University of Alberta

AEROBIC MICROBIAL METABOLISM OF CONDENSED THIOPHENES FOUND IN PETROLEUM

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled AEROBIC MICROBIAL METABOLISM OF CONDENSED THIOPHENES FOUND IN PETROLEUM submitted by KEVIN GLEN KROPP in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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To my parents, who taught me to do my best at whatever I set my hand to,

and then who accept and support me regardless of

what I choose or how things turn out.

ABSTRACT

The aerobic microbial metabolism of benzothiophene, six isomers of each of methyl- and dimethylbenzothiophene, dibenzothiophene, three isomers of dimethyldibenzothiophene, 1,2,3,4-tetrahydrodibenzothiophene, naphtho[2,1-*b*]thiophene, naphtho[2,3-*b*]thiophene, and 1-methylnaphtho[2,1-*b*]thiophene was studied. All 21 of these compounds are found in petroleum or synthetic fuels, but only four of them are commercially available. The other condensed thiophenes were synthesized by Dr. J. T. Andersson (University of Münster), for use in biodegradation studies. The study of methyl-substituted condensed thiophenes was motivated by recent research which showed that their resistance to biodegradation increases with increasing methyl-substitution.

The objective was to identify metabolites in pure cultures of aromatic hydrocarbon-degrading *Pseudomonas* spp. incubated in mineral medium in the presence of an aromatic growth substrate (i.e. 1-methylnaphthalene) and a pure condensed thiophene. As well, some studies investigated the biodegradation of condensed thiophenes in mineral medium with petroleum or the aromatic fraction of petroleum as the growth substrate for mixed cultures of petroleum-degrading bacteria or for cultures inoculated with an environmental sample (i.e. river water). These studies were done because mixed populations generally have greater degradative potential and more closely approximate the situation of an actual oil-contaminated environment. After appropriate incubation times, the cultures were extracted with organic solvent to recover substrates and products, and extracts were analyzed by gas chromatography with flame photometric, mass, and Fourier transform infrared detectors. This enabled the identification of biotransformation products, whose structures frequently were verified by comparisons with synthesized authentic standards. Other identifications required the purification of metabolites and analysis by nuclear magnetic resonance spectrometry. Gas chromatography analysis with an atomic emission

detector, which gives a linear response to sulfur in all organic forms, was used to quantify some of the observed metabolites.

By these methods, over 80 metabolites of the condensed thiophenes listed above were identified. These include sulfoxides, sulfones, hydroxy- and carboxyl-substituted benzothiophenes, hydroxy-substituted dibenzothiophenes, and substituted benzothiophene-2,3-diones and 3-hydroxy-2-formylbenzothiophenes. Benzonaphthothiophenes were also observed to form by an abiotic condensation of microbially-produced benzothiophene sulfoxides.

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LIST OF ABBREVIATIONS

AED	Atomic emission detector
BSA	*N,O*-bis(trimethylsilyl)acetamide
CFU	Colony forming units
DCM	Dichloromethane
FID	Flame ionization detector
FPD	Flame photometric detector
GC	Gas chromatography
GC-FTIR	Gas chromatography-Fourier transform infrared
GC-MS	Gas chromatography-mass spectrometry
HP	Hewlett-Packard
HPLC	High performance liquid chromatography
LB	Luria broth
1-MN	1-Methylnaphthalene
^{1}H-NMR	^{1}H-Nuclear magnetic resonance
PCA	Plate count agar
TMS	Trimethylsilyl
TSB	Trypticase soy broth

1. INTRODUCTION

1.1 Sulfur in petroleum

Petroleum is generally considered to be a naturally-occurring gaseous, liquid, or solid mixture predominantly composed of hydrocarbons. This definition includes conventional and heavy crude oils, mineral wax, asphalts (bitumens), and bituminous rock such as oil shales (Foght *et al.*, 1990).

After carbon and hydrogen, sulfur is typically the third most abundant element in petroleum, ranging from 0.05 to 5% (w/w) in crude oils, depending upon the source (Speight, 1980). In some rare cases, the sulfur content is actually much higher than these typical values. Crude oils from Utah, Germany, and California were reported by Thompson (1981) to have sulfur contents of 13.9, 9.6, and 7.5%, respectively. Sulfur contents of 0.2 to 12% and 0.4 to 11% are known for oil shales and tar sands, respectively (Czogalla and Boberg, 1983). Most of the sulfur present in crude oils is organically-bound sulfur, with hydrogen sulfide and elemental sulfur, dissolved in the crude oil, usually only representing a minor portion of the total (Sinninghe Damste´ and de Leeuw, 1990).

The sulfur content of typical crude oils is too high to attribute its presence solely to the biochemical incorporation of sulfur into the biological material that eventually formed the petroleum. As well, the structures of organosulfur compounds found in petroleum are largely different from those which are biosynthesized (Sinninghe Damste´ and de Leeuw, 1990). Thus, it is generally accepted that much of the organically-bound sulfur in petroleum was produced by reactions of organic matter with reduced inorganic sulfur species (sulfides) during the early stages of diagenesis (Sinninghe Damste´ and de Leeuw, 1990). The presence of sulfides from the bacterial reduction of sulfate during these early stages would have depended upon the amount of sulfate present in the marine or lacustrine environment in which the petroleum was being formed. As well, the amounts of reactive iron minerals that could compete with the organic matter for the reduced sulfur species and lead to the formation of low sulfur crudes even in marine environments that were rich in sulfate may have influenced the incorporation of sulfur into crude oils at different geographical locations (Sinninghe Damste´ and de Leeuw, 1990). Thus, the sulfur content of crude oils varies with geographical location depending upon the conditions prevalent at the time of formation.

1.2 Types of organosulfur compounds present in petroleum

As a general rule, the greater the density of a crude oil (or the lower the API gravity of the crude oil), the higher the sulfur content (Speight, 1980). Thus, it follows that the

distribution of sulfur within a particular crude oil is such that the sulfur compounds become progressively more concentrated as the boiling range of the fractions increases (Speight, 1980; Thompson, 1981). As well, the types of organosulfur compounds which are predominant varies with the boiling range (Czogalla and Boberg, 1983). Figure 1.1 shows structures that are representative of the various types of organosulfur compounds found in petroleum. In fractions boiling below 150°C, the sulfur is present primarily as alkane (compound 1, Figure 1.1) and cycloalkane (compound 2) thiols, dialkyl (compound 4) and alkyl cycloalkyl (compound 5) sulfides, disulfides (compound 7), 5- and 6-membered monocyclic sufides (compound 8), thiophene (compound 9), and thiophenes with one or two methyl groups. In fractions boiling between 150 to 250°C, many of these same compound types with slightly larger molecular weights dominate together with arene thiols (compound 3), alkyl aryl sulfides (compound 6), polysulfides (compound 7), and mono-, bi-, and tricyclic sulfides (compound 8). As well, thiaindanes (compound 10), thiophenes (compound 9) with up to four short side chains, and thiophenes condensed with one aromatic ring to form benzothiophenes (compound 11), thienothiophenes (compound 12), and thienopyridines (compound 13) are typically major sulfur-containing constituents in this boiling range (Czogalla and Boberg, 1983). The sulfur present in fractions boiling between 250 to 540°C exists primarily in substituted thiophenes (compound 9) and thiophene rings that are condensed to form substituted benzo- (compound 11), dibenzo- (compound 14), naphtho- (compound 15), benzonaphtho- (compound 16), and phenanthrothiophenes (compound 17). As well, many other complex compounds containing thiophene rings anellated with aromatic and naphthenoaromatic structures have been identified in this fraction (Czogalla and Boberg, 1983).

Speight (1980) reported that a substantial proportion (>60%) of the sulfur in higher-boiling fractions of various crudes is present as substituted benzothiophenes (compound 11, Figure 1.1). In some Texas oils, as much as 70% of the organic sulfur is present as dibenzothiophene (compound 14), and in some Middle East oils the alkyl-substituted benzo- and dibenzothiophenes contribute up to 40% of the organic sulfur present (Finnerty and Robinson, 1986). Thus, benzo- and dibenzothiophene and their alkylated derivatives are among the many condensed thiophenes which are an important form of organic sulfur in the heavier fractions of many crude oils. Condensed thiophenes are also the predominant form of sulfur identified in synthetic fuels derived from coal, oil shale, and tar sands (Later *et al.*, 1981; Nishioka *et al.*, 1985; Nishioka, 1988; Thompson, 1981; Willey *et al.*, 1981). Sulfur compounds in the extremely high boiling fractions of petroleum (>540°C) typically contain approximately half of the total sulfur content of crude oils. While these compounds are the most difficult to analyze and identify, approximately

80% of the sulfur is estimated to be thiophenic in nature as part of larger complex molecules (Czogalla and Boberg, 1983).

1.3 Toxicity, genotoxicity, and potential for bioaccumulation of condensed thiophenes

From an industrial viewpoint, the presence of organosulfur compounds of any of the types listed above is a concern because they cause corrosion and can poison catalysts during the refining of petroleum. The combustion of organosulfur compounds in petroleum products releases sulfur dioxide into the atmosphere which is an environmental concern because this leads to the problem of acid rain. This is also an industrial concern because legislation limits maximum allowable sulfur dioxide emissions. Thus, the refining process must be capable of removing sulfur from petroleum feedstocks to meet the requirements for subsequent use of the refined product.

Another environmental concern is focused particularly on the condensed thiophenes, which are predominant in the heavier fractions of petroleum where sulfur content is the highest. These polycyclic aromatic sulfur heterocycles consist of a thiophene ring fused with one or more benzene rings (compound 11, compounds 14 to 17; Figure 1.1). As such, these sulfur-containing analogs of the polycyclic aromatic hydrocarbons, whose carcinogenic and mutagenic potential are well known, may also have biological activity (Jacob, 1990). The mutagenicity, carcinogenicity, and acute toxicity of these organosulfur compounds in environments that become contaminated with condensed thiophene-containing crude oil will impact the organisms that live in contaminated ecosystems.

Pelroy *et al.* (1983) tested various 3- and 4-ring condensed thiophenes for mutagenic activity in the Ames' *Salmonella typhimurium* assay after metabolic activation with hepatic monooxygenase from rats. McFall *et al.* (1984) tested the methyl-substituted derivatives of these compounds in the same assay. The structures of many of these compounds are shown in Figure 1.2. Naphtho[1,2-*b*]thiophene (compound 15, Figure 1.2) was shown to have mutagenic potential whereas dibenzothiophene (compound 14) and the other two isomers of naphthothiophene (compounds 18 and 19) were inactive, even after metabolic activation. Methyl substitution of dibenzothiophene (compound 14) did not lead to mutagenic activity. Seven of the 13 unsubstituted 4-ring polycyclic aromatic sulfur heterocycles tested were shown to have mutagenic potential that was activated by treatment with hepatic monooxygenase from rats. The highest activity for the 4-ring compounds was observed for phenanthro[3,4-*b*]thiophene (compound 17), which was of approximately the same mutagenic potency as the polycyclic aromatic hydrocarbon benzo[*a*]pyrene. The

isomeric compound phenanthro[4,3-*b*]thiophene (compound 20) exhibited only low activity, even after metabolic activation, indicating that the position of the sulfur atom plays a key role in the biological activity. For the other unsubstituted 4-ring compounds with mutagenic potential, the mutagenic activities did not correlate with the mutagenicity of the isosteric polycyclic aromatic hydrocarbon. Several of the methyl-substituted benzo[*b*]naphtho[1,2-*d*]- (compound 16), -[2,1-*d*]- (compound 21), and -[2,3-*d*]thiophenes (compound 22) were found to have mutagenic potential (McFall *et al.*, 1984). The most potent of these was 6-methylbenzo[*b*]naphtho[2,1-*d*]thiophene which is isosteric to the potent mutagen and carcinogen 5-methylchrysene. For the other methyl-substituted 4-ring compounds there was no observable correlation between their mutagenic potential and that of the isosteric polycyclic aromatic hydrocarbon. Thus, numerous condensed thiophenes have been shown to possess mutagenic potential and will contribute to the overall impact of petroleum hydrocarbons on contaminated environments.

Jacob (1990) reviewed research on the carcinogenicity of numerous polycyclic aromatic sulfur heterocycles and methyl-substituted derivatives in rats and mice. Among the compounds which have been shown to be highly carcinogenic are the following (Figure 1.3): 7,11-dimethylphenanthro[2,3-*b*]thiophene (compound 23); 6,11-dimethylanthra[1,2-*b*]thiophene (compound 24); 6,12-dimethylbenzo[1,2-*b*:4,5-*b*']bis[1]benzothiophene (compound 25); 7,13-dimethylbenzo[*b*]phenanthro[3,2-*d*]thiophene (compound 26); 6,12-dimethylbenzo[1,2-*b*:5,4-*b*']bis[1]benzothiophene (compound 27); and, benzo[*b*]phenanthro[3,4-*d*]thiophene (compound 28). The carcinogenic potential of these compounds and the inactivity of other isomers does not correlate well with the carcinogenic activity of the polycyclic aromatic hydrocarbon isosters of these condensed thiophenes. In many cases, the condensed thiophenes were more potent carcinogens than their carbocyclic isosters.

A series of polycylic aromatic sulfur heterocycles were compared to their sterically and structurally similar polycyclic aromatic hydrocarbons for acute toxicity to the zooplankton *Daphnia magna* (Eastmond *et al.*, 1984). Dibenzothiophene (compound 14, Figure 1.2) was shown to be more toxic than phenanthrene and anthracene, and benzo[*b*]naphtho[1,2-*d*]thiophene (compound 16) was more toxic than benz[*a*]anthracene. However, benzothiophene (compound 11) was less toxic than its homocyclic isoster naphthalene and benzo[*b*]naphtho[2,1-*d*]thiophene (compound 21) did not exhibit any toxicity in this test.

The potential mutagenicity, carcinogenicity, and toxicity of condensed thiophenes is especially important in light of reports that these compounds are prone to bioaccumulation in tissues of living organisms with the subsequent potential for biomagnification in the food chain. Flat and Japanese oysters from marine environments contaminated with oil from the

Amoco Cadiz spill were shown to contain C_1-, C_2-, and C_3-substituted dibenzothiophenes with some of the more highly substituted isomers persistant for up to three years after the spill (Berthou *et al.*, 1981; Friocourt *et al.*, 1982; Laseter *et al.*, 1981). The enrichment of alkyl-dibenzothiophenes and phenanthrenes relative to unsubstituted parent compounds was also observed in tissues of clams and mussels impacted by the *Tsesis* oil spill (Boehm *et al.*, 1982). The accumulation of a series of polycyclic aromatic sulfur heterocycles was compared with their analogous polycyclic aromatic hydrocarbons by Eastmond *et al.* (1984). Benzothiophene (compound 11, Figure 1.2) and benzo[*b*]naphtho[2,1-*d*]thiophene (compound 21) were bioconcentrated by *D. magna* to a greater extent than naphthalene and chrysene, respectively, but dibenzothiophene (compound 14) and phenanthrene exhibited similar uptake curves. No clear trend was observed for elimination differences between polycyclic aromatic hydrocarbons and sulfur heterocycles in this system.

The bioaccumulation of condensed thiophenes in tissues of living organisms, coupled with the potential toxicity, carcinogenicity, and mutagenicity of these compounds suggests that they may make a significant contribution to the impact that oil spills have on contaminated environments. As low-sulfur feedstocks are depleted, the increased transport, refining, and use of higher-sulfur crude oils and synthetic fuels derived from coal, tar sands, and shale oils may lead to increased environmental contamination with condensed thiophenes. The fate and effect of these compounds in contaminated environments will be partly determined by the abilities of the microbial populations present to biodegrade these complex mixtures of polycyclic aromatic compounds.

1.4 Biodegradation of condensed thiophenes within the complex mixture of petroleum

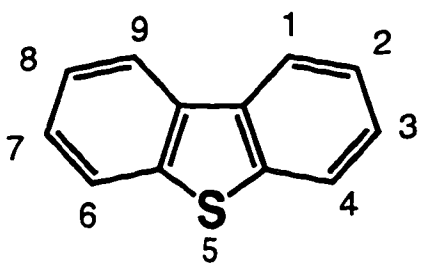
Most studies of the biodegradation of condensed thiophenes within the complex mixture of hydrocarbons that make up petroleum have been done using gas chromatography (GC) analysis with a sulfur-selective flame photometric detector (FPD) and GC-mass spectrometry (GC-MS) to follow the depletion of condensed thiophenes in the oil. The alkyl-substituted dibenzothiophenes have been reported to be among the compounds that are most resistant to biodegradation within the aromatic fraction of petroleum. The investigations described above which studied the accumulation and persistance of alkyldibenzothiophenes in tissues of oysters, mussels, and clams impacted by oil from the *Amoco Cadiz* and *Tsesis* oil spills also evaluated the persistance of petroleum in contaminated sediments (Berthou *et al.*, 1981; Boehm *et al.*, 1982; Laseter *et al.*, 1981). Berthou *et al.* (1981) observed that dibenzothiophene derivatives were the most persistant compounds in the environment 2 years after the *Amoco Cadiz* spill.

Dibenzothiophene and methyldibenzothiophenes were observed to be more rapidly biodegraded than C_2- and C_3-dibenzothiophenes. They stated that in weathered oil, "some results suggest that di- and tri-methyldibenzothiophene could represent up to about 50% of the total aromatic fraction" (Berthou *et al.*, 1981). In other studies, C_2- and C_3-dibenzothiophenes were also observed to be among the most persistant compounds in contaminated sediment (Boehm *et al.*, 1982; Laseter *et al.*, 1981). Boehm *et al.* (1981) listed alkyldibenzothiophenes with C_3- and C_4-phenanthrenes, naphthenoaromatic compounds, naphthenic compounds, and polycyclic aliphatics as the most persistant classes of compounds in residual *Amoco Cadiz* oil. Similar findings were reported by Atlas *et al.* (1981) in their studies of the same oil spill. Teal *et al.* (1978) reported that dibenzothiophene and methyldibenzothiophene concentrations decreased more slowly than those of the aromatic hydrocarbons including phenanthrene and methylphenanthrenes in intertidal marsh surface sediment from Buzzards Bay, Massachusetts contaminated with No. 2 fuel oil. The persistance of alkyldibenzothiophenes in sediments and tissues of organisms in contaminated environments has led to the suggestion that these compounds might serve as oil pollution markers (Friocourt *et al.*, 1982; Ogata and Fujisawa, 1985).

These studies of environments contaminated by oil spillage give indication of the recalcitrance of alkyldibenzothiophenes relative to other aromatic compounds present in crude oil and the relative increase in recalcitrance caused by increased alkyl-substitution of the dibenzothiophene nucleus. However, even in the studies described above, which focused on the persistence of dibenzothiophenes in contaminated environments and the resulting accumulation in tissues of living organisms, the dibenzothiophenes were indeed biodegraded to significant extents. Other studies have demonstrated the biodegradation of dibenzothiophenes in the complex mixture of crude oil in contaminated environments (Hostettler and Kvenvolden, 1994; Wang and Fingas, 1995), within petroleum reservoirs (Westlake, 1983; Williams *et al.*, 1985), as well as in laboratory mixed cultures of petroleum-degrading bacteria (Fayad and Overton, 1995; Fedorak and Westlake, 1983, 1984; Wang and Fingas, 1995) and pure cultures (Bayona *et al.*, 1986; Foght and Westlake, 1988). The primary findings of these investigations are highlighted below.

Geochemical changes in crude oil spilled from the *Exxon Valdez* supertanker into Prince William Sound (Alaska) were investigated by Hostettler and Kvenvolden (1994). They observed a decrease in the amounts of alkylated phenanthrenes relative to the alkylated dibenzothiophenes, suggesting that dibenzothiophenes are more resistant to biodegradation. However, the dibenzothiophenes were observed to disappear progressively until all that remained were trace levels of C_3-dibenzothiophenes in the most highly degraded samples.

Wang and Fingas (1995) reported a GC-MS method for the differentiation and source identification of crude and weathered oils by the use of methyldibenzothiophene ratios. They demonstrated that the ratios of isomeric methyldibenzothiophenes in crude oils did not significantly change as a result of evaporation or *in situ* burning of crude oil. However, the ratios of these isomers in samples from a 12-year-old controlled arctic oil spill at Baffin Island and in samples recovered from mixed petroleum-degrading laboratory cultures were affected by biodegradation. They reported that 2- and 3-methyldibenzothiophene were degraded the fastest, followed by 4-methyldibenzothiophene and 1-methyldibenzothiophene, which was the most recalcitrant isomer. The ring numbering convention for dibenzothiophene is shown below.



Westlake (1983) presented evidence that the saturate fraction and the aromatic fraction, including the condensed thiophenes, of Kumak crude oils were susceptible to biodegradation within their natural reservoir. The oils recovered from greater depths within the reservoir gave GC profiles consistent with increased recalcitrance to biodegradation. Westlake (1983) hypothesized that oxygen, nutrients, and microorganisms responsible for the biodegradation of oil would reach oil pools through meteoric water via faults, fractures, and other conduits. Presumably this effect would diminish at greater depths within the reservoir resulting in decreased biodegradation of the petroleum components. The increased temperature at greater depths within the reservoir would also likely limit microbial activity.

The biodegradation of South Texas Eocene oils in reservoirs ranging from approximately 350 m to 1300 m in depth was studied by Williams *et al.* (1986). Samples of 26 oils from 21 fields, representing various degradation stages, were analyzed. The depletion of substituted benzothiophenes and dibenzothiophenes was observed in the most extensively degraded samples, but information on the relative susceptibilities of these compounds was not obtained.

Fedorak and Westlake (1981) observed that the aromatic compounds in Prudhoe Bay crude oil were more readily attacked than the saturated compounds by the microbial populations in a pristine marine environment and a commercial harbour. Fayad and Overton (1995) observed a similar biodegradation pattern, for oil spilled during the 1991 Gulf war,

in microcosms containing Gulf seawater. The biodegradation of polycyclic aromatic compounds including the dibenzothiophenes was observed to proceed much faster than the degradation of the saturated *n*-alkane fraction of the crude oil. At oil concentrations of 20 g/L, dibenzothiophene was gone within 144 h incubation and the methyldibenzothiophenes had been significantly degraded. The 2- and 3-methyl-substituted isomers were the most susceptible to biodegradation, followed by 4-methyldibenzothiophene, and 1-methyldibenzothiophene, which was the most recalcitrant. This order of susceptibility of the methyldibenzothiophenes to biodegradation was the same as that observed by Wang and Fingas (1995) in contaminated environments and mixed petroleum-degrading cultures. At oil concentrations of 5 g/L, Fayad and Overton (1995) also examined the effect of nutrient amendment of seawater on degradation patterns. The rate of degradation of 1- and 2-methylnaphthalene, dibenzothiophene, and all four isomers of methyldibenzothiophene was greatly reduced by the nutrient amendment, while the degradation of saturated *n*-alkanes proceeded at a significantly greater rate. It seems that the activity of the microorganisms responsible for the degradation of the aromatic compounds in the crude oil was reduced by the nutrient amendment. This is an important observation because the addition of nutrients during bioremediation is proposed to stimulate biodegradation by altering the C:N:P ratio to one more favourable for bacterial growth. However, this may decrease the biodegradation of the aromatic components of crude oil which are of the most concern due to their known toxic, mutagenic, and carcinogenic properties.

Fedorak and Westlake reported the biodegradation of condensed thiophenes in Prudhoe Bay crude oil by mixed cultures of petroleum-degrading bacteria enriched from three different marine environments (Fedorak and Westlake, 1983) and greenhouse soil (Fedorak and Westlake, 1984). In both cases, the C_2-benzothiophenes were more susceptible to biodegradation than the C_3-benzothiophenes. The latter compounds were of approximately the same susceptibility as dibenzothiophene, which was degraded before the methyldibenzothiophenes. The C_2- and C_3-dibenzothiophenes increased in recalcitrance with increased substitution. In all of the enrichments, addition of N and P increased the rate and extent of degradation of condensed thiophenes. In fact, in the soil enrichment cultures, the addition of N and P promoted the degradation of most of the C_2-dibenzothiophenes and some of the C_3-dibenzothiophenes which persisted when N and P were not added to mineral medium.

Not only has the biodegradation of benzo- and dibenzothiophenes within crude oil been observed with complex microbial populations in contaminated environments and mixed cultures in laboratory studies, but it has also been demonstrated with pure cultures of petroleum-degrading *Pseudomonas* spp. Bayona *et al.* (1986) isolated a *Pseudomonas* sp.

from oil tanker ballast waters that grew on Arabian light crude oil. This strain degraded different fractions of petroleum at different rates: saturates > monocyclic aromatics > polycyclic aromatic hydrocarbons > polycyclic aromatic sulfur heterocycles. Increased methyl-substitution of dibenzothiophenes increased the recalcitrance towards biodegradation. Within a homologous series, the 2- and 3-methyldibenzothiophenes were degraded before the isomers substituted at positions 1 and 4. The general rule that methyl-substituted polycyclic aromatic compounds were preferentially degraded in compounds containing unsubstituted alpha and beta positions was observed to hold true for methyl-substituted phenanthrenes, dibenzothiophenes, pyrenes, and chrysenes.

Foght and Westlake (1988) isolated *Pseudomonas* sp. HL7b from a lake water enrichment that had grown with Norman Wells crude oil. This aromatic-hydrocarbon degrading bacterium grew on Prudhoe Bay crude oil and depleted C_2- and C_3-benzothiophenes, dibenzothiophene, methyldibenzothiophenes, and some C_2-dibenzothiophenes. As well, the isolate degraded naphthalene and alkylnaphthalenes, biphenyl and methylbiphenyls, and phenanthrene and alkylphenanthrenes, but not saturated hydrocarbons.

Thus, despite the fact that dibenzothiophenes are among the compounds in petroleum that are most resistant to biodegradation and that increased alkyl-substitution has been shown to give increased recalcitrance of dibenzothiophenes, there are numerous reports with pure cultures and mixed populations that demonstrate the biodegradation of condensed thiophenes within the complex mixture of compounds known as petroleum. These studies assessed the biodegradation of condensed thiophenes by the use of GC analysis to follow the loss of peaks from the chromatogram obtained, usually, with a sulfur-selective detector (FPD). However, because of the complex mixture of components present in petroleum, these methods are not amenable to the identification of metabolic intermediates, which gives an indication of the metabolic pathways whereby contaminants are degraded, nor do they allow for the identification of biotransformation products whose recalcitrance towards subsequent biodegradation and toxicity will influence the success of bioremediation (either intrinsic or engineered) to cleanup a contaminated environment.

The biodegradation of petroleum has been reported to cause a decrease in the content of saturate and aromatic fractions coupled with a corresponding increase in the content of polar N-, S-, and O-containing materials (Boehm *et al.*, 1981; Jobson *et al.*, 1972). This may be due to the accumulation of metabolic products which are not completely mineralized. Belkin *et al.* (1994) demonstrated that biodegradation of a polycyclic aromatic hydrocarbon mixture in percolating soil columns led to a corresponding increase in the genotoxic activity of column effluents. In some cases, the changes in

genotoxicity were paralleled with toxicity data. The possibility that the genotoxicity increases were due to accumulated intermediates from microbial metabolism of the polycyclic aromatic hydrocarbons in the soil columns was proposed by these researchers. Similar effects could result from the degradation of condensed thiophene-containing crude oils, with metabolites from the partial degradation of condensed thiophenes contributing to the overall effect. Studies of the microbial metabolism of condensed thiophenes which are focused on the identification of metabolites and biotransformation products have mostly been done using pure compounds, especially those which are commercially available, namely, benzothiophene and dibenzothiophene.

1.5 Aerobic microbial metabolism of pure condensed thiophenes

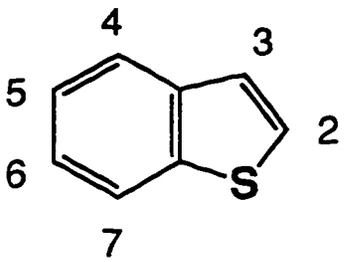
While the identification of metabolic pathways for the degradation of condensed thiophenes and of biotransformation products are most easily done with pure organosulfur compounds, many of the condensed thiophenes do not serve as sole carbon and energy sources for the growth of microorganisms (Fedorak, 1990). However, these condensed thiophenes are frequently metabolized in cultures of microorganisms grown on other organic compounds which do serve as carbon and energy sources. This phenomenon is known as cometabolism (Dalton and Stirling, 1982). Many of the studies of pure condensed thiophenes described below included other organic compounds as primary growth substrates.

1.5.1 Studies with benzothiophene and methylbenzothiophenes

Sagardía *et al.* (1975) described the biodegradation of benzothiophene by *Pseudomonas aeruginosa* PRG-1, isolated from oil-contaminated soil. Benzothiophene did not support the growth of the isolate, but was cometabolized in cultures grown on 0.05% yeast extract. The benzothiophene was supplied to cultures of the bacterium in a 5% light oil-basal medium system. This reduced the toxicity of benzothiophene to the bacterium, which had been observed when benzothiophene was supplied as a suspension directly in the aqueous phase. Growth of the culture resulted in emulsification of the oil phase and loss of 40% of the benzothiophene given within 6 days. Oxygen uptake rates of washed cell suspensions were increased when benzothiophene was included in the incubations. However, the identification of metabolites from bacterial oxidation of benzothiophene was not pursued in these studies.

Bohonos *et al.* (1977) observed the biodegradation of benzothiophene by mixed populations of microorganisms in water samples from eutrophic and oligotrophic freshwater environments and from aeration effluents of wastewater treatment plants. The

microorganisms present in the water samples were capable of biotransformation of benzothiophene only when naphthalene was included in the incubations. The authors were unable to obtain active enrichment cultures by subsequent transfer of the incubations containing benzothiophene and naphthalene. However, extracts of the water samples were analyzed by GC-MS, and the metabolites benzothiophene sulfoxide, 2,3-dihydroxy-2,3-dihydrobenzothiophene (both the *cis* and *trans* isomers), and benzothiophene-2,3-dione were tentatively identified. The ring numbering convention for benzothiophene is shown below.



Fedorak and Grbic´-Galic´ (1991) studied the aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene in a 1-methylnaphthalene (1-MN)-degrading mixed enrichment culture and in pure cultures of a *Pseudomonas* sp., designated strain BT1, which was isolated from the mixed culture. The 1-MN-degrading mixed enrichment culture was originally inoculated from a petroleum-degrading mixed culture derived from fuel-contaminated beach material from Shell Lake, Northwest Territories (Fedorak and Peakman, 1992). Neither of the heterocyclic compounds would support growth of the 1-MN-degrading mixed culture, but were biotransformed by the culture when it was grown on 1-MN, glucose, or peptone (Fedorak and Grbic´-Galic´, 1991). Cometabolism of benzothiophene yielded benzothiophene-2,3-dione, whereas cometabolism of 3-methyl-benzothiophene yielded the corresponding sulfoxide and sulfone. The identifications of the metabolites were made by GC-MS and GC-Fourier transform infrared (GC-FTIR) analyses and verified by comparisons with authentic standards of the dione and sulfone.

After several months of bi-weekly transfers of the mixed culture with 1-MN as growth substrate, the predominant microorganism in the mixed culture was isolated, designated strain BT1, and shown to mineralize the ^{14}C-containing aromatic hydrocarbons naphthalene, biphenyl, and phenanthrene, but not saturated hydrocarbons (Fedorak and Grbic´-Galic´, 1991). In pure cultures with isolate BT1 grown on 1-MN, the same metabolites were produced from benzothiophene and 3-methylbenzothiophene as had been observed previously with the mixed culture. Isolate BT1 was shown to be unable to

further metabolize 3-methylbenzothiophene sulfone in the presence of 1-MN, and the further metabolism of benzothiophene-2,3-dione was not tested due to its limited supply. When 3-methylbenzothiophene was added to Prudhoe Bay crude oil, it was oxidized to the sulfoxide and sulfone by strain BT1 as it grew on the aromatic hydrocarbons in the crude oil. Benzothiophene-2,3-dione was found to be chemically unstable when incubated with Prudhoe Bay crude oil, so its formation by isolate BT1 incubated with oil and benzothiophene could not be determined.

The results of these studies with isolate BT1 (Fedorak and Grbic´-Galic´, 1991) led to the prediction that of the other five possible isomers of methyl-substituted benzothiophene, those which have a methyl group on the benzene ring would be cometabolized to give methylbenzothiophene-2,3-diones, whereas those which have a methyl group on the thiophene ring would give the corresponding sulfoxides and sulfones. These methylbenzothiophenes were synthesized and tested in cometabolism studies with cultures of isolate BT1 grown on 1-MN or glucose (Saftic´ *et al.*, 1992). The prediction was observed to hold true for all isomers of methylbenzothiophene except for 7-methylbenzothiophene, which yielded the methylbenzothiophene-2,3-dione, sulfoxide, and sulfone, among other unidentified products. As well, 2,3-dimethylbenzothiophene was synthesized and cometabolism studies showed that it was oxidized to the sulfoxide and sulfone in cultures of isolate BT1.

Recent studies have provided insight into the oxidation of the sulfur atom of benzothiophenes to form sulfoxides and sulfones by aromatic hydrocarbon-degrading bacteria. Recombinant bacteria expressing toluene dioxygenase (Allen *et al.*, 1995) and naphthalene dioxygenase (Selifonov *et al.*, 1996) have been shown to catalyze monooxygenation reactions of numerous organosulfur compounds, including 3-methylbenzothiophene (Selifonov *et al.*, 1996), to form sulfoxides and sulfones, as dead-end products. Thus, sulfoxidation by aromatic hydrocarbon-degrading bacteria is likely a fortuitous oxidation by the dioxygenase enzymes. The primary function of these enzymes is the dioxygenation of aromatic rings to form dihydrodiols as precursors to aromatic ring cleavage (Gibson and Subramanian, 1984).

A recent study of the biotransformation of benzothiophene by the isopropylbenzene-degrading bacterium *Pseudomonas putida* RE204 has also contributed insight into the mechanism of formation of the benzothiophene-2,3-diones (Eaton and Nitterauer, 1994). Isopropylbenzene-2,3-dioxygenase (enzyme A, Figure 1.4) attack at positions 2 and 3 of benzothiophene (compound 11, Figure 1.4) gives *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene (compound 29). This thiohemiacetal (compound 29) can undergo spontaneous opening of the ring to yield 2'-mercaptomandelaldehyde (compound 30) and

subsequently recyclize to form *trans*-2,3-dihydroxy-2,3-dihydrobenzothiophene (compound 31) which was purified and identified (Eaton and Nitterauer, 1994). In a mutant strain of *P. putida* (designated RE213), which was deficient in 2,3-dihydroxy-2,3-dihydroisopropylbenzene dehydrogenase (enzyme B), this was the sole fate of *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene (compound 29). With strain RE204, a competing dehydrogenation reaction catalyzed by enzyme B converted *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene (compound 29) into 2-hydroxy-3-oxo-2,3-dihydrobenzothiophene (compound 32). This keto-tautomer of 2,3-dihydroxybenzothiophene is not a substrate for the ring cleavage dioxygenase (3-isopropylcatechol 2,3-dioxygenase, enzyme C), but spontaneously opens to form 2-mercaptophenylglyoxaldehyde (compound 33) and subsequently 2-mercaptophenylglyoxalate (compound 34). 2-Mercaptophenylglyoxalate (compound 34) was shown to undergo an acid-catalyzed dehydration reaction to form benzothiophene-2,3-dione (compound 35). Thus, in the studies described above (Bohonos *et al.*, 1977; Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992) the detection of benzothiophene-2,3-diones in extracts of acidified cultures suggests that thiophene ring cleavage had probably taken place. These findings also explain the result of Bohonos *et al.* (1977) that both *cis*- and *trans*-2,3-dihydroxy-2,3-dihydrobenzothiophene were detected in extracts of environmental water samples incubated with benzothiophene.

Eaton and Nitterauer (1994) also observed isopropylbenzene-2,3-dioxygenase (enzyme A, Figure 1.4) attack of the homocyclic ring of benzothiophene (compound 11, Figure 1.4) at positions 4 and 5 to yield *cis*-4,5-dihydroxy-4,5-dihydrobenzothiophene (compound 36). This compound 36 accumulated with the mutant strain RE213 that is deficient in 2,3-dihydroxy-2,3-dihydroisopropylbenzene dehydrogenase (enzyme B). However, with cell extracts of strain RE204, enzyme B converted this dihydrodiol (compound 36) to 4,5-dihydroxybenzothiophene (compound 37) and enzyme C (3-isopropylcatechol 2,3-dioxygenase) catalyzed meta cleavage of compound 37 to form *cis*-4-(3-oxo-2,3-dihydrothienyl)-2-hydroxybuta-2,4-dienoate (compound 38) which was purified and identified as *trans*-4-(3-hydroxy-2-thienyl)-2-oxobut-3-enoate (compound 39). Thus, the cometabolic oxidation of benzothiophene catalyzed by enzymes for the biodegradation of isopropylbenzene results in cleavage of both the homocyclic and heterocyclic rings of benzothiophene (Eaton and Nitterauer, 1994).

1.5.2 Studies with dibenzothiophene and methyldibenzothiophenes

The commercially available compound dibenzothiophene has been frequently used as a model compound for studies of the microbial desulfurization of petroleum or coal (Izumi *et al.*, 1994; Kayser *et al.*, 1993; Omori *et al.*, 1992; Wang and Krawiec, 1994).

The sulfur atom in the central thiophene ring is thought to be present in a sterically-hindered environment that is likely similar to that of the thiophenic sulfur in coal and higher boiling petroleum fractions. Furthermore, dibenzothiophene and its alkyl-derivatives are frequently among the most abundant organosulfur compounds actually present in crude oils.

The biocatalytic activity which is needed for a microbial desulfurization process will specifically cleave the sulfur atom from dibenzothiophene without breaking the hydrocarbon backbone of the molecule, because this would decrease the fuel value of the desulfurized product. This biocatalytic activity has been reported for numerous bacterial strains and has been named the "4S pathway" (Gallagher *et al.*, 1993; Olson *et al.*, 1993; Wang and Krawiec, 1994). The pathway proceeds via oxidation of the sulfur atom of dibenzothiophene to the sulfoxide and sulfone, with subsequent thiophene ring cleavage and release of sulfur yielding the desulfurized metabolite 2-hydroxybiphenyl (Izumi *et al.*, 1994; Kayser *et al.*, 1993; Olson *et al.*, 1993; Omori *et al.*, 1992; Wang and Krawiec, 1994). This compound accumulates in stoichiometric amounts and is not further degraded by bacteria which utilize the 4S pathway to metabolize dibenzothiophene as a sulfur source for growth.

However, in petroleum-contaminated environments, particularly marine environments, available sulfur is not likely to be the nutrient limiting the growth of microorganisms. Thus, it is not likely that the biodegradation of dibenzothiophenes in oil-contaminated environments will proceed by the 4S pathway. Rather, the oxidation of dibenzothiophenes is likely to proceed by oxidation of the carbon backbone as microorganisms scavenge for carbon and energy. The dibenzothiophene backbone may serve as a source of carbon and energy or it may be cometabolized as other aromatic compounds in petroleum serve as growth substrates. Thus, because the 4S pathway is not expected to be of significant relevance to the metabolism of dibenzothiophenes in petroleum-contaminated environments, the literature describing this pathway and the isolates reported to possess this capability are only briefly reviewed herein.

Rhodococcus sp. IGTS8, isolated for the ability to utilize organic sulfur in coal as its sole sulfur source for growth, has been shown to oxidize dibenzothiophene via the 4S pathway (Gallagher *et al.*, 1993; Kayser *et al.*, 1993; Olson *et al.*, 1993) and the genetic basis of this ability has been characterized (Denome *et al.*, 1993a, 1994; Piddington *et al.*, 1995). Other isolates reported to oxidize dibenzothiophene by this pathway include *Rhodococcus erythropolis* D-1 (Izumi *et al.*, 1994; Ohshiro *et al.*, 1994), *R. erythropolis* N1-36 (Wang *et al.*, 1996; Wang and Krawiec, 1994, 1996), and *Rhodococcus* sp. SY1 (Omori *et al.*, 1992, 1995). Biodesulfurization of 2,8-dimethyldibenzothiophene, 4,6-dimethyldibenzothiophene, and benzo[*b*]naphtho]2,1-*d*]thiophene by whole cells of *R.*

erythropolis H-2 (Ohshiro *et al.*, 1996) and of 4,6-diethyldibenzothiophene by *Arthrobacter* sp. (Lee *et al.*, 1995) have also been reported.

The thermophilic microorganism *Sulfolobus acidocaldarius*, which was also studied as a potential biodesulfurization catalyst, was shown to release sulfate from dibenzothiophene (Kargi, 1987; Kargi and Robinson, 1984), and from thianthrene and thioxanthine (Kargi, 1987). In those studies no other organic carbon source was supplied, but this facultative autotroph could also utilize carbon dioxide as sole carbon source (Kargi, 1987). Kankipati and Ju (1994) tentatively identified 2'-hydroxybiphenyl-2-sulfinic acid as an intermediate in the oxidation of the sulfur atom of dibenzothiophene to sulfate. They also tentatively identified 4-hydroxybenzoic acid as a metabolite, suggesting that the carbon backbone was further degraded after the sulfur atom was released. Thus, since the pathway used by this isolate does provide organic carbon to the microorganism it seems more relevant to an oil-contaminated environment than the 4S pathway. However, the temperature range of 60 to 90°C required for the growth of this thermophile likely means that this microorganism and the pathway it employs for dibenzothiophene oxidation do not accurately represent biodegradative processes that would occur in most oil-contaminated environments.

Other microorganisms have also been reported to initiate oxidation of dibenzothiophene by attack of the sulfur atom, although the 4S pathway is not utilized. The fungi *Cunninghamella elegans* (Crawford and Gupta, 1990) and *Pleurotus ostreatus* (Bezalel *et al.*, 1996) oxidize dibenzothiophene to the sulfoxide and sulfone, the latter of which accumulates as a dead-end product.

As well, van Afferden *et al.* (1990) isolated a *Brevibacterium* sp. which utilized dibenzothiophene as sole source of carbon, sulfur, and energy for growth. This isolate initiated attack of dibenzothiophene by oxidation to the sulfoxide and sulfone. The further metabolism of dibenzothiophene sulfone was reported to proceed via angular dioxygenation at the bridgehead carbon beside the sulfur atom and the adjacent methine carbon, with spontaneous decay of the dihydroxylated dibenzothiophene sulfone to form a sulfinated dihydroxybiphenyl structure (van Afferden *et al.*, 1993). Subsequent release of sulfite occurred concomitantly with degradation of the dihydroxylated biphenyl metabolite via benzoate. By this mechanism, the complete mineralization of dibenzothiophene was accomplished by this strain. This isolate has been studied primarily as a potential biodesulfurization catalyst, but the fact that this pathway for dibenzothiophene oxidation releases carbon and energy for the growth of the strain suggests that this may also be relevant to the biodegradation of dibenzothiophenes in oil-contaminated environments. This is even more likely if other microorganisms such as the fungi described above (Bezalel *et*

al., 1996; Crawford and Gupta, 1990) or aromatic hydrocarbon-degrading bacteria with aryl dioxygenases that fortuitously catalyze sulfoxygenation reactions (Allen *et al.*, 1995; Selifonov *et al.*, 1996) are present in contaminated environments and are actively involved in the oxidation of dibenzothiophenes to their corresponding sulfones which they do not further metabolize. Dahlberg *et al.* (1993) reported an *Arthrobacter* sp. that was capable of metabolizing dibenzothiophene sulfone via the same pathway reported by van Afferden *et al.* (1993), although this isolate could not catalyze the initial oxidation of dibenzothiophene to its sulfone.

Another pathway for dibenzothiophene oxidation which likely gives an accurate reflection of how dibenzothiophenes are metabolized in oil-contaminated environments in the presence of numerous abundant saturated and aromatic hydrocarbons is that which results from initial attack, not of the sulfur atom, but of the homocyclic ring. This pathway, called the "Kodama pathway" after the first researcher to study it, is shown in Figure 1.5 as it is currently understood, and results in degradation of one of the homocyclic rings of dibenzothiophene (compound 14) to form 3-hydroxy-2-formylbenzothiophene (compound 45).

Yamada *et al.* (1968) reported the isolation of numerous soil *Pseudomonas* spp., three of which were classified as two new species designated *Pseudomonas abikonensis* and *Pseudomonas jianii*. These soil bacteria were able to oxidize dibenzothiophene to water-soluble organic products. Nakatani *et al.* (1968) varied culture conditions to optimize the abilities of these isolates to oxidize dibenzothiophene. Kodama *et al.* (1970, 1973) identified compounds 42 through 45 (Figure 1.5) from dibenzothiophene oxidation in pure cultures of these *Pseudomonas* spp. and proposed the original Kodama pathway. The oxidation of dibenzothiophene via the Kodama pathway by *P. jianii* was shown to be cometabolic, because other substrates were required for growth and dibenzothiophene oxidation to occur (Kodama, 1977a, b).

The direct precursor to enzymatic formation of 3-hydroxy-2-formylbenzothiophene (compound 45) was shown by Kodama *et al.* (1973) to be compound 44. Compound 43 was thought to exist in chemical equilibrium with compound 42, which was thought to be the direct precursor to compound 44 (Kodama *et al.*, 1973). In Figure 1.5, compound 43, the hemiacetal of compound 42, has been included as a direct precursor to compound 44 because of the recent work of Denome *et al.* (1993b) which showed that a single genetic pathway controls the metabolism of dibenzothiophene (compound 14) to 3-hydroxy-2-formylbenzothiophene (compound 45) and the metabolism of naphthalene to salicylaldehyde. Recent studies of the upper pathway for naphthalene metabolism have shown that the hemiacetal 2-hydroxychromene-2-carboxylate, which is the analog of

compound 43 for naphthalene metabolism, is the direct precursor to *trans-o*-hydroxybenzylidenepyruvate, which is the analog of compound 44 for naphthalene metabolism (Eaton and Chapman, 1992). Thus, it is likely that the hemiacetal compound 43 is also the direct precursor to formation of *trans*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (compound 44) in the Kodama pathway of dibenzothiophene metabolism.

Laborde and Gibson (1977) also contributed to the understanding of the Kodama pathway by their study of dibenzothiophene metabolism in succinate-grown cultures of a *Beijerinckia* sp. This isolate also converted dibenzothiophene to 3-hydroxy-2-formylbenzothiophene (compound 45, Figure 1.5), but transiently accumulated (+)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene (compound 40) in the process. This compound was purified, identified, and shown to be converted by crude cell extracts and purified 1,2-dihydroxy-1,2-dihydronaphthalene dehydrodrogenase to 1,2-dihydroxydibenzothiophene (compound 41). The further enzymatic conversion of this compound to 3-hydroxy-2-formylbenzothiophene, which was not further metabolized, was also demonstrated.

The cometabolic oxidation of dibenzothiophene to 3-hydroxy-2-formylbenzothiophene by the Kodama pathway has also been reported to occur as a plasmid-mediated process in two *Pseudomonas* spp. (Monticello *et al.*, 1985). In both of these strains, dibenzothiophene oxidation was inducible by naphthalene, salicylate, or dibenzothiophene and was repressed by succinate. *Pseudomonas* sp. HL7b was also able to cometabolize dibenzothiophene via the Kodama pathway with the production of 3-hydroxy-2-formylbenzothiophene (Foght and Westlake, 1988). Strain HL7b was able to constitutively oxidize dibenzothiophene, although it would not utilize this compound as sole carbon source. In both of these studies, as well as in those of Kodama *et al.* (1970, 1973), a non-enzymatic dimerization product of 3-hydroxy-2-formylbenzothiophene was produced in culture or during the purification of metabolites, and identified as 3-oxo-[3'-hydroxy-thianaphthenyl-(2)-methylene]-dihydrothianaphthene.

Many of the isolates that have been reported to oxidize dibenzothiophene to 3-hydroxy-2-formylbenzothiophene by the Kodama pathway have also been observed to oxidize the sulfur atom of dibenzothiophene to give dibenzothiophene sulfoxide, which accumulates as a dead-end product (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977). Mormile and Atlas (1989) described a strain of *Pseudomonas putida* which oxidized dibenzothiophene to 3-hydroxy-2-formylbenzothiophene and dibenzothiophene sulfone. Neither of these metabolites were further degraded by this bacterium. The oxidation of the sulfur atom of dibenzothiophene to give sulfoxides and sulfones is likely a fortuitous sulfoxygenation catalyzed by the aryl dioxygenase enzymes responsible for the initial dioxygenation of dibenzothiophene (Allen *et al.*, 1995; Selifonov *et al.*, 1996).

Because 3-hydroxy-2-formylbenzothiophene, dibenzothiophene sulfoxide, and dibenzothiophene sulfone have been repeatedly reported as products accumulating from dibenzothiophene oxidation, Mormile and Atlas (1988) studied the further biodegradation of these metabolites in mixed cultures inoculated with soil and with sediment of a polluted creek. 3-Hydroxy-2-formylbenzothiophene was depleted from both of the mixed cultures relative to the sterile controls. As well, CO_2 production from dibenzothiophene sulfoxide and sulfone was observed in the mixed culture inoculated with sediment. However, the biodegradation of 3-hydroxy-2-formylbenzothiophene, dibenzothiophene sulfoxide, and dibenzothiophene sulfone did not lead to release of sulfate into the medium. Thus, the authors concluded that while these metabolites were further degraded, with the release of CO_2, they were not completely mineralized.

Saftic´ *et al.* (1993) studied the aerobic cometabolism of all four isomers of methyl-substituted dibenzothiophene in pure cultures of three *Pseudomonas* spp. The methyldibenzothiophenes were synthesized for use in those studies. The strains tested were isolate BT1 of Fedorak and Grbic´-Galic´ (1991), which was described above, and two new strains, designated as W1 and F, which were isolated from enrichment cultures inoculated with water from a tropical freshwater aquarium and with activated sludge from a municipal wastewater treatment system, respectively. These enrichments were done with 1-MN and dibenzothiophene provided as carbon and energy sources. The three pure strains were maintained by weekly transfers into fresh medium with 1-MN provided as carbon and energy source. The three isolates were shown to grow on 1-MN and cometabolize dibenzothiophene to benzothiophene-2,3-dione, 3-hydroxy-2-formylbenzothiophene, dibenzothiophene sulfoxide, and dibenzothiophene sulfone. Isolates W1 and F also fortuitously oxidized a small portion of the 1-MN to 1-naphthalenemethanol and 1-naphthoic acid which were dead-end products that were not further metabolized. Neither of these compounds would support the growth of either isolate. Isolate BT1 was able to survive repeated transfers with dibenzothiophene as sole carbon and energy source, indicating that the isolate was capable of growth on dibenzothiophene. However, neither isolate W1 nor isolate F were able to grow on dibenzothiophene. Isolate W1 was able to mineralize ^{14}C-labeled naphthalene and phenanthrene, where isolate F only mineralized naphthalene. The mineralization of these labeled compounds was tested with Norman Wells crude oil spiked with these compounds provided as the growth substrate.

Cultures of each of these three isolates grown on 1-MN or glucose in the presence of an isomer of methyldibenzothiophene were extracted under acidic conditions and the extracts were analyzed to identify biotransformation products. For all isomers, the unsubstituted ring was preferentially oxidized and cleaved by the Kodama pathway to form

methyl-substituted 3-hydroxy-2-formylbenzothiophenes and benzothiophene-2,3-diones. In addition, other oxidation products were detected for some of the isomers, including methyldibenzothiophene sulfones and dibenzothiophene methanols. Hydroxylated methyldibenzothiophenes which had a phenolic hydroxyl group were also detected and possibly resulted from acid-catalyzed dehydration of methyldibenzothiophene dihydrodiols.

1.5.3 The qualitative nature of most studies of the biodegradation of condensed thiophenes that are relevant to petroleum-contaminated environments

Quantitative investigations into the microbial metabolism of pure condensed thiophenes have been mostly limited to isolates which metabolize dibenzothiophene by the 4S pathway (Izumi *et al.*, 1994; Lee *et al.*, 1995; Ohshiro *et al.*, 1994, 1996; Omori *et al.*, 1992, 1995; Wang and Krawiec, 1996; Wang *et al.*, 1996). In those studies, the kinetics of dibenzothiophene depletion and the concomitant production of 2-hydroxybiphenyl and sometimes sulfate have been reported for many of the isolates with this activity. The potential of a biocatalytic desulfurization process depends upon maximizing the release of sulfur from dibenzothiophene and the accumulation of 2-hydroxybiphenyl without further metabolism. As well, to commercially develop a biodesulfurization process quantitative data are required to determine rates of reactions, estimate economic viability, and monitor strain development programs.

Brevibacterium sp. DO was shown to completely degrade 3 mM dibenzothiophene, with transient accumulation of dibenzothiophene sulfoxide and sulfone that were subsequently degraded, resulting in the release of 3 mM sulfate (van Afferden *et al.*, 1990). Analysis of culture fluid from stationary phase cultures did not detect any water-soluble organic metabolites so it was concluded that the hydrocarbon backbone of the molecule was also completely mineralized. This is the only report of the complete mineralization of dibenzothiophene, and, because the pathway used provides carbon and energy for the growth of the bacterium, it may represent a pathway for dibenzothiophene degradation that is relevant to petroleum-contaminated environments.

However, other studies of the microbial metabolism of pure condensed thiophenes, that are likely relevant to petroleum-contaminated environments because they result in degradation of the carbon backbone of the molecule, have seldom reported quantitative data. Rather, these studies have focused on the identification of metabolites which accumulate in cultures, either transiently or as dead-end products. When quantitative data have been presented, either substrate depletion or metabolite formation were monitored, but not both. For example, Sagardía *et al.* (1975) reported a 40% loss of benzothiophene over

6 days of incubation with *Pseudomonas aeruginosa* PRG-1, but the quantification of water-soluble metabolites was not pursued. Kodama *et al.* (1970, 1973) reported the yields of three of the purified metabolites from dibenzothiophene oxidation by the Kodama pathway and by my calculations these account for 31% of the dibenzothiophene added to the culture. However, the amount of dibenzothiophene remaining at the end of the incubation was not reported so it is not known if a mass balance existed. Conversely, Mormile and Atlas (1989) monitored the depletion of dibenzothiophene, but did not quantify the amounts of dibenzothiophene sulfone and 3-hydroxy-2-formylbenzothiophene that were produced. I am not aware of any other studies that have reported quantitative data for oxidation of dibenzothiophene by bacteria which utilize the Kodama pathway or for oxidation of benzothiophene, and none that have established a mass balance. Quantitative data of this nature is important in order to assess the significance of the metabolites which are detected and determine whether they are dead-end products or transiently accumulating intermediates.

Quantitative studies with benzothiophenes were not attempted by Fedorak and Grbic´-Galic´ (1991) or by Saftic´ *et al.* (1992), because the volatility of these compounds would have necessitated extensive sterile controls to account for evaporation of substrates. The limited supply of synthesized methylbenzothiophenes meant that this was not feasible. The limited supply of synthesized methyldibenzothiophenes also restricted the quantitative work that could have been done by Saftic´ *et al.* (1993), although volatility was not as serious a concern.

In addition to these factors, the depletion of benzo- and dibenzothiophenes and the formation of sulfur-containing metabolites were monitored in these previous studies (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992, 1993) by GC analysis of culture extracts with a flame ionization detector (FID) and a sulfur-selective FPD used to simultaneously analyze the column effluent which was split between the two detectors. GC analysis to quantify the amounts of metabolites produced would have required authentic standards of each metabolite to construct calibration curves, since the response of each of the detectors would vary for different compounds. The non-linear response of the FPD to organic sulfur further complicates its use for quantitative analysis (Wenzel and Aiken, 1979). A gas chromatograph with an atomic emission detector (AED), which gives a linear response to sulfur in all organic forms that are amenable to GC analysis (Andersson and Schmid, 1993), would have allowed quantification of the amount of sulfur-containing substrate and metabolites present in a culture extract without authentic standards of each metabolite. However, during these previous studies (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992, 1993) a GC with an AED was not available to that research program.

1.6 Research overview and objectives

The research described in the following six chapters of this dissertation began in September of 1992, and is a continuation of the research program that investigated the cometabolism of benzothiophene, all six isomers of methylbenzothiophene, and 2,3-dimethylbenzothiophene by *Pseudomonas* sp. BT1 (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992), and of dibenzothiophene and all four isomers of methyldibenzothiophene by *Pseudomonas* spp. BT1, W1, and F (Saftic´ *et al.*, 1993). Collaboration with a chemist, Dr. J. T. Andersson (University of Münster), who supplied 16 condensed thiophenes for the project described in this dissertation, provided a unique opportunity to study the biodegradation of compounds that are not commercially available. Most of this work was funded and driven by a contract through the Environment Canada, Groundwater and Soil Remediation Program (Contract KA168-2-2191). The objective of the contract was to identify metabolites from a variety of organosulfur compounds found in petroleum. The work scope of the contract precluded investigations into the biochemical mechanisms of formation of these metabolites.

