG. 5. GC-MS total ion current chromatogram of the diazomethane-derivatized extract of a *Pseudomonas* sp. strain BT1d culture grown on dibenzothiophene in mineral salts medium. The CH_2 addition to HFBT is due to a ring insertion of a methylene group during the diazomethane derivatization. The compound eluting at 45.7 min is thioindigo.

cause of (i) their high polarity, being dicarboxylic acids; (ii) the need to lower the pH of the aqueous medium to <1 to extract them into an organic solvent such as dichloromethane; (iii) their poor solubility in organic solvents, (iv) their removal from organic extracts of cultures during filtration through the commonly used drying agent anhydrous sodium sulfate, and (v) their high molecular weight. For example, although Frassinetti et al. (20) acidified their culture medium to pH 1 before extraction, their drying step, which used anhydrous sodium sulfate, may have removed the disulfides from the organic solvent.

In general, when investigators are studying the biodegradation of an organic compound, they look for products with fewer carbon atoms than the parent compound. The three disulfides have more carbon atoms than dibenzothiophene. For example, compound C has 16 carbon atoms and 6 oxygen atoms, whereas dibenzothiophene has 12 carbon atoms and no oxygen atoms. In addition, the molecular weight of compound C is 362, compared to 184 for dibenzothiophene, and the molecular weight of the dimethyl ester of compound C is 390 (Fig. 3). For studies in which GC or GC-MS is used to detect products of biodegradation, the very polar, high-molecular-weight disulfides require derivatization, high oven temperatures, and long analysis times to elute them from the column.

Previous studies in our laboratory (9, 28) failed to detect these disulfides. The procedures used included adjusting the pH to approximately 2, extracting with dichloromethane, drying the extract by filtering it through anhydrous sodium sulfate,

and analyzing the derivatized extract by GC-MS with an upper column temperature of 250°C . In retrospect, the combination of these experimental conditions would account for the disulfides escaping detection. Finkel'stein et al. (19) detected 2,2'-dithiosalicylic acid by thin-layer chromatography, and thus, vaporizing the analyte was not required. In the current study, it was the use of HPLC, especially HPLC-MS, which provided the initial indications that the disulfides existed.

Figure 4 summarizes the series of reactions that yielded the compounds shown in the chromatogram in Fig. 5. With the exception of the initial reactions which transform dibenzothiophene to HFBT, most of the other reactions are abiotic. Thioindigo, which was recently found in the culture extract of strain BT1d degrading dibenzothiophene (9), eluted from the GC column after the disulfides (Fig. 5) and is another example of a product which has a much higher molecular mass (296 Da) than the substrate (184 Da). The microbially mediated formation of benzo[*b*]naphtho[1,2-*d*]thiophene (234 Da) from benzothiophene (134 Da) has also been reported (32).

2-Mercaptophenylglyoxalate, detected as benzothiophene-2,3-dione in extracts of acidified cultures, has been found in cultures containing benzothiophene (6, 14, 16) or dibenzothiophene (6, 28). Eaton and Nitterauer (14) proposed possible pathways by which 2-mercaptophenylglyoxalate might arise from benzothiophene. After an initial dioxygenase attack on the thiophene ring, a series of abiotic reactions were suggested to yield 2-mercaptophenylglyoxalate.

FIG. 6. Disulfides detected when benzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione (100 mg/liter each) were incubated for 8 days in sterile sulfate-free medium. The bold moieties were derived from 3-methyl-2-mercaptophenylglyoxylate (Fig. 1). The dark-shaded peaks are from the homodimerization of 3-methyl-2-mercaptophenylglyoxylate, the lightly shaded peaks are from the heterodimerization of 3-methyl-2-mercaptophenylglyoxylate and 2-mercaptophenylglyoxylate, and the unshaded peaks are from the homodimerization of 2-mercaptophenylglyoxylate.