This study of the aerobic microbial metabolism of condensed thiophenes found in petroleum focused on numerous chemically-synthesized compounds, and so the extent of quantitative studies that could be done was again limited by the supply of these compounds. As well, for most of this project, a GC with an AED was not available for quantitative analysis. Due to these limitations, the research described in Chapters 2 to 5 of this dissertation was focused primarily on the qualitative identification of metabolites detected in extracts of bacterial cultures incubated with numerous chemically-synthesized condensed thiophenes. A recently-acquired GC-AED was available during the completion of the studies described in Chapters 5 and 6, and was used for preliminary quantitative investigations. The research described in Chapter 7 was begun after the GC-AED was acquired, and experiments throughout were designed to provide quantitative data. The specific objectives of the research described in each of these chapters are as follows:

Chapter 2. To determine if *Pseudomonas* spp. W1 and F (Saftic´ *et al.*, 1993) were able to oxidize benzothiophene and all six isomers of methylbenzothiophene and give the same transformation products as observed previously with *Pseudomonas* sp. BT1 (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992) and *Pseudomonas pseudoalcaligenes* strain SB(G) (Gonçalves, 1993).

Chapter 3. To identify and determine the mechanism of formation of some high-molecular-weight sulfur-containing products that were detected (Chapter 2) as products from benzothiophene and those methylbenzothiophenes that were substituted on the homocyclic ring.

Chapter 4. To determine if *Pseudomonas* spp. BT1, W1, and F were able to oxidize six isomers of dimethyl-substituted benzothiophene and to identify biotransformation products.

Chapter 5. To determine if *Pseudomonas* spp. BT1, W1, and F and numerous petroleum-degrading mixed cultures were able to oxidize three isomers of dimethyl-substituted dibenzothiophene and to identify biotransformation products.

Chapter 6. To determine if *Pseudomonas* spp. BT1, W1, and F and a cyclohexane-degrading bacterium were able to oxidize 1,2,3,4-tetrahydrodibenzothiophene and to identify biotransformation products. As well, quantitative studies of dibenzothiophene metabolism by *Pseudomonas* spp. BT1, W1, and F were done to determine if a sulfur mass balance could be achieved.

Chapter 7. To determine if *Pseudomonas* spp. BT1, W1, and F were able to grow on and/or to cometabolize naphtho[2,1-*b*]thiophene and naphtho[2,3-*b*]thiophene, and to identify and quantify biotransformation products.

1.7 Organization of this dissertation

This dissertation is prepared in "paper format" so that the results of the research described in each of Chapters 2 through 7 are presented together with a brief introduction, description of the materials and methods used, and discussion of the results. As with this introduction (Chapter 1), the relevant tables and figures which are cited in the text are grouped together at the end of each chapter, just before the list of literature citations. The overall discussion of the results and suggestions for future research are presented in Chapter 8. The appendices describe some research that was conducted to further characterize isolates W1, BT1, and F. These appendices are also presented in paper format.

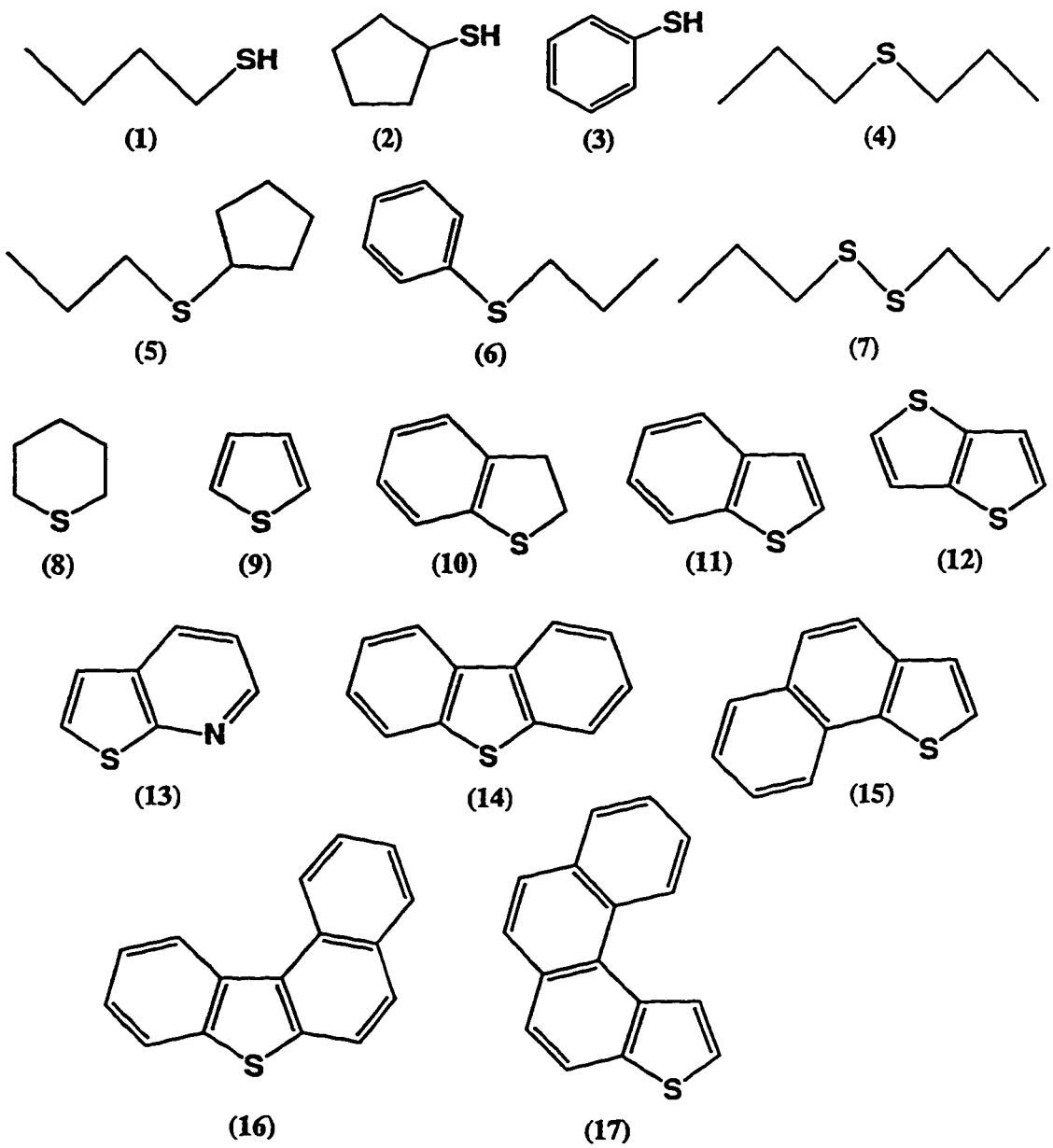


Figure 1.1 Structures that are representative of the various types of organosulfur compounds found in petroleum. Chemical designations: (1), alkane thiols; (2), cycloalkane thiols; (3), arene thiols; (4), dialkyl sulfides; (5), alkyl cycloalkyl sulfides; (6), alkyl aryl sufides; (7), polysulfides; (8), cyclic sulfides; (9), thiophenes; (10), thiaindanes; (11), benzothiophenes; (12), thienothiophenes; (13), thienopyridines; (14), dibenzothiophenes; (15), naphthothiophenes; (16), benzonaphthothiophenes; (17), phenanthrothiophenes (After Czogalla and Boberg, 1983; Foght *et al.*, 1990).

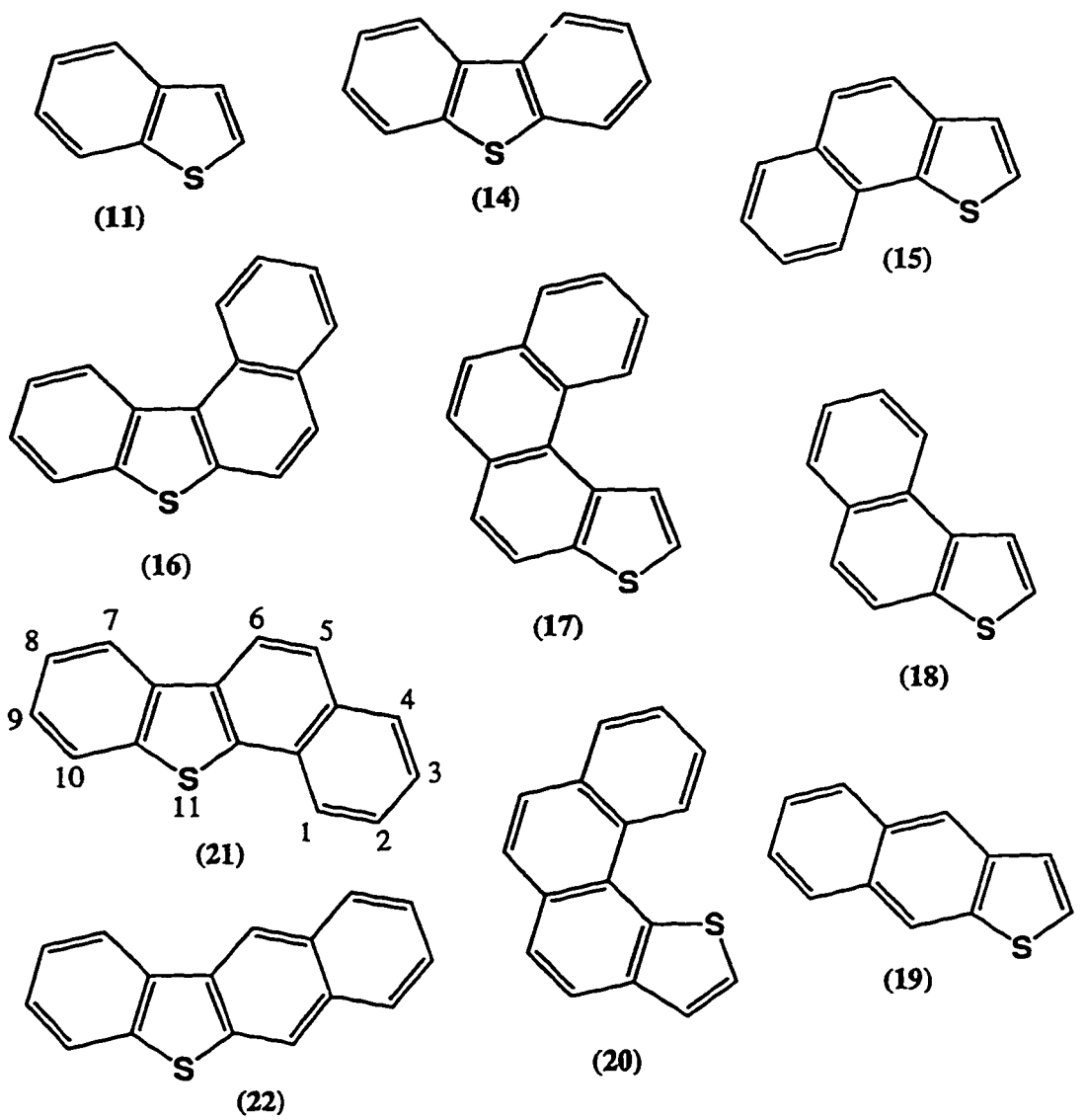


Figure 1.2 Structures of some of the condensed thiophenes that have been tested for toxicity, mutagenicity, and bioaccumulation potential. Chemical designations: (11), benzothiophene; (14), dibenzothiophene; (15), naphtho[1,2-*b*]thiophene; (16), benzo[*b*]naphtho[1,2-*d*]thiophene; (17), phenanthro[3,4-*b*]thiophene; (18), naphtho[2,1-*b*]thiophene; (19), naphtho[2,3-*b*]thiophene; (20), phenanthro[4,3-*b*]thiophene; (21), benzo[*b*]naphtho[2,1-*d*]thiophene; (22), benzo[*b*]naphtho[2,3-*d*]thiophene (After Jacob, 1990).

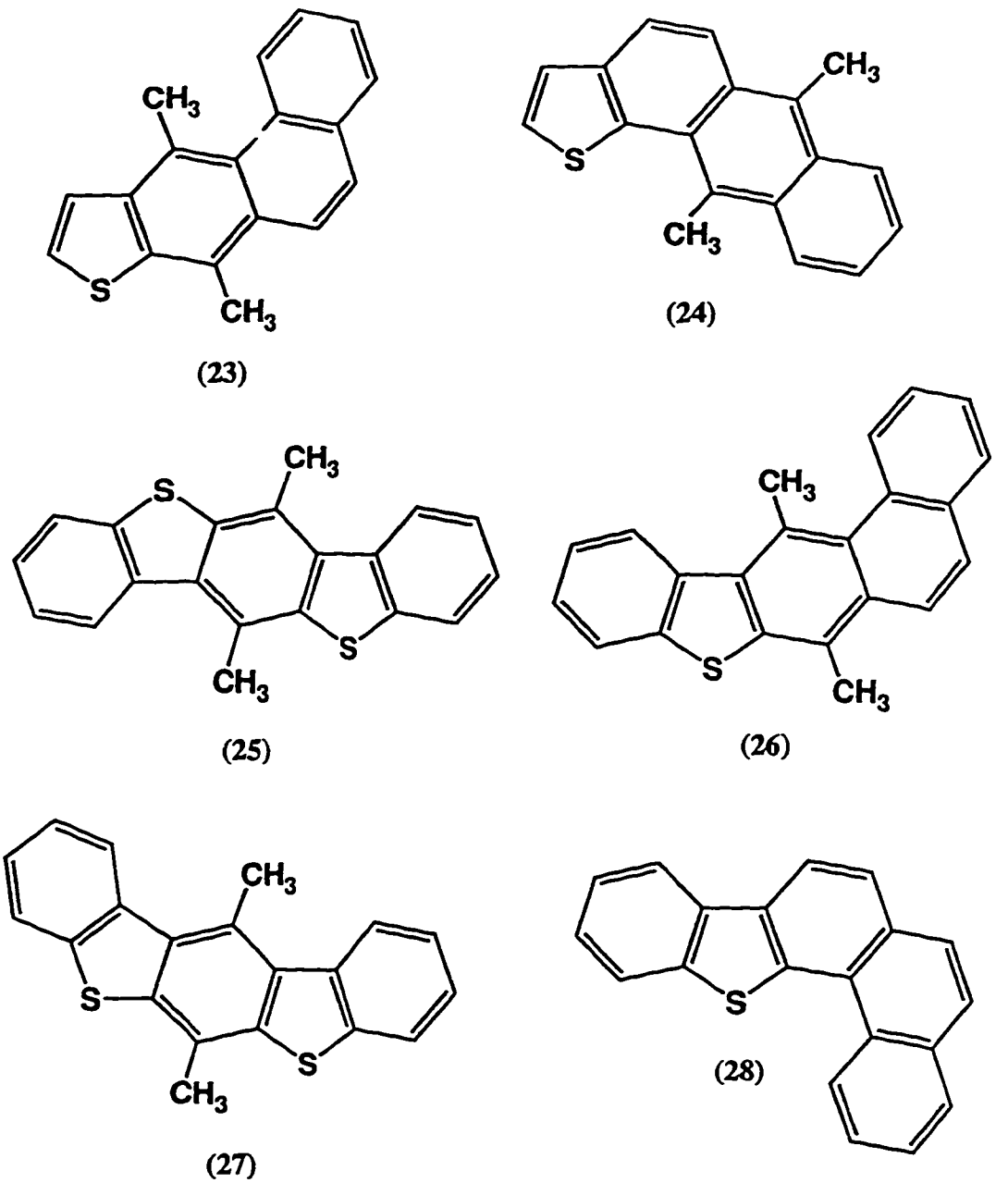


Figure 1.3 Structures of some of the condensed thiophenes that have been shown to be carcinogenic. Chemical designations: (23), 7,11-dimethylphenanthro[2,3-*b*]thiophene; (24), 6,11-dimethylanthra[1,2-*b*]thiophene; (25), 6,12-dimethylbenzo[1,2-*b*; 4,5-*b*']bis[1]benzothiophene; (26), 7,13-dimethylbenzo[*b*]phenanthro[3,2-*d*]thiophene; (27), 6,12-dimethylbenzo[1,2-*b*; 5,4-*b*']bis[1]benzothiophene; (28), benzo[*b*]phenanthro[3,4-*d*]thiophene (After Jacob, 1990).

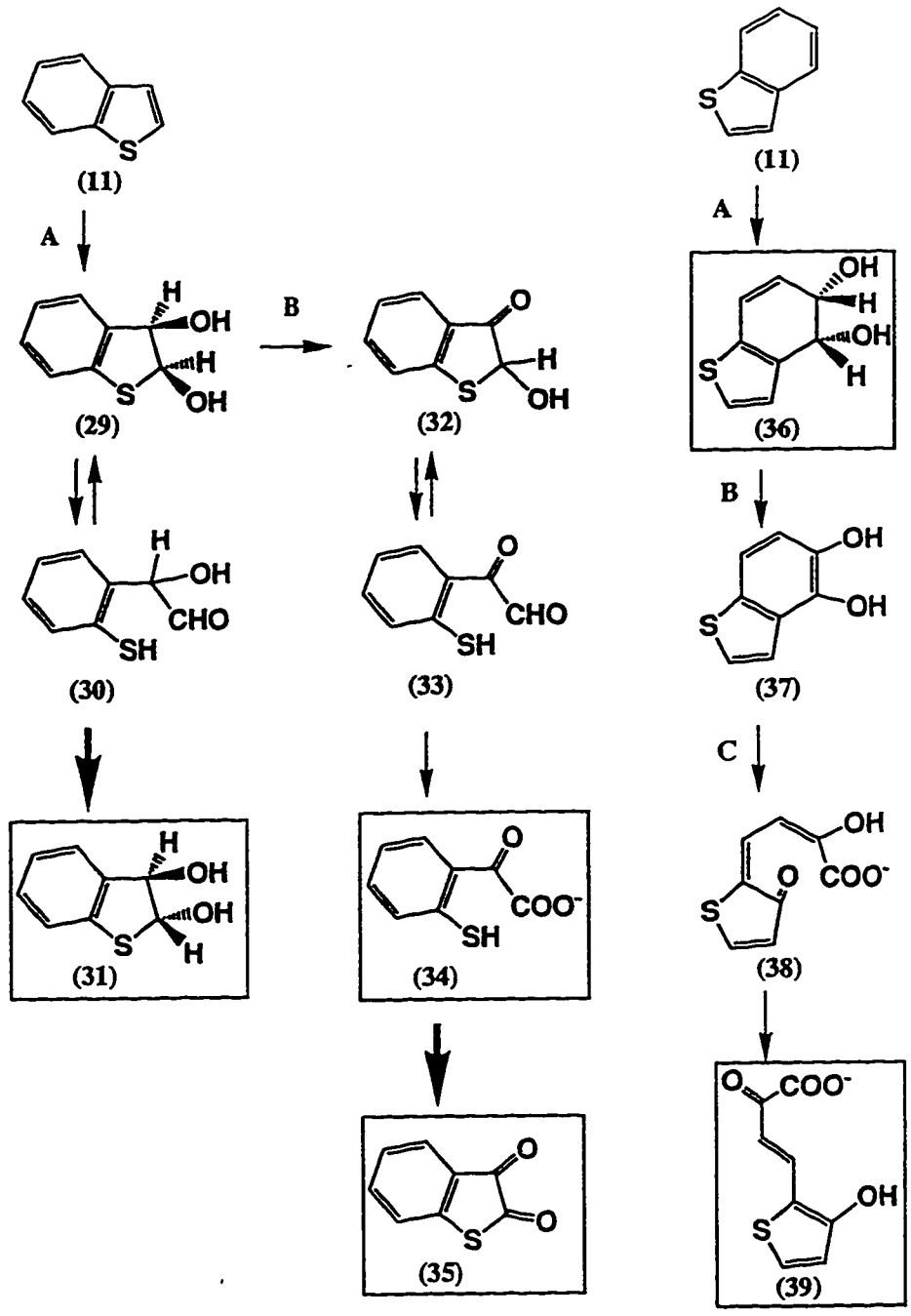


Figure 1.4 Pathways for the biotransformation of benzothiophene by *P. putida* RE204. Chemical designations: (11), benzothiophene; (29), *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene; (30), 2'-mercaptomandelaldehyde; (31), *trans*-2,3-dihydroxy-2,3-dihydrobenzothiophene; (32), 2-hydroxy-3-oxo-2,3-dihydrobenzothiophene; (33), 2-mercaptophenylglyoxaldehyde; (34), 2-mercaptophenylglyoxalate; (35), benzothiophene-2,3-dione; (36), *cis*-4,5-dihydroxy-4,5-dihydrobenzothiophene; (37), 4,5-dihydroxybenzothiophene; (38), *cis*-4-(3-oxo-2,3-dihydrothienyl)-2-hydroxybuta-2,4-dienoate; (39), *trans*-4-(3-hydroxy-2-thienyl)-2-oxobut-3-enoate. Enzymes: A, isopropylbenzene-2,3-dioxygenase; B, 2,3-dihydroxy-2,3-dihydroisopropylbenzene dehydrogenase; C, 3-isopropylcatechol 2,3-dioxygenase. Compounds in boxes were purified and identified; heavy arrows indicate transformations that occur during extraction of products (After Eaton and Nitterauer, 1994).

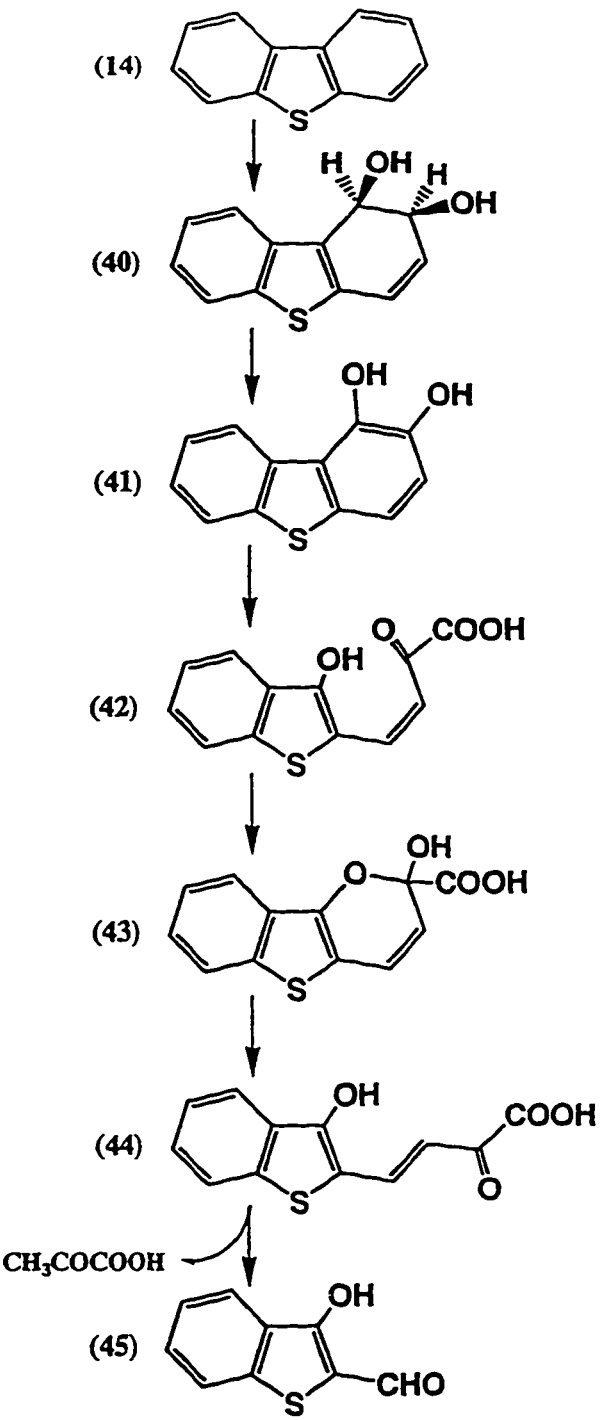


Figure 1.5 Kodama pathway for the biotransformation of dibenzothiophene by attack of the homocyclic ring. Chemical designations: (14), dibenzothiophene; (40), (+)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene; (41), 1,2-dihdyroxydibenzothiophene; (42), *cis*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid; (43), hemiacetal form of (42); (44), *trans*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid; (45), 3-hydroxy-2-formylbenzothiophene (After Foght *et al.*, 1990; Denome *et al.*, 1993b).

1.8 Literature cited

Allen, C. C. R., D. R. Boyd, H. Dalton, N. D. Sharma, S. A. Haughey, R. A. S. McMordie, B. T. McMurray, G. N. Sheldrake, and K. Sproule. 1995. Sulfoxides of high enantiopurity from bacterial dioxygenase-catalyzed oxidation. J. Chem. Soc. Chem. Commun. 1995: 119-120.

Andersson, J. T., and B. Schmid. 1993. The atomic emission detector in gas chromatographic trace analysis. Fesenius' J. Anal. Chem. 346: 403-409.

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.

Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald. 1986. Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures. Intern. J. Environ. Anal. Chem. 23: 289-303.

Belkin, S., M. Stieber, A. Tiehm, F. H. Frimmel, A. Abeliovich, P. Werner, and S. Ulitzur. 1994. Toxicity and genotoxicity enhancement during polycyclic aromatic hydrocarbons biodegradation. Environ. Toxicol. Water Qual. 9: 303-309.

Berthou, F., Y. Gourmelun, Y. Dreano, and M. P. Friocourt. 1981. Application of gas chromatography on glass capillary columns to the analysis of hydrocarbon pollutants from the *Amoco Cadiz* oil spill. J. Chromatogr. 203: 279-292.

Bezalel, L., Y. Hadar, P. P. Fu, J. P. Freeman, and C. E. Cerniglia. 1996. Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol. 62: 2554-2559.

Boehm, P. D., J. E. Barak, D. L. Fiest, and A. Elskus. 1982. A chemical investigation of the transport and fate of petroleum hydrocarbons in littoral and benthic environments: The *Tsesis* oil spill. Mar. Environ. Res. 6: 157-188.

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. In: Amoco Cadiz: Fates and effects of the oil spill. Proceedings of the International Symposium. Centre Oceanologique de Bretagne. Brest. Le Centre National pour l'Exploration des Oceans: Paris; p. 159-173.

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.

Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. Curr. Microbiol. 21: 229-231.

Czogalla, C.-D., and F. Boberg. 1983. Sulfur compounds in fossil fuels I. Sulfur Reports 3: 121-167.

Dahlberg, M. D., R. L. Rohrer, D. J. Fauth, R. Sprecher, and G. J. Olson. 1993. Biodesulfurization of dibenzothiophene sulfone by *Arthrobacter* sp. and studies with oxidized Illinois No. 6 coal. Fuel 72: 1645-1649.

Dalton, H., and D. I. Stirling. 1982. Co-metabolism. Phil. Trans. R. Soc. Lond. (B) 297: 481-496.

Denome, S. A., C. Oldfield, L. J. Nash, and K. D. Young. 1994. Characterization of the desulfurization genes from *Rhodococcus* sp. strain IGTS8. J. Bacteriol. 176: 6707-6716.

Denome, S. A., E. S. Olson, and K. D. Young. 1993a. Identification and cloning of genes involved in specific desulfurization of dibenzothiophene by *Rhodococcus* sp. strain IGTS8. Appl. Environ. Microbiol. 59: 2837-2843.

Denome, S. A., D. C. Stanley, E. S. Olson, and K. D. Young. 1993b. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: Complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. 175: 6890-6901.

Eastmond, D. A., G. M. Booth, and M. L. Lee. 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles in *Daphnia magna*. Arch. Environ. Contam. Toxicol. 13: 105-111.

Eaton, R. W., and P. J. Chapman. 1992. Bacterial metabolism of naphthalene: Construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. J. Bacteriol. 174: 7542-7554.

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

Fayad, N. M. and E. Overton. 1995. A unique biodegradation pattern of the oil spilled during the 1991 Gulf war. Mar. Pollut. Bull. 30: 239-246.

Fedorak, P. M. 1990. Microbial metabolism of organosulfur compounds in petroleum. In: Geochemistry of Sulfur in Fossil Fuels. Orr, W. L., and C. M. White, Eds. American Chemical Society: Washington, DC; p. 93-112.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., and T. M. Peakman. 1992. Aerobic microbial metabolism of some alkyl thiophenes found in petroleum. Biodegradation. 2: 223-236.

Fedorak, P. M., and D. W. S. Westlake. 1981. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can. J. Microbiol. 27: 432-443.

Fedorak, P. M., and D. W. S. Westlake. 1983. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. 29: 291-296.

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.

Finnerty, W. R., and M. Robinson. 1986. Microbial desulfurization of fossil fuels: A review. Biotechnol. Bioeng. Symp. 16: 205-221.

Foght, J. M., P. M. Fedorak, M. R. Gray, and D. W. S. Westlake. 1990. Microbial desulfurization of petroleum. In: Microbial Mineral Recovery. Ehrlich, H. L., and C. L. Brierley, Eds. McGraw-Hill Publishing Co.: New York; p. 379-407.

Foght, J. M., and D. W. S. Westlake. 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. Can. J. Microbiol. 34: 1135-1141.

Friocourt, M. P., F. Berthou, and D. Picart. 1982. Dibenzothiophene derivatives as organic markers of oil pollution. Toxicol. Environ. Chem. 5: 205-215.

Gallagher, J. R., E. S. Olson, and D. C. Stanley. 1993. Microbial desulfurization of dibenzothiophene: A sulfur specific pathway. FEMS Microbiol. Lett. 107: 31-36.

Gibson, D. T., and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons. In: Microbial Degradation of Organic Compounds. Gibson D. T., Ed. Marcel Dekker, Inc.: New York; p. 181-252.

Gonçalves, J. A. 1993. Oxidacion microbiana de compuestos organosulfurados puros y del petroleo. Ph.D. Thesis. Universidad Central de Venezuela, Caracas.

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.

Izumi, Y., T. Ohshiro, H. Ogino, Y. Hine, and M. Shimao. 1994. Selective desulfurization of dibenzothiophene by *Rhodococcus erythropolis* D-1. Appl. Environ. Microbiol. 60: 223-226.

Jacob, J. Sulfur Analogues of Polycyclic Aromatic Hydrocarbons (Thiaarenes). 1990. Cambridge University Press: Cambridge.

Jobson, A., F. D. Cook, and D. W. S. Westlake. 1972. Microbial utilization of crude oil. Appl. Microbiol. 23: 1082-1089.

Kankipati, P., and L.-K. Ju. 1994. Microbial desulfurization of petroleum: Fundamental study on dibenzothiophene desulfurization by *Sulfolobus acidocaldarius*. In: Environmental Issues and Solutions in Petroleum Exploration, Production, and Refining. Sublette, K. L., T. M. Harris, and F. S. Manning, Eds. PennWell Books: Tulsa, OK; p. 605-614.

Kargi, F. 1987. Biological oxidation of thianthrene,thioxanthene, and dibenzothiophene by the thermophilic organism *Sulfolobus acidocaldarius*. Biotechnol. Lett. 9: 478-482.

Kargi, F., and J. M. Robinson. 1994. Microbial oxidation of dibenzothiophene by the thermophilic organism *Sulfolobus acidocaldarius*. Biotechnol. Bioeng. 26: 687-690.

Kayser, K. J., B. A. Bielaga-Jones, K. Jackowski, O. Odusan, and J. J. Kilbane. 1993. Utilization of organosulphur compounds by axenic and mixed cultures of *Rhodococcus rhodochrous* IGTS8. J. Gen. Microbiol. 139: 3123-3129.

Kodama, K. 1977a. Induction of dibenzothiophene oxidation by *Pseudomonas jianii*. Agric. Biol. Chem. 41: 1193-1196.

Kodama, K. 1977b. Cometabolism of dibenzothiophene by *Pseudomonas jianii*. Agric. Biol. Chem. 41: 1305-1306.

Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. Part III. Isolation and identification of products from dibenzothiophene. Agr. Biol. Chem. 34: 1320-1324.

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. Agr. Biol. Chem. 37: 45-50.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* sp. Appl. Environ. Microbiol. 34: 783-790.

Laseter, J. L., G. C. Lawler, E. B. Overton, J. R. Patel, J. P. Holmes, M. I. Shields, and M. Maberry. 1981. Characterization of aliphatic and aromatic hydrocarbons in flat and Japanese type oysters and adjacent sediments collected from L'Aver Wrac'h following the Amoco Cadiz oil spill. In: Amoco Cadiz: Fates and effects of the oil spill. Proceedings of the International Symposium, Centre Oceanologique de Bretagne. Brest. Le Centre National pour l'Exploration des Oceans: Paris; p. 633-644.

Later, D. W., M. L. Lee, K. D. Bartle, R. C. Kong, and D. L. Vassilaros. 1981. Chemical class separation and characterization of organic compounds in synthetic fuels. Anal. Chem. 53: 1612-1620.

Lee, M. K., J. D. Senius, and R. J. Grossman. 1995. Sulfur-specific microbial desulfurization of sterically hindered analogs of dibenzothiophene. Appl. Environ. Microbiol. 61: 4362-4366.

McFall, T., G. M. Booth, M. L. Lee, Y. Tominaga, R. Pratap, M. Tedjamulia, and R. N. Castle. 1984. Mutagenic activity of methyl-substituted tri- and tetracyclic aromatic sulfur heterocycles. Mutat. Res. 135: 97-103.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Mormile, M. R., and R. M. Atlas. 1988. Mineralization of the dibenzothiophene biodegradation products 3-hydroxy-2-formyl benzothiophene and dibenzothiophene sulfone. Appl. Environ. Microbiol. 54: 3183-3184.

Mormile, M. R., and R. M. Atlas. 1989. Biotransformation of dibenzothiophene to dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. 35: 603-605.

Nakatani, S. T. Akasaki, K. Kodama, Y. Minoda, and K. Yamada. 1968. Microbial conversion of petro-sulfur compounds. Part II. Culture conditions of dibenzothiophene-utilizing bacteria. Agr. Biol. Chem. 32: 1205-1211.

Nishioka, M. 1988. Aromatic sulfur compounds other than condensed thiophenes in fossil fuels: Enrichment and identification. Energy Fuels 2: 214-219.

Nishioka, M., J. S. Bradshaw, M. L. Lee, Y. Tominaga, M. Tedjamulia, and R. N. Castle. 1985. Capillary column gas chromatography of sulfur heterocycles in heavy oils and tars using a biphenylpolysiloxane stationary phase. Anal. Chem. 57: 309-312.

Ogata, M., and K. Fujisawa. 1985. Organic sulfur compounds and polycyclic hydrocarbons transferred to oyster and mussel from petroleum suspension. Identification by gas chromatography and capillary mass chromatography. Water Res. 19: 107-118.

Ohshiro, T., Y. Hine, and Y. Izumi. 1994. Enzymatic desulfurization of dibenzothiophene by a cell-free system of *Rhodococcus erythropolis* D-1. FEMS Microbiol. Lett. 118: 341-344.

Ohshiro, T., T. Hirata, and Y., Izumi. 1996. Desulfurization of dibenzothiophene derivatives by whole cells of *Rhodococcus erythropolis* H-2. FEMS Microbiol. Lett. 142: 65-70.

Olson, E. S., D. C. Stanley, and J. R. Gallagher. 1993. Characterization of intermediates in the microbial desulfurizaton of dibenzothiophene. Energy Fuels 7: 159-164.

Omori, T., L. Monna, Y. Saiki, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Coynebacterium* sp. strain SY1. Appl. Environ. Microbiol. 58: 911-915.

Omori, T., Y. Saiki, K. Kasuga, and T. Kodama. 1995. Desulfurization of alkyl and aromatic sulfides and sulfonates by dibenzothiophene-desulfurizing *Rhodococcus* sp. strain SY1. Biosci. Biotech. Biochem. 59: 1195-1198.

Pelroy, R. A., D. L. Stewart, Y. Tominaga, M. Iwao, R. N. Castle, and M. L. Lee. 1983. Microbial mutagenicity of 3- and 4-ring polycyclic aromatic sulfur heterocycles. Mutat. Res. 117: 31-40.

Piddington, C. S., B. R. Kovacevich, and J. Rambosek. 1995. Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of *Rhodococcus* sp. strain IGTS8. Appl. Environ. Microbiol. 61: 468-475.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1992. Diones, sufoxides and sulfones from the aerobic cometabolism of methylbenzothiophenes by *Pseudomonas* strain BT1. Environ. Sci. Technol. 26: 1759-1764.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Sagardía, F., J. J. Rigau, A. Martínez-Lahoz, F. Fuentes, C. López, and W. Flores. Degradation of benzothiophene and related compounds by a soil *Pseudomonas* in an oil-aqueous environment. Appl. Microbiol. 29: 722-725.

Selifonov, S. A., M. Grifoll, R. W. Eaton, and P J. Chapman. 1996. Oxidation of naphthenoaromatic and methyl-substituted aromatic compounds by naphthalene 1,2-dioxygenase. Appl. Environ. Microbiol. 62: 507-514.

Sinninghe Damste´, J. S., and J. W. de Leeuw. 1990. Analysis, structure and geochemical significance of organically-bound sulphur in the geosphere: State of the art and future research. Org. Geochem. 16: 1077-1101.

Speight, J. G. 1980. The Chemistry and Technology of Petroleum. Marcel Dekker Inc.: New York.

Teal, J. M., K. Burns, and J. Farrington. 1978. Analyses of aromatic hydrocarbons in intertidal sediments resulting from two spills of no. 2 fuel oil in Buzzards Bay, Massachusetts. J. Fish. Res. Board Can. 35: 510-520.

Thompson, C. J. 1981. Identification of sulfur compounds in petroleum and alternative fossil fuels. In: Organic Sulfur Chemistry. Freidlina, R. Kh., and A. E. Skorova, Eds. Pergamon Press: Oxford; p. 189-208.

van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. Arch. Microbiol. 153: 324-328.

van Afferden, M., D. Tappe, M. Beyer, H. G. Trüper, and J. Klein. 1993. Biochemical mechanisms for the desulfurization of coal-relevant organic sulfur compounds. Fuel 72: 1635-1643.

Wang, Z., and M. Fingas. 1995. Use of methyldibenzothiophenes as markers for differentiation and source identification of crude and weathered oils. Environ. Sci. Technol. 29: 2842-2849.

Wang, P., A. E. Humphrey, and S. Krawiec. 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in continuous cultures. Appl. Environ. Microbiol. 62: 3066-3068.

Wang, P., and S. Krawiec. 1994. Desulfurization of dibenzothiophene to 2-hydroxybiphenyl by some newly isolated bacterial strains. Arch. Microbiol. 161: 266-271.

Wang, P., and S. Krawiec. 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in batch and fed-batch cultures. Appl. Environ. Microbiol. 62: 1670-1675.

Wenzel, B., and R. L. Aiken. 1979. Thiophenic sulfur distribution in petroleum fractions by gas chromatography with a flame photometric detector. J. Chromatogr. Sci. 17: 503-509.

Westlake, D. W. S. 1983. Microbial activities and changes in the chemical and physical properties of oil. In: Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Donaldson E. C. and J. B. Clark, Eds. Technology Transfer Branch, Bartlesville Energy Technology Centre: Bartlesville, Oklahoma; p. 102-111.

Willey, C., M. Iwao, R. N. Castle, and M. L. Lee. 1981. Determination of sulfur heterocycles in coal liquids and shale oils. Anal. Chem. 53: 400-407.

Williams, J. A., M. Bjorøy, D. L. Dolcater, and J. C. Winters. 1986. Biodegradation of South Texas Eocene oils-effects on aromatics and biomarkers. Org. Geochem. 10: 451-461.

Yamada, K., Y. Minoda, K. Kodama, S. Nakatani, and T. Akasaki. 1968. Microbial conversion of petro-sulfur compounds. Part I. Isolation and identification of dibenzothiophene-utilizing bacteria. Agr. Biol. Chem. 32: 840-845.

2. BACTERIAL TRANSFORMATIONS OF BENZOTHIOPHENE AND METHYLBENZOTHIOPHENES*

2.1 INTRODUCTION

Gas chromatographic analyses of residual petroleum from laboratory cultures (Bayona *et al.*, 1986; Fedorak and Westlake, 1983, 1984), petroleum-contaminated environments (Atlas *et al.*, 1981), and oil that has undergone biodegradation in the reservoir (Westlake, 1983; Williams *et al.*, 1986) have shown that alkylbenzothiophenes and alkyldibenzothiophenes are removed from the aromatic fraction of these oils by microbial activity. However, the identities of the metabolites were not determined in those studies because of the complexity of the mixture of compounds in the oil.

Biodegradation of petroleum leads to a decrease in the content of saturates and aromatics (Atlas *et al.*, 1981; Fedorak and Westlake, 1981; Patton *et al.*, 1981) and an increase in the content of polar materials (Jobson *et al.*, 1972). Laboratory studies have also shown the formation of a variety of polar products that accumulate during the microbial metabolism of organosulfur compounds. These products include sulfoxides, sulfones and 2,3-diones from benzothiophenes (Bohonos *et al.*, 1977; Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992); thiophene carboxylic acids from alkylthiophenes (Fedorak and Peakman, 1992); 3-hydroxy-2-formylbenzothiophene, dibenzothiophene sulfoxide and sulfone from dibenzothiophene (Kodama *et al.*, 1970; Laborde and Gibson, 1977; Monticello *et al.*, 1985); and methyl-3-hydroxy-2-formylbenzothiophenes, methylbenzothiophene-2,3-diones, hydroxylated methyldibenzothiophenes and sulfones from methyldibenzothiophenes (Saftic´ *et al.*, 1993).

A literature review (Fedorak, 1990) showed that of the hundreds of organosulfur compounds in petroleum, fewer than 20 of these compounds had been subjected to biodegradation studies. The best studied compounds are those that are commercially available. For example, two commercially available organosulfur compounds, benzothiophene and 3-methylbenzothiophene were transformed by a 1-MN-utilizing bacterium (*Pseudomonas* sp. BT1) and the predominant metabolites were benzothiophene-2,3-dione and 3-methylbenzothiophene sulfoxide, respectively (Fedorak and Grbic´-Galic´, 1991). 3-Methylbenzothiophene also yielded a small amount of its sulfone. These findings led to the prediction that if a methyl group was on the thiophene ring of benzothiophene, the corresponding sulfoxide and sulfone would be formed, whereas if a methyl group was on the benzene ring, the corresponding dione would be formed. In a systematic study with

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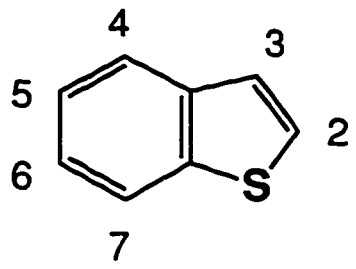
Pseudomonas sp. BT1 and synthesized methylbenzothiophenes, Saftic´ *et al.* (1992), observed that this prediction held true for the metabolism of 2-, 4-, 5-, and 6-methylbenzothiophenes, and 2,3-dimethylbenzothiophene. However, the metabolism of 7-methylbenzothiophene yielded the dione, sulfoxide and sulfone, along with several sulfur-containing metabolites which could not be identified. The ring numbering convention for benzothiophene is shown below.



The objective of the work described in this chapter was to determine whether two different bacterial strains (*Pseudomonas* spp. W1 and F) would give the same transformation products from benzothiophene and the methylbenzothiophenes as those observed with *Pseudomonas* sp. BT1 (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992) and *Pseudomonas pseudoalcaligenes* strain SB(G) (Gonçalves, 1993). These compounds would not support the growth of the two strains studied herein, but were biotransformed in cultures grown on 1-MN or glucose.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

1-MN was purchased from Fluka (Buchs, Switzerland). Benzothiophene and 3-methylbenzothiophene were purchased from Aldrich (Milwaukee, WI) and Lancaster Synthesis (Windham, NH), respectively. The methods for the syntheses of 2-, 4-, 5-, and 7-methylbenzothiophenes and a mixture of 4- and 6-methylbenzothiophenes are given by Andersson (1986). Sulfones of the benzothiophenes were synthesized by boiling the benzothiophene with hydrogen peroxide in acetic acid for 15 min. Benzothiophene sulfoxide was prepared in analytical amounts by oxidizing benzothiophene using horse heart cytochrome c and hydrogen peroxide (Vazquez-Duhalt *et al.*, 1993). 5-Methylbenzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione were synthesized according to the method of Hannoun *et al.* (1982). 2-Benzothiophenecarboxylic acid was

also synthesized (Shirley and Cameron, 1950), as was *m*-tolyl methyl sulfoxide (Cerniani and Modena, 1959).

2.2.2 Bacterial cultures

The isolation of *Pseudomonas* strain W1 and *Pseudomonas* strain F has been described previously, and cultures of these two bacteria accumulate 1-naphthalenemethanol and 1-naphthoic acid from 1-MN (Saftic´ *et al.*, 1993). To determine the amounts of these two compounds produced, isolates W1 and F were inoculated into flasks with 200 mL of mineral medium that contained 140 µmol of 1-MN. These flasks were sealed with Teflon-lined screw caps to reduce evaporation of the substrate, and three times each day the caps were loosened briefly to provide fresh air to ensure that oxygen would not become limiting. Turbidity of the culture was monitored and maximum growth reached after 24 h. After 3 days incubation, the cultures were acidified to pH<2 and extracted with dichloromethane (DCM). The extracts were analyzed by GC to determine the amounts of 1-MN, 1-naphthalenemethanol, and 1-naphthoic acid present.

2.2.3 Culture methods and medium

Cultures were routinely grown at 28°C in shake-flasks containing 200 mL of liquid mineral medium. The medium contained (per 0.9 L): NH_4Cl, 1.0 g; Na_2SO_4, 2.0 g; KNO_3, 2.0 g; $FeSO_4 \cdot 7H_2O$, trace; and 1 mL of trace metal solution (Fedorak and Grbic´-Galic´, 1991). To this was added 0.1 L of a buffer prepared by adding a solution of KH_2PO_4 (4 g/100 mL) into a solution of K_2HPO_4 (4 g/100 mL) until the pH was 7.0. After sterilizing in an autoclave, 1.0 mL of a separately sterilized solution of $MgSO_4 \cdot 7H_2O$ (4 g/100 mL) was added to each flask of 200 mL medium.

Growing cells of isolates W1 and F were used for biotransformation studies. Because the isolates would not grow on the benzothiophenes, the growth substrates used were 1-MN or glucose. When 1-MN was used, the cultures were inoculated with 10 mL of a 1-MN-grown maintenance culture that was transferred weekly. Often some of the metabolites of 1-MN interfered with the analysis of metabolites from the benzothiophenes. This problem did not arise when glucose was used. In this case, a glucose-grown culture that had incubated for 2 to 3 days was used as the source of inoculum (1 mL). This glucose-grown seed culture was inoculated from a single colony of the desired isolate that had grown 3 to 5 days on Plate Count Agar (PCA; Difco, Detroit, MI), after having been streaked from a maintenance culture grown with 1-MN. Each 200-mL portion of medium was supplemented with 50 mg of one of the growth substrates and 2 to 5 mg of the benzothiophene. These cultures were incubated for 7 days prior to extraction. For each

biotransformation experiment, appropriate sterile controls were incubated to account for any abiotic transformations.

A brief experiment was done to determine whether the microbial population in an unpolluted river water sample could produce oxidized metabolites from benzothiophene and 3-methylbenzothiophene. A sample of water was collected from the ice-covered North Saskatchewan River at Edmonton, upstream from the wastewater treatment plant discharge. A total of 200 mL of the river water were supplemented with 2 mL of a filter-sterilized nitrogen and phosphorus solution (which contained 59 g PO_4^{3-}/L and 100 g NH_4NO_3/L, pH 7.4) in a 500-mL Erlenmeyer flask. To this were added 200 µL of Prudhoe Bay crude oil, 8 mg of benzothiophene and 10 µL of 3-methylbenzothiophene. This culture was incubated for 14 days at 28°C with shaking, along with a sterile river water control.

2.2.4 Analytical methods

After incubation, the cultures were acidified with sulfuric acid to pH<2 and extracted with DCM (4 times 20 mL) to recover substrates and products. The extracts were dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. To screen for the presence of sulfur-containing metabolites, the extracts were analyzed by capillary GC using a 30-m DB-5 capillary column in an instrument equipped with a FID and a sulfur-selective FPD (Fedorak and Grbic´-Galic´, 1991). Details of the methods for GC-FPD, GC-MS, and GC-FTIR analyses have been described previously (Saftic´ *et al.*, 1993).

To facilitate GC-MS identification of some of the metabolites, trimethylsilyl (TMS) derivatives of compounds in culture extracts were made by silylating with N,O-bis(trimethylsilyl)acetamide (BSA) in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, IL; method 5).

2.3 RESULTS

2.3.1 Methyl group oxidation of 1-MN

When grown on 1-MN, isolates W1 and F produced 1-naphthalenemethanol and 1-naphthoic acid that accumulated in the medium, because neither isolate can grow on these two oxidation products. GC analyses of extracts from 3-day-old cultures of isolate W1 showed that of the 140 µmol of 1-MN added, 1 µmol remained, and 11 µmol of the methanol and 4 µmol of the acid accumulated. Similarly, 1 µmol of 1-MN remained in cultures of isolate F, in which 7 µmol of the methanol and 1 µmol of the acid accumulated. Analyses of the extracts from the sterile controls showed that only 58 µmol of 1-MN was recovered after the 3-day incubation period.

2.3.2 Biotransformations of benzothiophene

The GC-FPD chromatogram of an extract from a culture of strain W1 grown on glucose in the presence of benzothiophene showed numerous sulfur-containing metabolites. GC-MS analysis showed that one product had a molecular weight of 150 (Figure 2.1) which was consistent with the metabolite being benzothiophene sulfoxide. The base peak was m/z 134, and corresponds to the loss of an oxygen atom. The ion at m/z 121 $(M-29)^+$ would result from the loss of CHO which has been observed in the mass spectra of other sulfoxides (Glinzer *et al.*, 1983).

Sulfoxides of benzothiophenes are very difficult to chemically synthesize because the oxidation reactions usually proceed to yield the sulfone. However, Vazquez-Duhalt *et al.* (1993) recently demonstrated that in the presence of hydrogen peroxide, cytochrome c would oxidize benzothiophene to its sulfoxide. Thus, this reaction was used to produce enough of the sulfoxide for GC-MS confirmation that the metabolite was the sulfoxide. Indeed, the mass spectrum in Figure 2.1 matched that of the synthesized sulfoxide.

A second transformation product formed by isolate W1 from benzothiophene eluted on the tail of the sulfoxide peak, and its molecular ion at m/z 166 (Figure 2.2a) suggested that it was benzothiophene sulfone. The ions at m/z 137, 118 and 109 were observed by others who reported the mass spectrum of benzothiophene sulfone (Porter, 1967). GC-FTIR analysis (Figure 2.2b) showed two strong absorptions at 1342 and 1167 cm^{-1}. This was consistent with the metabolite being a sulfone because sulfones give two characteristic absorptions in the regions of 1350 to 1300 cm^{-1} and 1160 to 1120 cm^{-1} (Shriner *et al.*, 1980). Benzothiophene sulfone was synthesized and it gave the same mass spectrum as shown in Figure 2.2a.

GC-MS analysis of the extract from this glucose-grown isolate W1 culture also detected the presence of small amounts of benzothiophene-2,3-dione. The mass spectrum of this metabolite was identical to that reported by Fedorak and Grbic´-Galic´ (1991).

GC-FPD and GC-MS analyses showed the presence a high-molecular-weight product in the extracts of cultures of isolate W1 grown on either 1-MN or glucose in the presence of benzothiophene. The molecular weight of this product was 234 which is consistent with the formula $C_{16}H_{10}S$.

Isolate F grown on 1-MN with benzothiophene produced a single abundant sulfur-containing metabolite and GC-MS analysis showed that this compound had a molecular ion at m/z 234. When grown on glucose in the presence of benzothiophene, isolate F produced the high-molecular-weight compound (M^+=234), benzothiophene-2,3-dione, and the sulfoxide and sulfone of benzothiophene.

2.3.3 Biotransformations of 2-methylbenzothiophene

When isolates W1 and F were incubated individually with 2-methylbenzothiophene, they gave a product with a molecular weight of 164, and with a mass spectrum that was almost identical to that of 2-methylbenzothiophene sulfoxide which had been previously identified as a metabolite of 2-methylbenzothiophene (Saftic´*et al.*, 1992). GC-FTIR analysis showed that this compound gave a strong absorption at 1080 cm^{-1} which is also characteristic of 2-methylbenzothiophene sulfoxide. A less abundant, second metabolite produced by both of these isolates eluted immediately after the sulfoxide during GC analyses. GC-MS analysis showed this metabolite had a molecular ion at m/z 180, and GC-FTIR analysis showed strong absorptions at 1335 and 1162 cm^{-1}, consistent with the metabolite being 2-methylbenzothiophene sulfone. GC-MS analysis of chemically synthesized 2-methylbenzothiophene sulfone gave a mass spectrum that was identical to that of the metabolite.

When the extracts from cultures of isolates W1 and F grown on glucose in the presence of 2-methylbenzothiophene were reacted with BSA, new metabolites were detected by GC-MS. One gave a mass spectrum with abundant ions at m/z 250, 235, 191, 161, 133 and 89 (Figure 2.3a). These results suggested that the metabolite was 2-benzothiophenecarboxylic acid. The TMS-derivative is drawn in Figure 2.3a, and the fragmentations that would yield most of the major ions are also shown. The fragment at m/z 191 is the $(M-59)^+$ ion which is the result of the loss of a methyl group followed by skeletal rearrangement with the loss of CO_2. This fragmentation is a characteristic of TMS-esters of carboxylic acids (Pierce, 1968).

To verify the identity of this metabolite, 2-benzothiophenecarboxylic acid was synthesized and its TMS-derivative was prepared giving a mass spectrum (Figure 2.3b) that was identical to that of the metabolite (Figure 2.3a). The underivatized acid in the culture extract was not detected by GC-MS, presumably because it was too dilute and because the free carboxylic acid was poorly chromatographed under the conditions used.

Also in the extract from culture F, a trace amount of another TMS-derivative was detected by GC-MS. The mass spectrum showed the base peak at m/z 73 (the trimethylsilyl fragment), a strong M^+ at m/z 236, an (M^+-15) ion from the loss of methyl group, and an ion at m/z 147 from the loss of $OSi(CH_3)_3$. This spectrum was consistent with the metabolite being a hydroxy-substituted 2-methylbenzothiophene, presumably 2-benzothiophenemethanol because isolate F is known to oxidize the methyl group of 1-MN to 1-naphthalenemethanol and 1-naphthoic acid.

2.3.4 Biotransformations of 3-methylbenzothiophene

When the DCM-extract of isolate F grown on 1-MN in the presence of 3-methylbenzothiophene was analyzed by GC-FPD, two sulfur-containing metabolites were detected. The mass spectra and the FTIR spectra (data not shown) of these two metabolites were essentially the same as those of 3-methylbenzothiophene sulfoxide and sulfone (Fedorak and Grbic´-Galic´, 1991).

A culture of isolate F grown on glucose with 3-methylbenzothiophene also produced 3-methylbenzothiophene sulfoxide and 3-methylbenzothiophene sulfone based on their GC retention times and mass spectra. In addition to these metabolites, another compound was present which co-eluted with the sulfoxide. The molecular ion of this novel metabolite was at m/z 178, and the mass spectrum contained an ion at m/z 161, which corresponds to the loss of OH. Further interpretation of this mass spectrum was not possible because it contained many contaminating ions contributed by the 3-methylbenzothiophene sulfoxide. However, the molecular weight and loss of OH suggested that the metabolite was 3-benzothiophenecarboxylic acid.

To verify this, the culture extract was treated with BSA to produce a TMS-derivative of the metabolite. GC-MS analysis showed that the derivative was well separated from the sulfoxide and that the mass spectrum of the TMS-derivative was virtually identical to those in Figure 2.3. These results strongly suggest the formation of 3-benzothiophenecarboxylic acid from 3-methylbenzothiophene by isolate F.