It is not known whether benzothiophene-2,3-dione or 2-mercaptophenylglyoxalate might occur first in the transformation of HFBT. If the 2,3-dione forms first, the sulfur-containing ring would quickly open at neutral pH to yield 2-mercaptophenylglyoxalate. Alternatively, if the latter compound forms directly from HFBT, the 2,3-dione is an artifact created by the acidification of the culture medium. We suggest that the 2,3-dione is actually formed as a short-lived intermediate. Figure 7 proposes a mechanism by which benzothiophene-2,3-dione might be formed in the Kodama pathway for dibenzothiophene degradation. Under aerobic conditions, a free radical (F) may be generated from HFBT. Molecular oxygen then attacks carbon 2, forming a second free radical (G). The unpaired electron is passed to another molecule of HFBT, and the intermediate (H) loses HCHO and H₂O, forming the 2,3-dione. Although this reaction scheme has not been proven, it is consistent with the observation of benzothiophene-2,3-diones being found in a sterile control that contained pure HFBT (9). Once the 2,3-dione is formed, it would spontaneously open to yield 2-mercaptophenylglyoxylate (Fig. 4, compound B).

In an earlier study (16), benzothiophene was mixed with crude oil and inoculated with a bacterial strain known to metabolize benzothiophene, yielding benzothiophene-2,3-dione in acidified culture extracts. After 14 days of incubation, no trace of the 2,3-dione was found in the acidified culture extract. When the 2,3-dione was added to a sterile control with crude oil, it could not be recovered after 14 days of incubation. The abiotic formation of the disulfides provides an explanation of why the 2,3-dione could not be recovered.

Kropp et al. (28) could not account for all of the sulfur from dibenzothiophene in cultures that degraded it via the Kodama pathway, leading them to predict that the missing sulfur must exist as highly polar organic compounds in the culture supernatants. The disulfides detected in the present study fit that

description. During the analyses of the disulfides, it was apparent that upon acidification of the culture, the disulfides became insoluble in aqueous phase and only sparingly soluble in all other organic phases commonly used for extractions. The disulfides precipitated out of solution and often associated with the emulsion between the aqueous and organic layers. Quantitative recovery of the disulfides from the emulsion that also contained bacterial cells was not possible, preventing accurate determination of the amount of dibenzothiophene converted to disulfides by strain BT1d.

Petroleum contains a variety of methyl-substituted benzothiophenes and dibenzothiophenes that can be attacked by bacteria. Methylbenzothiophene-2,3-diones have been identified in acidified extracts from cultures degrading methylbenzothiophenes (31, 44), methyl-dibenzothiophenes (45), and dimethyldibenzothiophenes (29, 37). Similarly, dimethylbenzothiophene-2,3-diones have been identified in acidified extracts from cultures degrading some dimethylbenzothiophenes (33). Thus, at neutral pH, a wide range of methyl- and dimethyl-substituted 2-mercaptophenylglyoxylates would presumably be produced while petroleum undergoes biodegradation. This mixture of substituted 2-mercaptophenylglyoxylates would likely yield a vast array of disulfides in the residual oil.

The experiment in which benzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione (which has been found in extracts of acidified cultures containing 7-methylbenzothiophene [44], 4-methyldibenzothiophene [45], or 4,6-dimethyldibenzothiophene [37]) were incubated in sterile medium illustrates the likelihood of a variety of disulfides being formed. Figure 6 shows seven disulfides formed from this simple mixture of two compounds that yield 2-mercaptophenylglyoxylates. Laboratory experiments (24, 52) and analyses of spilled oil from environmental samples (7, 51) have shown that biodegradation increases the proportion of the polar fraction

FIG. 7. Proposed mechanism for the abiotic formation of benzo[thiophene]-2,3-dione from HFBT under aerobic conditions.

found in the biodegraded residual oils. The formation of a myriad of disulfides from the 2-mercaptophenylglyoxylates would contribute to the increased proportion of polar compounds. In some cases, the polar fraction of residual petroleum has been found to be the most toxic fraction (54).