A trace amount of another sulfur-containing metabolite was detected in the extract of this culture of isolate F grown on glucose in the presence of 3-methylbenzothiophene. The molecular ion of this product was at m/z 164 and it had a base peak at m/z 147 (Figure 2.4a). This fragmentation $(M-17)^+$ corresponds to the loss of OH. The TMS-derivative was prepared and its mass spectrum is shown in Figure 2.4b. These spectra suggest that this product is a hydroxy-substituted 3-methylbenzothiophene. The structures shown in Figure 2.4 assume that the product was 3-benzothiophenemethanol because oxidation of the methyl group of 2-methylbenzothiophene to a carboxyl group has been demonstrated (Figure 2.3).

Isolate W1 grown on glucose in the presence of 3-methylbenzothiophene produced the same four metabolites: 3-methylbenzothiophene sulfoxide, 3-methylbenzothiophene sulfone, 3-benzothiophenecarboxylic acid and 3-benzothiophenemethanol.

2.3.5 Biotransformations of 4-methylbenzothiophene

GC-FPD analysis of the extract of a culture of isolate W1 grown on glucose-containing medium with 4-methylbenzothiophene showed a single, large sulfur-containing

peak. The mass spectrum (Figure 2.5a) showed a weak molecular ion at m/z 178, a base peak at m/z 150, and other fragments at m/z 121, 78, and 69. This fragmentation pattern was consistent with the metabolite being 4-methylbenzothiophene-2,3-dione, but an authentic sample of this compound was not available. However, 5-methylbenzothiophene-2,3-dione was synthesized and its mass spectrum (Figure 2.5b) showed the same fragmentation pattern as the metabolite from 4-methylbenzothiophene, and authentic 7-methylbenzothiophene-2,3-dione (Saftić *et al.*, 1993).

When this extract was treated with BSA and analyzed by GC-MS, the TMS-derivatives of a carboxylic acid and a hydroxy-substituted 4-methylbenzothiophene were observed. The mass spectrum of the acid had the same fragmentation pattern as those shown in Figure 2.3 (M^+=250, other abundant ions at 191, 161, 133 and 89) with the exception that the base peak was at m/z 235. The other TMS-derivative had a molecular ion at m/z 236, the base peak at m/z 73, and an abundant $(M-15)^+$ at m/z 221. Unlike the mass spectrum shown in Figure 2.4b, the ion at m/z 147 from this TMS-derivative was not very abundant. The hydroxy-substituted metabolite was presumed to be 4-benzothiophenemethanol.

Isolate F also produced 4-methylbenzothiophene-2,3-dione as the most abundant sulfur-containing metabolite when it was grown on either glucose or 1-MN in the presence of 4-methylbenzothiophene. In addition, GC-MS analysis of the extract of the culture of isolate F grown on glucose in the presence of 4-methylbenzothiophene showed a high-molecular-weight compound. Its molecular ion (m/z 262) was consistent with the molecular formula $C_{18}H_{14}S$.

2.3.6 Biotransformations of 5-methylbenzothiophene

GC-FPD analysis of the extract of a culture of isolate W1 grown on 1-MN in the presence of 5-methylbenzothiophene showed a single sulfur-containing metabolite. GC-MS analysis showed that the compound had a molecular ion at m/z 178 (Figure 2.6a). The fragments with m/z 161 $(M-17)^+$ and 133 $(M-45)^+$ would result from the losses of OH and COOH, respectively, suggesting that the metabolite was 5-benzothiophenecarboxylic acid. 2-Benzothiophenecarboxylic acid was synthesized and its mass spectrum (Figure 2.6b) showed the same fragmentation pattern as the metabolite from 5-methylbenzothiophene. Both free acids were amenable to GC-MS analysis. In addition, the TMS-derivative of this metabolite was prepared and its mass spectrum was virtually identical to that shown in Figure 2.3b, providing further evidence that 5-benzothiophenecarboxylic acid was produced in this culture of isolate W1.

Two abundant sulfur-containing metabolites were detected by GC-FPD analysis of the extract of a culture of isolate F grown on 1-MN in the presence of 5-methylbenzothiophene. One of these had the same retention time and mass spectrum as 5-benzothiophenecarboxylic acid. The mass spectrum of the second metabolite showed it was a high-molecular-weight product with a strong molecular ion at m/z 262, consistent with the molecular formula $C_{18}H_{14}S$.

When isolate F was grown on glucose in the presence of 5-methylbenzothiophene, 5-benzothiophenecarboxylic acid and the high-molecular-weight product were detected by GC-MS analysis. In addition, a metabolite with the same GC retention time and identical mass spectrum as 5-methylbenzothiophene-2,3-dione (Figure 2.5b) was detected.

2.3.7 Biotransformations of 4- and 6-methylbenzothiophenes

The synthesis method for 6-methylbenzothiophene (Andersson, 1986) yielded a mixture of two isomers, 4- and 6-methylbenzothiophenes, and this mixture was used in the studies with the two bacterial isolates.

GC-FPD analysis of an extract of a culture of isolate W1 grown in medium containing glucose and the mixture of these two isomers of methylbenzothiophene showed several sulfur-containing products. The earliest of these to elute had a retention time and mass spectrum (M^+=154, base peak at m/z 139, other fragments at 111, 108, 91, 77, and 65) that matched those of authentic *m*-tolyl methyl sulfoxide, and the metabolite produced by the activity of isolate BT1 on a mixture of 4- and 6-methylbenzothiophenes when grown on 1-MN (Saftić *et al.*, 1992). This ring-cleavage product could arise from either 4-methylbenzothiophene or 6-methylbenzothiophene. However, because it was not detected in the cultures of isolates W1 or F grown in the presence of 4-methylbenzothiophene, it is most likely that the *m*-tolyl methyl sulfoxide is a product of 6-methylbenzothiophene.

Two other sulfur-containing products had nearly identical mass spectra (weak M^+=178, base peak at m/z 150, other fragments at 121 and 78), and these matched that of 5-methylbenzothiophene-2,3-dione (Figure 2.5b). The GC retention time of the first dione to elute matched that of the dione observed in cultures that contained only 4-methylbenzothiophene. Therefore this was 4-methylbenzothiophene-2,3-dione, and the second dione to elute was 6-methylbenzothiophene-2,3-dione.

Also present in this extract was a compound with a mass spectrum that gave a molecular ion that was the base peak at m/z 178, with fragments at m/z 161 $(M-17)^+$ and 133 $(M-45)^+$ corresponding to the loss of OH and COOH, respectively. The mass spectrum closely resembled that of 2-benzothiophenecarboxylic acid (Figure 2.6b). The TMS-derivative of this metabolite was prepared and its mass spectrum matched that of 2-

benzothiophenecarboxylic acid (Figure 2.3b). Thus, a single isomer of benzothiophene-carboxylic acid was detected, but it is not known which isomer of methylbenzothiophene was oxidized to give this product.

Finally, two high-molecular-weight, sulfur-containing products were detected in the extract of this culture of isolate W1 containing 4- and 6-methylbenzothiophenes. These compounds both had molecular ions at m/z 262, consistent with the molecular formula $C_{18}H_{14}S$.

A culture of isolate F grown on glucose-containing medium in the presence of 4- and 6-methylbenzothiophenes showed all the same sulfur-containing metabolites previously described for isolate W1, with one exception: no 6-methylbenzothiophene-2,3-dione was detected in this culture of isolate F.

2.3.8 Biotransformations of 7-methylbenzothiophene

Several sulfur-containing metabolites were detected by GC-FPD analysis of an extract of a culture of isolate F grown on glucose in the presence of 7-methylbenzothiophene. The earliest of these to elute during GC-MS analysis had a molecular ion at m/z 154 (Figure 2.7a). This molecular weight and the major fragment ions at m/z 65, 77, 91, and 111 were also found in the mass spectrum of *m*-tolyl methyl sulfoxide published by Saftić *et al.* (1992). One notable difference between the mass spectrum in Figure 2.7 and that of *m*-tolyl methyl sulfoxide (Saftić *et al.*, 1992) was the ion at m/z 137 $(M-17)^+$ (Figure 2.7a) that would result from the loss of OH. This ion was not found in the mass spectrum of *m*-tolyl methyl sulfoxide (Saftić *et al.*, 1992) in which the base peak was at m/z 139 $(M-15)^+$ resulting from the loss of a methyl group. The FTIR spectrum of the metabolite from 7-methylbenzothiophene (Figure 2.7b) shows a strong absorption at 1095 cm^{-1} which is characteristic of a sulfoxide (Shriner *et al.*, 1980) and is very similar to the FTIR spectrum of *m*-tolyl methyl sulfoxide (Saftić *et al.*, 1992), which showed a strong absorption at 1099 cm^{-1}. A portion of the culture extract was oxidized (Bordwell *et al.*, 1949), and GC-MS analysis of this material showed that the sulfoxide was absent and that a new product with a molecular weight of 170 had formed. This was 16 mass units greater than the sulfoxide, and was the corresponding sulfone. These results indicate that this metabolite from 7-methylbenzothiophene was *o*-tolyl methyl sulfoxide, which resulted from the cleavage of the thiophene ring.

In the unoxidized extract, the second sulfur-containing metabolite to elute during GC analysis gave a mass spectrum similar to those in Figure 2.5. The weak molecular ion at m/z 178, the base peak at m/z 150, other fragments at 121, and 78 were consistent with

the metabolite being 7-methylbenzothiophene-2,3-dione. In addition, the FTIR spectrum of this metabolite was identical to that of authentic 7-methylbenzothiophene-2,3-dione.

Two other sulfur-containing products were detected, one of which eluted on the tail of the first. The mass spectra of these compounds showed molecular ions at m/z 164 and 180, respectively. GC-FTIR spectra of these two metabolites from isolate F showed that the first product to elute gave a strong absorption at 1072 cm^{-1}, characteristic of a sulfoxide (Shriner *et al.*, 1980), and the second product gave strong absorptions at 1334 and 1163 cm^{-1}, characteristic of a sulfone. Thus these metabolites were 7-methylbenzothiophene sulfoxide and 7-methylbenzothiophene sulfone, respectively.

A high-molecular-weight, sulfur-containing metabolite was also detected in the extract of this glucose-grown culture of isolate F. Its mass spectrum showed a strong molecular ion at m/z 262, corresponding to a molecular formula of $C_{18}H_{14}S$.

When the extract from the culture of isolate F grown on glucose in the presence of 7-methylbenzothiophene was reacted with BSA, two new metabolites were detected by GC-MS. One gave a mass spectrum with abundant ions at m/z 250, 235, 191, 161, 133 and 89. This is characteristic of a TMS-derivatized benzothiophenecarboxylic acid (Figure 2.3), suggesting that the metabolite was 7-benzothiophenecarboxylic acid.

The TMS-derivatization also lead to the detection of another metabolite that was present in very low concentration. The mass spectrum of this TMS-derivatized metabolite had a weak molecular ion at m/z 236 with a small fragment at m/z 221 (corresponds to loss of CH_3) and the base peak at m/z 147. This fragmentation is the same as that observed in Figure 2.4b suggesting that the metabolite was a monohydroxy-substituted 7-methylbenzothiophene, presumably, a methanol because the corresponding carboxylic acid was detected in this extract.

The sulfur-containing metabolites from isolate W1 grown on glucose in the presence of 7-methylbenzothiophene were identified by GC-FPD and GC-MS analyses by comparison with the results obtained for isolate F grown under identical conditions. Products with the same retention times and mass spectra as those identified in the extracts from isolate F were: *o*-tolyl methyl sulfoxide, 7-methylbenzothiophene-2,3-dione, 7-methylbenzothiophene sulfoxide, 7-methylbenzothiophene sulfone, 7-benzothiophene-carboxylic acid, 7-benzothiophenemethanol, and a high-molecular-weight product (M^+=262), $C_{18}H_{14}S$.

2.3.9 Analysis of oil extracted from river water culture

Prudhoe Bay crude oil was supplemented with the two commercially available compounds, benzothiophene and 3-methylbenzothiophene, to provide sufficient

concentrations to enable the detection of any metabolite that might be formed. GC analysis of the extract from the 14-day-old culture that was inoculated with river water showed that the *n*-alkanes had been degraded. The FPD showed that the two organosulfur compounds that had been added were still present and a metabolite peak with the retention time of benzothiophene sulfoxide was detected. GC-MS analysis showed that the mass spectrum of the metabolite was very similar to that of benzothiophene sulfoxide (Figure 2.1), but contaminated with numerous ions from background components in the oil extract. Nonetheless, the mass spectrum of the metabolite showed the molecular ion at m/z 150, the base peak at m/z 134, and other abundant ions at m/z 122, 121, 89, 78, 69 and 63.

2.4 DISCUSSION

The objective of this study was to determine if other bacterial strains would yield the same products from benzothiophene and the methylbenzothiophenes as *Pseudomonas* strains BT1 and SB(G). Table 2.1 summarizes the results from the survey with *Pseudomonas* strains W1 and F, and compares these to previously reported results obtained with strain BT1 (Saftić *et al.*, 1992) and strain SB(G) (Gonçalves, 1993). None of the compounds listed in Table 2.1 was detected in the corresponding sterile controls. In general, the major products observed with isolate BT1 were (a) sulfoxides when there was a methyl group on the thiophene ring, and (b) 2,3-diones when there was no methyl group on the thiophene ring. 7-Methylbenzothiophene was a notable exception, yielding the sulfoxide, sulfone and 2,3-dione. In general, the major products observed with strain SB(G) were sulfoxides, sulfones, and high-molecular-weight products, although not all the isomers of methylbenzothiophene were tested with this isolate.

Both of the new isolates produced the sulfoxide and sulfone of benzothiophene (Table 2.1) as was previously observed with strain SB(G). However these compounds were not produced by isolate BT1, which gave benzothiophene-2,3-dione. This dione was also produced by isolates W1 and F. No 2,3-diones were produced from any of the methylbenzothiophenes by isolate SB(G), whereas 2,3-diones were commonly found in extracts of isolates W1 and F grown in the presence of methylbenzothiophenes that had the methyl group on the benzene ring. Methylbenzothiophene-2,3-diones were identified as metabolites of methyldibenzothiophenes (Saftić *et al.*, 1993).

The detection of 2,3-diones as metabolites from oxidation of benzothiophene, methylbenzothiophenes, and methyldibenzothiophene was originally interpreted as evidence for the resistance of the thiophene ring to microbial attack. Indeed, several investigations have shown that the microbial metabolism of dibenzothiophene (Foght and Westlake, 1988; Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977) and

methyldibenzothiophenes (Saftic´ *et al.*, 1993) leads to the removal of carbon atoms from one ring, leaving substituted benzothiophenes that accumulate in the medium. However, recently Eaton and Nitterauer (1994) demonstrated that benzothiophene was microbially oxidized via dioxygenase attack at positions 2 and 3, and that subsequent reactions, including thiophene ring cleavage, led to the formation of 2-mercaptophenylglyoxalate which cyclized to give benzothiophene-2,3-dione by acid catalyzed dehydration. Thus, the detection of benzothiophene-2,3-diones in my studies, which routinely involved acidification of the culture medium prior to extraction, gives indication that the thiophene ring is being cleaved. Eaton and Nitterauer (1994) also observed the formation of *trans*-4-[3-hydroxy-2-thienyl]-2-oxobut-3-enoic acid which resulted from the cleavage of 4,5-dihydroxybenzothiophene. Besides the 2,3-diones, the only ring cleavage products detected in the current study were *m*-tolyl methyl sulfoxide, which was produced from 6-methylbenzothiophene by both isolates W1 and F as had been previously observed with strains BT1 and SB(G), and *o*-tolyl methyl sulfoxide, which was produced from 7-methylbenzothiophene by only isolates W1 and F (Table 2.1). The mechanism for these ring cleavages is unknown.

Benzothiophene-2,3-dione was the first product identified during the photooxidation of benzothiophene (Andersson and Bobinger, 1992), and subsequent reactions lead to near stoichiometric formation of 2-sulfobenzoic acid. The microbially produced 2,3-diones observed in this study (Table 2.1) may also undergo abiotic reactions yielding sulfobenzoic acids. However, these compounds are too polar to be extracted from the medium by the method used in this study. Indeed, this investigation focused on the identification of metabolites that could be partitioned into DCM, that were amenable to the GC analysis used, and that were detected by the sulfur-selective FPD.

Neither isolate BT1 nor SB(G) oxidized the methyl groups of any of the methylbenzothiophenes. In contrast, isolates W1 and F produced carboxylic acids from several of the methylbenzothiophenes (Table 2.1). This was consistent with the latter two isolates being able to oxidize the methyl group of 1-MN to 1-naphthalenemethanol and 1-naphthoic acid. In addition, these isolates oxidized the methyl groups of selected methyldibenzothiophenes to give dibenzothiophenemethanols (Saftic´ *et al.*, 1993). Thus, it was presumed that the hydroxylated compounds produced from the 2-, 3-, 4- and 7-methylbenzothiophenes by isolates W1 and F were methanols.

GC-MS analyses detected high-molecular-weight, sulfur-containing products in several of the culture extracts. In the case of benzothiophene, the molecular formula is consistent with the product being a benzonaphthothiophene. Indeed, photochemical reactions of benzothiophene have been shown to yield benzo[*b*]naphtho[2,1-*d*]thiophene

(Andersson and Bobinger, 1992; Haines *et al.*, 1956). The high-molecular-weight metabolite from benzothiophene was identified as benzo[*b*]naphtho[1,2-*d*]thiophene, and the mechanism of its formation was determined (Kropp *et al.*, 1994; Chapter 3).

Several factors precluded quantitative studies of the metabolism of these sulfur heterocyclic compounds. These included the volatility of the benzothiophenes, the limited amounts of the synthesized methylbenzothiophenes, the lack of authentic standards of some of the metabolites (in particular, the sulfoxides), the decomposition of the sulfoxides in the GC injection port (Fedorak and Andersson, 1992), the non-linear response of the FPD, and its susceptibility to quenching (Wenzel and Aiken, 1979). Nonetheless, some general comments on the relative amounts of conversion of the substrates can be presented. In each of the culture extracts, the original benzothiophene being studied was detected. Thus, the cultures did not convert all of the organosulfur compound to metabolites. Sulfoxides and sulfones were the most abundant products from 2- and 3-methylbenzothiophenes. Carboxylic acids were abundant products from 3-, 5- and 6-methylbenzothiophenes, and although benzothiophenemethanols were often detected, they were usually found in trace amounts.

The long-term goal of this research program is to gain an understanding of the fates and consequences of organosulfur compounds in petroleum- or creosote-contaminated environments. The results of the current study and other related investigations (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992, 1993) raise the following questions. Are these metabolites produced by microbial populations in the presence of petroleum or creosote? Will the sulfur-containing metabolites persist in the environment? Are the metabolites more toxic than the parent compounds? Few answers to these questions are available.

Previous studies with 3-methylbenzothiophene and isolate BT1 (Fedorak and Grbic´-Galic´, 1991) showed that the sulfoxide and the sulfone were produced in the presence of Prudhoe Bay crude oil. In the same study, Fedorak and Grbic´-Galic´ (1991) demonstrated that only 50% of the authentic benzothiophene-2,3-dione added to sterile medium containing Prudhoe Bay crude oil could be recovered after a few minutes of incubation. These results suggest that the dione reacts quickly with some compounds in the oil.

In the current study, a preliminary investigation was undertaken to determine whether the mixed microbial population in a sample of the North Saskatchewan River water could yield any of the metabolites detected in the pure culture studies. The results showed that after the culture had been incubated for 14 days with Prudhoe Bay crude oil supplemented with benzothiophene and 3-methylbenzothiophene, benzothiophene

sulfoxide could be detected in the residual oil extracted from the cultures. These preliminary findings suggest that the metabolites identified in the pure culture, pure substrate systems may be formed in environments contaminated with petroleum.

Many studies with thiophenes have shown that microorganisms can oxidize them to sulfoxides (Bohonos *et al.*, 1977; Fedorak *et al.*, 1988; Kodama *et al.*, 1973; Laborde and Gibson, 1977) and sulfones (Crawford and Gupta, 1990; Fedorak *et al.*, 1988; Mormile and Atlas, 1989; Omori *et al.*, 1992). Recently, Vazquez-Duhalt *et al.* (1993) demonstrated that eight of ten organosulfur compounds tested reacted with cytochrome c and hydrogen peroxide yielding the corresponding sulfoxides. There is little information on the relative toxicities of these oxidized products and their parent compounds. When administered orally to rats and mice, the LD_{50} values of benzothiophene were 1.26 and 0.96 g/kg, respectively (Lagno and Sviridov, 1975). Whereas the LD_{50} values of benzothiophene sulfone in the same experiments were 3.75 and 1.57 g/kg, respectively, but in contrast to benzothiophene, its sulfone had significant cumulative toxicity. Jacob (1990) reviewed a study in which the mutagenicity of dibenzothiophene, the three isomers of benzonaphthothiophene, and triphenyleno[1,12-*bcd*]thiophene along with some of their sulfoxides and sulfones were tested using *Salmonella typhimurium* strains TA 98 and TA 100. No mutagenicity was observed for the parent compounds, but all five sulfoxides and dibenzothiophene sulfone were weakly mutagenic and benzo[*b*]naphtho[2,1-*d*]thiophene sulfone was a potent mutagen. Interestingly, dibenzothiophene sulfoxide has been patented as a herbicide (Schlesinger, 1953).

In general, the types of abundant metabolites detected in the extracts from *Pseudomonas* strains W1 and F were similar to those that had been previously reported from isolates BT1 and SB(G) (Table 2.1). However, the occurrence of sulfoxides and sulfones was more prevalent with isolates SB(G), W1, and F. The oxidation of the methyl groups to carboxylic acids and presumably methanols, was restricted to isolates W1 and F. 2,3-Diones were detected from benzothiophene and those methylbenzothiophenes with a methyl group on the benzene ring by isolates BT1, W1, and F, but not by isolate SB(G). Two ring cleavage products, isomers of tolyl methyl sulfoxide, arose from 6- and 7-methylbenzothiophene. Some novel high-molecular-weight metabolites were detected and these are the subject of the following chapter.

Table 2.1 Summary of the sulfur-containing metabolites produced from benzothiophene and all methylbenzothiophenes by four bacterial isolates.[a]

Substrate[b]	Products found in cultures of each bacterial strain			
	BT1	SB(G)	W1	F
BT	2,3-dione	sulfoxide sulfone $C_{16}H_{10}S$[c]	sulfoxide sulfone 2,3-dione $C_{16}H_{10}S$	sulfoxide sulfone 2,3-dione $C_{16}H_{10}S$
2-MBT	sulfoxide sulfone	sulfoxide sulfone	sulfoxide sulfone carboxylic acid	sulfoxide sulfone methanol carboxylic acid
3-MBT	sulfoxide sulfone	sulfoxide sulfone	sulfoxide sulfone methanol carboxylic acid	sulfoxide sulfone methanol carboxylic acid
4-MBT	2,3-dione	NT[d]	2,3-dione methanol carboxylic acid	2,3-dione $C_{18}H_{14}S$
5-MBT	2,3-dione	sulfone $C_{18}H_{14}S$	carboxylic acid	carboxylic acid 2,3-dione $C_{18}H_{14}S$
4-MBT & 6-MBT	*m*-tolyl methyl sulfoxide 2,3-diones	*m*-tolyl methyl sulfoxide sulfones $C_{18}H_{14}S$	*m*-tolyl methyl sulfoxide 2,3-diones carboxylic acid $C_{18}H_{14}S$	*m*-tolyl methyl sulfoxide 4-MBT-2,3-dione carboxylic acid $C_{18}H_{14}S$
7-MBT	sulfoxide sulfone 2,3-dione several unidentified compounds	NT	sulfoxide sulfone 2,3-dione *o*-tolyl methyl sulfoxide methanol carboxylic acid $C_{18}H_{14}S$	sulfoxide sulfone 2,3-dione *o*-tolyl methyl sulfoxide methanol carboxylic acid $C_{18}H_{14}S$

[a]The results for isolate BT1 were reported by Saftić *et al.* (1992) and the results for isolate SB(G) were reported by Gonçalves (1993).

[b] BT = benzothiophene, MBT = methylbenzothiophene

[c] Formula of high-molecular-weight product

[d] NT = Not tested

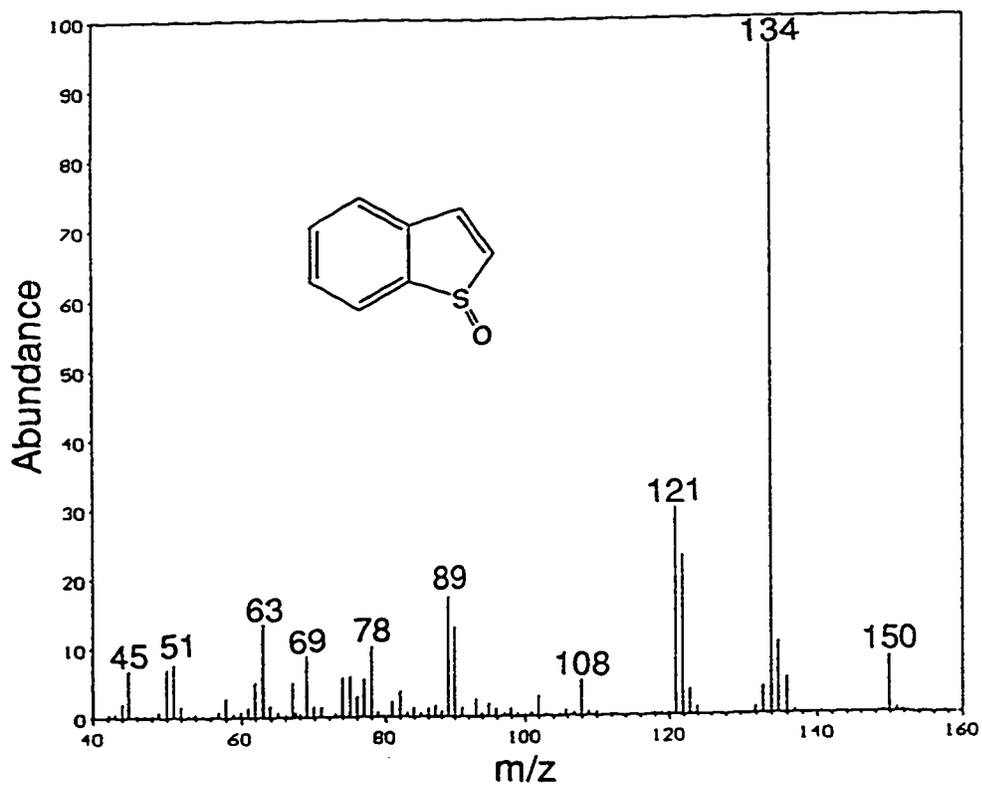


Figure 2.1 Mass spectrum of a metabolite produced from benzothiophene by a culture of isolate W1.

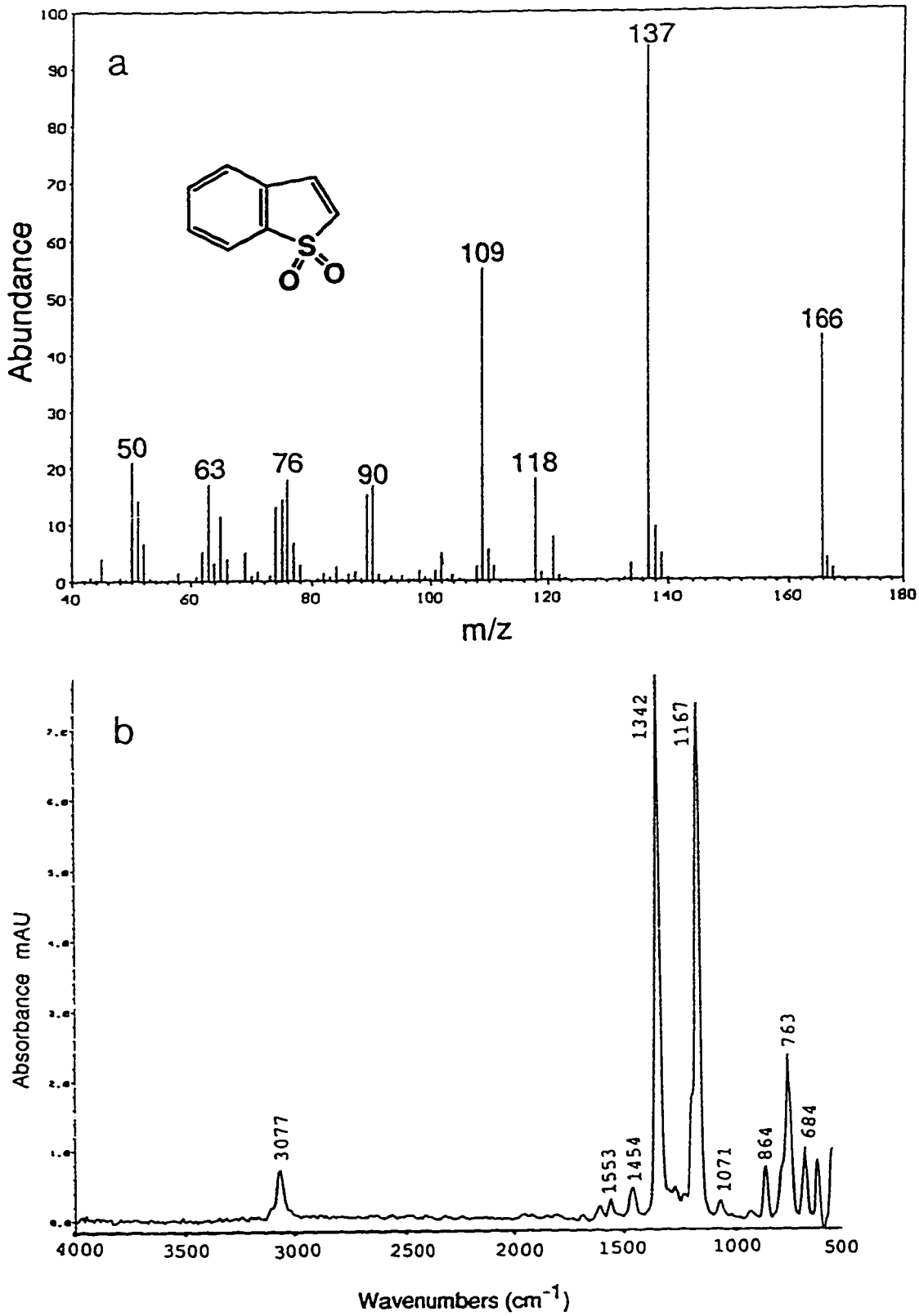


Figure 2.2 Mass spectrum (a) and FTIR spectrum (b) of one of the metabolites from benzothiophene produced by isolate W1.

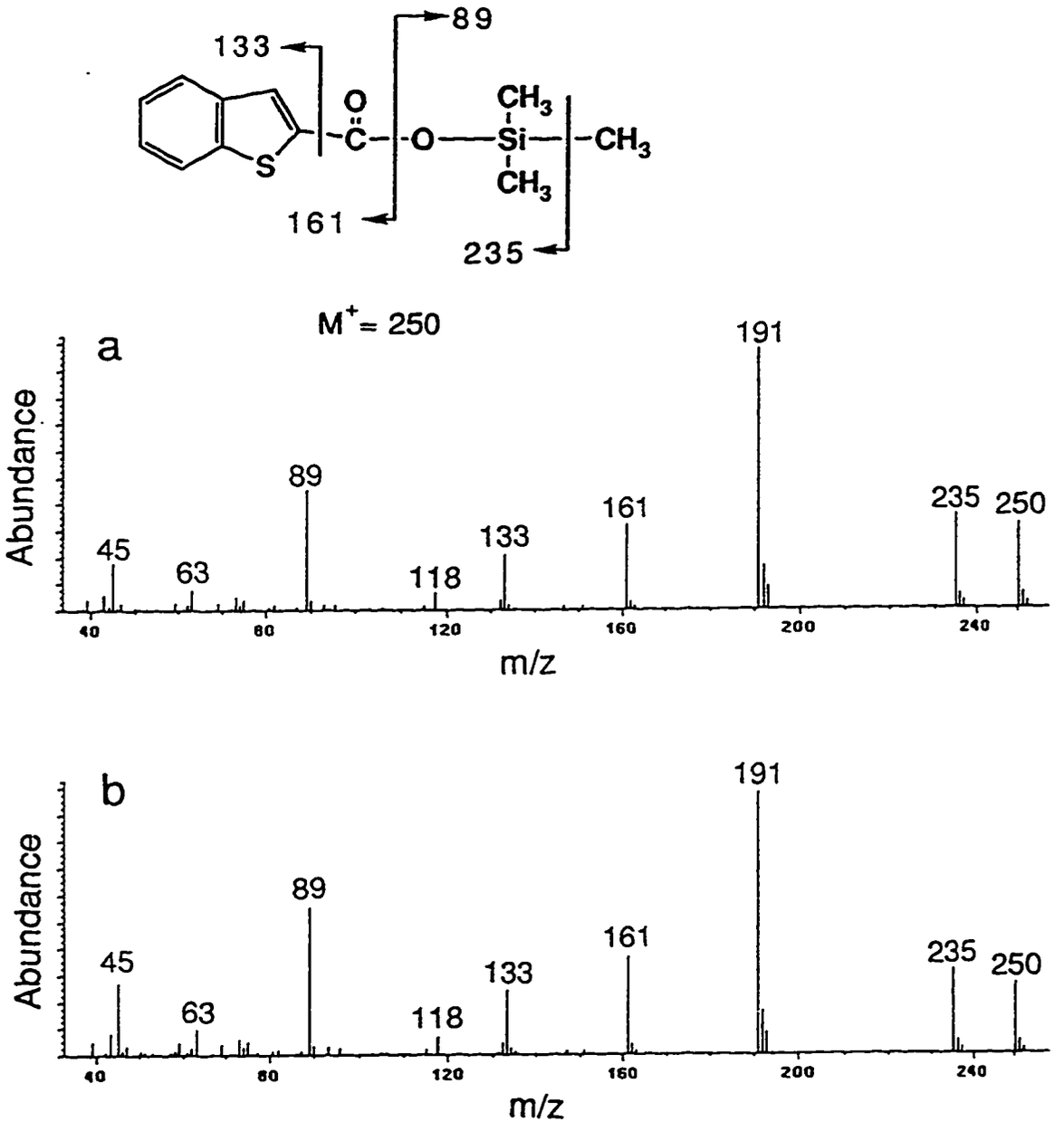


Figure 2.3 Mass spectra of the TMS-derivative of a sulfur-containing metabolite from a culture of isolate W1 grown on glucose in the presence of 2-methylbenzothiophene (a) and of the TMS-derivative of authentic 2-benzothiophenecarboxylic acid (b).

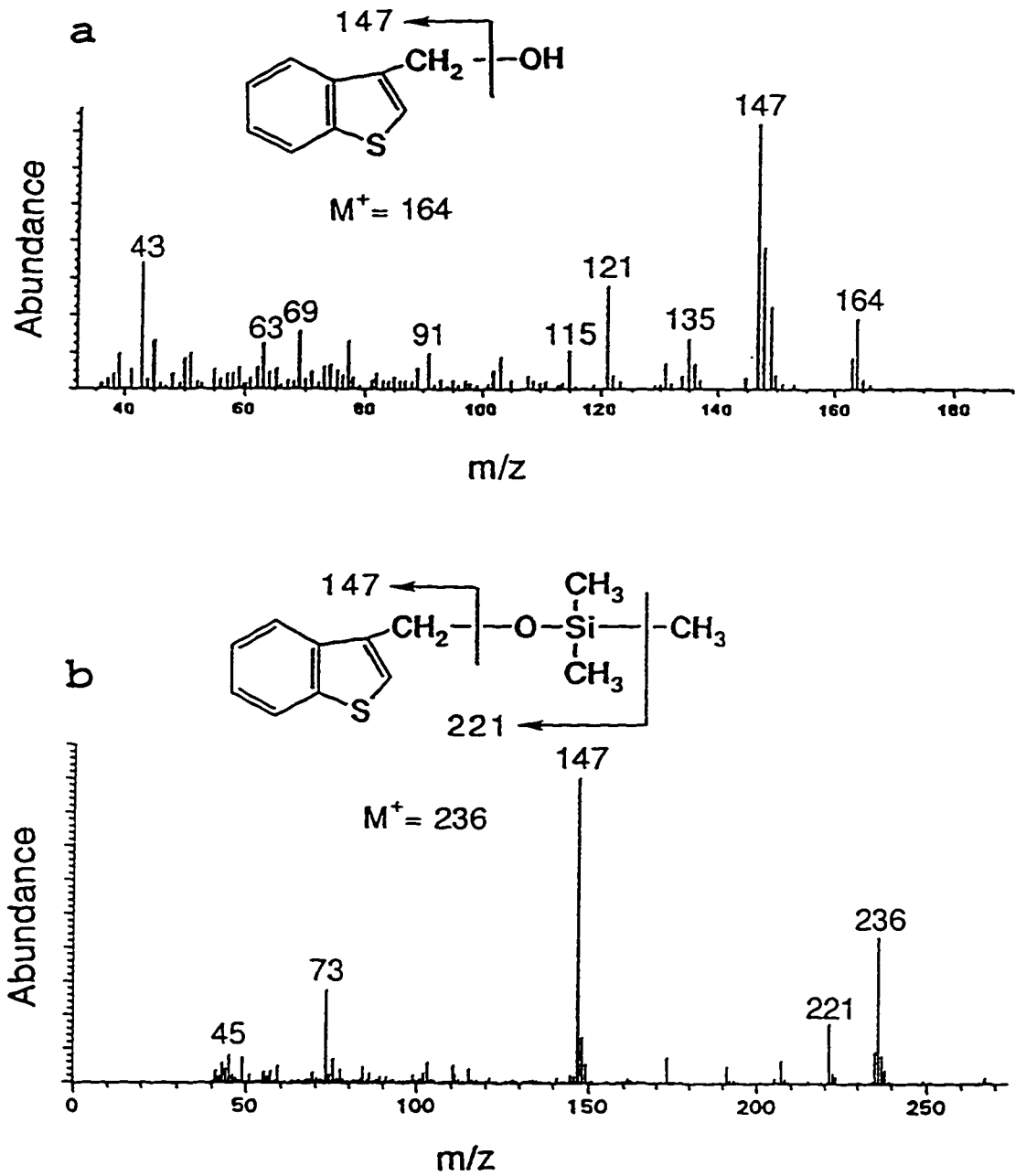


Figure 2.4 The mass spectra of a sulfur-containing metabolite from a culture of isolate F grown on glucose in the presence of 3-methylbenzothiophene (a) and of the TMS-derivative of the metabolite (b).

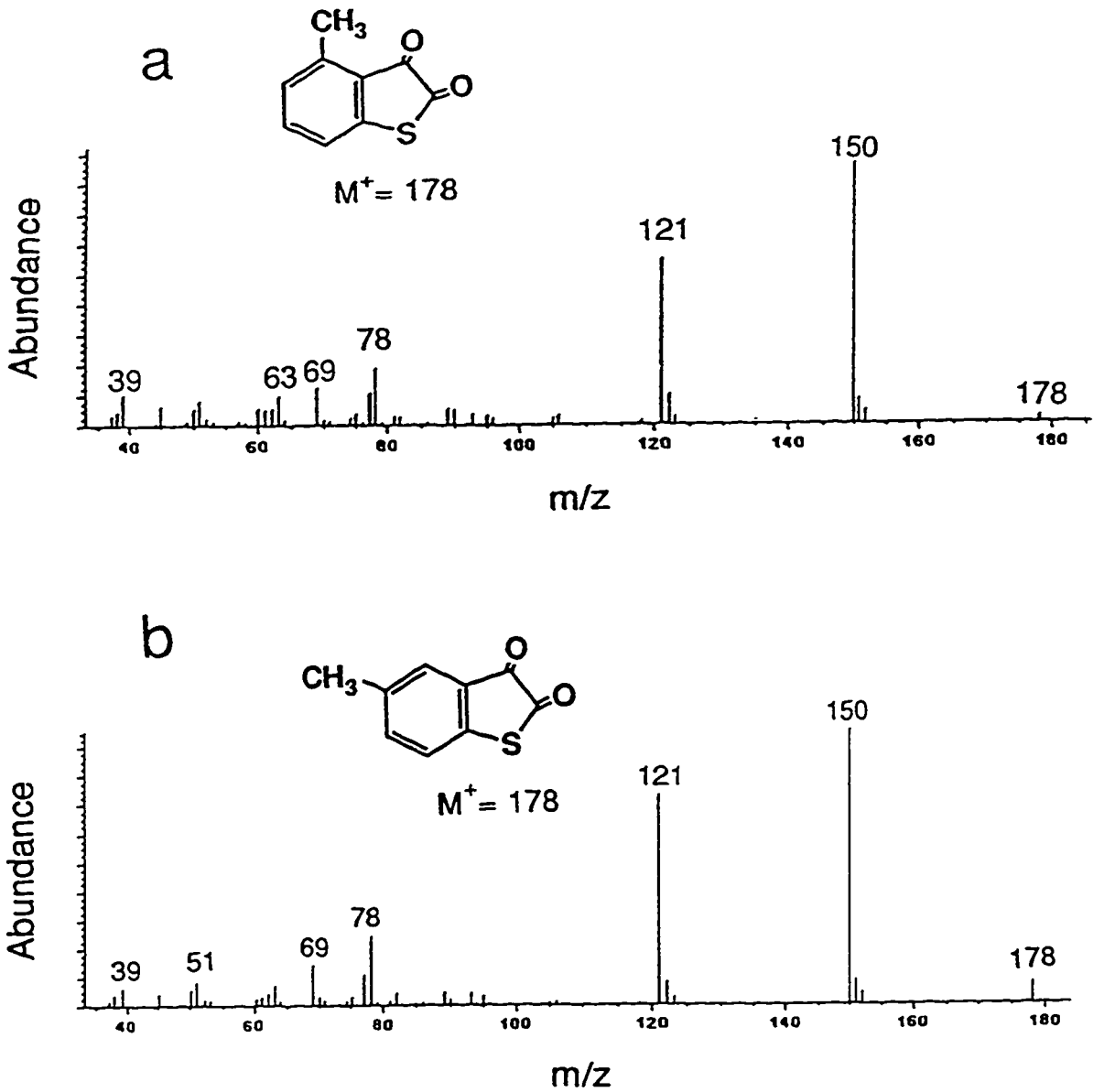


Figure 2.5 Mass spectra of a sulfur-containing metabolite from a culture of isolate W1 grown on glucose in the presence of 4-methylbenzothiophene (a) and of authentic 5-methylbenzothiophene-2,3-dione (b).

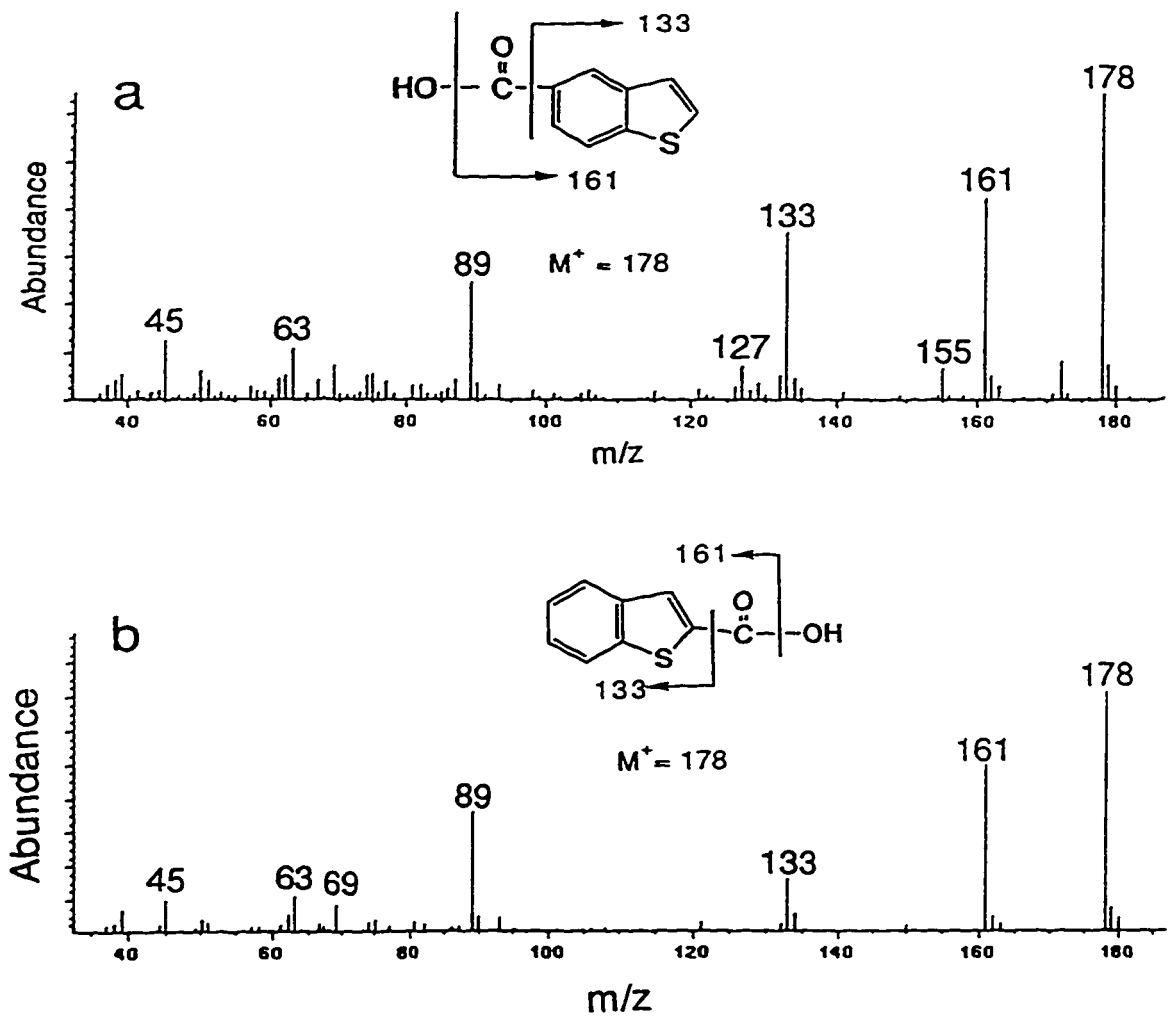


Figure 2.6 Mass spectra of a sulfur-containing metabolite from a culture of isolate W1 grown on 1-MN in the presence of 5-methylbenzothiophene (a) and of authentic 2-benzothiophenecarboxylic acid (b).

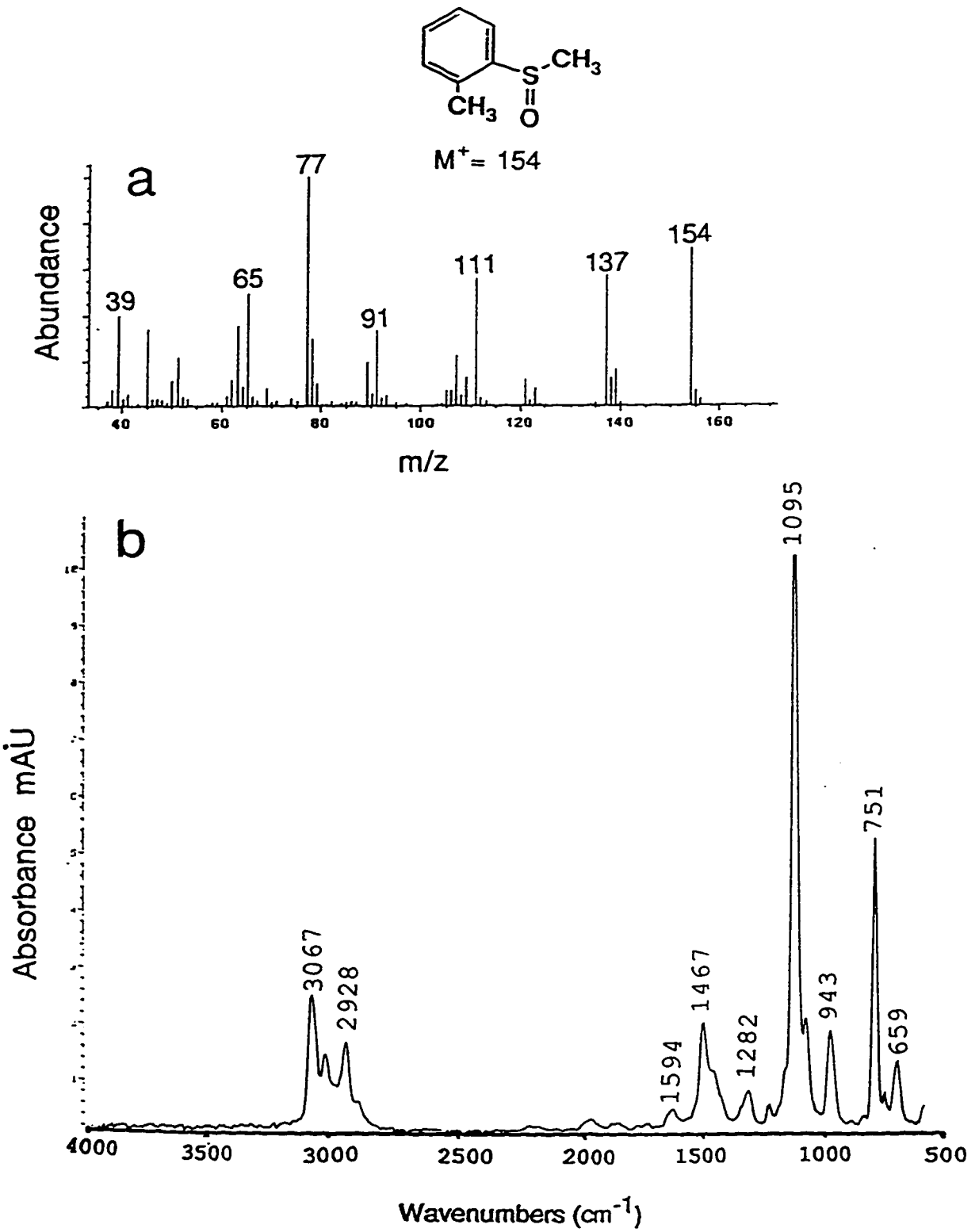


Figure 2.7 Mass spectrum (a) and FTIR spectrum (b) of one of the sulfur-containing metabolites from a culture of isolate F grown on glucose in the presence of 7-methylbenzothiophene.

2.5 LITERATURE CITED

Andersson, J. T. 1986. Gas chromatographic retention indices for all C_1- and C_2-alkylated benzothiophenes and their dioxides on three different stationary phases. J. Chromatogr. 354: 83-98.

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.

Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald. 1986. Selective aerobic degradation of methyl-substituted polycylic aromatic hydrocarbons in petroleum by pure microbial cultures. Intern. J. Environ. Anal. Chem. 23: 289-303.

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.

Bordwell, F. G., B. B. Lampert, and W. H. McKellin. 1949. Thianaphthene chemistry. III. The reaction of 2,3-dibromo-2,3-dihydro- and 2-bromothianaphthene-1-dioxides with secondary amines. J. Am. Chem. Soc. 71: 1702-1705.

Cerniani, A., and G. Modena. 1959. Oxidation of aryl- alkyl- and diarylsulfoxides to sulfones. Gazz. Chim. Ital. 89: 843-853; Chem. Abstr. 1960. 54: 22446i.

Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. Current Microbiol. 21: 229-231.

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

Fedorak, P. M. 1990. Microbial metabolism of organosulfur compounds in petroleum. In: Geochemistry of Sulfur in Fossil Fuels. Orr, W. L., and C. M. White, Eds. ACS Symposium Series 429; American Chemical Society: Washington, DC; p. 93-112.

Fedorak, P. M., and J. T. Andersson. 1992. Decomposition of two methylbenzothiophene sulfoxides in a commercial gas chromatography injection port liner. J. Chromatogr. 591: 362-366.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., J. D. Payzant, D. S. Montgomery, and D. W. S. Westlake. 1988. Microbial degradation of *n*-alkyl tetrahydrothiophenes found in petroleum. Appl. Environ. Microbiol. 54: 1243-1248.

Fedorak, P. M., and T. M. Peakman. 1992. Aerobic microbial metabolism of some alkylthiophenes found in petroleum. Biodegradation 2: 223-236.

Fedorak, P. M., and D. W. S. Westlake. 1981. Degradation of aromatics and saturates in crude oil by soil enrichments. Water, Air Soil Pollut. 16: 367-375.

Fedorak, P. M., and D. W. S. Westlake. 1983. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. 29: 291-296.

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.

Foght, J. M., and D. W. S. Westlake. 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. Can. J. Microbiol. 34: 1135-1141.

Glinzer, O., D. Severin, C.-D. Czogalla, C. Bedürftig, and U. Puttins. 1983. Mass spectrometry of bisanellated thiophene-S-oxides as model substances for oxidation products in crude oil. Fresenius Z. Anal. Chem. 315: 345-349.

Gonçalves, J. A. 1993. Oxidacion microbiana de compuestos organosulfurados puros y del petroleo. Ph.D. Thesis. Universidad Central de Venezuela, Caracas.

Haines, W. E., R. V. Helm, G. L. Cook, and J. S. Ball. 1956. Purification and properties of ten organic sulfur compounds-second series. J. Phys. Chem. 60: 549-555.

Hannoun, M., N. Blazevic´, D. Kolbah, M. Mihalic´, and F. Kajfez. 1982. *alpha*-Phenylpropionic acid derivatives. Synthesis and dethiation of 5-benzoylbenzo[*b*]thiophene-3-carboxylic acid. J. Heterocyclic Chem. 19: 1131-1136.

Jacob, J. 1990. Sulfur Analogues of Polycyclic Aromatic Hydrocarbons (Thiaarenes). Cambridge University Press: Cambridge; p. 70-87.

Jobson, A., F. D. Cook, and D. W. S. Westlake. 1972. Microbial utilization of crude oil. Appl. Microbiol. 23: 1082-1089.

Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. Part III. Isolation and identification of products from dibenzothiophene. Agr. Biol. Chem. 34: 1320-1324.

Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. 1973. Identification of microbial oxidation products from dibenzothiophene and its proposed oxidation pathway. Agr. Biol. Chem. 37: 45-50.

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.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* species. Appl. Environ. Microbiol. 34: 783-790.

Lagno, Z. Y., and S. M. Sviridov. 1972. Toxicity of benzothiophene and benzothiophene sulfone. Khim. Seraorg. Soedin., Soderzh. Neftyakh Neftprod. 9: 580-583; Chem. Abstr. 1975. 82: 119738e.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Mormile, M. R., and R. M. Atlas. 1989. Biotransformation of dibenzothiophene to dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. 35: 603-605.

Omori, T., L. Monna, Y. Saiki, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. SY1. Appl. Environ. Microbiol. 58: 911-915.

Patton, J. S., M. W. Rigler, P. D. Boehm, and D. L. Fiest. 1981. Ixtoc 2 oil spill: flaking of surface mousse in the Gulf of Mexico. Nature 290: 235-238.

Pierce, A. E. 1968. Silylation of Organic Compounds. Pierce Chemical Company: Rockford, Illinois; p. 33-39.

Porter, Q. N. 1967. Mass-spectrometric studies. I. Benzo[*b*]thiophen, some alkyl and aryl derivatives, and the 1,1-dioxide and 2,3-dihydro-1,1-dioxide. Aust. J. Chem. 20: 103-116.

Saftic´, 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.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Schlesinger, A. H. January 1953. U.S. Patent 2,624,665; Chem. Abstr. 1953. 47: 6596e.

Shirley, D. A., and M. D. Cameron. 1950. Metalation of thianaphthene by *n*-butyllithium. J. Am. Chem. Soc. 72: 2788-2789.

Shriner, R. L., R. C. Fuson, D. Y. Curtin, and T. C. Morrill. 1980. The Systematic Identification of Organic Compounds. 6th Ed. John Wiley & Sons: New York; p. 333.

Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak. 1993. Cytochrome c as a biocatalyst for the oxidation of thiophenes and organosulfides. Enzyme Microb. Technol. 15: 494-499.

Wenzel, B., and R. L. Aiken. 1979. Thiophenic sulfur distribution in petroleum fractions by gas chromatography with a flame photometric detector. J. Chromatogr. Sci. 17: 503-509.

Westlake, D. W. S. 1983. Microbial activities and changes in the chemical and physical properties of oil. In: Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Donaldson, E. C. and J. B. Clark, Eds. Technology

Transfer Branch, Bartlesville Energy Technology Centre: Bartlesville, Oklahoma; p. 102-111.

Williams, J. A., M. Bjorøy, D. L. Dolcater, and J. C. Winters. 1986. Biodegradation in south Texas eocene oils-effects on aromatics and biomarkers. Org. Geochem. 10: 451-461.

3. MICROBIALLY MEDIATED FORMATION OF BENZONAPHTHOTHIOPHENES FROM BENZO[*b*]THIOPHENES*

3.1 INTRODUCTION

The microbial removal of alkylbenzothiophenes and alkyldibenzothiophenes from the aromatic fraction of crude oil present in laboratory cultures (Bayona *et al.*, 1986; Fedorak and Westlake, 1983, 1984) and from the aromatic fraction of crude oil spilled into the environment (Atlas *et al.*, 1981) or biodegraded within its natural reservoir (Westlake, 1983; Williams *et al.*, 1986) has been demonstrated. Because of the complexity of the mixture of compounds present in crude oil, the metabolites of the biodegradation of these petroleum organosulfur compounds were not determined in those studies.

Various investigations using pure bacterial cultures and pure organosulfur compounds have demonstrated that benzothiophene and methylbenzothiophenes are susceptible to microbial attack. For example, *Pseudomonas aeruginosa* PRG-1 oxidized benzothiophene dissolved in light oil, but could not use this compound as a sole carbon source (Sagardía *et al.*, 1975), and a dibenzothiophene-oxidizing isolate, *Pseudomonas alcaligenes* DBT2, oxidized benzothiophene to water-soluble products (Finnerty *et al.*, 1983). However, the metabolites of benzothiophene oxidation were not identified in either of these studies.

In another investigation (Bohonos *et al.*, 1977), enrichment cultures from several aquatic environments and from wastewater treatment plant effluents were able to oxidize benzothiophene when naphthalene was provided as a carbon source. Tentative identifications of the benzothiophene metabolites as benzothiophene sulfoxide, 2,3-dihydrobenzothiophene-2,3-diol, and benzothiophene-2,3-dione were made on the basis of GC-MS analysis. Fedorak and Grbic´-Galic´ (1991) identified the products of biotransformation of benzothiophene and 3-methylbenzothiophene by a 1-MN-utilizing bacterium, *Pseudomonas* sp. BT1, as benzothiophene-2,3-dione and 3-methylbenzothiophene sulfoxide, respectively. A small amount of the sulfone of 3-methylbenzothiophene was also produced.

These findings led to the prediction that if benzothiophene was substituted with a methyl group on the thiophene ring, then the corresponding sulfoxide and sulfone would be formed whereas if there was a methyl group on the benzene ring, the corresponding 2,3-dione would be formed (Fedorak and Grbic´-Galic´, 1991). In a systematic study with

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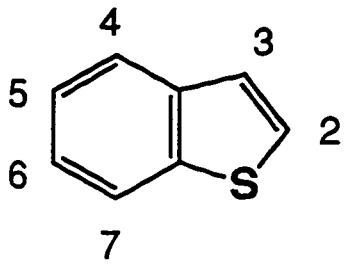
Pseudomonas sp. BT1 and synthesized methylbenzothiophenes, Saftic´ *et al.* (1992) observed that this prediction held true for the metabolism of 2-, 3-, 4-, 5-, and 6-methylbenzothiophene and 2,3-dimethylbenzothiophene. However, the metabolism of 7-methylbenzothiophene yielded the 2,3-dione, sulfoxide, and sulfone, in addition to several unidentified products. The ring numbering convention for benzothiophene is shown below.



A subsequent report (Kropp *et al.*, 1994) described the abilities of three other *Pseudomonas* strains to transform benzothiophene and each of the six isomers of methylbenzothiophene. Sulfoxides and sulfones were frequently detected and these were the most abundant products from 2- and 3-methylbenzothiophene. 2,3-Diones were observed as metabolites of benzothiophene and methylbenzothiophenes with a methyl group on the benzene ring. However, these new strains also oxidized the sulfur atom of benzothiophene to give the sulfoxide and sulfone. Two of the isolates oxidized the methyl groups of the methylbenzothiophenes producing benzothiophenemethanols and benzothiophenecarboxylic acids. Isomers of tolyl methyl sulfoxide were also observed as thiophene ring cleavage products from 6- and 7-methylbenzothiophene. Recently, Eaton and Nitterauer (1994) identified several metabolites of benzothiophene produced by isopropylbenzene-degrading bacteria. These included 2-mercaptophenylglyoxalate and *trans*-4-[3-hydroxy-2-thienyl]-2-oxobut-3-enoate that resulted from the cleavage of the thiophene ring and the benzene ring, respectively.

Kropp *et al.* (1994) observed that some high-molecular-weight products were formed in cultures that were incubated in the presence of benzothiophene and those methylbenzothiophenes that had the methyl group on the benzene ring. The molecular weight of the product from benzothiophene was 234, consistent with the chemical formula $C_{16}H_{10}S$. Methylbenzothiophenes gave products which were 28 mass units larger than the product from benzothiophene, consistent with the formula $C_{18}H_{14}S$. This chapter describes the identification of these high-molecular-weight compounds and the mechanism of their formation.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Benzo[*b*]thiophene, benzo[*b*]naphtho[2,1-*d*]thiophene, and 1-phenylnaphthalene were purchased from Aldrich (Milwaukee, WI). 3-Methylbenzo[*b*]thiophene was purchased from Lancaster Synthesis (Windham, NH). 1-MN and 2-phenylnaphthalene were purchased from Fluka (Buchs, Switzerland). The methods given by Andersson (1986) were used for the syntheses of 2-, 4-, 5-, and 7-methylbenzothiophene and a mixture of 4- and 6-methylbenzothiophene. Sulfones of the benzothiophenes were synthesized by boiling the sulfur-containing compound with H_2O_2 in acetic acid for 15 min (Bordwell *et al.*, 1949).

Crude preparations of the sulfoxides were prepared by oxidizing the benzothiophenes with horse heart cytochrome c (Sigma, St. Louis, MO) and H_2O_2 by scaling up the method of Vazquez-Duhalt *et al.* (1993). Specifically, benzothiophene or 5-methylbenzothiophene was dissolved in acetonitrile to 10 mM. This was diluted to 1 mM with 6 mM phosphate buffer (pH 6.1), and then cytochrome c, in the same buffer solution, and H_2O_2 were added to concentrations of 400 nM and 1 mM, respectively. The reaction was stirred briefly and left to sit for 1 h at room temperature (20 to 22°C). Because H_2O_2 destroys the activity of cytochrome c, multiple hourly additions of the cytochrome and H_2O_2 were made over the course of two working days. The reaction mixture sat at room temperature between additions. On the third day, the reaction mixture was saturated with sodium chloride and extracted with diethylether to recover the products. In an attempt to produce enough benzothiophene sulfoxide to purify by column chromatography, 94 mg of benzothiophene was used for the reaction described above, with a total of 15 additions of the cytochrome c and H_2O_2 solutions.

3.2.2 Bacterial cultures

The isolation and characterization of *Pseudomonas* strain W1 and *Pseudomonas* strain F (Kropp *et al.*, 1994; Satic´ *et al.*, 1993) have been described previously. A mixed culture of petroleum-degrading bacteria, designated SLPB (Fedorak and Peakman, 1992) was also used.

3.2.3 Culture methods and media

The mineral medium used for growing cultures of isolates W1 and F in biotransformation studies was modified from that of Fedorak and Westlake (1984) by increasing the phosphate concentration 8-fold to provide greater buffer capacity at pH 7.0.

The modified medium contained (per 900 mL): NH_4Cl, 1.0 g; Na_2SO_4, 2.0 g; KNO_3, 2.0 g; $FeSO_4 \cdot 7H_2O$, trace; trace metal solution (Fedorak and Grbic´-Galic´, 1991), 1 mL. To this was added 100 mL of a buffer prepared by adding a solution of KH_2PO_4 (4 g/100 mL) into a solution of K_2HPO_4 (4 g/100 mL) until the pH was 7.0. The buffered medium was then sterilized by autoclaving. Prior to inoculation, 1.0 mL of a separately sterilized solution of $MgSO_4 \cdot 7H_2O$ (4 g/100 mL) was added to each 200-mL portion of medium.

The benzothiophenes studied in biotransformation experiments with strains W1 and F would not support the growth of these isolates. Thus, 1-MN or glucose was used as the growth substrate and the biotransformations of the benzothiophenes were observed. When 1-MN was used, the cultures were inoculated with 10 mL of a 1-MN-grown maintenance culture that was transferred weekly. When glucose was used, a glucose-grown culture that had incubated for 2 to 3 days was used as the source of inoculum (1 mL). This glucose-grown seed culture was inoculated from a single colony of the desired isolate that had grown for 3 to 5 days on PCA (Difco, Detroit, MI), after having been streaked from a maintenance culture grown with 1-MN. The growth substrates were sterilized separately and 50 mg were added per 200 mL of modified medium. Each 200-mL portion of medium also received 2 to 5 mg of benzothiophene or a methylbenzothiophene, and the culture was incubated for 7 days (unless otherwise stated) prior to extraction. Occasionally, the amount of benzothiophene added to cultures was increased to as high as 45 mg per 200-mL culture. For each biotransformation experiment, appropriate sterile controls were incubated to account for any abiotic transformations. All cultures and controls were incubated at 28°C with shaking at 200 rpm.

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

A mixed culture, designated SLPB, was used in experiments to determine if the high-molecular-weight product would be formed in the presence of crude oil. This culture was grown in 200 mL of mineral medium (Fedorak and Westlake, 1984) with 0.2 mL of Prudhoe Bay crude oil supplemented with 8 mg of benzothiophene.

3.2.4 Analytical methods

After incubation, the cultures were acidified with sulfuric acid to pH<2 and extracted with DCM (4 times 20 mL) to recover substrates and products. The extracts were dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. To screen for the presence of sulfur-containing products, the extracts were analyzed by GC using a 30 m DB-5 capillary column in an instrument equipped with a FID and a sulfur-selective FPD (Fedorak and Grbic´-Galic´, 1991).

Routine GC-MS analyses were done with a Hewlett-Packard (HP; Mississauga, ON) model 5890 GC fitted with a 30 m DB-1 or DB-5 capillary column and coupled to a 5970 series mass selective detector (Saftic´ *et al.*, 1993). In some instances, GC-MS was done by the personnel in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta. The instrument and conditions have been described previously (Fedorak and Westlake, 1986).

3.2.5 Column chromatography procedure

To purify the polar products from the cytochrome c-mediated oxidation of benzothiophene, the silica gel column chromatography method of Fedorak and Andersson (1992) was followed with a few modifications. Specifically, the prepared column was developed with 5 mL of *n*-pentane, 5 mL of DCM:*n*-pentane (20:80), and 25 mL of DCM:*n*-pentane (50:50) as previously reported (Fedorak and Andersson, 1992), but the volume of methanol:benzene (50:50) used as the final solvent was increased from 30 mL to 45 mL. The first 50 mL to elute from the column were collected as the aromatic fraction. The next 5-mL fraction was collected and concentrated to 100 µL, and analyzed by GC to ensure that no thiophenes or polar compounds were present, confirming a clean separation of these compounds. The final 20 mL were collected to contain the polar compounds, namely the sulfoxide and sulfone of benzothiophene.

3.2.6 Nickel boride desulfurization procedure

To help determine the structure of some sulfur-containing, high-molecular-weight products, the nickel boride desulfurization reaction of Back *et al.* (1992) was used. The procedure was verified using dibenzothiophene and benzo[*b*]naphtho[2,1-*d*]thiophene which yielded biphenyl and 2-phenylnaphthalene, respectively.

3.3 RESULTS

3.3.1 Identification of the high-molecular-weight compound from benzothiophene

DCM-extracts of the benzothiophene-containing medium that had been incubated with either of the *Pseudomonas* isolates showed the presence of a high-molecular-weight, sulfur-containing compound that eluted from the GC column at a late retention time. GC-MS analysis gave a mass spectrum for the compound with a strong molecular ion at m/z 234 (Figure 3.1), corresponding to the empirical formula $C_{16}H_{10}S$. Figure 3.2 shows four products with this chemical formula that could arise from a condensation reaction between two molecules of benzothiophene with the loss of two atoms of H and one atom of S. These include the three isomers with a central thiophene ring, the benzonaphtho-thiophenes, and one example of a condensation product with a peripheral thiophene ring, phenanthro[2,1-*b*]thiophene. The mass spectrum of the compound found in culture extracts was very similar to that of benzo[*b*]naphtho[1,2-*d*]thiophene and the other two benzonaphthothiophenes (Karcher *et al.*, 1985).

The GC retention time of the high-molecular-weight metabolite did not match that of an authentic standard of benzo[*b*]naphtho[2,1-*d*]thiophene (compound III), therefore it was not this isomer of benzonaphthothiophene. No authentic standards of the other possible sulfur-containing compounds shown in Figure 3.2 were available, so the nickel boride desulfurization reaction was used to identify the high-molecular-weight compound. This reaction distinguishes between benzo[*b*]naphtho[1,2-*d*]thiophene (compound I) and benzo[*b*]naphtho[2,3-*d*]thiophene (compound II) because the former gives 1-phenyl-naphthalene (compound V) and the latter gives 2-phenylnaphthalene (compound VI) upon desulfurization. These two isomers of phenylnaphthalene were readily separated by the GC method used.

Desulfurization of the high-molecular-weight metabolite yielded a product with the same GC retention time as 1-phenylnaphthalene (compound V, Figure 3.2). The mass spectrum of the desulfurized metabolite is shown in Figure 3.3a and it matches that of authentic 1-phenylnaphthalene shown in Figure 3.3b. Thus, the high-molecular-weight product was benzo[*b*]naphtho[1,2-*d*]thiophene (compound I, Figure 3.2).

3.3.2 Verification that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene was microbially-mediated

Evidence suggested that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene in 1-MN- or glucose-grown cultures of isolates W1 and F was microbially-mediated. For

example, the high-molecular-weight compound never formed in the sterile controls that contained medium and benzothiophene. Furthermore, Gonçalves (1993) reported that when 100 μg/mL of chloramphenicol was added to the cell suspension of isolate SB(G), the high-molecular-weight compound was not formed from benzothiophene.

Haines *et al.* (1956) and Andersson and Bobinger (1992) identified benzo[*b*]naphtho[2,1-*d*]thiophene as a photooxidation product of benzothiophene. To verify that the condensation product was not the result of a photochemical reaction, duplicate cultures of isolate W1 were grown on 1-MN in the presence of benzothiophene and duplicate sterile controls were incubated with these cultures. One flask of the inoculated cultures and one of the sterile controls were wrapped in aluminum foil to prevent light from reaching the benzothiophene-containing medium, and the remaining two flasks were left uncovered. After 7 days of incubation, the contents of the flasks were extracted and analyzed by GC-FPD. The sterile controls contained no sulfur-containing compounds other than the benzothiophene originally added. On the other hand, GC analyses of the extracts from both of the cultures of isolate W1 showed the presence of the high-molecular-weight product regardless of whether or not the culture was incubated with the exclusion of light. These results clearly indicate that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene in cultures containing benzothiophene was microbially-mediated and was not the result of a photochemical reaction of benzothiophene or of a photochemical reaction of the microbial metabolites of benzothiophene oxidation.

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

To ensure that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene was not an artifact of some other aspect of the liquid-liquid extraction procedure which was routinely used, another method to recover the high-molecular-weight product from aqueous cultures was used. In this case, a 200-mL culture of isolate F grown on 1-MN in the presence of 25 mg of benzothiophene for 7 days, was freeze-dried overnight. The residue was washed with acetonitrile, and after concentrating under nitrogen, the wash was analyzed by GC which confirmed the presence of benzo[*b*]naphtho[1,2-*d*]thiophene. A 200-mL sterile control containing 1-MN and 25 mg of benzothiophene was carried through the same

procedure and no condensation product was detected in the acetonitrile wash of the freeze-dried material. These experiments confirmed that the formation of benzo[*b*]naphtho[1,2-*d*]thiophene was not caused by the conditions of the liquid-liquid extraction procedure that was routinely used.

3.3.3 Dimethylbenzonaphthothiophenes from methylbenzothiophenes

In addition to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene from benzothiophene, the formation of high-molecular-weight products from 4-, 5-, 6-, and 7-methylbenzothiophenes in cultures of isolates SB(G), W1, and F was observed (Kropp *et al.*, 1994). All of these products gave very similar mass spectra with an abundant molecular ion at m/z 262, with few fragmentations. This molecular weight is consistent with the empirical formula $C_{18}H_{14}S$, which is 28 mass units greater than benzo[*b*]naphtho[1,2-*d*]thiophene, suggesting dimethyl-substituted products. The mass spectrum shown in Figure 3.4a is that of the product which was formed from 5-methylbenzothiophene in a 1-MN-grown culture of isolate F. The proposed structure shown in Figure 3.4a has the methyl groups substituted at positions 3 and 10 of the benzo[*b*]naphtho[1,2-*d*]thiophene nucleus, which is consistent with the condensation mechanism discussed later. If 5-methylbenzothiophene condenses by the same mechanism which gives benzo[*b*]naphtho[1,2-*d*]thiophene from benzothiophene, then 3,10-dimethylbenzo[*b*]naphtho[1,2-*d*]thiophene is postulated to be the condensation product from 5-methylbenzothiophene. This high-molecular-weight product was desulfurized with nickel boride to give a compound with a molecular weight of 232 (Figure 3.4b). This is consistent with a dimethyl-substituted 1-phenylnaphthalene. The fragments at m/z 217 and m/z 202 represent the loss of one and two methyl groups, respectively. However, no authentic standard was available to positively identify this compound.

3.3.4 Methyl-substituted benzonaphthothiophenes from a mixture of benzothiophene and 5-methylbenzothiophene

When isolate F was incubated with 1-MN in the presence of both benzothiophene and 5-methylbenzothiophene, four high-molecular-weight products were detected. Two of these had the same retention times and mass spectra as benzo[*b*]naphtho[1,2-*d*]thiophene and 3,10-dimethylbenzo[*b*]naphtho[1,2-*d*]thiophene which were previously identified as products from benzothiophene and 5-methylbenzothiophene, respectively. The other two products eluted between the unsubstituted and dimethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes during GC analysis. These both gave very similar mass spectra, so only one is shown (Figure 3.5), with a strong molecular ion at m/z 248. This molecular weight is

consistent with the two possible monomethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes which could form by the condensation of a molecule of benzothiophene with a molecule of 5-methylbenzothiophene.

3.3.5 Mechanism of formation of benzo[*b*]naphtho[1,2-*d*]thiophene

Figure 3.6 shows six possible schemes that might lead to an initial Diels-Alder condensation reaction, the product of which could undergo subsequent reactions leading to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene. The goal of this work was to determine which of these schemes was the most likely mechanism for the initial condensation step. Benzo[*b*]naphtho[1,2-*d*]thiophene was never formed in sterile controls containing benzothiophene, eliminating Scheme A and indicating the important role of the bacterial isolates in mediating the formation of these condensation products. It was postulated that after microbial oxidation of benzothiophene to the sulfoxide or sulfone, an abiotic condensation reaction occurred, leading to the formation of the high-molecular-weight product (Schemes B to F). Extracts of benzothiophene-containing cultures of isolates SB(G), W1, and F, in which benzo[*b*]naphtho[1,2-*d*]thiophene had been first identified, always contained benzothiophene and its sulfoxide and sulfone (Kropp *et al.*, 1994).

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

Hence, it appeared that the condensation reaction to give benzo[*b*]naphtho[1,2-*d*]thiophene involved benzothiophene sulfoxide (Scheme B, D, or E) which could not be synthesized by chemical means. Thus, the method of Vazquez-Duhalt *et al.* (1993), using cytochrome c and H_2O_2, was scaled up to synthesize a modest amount of sulfoxide from 94 mg of benzothiophene. However GC-FPD analysis of ether extracts of the reaction mixture showed that little of the benzothiophene had been oxidized and that under these reaction conditions both the sulfoxide and sulfone of benzothiophene, along with several other sulfur-containing compounds were formed. Interestingly, GC-MS analysis also showed that benzo[*b*]naphtho[1,2-*d*]thiophene was present in the extract of the reaction mixture. GC-FID analysis, using commercially available benzo[*b*]naphtho[2,1-*d*]thiophene as a quantitative standard, showed that 1.4 mg of the high-molecular-weight product had

formed in this reaction. This observation demonstrated that active microbial growth was not required for the formation of the condensation product. The cytochrome c-catalyzed oxidation of benzothiophene was sufficient to promote the formation of benzo[*b*]naphtho[1,2-*d*]thiophene.

The polar compounds benzothiophene sulfoxide and benzothiophene sulfone were separated from the excess benzothiophene and the condensation product by silica gel column chromatography. GC analysis revealed that the first 50 mL of solvent collected from the column contained the benzothiophene and benzo[*b*]naphtho[1,2-*d*]thiophene that had been present in the extract of the cytochrome c reaction mixture. The next 5 mL of solvent to elute from the column was concentrated approximately 50-fold, and GC analysis of the concentrate showed no sulfur-containing compounds. This confirmed that all the benzothiophene and benzo[*b*]naphtho[1,2-*d*]thiophene had eluted in the first 50 mL of eluent and that none of the oxidized products had eluted. GC-MS analysis showed that the last 20 mL of methanol-benzene to elute contained benzothiophene sulfoxide and sulfone. Furthermore, GC-MS analysis showed benzo[*b*]naphtho[1,2-*d*]thiophene in this polar fraction. This suggested that the high-molecular-weight product was formed by abiotic condensation of the compounds present in the polar fraction thereby ruling out Scheme B. The benzo[*b*]naphtho[1,2-*d*]thiophene formation during silica gel column chromatography is consistent with the enhancement of the rate of Diels-Alder reactions by absorption onto silica gel as has been previously reported (Veselovsky *et al.*, 1988).

The cytochrome c-catalyzed oxidation procedure was also done with a mixture of benzothiophene and 5-methylbenzothiophene. GC-MS analysis of the ether extract of the reaction mixture showed that it contained benzothiophene, 5-methylbenzothiophene, the corresponding sulfones, benzothiophene sulfoxide, benzo[*b*]naphtho[1,2-*d*]thiophene, two isomers of mono-methyl benzo[*b*]naphtho[1,2-*d*]thiophene and a dimethylbenzo[*b*]naphtho[1,2-*d*]thiophene. Interestingly, no 5-methylbenzothiophene sulfoxide was detected in the extract, presumably because it readily reacted to form the condensation products or was readily oxidized further to the respective sulfone. Alternatively, all of this sulfoxide may have decomposed in the GC injection port liner (Fedorak and Andersson, 1992).

The observations from the cytochrome-c oxidation of the mixture of benzothiophene and 5-methylbenzothiophene provided a simple means to determine whether or not the sulfones played a role in the condensation mechanism (Scheme E). Benzothiophene (14 mg) and 3 mg of chemically synthesized 5-methylbenzothiophene sulfone were added to a reaction mixture containing cytochrome c and H_2O_2. At the end of the reaction time, the mixture was extracted and analyzed by GC-MS which showed that the only high-molecular-weight product that formed was benzo[*b*]naphtho[1,2-

d]thiophene. This indicated that the condensation did not involve the sulfone, because if it had, the 5-methylbenzothiophene sulfone would have reacted with the benzothiophene sulfoxide (formed by the cytochrome c oxidation) to give a mono-methyl benzo[*b*]naphtho[1,2-*d*]thiophene. To further verify this, the cytochrome c oxidation procedure was done with 5 mg of 5-methylbenzothiophene and 13 mg of chemically synthesized benzothiophene sulfone. If the sulfone was involved in the condensation mechanism, then a mono-methylated condensation product would have been detected. However, the only condensation product present was a dimethylbenzo[*b*]naphtho[1,2-*d*]thiophene which formed through the condensation of two molecules of 5-methylbenzothiophene sulfoxide, produced by the cytochrome c oxidation. Thus, Scheme D describes the formation of benzo[*b*]naphtho[1,2-*d*]thiophene from benzothiophene sulfoxide.

Based on these observations, the mechanism shown in Figure 3.7 was proposed, whereby two molecules of benzothiophene sulfoxide, produced by bacterial or cytochrome c oxidation of benzothiophene, condense by a Diels-Alders type mechanism with the subsequent loss of one atom of sulfur, two atoms of hydrogen and two atoms of oxygen resulting in the formation of benzo[*b*]naphtho[1,2-*d*]thiophene. The sulfoxide of benzothiophene functions as both diene and dienophile in this reaction. The last two steps in the mechanism are considered to be abiotic because of the observed formation of the condensation product in the polar fraction obtained by silica gel chromatography.

The methyl- and dimethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes observed in cultures of isolates SB(G), W1, and F (Kropp *et al.*, 1994) are believed to form by the same mechanism. Although the sulfoxides of 2-methylbenzothiophene and 3-methylbenzothiophene have been observed in other studies (Fedorak and Grbic´-Galic´, 1991; Kropp *et al.*, 1994; Saftic´ *et al.*, 1992), condensation products from these isomers were never detected (Kropp *et al.*, 1994). This is consistent with the mechanism shown in Figure 3.7, because the condensation would be hindered by a methyl group on the thiophene ring.

Table 3.1 summarizes the high-molecular-weight products found in various cultures incubated with methylbenzothiophenes. Assuming the mechanism given in Figure 3.7 applies to all of these condensation reactions, the postulated identities of the methyl- and dimethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophenes are given in Table 3.1. However, no authentic standards were available to unequivocally identify these compounds. Although it is possible that four isomers of dimethyl-substituted benzo[*b*]naphtho[1,2-*d*]thiophene could be formed from the mixture of 4- and 6-methylbenzothiophene, only two peaks on

the gas chromatogram were detected. However, these peaks may have contained co-eluting isomers.

3.3.6 Quantification of the amount of benzo[*b*]naphtho[1,2-*d*]thiophene produced by isolates W1 and F

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

3.3.7 Formation of benzo[*b*]naphtho[1,2-*d*]thiophene by a mixed culture incubated with Prudhoe Bay crude oil

The DCM extract from a 14-day-old culture of an oil-degrading mixed culture, SLPB, grown on benzothiophene-supplemented crude oil was analyzed by GC-FPD, and the extract from a sterile control was analyzed in the same manner (Figure 3.8). GC-MS analysis of the extract from the viable culture confirmed the presence of the sulfoxide and sulfone of benzothiophene, and benzo[*b*]naphtho[1,2-*d*]thiophene. GC-FID analyses, using benzo[*b*]naphtho[2,1-*d*]thiophene as a quantitative standard, showed that 0.04 mg of the condensation product was formed from the 8 mg of benzothiophene added to the oil. This amount of benzothiophene was approximately 5% of the weight of the oil added. In another experiment, 1 mg of benzothiophene was added, and the benzo[*b*]naphtho[1,2-*d*]thiophene peak was just detectable in the culture extract.

3.4 DISCUSSION

The oxidation of some of the benzothiophenes to their respective sulfoxides by the activity of the three *Pseudomonas* isolates SB(G) (Gonçalves *et al.*, 1993), W1, and F, or by the cytochrome c-catalyzed oxidation reaction, resulted in the formation of benzo[*b*]naphtho[1,2-*d*]thiophenes. Proving the involvement of sulfoxides in the

condensation mechanism was complicated by the difficulty experienced in synthesizing and isolating pure benzothiophene sulfoxide. Chemical methods of oxidizing the sulfur atom do not stop at the sulfoxide, but proceed to the sulfone. Vazquez-Duhalt *et al.* (1993) reported the oxidation of a number of organosulfur compounds, including benzothiophene, to their respective sulfoxides by horse heart cytochrome c, in small-scale reactions. Sulfones were not detected by GC-MS and high performance liquid chromatography analyses in that report. In the current study, the synthesis of about 100 mg of benzothiophene sulfoxide was attempted using a scaled up version of their method. However, despite multiple additions of cytochrome c and H_2O_2, quantitative oxidization of the benzothiophene was not achieved. As well, the scaled up method yielded the sulfoxide and sulfone as determined by GC-MS analyses. The isolation of pure benzothiophene sulfoxide was further complicated by the formation of the condensation product, benzo[*b*]naphtho[1,2-*d*]thiophene, from the benzothiophene sulfoxide in the reaction mixture and on the silica gel column. Although the attempted synthesis of pure benzothiophene sulfoxide was unsuccessful, the formation of the condensation product in the cytochrome c-mediated oxidation reaction made it possible to determine that only the sulfoxides (Scheme D, Figure 3.6) were involved in the condensation reaction.

GC analyses of some of the extracts from cultures of isolates SB(G), W1, and F incubated in the presence of methylbenzothiophenes failed to detect the corresponding sulfoxide intermediates, although the dimethylbenzonaphthothiophenes were detected (Kropp *et al.*, 1994). Likely, the sulfoxide had condensed to form the high-molecular-weight product, or it had been oxidized by the bacterial culture to the sulfone. However, it is possible that the undetected sulfoxides decomposed in the GC injection port liner (Fedorak and Andersson, 1992).

There are a few reports on methods for the chemical synthesis of benzo[*b*]naphtho[1,2-*d*]thiophene. For example, Davies *et al.* (1952) observed its formation as a minor product when the reaction mixture from the oxidation of benzothiophene to benzothiophene sulfone by H_2O_2 and acetic acid (Bordwell *et al.*, 1949) was extracted with chloroform, dried and distilled under reduced pressure (boiling point 280°C at 15 mm Hg). Minor amounts of the sulfone of benzo[*b*]naphtho[1,2-*d*]thiophene were also produced. In other studies (Bordwell *et al.*, 1951; Davies *et al.*, 1952), 6a,11b-dihydrobenzo[*b*]naphtho[1,2-*d*]thiophene sulfone was obtained in 80-90% yield from the controlled pyrolysis of benzothiophene sulfone (180-200°C in tetralin). The aromatization of this compound by sequential bromination and treatment with alcoholic potassium hydroxide followed by reduction of the sulfone with $LiAlH_4$ has been used for the synthesis of benzo[*b*]naphtho[1,2-*d*]thiophene (Davies *et al.*, 1952). This synthesis requires reaction

conditions that are much more severe than those that lead to the formation of benzo[*b*]naphtho[1,2-*d*]thiophene from the microbially-produced benzothiophene sulfoxide reported here. Indeed, the cytochrome c-catalyzed oxidation of benzothiophenes to their sulfoxides, that spontaneously condense, should serve as a simple method for the synthesis of small amounts of benzo[*b*]naphtho[1,2-*d*]thiophenes, although isolation of the condensation product from other sulfur-containing products will be required.

The microbially-mediated formation of benzonaphthothiophenes from benzothiophenes will produce compounds that are less volatile, less water-soluble, and less mobile than the parent compounds. Whether this occurs in petroleum- or creosote-contaminated environments has yet to be determined. However, the laboratory tests using Prudhoe Bay crude oil supplemented with benzothiophene, to a concentration that was sufficient to allow detection of the metabolites, showed that a mixed culture produced benzothiophene sulfoxide, benzothiophene sulfone, and benzo[*b*]naphtho[1,2-*d*]thiophene. Kropp *et al.* (1994) demonstrated that the microbial population in a river water sample also produced the benzothiophene sulfoxide from benzothiophene supplemented in crude oil. The presence of the microbially-produced sulfoxide may lead to the abiotic formation of the benzonaphthothiophene. These transformations would likely increase the recalcitrance of the residual petroleum or creosote and may affect its toxicity.

As a general rule, the larger the number of aromatic rings in a polycyclic aromatic molecule, the more recalcitrant it is to biodegradation. The condensation described in this chapter transforms a 2-ring sulfur heterocycle into a 4-ring sulfur heterocycle, which its presumably quite resistant to microbial attack. For example, although the commercially available benzo[*b*]naphtho[2,1-*d*]thiophene has been oxidized to its sulfoxide by cytochrome c (Vazquez-Duhalt *et al.*, 1993), repeated attempts to demonstrate microbial transformation of this compound have failed (S. Saftic´ and P.M. Fedorak, unpublished results). In addition, the greater the number of alkyl carbons on an aromatic ring system, the more recalcitrant the molecule. For instance, naphthalene is more susceptible to biodegradation than are the C_1-naphthalenes which are more susceptible to biodegradation than are the C_2-naphthalenes (Fedorak and Westlake, 1981). Similarly, dibenzothiophene is more susceptible to biodegradation than are the C_1-dibenzothiophenes which are more susceptible to biodegradation than are the C_2-dibenzothiophenes (Bayona *et al.*, 1986; Fedorak and Westlake, 1983, 1984). The condensation reactions involving methylbenzothiophenes, that were demonstrated in the current study, yielded C_1- and C_2-benzo[*b*]naphtho[1,2-*d*]thiophenes, which are likely more resistant to microbial degradation than benzo[*b*]naphtho[1,2-*d*]thiophene.

Eastmond *et al.* (1984) tested a variety of polycyclic aromatic hydrocarbons and structurally similar polycyclic aromatic sulfur heterocycles for their toxicity to the zooplankton *Daphnia magna*. They observed LC_{50} values of 0.22 mg/L for benzo[*b*]naphtho[1,2-*d*]thiophene and 63.7 mg/L for benzothiophene. Thus, the product of the microbially-mediated condensation is more toxic than benzothiophene. However, benzo[*b*]naphtho[1,2-*d*]thiophene showed no mutagenicity in the standard Ames or pre-incubation Ames test (Pelroy *et al.*, 1983).

Table 3.1 Postulated identities of methyl- and dimethyl-substituted benzonaphthothiophenes produced in cultures incubated with various methylbenzothiophenes.

Substrates[a]	Number of high-molecular-weight products detected by GC-MS	Postulated identities of products[b]	Bacterial strains studied[c]
4-MethylBT	1	4,11-dimethylBNT	F
5-MethylBT	1	3,10-dimethylBNT	SB(G), F
7-MethylBT	1	1,8-dimethylBNT	W1, F
4- & 6-MethylBTs[d]	2	4,9-dimethylBNT 4,11-dimethylBNT	W1, SB(G), F
BT & 5-methylBT	4[e]	2,9-dimethylBNT 2,11-dimethylBNT 3-methylBNT 10-methylBNT 3,10-dimethylBNT	F

[a]BT=benzothiophene.

[b]Presuming the mechanism shown in Figure 3.7 applies. BNT = benzo[*b*]naphtho[1,2-*d*]thiophene.

[c]Not all strains were tested on each substrate. The results with strain SB(G) were reported by Gonçalves (1993).

[d]The synthesis for 6-methylbenzothiophene yielded a mixture of this compound and 4-methylbenzothiophene.

[e]Unsubstituted benzo[*b*]naphtho[1,2-*d*]thiophene was also detected in these culture extracts.

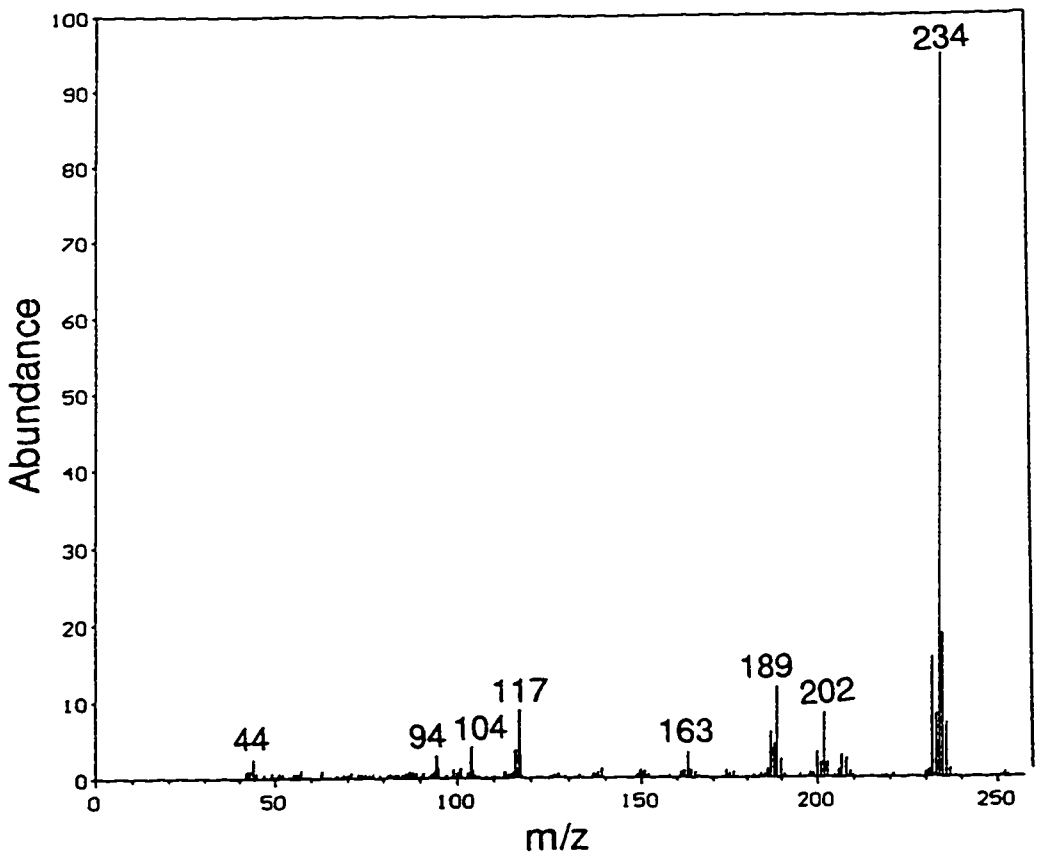


Figure 3.1 From GC-MS analysis, the mass spectrum of the high-molecular-weight compound found in the extract of a culture of isolate W1 incubated with benzothiophene.

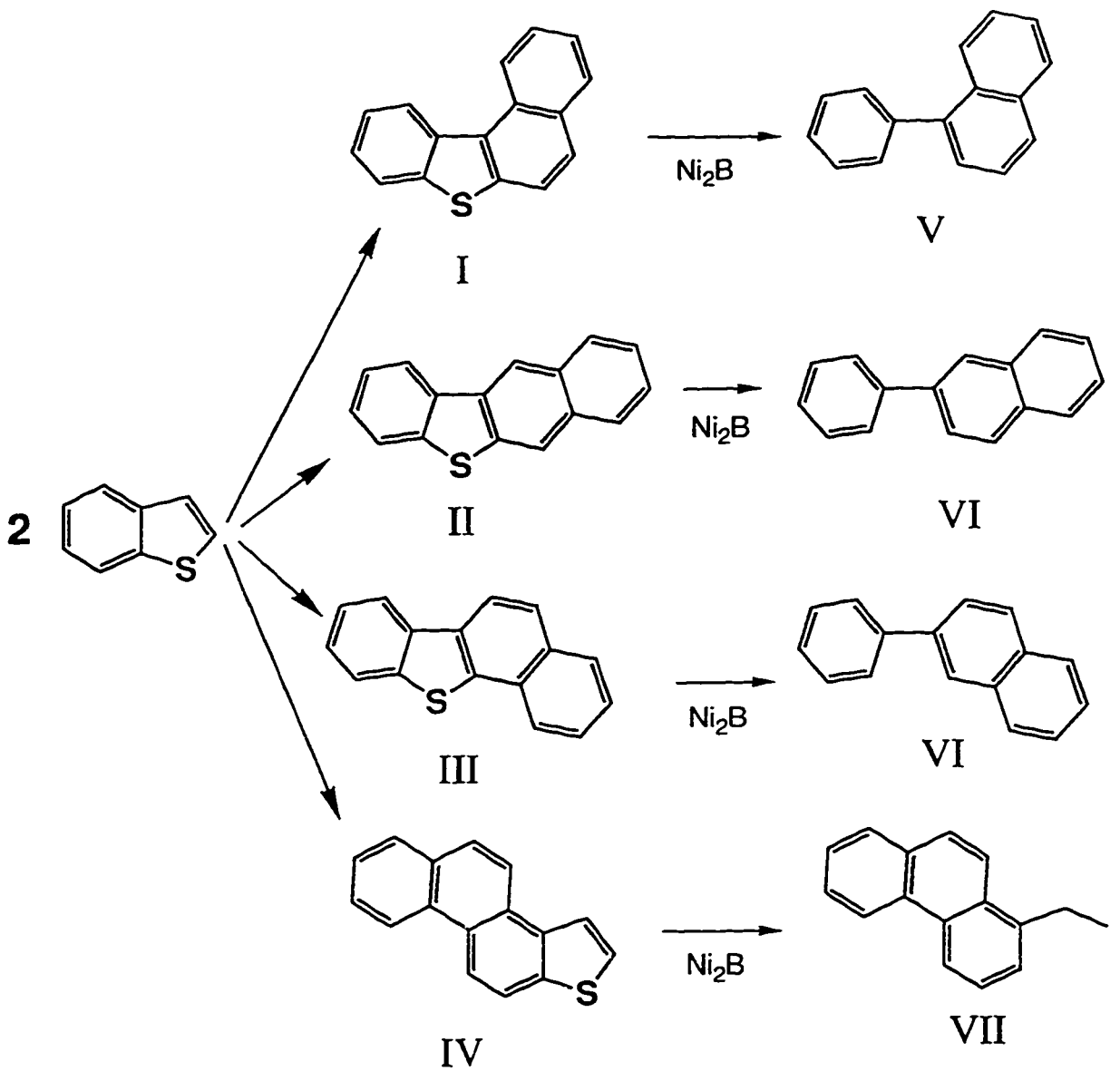


Figure 3.2 Possible structures of the sulfur-containing metabolite with molecular weight 234, and the compounds that would be produced after desulfurization with nickel boride. I, Benzo[*b*]naphtho[1,2-*d*]thiophene; II, benzo[*b*]naphtho[2,3-*d*]thiophene; III, benzo[*b*]naphtho[2,1-*d*]thiophene; IV, phenanthro[2,1-*b*]thiophene; V, 1-phenylnaphthalene; VI, 2-phenylnaphthalene; VII, 1-ethylphenanthrene.

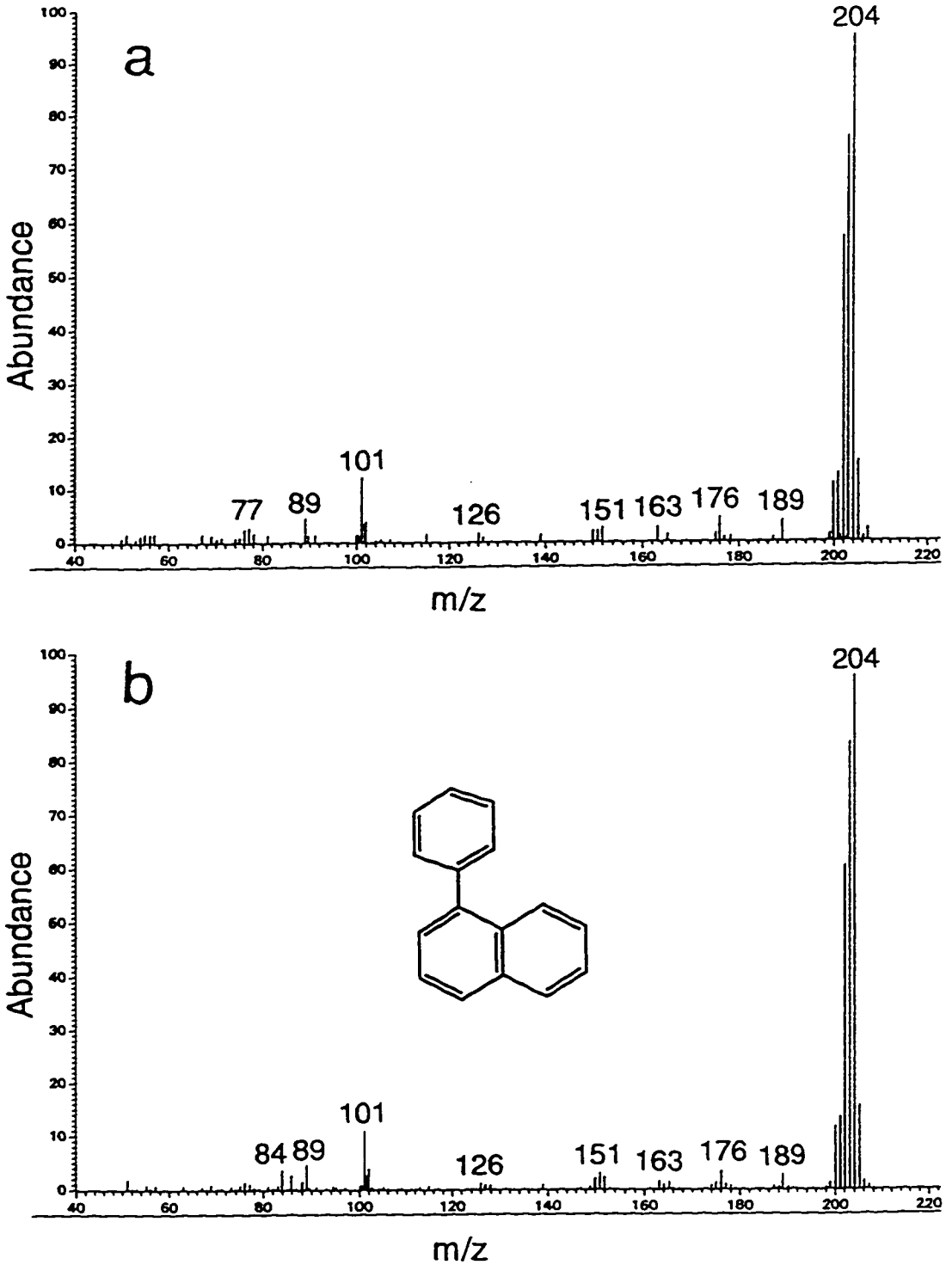


Figure 3.3 From GC-MS analyses, the mass spectrum of the desulfurized metabolite from isolate W1 incubated in the presence of benzothiophene (a) and the mass spectrum of authentic 1-phenylnaphthalene (b).

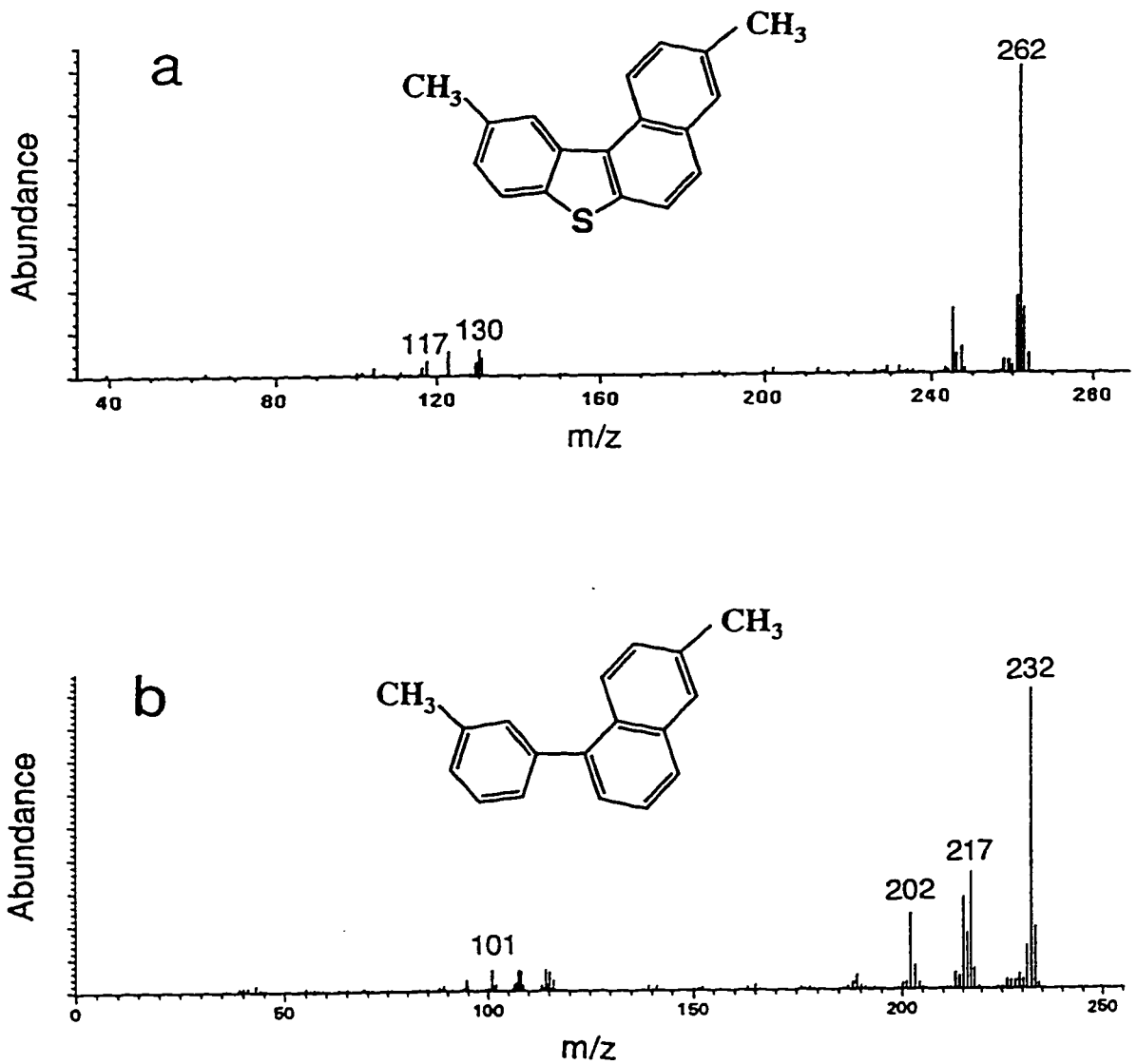


Figure 3.4 From GC-MS analyses, the mass spectra of a sulfur-containing, high-molecular-weight compound found in the extract of a culture of isolate F grown on 1-MN in the presence of 5-methylbenzothiophene (a), and of the product after nickel boride desulfurization of the high-molecular-weight compound (b).

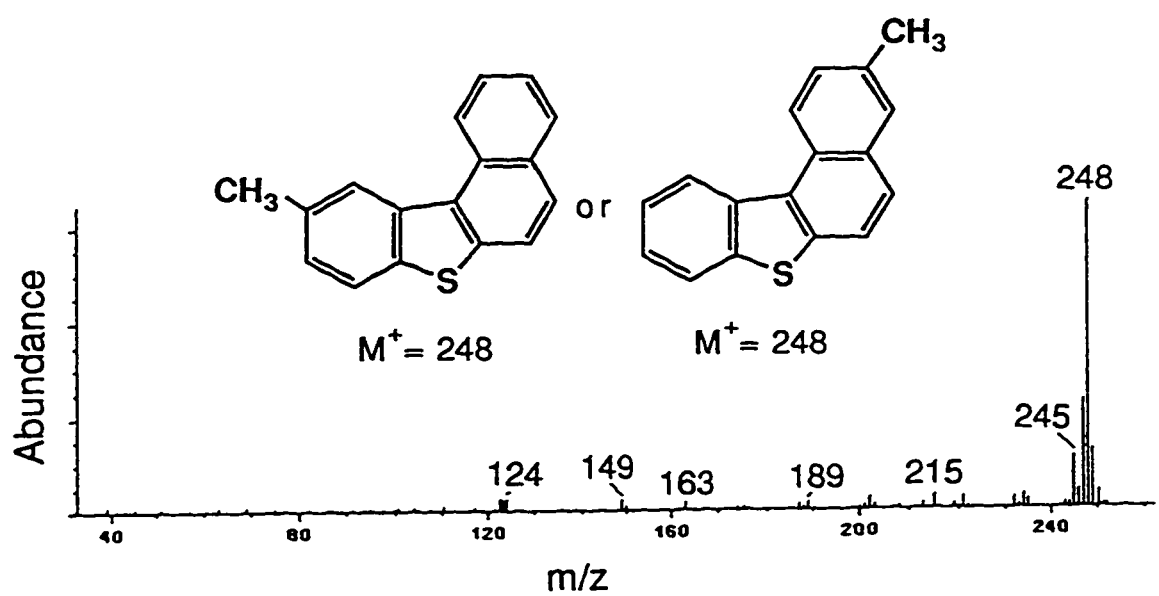


Figure 3.5 From GC-MS analysis, the mass spectrum of one of the high-molecular-weight sulfur-containing metabolites detected in a culture of isolate F grown on 1-MN in the presence of a mixture of benzothiophene and 5-methylbenzothiophene.

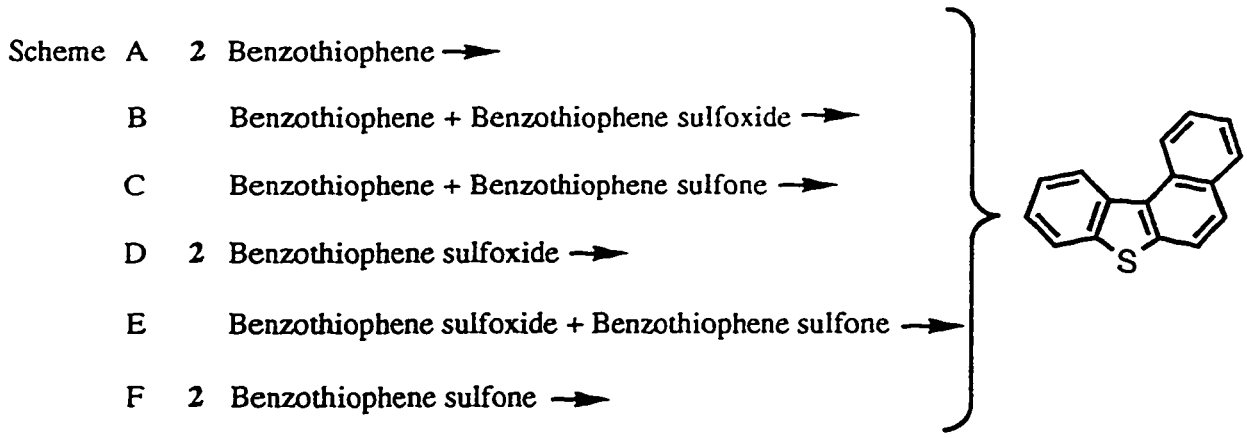


Figure 3.6 Six possible schemes that might lead to a Diels-Alder condensation resulting in the formation of benzo[*b*]naphtho[1,2-*d*]thiophene.

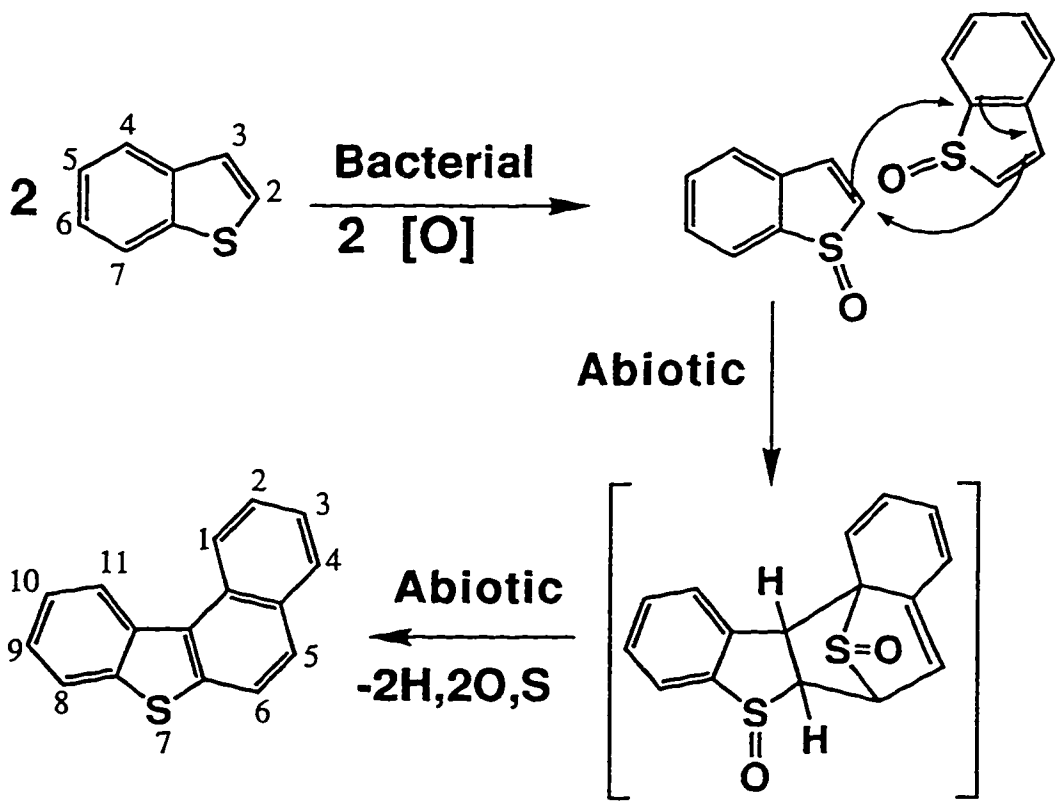


Figure 3.7 Proposed mechanism for the formation of benzo[*b*]naphtho[1,2-*d*]thiophene by abiotic condensation of microbially-produced benzothiophene sulfoxide.