The major goals of bioremediation are the removal of contaminants and the reduction of toxicity in a contaminated area. The formation of products that are more toxic than the original contaminants is undesirable. No information on the toxicity of compounds C and D could be found. However, 2,2'-dithiosalicylic acid, the final disulfide in the reaction series shown in Fig. 4, is likely not very toxic to humans because its magnesium salt was clinically compared with aspirin as a medication for pa-

tients with rheumatoid arthritis (13). 2,2'-Dithiosalicylic acid has also been detected as a decomposition product of thimerosal (43, 49), which is widely used in pharmaceutical products such as eye drops, in which it serves as an antibacterial and antifungal preservative (43).

Although 2,2'-dithiosalicylic acid has antimicrobial properties, we have observed that it can serve as the sole carbon and sulfur source in aerobic soil enrichment cultures (this will be the topic of a future paper). Thus, it is possible that the other two disulfides (compounds C and D) may be biodegraded. If these activities can be detected, it should be possible to assemble a microbial consortium to mineralize dibenzothiophene via the Kodama pathway. Strain BT1d would initiate the process through the pathway shown in Fig. 4. The abiotic reactions in the lower portion of Fig. 4 will provide the substrates for the disulfide-degrading population, and dibenzothiophene would be mineralized to carbon dioxide and sulfate. To date, there have been no reports on the mineralization of dibenzothiophene via the Kodama pathway. The experimental approach described above may demonstrate this mineralization.

ACKNOWLEDGMENTS

This work was funded by the Natural Sciences and Engineering Research Council of Canada and by a University of Alberta Dissertation Fellowship.

We acknowledge the assistance of the personnel in Mass Spectrometry Laboratory and thank J.C. Vederas and A. Sutherland of the Department of Chemistry at the University of Alberta for valuable discussions.

REFERENCES

- 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.
- 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.
- Bence, A. E., K. A. Kvenvolden, and M. C. Kennicutt II. 1996. Organic geochemistry applied to environmental assessments of Prince William Sound, Alaska, after the *Exxon Valdez* oil spill—a review. *Org. Geochem.* **24**:7–42.
- Bodini, M. E., and M. A. Del Valle. 1990. Redox chemistry and spectroscopy of 2-mercaptobenzoic acid and its manganese(II) and (III) complexes in dimethylsulphoxide. *Polyhedron* **9**:1181–1186.
- Boehm, P. D., D. L. Fiest, and A. Elskus. 1981. Comparative weathering patterns of hydrocarbons from the *Amoco Cadiz* oil spill observed at a variety of coastal environments. *Amoco Cadiz: fates and effects of the oil spill*, p. 159–173. In *Proceedings of the International Symposium Centre Oceanologique de Bretagne. Le Centre National pour l'Exploitation des Oceans*, Paris, France.
- 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.
- Bragg, J. R., R. C. Prince, E. J. Harner, and R. M. Atlas. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. *Nature* **368**:413–418.
- Bressler, D. C., and P. M. Fedorak. 2000. Bacterial metabolism of fluorene, dibenzofuran, dibenzothiophene and carbazole. *Can. J. Microbiol.* **46**:397–409.
- Bressler, D. C., and P. M. Fedorak. 2001. Purification, stability and mineralization of 3-hydroxy-2-formylbenzothiophene, a metabolite of dibenzothiophene. *Appl. Environ. Microbiol.* **67**:821–826.
- Bressler, D. C., B. K. Leskiw, and P. M. Fedorak. 1999. Biodegradation of benzothiophene sulfones by a filamentous bacterium. *Can. J. Microbiol.* **45**:360–368.
- Chen, L.-T., G.-J. Chen, and X.-Y. Fu. 1995. Theoretical studies on the mechanism of the thermal decarboxylation and decarbonylation of a number of α -keto acids. *Chin. J. Chem.* **13**:487–492.
- Dalglish, C. E., and F. G. Mann. 1945. The comparative reactivity of the carbonyl groups in the thionaphthenquinones. II. The influence of substituent groups in the thionaphthenquinones. *J. Chem. Soc.* 893–909.
- Dequeker, J., E. Stevens, and L. Wuyts. 1980. A controlled trial of magnesium dithiosalicylate compared with aspirin in rheumatoid arthritis. *Curr. Med. Res. Opin.* **6**:589–592.

14. Eaton, R. W., and J. D. Nitterauer. 1994. Biotransformation of benzothiophene by isopropylbenzene-degrading bacteria. *J. Bacteriol.* **176**:3992–4002.
15. Eistert, B., R. Müller, H. Selzer and E.-A. Hackmann. 1964. Über die Umsetzung von α -Oximino-carbonylverbindungen mit Diazomethan. *Chem. Ber.* **97**:2469–2478.
16. Fedorak, P. M., and D. Grbić-Galić. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. *Appl. Environ. Microbiol.* **57**:932–940.
17. Fedorak, P. M., and D. W. S. Westlake. 1982. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. *Can. J. Microbiol.* **29**:291–296.
18. 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.
19. Finkel'stein, Z. I., B. P. Baskunov, L. N. Vavilova, and L. A. Golovleva. 1997. Microbial transformation of dibenzothiophene and 4,6-dimethyldibenzothiophene. *Microbiologiya* **66**:481–487.
20. Frassinetti, S., L. Setti, A. Corti, P. Farrinelli, P. Montevecchi, and G. Vallini. 1998. Biodegradation of dibenzothiophene by a nodulating isolate of *Rhizobium meliloti*. *Can. J. Microbiol.* **44**:289–297.
21. Furniss, B. S., A. J. Hannaford, P. W. G. Smith, and A. R. Tatchell. 1989. Vogel's textbook of practical organic chemistry, 5th ed., p. 1111. John Wiley & Sons, Inc., New York, N.Y.
22. Hannoun, M., N. Blazevic, D. Kolbah, M. Mihalic, and F. Kaijfez. 1982. α -Phenylpropionic acid derivatives: synthesis and dethiation of benzoylbenzo[b]thiophene-3-carboxylic acid. *J. Heterocyclic Chem.* **19**:1131–1136.
23. Hostettler, F. D., and K. A. Kvenvolden. 1994. Geochemical changes in crude oil spilled from the *Exxon Valdez* supertanker into Prince William Sound, Alaska. *Org. Geochem.* **21**:927–936.
24. Jobson, A., F. D. Cook, and D. W. S. Westlake. 1972. Microbial utilization of crude oil. *Appl. Microbiol.* **23**:1082–1089.
25. Kargi, F., and J. M. Robinson. 1984. Microbial oxidation of dibenzothiophene by the thermophilic organism *Sulfolobus acidocaldarius*. *Biotechnol. Bioeng.* **26**:687–690.
26. Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. III. Isolation and identification of products from dibenzothiophene. *Agric. Biol. Chem.* **34**:1320–1324.
27. Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. 1973. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. *Agric. Biol. Chem.* **37**:45–50.
28. Kropp, K. G., J. T. Anderson, and P. M. Fedorak. 1997. Bacterial transformations of 1,2,3,4-tetrahydrodibenzothiophene and dibenzothiophene. *Appl. Environ. Microbiol.* **63**:3032–3042.
29. Kropp, K. G., J. T. Anderson, and P. M. Fedorak. 1997. Biotransformations of three dimethyldibenzothiophenes by pure and mixed bacterial cultures. *Environ. Sci. Technol.* **31**:1547–1554.
30. Kropp, K. G., and P. M. Fedorak. 1998. A review of the occurrence, toxicity and biodegradation of condensed thiophenes found in petroleum. *Can. J. Microbiol.* **44**:605–622.
31. Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994. Microbial transformations of benzothiophene and methylbenzothiophenes. *Environ. Sci. Technol.* **28**:1348–1356.
32. Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994. Microbially mediated formation of benzonaphthothiophenes from benzo[b]thiophenes. *Appl. Environ. Microbiol.* **60**:3624–3631.
33. Kropp, K. G., S. Saffić, J. T. Andersson, and P. M. Fedorak. 1996. Transformations of six isomers of dimethylbenzothiophenes by three *Pseudomonas* strains. *Biodegradation* **7**:203–221.