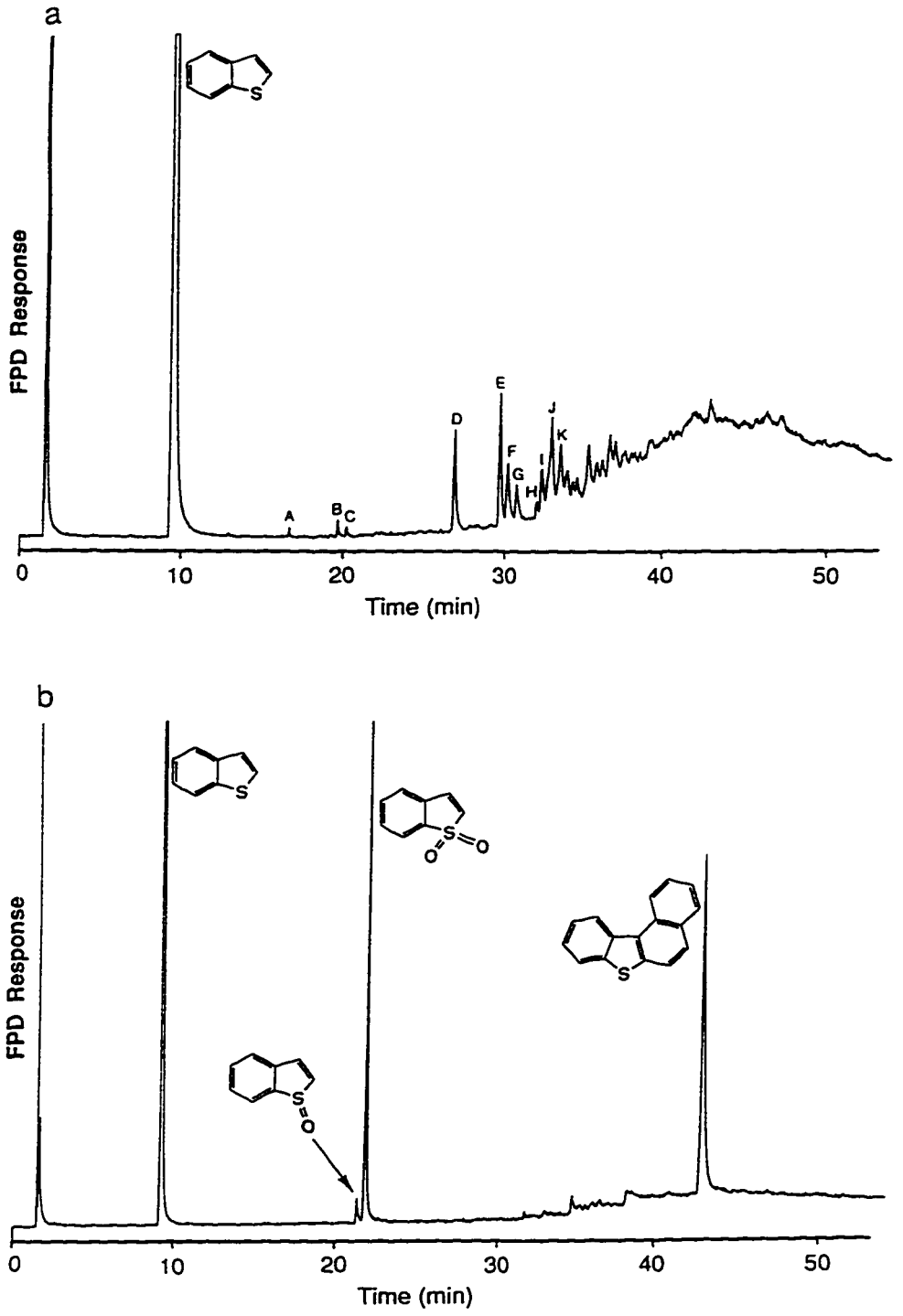


Figure 3.8 GC-FPD analysis of benzothiophene-supplemented Prudhoe Bay crude oil extracted from a sterile control (a) and from a culture of the oil degrading mixed culture SLPB after 14 days incubation (b). Peak designation: A, C_2-benzothiophene; B & C, C_3-benzothiophenes; D, dibenzothiophene; E, F, & G, C_1-dibenzothiophenes; H, I, J, & K, C_2-dibenzothiophenes.

3.5 LITERATURE CITED

Andersson, J. T. 1986. Gas chromatographic retention indices for all C_1- and C_2-alkylated benzothiophenes and their dioxides on three different stationary phases. J. Chromatogr. 354: 83- 98.

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.

Back, T. G., K. Yang, and H. R. Krouse. 1992. Desulfurization of benzo- and dibenzothiophenes with nickel boride. J. Org. Chem. 57: 1986-1990.

Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald. 1986. Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures. J. Environ. Anal. Chem. 23: 289-303.

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.

Bordwell, F. G., B. B. Lampert, and W. H. McKellin. 1949. Thianaphthene chemistry. III. The reaction of 2,3-dibromo-2,3-dihydro- and 2-bromo-thianaphthene-1-dioxide with secondary amines. J. Am. Chem. Soc. 71: 1702-1705.

Bordwell, F. G., W. H. McKellin, and D. Babcock. 1951. Benzothiophene chemistry. V. The pyrolysis of benzothiophene 1-dioxide. J. Am. Chem. Soc. 73: 5566-5568.

Davies, W., N. W. Gamble, and W. E. Savige. 1952. The direct conversion of thionaphthen into derivatives of 9-thiafluorene. J. Chem. Soc. 1952: 4678-4683.

Eastmond, D. A., G. M. Booth, and M. L. Lee. 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles in *Daphnia magna*. Arch. Environ. Contam. Toxicol. 13: 105-111.

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

Fedorak, P. M., and J. T. Andersson. 1992. Decomposition of two methylbenzothiophene sulphoxides in a commercial GC injection port liner. J. Chromatogr. 591: 362-366.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., and T. M. Peakman. 1992. Aerobic microbial metabolism of some alkyl thiophenes found in petroleum. Biodegradation. 2: 223-236.

Fedorak, P. M., and D. W. S. Westlake. 1981. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can. J. Microbiol. 27: 432-443.

Fedorak, P. M., and D. W. S. Westlake. 1983. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. 29: 291-296.

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.

Fedorak, P. M., and D. W. S. Westlake. 1986. Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. 51: 435-437.

Finnerty, W. R., K. Shockley, and H. Attaway. 1983. Microbial desulfurization and denitrification of hydrocarbons, In: Microbial enhanced oil recovery. Zajic, J. E., D.C. Cooper, T.R. Jack, and N. Kosaric Eds. PennWell Publishing Co.: Tulsa, Oklahoma; p. 83-91.

Gonçalves, J. A. 1993. Oxidacion microbiana de compuestos organosulfurados puros y del petroleo. Ph.D. Thesis. Universidad Central de Venezuela, Caracas.

Haines, W. E., R. V. Helm, G. L. Cook, and J. S. Ball. 1956. Purification and properties of ten organic sulfur compounds-second series. J. Phys. Chem. 60: 549-555.

Karcher, W., R. J. Fordham, J. J. Dubois, P. G. J. M. Glaude, and J. A. M. Ligthart. 1985. Spectral Atlas of Polycyclic Aromatic Compounds. D. Reidel Publishing Co.; Boston.

Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994. Bacterial transformations of benzothiophene and methylbenzothiophenes. Environ. Sci. Technol. 28: 1348-1356.

Pelroy, R. A., D. L. Stewart, Y. Tominaga, M. Iwao, R. N. Castle, and M. L. Lee. 1983. Microbial mutagenicity of 3- and 4-ring polycyclic aromatic sulfur heterocycles. Mutation Res. 117: 31-40.

Saftic´, 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.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Sagardía, F., J. J. Rigau, A. Martínez-Lahoz, F. Fuentes, C. López, and W. Flores. 1975. Degradation of benzothiophene and related compounds by a soil *Pseudomonas* in an oil-aqueous environment. Appl. Microbiol. 29: 722-725.

Vazquez-Duhalt, R., D. W. S. Westlake, and P. M. Fedorak. 1993. Cytochrome c as a biocatalyst for the oxidation of thiophenes and organosulfides. Enzyme Microb. Technol. 15: 494-499.

Veselovsky, V. V., A. S. Gybin, A. V. Lozanova, A. M. Moiseenkov, and W. A. Smit. 1988. Dramatic acceleration of the Diels-Alder reaction by adsorption on chromatography adsorbents. Tetrahedron Lett. 29: 175-178.

Westlake, D. W. S. 1983. Microbial activities and changes in the chemical and physical properties of oil. In: Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Donaldson, E. C. and J.B. Clark, Eds. Technology Transfer Branch, Bartlesville Energy Technology Centre: Bartlesville, Oklahoma; p.102-111.

Williams, J. A., M. Bjorøy, D. L. Dolcater, and J. C. Winters. 1986. Biodegradation of South Texas Eocene oil-effects on aromatics and biomarkers. Org. Geochem. 10: 451-461.

4. TRANSFORMATIONS OF SIX ISOMERS OF DIMETHYLBENZOTHIOPHENE BY THREE *PSEUDOMONAS* STRAINS*

4.1 INTRODUCTION

After carbon and hydrogen, sulfur is typically the third most abundant element in petroleum, ranging from about 0.04 to 5 weight percent in conventional oils (Speight, 1980). Sulfur is found in several organic forms, including thiols, sulfides and thiophenes.

Biodegradation studies of organosulfur compounds have primarily focused on those compounds found in the aromatic fraction of petroleum or coal-derived liquids. These include benzothiophene, alkylated benzothiophenes, dibenzothiophene and alkylated dibenzothiophenes. There have been numerous investigations with dibenzothiophene, which is commercially available to serve as a model condensed thiophene (Crawford and Gupta, 1990; Hou and Laskin, 1976; Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985). Dibenzothiophene and its alkyl-substituted analogues have been shown to be removed from the aromatic fraction of petroleum by microbial activity (Atlas *et al.*, 1981; Bayona *et al.*, 1986; Fedorak and Westlake, 1983, 1984; Hostettler and Kvenvolden, 1994), although the identities of their metabolites were not determined in those studies.

Analyses using GC-MS have demonstrated the presence of C_2-benzothiophenes in crude oils (Fedorak and Westlake, 1983; Westlake, 1983; Williams *et al.*, 1986), shale oils (Andersson, 1992; Willey *et al.*, 1981), and coal tar (Burchill *et al.*, 1982). These may be ethylbenzothiophenes or dimethylbenzothiophenes, which have the same molecular weight and are difficult to distinguish by GC-MS analysis. Microbial activity has been shown to remove the C_2-benzothiophenes from crude oil (Fedorak and Westlake, 1983, 1984; Westlake, 1983; Williams *et al.*, 1986), however, little is known about the metabolites of these compounds, since C_2-benzothiophenes are not commercially available.

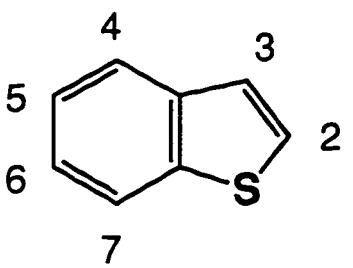
Benzothiophene and the methylbenzothiophenes do not appear to serve as growth substrates for aerobic bacteria, but biotransformation products have been observed in cultures grown on other substrates. For example, Bohonos *et al.* (1977) used naphthalene as a growth substrate for mixed cultures and tentatively identified 2,3-dihydro-benzothiophene-2,3-diol, benzothiophene-2,3-dione and benzothiophene sulfoxide as metabolites of benzothiophene. The 2,3-dione was identified in the extracts of cultures of *Pseudomonas* strain BT1 grown on 1-MN, glucose or peptone (Fedorak and Grbić-

* A version of this chapter has been previously published. Kropp, K. G., S. Saftić, J. T. Andersson, and P. M. Fedorak. 1996. Biodegradation 7: 203-221.

Galic´, 1991). Eaton and Nitterauer (1994) grew *Pseudomonas putida* RE204, an isopropylbenzene-degrading bacterium, on succinate and yeast extract and identified *trans*-4-[3-hydroxy-2-thienyl]-2-oxobut-3-enoate as a product of cleavage of the homocyclic ring, and 2-mercaptophenylglyoxalate as a product of cleavage of the heterocyclic ring. They demonstrated that the latter compound cyclized to benzothiophene-2,3-dione when treated with acid.

Sulfoxides, sulfones and 2,3-diones were commonly found as metabolites of benzothiophene and six methylbenzothiophenes in the DCM-extracts of acidified cultures (Fedorak and Grbic´-Galic´, 1991; Kropp *et al.*, 1994a; Saftic´ *et al.*, 1992). Some of these cultures were also capable of oxidizing the methyl groups of the methylbenzothiophenes, yielding benzothiophene-methanols and benzothiophene-carboxylic acids (Kropp *et al.*, 1994a). Benzo[*b*]naphtho[1,2-*d*]thiophenes were identified as products of the abiotic condensation of microbially produced benzothiophene sulfoxide and some methylbenzothiophene sulfoxides (Kropp *et al.*, 1994b). The only report on the microbial metabolism of a dimethylbenzothiophene was by Saftic´ *et al.* (1992) who demonstrated that 2,3-dimethylbenzothiophene was oxidized to its sulfone and sulfoxide by *Pseudomonas* strain BT1.

The objective of this work was to detect and identify sulfur-containing metabolites of six isomers of dimethylbenzothiophenes that were synthesized for this study. Three bacterial isolates were used for these investigations in which 1-MN or glucose served as the growth substrate. The ring numbering convention for benzothiophene is shown below.



4.2 MATERIALS AND METHODS

4.2.1 Chemicals

The methods for the syntheses of 2,3-, 2,7-, 3,5-, 3,7-, 4,6-, and 4,7-dimethylbenzothiophenes are given by Andersson (1986). These synthesized compounds were shown to be ≥99% pure by GC analyses. Sulfones of some of the dimethylbenzothiophenes were synthesized by boiling the dimethylbenzothiophene with H_2O_2 in acetic

acid for 15 min. The structures of the sulfones were verified by GC-MS analyses which showed the correct molecular weight and by GC-FTIR analyses which showed strong absorptions in the regions of 1350-1300 cm^{-1} and 1160-1120 cm^{-1} that are characteristic of sulfones (Shriner *et al.* 1980). 3-Methylbenzothiophene, 5-methylbenzothiophene and 2,5-thiophenedicarboxylic acid were purchased from Lancaster Synthesis (Windham, NH), and 1-MN was purchased from Fluka (Buchs, Switzerland).

2-Methylbenzothiophene-3-carboxylic acid was synthesized from 2-methylbenzothiophene using a Friedel-Craft acylation with oxalyl chloride and aluminum chloride as catalyst, according to the procedure of Sokol (1973). 2-Methyl-3-hydroxymethylbenzothiophene was prepared by chloromethylating 2-methylbenzothiophene (Grummit and Buck, 1955) and hydrolysing the resulting chloride by the procedure of Baciocchi and Mandolini (1968). 2-Hydroxymethyl-3-methylbenzothiophene was synthesized by bromination of 2,3-dimethylbenzothiophene and hydrolysis (Baciocchi and Mandolini, 1968).

5-Methylbenzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione were synthesized by the procedure of Hannoun *et al.* (1982). The structure and purity of the synthesized diones were confirmed by determination of the melting points, which were the same as literature values. Using the general methods of Dickinson and Iddon (1970) and Stridsberg and Allenmark (1974), authentic standards of 4,7-dimethylbenzothiophene-2(3*H*)-one and 4,7-dimethylbenzothiophene-3(2*H*)-one were prepared. The 2(3*H*)-one was synthesized by treating 4,7-dimethylbenzothiophene (50 mg) with *n*-butyllithium, tri-(*n*-butyl)borate, and H_2O_2, whereas the 3(2*H*)-one was synthesized from 2 g of 2,5-dimethylthiophenol (Aldrich, Milwaukee, WI) by treating it with chloroacetic acid, thionyl chloride, and $AlCl_3$. Similarly, the 2(3*H*)-one and 3(2*H*)-one of 4,6-dimethylbenzothiophene were synthesized from 4,6-dimethylbenzothiophene (20 mg) and from 3,5-dimethylthiophenol (1 g, Aldrich), respectively. The 2(3*H*)-ones, present in analytical amounts, and the 3(2*H*)-ones of 4,6- and 4,7-dimethylbenzothiophene were recovered from reaction mixtures by extraction and maintained as solutions in DCM to minimize the chance of air oxidation of the products (Friedländer, 1906). GC-MS analyses of these solutions showed that extracts of reaction mixtures from each synthesis contained a single abundant compound with the molecular weight of the desired product.

4.2.2 Bacterial cultures

The isolation and characteristics of *Pseudomonas* strain BT1 were described by Fedorak and Grbic´-Galic´ (1991). The isolation of *Pseudomonas* strain W1 and *Pseudomonas* strain F has also been described previously (Saftic´ *et al.*, 1993).

4.2.3 Culture methods and medium

Growing cells of isolates BT1, W1 and F were used for biotransformation studies. Because these isolates would not grow on the benzothiophenes, the growth substrates used were 1-MN or glucose. When 1-MN was used, the cultures were inoculated with 10 mL of a 1-MN-grown maintenance culture that was transferred weekly. When glucose was used, a glucose-grown culture that had been incubated for 2 to 3 days was used as the source of inoculum (1 mL). This glucose-grown seed culture was inoculated from a single colony of the desired isolate that had been streaked onto PCA (Difco, Detroit, MI) 3 to 5 days earlier from a maintenance culture grown with 1-MN. Cultures were grown at 28°C in 500-mL shake-flasks containing 200 mL of liquid mineral medium supplemented with a trace metals solution (Kropp *et al.*, 1994b). Each 200-mL portion of medium was supplemented with 50 mg of one of the growth substrates and 2 to 5 mg of the dimethylbenzothiophene. Unless otherwise noted, these cultures were incubated for 7 days prior to extraction. For each biotransformation experiment, appropriate sterile controls were incubated to account for any abiotic transformations.

4.2.4 Analytical methods

After incubation, the cultures were acidified with sulfuric acid to pH<2 and extracted with DCM (4 times 20 mL) to recover substrates and products. The extracts were dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. To screen for the presence of sulfur-containing metabolites, the extracts were analyzed by capillary GC using a 30-m DB-5 capillary column in a HP (Mississauga, ON) model 5890 equipped with a FID and a sulfur-selective FPD. Details of the operating conditions were given by Fedorak and Grbic´-Galic´ (1991). Details of the methods for GC-MS and GC-FTIR analyses have been described previously (Saftic´ *et al.*, 1993).

To facilitate GC-MS identification of some of the metabolites, TMS-derivatives of compounds in culture extracts were made by silylating with BSA in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, IL; method 5).

Some culture extracts containing compounds suspected of being carboxylic acids (based on GC-MS analysis) were methyl-esterified using BF_3 in methanol (Aldrich). Specifically, a portion of the extract was evaporated to dryness under nitrogen. The residue was then dissolved in 1.0 mL of BF_3-methanol reagent and heated at 65°C for 9 min. After cooling, the mixture was added to 10 mL of DCM and washed with 10 mL of 4 M aqueous NaCl solution. The aqueous phase was separated and extracted with 10 mL of DCM. The two organic extracts were pooled, dried over anhydrous sodium sulfate, concentrated and analyzed by GC.

4.3 RESULTS

Nearly 30 sulfur-containing metabolites, from the six isomers of dimethylbenzothiophenes, were detected and identified during this study. None of the transformation products described below were detected in the sterile controls, thus they were all products of bacterial oxidations. Active growth of the cultures on 1-MN and glucose, as indicated by turbidity increases, occurred within the first 3 days of incubation. However, most of the cultures were incubated for 7 days prior to extraction, and those sulfur-containing metabolites that remained were identified. With the exception of the cultures of isolate BT1 grown in medium containing 3,5-dimethylbenzothiophene, all of the extracts of the 7-day-old cultures contained residual dimethylbenzothiophene that had not been oxidized by the *Pseudomonas* strains.

4.3.1 Metabolites from 2,3-dimethylbenzothiophene

GC-FPD analysis of an extract of a culture of isolate W1 grown on glucose in liquid medium containing 2,3-dimethylbenzothiophene revealed several sulfur-containing metabolites. GC-MS analysis showed that the most abundant of these gave a mass spectrum that was the same as that of 2,3-dimethylbenzothiophene sulfoxide identified by Saftic´ *et al.* (1992). Eluting immediately after the sulfoxide was a smaller peak which gave a mass spectrum with a molecular ion of m/z 194 and base peak at m/z 151. This spectrum was the same as that of an authentic standard of 2,3-dimethylbenzothiophene sulfone.

Two other sulfur-containing metabolites eluted before the sulfoxide of 2,3-dimethylbenzothiophene. These two products eluted within 0.5 min of each other and gave identical mass spectra (Figures 4.1a and 4.1b). The molecular ion for each of these was at m/z 178 which was consistent with the structures of hydroxy-substituted 2,3-dimethylbenzothiophenes. Loss of OH $(M-17)^+$ would give the fragment at m/z 161. An authentic standard of 2-methyl-3-hydroxymethylbenzothiophene had an identical mass spectrum (Figure 4.1c) to those of the metabolites (Figures 4.1a and 4.1b). The retention time of this standard matched that of the metabolite that was first to elute, indicating that the hydroxy substitution was on the methyl group at position 3. The metabolite that was later to elute is likely 2-hydroxymethyl-3-methylbenzothiophene. These types of compounds are referred to as methanols.

TMS-derivatives were prepared using BSA, and the mass spectrum and retention time of the derivatized methanol with the shorter retention time matched that of the TMS-derivative of an authentic standard of 2-methyl-3-hydroxymethylbenzothiophene. The other methanol metabolite also reacted to give a TMS-derivative.

In the extract of a second glucose-grown culture of isolate W1, the sulfoxide of 2,3-dimethylbenzothiophene was the most abundant product, and the sulfone and two methanols were again detected. However, when this extract was treated with BSA to derivatize the two methanols, two new derivatized metabolites were detected by GC-MS analysis. These were present in small amounts and both eluted after the sulfone of 2,3-dimethylbenzothiophene. The molecular ion of these products was at m/z 264 (Figures 4.2a and 4.2b). The fragmentations to give the abundant ions are shown in Figure 4.2 except for the ion at m/z 205 which is the result of the loss of a methyl group from the TMS substituent, followed by skeletal rearrangement with the loss of CO_2. This is a characteristic fragmentation pattern for TMS-esters of carboxylic acids (Pierce, 1968). The fragmentation pattern of these derivatized methylbenzothiophene-carboxylic acid metabolites closely matched that of a TMS-derivatized authentic standard of 2-methylbenzothiophene-3-carboxylic acid (Figure 4.2c).

GC-MS analyses of the extracts of cultures of isolate F grown in medium containing 1-MN and 2,3-dimethylbenzothiophene showed that they contained 2,3-dimethylbenzothiophene sulfoxide and sulfone, both methanol isomers and a carboxylic acid that yielded a TMS-dervative that gave the same mass spectrum as that shown in Figure 4.2b.

An extract of a culture of isolate F grown on glucose in the presence of 2,3-dimethylbenzothiophene was also analyzed by GC-MS. Again, 2,3-dimethylbenzothiophene sulfoxide was the most abundant metabolite, and a methanol with the same retention time as authentic 2-methyl-3-hydroxymethylbenzothiophene was detected. In this extract, there was enough of a carboxylic acid present that it was detected in its free form, despite the fact that it was very poorly chromatographed. The mass spectrum (Figure 4.3a) showed the molecular ion at m/z 192 and abundant fragments at m/z 174 and 147 from the loss of H_2O and COOH, respectively. This methylbenzothiophene-carboxylic acid loses H_2O to give the ion at m/z 174 rather than losing OH to give an abundant fragment at m/z 175 because regardless of which methyl group is oxidized to a carboxy group by isolate F, there will be a methyl group ortho to it on the thiophene ring. By proton transfer from the methyl group and elimination of H_2O from the carboxy group, the abundant ion $(M-18)^+$ is formed (Silverstein *et al.*, 1991). This is consistent with the mass spectrum that is observed for an authentic standard of 2-methylbenzothiophene-3-carboxylic acid as shown in Figure 4.3b.

Extracts from isolate BT1 grown on glucose or 1-MN in the presence of 2,3-dimethylbenzothiophene contained the sulfoxide as the major metabolite and the sulfone as

a minor metabolite. Small amounts of both methanol isomers were found in the extract of the glucose-grown culture.

4.3.2 Metabolites from 2,7-dimethylbenzothiophene

Three abundant sulfur-containing metabolites were detected during the GC-FPD analysis of an extract of a culture of isolate W1 grown on glucose in medium containing 2,7-dimethylbenzothiophene. The metabolite that eluted last gave the mass spectrum shown in Figure 4.4. The molecular ion was at m/z 192 with a major fragment at m/z 147 $(M-45)^+$, corresponding to the loss of COOH, suggesting that one of the methyl groups had been oxidized to a carboxylic acid. The TMS-derivative was prepared and its mass spectrum was very similar to that shown in Figure 4.2b with the molecular ion at m/z 264, and abundant ions at m/z 249 $(M-15)^+$, 175 $(M-89)^+$, and 147 $(M-117)^+$. There was also an abundant fragment at m/z 205 $(M-59)^+$, a characteristic fragmentation of TMS esters of carboxylic acids.

GC-MS analysis revealed that a second sulfur-containing metabolite in the extract of this culture gave a mass spectrum (Figure 4.5a) with the molecular ion at m/z 178, consisent with the metabolite being the sulfoxide of 2,7-dimethylbenzothiophene. The fragments at m/z 163, 162 and 161 correspond to the losses of CH_3, O and OH, respectively. The base peak at m/z 135 may be the result of the loss of the oxygen atom, the C-2 atom and the C-2 methyl group $(M-43)^+$. Porter (1967) demonstrated the loss of the C-2 atom and the C-2 methyl group $(M-27)^+$ from 2-methylbenzothiophene. GC-FTIR analysis of this extract showed that the metabolite gave a strong absorption at 1089 cm^{-1} (Figure 4.5b) which is characteristic of a sulfoxide (Shriner *et al.*, 1980).

GC-MS analysis showed that the third sulfur-containing metabolite in this extract had a molecular ion, which was the base peak, at m/z 178. Other abundant ions were at m/z 161, 149, 134 and 115. These ions were found in the spectrum of 2-methyl-3-hydroxymethylbenzothiophene (Figure 4.1), suggesting that this metabolite was the result of the microbial oxidation of one of the methyl groups of 2,7-dimethylbenzothiophene to yield a methanol. GC-MS analysis of this extract after it was treated with BSA showed the loss of this peak and the apearance of a new peak with a molecular ion at m/z 250, and a base peak at m/z 161, resulting from the loss of $OSi(CH_3)_3$. This is consistent with the formation of a TMS-derivative of a hydroxymethyl-methylbenzothiophene.

GC-FPD analysis of the DCM extract of isolate BT1 grown on 1-MN with 2,7-dimethylbenzothiophene showed a single, major sulfur-containing metabolite. The mass and FTIR spectra of this compound were nearly identical to those shown in Figure 4.5. Thus, the metabolite was 2,7-dimethylbenzothiophene sulfoxide. GC-FTIR analysis

showed two different spectra existed within the peak of the sulfoxide. Closer examination showed that a second compound eluted on the tail of the sulfoxide peak. Its FTIR spectrum had two strong absorptions at 1327 and 1160 cm^{-1} which are the same as an authentic standard of 2,7-dimethylbenzothiophene sulfone. The sulfone could not be detected by GC-MS analysis of this extract. Isolate BT1 also produced a small amount of a single isomer of methylbenzothiophene-carboxylic acid with a mass spectrum similar to that of the acid product from isolate W1 shown in Figure 4.4.

GC-MS analysis of an extract of a culture of isolate F grown on 1-MN in liquid medium containing 2,7-dimethylbenzothiophene showed four sulfur-containing products. The metabolites were: a methylbenzothiophene-carboxylic acid; 2,7-dimethyl-benzothiophene sulfoxide; and two isomers of the hydroxymethyl-methylbenzothiophenes. The same metabolites were detected when isolate F was grown on glucose in the presence of 2,7-dimethylbenzothiophene. The sulfone of 2,7-dimethylbenzothiophene was not detected in any of the extracts of isolates F or W1.

4.3.3 Metabolites from 3,5-dimethylbenzothiophene

Four metabolites were detected by GC-FPD analysis of an extract of a culture of isolate W1 grown in medium with glucose and 3,5-dimethylbenzothiophene. GC-MS analysis showed that two of these compounds had virtually identical mass spectra (Figure 4.6), with the molecular ion at m/z 192. The major ions at m/z 175 and 147 are the $(M-17)^+$ and $(M-45)^+$ ions, corresponding to the loss of OH and COOH, respectively. These data suggested that the metabolites were the results of oxidation of each of the two methyl substituents to carboxyl groups resulting in two methylbenzothiophene-carboxylic acids. The ion at m/z 147 was observed in Figures. 4.3, 4.4 and 4.6, however the ion at m/z 175 was observed in the carboxylic acids from 3,5-dimethylbenzothiophene (Figure 4.6) and 2,7-dimethylbenzothiophene (Figure 4.4), but was not as abundant as the ion at m/z 174 $(M-18)^+$ in the acids from 2,3-dimethylbenzothiophene (Figure 4.3). The methyl group adjacent to the carboxy group in the acids from 2,3-dimethylbenzothiophene facilitated the loss of H_2O, rather than OH.

The methyl esters of these acids from 3,5-dimethylbenzothiophene were prepared by treating the culture extract with BF_3 in methanol. GC-MS analysis of these methyl esters showed that they had a molecular ion at m/z 206, and major fragments at m/z 175 $(M-CH_3O)^+$, and 147 $(M-CH_3OCO)^+$. These results are consistent with the metabolites being 3-methylbenzothiophene-5-carboxylic and 5-methylbenzothiophene-3-carboxylic acids. No standards of these two acids were available, but the methyl ester of authentic 2-

methylbenzothiophene-3-carboxylic acid was prepared and its mass spectrum showed a molecular ion at m/z 206, and abundant ions at m/z 175 and 147.

GC-MS analysis showed the other two sulfur-containing metabolites from 3,5-dimethylbenzothiophene had very similar mass spectra. The molecular ion was at m/z 178, and the loss of OH gave an $(M-17)^+$ fragment at m/z 161. These results suggested that the metabolites were the products of oxidation of a methyl group to the corresponding methanol. Other abundant ions were at m/z 149, 134 and 115. These ions were observed in the spectrum of authentic 2-methyl-3-hydroxymethylbenzothiophene (Figure 4.1). TMS-derivatives of these metabolites were prepared and their mass spectra showed a molecular ion at m/z 250, and the fragments at m/z 235, from the loss of CH_3, and m/z 161, from the loss of $OSi(CH_3)_3$. Although these two products may be phenols, it is more likely that they are the products of the oxidation of the two methyl groups to methanols because two methylbenzothiophene-carboxylic acids were detected as metabolites from 3,5-dimethyl-benzothiophene (Figure 4.6).

Sulfur-containing metabolites detected from 3,5-dimethylbenzothiophene in a 1-MN-grown culture of isolate F were the two methanols and a monocarboxylic acid. In the extract from a glucose-grown culture of isolate F (with 3,5-dimethylbenzothiophene in the medium), a single methanol was detected, along with two carboxylic acids arising from the oxidation of each of the two methyl groups.

Surprisingly, no sulfur-containing metabolites were found in the extracts of isolate BT1 grown for 7 days in the presence of 3,5-dimethylbenzothiophene. In addition, only trace amounts of residual 3,5-dimethylbenzothiophene were found in these extracts.

4.3.4 Metabolites from 3,7-dimethylbenzothiophene

GC analysis of an extract of a culture of isolate W1 grown in glucose-containing medium with 3,7-dimethylbenzothiophene showed five sulfur-containing metabolites. The last two of these to elute gave mass spectra with base peaks at m/z 192, which were the molecular ions. The next most abundant fragments were at m/z 147 and 175. These were consistent with the metabolites being methylbenzothiophene-carboxylic acids. The TMS-derivatives were prepared and the GC-MS analysis showed molecular ions at m/z 264 with major fragments at 249, 205 (base peak) 175 and 147. With the exception of the mass to charge ratio of the base peak, these spectra matched those of the TMS-derivatives of the methylbenzothiophene-carboxylic acids from 2,3-dimethylbenzothiophene (Figure 4.2).

The metabolite that eluted just prior to the carboxylic acids had a molecular ion at m/z 178. This molecular weight results from the incorporation of a single oxygen atom into 3,7-dimethylbenzothiophene. However, this metabolite did not react with the TMS-

derivatizing reagents so it was not an alcohol or phenol. GC-FTIR analysis showed a strong absorption at 1069 cm^{-1} which is characteristic of a sulfoxide. Thus, the metabolite was 3,7-dimethylbenzothiophene sulfoxide. Interestingly, no sulfone was detected.

The first two metabolites to elute from the GC-MS also had base peaks that were the molecular ions at m/z 178 and very similar mass spectra with major fragments at m/z 161, 149, 134, and 115, like those in Figure 4.1. Both of these products reacted with BSA giving products with molecular ions at m/z 250. The data suggest that these two products are hydroxy-substituted 3,7-dimethylbenzothiophenes, presumably methanols.

GC-MS analyses of extracts of cultures of isolate F grown on glucose or 1-MN in medium containing 3,7-dimethylbenzothiophene showed that it produced the same five metabolites as isolate W1. Under the same growth conditions, the major metabolite produced by isolate BT1 was 3,7-dimethylbenzothiophene sulfoxide. In addition, a small amount of a metabolite with the same mass spectrum and retention time as authentic 3,7-dimethylbenzothiophene sulfone (M^+ = 194) was detected in these extracts. When grown on glucose, isolate BT1 oxidized the methyl groups producing the two methanols from 3,7-dimethylbenzothiophene in addition to the sulfoxide and sulfone.

4.3.5 Metabolites from 4,7-dimethylbenzothiophene

GC-FPD analysis of an extract of a culture of isolate BT1 grown on 1-MN in the presence of 4,7-dimethylbenzothiophene showed an abundant sulfur-containing metabolite and several other minor products. The mass spectrum of the most abundant metabolite (Figure 4.7a) showed a weak molecular ion at m/z 192. This molecular weight was consistent with the structure of 4,7-dimethylbenzothiophene-2,3-dione. The base peak (m/z 164) resulted from the loss of CO $(M-28)^+$. Subsequent loss of CHO gave the abundant fragment at m/z 135 $(M-57)^+$. Fragments at $(M-28)^+$ were found in the mass spectra of benzothiophene-2,3-dione (Fedorak and Grbic´-Galic´, 1991) and 5-methylbenzothiophene-2,3-dione (Saftic´ *et al.*, 1992). Similarly, the $(M-57)^+$ was observed in the mass spectra of 5-methyl- and 7-methylbenzothiophene-2,3-diones (Saftic´ *et al.*, 1992). GC-FTIR analysis of this metabolite (Figure 4.7b) showed a single strong absorption at 1726 cm^{-1}, similar to the absorptions at 1739 cm^{-1} and 1735 cm^{-1} observed for 5-methyl- and 7-methylbenzothiophene-2,3-diones, respectively. These data indicate that the most abundant metabolite was 4,7-dimethylbenzothiophene-2,3-dione.

Eluting before the 2,3-dione, and within 0.5 min of each other, were two sulfur-containing metabolites that each had a molecular weight of 178. The product with the shorter retention time fragmented to give ions at m/z 163 $(M-15)^+$ and 149 $(M-29)^+$ (Figure 4.8a), whereas the product with the longer retention time showed an abundant

fragment ion at m/z 150 $(M-28)^+$ (Figure 4.8b). Although these products had a molecular weight that was consistent with the incorporation of a single atom of oxygen into 4,7-dimethylbenzothiophene, they did not show fragmentation patterns that were commonly observed with hydroxy-substituted dimethylbenzothiophenes, such as the loss of OH $(M-17)^+$ (Figure 4.1). Furthermore, GC-FTIR analysis showed that the product with the shorter retention time gave a strong absorption at 1710 cm^{-1} (Figure 4.9a) whereas the other product gave a strong absorption at 1746 cm^{-1} (Figure 4.9b). Thus, the oxygen atom in each of these products was in a carbonyl group.

It was speculated that these two products were 4,7-dimethylbenzothiophene-3(2*H*)-one and 4,7-dimethylbenzothiophene-2(3*H*)-one, and these two compounds were synthesized. The former compound had the same retention time as the metabolite with the shorter retention time, and the same mass and FTIR spectra as shown in Figures 4.8a and 4.9a, respectively. The second metabolite (Figures 4.8b and 4.9b) had identical characteristics to the authentic standard of 4,7-dimethylbenzothiophene-2(3*H*)-one. Thus, these two products were the keto tautomers of 3-hydroxy-4,7-dimethylbenzothiophene and 2-hydroxy-4,7-dimethylbenzothiophene. The equilibrium for the keto-enol tautomerism lies strongly toward the keto form so that the oxygen atom is observed to be in a carbonyl group during GC-FTIR analysis.

In addition to these metabolites, there were several high-molecular-weight, sulfur-containing products in this extract that could not be identified. One had a molecular weight of 290 (Figure 4.10), and was likely a tetramethylbenzo[*b*]naphtho[1,2-*d*]thiophene ($C_{20}H_{18}S$) which could arise from the condensation of two molecules of 4,7-dimethylbenzothiophene in an analogous manner to that by which benzo[*b*]naphtho[1,2-*d*]thiophene was formed from benzothiophene (Kropp *et al.*, 1994b).

Other high-molecular-weight compounds had molecular ions at m/z 292, 308, 322, 324, 340, and 342. Many of these products gave spectra in which the loss of methyl groups from the molecular ion was an abundant fragmentation, suggesting that these were some forms of condensation products with numerous methyl groups arising from the dimethylbenzothiophene. These products may be intermediates in the condensation reaction which leads to formation of the tetramethylbenzonaphthothiophene. They may also be products of bacterial oxidation of the tetramethylbenzonaphthothiophene. The identification of these minor products was not pursued.

When strains F and W1 were grown on 1-MN in the presence of 4,7-dimethylbenzothiophene, they produced 4,7-dimethylbenzothiophene-2,3-dione, 4,7-dimethylbenzothiophene-3(2*H*)-one, 4,7-dimethylbenzothiophene-2(3*H*)-one and high-molecular-weight sulfur-containing products with molecular ions at m/z 290

(corresponding to $C_{20}H_{18}S$), 292, 308, 322, 340, and 342. In addition, each strain produced two metabolites that had mass spectra similar to those shown in Figure 4.6, with $M^+ = 192$, and major fragments at m/z 175 $(M-17)^+$ and 147 $(M-45)^+$. These fragments corresponded to the loss of OH and COOH, respectively, suggesting that the metabolites were the two possible monocarboxylic acids. To verify this, the TMS-esters were prepared and GC-MS analysis gave the mass spectra with $M^+ = 264$ and the fragmentation pattern: m/z 249 $(M-15)^+$, 205 $(M-59)^+$, 175, and 147 as observed in Figure 4.2.

4.3.6 Metabolites from 4,6-dimethylbenzothiophene

In the extract of a 1-MN-grown culture of isolate BT1 incubated with 4,6-dimethylbenzothiophene was a metabolite with a weak molecular ion at m/z 192, a base peak at m/z 164 $(M-28)^+$ and another abundant fragment at m/z 135 $(M-57)^+$. This spectrum suggested that the metabolite was 4,6-dimethylbenzothiophene-2,3-dione because it was very similar to that of 4,7-dimethylbenzothiophene-2,3-dione identified by GC-MS and GC-FTIR analyses (Figure 4.7) of extracts of cultures of isolates W1, BT1, and F when grown on 1-MN in the presence of 4,7-dimethylbenzothiophene.

Isolate BT1 also oxidized 4,6-dimethylbenzothiophene to give two minor products which nearly coeluted during GC analysis. These metabolites had identical mass spectra to the 3(2*H*)-one and 2(3*H*)-one of 4,7-dimethylbenzothiophene (see Figure 4.8). To prove the identities of these two metabolites, 4,6-dimethylbenzothiophene-3(2*H*)-one and 4,6-dimethylbenzothiophene-2(3*H*)-one were synthesized. The metabolite that eluted first had the same retention time and mass spectrum as the authentic 3(2*H*)-one. Similarly, the GC retention time and mass spectrum of the second metabolite matched those of the authentic 2(3*H*)-one.

This extract from isolate BT1 also contained several high-molecular-weight, sulfur-containing products that were not identified. Among these were products with abundant molecular ions at m/z 290, 322, 324 and 340. The product with a molecular ion at m/z 290 is consistent with the structure of a tetramethylbenzo[*b*]naphtho[1,2-*d*]thiophene ($C_{20}H_{18}S$) and had a mass spectrum similar to the analagous high-molecular-weight product from 4,7-dimethylbenzothiophene (Figure 4.10).

4,6-Dimethylbenzothiophene-2,3-dione was the major metabolite found in extracts of isolates F and W1 grown on 1-MN in the presence of 4,6-dimethylbenzothiophene. Also found in these extracts were minor amounts of the 3(2*H*)-one and 2(3*H*)-one of 4,6-dimethylbenzothiophene. A trace amount of the high-molecular-weight product with a molecular weight of 290 ($C_{20}H_{18}S$) was detected in the extract from isolate F, but not in that of isolate W1.

4.3.7 Additional investigations with isolate BT1

Further studies with strain BT1 were prompted by the observations that this bacterium completely removed 3,5-dimethylbenzothiophene from its growth medium after 7 days of incubation and that no oxidation products were detected. When a culture was incubated for 1 day, its extracts contained two isomers of hydroxymethyl-methylbenzothiophene and two isomers of methylbenzothiophene-carboxylic acid. No metabolites were found in a culture that was incubated for 3 days. Thus, isolate BT1 oxidized the methyl groups of 3,5-dimethylbenzothiophene to methanols and carboxylic acids which were transient metabolites.

It was hypothesized that this strain might oxidize both methyl groups, yielding a dicarboxylic acid that was too polar to be recovered by the extraction method used. Thus, 3-, 5-, and 7-day-old cultures were adjusted to pH 12, freeze-dried and the residue from each culture was refluxed with methyl alcohol and a catalytic amount of sulfuric acid to form the methyl esters of any carboxylic acids present. However, the hypothesized product, 3,5-benzothiophenedicarboxylic acid, was not detected by GC-MS analyses in any of the cultures. The validity of this method was verified using 2,5-thiophenedicarboxylic acid, which is too polar to extract into DCM from an aqueous solution at pH 2. Indeed, the dimethyl ester of this acid was readily detected by GC analysis after the freeze-drying and esterification procedures.

To determine whether strain BT1 could oxidize the methyl groups of 3-methylbenzothiophene and 5-methylbenzothiophene, it was also grown on either 1-MN or glucose with one of these condensed thiophenes in the growth medium for 1, 3, or 14 days. Interestingly, neither the methanol nor the carboxylic acid of these two isomers was detected after any incubation period.

The ability of isolate BT1 to grow on 1-naphthalenemethanol and 1-naphthoic acid (50 mg per 200-mL culture) was tested. Lag periods of 2 days and 6 days, respectively, were observed with these substrates. Stationary phase (OD_{600} = 0.3) was reached after 5 days of incubation with 1-naphthalenemethanol, and after 8 days of incubation with 1-naphthoic acid. In contrast, stationary phase was reached after 1 day of incubation with 1-MN.

4.4 DISCUSSION

Six of the possible 15 isomers of dimethylbenzothiophene were used in this investigation. The isomers were chosen to have a variety of substitution patterns: both methyl groups on the thiophene ring (the 2,3- isomer); a methyl group on each of the rings

(the 2,7-, 3,5- and 3,7- isomers); and both methyl groups on the benzene ring (the 4,6- and 4,7- isomers).

Table 4.1 summarizes the sulfur-containing metabolites detected in the extracts of 7-day-old cultures of the three *Pseudomonas* strains studied. The plural entries "methanols" and "carboxylic acids" indicate that two isomers of these compounds were found in the extracts.

Sulfoxides and sulfones were detected from only those compounds with a methyl group on the thiophene ring (i.e. the 2,3-, 2,7- and 3,7- isomers). In contrast, 2,3-diones were detected from those compounds with no methyl groups on the thiophene ring (i.e. the 4,6- and 4,7- isomers). These findings are consistent with the predictions of Fedorak and Grbic´-Galic´ (1991) and observations of Saftic´ *et al.* (1992) with monomethyl-benzothiophenes, although in the latter study and in that of Kropp *et al.* (1994a) sulfoxides and sulfones were observed from some methylbenzothiophenes with unsubstituted thiophene rings. The 2,3-diones detected in these cultures (Table 4.1) likely existed as dimethyl-substituted 2-mercaptophenylglyoxalates in the culture medium at neutral pH. Indeed, Eaton and Nitterauer (1994) showed that benzothiophene was microbially oxidized to 2-mercaptophenylglyoxalate which cyclized to benzothiophene-2,3-dione when acidified. In this study, the cultures were routinely acidified prior to extraction.

Although no sulfoxides were detected in the cultures incubated with 4,6- or 4,7-dimethylbenzothiophenes, they were very likely produced, and then subsequently reacted to give the high-molecular-weight products with the empirical formula $C_{20}H_{18}S$. Kropp *et al.* (1994b) demonstrated that two molecules of benzothiophene sulfoxide undergo an abiotic Diel-Alder-type condensation to form benzo[*b*]naphtho[1,2-*d*]thiophene. Kropp *et al.* (1994a) detected dimethylbenzo[*b*]naphtho[1,2-*d*]thiophenes in cultures incubated with 4-, 5-, 6- or 7-methylbenzothiophene. However, no high-molecular-weight products were detected from 2- or 3-methylbenzothiophene because the methyl group on the thiophene ring sterically hinders the condensation reaction. The empirical formula $C_{20}H_{18}S$ is consistent with tetramethylbenzo[*b*]naphtho[1,2-*d*]thiophenes, and these products were not found in the extracts of the cultures incubated with isomers of dimethylbenzothiophene with a methyl group on the thiophene ring (2,3-, 2,7-, 3,5-, and 3,7-dimethylbenzothiophenes). The sulfoxides of these isomers, with the exception of the 3,5-isomer, were detected in culture extracts (Table 4.1).

The metabolism of 3,5-dimethylbenzothiophene was unique among the six isomers studied (Table 4.1). In the 7-day-old cultures of strains W1 and F, methanols and carboxylic acids were found, but no sulfoxide was detected. No metabolites were found in

the extract of a 7-day-old culture of strain BT1. Methanols and carboxylic acids were transient intermediates in cultures of strain BT1.

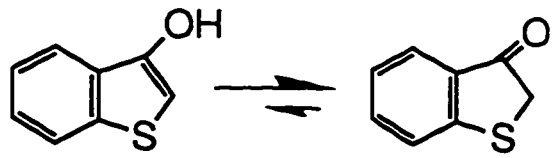
With the exception of 4,6-dimethylbenzothiophene, methyl group oxidation products were found in culture extracts from isolates W1 and F (Table 4.1). These isolates were known to produce methanols and carboxylic acids from methylbenzothiophenes (Kropp *et al.*, 1994a) and methanols from some isomers of methyldibenzothiophene (Saftic´ *et al.*, 1993). In addition, 1-naphthalenemethanol and 1-naphthoic acid were found to accumulate in cultures of strains W1 and F grown on 1-MN (Kropp *et al.*, 1994a), and neither of these oxidation products serve as growth substrates for these two isolates. In contrast, methanols or carboxylic acids were found in fewer extracts from 7-day-old cultures of isolate BT1 (Table 4.1). Furthermore, 1-naphthalenemethanol and 1-naphthoic acid were produced by isolate BT1 but they only transiently accumulated in cultures of this isolate grown on 1-MN before they were further degraded. Indeed, 1-naphthalenemethanol and 1-naphthoic acid serve as growth substrates for strain BT1.

Studies on the bacterial metabolism of dimethylnaphthalenes have shown that the methyl groups are also susceptible to oxidation. For example, Dean-Raymond and Bartha (1975) showed that although dimethylnaphthalenes would not support growth of their bacterial isolates that grew on naphthalene and methylnaphthalenes, 1,5- and 2,6-dimethylnaphthalenes were oxidized to their corresponding monocarboxylic acids by naphthalene-grown resting cells. Similarly, Barnsley (1988) identified 2-hydroxymethyl-6-methylnaphthalene and 6-methyl-2-naphthalenecarboxylic acid as metabolites from 2,6-dimethylnaphthalene. In addition to the metabolites found by Barnsley (1988), Miyachi *et al.* (1993) detected 2,6-naphthalene dicarboxylic acid by HPLC analysis of fluids from cultures grown on 2,6-dimethylnaphthalene.

Because of the transient nature of the methanols and carboxylic acids produced from 3,5-dimethylbenzothiophene by strain BT1, attempts were made to detect the corresponding dicarboxylic acid. Since the methanols and carboxylic acids were present after 1 and 2 days incubation but had been depleted from the medium by the third day, I tried to detect the dicarboxylic acid over incubation periods of 3, 5 and 7 days. The dicarboxylic acid was not detected, nor were any other sulfur-containing metabolites. Thus, the fates of the methanols and carboxylic acids from 3,5-dimethylbenzothiophene are unknown, and further investigations are required to determine the identities of subsequent metabolites.

The 3(2*H*)-ones and 2(3*H*)-ones of 4,6- and 4,7-dimethylbenzothiophene (Table 4.1) are novel products. 3-Hydroxybenzothiophene (the enol form shown below) and 2-hydroxybenzothiophene are known to exist almost exclusively as their keto tautomers

(Iddon and Scrowston, 1970) and therefore are more appropriately called benzothiophene-3(2*H*)-one and benzothiophene-2(3*H*)-one, respectively.



enol form keto form

These compounds react as if they have a hydroxyl group on the thiophene ring. For example, the methyl ethers of the 2(3*H*)-one and the 3(2*H*)-one can be formed by the methods of Dickinson and Iddon (1970) and Friedländer (1907), respectively. In this study, the 3(2*H*)-ones and 2(3*H*)-ones of the dimethylbenzothiophenes tautomerized to the enol forms which reacted with BSA giving TMS derivatives.

In their studies on bacterial metabolism of benzothiophene, Eaton and Nitterauer (1994) identified 2-hydroxybenzothiophene and 3-hydroxybenzothiophene. They proposed that these phenols were the result of dehydration of 2,3-dihydroxy-2,3-dihydrobenzothiophene. Phenol formation resulting from dehydration of dihydrodiols under acidic conditions has been observed in studies with dibenzothiophene (Laborde and Gibson, 1977), phenanthrene and anthracene (Jerina *et al.*, 1976) and carbazole (Resnick *et al.*, 1993). Thus, the 2(3*H*)-ones and the 3(2*H*)-ones found in the extracts of cultures incubated with 4,6- and 4,7-dimethylbenzothiophene are likely the dehydration products of undetected 2,3-dihydrodiols.

During this investigation, quantitative analyses of the sulfur-containing metabolites were precluded for several reasons. For example, routine analyses were done with a FPD which gives nonlinear response and is susceptible to quenching. In addition, authentic standards of some products, such as the sulfoxides and the tetramethylbenzo[*b*]naphtho[1,2-*d*]thiophenes, were not available, and the quantities of other reference compounds that were synthesized, such as the 3(2*H*)- and 2(3*H*)-ones, were not sufficient for the preparation of calibration curves for quantitation using a FID. The analyses of sulfoxides is also complicated by their decomposition in GC injection port liners (Fedorak and Andersson, 1992).

The focus of these investigations was the identification of sulfur-containing metabolites that could be partitioned from an acidified culture into DCM and that were amenable to GC analysis. Other metabolites may have been produced but not detected if they were too polar to be extracted or chromatographed. Nonetheless, nearly 30 sulfur-containing products were identified during this survey. The metabolism of 3,5-

dimethylbenzothiophene by *Pseudomonas* strain BT1 deserves further study because of its complete removal of this compound from the medium and the absence of metabolites after 7 days of incubation.

Whether microbial oxidations of C_2-benzothiophenes in petroleum- or creosote-contaminated environments lead to the same metabolites that were identified in this laboratory study is yet to be determined. Similarly, the fates of the identified oxidation products in diverse microbial populations found in the environment have not been determined.

Table 4.1 Summary of the sulfur-containing products found in the extracts of three bacterial cultures after incubation with various dimethylbenzothiophenes for 7 days. The products were found in cultures grown on 1-MN or glucose.

Substrate	Products found in cultures of *Pseudomonas* strain		
	BT1	W1	F
2,3-dimethyl-benzothiophene	sulfoxide sulfone methanols	sulfoxide sulfone methanols carboxylic acids	sulfoxide sulfone methanols carboxylic acids
2,7-dimethyl-benzothiophene	sulfoxide sulfone carboxylic acid	sulfoxide methanol carboxylic acid	sulfoxide methanols carboxylic acid
3,5-dimethyl-benzothiophene	none	methanols carboxylic acids	methanols carboxylic acids
3,7-dimethyl-benzothiophene	sulfoxide sulfone methanols	sulfoxide methanols carboxylic acids	sulfoxide methanols carboxylic acids
4,6-dimethyl-benzothiophene	2,3-dione[a] 3(2*H*)-one 2(3*H*)-one $C_{20}H_{18}S$[b]	2,3-dione[a] 3(2*H*)-one 2(3*H*)-one	2,3-dione[a] 3(2*H*)-one 2(3*H*)-one $C_{20}H_{18}S$
4,7-dimethyl-benzothiophene	2,3-dione[a] 3(2*H*)-one 2(3*H*)-one $C_{20}H_{18}S$[b]	2,3-dione[a] 3(2*H*)-one 2(3*H*)-one carboxylic acids $C_{20}H_{18}S$[b]	2,3-dione[a] 3(2*H*)-one 2(3*H*)-one carboxylic acids $C_{20}H_{18}S$[b]

[a] exist as dimethyl-substituted 2-mercaptophenylglyoxalates at neutral pH
[b] and several other high molecular weight products

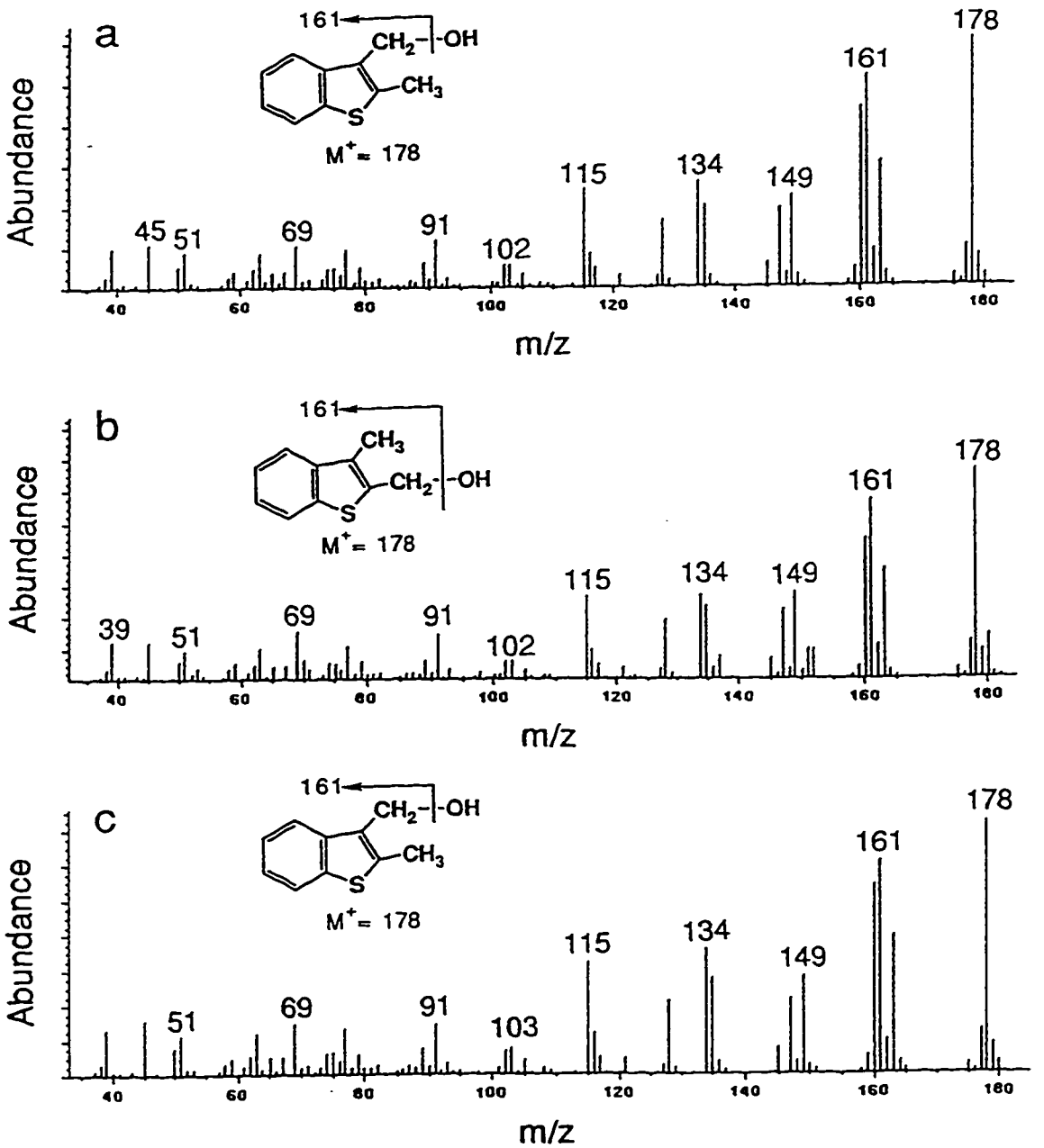


Figure 4.1 From GC-MS analyses, the mass spectra of two sulfur-containing metabolites from a culture of isolate W1 grown on glucose in the presence of 2,3-dimethylbenzothiophene. The metabolite with the shorter retention time (a) and the metabolite with the longer retention time (b). The mass spectrum of authentic 2-methyl-3-hydroxymethylbenzothiophene (c).

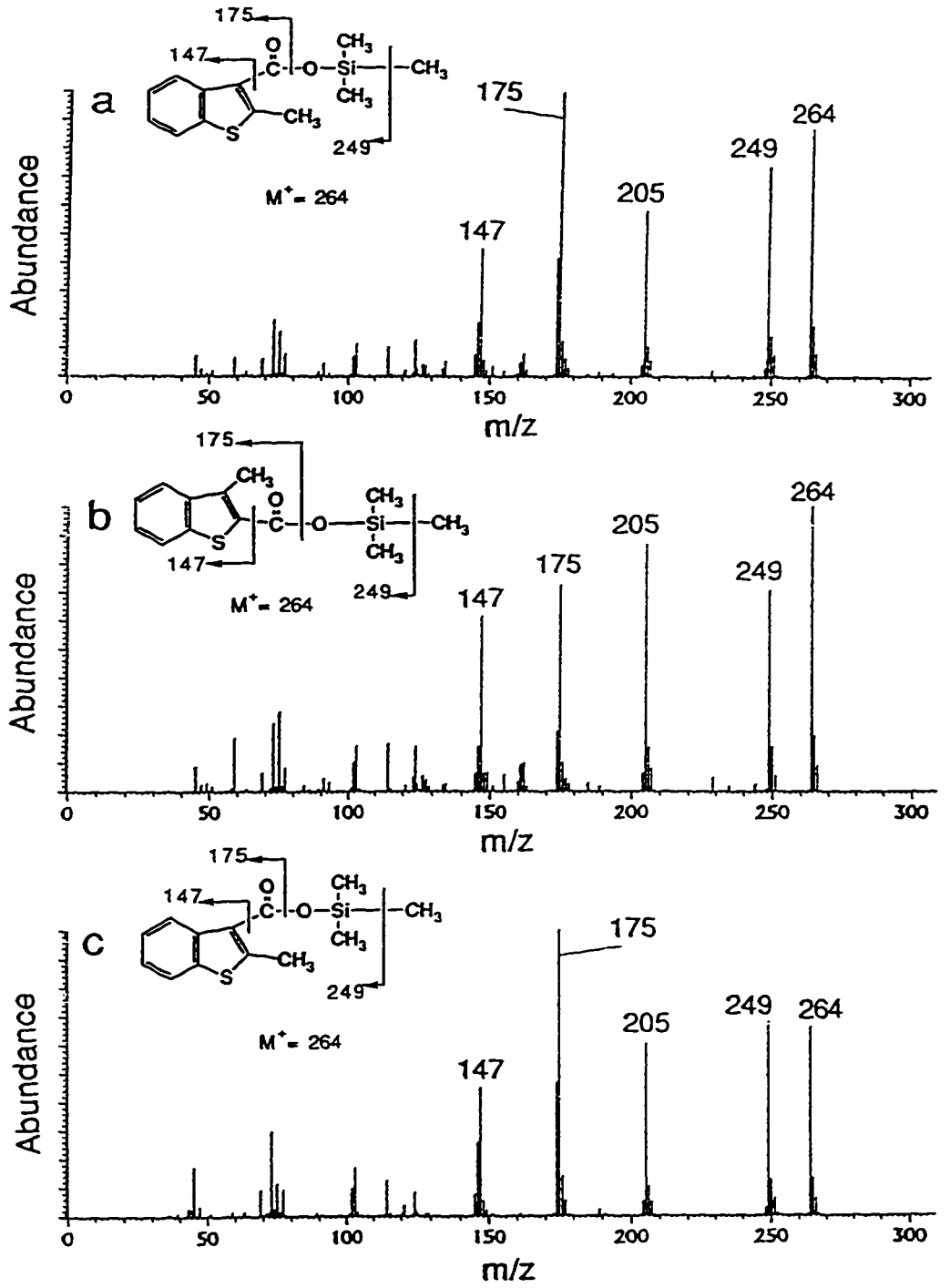


Figure 4.2 From GC-MS analyses, the mass spectra of the TMS-derivatives of two metabolites of 2,3-dimethylbenzothiophene (a,b) from a culture of isolate W1 grown on glucose. The mass spectrum of the TMS-derivative of authentic 2-methylbenzothiophene-3-carboxylic acid (c).

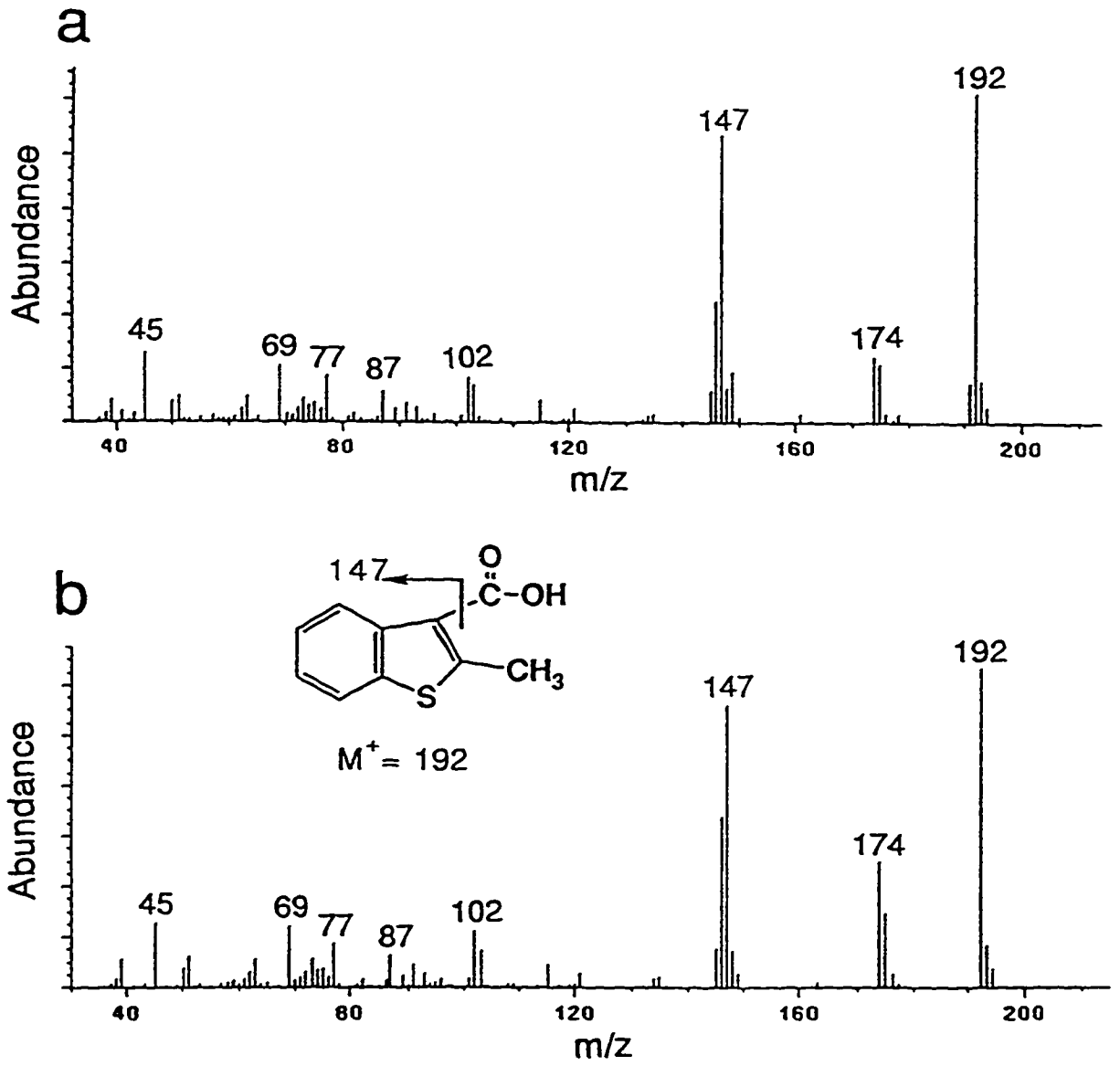


Figure 4.3 From GC-MS analyses, the mass spectra of a sulfur-containing metabolite (a) from a culture of isolate F grown on glucose in the presence of 2,3-dimethylbenzothiophene and authentic 2-methylbenzothiophene-3-carboxylic acid (b).

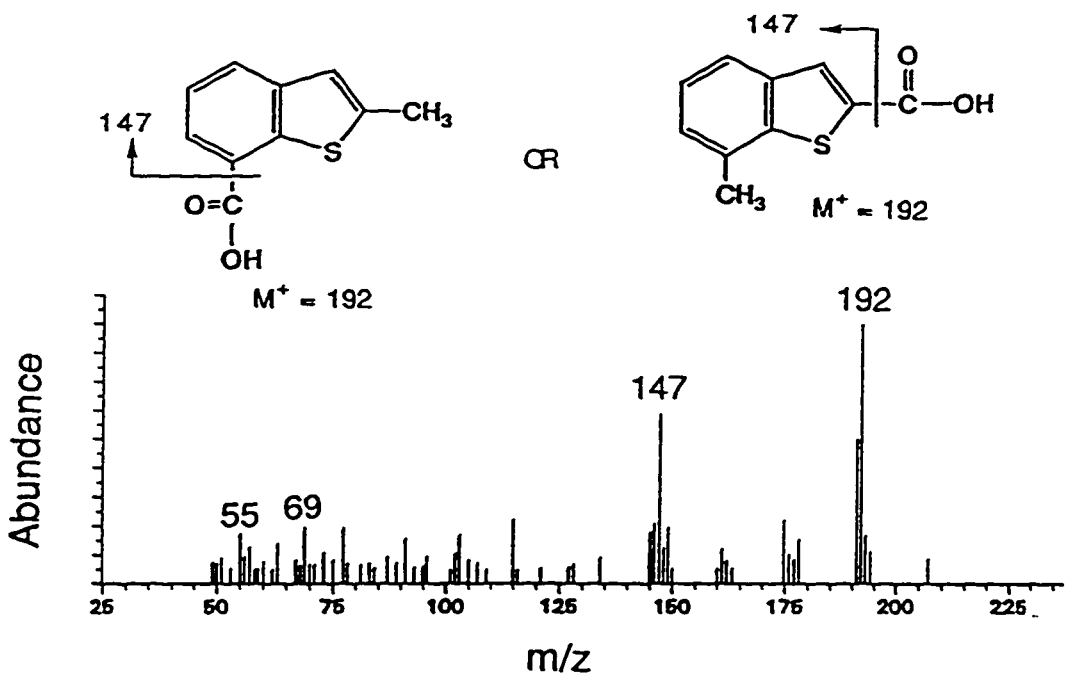


Figure 4.4 From GC-MS analysis, the mass spectrum of a sulfur-containing metabolite from a culture of isolate W1 grown on glucose in the presence of 2,7-dimethylbenzothiophene.

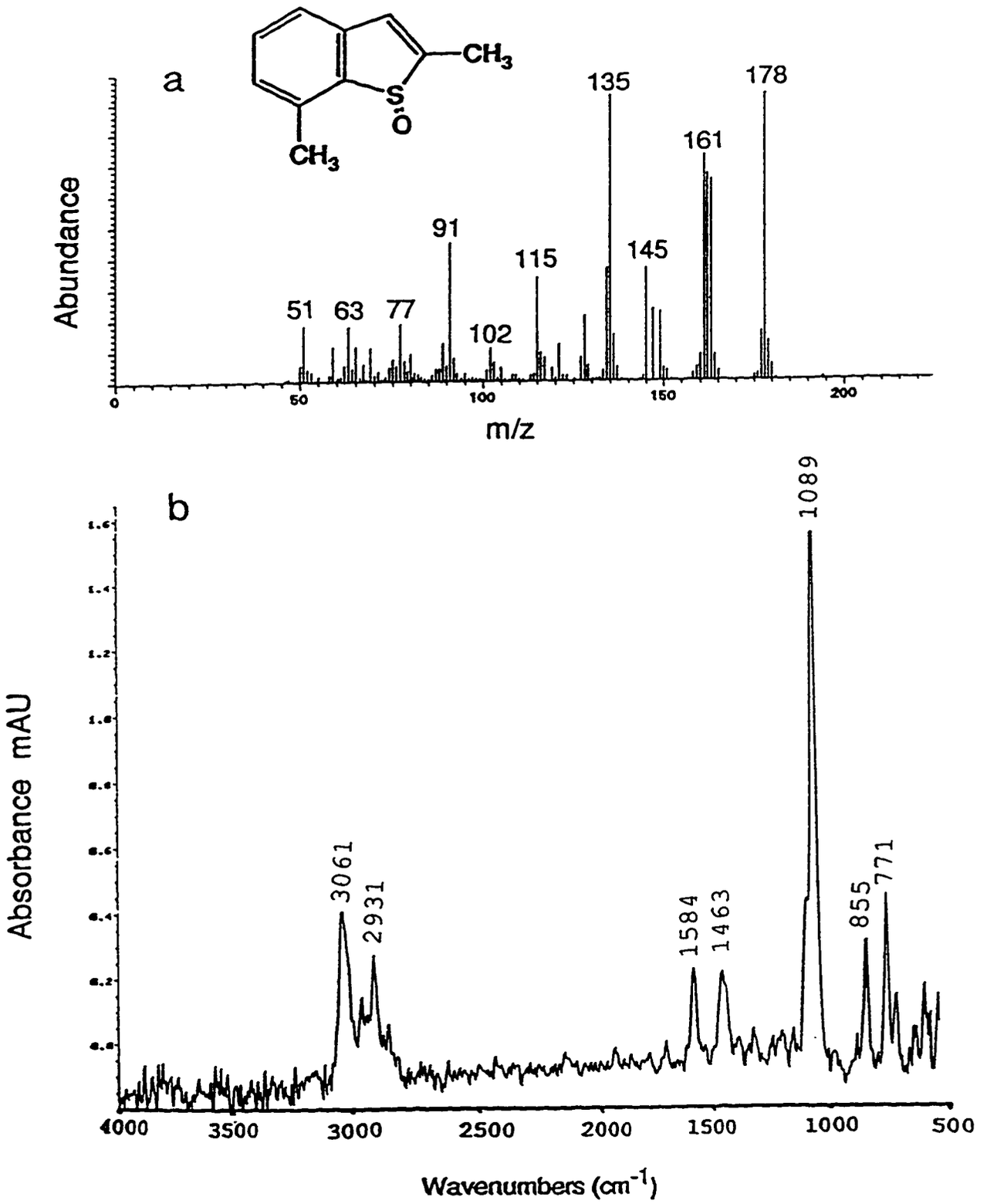


Figure 4.5 Mass spectrum (a) and FTIR spectrum (b) of a sulfur-containing metabolite from a culture of isolate W1 grown on glucose in the presence of 2,7-dimethylbenzothiophene.

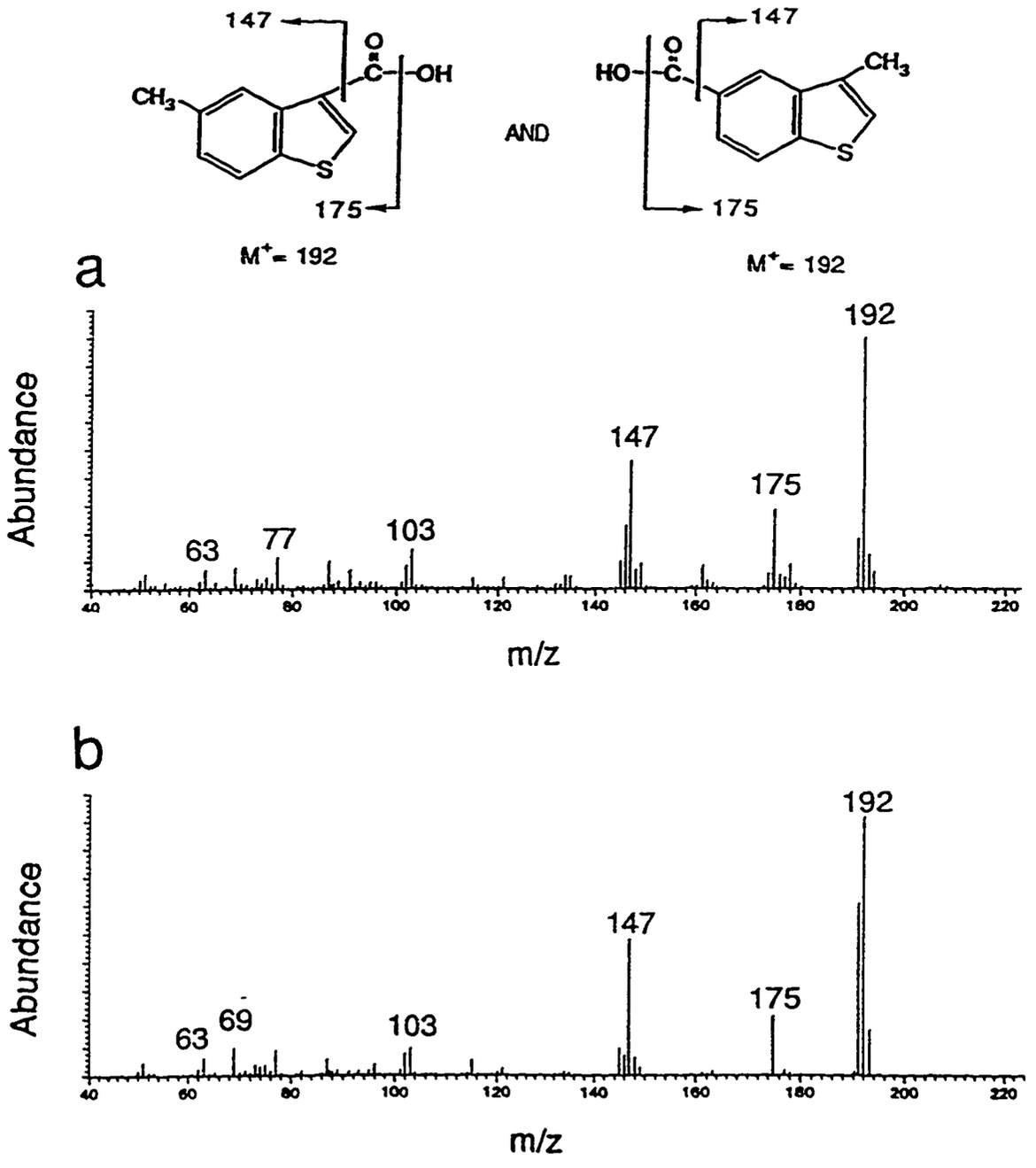


Figure 4.6 From GC-MS analysis, the mass spectra of two sulfur-containing metabolites from a culture of isolate W1 grown on glucose in the presence of 3,5-dimethylbenzothiophene. The metabolite with the shorter retention time (a) and the metabolite with the longer retention time (b).

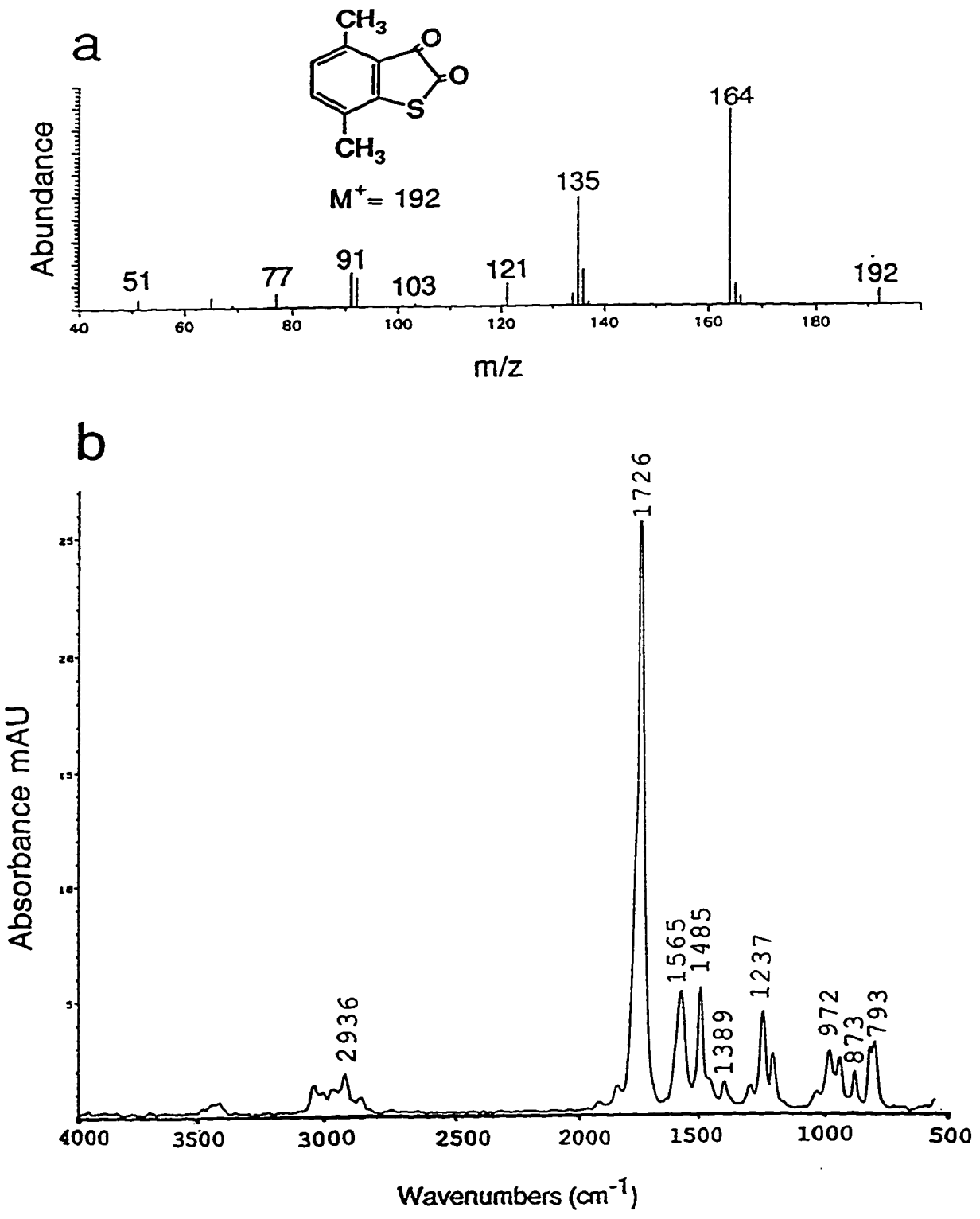


Figure 4.7 Mass spectrum (a) and FTIR spectrum (b) of a sulfur-containing metabolite from a culture of isolate BT1 grown on 1-MN in the presence of 4,7-dimethylbenzothiophene. The metabolite was identified as 4,7-dimethyl-benzothiophene-2,3-dione.

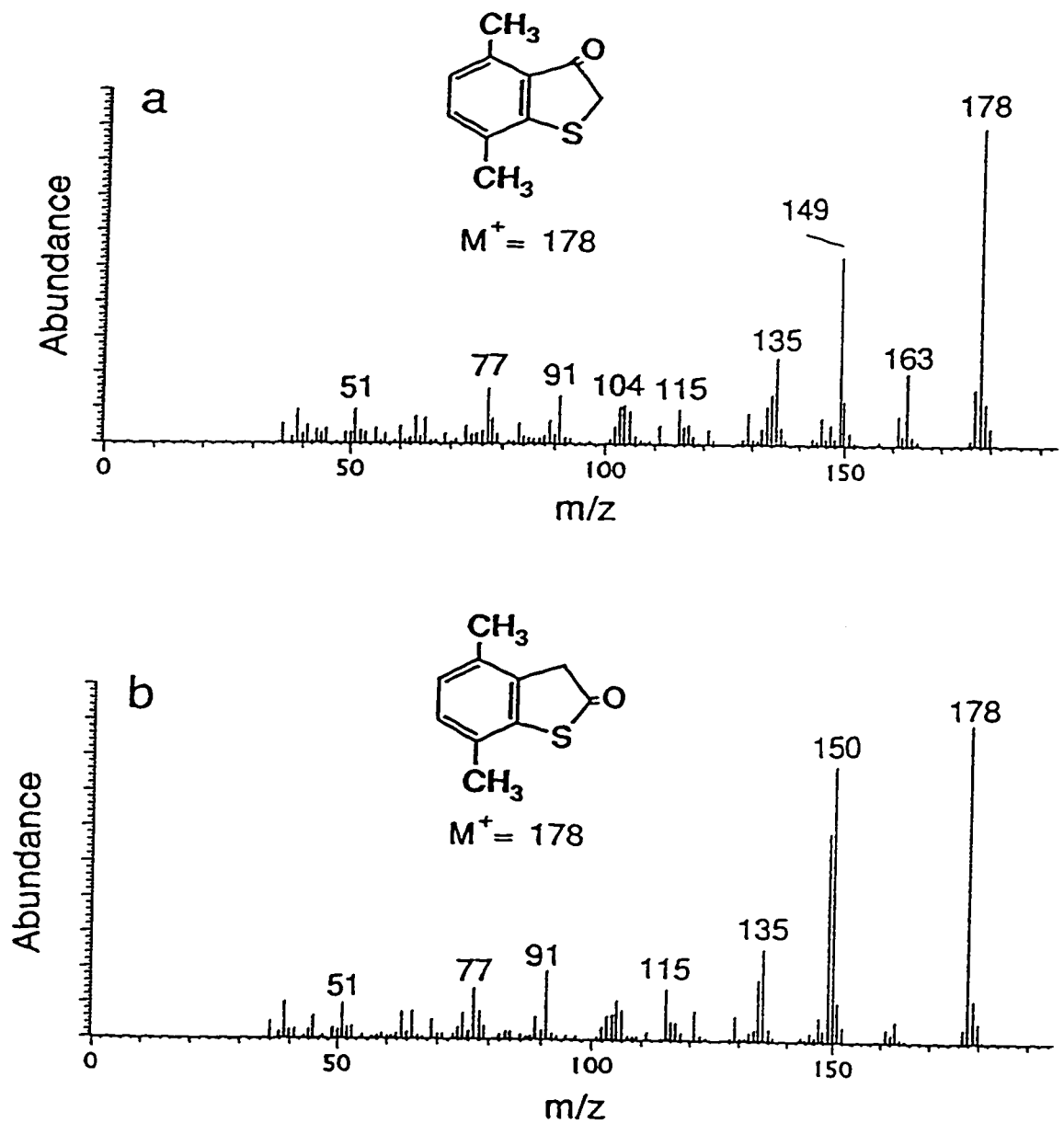


Figure 4.8 From GC-MS analysis, the mass spectra of two sulfur-containing metabolites from a culture of isolate BT1 grown on 1-MN in the presence of 4,7-dimethylbenzothiophene. The metabolite with the shorter retention time (a) and the metabolite with the longer retention time (b).

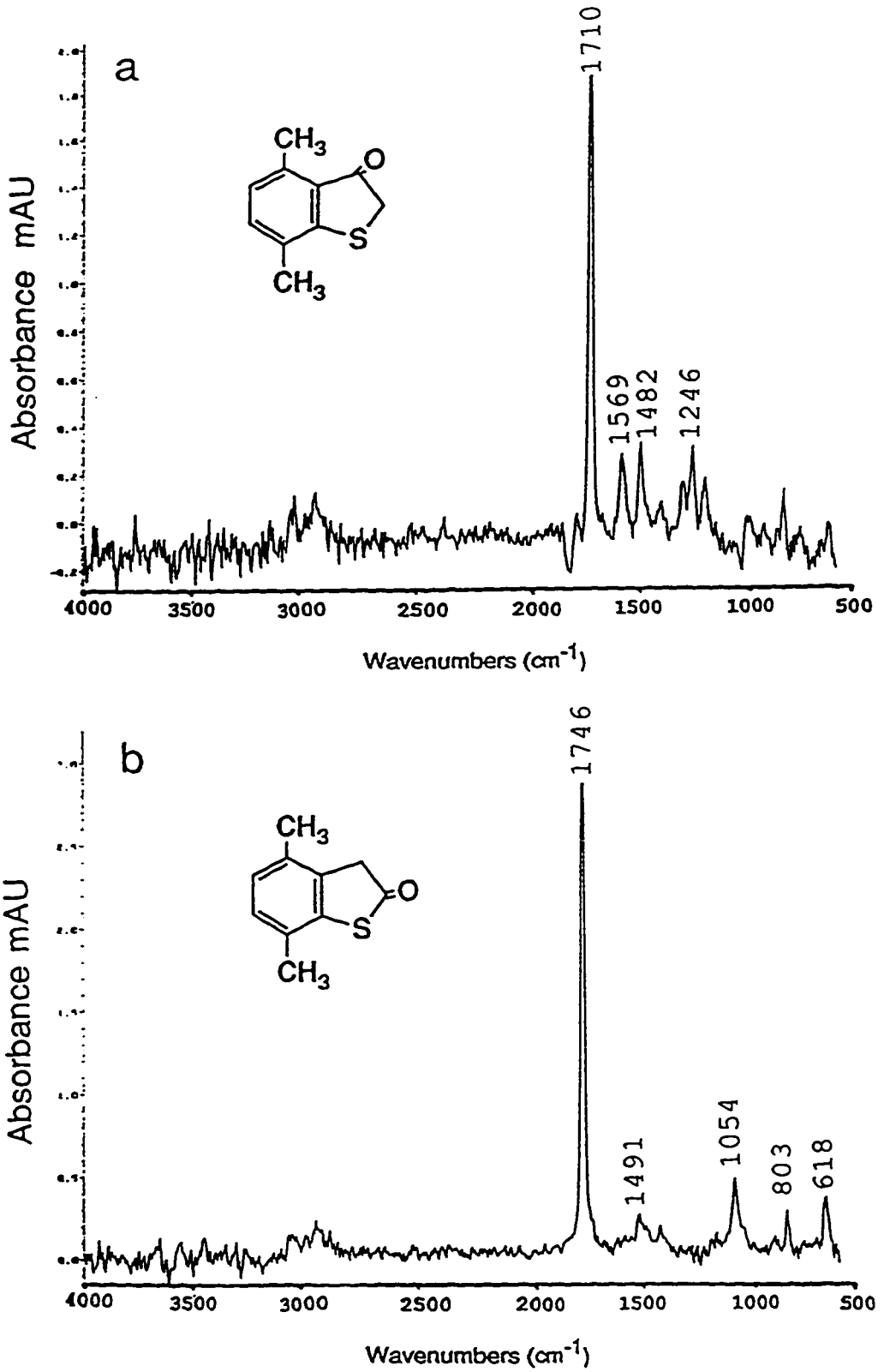


Figure 4.9 From GC-FTIR analysis, the FTIR spectra of two sulfur-containing metabolites from a culture of isolate BT1 grown on 1-MN in the presence of 4,7-dimethylbenzothiophene. The metabolite with the shorter retention time (a) and the metabolite with the longer retention time (b).

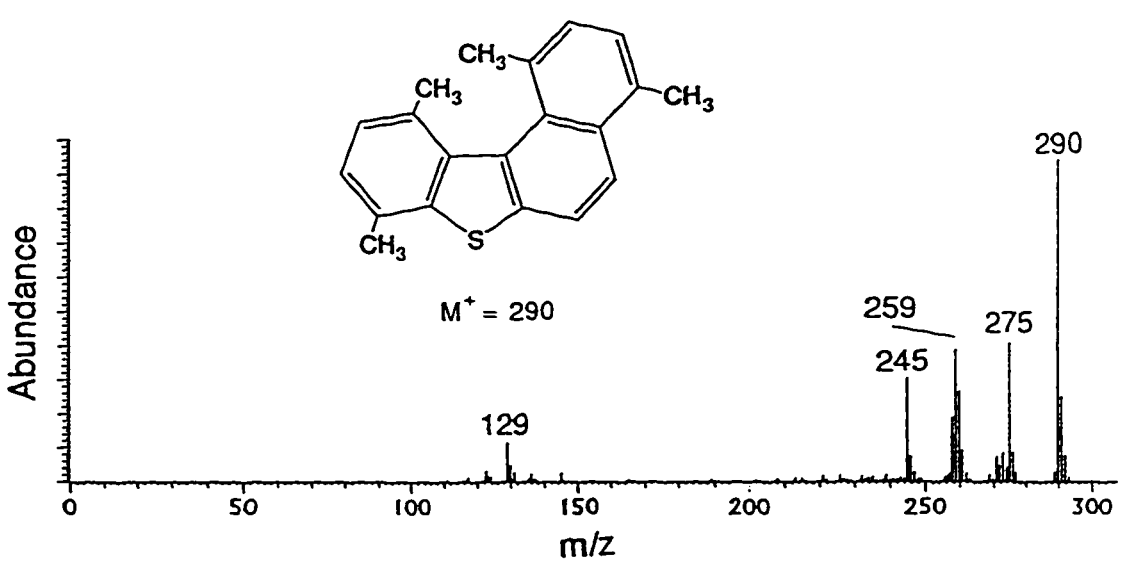


Figure 4.10 From GC-MS analysis, the mass spectrum of a high-molecular-weight sulfur-containing metabolite from a culture of isolate BT1 grown on 1-MN in the presence of 4,7-dimethylbenzothiophene.

4.5 LITERATURE CITED

Andersson, J. T. 1986. Gas chromatographic retention indices for all C_1- and C_2-alkylated benzothiophenes and their dioxides on three different stationary phases. J. Chromatogr. 354: 83-98.

Andersson, J. T. 1992. Polycyclic aromatic sulfur heterocycles. I. Use of hydrogen peroxide oxidation for the group separation of polycyclic aromatic hydrocarbons and their sulfur analogs. Intern. J. Environ. Anal. Chem. 48: 1-15.

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.

Baciocchi, E., and L. Mandolini. 1968. Products, kinetics, and mechanism of the chlorination of 2,3-dimethylbenzothiophene in acetic acid. J. Chem. Soc. (B). 1968: 397-400.

Barnsley, E. A. 1988. Metabolism of 2,6-dimethylnaphthalene by Flavobacteria. Appl. Environ. Microbiol. 54: 428-433.

Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald. 1986. Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures. Intern. J. Environ. Anal. Chem. 23: 289-303.

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.

Burchill, P., A. A. Herod, and E. Pritchard. 1982. Identification of sulphur heterocycles in coal tar and pitch. J. Chromatogr. 242: 51-64.

Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. Current Microbiol. 21: 229-231.

Dean-Raymond, D., and R. Bartha. 1975. Biodegradation of some polynuclear aromatic petroleum components by marine bacteria. Dev. Ind. Microbiol. 16: 97-110.

Dickinson, R. P., and B. Iddon. 1970. Condensed thiophen ring systems. Part III. A new synthesis of benzo[*b*]thiophen-2(3*H*)- and -3(2*H*)-ones and some reactions of benzo[*b*]thiophen-2(3*H*)-one with dimethylsulfate in the presence of base. J. Chem. Soc. (C). p. 1926-1928.

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

Fedorak, P. M., and J. T. Andersson. 1992. Decomposition of two methylbenzothiophene sulphoxides in a commercial GC injection port liner. J. Chromatogr. 591: 362-366.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., and D. W. S. Westlake. 1983. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. 29: 291-296.

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.

Friedländer, P. 1906. Ueber schwefelhaltige Analoga der Indigogruppe. Chem. Ber. 39: 1060-1066.

Friedländer, P. 1907. Ueber Thionaphtenderivate und Thioindigo. Annalen. 351: 390-420.

Grummitt, O., and A. Buck. 1955. Organic Syntheses Coll. Vol. 3. John Wiley & Sons, Inc.: New York; p. 195-197.

Hannoun, M., N. Blazevic', D. Kolbah, M. Mihalic', and F. Kajfez. 1982. *alpha*-Phenylpropionic acid derivatives. Synthesis and dethiation of 5-benzoylbenzo[*b*]thiophene-3-carboxylic acid. J. Heterocyclic Chem. 19: 1131-1136.

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.

Hou, C. T., and A. I. Laskin. 1976. Microbial conversion of dibenzothiophene. Dev. Ind. Microbiol. 17: 351-362.

Iddon, B., and R. M. Scrowston. 1970. Recent advances in the chemistry of benzo[*b*]thiophenes. In: Advances in Heterocyclic Chemistry, Vol. 11. Katritzky, A. R. and A. J. Boulton, Eds. Academic Press: New York; p. 177-381.

Jerina, D. M., H. Delander, H. Yagi, M. C. Wells, J. F. Davey, V. Mahadevan, and D. T. Gibson. 1976. Dihydrodiols from anthracene and phenanthrene. J. Am. Chem. Soc. 98: 5988-9596.

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.

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.

Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994a. Bacterial transformations of benzothiophene and methylbenzothiophenes. Environ. Sci. Technol. 28: 1348-1356.

Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994b. Microbially mediated formation of benzonaphthothiophenes from benzo[*b*]thiophenes. Appl. Environ. Microbiol. 60: 3624-3631.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinkia* species. Appl. Environ. Microbiol. 34: 783-790.

Miyachi, N., T. Tanaka, T. Suzuki, Y. Hotta, and T. Omori. 1993. Microbial oxidation of dimethylnaphthalene isomers. Appl. Environ. Microbiol. 59: 1504-1506.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Pierce, A. E. 1968. Silylation of Organic Compounds. Pierce Chemial Company: Rockford, Illinois; p. 33-39.

Porter, Q. N. 1967. Mass-spectrometric studies. I. Benzo[*b*]thiophen, some alkyl and aryl derivatives, and the 1,1-dioxide and 2,3-dihydro-1,1-dioxide. Aust. J. Chem. 20: 103-116.

Resnick, S. M., D. S. Torok, and D. T. Gibson. 1993. Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase. FEMS Microbiol Lett. 113: 297-302.

Saftic´, 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.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Shriner, R. L., R. C. Fuson, D. Y. Curtin, and T. C. Morrill. 1980. The Systematic Identification of Organic Compounds. 6th Ed. John Wiley & Sons, Inc.: New York; p. 333.

Silverstein, R. M., G. C. Bassler, and T. C. Morrill. 1991. Spectrometric Identification of Organic Compounds. 5th Ed. John Wiley & Sons, Inc.: New York; p. 27.

Sokol, P. E. 1973. Organic Syntheses Coll. Vol. 5. John Wiley & Sons, Inc.: New York; p. 706-708.

Speight, J. G. 1980. The Chemistry and Technology of Petroleum. Marcel Dekker Inc.: New York.

Stridsberg, R., and S. Allenmark. 1974. Studies of the benzo[*b*]thiophene-3(2*H*)-one system. Chemica Scripta 6: 184-192.

Westlake, D. W. S. 1983. Microbial activities and changes in the chemical and physical properties of oil. In: Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Donaldson E. C. and J. B. Clark, Eds. Technology Transfer Branch, Bartlesville Energy Technology Centre: Bartlesville, Oklahoma; p. 102-111.

Willey, C., M. Iwao, R. N. Castle, and M. Lee. 1981. Determination of sulfur heterocycles in coal liquids and shale oils. Anal. Chem. 53: 400-407.

Williams, J. A., M. Bjorøy, D. L. Dolcater, and J. C. Winters. 1986. Biodegradation of South Texas Eocene oils-effects on aromatics and biomarkers. Org. Geochem. 10: 451-461.

5. BIOTRANSFORMATIONS OF THREE DIMETHYLDIBENZOTHIOPHENES BY PURE AND MIXED BACTERIAL CULTURES*

5.1 INTRODUCTION

Studies of the biodegradation of petroleum or other fossil fuel derivatives, and of pure compounds typically found in these complex mixtures, are done to better understand the feasibility of bioremediation in environmental cleanup. Numerous organosulfur compounds are present in petroleum, making sulfur the third most abundant element in a typical crude oil, after carbon and hydrogen (Speight, 1980). The organosulfur compounds contain thiol, sulfide, and thiophene moieties. However, in crude oils of higher density, where sulfur content is typically the highest (Speight, 1980), the sulfur exists primarily in the form of condensed thiophenes. Thus, most studies of the biodegradation of organosulfur compounds have focused on those compounds found in the aromatic fraction of petroleum, namely the benzothiophenes and dibenzothiophenes.

Dibenzothiophene and its alkylated derivatives are present in most crude oils, and analyses of residual oil that has undergone biodegradation at contaminated sites has shown that these derivatives are among the most recalcitrant compounds in the aromatic fraction that can be analyzed by GC (Berthou *et al.*, 1981; Boehm *et al.*, 1982; Teal *et al.*, 1978). This recalcitrant nature is also reflected by studies showing the accumulation of dibenzothiophenes in sediments (Boehm *et al.*, 1981) and in tissues of shellfish (Laseter *et al.*, 1981; Ogata and Fujisawa, 1985) in contaminated marine environments. The persistence and accumulation of these compounds has led to the suggestion that they might serve as oil pollution markers (Friocourt *et al.*, 1982; Ogata and Fujisawa, 1985) and are a concern since these are potentially harmful environmental pollutants (Jacob, 1990).

Despite the recalcitrance of the alkyldibenzothiophenes, their biodegradation in the aromatic fraction of crude oil has been observed in laboratory cultures (Bayona *et al.*, 1986; Fedorak and Westlake, 1983a, 1984), in contaminated environments (Atlas *et al.*, 1981; Hostettler and Kvenvolden, 1994), and within natural petroleum reservoirs (Westlake, 1983; Williams *et al.*, 1986). Fedorak and Westlake (1983a, 1984) showed that the susceptibility of dibenzothiophenes in Prudhoe Bay crude oil to biodegradation decreased with increasing alkyl substitution. Biodegradation studies with complex mixtures of compounds such as crude oil allow one to follow the depletion of condensed thiophenes by GC analysis. However, the identification of metabolic intermediates, metabolic pathways,

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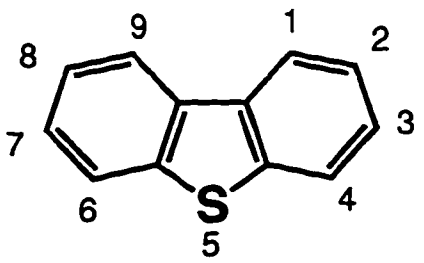
* A version of this chapter has been previously published. Kropp, K. G., J. T. Andersson, and P. M. Fedorak. 1997. Environ. Sci. Technol. 31: 1547-1554.

or biotransformation products, whose recalcitrance and toxicity may have implications for a contaminated environment and its bioremediation, are not easily done in these complex systems.

The identification of metabolites is most easily accomplished using pure compounds in microbial cultures. Thus, there have been numerous studies of the microbial metabolism of dibenzothiophene as a model condensed thiophene (Crawford and Gupta, 1990; Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989), even though it is the least recalcitrant of the dibenzothiophenes. Bacteria have been reported to metabolize dibenzothiophene to 3-hydroxy-2-formylbenzothiophene as the major product by oxidation and cleavage of one of the benzene rings of dibenzothiophene (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989). Microbial oxidation of the sulfur atom gives dibenzothiophene sulfoxide and sulfone (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Mormile and Atlas, 1989), which appears to be a dead-end pathway in these aromatic-degrading bacteria. Other studies used isolates that utilize dibenzothiophene as a sulfur source by oxidizing dibenzothiophene via the sulfoxide and sulfone and then subsequently release the sulfur atom as sulfate leaving 2-hydroxybiphenyl (Gallagher *et al.*, 1993; Izumi *et al.*, 1994; Omori *et al.*, 1992). This pathway does not result in the degradation of the carbon backbone of dibenzothiophene and is potentially useful in the development of a microbial process for the biodesulfurization of petroleum.

The bacterial metabolism of all four isomers of methyldibenzothiophene was investigated by Saftic´ *et al.* (1993). The metabolites detected resulted from degradation of the unsubstituted benzene ring of each of the methyldibenzothiophenes to give the respective methyl-substituted 3-hydroxy-2-formylbenzothiophenes and benzothiophene-2,3-diones. They also detected dibenzothiophenemethanols and methyldibenzothiophene sulfones.

The objective of this work was to study the cometabolic degradation of three dimethyldibenzothiophenes by three *Pseudomonas* spp. and by some petroleum-degrading mixed bacterial cultures, since mixed cultures generally have greater degradative potential and more closely approximate the situation of an actual oil-contaminated environment. One isomer of dimethyldibenzothiophene studied (3,4-) has both methyl groups on the same benzene ring of dibenzothiophene and the other two isomers (2,8- and 4,6-) have a single methyl group on each external homocyclic ring. The ring numbering convention for dibenzothiophene is shown below.



These studies focused on the identification of metabolites detected from bacterial oxidation of the dimethyldibenzothiophenes, but near the completion of this investigation, access to a recently-acquired gas chromatograph with an AED allowed preliminary quantification of the observed transformations.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

2,8-Dimethyldibenzothiophene (Gerdil and Lucken, 1965), and 3,4-dimethyl- and 4-methyldibenzothiophene (Tedjamulia *et al.*, 1983) were synthesized (≥99% pure by GC). 4,6-Dimethyldibenzothiophene (Gerdil and Lucken, 1965) (97% pure by GC) was a gift from Dr. T.G. Back (University of Calgary, Canada). 1-MN and dibenzothiophene were purchased from Fluka (Buchs, Switzerland). 5-Methylbenzothiophene-2,3-dione and 7-methylbenzothiophene-2,3-dione were synthesized (Hannoun *et al.*, 1982) and their structure and purity were confirmed by determination of their melting points which were the same as the literature values. 6,7-Dimethylbenzothiophene-3(2*H*)-one and 5-methylbenzothiophene-3(2*H*)-one were synthesized (Stridsberg and Allenmark, 1974) from 2,3-dimethylthiophenol (Tedjamulia *et al.*, 1983) and 4-thiocrescol (Aldrich, Milwaukee, WI), respectively. 6,7-Dimethylbenzothiophene-2(3*H*)-one and 5-methylbenzothiophene-2(3*H*)-one were synthesized (Dickinson and Iddon, 1970) from 6,7-dimethylbenzothiophene (prepared by Andersson (1986)) and 5-methylbenzothiophene (Lancaster Synthesis, Windham, NH), respectively. These 3(2*H*)-ones and 2(3*H*)-ones were recovered from reaction mixtures by extraction and kept in DCM to minimize the chance of air oxidation of the products (Friedländer, 1906). GC-MS analyses of these solutions showed that extracts of reaction mixtures from each synthesis contained a single abundant compound with the molecular weight of the desired product.

5.2.2 Silica gel column chromatography

The aromatic fraction of Prudhoe Bay crude oil was obtained by column chromatography using 35 g of silica gel (100-200 mesh, Type 150A, Grade 644, Fisher Scientific, Fair Lawn, NJ) that had been activated at 125°C for 24 h. The silica gel was poured as a slurry in DCM into a 62 cm × 2.25 cm ID column. After displacement of the DCM with *n*-pentane and application of 1.0 mL of crude oil, the column was developed with 25 mL of *n*-pentane, 25 mL of 20% (v/v) DCM in *n*-pentane, and 125 mL of 50% (v/v) DCM in *n*-pentane. The first 65 mL to elute was the void volume of the column, the next 60 mL collected contained the saturated hydrocarbons, and the remaining solvent to elute contained the aromatic fraction, which was collected and concentrated. The solvent was removed using a rotary evaporator at 65°C, and when the aromatic fraction was reduced to a volume of 1 to 3 mL, the sample was transferred to a vial and further concentrated to near dryness under a stream of N_2 at room temperature. The aromatic fraction was then dissolved in 1.0 mL DCM. GC analysis of this fraction gave a chromatogram that was nearly identical to one previously published (Fedorak and Westlake, 1981), showing that volatile compounds such as the alkylbenzenes, naphthalene, and methylnaphthalenes remained in the fraction after this concentrating procedure.

To ensure a clean separation of the aromatic and the saturated fractions, each batch of silica gel was tested with crude oil supplemented with [1-^{14}C]hexadecane and [9-^{14}C]phenanthrene, and the volumes of the first two solvents (*n*-pentane, and 20% DCM in *n*-pentane) were adjusted to give the separation of the radioactive compounds shown by Fedorak and Westlake (1981).

For experiments with the oil-degrading mixed cultures grown on the aromatic fraction of petroleum, the silica gel column chromatography method was modified and used to cleanup extracts prior to high resolution and chemical ionization GC-MS, GC-FTIR, and GC-AED analyses. The method was scaled down using a 30 cm × 1.1 cm ID column with one-fifth the amounts of silica and developing solvents. In addition, 100 mL of a fourth developing solvent (benzene:methanol, 1:1) was used to elute the polar fraction which contained the dimethyldibenzothiophene oxidation products that eluted immediately after the aromatic fraction.

5.2.3 Bacterial cultures and culture methods

All cultures were incubated on a rotary shaker at 28°C in 500-mL Erlenmeyer flasks containing 200 mL of mineral medium supplemented with a trace metals solution (Kropp *et al.*, 1994b). Following inoculation and addition of the growth substrate, each culture received 2 to 5 mg of a dimethyldibenzothiophene dispensed either as a solid for qualitative

studies aimed at detection and identification of oxidation products, or as a solution in 200 to 300 μL of acetonitrile or DCM for quantitative studies. The amounts of dimethyldibenzothiophenes added exceeded the aqueous solubilities of these compounds, so that they were present in cultures as a fine suspension or partitioned into the growth substrate 1-MN, when it was present. For each experiment, appropriate sterile controls were included to account for any abiotic transformations. With some cultures that did not yield detectable metabolites from certain dimethyldibenzothiophenes, heat-killed controls were used in quantitative experiments to determine if loss of the dimethyldibenzothiophene occurred in the growing culture relative to the heat-killed control. For these heat-killed controls, flasks were inoculated with the appropriate culture, incubated for 5 days with the growth substrate, autoclaved, and then given the dimethyldibenzothiophene at the same time as the test culture was inoculated and given the growth substrate and dimethyldibenzothiophene. No dimethyldibenzothiophene oxidation products were found in the heat-killed controls.

The isolation and characteristics of the three *Pseudomonas* spp. BT1, W1, and F have been described previously (Fedorak and Grbic´-Galic´, 1991; Kropp *et al.*, 1994a, 1996; Saftic´ *et al.*, 1992, 1993). The 10-mL inocula used to establish cultures for biodegradation studies with each of these isolates came from 1-MN-grown maintenance cultures that were transferred weekly. Following inoculation, the cultures received 50 μL of 1-MN as growth substrate together with the dimethyldibenzothiophene.

The petroleum-degrading mixed cultures used in this study and the nature of the samples from which they were enriched are as follows: SLPB, enriched from fuel-contaminated beach material from Shell Lake, Northwest Territories (Fedorak and Peakman, 1992); 5W/5B, enriched from sea water and beach material from the Washington state coast; and, ESSO AG and ERN BIO, both enriched from petroleum refinery wastewater treatment systems in Germany. These mixed cultures were all screened to verify that they were capable of degrading the C_2-dibenzothiophenes naturally present in Prudhoe Bay crude oil before they were selected for this study. These cultures have been maintained in this laboratory for several years by monthly transfers into mineral medium with Prudhoe Bay crude oil as the growth substrate. The medium for the dimethyldibenzothiophene biodegradation studies received the aromatic fraction from 0.1 mL of Prudhoe Bay crude oil as growth substrate (added as 100 μL of the DCM solution from the silica gel fractionation) together with the dimethyldibenzothiophene. This was inoculated with 10 mL of these maintenance cultures and the DCM was allowed to evaporate as the cultures incubated on the shaker.

5.2.4 Extraction and analyses

After incubation, cultures and controls were acidified with 2 M H_2SO_4 to pH<2 and extracted with DCM (4 times 20 mL) to recover substrates and products. The DCM extracts were dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. For qualitative experiments with the aromatic fraction of crude oil, 50 µL of a solution of chrysene (2 g/L) in DCM was added as an internal standard prior to extraction to help in the assessment of degradation (loss) of the aromatic compounds and added dimethyldibenzothiophene by GC-FID analysis. For quantitative experiments with the AED, known amounts of phenyl sulfide (Aldrich, 98%) and/or dibenzothiophene sulfone (Aldrich, 97%), dissolved in acetonitrile or DCM, were added as internal standards prior to extraction. For studies with oil-degrading cultures, these two standards were useful because phenyl sulfide eluted from the silica gel column in the aromatic fraction and thus could be used to quantify the amount of dimethyldibenzothiophene substrate that remained, and dibenzothiophene sulfone eluted in the polar fraction and could be used to quantify the amounts of the polar oxidation products. The amounts of these standards added were nearly equimolar to the amounts of the dimethyldibenzothiophene or its oxidation products that they were meant to be used to quantify.

To screen for the presence of sulfur-containing metabolites, the extracts were analyzed by capillary GC using a 30-m DB-5 column in a HP (Mississauga, ON) model 5890 equipped with a FID and a sulfur-selective FPD (Fedorak and Grbic´-Galic´, 1991). The methods routinely used for GC-MS and GC-FTIR have been described (Saftic´ *et al.*, 1992, 1993). To obtain high resolution and chemical ionization GC-MS data, analysis was done in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta (Fedorak and Westlake, 1986).

Quantitative GC analyses were done using a HP 5921A AED, that gives an equimolar response to sulfur in all organic forms (Andersson and Schmid, 1993), connected to a HP 5890 gas chromatograph. The gas chromatograph was equipped with a split/splitless injection port and injections were made by an automatic sampler (model HP 7673). The column used was a HP-5MS with dimensions of 0.25 mm ID × 30 m (0.25 µm film thickness). The temperature program held the initial temperature of 90°C for 2 min before increasing it at 4°C/min to 250°C where it was held for 18 min. Helium (99.996% pure), after further purification with a VD-1200 helium purifier from VICI Valco Instruments (Houston, TX), was used as carrier and plasma gas. Hydrogen and oxygen were used as auxiliary gases as required. The system was controlled by a HP Chem Station 382 using the HP 35920A software package. Gas selection and detector tuning were computer controlled, whereas the plasma gas flow rate was set manually to 60 mL/min for

optimal sensitivity and peak shape in the sulfur trace. The data presented are based on the moles of sulfur in each compound detected by the GC-AED analysis.

To facilitate GC-MS identification of some of the metabolites, TMS derivatives of compounds in culture extracts were made by silylating with BSA in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, IL; method 5).

5.3 RESULTS

5.3.1 Pure culture studies with 3,4-dimethyldibenzothiophene

The most abundant product in extracts of pure cultures of isolates BT1, W1, and F that had been incubated for 3 days with 3,4-dimethyldibenzothiophene gave the spectrum shown in Figure 5.1. The molecular ion at m/z 206 was the only abundant ion and was consistent with the structure shown of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene. To verify that this product contained a hydroxy group, the BT1 culture extract was treated with BSA. GC-MS analysis of the derivatized extract showed that the metabolite did react to form a TMS-ether. The mass spectrum showed the molecular ion at m/z 278 (23%), the $(M-15)^+$ fragment at m/z 263 (100%) which is characteristic of the loss of a methyl group from TMS, and the ion at m/z 73 (25%) which is the TMS substituent. These ions are all consistent with the TMS-ether of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene.