34. Kuhn, H. J., and H. Görner. 1988. Triplet state and photodecarboxylation of phenylglyoxylic acid in the presence of water. *J. Phys. Chem.* **92**:6208–6219.
35. Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* species. *Appl. Environ. Microbiol.* **34**:783–790.
36. Leermakers, P. A., and G. F. Vesley. 1963. Photochemistry of α -keto acids and α -keto esters. I. Photolysis of pyruvic acid and benzoylformic acid. *J. Am. Chem. Soc.* **85**:3776–3779.
37. Lu, J., T. Nakajima-Kambe, T. Shigeno, A. Ohbo, N. Nomura, and T. Nakahara. 1999. Biodegradation of dibenzothiophene and 4,6-dimethyldibenzothiophene by *Sphingomonas paucimobilis* strain TZS-7. *J. Biosci. Bioeng.* **3**:293–299.
38. March, J. 1985. Advanced organic chemistry—reactions, mechanisms, and structure, p. 341–342. John Wiley & Sons, New York, N.Y.
39. Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. *Appl. Environ. Microbiol.* **49**:756–760.
40. Mormile, M. R., and R. M. Atlas. 1988. Mineralization of the dibenzothiophene biodegradation products 3-hydroxy-2-formylbenzothiophene and dibenzothiophene sulfone. *Appl. Environ. Microbiol.* **54**:3183–3184.
41. Munday, R. 1985. Toxicity of aromatic disulfides. I. Generation of superoxide radicals and hydrogen peroxide by aromatic disulfides in vitro. *J. Appl. Toxicol.* **5**:402–408.
42. Munday, R. 1985. Toxicity of aromatic disulfides. II. Intraerythrocytic hydrogen peroxide formation and oxidative damage by aromatic disulfides. *J. Appl. Toxicol.* **5**:409–413.
43. Reader, M. J., and C. B. Lines. 1982. Decomposition of Thimerosal in aqueous solution and its determination by high-performance liquid chromatography. *J. Pharm. Sci.* **72**:1406–1409.
44. Saffić, 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.
45. Saffić, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methylbenzothiophenes by three *Pseudomonas* isolates. *Environ. Sci. Technol.* **27**:2577–2584.
46. Siegel, B., and J. Lanphear. 1979. Iron-catalyzed oxidative decarboxylation of benzoylformic acid. *J. Am. Chem. Society.* **101**:2221–2222.
47. Siegel, B., and J. Lanphear. 1979. Kinetics and mechanisms for the acid-catalyzed oxidative decarboxylation of benzoylformic acid. *J. Org. Chem.* **44**:942–946.
48. Speight, J. G. 1980. The chemistry and technology of petroleum. Marcel Dekker Inc., New York, N.Y.
49. Tan, M., and J. E. Parkin. 2000. Route of decomposition of thiomersal (thimerosal). *Int. J. Pharm.* **208**:23–34.
50. van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. *Arch. Microbiol.* **153**:324–328.
51. Westlake, D. W. S., A. M. Jobson, and F. D. Cook. 1978. *In situ* degradation of oil in a soil of the boreal region of the Northwest Territories. *Can. J. Microbiol.* **24**:254–260.
52. Westlake, D. W. S., A. M. Jobson, R. Phillippe, and F. D. Cook. 1974. Biodegradability and crude oil composition. *Can. J. Microbiol.* **20**:915–928.
53. Yonezawa, N., T. Hino, K. Matsuda, T. Matsuki, D. Narushima, M. Kobayashi, and T. Ikeda. 2000. Specific and chemoselective multi- α -arylation reactions of benzoylformic acid with or without decarbonylation in P_2O_5 -MsOH and related acidic media. *J. Org. Chem.* **65**:941–944.
54. Zemanek, M. G., S. J. Pollard, S. L. Kenefick, and S. E. Hrudefy. 1997. Toxicity and mutagenicity of component classes of oils isolated from soils at petroleum- and creosote-contaminated sites. *J. Air Water Manage. Assoc.* **47**:1250–1258.