Further support for this identification of the product was obtained by GC-FTIR analysis of the underivatized BT1 culture extract. The metabolite from 3,4-dimethyldibenzothiophene gave strong absorptions at 1644, 1523, and 1282 cm^{-1} which is very similar to the strong absorptions reported for 3-hydroxy-2-formylbenzothiophene at 1640, 1526, and 1272 cm^{-1} and 6-methyl-3-hydroxy-2-formylbenzothiophene formed by bacterial cometabolism of 3-methyldibenzothiophene at 1647, 1513, and 1268 cm^{-1} (Saftic´ *et al.*, 1993). Non-methylated 3-hydroxy-2-formylbenzothiophene, that could potentially form by cleavage of the dimethyl-substituted ring of 3,4-dimethyldibenzothiophene, was not detected in the extracts. Thus, it appears that the isolates oxidize and cleave only the unsubstituted benzene ring of 3,4-dimethyldibenzothiophene leading to the formation of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene.

A second product that was detected in small amounts in the extract of only the BT1 culture gave the mass spectrum shown in Figure 5.2. The weak molecular ion at m/z 192 is consistent with the structure shown of 6,7-dimethylbenzothiophene-2,3-dione. The base peak at m/z 164 $(M-28)^+$ results from the loss of CO from the molecular ion and the other abundant ion at m/z 135 $(M-57)^+$ results from a subsequent loss of CHO. A weak molecular ion and the same fragmentation patterns were observed for 5-methylbenzo-

thiophene-2,3-dione (Saftic´ *et al.*, 1992) and 7-methylbenzothiophene-2,3-dione (Saftic´ *et al.*, 1993). Furthermore, GC-FTIR analysis showed that the metabolite from 3,4-dimethyldibenzothiophene gave a strong absorption at 1733 cm^{-1} indicative of the carbonyl groups (Silverstein *et al.*, 1991) and similar to 5- and 7-methylbenzothiophene-2,3-diones which absorb strongly at 1739 and 1735 cm^{-1}, respectively (Saftic´ *et al.*, 1992). Thus, the extract contained 6,7-dimethylbenzothiophene-2,3-dione.

GC-FPD analyses of extracts of cultures of all three isolates that had been incubated for 7 days prior to extraction showed that they each contained 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and 6,7-dimethylbenzothiophene-2,3-dione. After 30 days of incubation, only the 2,3-dione was detected in the W1 and BT1 culture extracts, and both the 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and 2,3-dione were detected in the isolate F culture extract. Even after 30 days of incubation, 3,4-dimethyldibenzothiophene was still present in all the cultures.

Quantitative analyses were done on cultures that had been incubated for 7 days. The recovery of 3,4-dimethyldibenzothiophene from the sterile control was 94%. In the isolate W1 culture extract, 74% of the 3,4-dimethyldibenzothiophene remained, and 2.8% and 0.8% of the substrate added was detected as the 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and 6,7-dimethylbenzothiophene-2,3-dione, respectively. With isolate BT1, 46% of the 3,4-dimethyldibenzothiophene was recovered from the culture as unoxidized substrate, and 1.1% and 3.7% as 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and 6,7-dimethylbenzothiophene-2,3-dione, respectively. Finally, 61% of the 3,4-dimethyldibenzothiophene was recovered unchanged in the isolate F culture extract, and 1.1% and 4.1% of the substrate added was recovered as the hydroxyformylbenzothiophene and 2,3-dione, respectively.

5.3.2 Mixed culture studies with 3,4-dimethyldibenzothiophene

The mixed cultures tested with 3,4-dimethyldibenzothiophene were SLPB and ERN BIO. GC-FPD analyses of the extracts of these cultures showed that the aromatic fraction, including the condensed thiophenes naturally present, was extensively degraded and that the added 3,4-dimethyldibenzothiophene was also oxidized. Although much 3,4-dimethyldibenzothiophene remained in the extract of a 4-day-old SLPB culture, there were two abundant sulfur-containing products detected which had the same GC retention times and mass spectra as the 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene (Figure 5.1) and 6,7-dimethylbenzothiophene-2,3-dione (Figure 5.2) detected in the pure culture studies.

By day 7, some of the alkylbenzenes had evaporated from the sterile control, but the GC-FID chromatogram showed that naphthalene and the methylnaphthalenes were still

present (data not shown). Figure 5.3 shows the FID and FPD chromatograms from GC analysis of the extract of a 7-day-old ERN BIO culture. The largest peak that was observed among the isomers of C_2-dibenzothiophene naturally present in Prudhoe Bay crude oil was the added 3,4-dimethyldibenzothiophene. The six sulfur-containing metabolites detected (labeled A to F) were too abundant to have formed from any of the condensed thiophenes naturally present in Prudhoe Bay crude oil and so must have formed from oxidation of the added 3,4-dimethyldibenzothiophene. The peaks E and D (Figure 5.3) have the same retention times, mass spectra (Figures 5.1 and 5.2), and FTIR spectra as 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and 6,7-dimethylbenzothiophene-2,3-dione, respectively, which were identified in pure cultures. Metabolite C was not detected by GC-MS analysis, so its identification was not pursued.

GC-MS analysis gave the spectra for metabolites A and B shown in Figure 5.4 with strong molecular ions, which were the base peaks, at m/z 178 for both compounds. The chemical formula for both of these metabolites, determined by high resolution GC-MS, was $C_{10}H_{10}OS$. GC-FTIR analysis showed that the oxygen atom present in each of metabolites A and B was present in the form of a carbonyl group with strong absorptions at 1748 and at 1719 cm^{-1}, respectively. However, by keto-enol tautomerism, these metabolites were both capable of reacting with one equivalent of BSA to give TMS-ethers of the enol tautomers with molecular ions at m/z 250. The mass spectra of both derivatives showed strong ions at m/z 73 from the TMS substituent and the spectrum of derivatized metabolite B also showed a strong $(M-15)^+$ ion from loss of a methyl group from the TMS substituent. Metabolites A and B were identified as 6,7-dimethylbenzothiophene-2(3*H*)-one and 6,7-dimethylbenzothiophene-3(2*H*)-one, respectively, because they had identical retention times, mass and FTIR spectra, and TMS-derivatives with identical retention times and mass spectra as authentic standards of these compounds.

Metabolite F gave the mass spectrum shown in Figure 5.5 with a molecular ion at m/z 194 which is consistent with the chemical formula $C_{10}H_{10}O_2S$ obtained by high resolution GC-MS. The GC-FTIR analysis showed that at least one of the oxygen atoms was present in the form of a carbonyl group since the metabolite absorbed strongly at 1729 cm^{-1}. However, metabolite F reacted with two equivalents of BSA to form a di-TMS ether with a strong molecular ion at m/z 338 (60%) and only one other abundant fragment at m/z 73 (100%). The oxygen atom that was present in the carbonyl group must undergo a keto-enol tautomerism for metabolite F to be able to react with two equivalents of BSA. Thus, metabolite F appears to be the keto tautomer of 2,3-dihydroxy-6,7-dimethylbenzothiophene. A hydroxy group at position 3, adjacent to the carbonyl group of authentic 6,7-dimethylbenzothiophene-2(3*H*)-one which absorbs strongly at 1748 cm^{-1}, would be

expected to decrease the wavenumber at which the carbonyl group absorbs by hydrogen bonding to near the carbonyl absorption observed for metabolite F at 1729 cm^{-1}. Thus, metabolite F is most likely 3-hydroxy-6,7-dimethylbenzothiophene-2(3*H*)-one. The other possible isomer, 2-hydroxy-6,7-dimethylbenzothiophene-3(2*H*)-one, would be expected to give, by hydrogen bonding, a carbonyl absorption at a lower wavenumber than authentic 6,7-dimethylbenzothiophene-3(2*H*)-one which absorbed strongly at 1719 cm^{-1}. This was not observed for metabolite F, so the keto tautomer of 2,3-dihydroxy-6,7-dimethylbenzothiophene that was present was most likely 3-hydroxy-6,7-dimethylbenzothiophene-2(3*H*)-one. This structural assignment for metabolite F is consistent with the available data, but was not proven by comparison with an authentic standard.

These metabolites were characterized and identified in the extract from the 7-day-old culture of ERN BIO (Figure 5.3). The GC-FID and FPD chromatograms from the extract of a 3-day-old culture are very similar to those of the 7-day extract (Figure 5.3) except that at day 3 the peak of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene (peak E) was larger than the peak of 6,7-dimethylbenzothiophene-2,3-dione (peak D). This suggests that larger amounts of the 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene were present earlier in the incubation, and later more of the 2,3-dione accumulated. GC-FPD analysis of an extract of a 13-day-old culture revealed that the 3,4-dimethyldibenzothiophene was absent and only trace amounts of a few sulfur-containing metabolites were detected. GC-AED analysis was performed on the extract of a 7-day-old culture of ERN BIO. The amount of substrate remaining in the culture was 34% of the amount of 3,4-dimethyldibenzothiophene that had been given to the culture and 6% of the substrate given was present as metabolites A through F. In the corresponding sterile control, the recovery of 3,4-dimethyldibenzothiophene was 116%.

5.3.3 Pure culture studies with 4,6-dimethyldibenzothiophene

Although pure cultures of isolates BT1, W1, and F grew and depleted the 1-MN from the medium in the presence of 4,6-dimethyldibenzothiophene, they did not deplete the 4,6-dimethyldibenzothiophene from the medium relative to the sterile controls. No oxidation products were detected in cultures incubated for 3, 11, 26, and 63 days.

Another attempt to demonstrate oxidation of 4,6-dimethyldibenzothiophene used isolate BT1, which is the only one of the three *Pseudomonas* spp. capable of growth on dibenzothiophene as its sole source of carbon and energy (Saftic´ *et al.*, 1993), to test its ability to transform 4,6-dimethyldibenzothiophene while growing on dibenzothiophene (8 mg) or a mixture of dibenzothiophene (3 mg) and 4-methyldibenzothiophene (6 mg). Over incubation times ranging from 3 to 12 days, the cultures grew on the dibenzothiophene and

3-hydroxy-2-formylbenzothiophene was produced as an abundant metabolite, as was 7-methyl-3-hydroxy-2-formylbenzothiophene from 4-methyldibenzothiophene. However, no metabolites from oxidation of 4,6-dimethyldibenzothiophene were detected and GC-AED analyses revealed that the 4,6-dimethyldibenzothiophene was not depleted from the medium relative to the heat-killed controls.

5.3.4 Mixed culture studies with 4,6-dimethydibenzothiophene

GC-FPD analysis revealed that the mixed cultures SLPB, ESSO AG, and ERN BIO extensively degraded the aromatic fraction of Prudhoe Bay crude oil including the dimethyldibenzothiophenes naturally present within a 13-day incubation period. However, no products from 4,6-dimethyldibenzothiophene oxidation were detected, and GC-AED analysis revealed that only a small fraction of the 4,6-dimethyldibenzothiophene had been removed from the culture medium. The recovery of 4,6-dimethyldibenzothiophene from active cultures of SLPB, ESSO AG, and ERN BIO was 65%, 82%, and 79%, respectively, whereas the corresponding heat-killed controls showed recovery of between 88% and 90% of the 4,6-dimethyldibenzothiophene given.

5.3.5 Pure culture studies with 2,8-dimethyldibenzothiophene

GC-FPD analyses of extracts of cultures of isolates W1 and F that had been incubated with 2,8-dimethyldibenzothiophene for up to 6 weeks did not detect any sulfur-containing metabolites in the extracts and GC-AED analyses of extracts of cultures incubated for 2 weeks confirmed that there was no depletion of the 2,8-dimethyl-dibenzothiophene relative to the heat-killed controls. However, in cultures of isolate BT1 that had been incubated for 1 to 6 weeks, three abundant, sulfur-containing metabolites (designated G, H, I) and some unoxidized 2,8-dimethyldibenzothiophene were always detected. The characterization and identification of these three products was done using extracts of numerous cultures that had been incubated for 1 to 3 weeks.

The first metabolite to elute from the GC (metabolite G) had a molecular ion at m/z 164 which was the base peak and was consistent with the chemical formula C_9H_8OS obtained by high resolution GC-MS (Figure 5.6a). GC-FTIR analysis gave a strong absorption at 1723 cm^{-1}, indicating that the oxygen atom was present in the form of a carbonyl group. However, by keto-enol tautomerism metabolite G was capable of reacting with BSA to give a TMS-ether of the enol form with a molecular ion at m/z 236 (100%) and abundant fragment ions at m/z 221 $(M-15)^+$ (68%) and m/z 73 (50%). Metabolite G was identified by comparison with an authentic standard of 5-methylbenzothiophene-3(2*H*)-one which had the same retention time and mass (Figure 5.6a) and FTIR spectra as

metabolite G, and gave a TMS-ether with the same retention time and mass spectrum as derivatized metabolite G. 5-Methylbenzothiophene-2(3*H*)-one was also synthesized and analyses of this compound showed that its retention time, mass spectrum, and FTIR spectrum were different from 5-methylbenzothiophene-3(2*H*)-one, proving that metabolite G was 5-methylbenzothiophene-3(2*H*)-one.

Metabolite H was easily recognized during GC-MS analysis by its weak molecular ion at m/z 178 (8%) and abundant fragment ions at m/z 150 $(M-28)^+$ (100%) and m/z 121 $(M-57)^+$ (70%) to be 5-methylbenzothiophene-2,3-dione (Saftić *et al.*, 1992). Comparison with the authentic standard showed that the metabolite had the same mass and FTIR spectra and retention time as 5-methylbenzothiophene-2,3-dione.

Figure 5.6b shows the mass spectrum of metabolite I with a weak molecular ion at m/z 180, consistent with the chemical formula $C_9H_8O_2S$ determined by high resolution GC-MS. The fragmentation pattern of metabolite I was very similar to that of metabolite F from 3,4-dimethyldibenzothiophene (Figure 5.5) with fragments at m/z 164 $(M-16)^+$, m/z 150 $(M-30)^+$, m/z 134 $(M-46)^+$, m/z 121 $(M-59)^+$, m/z 106 $(M-74)^+$, and m/z 89 $(M-91)^+$, all of which were the fragment losses observed for metabolite F. GC-FTIR analysis showed a strong absorption at 1733 cm^{-1} for metabolite I suggesting that one of the oxygen atoms was present as a carbonyl group. By keto-enol tautomerism, metabolite I reacted with two equivalents of BSA to give a di-TMS ether with a molecular ion at m/z 324 (46%) and abundant ions in the mass spectrum at m/z 309 (76%), m/z 147 (100%), and m/z 73 (64%). Thus, metabolite I, a product analogous to metabolite F from 3,4-dimethyldibenzothiophene, appears to be the keto tautomer of 2,3-dihydroxy-5-methylbenzothiophene which is 3-hydroxy-5-methylbenzothiophene-2(3*H*)-one. Although this structural assignment is consistent with the available data, it was not confirmed by comparison with an authentic standard.

GC-AED analyses for sulfur compounds in extracts of 3-week-old BT1 cultures showed that 9.2% of the added 2,8-dimethyldibenzothiophene remained in the culture and that 2.9% of the substrate sulfur was present in the three metabolites detected. The corresponding sterile control gave 90% recovery of the 2,8-dimethyldibenzothiophene.

5.3.6 Mixed culture studies with 2,8-dimethyldibenzothiophene

The mixed cultures SLPB, 5W/5B, ESSO AG, and ERN BIO were incubated with Prudhoe Bay aromatics and 2,8-dimethyldibenzothiophene. GC-FPD analyses of extracts of 3-week-old SLPB and 5W/5B cultures showed that they degraded some of the 2,8-dimethyldibenzothiophene and a single sulfur-containing product was detected in both cultures. It had the same retention time and mass spectrum as 5-methylbenzothiophene-2,3-

dione (metabolite H). Although no metabolites were detected in extracts of the ESSO AG and ERN BIO cultures incubated with 2,8-dimethyldibenzothiophene for 2 weeks, GC-AED analyses showed that 54% of the 2,8-dimethyldibenzothiophene was recovered from each of these cultures while the heat-killed controls gave recoveries of 94% and 92%, respectively.

5.4 DISCUSSION

The metabolites observed from the oxidation of the three isomers of dimethyldibenzothiophene are summarized in Figure 5.7. The susceptibility of the isomers of dimethyldibenzothiophene to bacterial degradation was dependent upon the positions of the methyl groups. The 3,4- isomer was oxidized by all three aromatic hydrocarbon-degrading *Pseudomonas* spp., whereas the 4,6- isomer was not oxidized by any of the three strains and the 2,8- isomer was only oxidized by isolate BT1. Furthermore, both mixed cultures tested (SLPB and ERN BIO) extensively degraded 3,4-dimethyldibenzothiophene producing numerous metabolites, and the culture of ERN BIO completely removed the 3,4-dimethyldibenzothiophene from the medium within a 13-day incubation period. The amounts of 4,6-dimethyldibenzothiophene remaining after 14 days incubation with this ERN BIO culture, and with SLPB and ESSO AG were much higher at 79%, 65%, and 82%, respectively. Fifty-four percent of the 2,8-dimethyldibenzothiophene was recovered from each of the cultures of ERN BIO and ESSO AG incubated with this isomer for 2 weeks. Thus, lines of evidence from both the pure and mixed culture studies suggest that the order of susceptibility of these three isomers to degradation is 3,4-dimethyldibenzothiophene > 2,8-dimethyldibenzothiophene > 4,6-dimethyldibenzo-thiophene.

Of the two dimethyldibenzothiophenes studied which were substituted on both benzene rings, 2,8-dimethyldibenzothiophene is more susceptible to degradation. This is consistent with observations from studies with methyldibenzothiophenes in petroleum that 2- and 3-methyldibenzothiophene were more easily degraded than 1- and 4-methyldibenzothiophene (Bayona *et al.*, 1986; Fayad and Overton, 1995; Wang and Fingas, 1995). 2,8-Dimethyldibenzothiophene bears the methyl groups on both benzene rings at the same position *beta* to the thiophene ring as in 2-methyldibenzothiophene. 4,6-Dimethyldibenzothiophene bears the methyl groups on both benzene rings at the same position *alpha* to the thiophene ring as in 4-methyldibenzothiophene. The relative recalcitrance of the symmetrical dimethyldibenzothiophenes reported herein reflects that previously reported for the methyldibenzothiophenes which have the methyl-substitution at the same location of only one of the homocyclic rings.

The ease with which 3,4-dimethyldibenzothiophene was degraded is because it has an unsubstituted benzene ring which is preferentially attacked by aromatic hydrocarbon-degrading bacteria. This was observed for the bacterial metabolism of aromatic hydrocarbons, including 1- and 2-methylnaphthalene (Dean-Raymond and Bartha, 1975; Williams *et al.*, 1975), 1- and 2-ethylnaphthalene (Dean-Raymond and Bartha, 1975), 1,3- and 2,3-dimethylnaphthalene (Dean-Raymond and Bartha, 1975), and 3- and 4-methylbiphenyl (Fedorak and Westlake, 1983b).

The C_1-dibenzothiophenes are more susceptible to biodegradation than the C_2-dibenzothiophenes (Atlas *et al.*, 1981; Fedorak and Westlake, 1983a, 1984). Among the dimethyldibenzothiophenes found in crude oil are some isomers that bear a methyl group on each of the homocyclic rings (Budzinski *et al.*, 1992). Thus, in environments contaminated with crude oil, dibenzothiophene and methyldibenzothiophenes would be depleted from the crude oil before the isomers of dimethyldibenzothiophene that are substituted on one of the homocyclic rings, which will in turn be degraded before the isomers that contain methyl groups on both benzene rings.

The 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene detected in pure and mixed cultures incubated with 3,4-dimethyldibenzothiophene resulted from oxidation, cleavage, and degradation of the unsubstituted benzene ring in a manner analogous to that reported for the degradation of dibenzothiophene which yields 3-hydroxy-2-formylbenzothiophene as a major product (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989). This is consistent with the observation that the unsubstituted homocyclic ring of the methyldibenzothiophenes was attacked and degraded to give methyl-3-hydroxy-2-formylbenzothiophenes (Saftić *et al.*, 1993).

Extracts of cultures incubated with 3,4-dimethyldibenzothiophene also contained 6,7-dimethylbenzothiophene-2,3-dione. Methylbenzothiophene-2,3-diones were detected in the studies with methyldibenzothiophenes (Saftić *et al.*, 1993), and benzothiophene-2,3-dione was reported as a product from dibenzothiophene oxidation in cultures that also yielded 3-hydroxy-2-formylbenzothiophene (Bohonos *et al.*, 1977). In the present study, 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene was more frequently detected as a product and was more abundant in extracts of cultures that had been incubated for shorter times, whereas the 2,3-dione was more frequently detected and was present in greater amounts over longer incubations. The 2,3-dione appears to result from further degradation of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and is the most extensively degraded metabolite of 3,4-dimethyldibenzothiophene that was detected.

Eaton and Nitterauer (1994) showed that benzothiophene was microbially oxidized via dioxygenase attack at positions 2 and 3 and that subsequent reactions, including

thiophene ring cleavage, led to the formation of 2-mercaptophenylglyoxalate which cyclized to give benzothiophene-2,3-dione by acid-catalyzed dehydration. In the present study, cultures were routinely acidified prior to extraction and so the 6,7-dimethylbenzothiophene-2,3-dione detected in extracts of cultures incubated with 3,4-dimethyldibenzothiophene was likely present in the culture at neutral pH as the dimethyl-substituted 2-mercaptophenylglyoxalate. Thus, identification of this dione in acidified culture extracts suggests that degradation of 3,4-dimethyldibenzothiophene includes cleavage of the thiophene ring.

It is likely that metabolites A, B and F are transiently accumulating intermediates in the further degradation of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene to the corresponding 2,3-dione. Metabolites A and B are the keto-tautomers of 2-hydroxy- and 3-hydroxy-6,7-dimethylbenzothiophene, respectively. They are analogous to 2-hydroxy- and 3-hydroxybenzothiophene found as metabolites of benzothiophene (Eaton and Nitterauer, 1994). It was proposed that these resulted from chemical dehydration of 2,3-dihydroxy-2,3-dihydrobenzothiophene that was produced by microbial dioxygenase-catalyzed oxidation of benzothiophene (Eaton and Nitterauer, 1994). Enzymatic dehydrogenation of this dihydrodiol was proposed to give the keto tautomer of 2,3-dihydroxybenzothiophene, namely 2-hydroxybenzothiophene-3(2*H*)-one (Eaton and Nitterauer, 1994). Metabolite F, 3-hydroxy-6,7-dimethylbenzothiophene-2(3*H*)-one, is the keto tautomer of 2,3-dihydroxy-6,7-dimethylbenzothiophene, and is analogous to the other tautomer of 2,3-dihydroxy-benzothiophene (Eaton and Nitterauer, 1994). Clearly the structures of metabolites A, B, and F are comparable to compounds found from benzothiophene, suggesting that the pathway for biodegradation of 3,4-dimethyldibenzothiophene converges with the pathway previously reported for bacterial metabolism of benzothiophene.

The formation of metabolites A, B, and F by further degradation of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene requires removal of the formyl group by an as yet uncharacterized mechanism. A possible mechanism was that proposed to explain the formation of 1-indanone from the degradation of fluorene via 2-formyl-1-indanone by *Pseudomonas cepacia* F297 (Grifoll *et al.*, 1995). This mechanism may also explain the formation of 7-acenaphthenone as a metabolite from fluoranthene degradation (Weissenfels *et al.*, 1991). Alternatively, since metabolites A, B, and F were detected in extracts of a mixed culture, they may not truly represent a single biochemical pathway for the biodegradation of 3,4-dimethyldibenzothiophene as is often proposed from the results of pure culture-pure compound studies.

The three *Pseudomonas* spp. were unable to oxidize 4,6-dimethyldibenzothiophene and the three mixed cultures tested only degraded a small portion of the 4,6-dimethyl-

dibenzothiophene (between 7% and 24% after accounting for losses in the heat-killed controls) over a 2-week incubation period, with no metabolites detected. However, cultures grew in the presence of this compound, as indicated by increased culture turbidity and depleted the 1-MN or Prudhoe Bay aromatics, including the condensed thiophenes naturally present, which had been provided as the growth substrates. Furthermore, the isolate BT1 metabolized dibenzothiophene and 4-methyldibenzothiophene producing 3-hydroxy-2-formylbenzothiophene and 7-methyl-3-hydroxy-2-formylbenzothiophene in the presence of 4,6-dimethyldibenzothiophene. Thus, the recalcitrance of 4,6-dimethyldibenzothiophene is not due to toxicity.

Bacteria that extract the sulfur atom from dibenzothiophenes have been shown to attack 4,6-diethyldibenzothiophene (Lee *et al.*, 1995) and 4,6-dimethyldibenzothiophene (Ohshiro *et al.*, 1996) yielding diethyl- and dimethyl-substituted monohydroxybiphenyls. The initial reaction rate for desulfurization of 4,6-dimethyldibenzothiophene by *Rhodococcus erythropolis* H-2 was 60% of that for dibenzothiophene (Ohshiro *et al.*, 1996). Biodesulfurization of 2,8-dimethyldibenzothiophene has also been reported (Ohshiro *et al.*, 1996).

In this study, 2,8-dimethyldibenzothiophene was oxidized by one of the *Pseudomonas* spp. and all of the mixed cultures. Although the expected 5-methyl-3-hydroxy-2-formylbenzothiophene was not detected from oxidation of 2,8-dimethyldibenzothiophene, the metabolites that were detected were analogous to some of those observed from 3,4-dimethyldibenzothiophene. The 5-methylbenzothiophene-3(2*H*)-one (metabolite G) is analogous to metabolite B from 3,4-dimethyldibenzothiophene and could have formed from the 5-methyl-3-hydroxy-2-formylbenzothiophene by the decarboxylation mechanism cited above (Grifoll *et al.*, 1995). The analogue of metabolite A, 5-methylbenzothiophene-2(3*H*)-one, was not produced from 2,8-dimethyldibenzothiophene. However, metabolite I, the keto tautomer of 2,3-dihydroxy-5-methylbenzothiophene, which is analogous to metabolite F, and metabolite H, which is 5-methylbenzothiophene-2,3-dione and whose presence gives indication that the thiophene ring of 2,8-dimethyldibenzothiophene was cleaved, were detected.

Methyl group oxidations by bacteria yielding methanols and carboxylic acids from 2,6-dimethylnaphthalene (Barnsley, 1988; Dean-Raymond and Bartha, 1975; Miyachi *et al.*, 1993), 1,5-dimethylnaphthalene (Dean-Raymond and Bartha, 1975), and methyldibenzothiophenes (Saftic´ *et al.*, 1993) have been reported. The oxidation of the methyl groups of methyl- and dimethylbenzothiophenes by the three *Pseudomonas* spp. used in this study was observed (Kropp *et al.*, 1994a, 1996). In the current study, oxidation of the methyl groups of dimethyldibenzothiophenes to give methanol- or carboxy-substituted

dibenzothiophenes was not observed. However, if oxidation of both methyl groups of a dimethyldibenzothiophene were to give dicarboxylic acids, these would likely be too polar to extract and analyze by the methods used in this study.

The metabolites detected and identified during this study were those sulfur-containing metabolites that could be partitioned from an acidified culture into DCM and that were amenable to GC analysis. GC-AED analysis was done to quantify the amounts of sulfur-containing substrate and products present in extracts of some of the cultures that were capable of oxidation of the individual dimethyldibenzothiophenes. In all cases tested, a significantly larger portion of the added substrate had been depleted than was present in the sulfur-containing metabolites detected. For example, in extracts of 7-day-old cultures of ERN BIO incubated with 3,4-dimethyldibenzothiophene, 34% was recovered unoxidized and 6% was present in the metabolites A through F. Thus, the detected metabolites are likely transiently accumulating intermediates that are further degraded to products too polar to extract or analyze by GC. It is also possible that some of the substrate was oxidized to highly polar intermediates in the formation of the detected metabolites. The metabolites that were detected from dimethyldibenzothiophene oxidation and those that remain to be determined are of concern because they may form in environments contaminated with condensed thiophene-containing crude oil. If so, the polarity of these metabolites will mean that they are likely more mobile than the parent compounds in groundwater and surface water, and the toxicity and susceptibility of these metabolites to further degradation will influence the success of bioremediation as a cleanup technology. In an ideal situation, the sulfur-containing metabolites detected would be intermediates in the complete mineralization of the condensed thiophenes with the missing substrate sulfur existing as a relatively innocuous product such as sulfate. Unfortunately, the extent of the quantitative studies that could be done with the dimethyldibenzothiophenes was limited by the small supply of these synthesized compounds.

Dibenzothiophene has been used in quantitative studies of the metabolism of condensed thiophenes. Wang and Krawiec (1996) reported kinetic data of the metabolism of dibenzothiophene by *R. erythropolis*. However, this isolate utilized the desulfurization pathway to enable dibenzothiophene to serve as a sulfur source, but did not oxidize the hydrocarbon backbone (Wang and Krawiec, 1996). This pathway is not likely to be prevalent in oil-contaminated environments where available sulfur is not expected to be a growth-limiting nutrient. van Afferden *et al.* (1990) observed stoichiometric release of sulfate from dibenzothiophene by a *Brevibacterium* sp. that oxidized dibenzothiophene via the sulfoxide and sulfone, released sulfite, and then subsequently degraded the desulfurized hydrocarbon. Kodama *et al.* (1970, 1973) identified dibenzothiophene metabolites from

pure cultures of *Pseudomonas jianii* and proposed the so-called "Kodama pathway". The yields of three of the purified metabolites were reported (Kodama *et al.*, 1970, 1973) and by my calculations account for 31% of the dibenzothiophene added to the culture. However, the amount of dibenzothiophene remaining at the end of the incubation period was not reported so it is not known if a mass balance existed.

As far as I am aware, quantitative studies which establish a mass balance for bacteria which utilize the Kodama pathway have not been reported. Such investigations are the focus of ongoing studies in this laboratory and will form the basis of a separate report. The results of these studies will provide insight into the significance of the metabolites identified from oxidation of dibenzothiophene and its alkyl-derivatives and the implications that the formation of these metabolites might have for bioremediation.

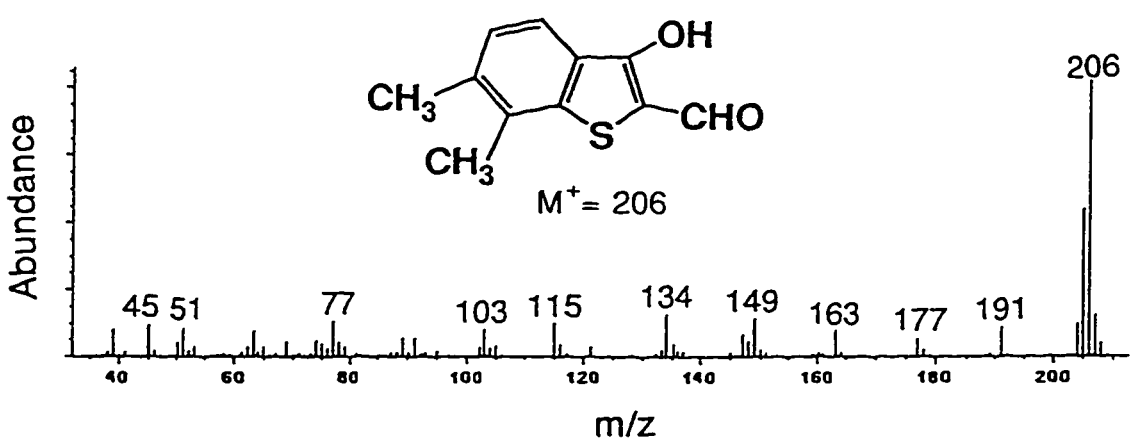


Figure 5.1 From GC-MS analysis, the mass spectrum of a sulfur-containing metabolite from a culture of isolate BT1 grown for 3 days on 1-MN in the presence of 3,4-dimethyldibenzothiophene. This metabolite was identified as 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene.

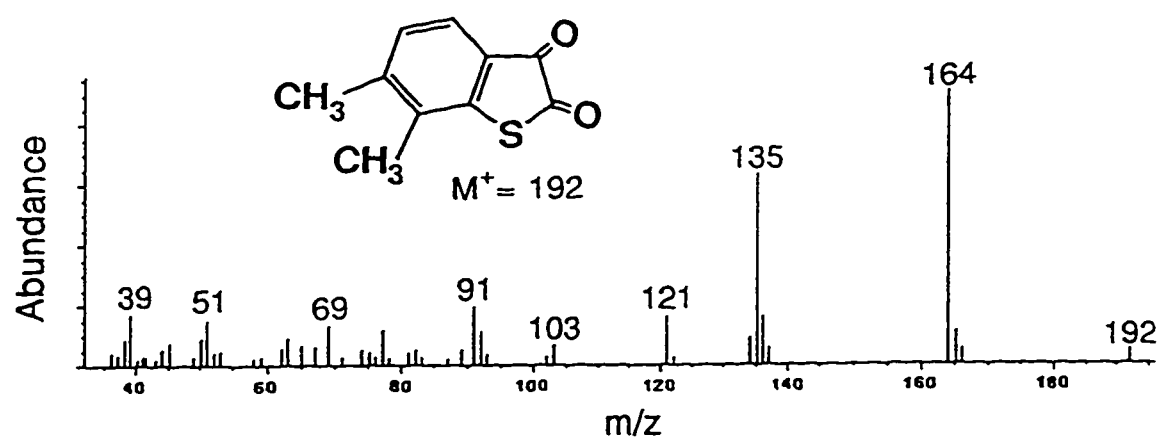


Figure 5.2 From GC-MS analysis, the mass spectrum of a sulfur-containing metabolite from a culture of isolate BT1 grown for 3 days on 1-MN in the presence of 3,4-dimethyldibenzothiophene. This metabolite was identified as 6,7-dimethylbenzothiophene-2,3-dione.

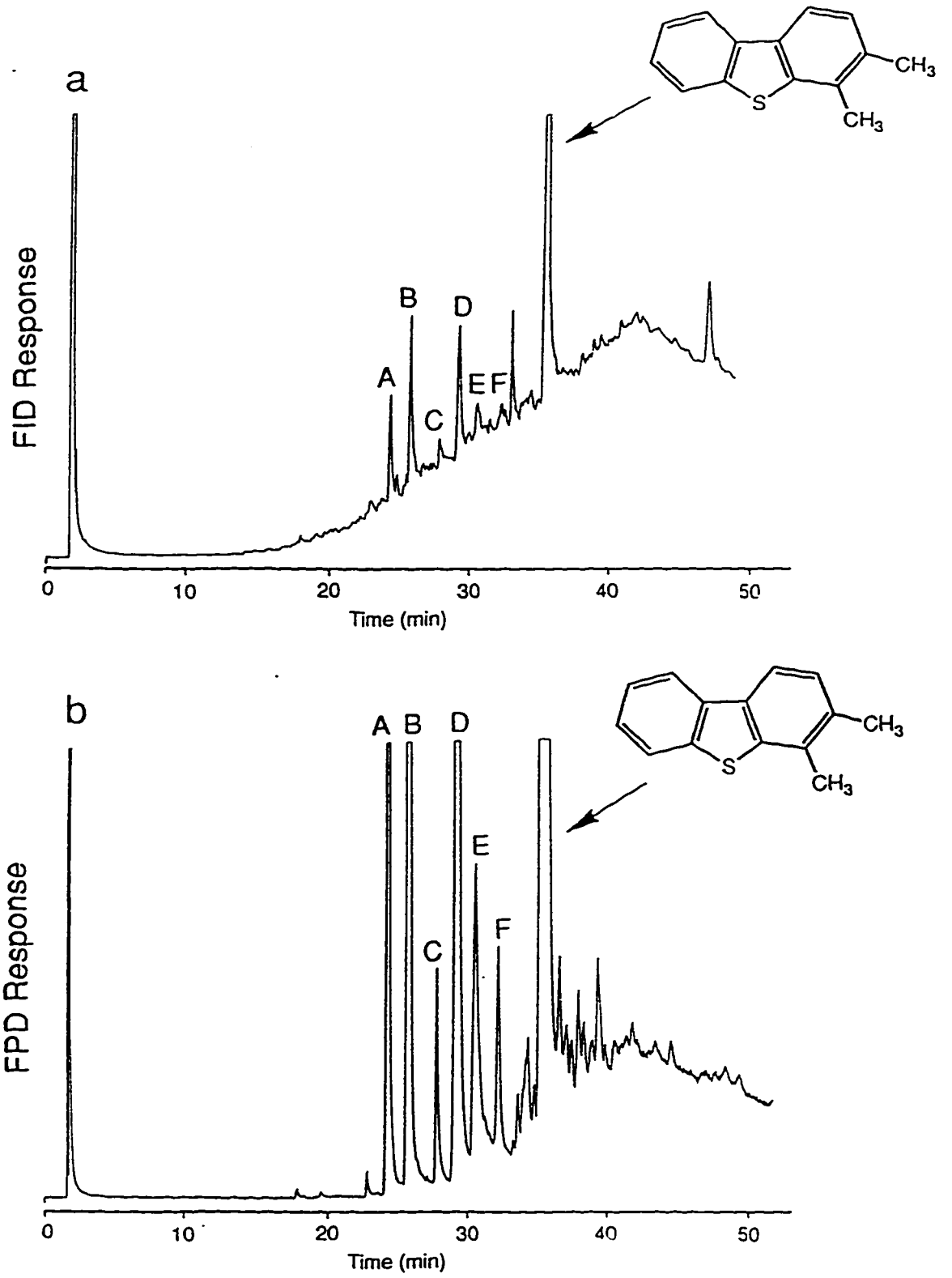


Figure 5.3 GC-FID (a) and GC-FPD (b) chromatograms from GC analysis of the extract of a culture of ERN BIO that was incubated for 7 days with the aromatic fraction of Prudhoe Bay crude oil and 3,4-dimethyldibenzothiophene.

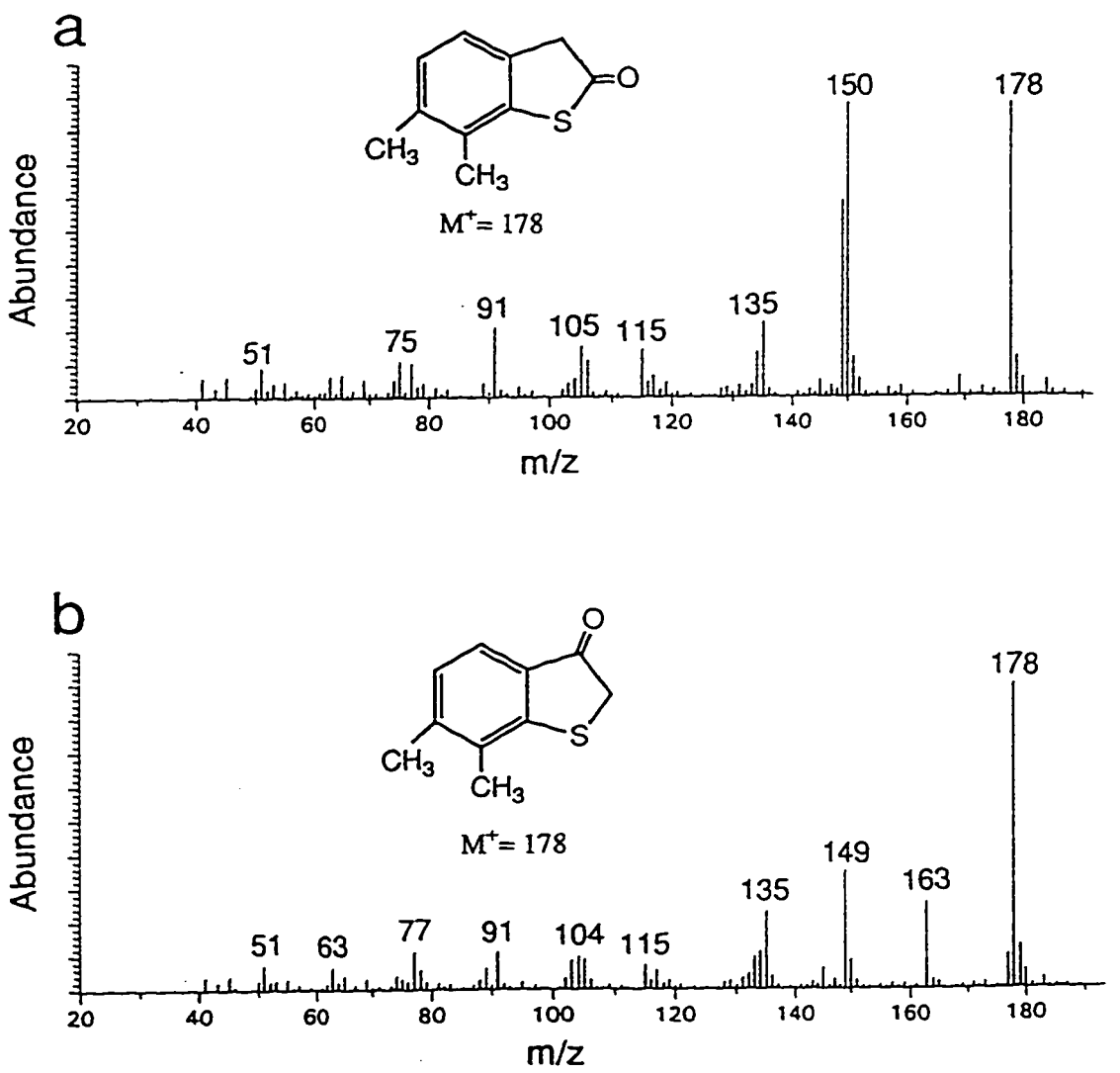


Figure 5.4 From GC-MS analysis, the mass spectra of metabolite A (a) and metabolite B (b) detected in an extract of a culture of ERN BIO that was incubated for 7 days with the aromatic fraction of Prudhoe Bay crude oil and 3,4-dimethyldibenzothiophene. Metabolites A and B were identified as 6,7-dimethylbenzothiophene-2(3*H*)-one and 6,7-dimethylbenzothiophene-3(2*H*)-one, respectively.

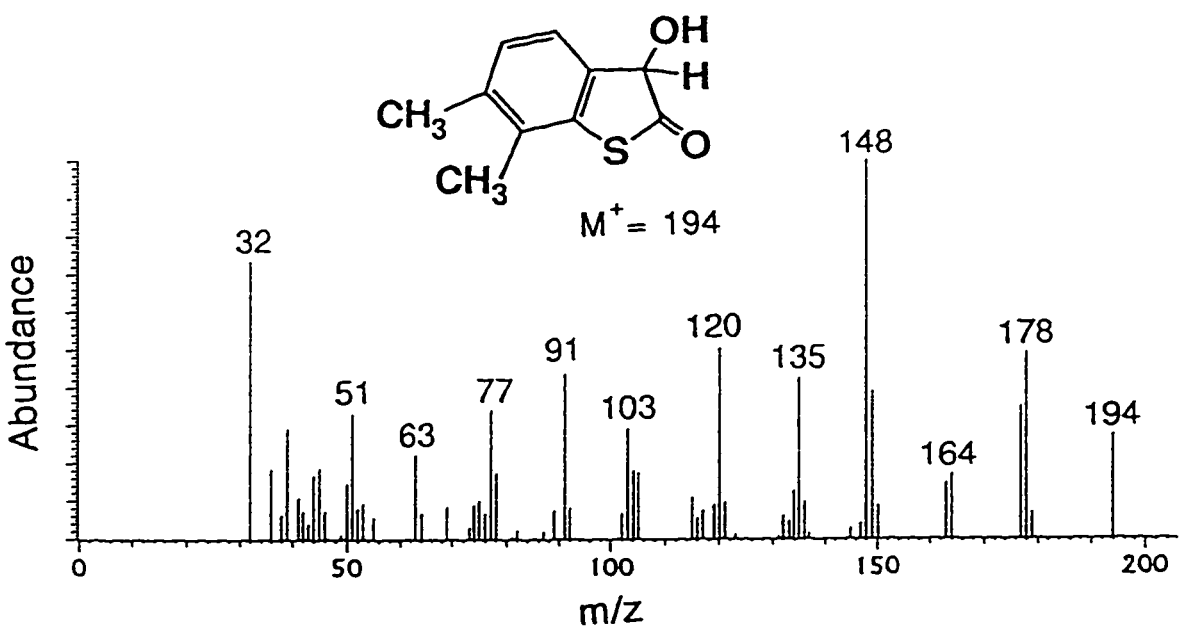


Figure 5.5 From GC-MS analysis, the mass spectrum of metabolite F detected in an extract of a culture of ERN BIO that was incubated for 7 days with the aromatic fraction of Prudhoe Bay crude oil and 3,4-dimethyldibenzothiophene. This metabolite was identified as 3-hydroxy-6,7-dimethylbenzothiophene-2(3*H*)-one.

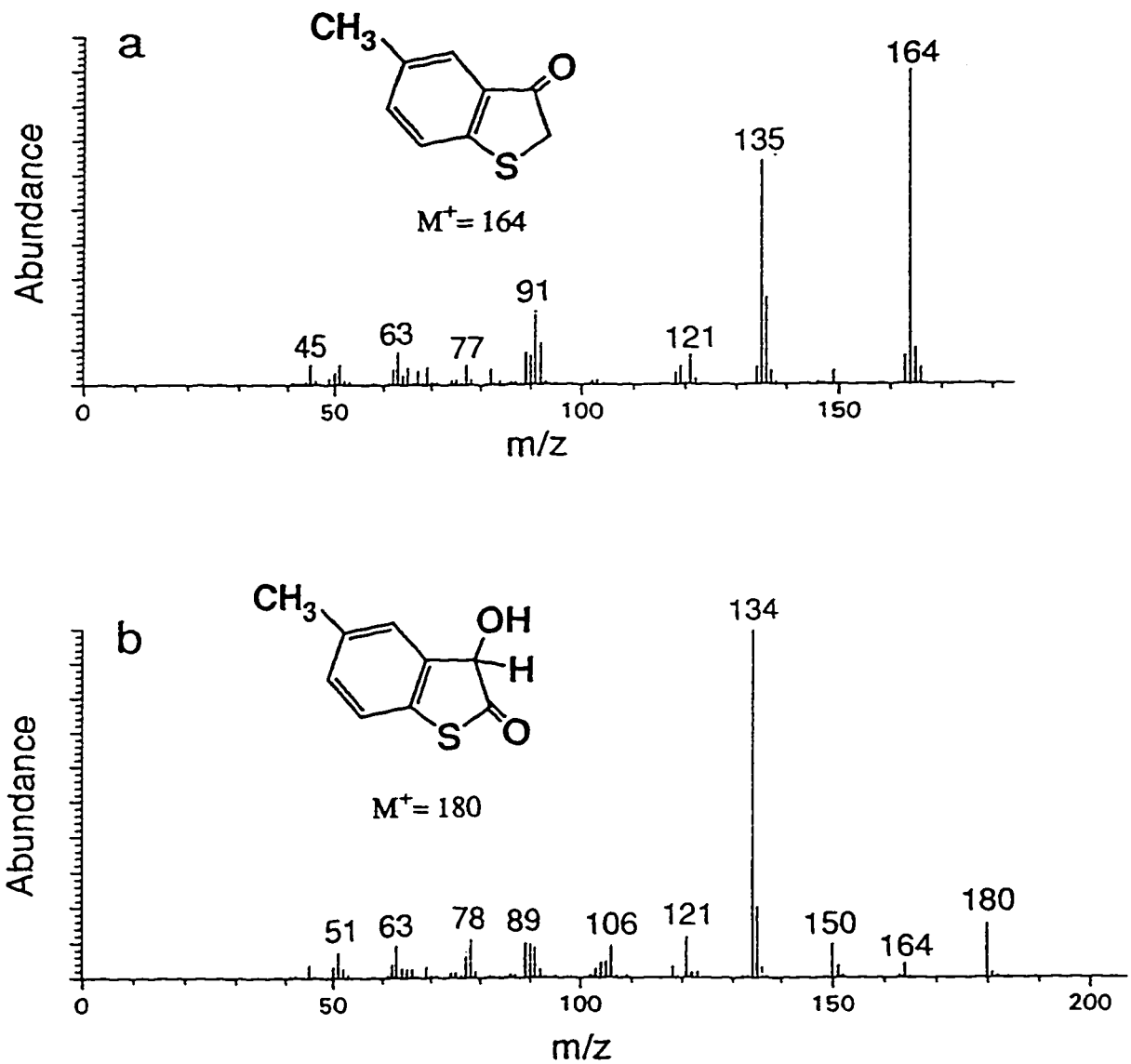


Figure 5.6 From GC-MS analysis, the mass spectra of metabolite G (a) and metabolite I (b) from a culture of isolate BT1 grown for 7 days on 1-MN in the presence of 2,8-dimethyldibenzothiophene. Metabolite G was identified as 5-methyl-benzothiophene-3(2*H*)-one and metabolite I was identified as 3-hydroxy-5-methylbenzothiophene-2(3*H*)-one.

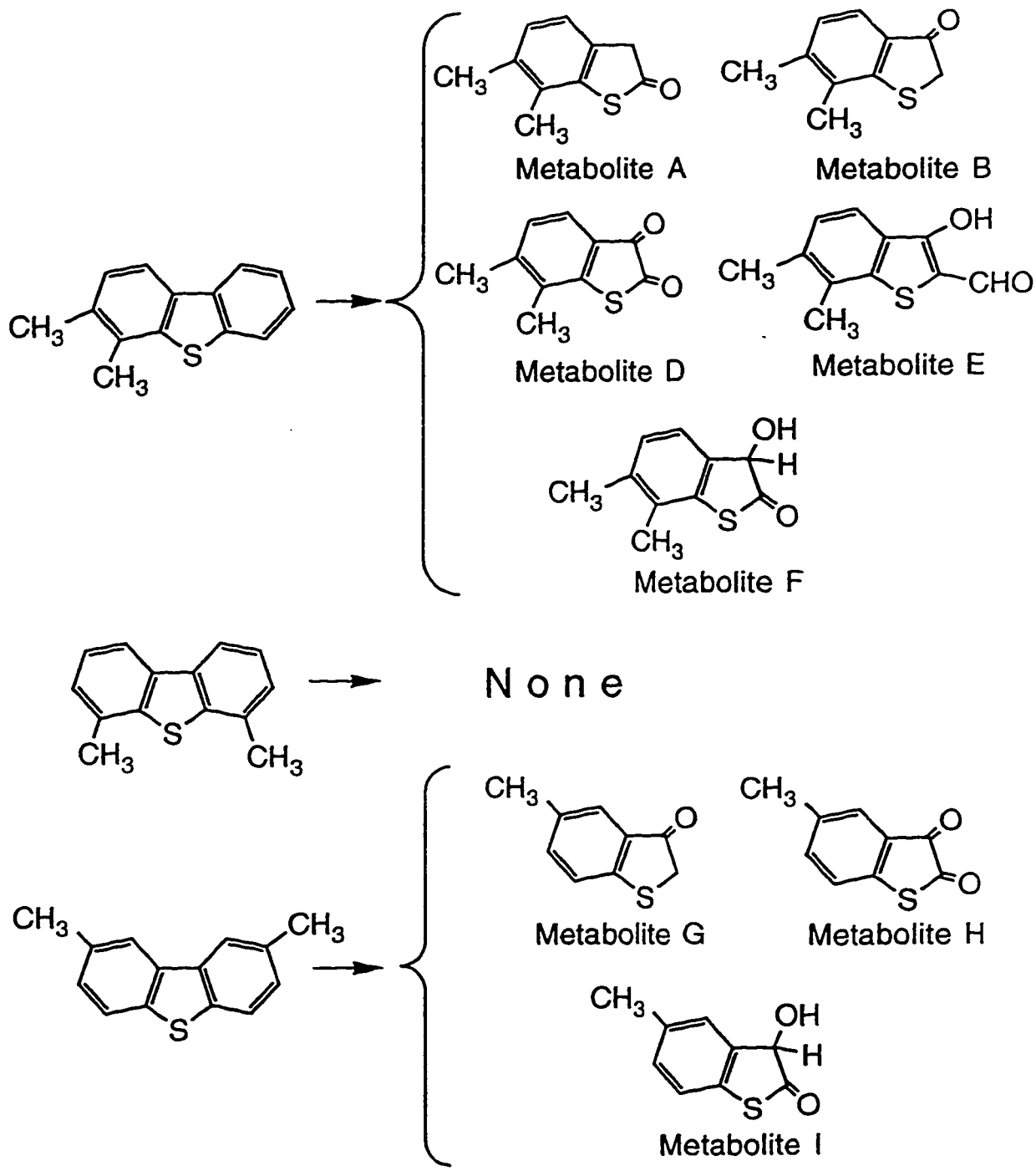


Figure 5.7 Summary of the metabolites detected from the oxidation of dimethyldibenzothiophenes by pure and mixed bacterial cultures.

5.5 LITERATURE CITED

Andersson, J. T. 1986. Gas chromatographic retention indices for all C_1- and C_2-alkylated benzothiophenes and their dioxides on three different stationary phases. J. Chromatogr. 354: 83-98.

Andersson, J. T., and B. Schmid. 1993. The atomic emission detector in gas chromatographic trace analysis. Fresenius' J. Anal. Chem. 346: 403-409.

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.

Barnsley, E. A. 1988. Metabolism of 2,6-dimethylnaphthalene by Flavobacteria. Appl. Environ. Microbiol. 54: 428-433.

Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald. 1986. Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures. Intern. J. Environ. Anal. Chem. 23: 289-303.

Berthou, F., Y. Gourmelun, Y. Dreano, and M. P. Friocourt. 1981. Application of gas chromatography on glass capillary columns to the analysis of hydrocarbon pollutants from the *Amoco Cadiz* oil spill. J. Chromatogr. 203: 279-292.

Boehm, P. D., J. E. Barak, D. L. Fiest, and A. Elskus. 1982. A chemical investigation of the transport and fate of petroleum hydrocarbons in littoral and benthic environments: The *Tsesis* oil spill. Mar. Environ. Res. 6: 157-188.

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. In: Amoco Cadiz: Fates and effects of the oil spill. Proceedings of the International Symposium. Centre Oceanologique de Bretagne. Brest. Le Centre National pour l'Exploration des Oceans: Paris; p.159-173.

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.

Budzinski, H., P. Garrigues, and J. Bellocq. 1992. Gas chromatographic retention behavior of dibenzothiophene derivatives on a smectic liquid crystalline polysiloxane stationary phase. J. Chromatogr. 590: 297-303.

Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. Current Microbiol. 21: 229-231.

Dean-Raymond, D., and R. Bartha. 1975. Biodegradation of some polynuclear aromatic petroleum components by marine bacteria. Dev. Ind. Microbiol. 16: 97-110.

Dickinson, R. P., and B. Iddon. 1970. Condensed thiophen ring systems. Part III. A new synthesis of benzo[*b*]thiophen-2(3*H*)- and -3(2*H*)-ones and some reactions of benzo[*b*]thiophen-2(3*H*)-one with dimethylsulfate in the presence of base. J. Chem. Soc. (C). p. 1926-1928.

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

Fayad, N. M., and E. Overton. 1995. A unique biodegradation pattern of the oil spilled during the 1991 Gulf war. Mar. Pollut. Bull. 30: 239-246.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., and T. M. Peakman. 1992. Aerobic microbial metabolism of some alkyl thiophenes found in petroleum. Biodegradation. 2: 223-236.

Fedorak, P. M., and D. W. S. Westlake. 1981. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. Can. J. Microbiol. 27: 432-443.

Fedorak, P. M., and D. W. S. Westlake. 1983a. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. 29: 291-296.

Fedorak, P. M., and D. W. S. Westlake. 1983b. Selective degradation of biphenyl and methylbiphenyls in crude oil by two strains of marine bacteria. Can. J. Microbiol. 29: 497-503.

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.

Fedorak, P. M., and D. W. S. Westlake. 1986. Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. 51: 435-437.

Friedländer, P. 1906. Ueber schwefelhaltige Analoga der Indigogruppe. Chem. Ber. 39: 1060-1066.

Friocourt, M. P., F. Berthou, and D. Picart. 1982. Dibenzothiophene derivatives as organic markers of oil pollution. Toxicol. Environ. Chem. 5: 205-215.

Gallagher, J. R., E. S. Olson, and D. C. Stanley. 1993. Microbial desulfurization of dibenzothiophene: A sulfur specific pathway. FEMS Microbiol. Lett. 107: 31-36.

Gerdil, R., and E. A. C. Lucken. 1965. The electron spin resonance spectra of the dibenzothiophene radical anion and its isologs and the electronic structure of conjugated sulfur-containing heterocycles. J. Am. Chem. Soc. 87: 213-217.

Grifoll, M., S. A. Selifonov, C. V. Gatlin, and P. J. Chapman. 1995. Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Appl. Environ. Microbiol. 61: 3711-3723.

Hannoun, M., N. Blazevic´, D. Kolbah, M. Mihalic´, and F. Kajfez. 1982. *alpha*-Phenylpropionic acid derivatives. Synthesis and dethiation of 5-benzoylbenzo[*b*]thiophene-3-carboxylic acid. J. Heterocyclic Chem. 19: 1131-1136.

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.

Izumi, Y., T. Oshiro, H. Ogino, Y. Hine, and M. Shimao. 1994. Selective desulfurization of dibenzothiophene by *Rhodococcus erythropolis* D-1. Appl. Environ. Microbiol. 60: 223-226.

Jacob, J. Sulfur Analogues of Polycyclic Aromatic Hydrocarbons (Thiaarenes). 1990. Cambridge University Press: Cambridge; p. 70-87.

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.

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.

Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994a. Bacterial transformations of benzothiophene and methylbenzothiophenes. Environ. Sci. Technol. 28: 1348-1356.

Kropp, K. G., J. A. Gonçalves, J. T. Andersson, and P. M. Fedorak. 1994b. Microbially mediated formation of benzonaphthothiophenes from benzo[*b*]thiophenes. Appl. Environ. Microbiol. 60: 3624-3631.

Kropp, K. G., S. Saftic´, J. T. Andersson, and P. M. Fedorak. 1996. Transformations of six isomers of dimethylbenzothiophenes by three *Pseudomonas* strains. Biodegradation 7: 203-221.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinkia* species. Appl. Environ. Microbiol. 34: 783-790.

Laseter, J. L., G. C. Lawler, E. B. Overton, J. R. Patel, J. P. Holmes, M. I. Shields, and M. Maberry. 1981. Characterization of aliphatic and aromatic hydrocarbons in flat and Japanese type oysters and adjacent sediments collected from L'Aver Wrac'h following the Amoco Cadiz oil spill. In: Amoco Cadiz: Fates and effects of the oil spill. Proceedings of the International Symposium, Centre Oceanologique de Bretagne. Brest. Le Centre National pour l'Exploration des Oceans: Paris; p. 633-644.

Lee, M. K., J. D. Senius, and M. J. Grossman. 1995. Sulfur-specific microbial desulfurization of sterically hindered analogs of dibenzothiophene. Appl. Environ. Microbiol. 61: 4362-4366.

Miyachi, N., T. Tanaka, T. Suzuki, Y. Hotta, and T. Omori. 1993. Microbial oxidation of dimethylnaphthalene isomers. Appl. Environ. Microbiol. 59: 1504-1506.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Mormile, M. R., and R. M. Atlas. 1989. Biotransformation of dibenzothiophene to dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. 35: 603-605.

Ogata, M., and K. Fujisawa. 1985. Organic sulfur compounds and polycyclic hydrocarbons transferred to oyster and mussel from petroleum suspension. Identification by gas chromatography and capillary mass chromatography. Water Res. 19: 107-118.

Ohshiro, T., T. Hirata, and Y. Izumi. 1996. Desulfurization of dibenzothiophene derivatives by whole cells of *Rhodococcus erythropolis* H-2. FEMS Microbiol. Lett. 142: 65-70.

Omori, T., L. Monna, Y. Saiki, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1. Appl. Environ. Microbiol. 58: 911-915.

Saftic´, 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.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Silverstein, R. M., G. C. Bassler, and T. C. Morrill. 1991. Spectrometric Identification of Organic Compounds. 5th Ed. John Wiley & Sons, Inc.: New York.

Speight, J. G. 1980. The Chemistry and Technology of Petroleum. Marcel Dekker Inc.: New York.

Stridsberg, R., and S. Allenmark. 1974. Studies of the benzo[*b*]thiophene-3(2*H*)-one system. Chemica Scripta 6: 184-192.

Teal, J. M., K. Burns, and J. Farrington. 1978. Analyses of aromatic hydrocarbons in intertidal sediments resulting from two spills of no. 2 fuel oil in Buzzards Bay, Massachusetts. J. Fish. Res. Board Can. 35: 510-520.

Tedjamulia, M. L., Y. Tominaga, and R. N. Castle. 1983. The synthesis of all of the dimethyldibenzothiophenes and monoethyldibenzothiophenes. J. Heterocycl. Chem. 30: 1485-1495.

van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. Arch. Microbiol. 153: 324-328.

Wang, Z., and M. Fingas. 1995. Use of methyldibenzothiophenes as markers for differentiation and source identification of crude and weathered oils. Environ. Sci. Technol. 29: 2842-2849.

Wang, P., and S. Krawiec. 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in batch and fed-batch cultures. Appl. Environ. Microbiol. 62: 1670-1675.

Weissenfels, W. D., M. Beyer, J. Klein, and H. J. Rehm. 1991. Microbial metabolism of fluoranthrene: isolation and identification of ring fission products. Appl Microbiol. Biotechnol. 34: 528-535.

Westlake, D. W. S. 1983. Microbial activities and changes in the chemical and physical properties of oil. In: Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Donaldson E. C. and J. B. Clark, Eds. Technology Transfer Branch, Bartlesville Energy Technology Centre: Bartlesville, Oklahoma; p. 102-111.

Williams, J. A., M. Bjorøy, D. L. Dolcater, and J. C. Winters. 1986. Biodegradation of South Texas Eocene oils-effects on aromatics and biomarkers. Org. Geochem. 10: 451-461.

Williams, P. A., F. A. Catterall, and K. Murray. 1975. Metabolism of naphthalene, 2-methylnaphthalene, salicylate, and benzoate by *Pseudomonas* PG: Regulation of tangential pathways. J. Bacteriol. 124: 679-685.

6. QUALITATIVE AND QUANTITATIVE STUDIES OF BACTERIAL TRANSFORMATIONS OF 1,2,3,4-TETRAHYDRODIBENZOTHIOPHENE AND DIBENZOTHIOPHENE*

6.1 INTRODUCTION

Organosulfur compounds are a small but significant component of petroleum and coal-derived liquids. Conventional crude oils contain between 0.04 and 5% (w/w) sulfur (Speight, 1980) and in general, crudes of higher density (lower API gravity) contain a higher percent sulfur. The organosulfur compounds present include thiols, sulfides, and thiophenes, but the sulfur compounds which predominate in the so-called heavy fractions, where sulfur content is the highest, are primarily the condensed thiophenes. Of these, the dibenzothiophenes were recognized to be among the compounds that were most resistant to biodegradation in sediments contaminated with oil from the *Amoco Cadiz* spill (Boehm *et al.*, 1981). The recalcitrance of the dibenzothiophenes, relative to the other aromatic compounds found in crude oil that are amenable to GC analysis, has also been observed in other studies (Berthou *et al.*, 1981; Boehm *et al.*, 1982; Teal *et al.*, 1978) and contributes to the potential of condensed thiophenes to accumulate in the tissues of shellfish in marine environments that become contaminated with crude oil (Laseter *et al.*, 1981; Ogata and Fujisawa, 1985). This has led to the suggestion that the dibenzothiophenes might serve as oil pollution markers (Friocourt *et al.*, 1982; Ogata and Fujisawa, 1985).

There have been numerous biodegradation studies using dibenzothiophene as a model condensed thiophene (Crawford and Gupta, 1990; Hou and Laskin, 1976; Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989). Since the initial studies of Kodama *et al.* (1970, 1973) with *Pseudomonas* strains, there have been several reports of bacterial oxidation and cleavage of one of the benzene rings of dibenzothiophene to yield 3-hydroxy-2-formylbenzothiophene by the so-called "Kodama pathway" (Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989). Many of these isolates also oxidize the sulfur atom giving dibenzothiophene sulfoxide and sulfone (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Mormile and Atlas, 1989), which appears to be a dead-end pathway in these aromatic-degrading bacteria.

Other isolates are reported to use dibenzothiophene as a sulfur source by oxidizing it via the sulfoxide and sulfone and then subsequently releasing the sulfur atom as sulfate

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leaving 2-hydroxybiphenyl (Gallagher *et al.*, 1993; Izumi *et al.*, 1994; Omori *et al.*, 1992). This pathway is of potential use in the development of a microbial process for the biodesulfurization of petroleum because it does not result in extensive degradation of the hydrocarbon. Kinetic data of the metabolism of dibenzothiophene by *Rhodococcus erythropolis*, which uses this pathway, have been reported (Wang and Krawiec, 1996). van Afferden *et al.* (1990) observed stoichiometric release of sulfate from dibenzothiophene by a *Brevibacterium* sp. that oxidized dibenzothiophene via the sulfoxide and sulfone, released sulfite, and then subsequently degraded the desulfurized hydrocarbon. The only metabolite detected in the degradation of the carbon backbone was benzoic acid. Although quantitative data have been reported for isolates which oxidize dibenzothiophene to the sulfoxide and sulfone, and then further degrade dibenzothiophene sulfone by one of the two pathways mentioned above, I am not aware of any quantitative studies which establish a mass balance for bacterial degradation of dibenzothiophene by the Kodama pathway.

As part of a series of investigations into the metabolism of condensed thiophenes (Fedorak and Grbic´-Galic´, 1991; Kropp *et al.*, 1994a, b, 1996, 1997; Saftic´ *et al.*, 1992, 1993), I now report on studies of the biotransformation of 1,2,3,4-tetrahydrodibenzothiophene. This compound is formed during hydroprocessing reactions aimed at the hydrodesulfurization of dibenzothiophenes found in petroleum (Nagai *et al.*, 1986; Rankel, 1991), and has been detected as a minor constituent of solvent refined coal liquids (Later *et al.*, 1981; Nishioka, 1988). Tetrahydrodibenzothiophene consists of a benzene ring and a cycloparaffin ring fused to opposite sides of a thiophene ring, and as such there are various potential mechanisms that could be utilized for its oxidation by microorganisms.

This report describes the biotransformations of tetrahydrodibenzothiophene by three *Pseudomonas* strains while growing on 1-MN and by cell suspensions of a cyclohexane-degrading bacterium. The use of a GC-AED, which gives an equimolar response to sulfur in all organic forms that are amenable to GC analysis (Andersson and Schmid, 1993), allowed quantitative analyses of the metabolites. However, the limited amount of synthesized tetrahydrodibenzothiophene did not allow additional investigations. Subsequently, quantitative studies were done with dibenzothiophene in an attempt to establish a sulfur mass balance for dibenzothiophene oxidation by four *Pseudomonas* strains, which utilize the Kodama pathway of dibenzothiophene metabolism. These quantitative studies with dibenzothiophene were motivated by the difficulty experienced in achieving a sulfur mass balance in studies with aromatic-hydrocarbon-degrading bacteria and tetrahydrodibenzothiophene (this report) or dimethyldibenzothiophenes (Kropp *et al.*, 1997).

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

1,2,3,4-Tetrahydrodibenzothiophene (≥99% pure by GC) was synthesized by the method of Wilputte and Martin (1956). Dibenzothiophene (98%) and 1-MN (97%) were purchased from Fluka (Buchs, Switzerland). Cyclohexane (pesticide grade) was purchased from Fisher Scientific (Fair Lawn, NJ). Dibenzothiophene sulfone (97%) was purchased from Aldrich (Milwaukee, WI). Tetrahydrodibenzothiophene sulfone was synthesized by refluxing tetrahydrodibenzothiophene with H_2O_2 (30%) in acetic acid for 15 min.

6.2.2 Bacterial cultures and culture methods

Biotransformation experiments were done in 500-mL Erlenmeyer flasks containing 200 mL of mineral medium supplemented with a trace metals solution (Kropp *et al.*, 1994b) and these were incubated at 28°C on a rotary shaker. Following inoculation, each flask received 2 to 4 μL of liquid tetrahydrodibenzothiophene (1 μL = 1.0 mg) or 4 mg of dibenzothiophene dissolved in 100 μL of acetonitrile. 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.

The isolation and characterization of the three 1-MN-degrading *Pseudomonas* strains BT1, W1, and F were described previously (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1993). The 10-mL inoculum used for biotransformation experiments with each of these isolates was obtained from 1-MN-grown maintenance cultures that were transferred weekly. Following inoculation, the biotransformation cultures received 50 μL of 1-MN as growth substrate together with the cosubstrate tetrahydrodibenzothiophene or dibenzothiophene. Because isolate BT1 can grow on dibenzothiophene as its sole carbon and energy source (Saftic´ *et al.*, 1993), some experiments tested this isolate with dibenzothiophene, omitting 1-MN from the medium. The inoculum used for these experiments (10 mL) also came from the 1-MN-grown BT1 maintenance culture. As well, a fourth strain, designated BT1d, was tested in some experiments with dibenzothiophene. This strain originated from isolate BT1 that was maintained for over 2 years by weekly transfers into mineral medium with 4 mg dibenzothiophene as sole carbon and energy source. Although the colonial morphologies of isolates BT1 and BT1d were identical, their metabolism of dibenzothiophene was found to be different, as discussed later. In all of the studies with dibenzothiophene, the maintenance cultures used as inocula were 7 days old and had reached the stationary phase of growth.

The cyclohexane-degrader CB1323, a gift from Celgene Inc. (Warren, NJ), was capable of growth on plates of mineral medium containing 1.5% Noble agar (Difco, Detroit, MI) incubated with cyclohexane vapors in a sealed 3-L container. However, better growth was obtained on plates of solidified Luria Broth (LB; per litre of distilled water: 15 g Difco agar; 10 g Bacto-Tryptone; 5 g Bacto-yeast extract; 10 g NaCl; adjust pH to 7.5) which were also incubated in the presence of cyclohexane vapors. The inoculum of strain CB1323 for biotransformation experiments was obtained by aseptically washing the growth from a 3-day-old culture from an LB agar plate with 9 mL of sterile phosphate buffer (pH 7.2, 10 mM) into each flask for the biotransformation experiment. Five replicate cell suspensions were incubated at the same time. Tetrahydrodibenzothiophene was the only carbon source added to these cell suspensions and after 2 days of incubation they were pooled prior to extraction.

6.2.3 Solvent extraction

Cultures, cell suspensions, and sterile controls were acidified with 2 M H_2SO_4 to pH<2 and extracted with DCM (4 times 20 mL) to recover substrates and products. The DCM extract was dried over anhydrous Na_2SO_4 and concentrated on a rotary evaporator. For quantitative experiments by GC-AED analysis, known amounts of one or two of the following commercially available organosulfur compounds (Aldrich, Milwaukee, WI) were added as internal standards prior to extraction: thianthrene (99%); phenyl sulfoxide (97%); and phenyl sulfide (98%). The standards used were chosen so that they would not co-elute during GC analysis with tetrahydrodibenzothiophene, dibenzothiophene, or the products detected in preliminary screenings.

6.2.4 GC analyses

To screen for the presence of sulfur-containing metabolites, the extracts were analyzed by capillary GC using a 30-m DB-5 column in a HP (Mississauga, ON) model 5890 equipped with a FID and a sulfur-selective FPD. Details of the operating conditions were given previously (Fedorak and Grbic´-Galic´, 1991). The methods routinely used for GC-MS and GC-FTIR have also been described previously (Saftic´ *et al.*, 1992, 1993). To obtain high resolution GC-MS data, analyses were done in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta (Fedorak and Westlake, 1986). The operation of the GC-AED for quantitative analyses was outlined by Kropp *et al.* (1997).

To facilitate GC-MS identification of some of the metabolites, TMS derivatives of compounds in culture extracts were made by silylating with BSA in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, IL; method 5).

6.2.5 Total sulfur analysis

The DCM extracts of cultures of the *Pseudomonas* strains incubated with dibenzothiophene were diluted to 1.0 mL with DCM in volumetric flasks and 200-µL portions of these were applied to filter paper (Whatman #42). After evaporation of the DCM, the filter paper samples were combusted in sealed Schöninger oxygen flasks and the SO_2 produced was trapped as H_2SO_4 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 $BaClO_4$ (0.01077 M) using Thorin as the endpoint indicator (Fritz and Yamamura, 1955).

6.2.6 Assessment of sulfate release from dibenzothiophene

Cultures of the three 1-MN-degrading *Pseudomonas* strains were maintained for three sequential 10-mL transfers in 500-mL Erlenmeyer flasks containing 200-mL sulfate-free medium (Gonçalves and Fedorak, 1996). The transfers were done every 5 days after the cultures had grown and at each transfer the three isolates received 50 µL 1-MN and 4 mg dibenzothiophene. In addition, isolate BT1d was grown on 4 mg dibenzothiophene without 1-MN. At the time of the third transfer, the cultures were transferred into flasks of medium that contained 50 mg of dibenzothiophene. After 6 days of incubation, the cultures were acidified with 2 mL of 4 M HCl and centrifuged at 16 300 × g for 15 min to remove cells and undissolved dibenzothiophene. The supernatant was then passed through an ENVI Chrom P solid phase extraction tube (Supelco, Bellefonte, PA) that had been pre-conditioned with 10 mL methanol and 10 mL acidified sulfate-free medium. The solid phase extraction procedure effectively removed the colored dibenzothiophene oxidation products from the supernatant. The color of these metabolites would have interferred with the turbidometric method used to assay for sulfate (American Public Health Association, 1989). The solid phase extraction procedure did not retain sulfate when a standard solution of Na_2SO_4 (15 mg/L) was passed through the procedure.

6.2.7 Lyophilization and methyl-esterification

Cultures of the *Pseudomonas* strains incubated with dibenzothiophene were made alkaline (pH>12) by the addition of 0.5 g 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 H_2SO_4 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.

6.3 RESULTS

6.3.1 Transformations of tetrahydrodibenzothiophene by *Pseudomonas* strains

GC-FPD analysis of an extract of an acidified culture of isolate F grown in the presence of tetrahydrodibenzothiophene for 3 days revealed the presence of three abundant sulfur-containing metabolites. GC-MS analysis (Figure 6.1) showed that metabolite A, with the shortest retention time, had a molecular ion at m/z 182 and a base peak at m/z 154, that could result from the loss of CO or C_2H_4 $(M-28)^+$, both of which would be fragmentations consistent with the structure shown (Figure 6.1). Loss of CO $(M-28)^+$ is characteristic of phenols and loss of C_2H_4 $(M-28)^+$ is characteristic of cyclic alkanes (Silverstein *et al.*, 1991). The molecular weight of metabolite A is 4 mass units greater than that of 3-hydroxy-2-formylbenzothiophene, which has been previously reported as a product of dibenzothiophene biotransformation (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989), and is consistent with the metabolite being 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene, as shown in Figure 6.1. The presence of the hydroxy functional group was verified by preparing the TMS-ether of the metabolite which gave a weak molecular ion at m/z 254 (3%) during GC-MS analysis. The derivative characteristically lost a methyl group $(M-15)^+$ from the molecular ion (Pierce, 1968) to give the base peak at m/z 239.

GC-FTIR analysis of the underivatized metabolite A gave the FTIR spectrum shown in Figure 6.2a. The aromatic C–H stretching absorption in the FTIR spectrum of tetrahydrodibenzothiophene at 3069 cm^{-1} (Figure 6.2b) is missing from the FTIR spectrum of metabolite A (Figure 6.2a) whereas the saturated C–H stretching absorptions observed at 2863 and 2945 cm^{-1} in the parent compound (Figure 6.2b) are still present in the FTIR spectrum of the metabolite (Figure 6.2a). This suggests that the aromatic ring of tetrahydrodibenzothiophene has been degraded while the saturated ring has been left unaltered to give metabolite A. However, the saturated C–H stretching absorption at 2863 cm^{-1} in the metabolite also overlaps with the aldehydic C–H stretching absorption observed in Figure 6.2a as a doublet at 2844 cm^{-1} and approximately 2770 cm^{-1} (Silverstein *et al.*, 1991). The other strong absorption of metabolite A (Figure 6.2a) at 1638 cm^{-1} is due to the carbonyl group and is similar to the strong absorption previously reported for 3-hydroxy-2-formylbenzothiophene at 1640 cm^{-1} (Saftić *et al.*, 1993). Thus, this evidence further

suggests that metabolite A from tetrahydrodibenzothiophene is 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene.

The second and third sulfur-containing metabolites detected by GC-FPD analysis of the isolate F culture extract nearly co-eluted with one another, but were sufficiently resolved for GC-MS and GC-FTIR analyses. The mass spectrum of the more abundant of these two metabolites (metabolite B) showed a molecular ion at m/z 204 (Figure 6.3a), which is 16 mass units greater than that of tetrahydrodibenzothiophene, suggesting the incorporation of a single atom of oxygen into the substrate. However, no TMS-ether of this metabolite was formed when the extract was treated with BSA, ruling out the possibility that it is a hydroxy-substituted isomer of tetrahydrodibenzothiophene. Thus, the metabolite was thought to be tetrahydrodibenzothiophene sulfoxide. This conclusion was verified by GC-FTIR analysis which showed that metabolite B absorbed strongly at 1081 cm^{-1}, an absorption characteristic of sulfoxides (Silverstein *et al.*, 1991).

The later of these two metabolites to elute (metabolite C) gave the mass spectrum shown in Figure 6.3b. This spectrum and the GC retention time of the metabolite were the same as an authentic standard of tetrahydrodibenzothiophene sulfone. GC-FTIR analysis of metabolite C showed that it gave strong absorptions at 1331 and 1167 cm^{-1}, which are characteristic of sulfones (Silverstein *et al.*, 1991). Thus, metabolite C is tetrahydrodibenzothiophene sulfone.

Isolate W1 produced the same three abundant metabolites from tetrahydrodibenzothiophene as the isolate F culture, and the extracts of both of these isolates showed similar amounts of metabolites and an abundant peak in the chromatograms corresponding to unoxidized tetrahydrodibenzothiophene. However, GC-FPD analyses of the extracts of 3-day-old cultures of isolate BT1 incubated with tetrahydrodibenzothiophene showed only trace amounts of residual tetrahydrodibenzothiophene and of a number of novel sulfur-containing metabolites that were not abundant enough to identify. 3-Hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene (metabolite A), which was also present in trace amounts, was detected in 3-day-old BT1 culture extracts. In extracts of BT1 cultures that had been incubated for 1 or 2 days, 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene was present in greater abundance, but only trace amounts of the novel sulfur-containing metabolites were ever observed, even with the shorter incubation times, and so they could not be identified.

The sulfur-selective GC-AED analyses of extracts of cultures that had been incubated for 3 days and extracted after the addition of internal standards showed that 92% of the 16.5 μmol of tetrahydrodibenzothiophene added was recovered from the sterile control. After 3 days of incubation, recovery of residual tetrahydrodibenzothiophene was

7.4% (1.2 μmol) for isolate F; 13% (2.1 μmol) for isolate W1; and 0.7% (0.1 μmol) for isolate BT1. In the isolate F culture extract, 3.3% (0.5 μmol) of the tetrahydrodibenzothiophene added was detected as 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene and 21% (3.5 μmol) as the sulfoxide and sulfone, which were quantified together because they were not adequately resolved during GC-AED analyses. Similarly, in the extract of the isolate W1 culture, 4.8% (0.8 μmol) of the tetrahydrodibenzothiophene added was present as 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene and 16% (2.6 μmol) was present as the sulfoxide and sulfone. In a 3-day-old BT1 culture extract, 0.2% (.03 μmol) of the tetrahydrodibenzothiophene added was detected as 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene, and the sulfoxide and sulfone were not detected.

Assuming that the evaporative loss from the viable cultures was the same as from the sterile control, 1.3 μmol of tetrahydrodibenzothiophene would have been lost abiotically. This value likely overestimates the loss due to evaporation in the viable cultures because the amount of tetrahydrodibenzothiophene present decreased over the incubation period due to microbial oxidation, and the extent of evaporation likely also decreased. Totaling the estimated loss due to evaporation, the remaining tetrahydrodibenzothiophene, and the identified metabolites, the analytical method used accounted for 6.5 μmol (39%) of the sulfur from the initial amount of tetrahydrodibenzothiophene added to the cultures of strain F; 6.8 μmol (41%) of the organosulfur added to cultures of strain W1; and 1.4 μmol (9%) of the organosulfur added to cultures of strain BT1. Clearly, other metabolites were produced but not detected by these analytical methods.

6.3.2 Transformations of tetrahydrodibenzothiophene by strain CB1323

Cell suspensions of strain CB1323 that had been grown in the presence of cyclohexane vapors were shown to oxidize tetrahydrodibenzothiophene, producing three sulfur-containing metabolites (referred to as D, E, and F below) over a 2-day incubation.

GC-MS analysis gave the mass spectrum shown in Figure 6.4a for metabolite D. The strong molecular ion at m/z 202 is consistent with the chemical formula of $C_{12}H_{10}OS$ that was determined by high resolution GC-MS analysis. The base peak at m/z 160 $(M-42)^+$ results from loss of CH_2CO. GC-FTIR analysis gave the spectrum shown in Figure 6.4b for metabolite D. The presence of C–H stretching between 2850 and 2979 cm^{-1}, and at 3072 cm^{-1} indicates that both saturate and aromatic C–H bonds were present in the molecule although the relative intensity of the saturate C–H stretching was decreased relative to unoxidized tetrahydrodibenzothiophene (Figure 6.2b). The strong absorption at 1740 cm^{-1} (Figure 6.4b) is due to the presence of the oxygen atom in a carbonyl group. Because the chemical formula for metabolite D of $C_{12}H_{10}OS$ is one oxygen atom greater

and two hydrogen atoms less than that of tetrahydrodibenzothiophene, it is likely that one of the methylene carbon atoms in the saturated ring was oxidized to a ketone. This is indicated by the structure shown for metabolite D in Figure 6.4, although it is not known which of the methylene groups was oxidized to give the cyclic ketone. However, the loss of CH_2CO to give the fragment at m/z 160 $(M-42)^+$ in the mass spectrum of metabolite D (Figure 6.4a) has also been observed in the mass spectrum of 3,4-dihydro-2(1*H*)-naphthalenone, but not in the mass spectrum of 3,4-dihydro-1(2*H*)-naphthalenone (Heller and Milne, 1978). This may indicate that the carbonyl group of metabolite D is not located alpha to the thiophene ring of tetrahydrodibenzothiophene (i.e. positions 1 or 4), but rather is located at a position beta to the thiophene ring (i.e. positions 2 or 3). This assignment is further supported by the carbonyl absorption of metabolite D at 1740 cm^{-1} which is consistent with the carbonyl absorption of saturated ketones (1725 to 1745 cm^{-1} in vapor phase), but not consistent with the carbonyl absorption of conjugated ketones (1690 to 1720 cm^{-1} in vapor phase) (Lin-Vien *et al.*, 1991).

Metabolite E gave the mass spectrum shown in Figure 6.5a with a strong molecular ion at m/z 204, consistent with the chemical formula $C_{12}H_{12}OS$ determined by high resolution GC-MS analysis. This product resulted from the incorporation of a single atom of oxygen into tetrahydrodibenzothiophene ($C_{12}H_{12}S$) and GC-FTIR analysis proved that it was present as a hydroxyl group since the metabolite absorbs strongly at 3653 cm^{-1} (Figure 6.5b). The FTIR spectrum (Figure 6.5b) also suggests that the hydroxyl group is located on the saturated ring because of the relatively strong intensity of the aromatic C–H stretching at 3070 cm^{-1}. Other evidence for this assignment comes from the fragmentation pattern of metabolite E observed in Figure 6.5a. The fragment at m/z 186 results from the loss of H_2O $(M-18)^+$and this fragmentation is also observed in the mass spectrum of cyclohexanol (Silverstein *et al.*, 1991) and 1,2,3,4-tetrahydro-1-naphthalenol (Heller and Milne, 1978). The base peak at m/z 160 $(M-44)^+$ results from loss of CH_2CHOH. Thus, metabolite E appears to be a hydroxy-substituted tetrahydrodibenzothiophene which bears the hydroxy group on the saturated ring, although the exact position is not known. However, metabolite E likely bears the hydroxy substituent at the same position beta to the thiophene ring (position 2 or 3) as was suggested above for the further oxidized metabolite D.

Metabolite F also appears to be a hydroxy-substituted tetrahydrodibenzothiophene. It has the same molecular weight of 204 (Figure 6.6a) and the same chemical formula of $C_{12}H_{12}OS$. As well, GC-FTIR analysis shows a strong O–H stretching absorption at 3653 cm^{-1} (Figure 6.6b). However, the data suggest that the hydroxy group is located on the benzene ring of tetrahydrodibenzothiophene, not on the saturated ring as proposed for

metabolite E. The base peak at m/z 176 in the mass spectrum of metabolite F (Figure 6.6a) results from the loss of CO $(M-28)^+$. This fragmentation is commonly observed in the mass spectra of phenols (Silverstein *et al.*, 1991) and has been observed in the mass spectra of 5,6,7,8-tetrahydro-1-naphthalenol and 5,6,7,8-tetrahydro-2-naphthalenol (Heller and Milne, 1978). Furthermore, the aromatic C–H stretching at 3034 cm^{-1} in the FTIR spectrum of metabolite F (Figure 6.6b) is weaker and broader than in metabolite E (Figure 6.5b). Thus, metabolite F appears to be a hydroxy-substituted tetrahydrodibenzothiophene which bears the hydroxy group on the benzene ring, although the exact position is unknown.

When the extract of the cell suspension of strain CB1323 was treated with BSA, the two hydroxy-substituted products (metabolites E and F) both reacted to give the respective TMS-ethers with molecular ions at m/z 276 observed in the mass spectra from GC-MS analysis of the derivatized extract. The earlier derivative to elute was presumed to be that of metabolite E since the abundant ions at m/z 186 (68%) and m/z 160 (100%) were also observed in the mass spectrum of the underivatized metabolite E (Figure 6.5a). The mass spectrum of the TMS-ether of metabolite F gave the parent peak at m/z 276 (100%), with fragments at m/z 261 $(M-15)^+$ (17%), m/z 248 $(M-28)^+$ (40%), and m/z 233 $(M-43)^+$ (10%).

GC-AED analysis was used to quantify the amounts of metabolites D, E, and F produced by five replicate cell suspensions of strain CB1323 that each contained 16.5 µmol tetrahydrodibenzothiophene. These were incubated for 2 days and pooled prior to extraction. Results showed that 44.6 µmol (54%) of the initial 82.5 µmol tetrahydrodibenzothiophene added remained in the extract and 8 µmol (9.6%), 3.2 µmol (3.9%), and 4.6 µmol (5.6%) of the added tetrahydrodibenzothiophene was present as metabolites D, E, and F, respectively. The recovery of tetrahydrodibenzothiophene from a single sterile control that received 16.5 µmol tetrahydrodibenzothiophene was 73% (12 µmol). Assuming the evaporative loss from the individual cell suspensions was the same as from the sterile control (27%), the analytical method accounted for 82.7 µmol (100%) of sulfur added as tetrahydrodibenzothiophene.

6.3.3 Transformations of dibenzothiophene by *Pseudomonas* strains

The metabolites from dibenzothiophene oxidation that were detected and identified by GC analyses of extracts of acidified cultures of *Pseudomonas* strains W1, F, BT1, and BT1d were the same as those identified in previous studies, so their identification is not described here. The metabolites detected were 3-hydroxy-2-formylbenzothiophene (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile

and Atlas, 1989; Saftic´ *et al.*, 1993), benzothiophene-2,3-dione (Bohonos *et al.*, 1977), and dibenzothiophene sulfoxide and sulfone (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Mormile and Atlas, 1989). Strains W1, F, and BT1 have also been reported to oxidize methyldibenzothiophenes to give methyl-substituted 3-hydroxy-2-formylbenzothiophenes and benzothiophene-2,3-diones (Saftic´ *et al.*, 1993). As well, some of the methyldibenzothiophenes were oxidized to the corresponding sulfones (Saftic´ *et al.*, 1993)

GC-AED analyses of extracts of cultures of each of the *Pseudomonas* strains incubated with dibenzothiophene for 2 to 9 days gave quantitative data on the kinetics of dibenzothiophene oxidation to the observed metabolites. Figure 6.7a shows the oxidation of dibenzothiophene by a culture of isolate W1 growing on 1-MN in the presence of dibenzothiophene. This isolate and isolate F, which gave results similar to isolate W1 so they are not shown, cannot use dibenzothiophene as a growth substrate, but cometabolize it while growing on 1-MN. The recoveries of dibenzothiophene and the various metabolites after 7 days incubation are summarized in Table 6.1 for both isolates. Neither isolate W1 nor isolate F completely degraded the dibenzothiophene, with 9.6% (2.0 μmol) and 12% (2.5 μmol) remaining after 7 days, respectively. As well, while 3-hydroxy-2-formylbenzothiophene concentrations were appreciable after 2 days incubation (Figure 6.7a), this accumulation was transient resulting in only 0.6% (0.13 μmol) and 0.7% (0.15 μmol) of the sulfur from dibenzothiophene persisting as this product in cultures of isolates W1 and F, respectively, after 7 days. Thus, the metabolite 3-hydroxy-2-formylbenzothiophene is further degraded by these isolates. Only small amounts of the benzothiophene-2,3-dione were ever detected over the 7-day incubation (Figure 6.7a, Table 6.1). The major products of these isolates were the sulfoxide and sulfone of dibenzothiophene which together accounted for 17% (3.6 μmol) and 24% (5.0 μmol) of the sulfur from dibenzothiophene in cultures of isolate W1 and F, respectively (Table 6.1). The sulfoxide and sulfone were quantitated together because they co-eluted from the GC column.

Isolate BT1 is capable of growth with either 1-MN or dibenzothiophene as sole carbon and energy source, so the transformation of dibenzothiophene by this strain was tested both with and without 1-MN in the medium. The results of these tests after 7 days are summarized in Table 6.1, and Figure 6.7b shows the oxidation of dibenzothiophene as sole carbon source over 9 days. A notable difference between isolates W1 and F and isolate BT1, was that dibenzothiophene degradation proceeded to a greater extent in the cultures of isolate BT1, regardless of whether or not 1-MN was included in the medium. For example, only 0.8% (0.18 μmol) of the dibenzothiophene remained when 1-MN was included (Table 6.1, line 3), and 1.3% (0.29 μmol) remained when 1-MN was omitted (Table 6.1, line 4).

As was observed with isolates W1 and F, the accumulation of 3-hydroxy-2-formyl-benzothiophene in BT1 cultures was transient (Figure 6.7b), with further degradation yielding only trace amounts of this product after 7 days incubation regardless of whether or not 1-MN was included in the culture (Table 6.1, lines 3 and 4). As well, only trace amounts of benzothiophene-2,3-dione were ever detected (Figure 6.7b, Table 6.1).

When isolate BT1, pregrown on 1-MN, was incubated with dibenzothiophene as sole carbon source, oxidation of the sulfur atom gave the sulfoxide and sulfone as the major products (9.4%, 2.1 μmol) (Table 6.1, line 4), as was observed with isolates W1 and F. However, when 1-MN and dibenzothiophene were in the medium, only 1.6% (0.35 μmol) of the sulfur originally present as dibenzothiophene accumulated as the sulfoxide and sulfone (Table 6.1, line 3). Thus, with 1-MN in the BT1 cultures, either less dibenzothiophene was sulfoxygenated, or further degradation of the sulfoxygenated metabolites was stimulated, so that less of these products accumulated over the 7-day incubation.

The ability of strains W1, F, and BT1 to further transform dibenzothiophene sulfone (17 μmol) was tested. Isolates W1 and F were unable to transform this sulfone over a 7-day incubation period when grown with 1-MN and the sulfone in the medium. The same was true for isolate BT1 incubated with dibenzothiophene sulfone as sole carbon and energy source. However, when isolate BT1 was grown on 1-MN in the presence of dibenzothiophene sulfone, 55% (9.4 μmol) of the sulfone was depleted from the medium in 7 days. However, metabolites from oxidation of dibenzothiophene sulfone were not detected by the extraction and analytical methods used. The ability of isolate BT1 to further degrade dibenzothiophene sulfone, when provided with 1-MN as growth substrate, likely contributes to the large portion of organosulfur from dibenzothiophene (85.2%, Table 6.1, line 3) that was not detected by the DCM extraction and GC analytical methods used. The increase in the amount of sulfoxide and sulfone detected in cultures of isolate BT1 without 1-MN over that detected in cultures which included 1-MN (7.8%, 1.7 μmol) accounted for the smaller portion of organosulfur from dibenzothiophene that was undetected in cultures where 1-MN was excluded (74.8%, Table 6.1, line 4).

Large fractions of organosulfur from dibenzothiophene were not detected by DCM extraction and GC analysis of cultures of isolates W1, F, and BT1 (between 50.0% and 85.2%, Table 6.1). With 1-MN-grown cultures of isolate BT1, this was partly due to the further degradation of dibenzothiophene sulfone. Evidence that a large fraction of this undetected sulfur from dibenzothiophene resulted from the further degradation of 3-hydroxy-2-formylbenzothiophene by isolates W1, F, and BT1 was obtained using strain BT1d. The DCM extraction and GC analysis methods used accounted for 81.6% of the

sulfur from dibenzothiophene provided as sole carbon source to cultures of strain BT1d after 7 days incubation (Table 6.1, line 5). This 81.6% recovery is determined by totaling the recovery of unoxidized dibenzothiophene (5.9%) with the recovery of the detected metabolites (61.7%) and the evaporative losses of dibenzothiophene observed in the sterile control (14%). A large portion of this (52%, 11 μmol) was present in culture extracts as 3-hydroxy-2-formylbenzothiophene which accumulated in cultures (Figure 6.7c). While some further degradation of 3-hydroxy-2-formylbenzothiophene did occur to yield the trace amount of benzothiophene-2,3-dione that was detected (Figure 6.7c, Table 6.1), clearly much greater amounts of 3-hydroxy-2-formylbenzothiophene accumulated with this strain. The direct result is that for strain BT1d only 18.4% of the sulfur from dibenzothiophene is unaccounted for by the methods used. Thus, for strains W1, F, and BT1, which accumulated 3-hydroxy-2-formylbenzothiophene only transiently, the further degradation of 3-hydroxy-2-formylbenzothiophene is likely a major contributing factor to the large portions of organosulfur from dibenzothiophene that were not detected by the methods used (50.0% to 85.2%).

The sulfur from dibenzothiophene that escaped detection by GC-AED analysis of culture extracts could exist as metabolites that are extractable with DCM under the conditions used but are too polar to be analyzed by the GC method used. This was tested in a separate experiment comparing the recovery of dibenzothiophene and its metabolites as determined by GC-AED analysis with the recovery determined by total sulfur analysis of DCM extracts of acidified cultures. Table 6.2 shows that the two methods of analysis gave recoveries that were within 12% of each other for the five cultures used in this comparison. In addition, neither method gave consistently higher recoveries. Thus there was not a large amount of DCM-extractable organosulfur from dibenzothiophene that was not amenable to detection by the GC-AED method.

Because some sulfur from dibenzothiophene escaped detection by the methods used, the isolates were tested to detemine if sulfate was being released from dibenzothiophene. The cultures were incubated for three serial transfers in sulfate-free medium to dilute the sulfate in the maintenance medium to negligible background levels, and the amount of dibenzothiophene added at the time of the third transfer was increased to 50 mg so that if sulfate was released, it would be more likely to be detected above the background levels. The detection limit of the turbidometric assay used was 1 mg/l sulfate which would have detected sulfate release from ≥0.8% of the 50 mg of dibenzothiophene. However, no sulfate was detected with any of the isolates incubated with 1-MN in the presence of dibenzothiophene or with isolate BT1d incubated with dibenzothiophene alone.

Thus, it appeared that the sulfur from dibenzothiophene that could not be detected

was present in organic forms in aqueous cultures, but was too polar to extract with DCM. To test this hypothesis, duplicate cultures of all three isolates grown on 1-MN in the presence of dibenzothiophene, and of isolates BT1 and BT1d grown with dibenzothiophene as sole carbon and energy source were established using the usual culture methods. After 7 days of incubation, one of each of the cultures was acidified and extracted with DCM and the other was lyophilized and the residue methyl-esterified prior to extraction. Total sulfur analysis of the extracts was done to compare the recovery of sulfur from dibenzothiophene by the two methods (Table 6.3). A series of controls, which consisted of each of the isolates grown on 1-MN without dibenzothiophene, were also taken through the lyophilization and methyl-esterification procedure to account for any organosulfur products recovered by this method that result from microbial incorporation of sulfate provided in the medium into organic compounds.

As can be seen from Table 6.3, lyophilization and methyl-esterification gave increased recovery of organosulfur over that obtained by DCM extraction of acidified cultures. The increases ranged from 2.2 to 13.1 μmol of sulfur, or from 10% to 60% of the initial 22 μmol of dibenzothiophene added. Thus, there was a considerable portion of organosulfur that was not recovered by DCM extraction of acidified cultures but is recovered by lyophilization and methyl-esterification.

6.4 DISCUSSION

Tetrahydrodibenzothiophene is a minor constituent of some fossil fuel derivatives (Later *et al.*, 1981; Nishioka, 1988) and, while it is not itself a significant environmental contaminant or priority pollutant, its chemical structure incorporates a variety of molecular features offering an interesting study of bacterial transformations. The oxidation of tetrahydrodibenzothiophene by cell suspensions of the cyclohexane degrader CB1323 that had been pre-grown in the presence of cyclohexane vapors yielded products analogous to those reported for the initial steps in the metabolism of cyclohexane (Perry, 1984). Just as monooxygenation of cyclohexane and subsequent dehydrogenation yields cyclohexanol and cyclohexanone as intermediates in the bacterial metabolism of cyclohexane, strain CB1323 also oxidized a methylene group in the alicyclic ring of tetrahydrodibenzothiophene to give a hydroxy-substituted tetrahydrodibenzothiophene (metabolite E), and subsequent dehydrogenation yielded the corresponding ketone (metabolite D). It is not known which of the methylene groups in the saturated ring was oxidized to give these products.

In the metabolism of cyclohexane, these reactions are followed by a biological Baeyer-Villiger monooxygenation to give a lactone which is subsequently hydrolyzed to

give adipic acid (Perry, 1984). However, the analogous ring cleavage reactions were not evident in the oxidation of tetrahydrodibenzothiophene by strain CB1323. The quantitative data obtained by GC-AED analysis showed that 100% of the sulfur in the substrate tetrahydrodibenzothiophene was accounted for, assuming that the evaporative loss in the viable cultures was the same as in the sterile control (27%).

Other compounds that contain both an aromatic and an alicyclic ring have been used in biodegradation studies which have focused primarily on bacteria that are able to degrade aromatic compounds. For example, the carbazole-degrading bacterium *Pseudomonas* sp. LD2 cometabolized 1,2,3,4-tetrahydrocarbazole when carbazole was provided as a growth substrate, but could not utilize this compound as sole carbon and energy source (Gieg *et al.*, 1996). Although over 60% loss of the 1,2,3,4-tetrahydrocarbazole was observed, no metabolites from its oxidation were detected by GC analysis.

Schreiber and Winkler (1983) studied *Pseudomonas stutzeri* AS39 that was capable of growth on tetralin (1,2,3,4-tetrahydronaphthalene) and naphthalene, but not cyclohexane, cyclohexanol, or cyclohexanone. They reported the oxidation of tetralin to 1-tetralone (3,4-dihydro-1(2*H*)-naphthalenone) and 1-tetralol (1,2,3,4-tetrahydro-1-naphthalenol) which are analogous to metabolites D (Figure 6.4) and E (Figure 6.5) found from tetrahydrodibenzothiophene.

Sikkema and de Bont (1991a) described the ability of eight bacterial isolates to utilize tetralin as sole carbon and energy source. All but one of the strains were able to grow on one or more aromatic hydrocarbons, but none could utilize cyclohexane as sole carbon and energy source. Reported metabolites included products of oxidation of the alicyclic ring: 1,2,3,4-tetrahydro-1-naphthalenol and 3,4-dihydro-1(2*H*)-naphthalenone; and products of oxidation of the aromatic ring: 5,6,7,8-tetrahydro-1-naphthalenol and 5,6,7,8-tetrahydro-2-naphthalenol (Sikkema and de Bont, 1991b, 1993). Strain CB 1323, used in this study, also oxidized both the aromatic and alicyclic rings of tetrahydrodibenzothiophene, yielding metabolites D, E, and F.

The cometabolism of tetrahydrodibenzothiophene by the 1-MN-degrading *Pseudomonas* strains resulted in oxidation, cleavage, and degradation of the benzene ring of tetrahydrodibenzothiophene to form 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene (Figure 6.1). The assignment of these functional groups to positions 3- and 2- is based on analogy to the published Kodama pathway of dibenzothiophene metabolism (Kodama *et al.*, 1970, 1973) which proceeds via dioxygenase attack at positions 1- and 2- of dibenzothiophene to give 1,2-dihydroxy-1,2-dihydrodibenzothiophene, followed by dehydrogenation to give 1,2-dihydroxydibenzothiophene. This intermediate then undergoes meta-cleavage to open the benzene ring of dibenzothiophene and degradation of the opened

ring yields a molecule each of pyruvate and 3-hydroxy-2-formylbenzothiophene (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989). In the current study, the data suggest that *Pseudomonas* strains W1, F, and BT1 utilize an analogous pathway to oxidize the benzene ring of tetrahydrodibenzothiophene. This is not surprising since these isolates metabolize dibenzothiophene via 3-hydroxy-2-formylbenzothiophene using the Kodama pathway with further transformation yielding benzothiophene-2,3-dione. These isolates degrade the unsubstituted ring of methyldibenzothiophenes (Saftic´ *et al.*, 1993) and 3,4-dimethyldibenzothiophene (Kropp *et al.*, 1997) to give methyl and dimethyl-substituted isomers of 3-hydroxy-2-formylbenzothiophene and benzothiophene-2,3-dione. The dibenzothiophene-oxidizing cultures of Bohonos *et al.* (1977) also transformed dibenzothiophene to 3-hydroxy-2-formylbenzothiophene and benzothiophene-2,3-dione. However, 4,5,6,7-tetrahydrobenzothiophene-2,3-dione was not observed in these studies with tetrahydrodibenzothiophene.

The pathway used by these *Pseudomonas* strains to oxidize dibenzothiophenes to analogs of 3-hydroxy-2-formylbenzothiophene is biochemically similar to the pathway reported for naphthalene catabolism (Eaton and Chapman, 1992). Other research has shown that a single genetic pathway controls the metabolism of dibenzothiophene, naphthalene, and phenanthrene in a soil *Pseudomonas* sp. (Denome *et al.*, 1993). The oxidative activity of naphthalene dioxygenase is not restricted to aromatic ring dioxygenation with the subsequent dehydrogenation and ring cleavage reactions, but has also been reported to catalyze monooxygenations of benzylic methyl or methylene moities and sulfoxygenations of the sulfur atom of organic sulfur compounds (Allen *et al.*, 1995; Selifonov *et al.*, 1996). The monooxygenation of the C-1 and C-4 methylene groups of tetrahydrodibenzothiophene, which are not benzylic but are alpha to the aromatic thiophene ring, by the dioxygenases of the three *Pseudomonas* strains was not observed to occur as might be expected from previous reports of monoxygenation of benzylic methylene groups by naphthalene dioxygenase cloned from NAH7 (Selifonov *et al.*, 1996) and from studies of tetralin metabolism (see above). However, the oxidation of the sulfur atom of tetrahydrodibenzothiophene to give the sulfoxide and sulfone was observed with 1-MN-grown cultures of isolates W1 and F, and these were the most abundant products detected. The sulfoxide and sulfone of tetrahydrodibenzothiophene were not detected with isolate BT1. However, all four isolates were shown to oxidize dibenzothiophene to the sulfoxide and sulfone as has been reported as an alternate dead-end route for other bacteria that utilize the Kodama pathway to metabolize dibenzothiophene (Kodama *et al.*, 1970, 1973; Laborde and Gibson, 1977; Mormile and Atlas, 1989). This oxidation of dibenzothiophene to the sulfoxide and sulfone was a dead-end pathway for isolates W1 and F, however, isolate

BT1 was capable of further degradation of dibenzothiophene sulfone if 1-MN was present as a growth substrate. It is possible that the sulfoxide and sulfone were not detected in cultures of isolate BT1 grown on 1-MN in the presence of tetrahydrodibenzothiophene because the sulfone of tetrahydrodibenzothiophene was also further metabolized.

Quantitative GC-AED analyses of extracts of the three *Pseudomonas* strains incubated for 3 days with tetrahydrodibenzothiophene and 1-MN showed that only 9% to 41% of the sulfur in tetrahydrodibenzothiophene was detected by the analytical methods used. GC-FPD analyses of extracts from cultures incubated for shorter times suggested that 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene was more abundant prior to day 3. However, the non-linear response of the FPD does not allow quantitation, and GC-AED analysis was not done for these earlier sample times. However, it appeared that 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene accumulated transiently and was subsequently degraded by isolate BT1. Unfortunately, the amount of the synthesized tetrahydrodibenzothiophene available was insufficent to do further quantitative studies with this substrate.

Because a sulfur mass balance was not achieved in these studies with aromatic-hydrocarbon-degrading bacteria and tetrahydrodibenzothiophene, and in other studies with dimethyldibenzothiophenes (Kropp *et al.*, 1997), in depth quantitative studies were done with commercially available dibenzothiophene. In general, the amounts of dibenzothiophene remaining and the amounts of the 3-hydroxy-2-formylbenzothiophene, sulfoxide and sulfone that were produced from dibenzothiophene were comparable with the amounts of tetrahydrodibenzothiophene and the analogous metabolites that were observed for each isolate incubated with 1-MN as growth substrate. The amount of 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene that was observed to form with isolates W1 and F was slightly larger than the amount of 3-hydroxy-2-formylbenzothiophene. Although the analog of benzothiophene-2,3-dione was not observed to form from tetrahydrodibenzothiophene, this metabolite from dibenzothiophene was only observed in very small amounts (0.2% to 1.3% of the dibenzothiophene added to the cultures) so it does not significantly influence the sulfur mass balance. As well, the fact that the sulfoxide and sulfone of tetrahydrodibenzothiophene were not observed in 1-MN-grown cultures of isolate BT1 did not significantly influence the sulfur mass balance, because in 1-MN-grown cultures of isolate BT1 incubated with dibenzothiophene, only 1.6% of the dibenzothiophene was found in the form of the sulfoxide and sulfone. Therefore, similar results were obtained with dibenzothiophene as were observed with tetrahydrodibenzothiophene. That is, the sulfur present as the observed metabolites was much less than the

amount of substrate organosulfur that was depleted in cultures that were grown on 1-MN (e.g. Table 6.1).

As far as I am aware, quantitative studies that establish a sulfur mass balance for bacterial degradation of dibenzothiophene via the Kodama pathway have not been reported. Kodama *et al.* (1970, 1973) identified metabolites from pure cultures of *Pseudomonas jianii*, and the yields of three of the purified metabolites were reported. By my calculations the metabolites accounted for 31% of the dibenzothiophene added to the culture. However, the amount of dibenzothiophene remaining at the end of the incubation period was not reported so it is not known if a mass balance existed.

Maintaining isolate BT1 on dibenzothiophene as sole carbon source for over 2 years yielded a strain, designated BT1d, that had altered dibenzothiophene-degrading activity, leading to a difference in the abundance of 3-hydroxy-2-formylbenzothiophene remaining in batch cultures. The highest recovery of organosulfur was in the culture of isolate BT1d grown with 21 μmol of dibenzothiophene as sole carbon and energy source (Table 6.1). After 7 days incubation, 3-hydroxy-2-formylbenzothiophene was the most abundant product, accounting for 52% of the sulfur from dibenzothiophene. Although some of the 3-hydroxy-2-formylbenzothiophene was degraded further, presumably yielding a small amount of benzothiophene-2,3-dione (0.8%), 3-hydroxy-2-formylbenzothiophene accumulated in the culture without extensive further degradation taking place (Figure 6.7c). This accumulation of 3-hydroxy-2-formylbenzothiophene resulted in a high total recovery of sulfur from dibenzothiophene by the DCM extraction and GC methods used in this study (81.6% after accounting for evaporative loss). The low total recoveries of sulfur from dibenzothiophene (14.8% to 50.0% after accounting for evaporative losses) found with cultures that accumulated 3-hydroxy-2-formylbenzothiophene transiently suggest that further degradation of 3-hydroxy-2-formylbenzothiophene leads to the formation of metabolites that are too polar to extract with DCM and analyze by the GC methods used. However, the further degradation of 3-hydroxy-2-formylbenzothiophene did not lead to a corresponding increase in the amount of benzothiophene-2,3-dione that was detected (Table 6.1). There is only one report on 3-hydroxy-2-formylbenzothiophene biodegradation (Mormile and Atlas, 1988) which demonstrated CO_2 release from this compound, but no sulfate release was detected.

Eaton and Nitterauer (1994) showed that benzothiophene was microbially oxidized via dioxygenase attack at positions 2 and 3, and that subsequent reactions, including ring cleavage, led to the formation of 2-mercaptophenylglyoxalate which cyclized to give benzothiophene-2,3-dione by acid-catalyzed dehydration. In this study, cultures were routinely acidified prior to extraction and so the benzothiophene-2,3-dione detected in these

extracts was likely present in the culture at neutral pH as 2-mercaptophenylglyoxalate. Thus, detection of this dione indicates that cleavage of the thiophene ring has occurred. The small amounts of benzothiophene-2,3-dione that were detected, even in culture conditions where further degradation of 3-hydroxy-2-formylbenzothiophene did occur, suggest that 2-mercaptophenylglyoxalate may also be further degraded to compounds that are not detected by the methods used in this study.

These experimental results show that the amounts of metabolites detected from dibenzothiophene by DCM extraction and GC-AED analysis did not account for the amounts of the substrate depleted. They also suggest that 3-hydroxy-2-formylbenzothiophene, 2-mercaptophenylglyoxalate (detected as benzothiophene-2,3-dione), and dibenzothiophene sulfone were likely biodegraded under certain conditions. Thus, additional experiments were undertaken to determine the fate of the sulfur in dibenzothiophene. Total sulfur analyses of DCM extracts indicated that there was not a large portion of sulfur from dibenzothiophene in the extract that was not detectable by GC-AED analysis. In addition, there was no evidence of sulfate release from dibenzothiophene by any of the *Pseudomonas* strains. Thus, it appears that the missing sulfur from dibenzothiophene is present as organic metabolites that are too polar to recover by liquid-liquid extraction with DCM. This hypothesis was supported by the fact that lyophilization and methyl-esterification gave increased recovery of total sulfur over that obtained by simple DCM extraction of acidified cultures (Table 6.3). Some of the missing organic sulfur likely results from further degradation of 3-hydroxy-2-formylbenzothiophene, 2-mercaptophenylglyoxalate, and dibenzothiophene sulfone, and this possibility is the subject of ongoing investigations in this laboratory.

The amounts of dibenzothiophene sulfoxide and sulfone that were produced in cultures of strain BT1 incubated with and without 1-MN were quite different (Table 6.1), suggesting that cosubstrates may have a significant effect on the metabolism of organic contaminants. Of course, in petroleum-contaminated environments, dibenzothiophenes are present with numerous saturated and aromatic hydrocarbons. The complex mixture of compounds present in petroleum will likely influence the fate of the sulfur present as condensed thiophenes. Biodegradation studies that use pure cultures and pure compounds are useful for the determination of metabolic pathways for the destruction of organic contaminants, and are a step towards the identification of metabolites whose possible formation in contaminated environments may influence the ability of bioremediation to reduce the toxicity of a contaminated site. However, this work demonstrates that cosubstrates may significantly influence the degradation and the environmental fate of contaminants, such as condensed thiophenes, typically found in a complex aromatic matrix.

While the same metabolites were detected regardless of whether or not 1-MN was included in the pure culture studies described in this report, the amounts of metabolites present and the effect on the overall sulfur mass balance was significant. This emphasizes the importance of taking a quantitative approach and pursuing a mass balance in studies of the biodegradation of organic contaminants. There are relatively few studies of the biodegradation of condensed thiophenes that have emphasized this approach.

Table 6.1 Quantification of dibenzothiophene and sulfur-containing metabolites as determined by GC-AED analyses of DCM extracts of acidified cultures of four *Pseudomonas* strains incubated for 7 days.

		Percent of sulfur from DBT[a] recovered as						
Strain	Growth Substrate	DBT	HFBT[b]	DBT Sulfoxide & Sulfone	Benzothio-phene-2,3-dione	Total Recovery (%)	Recovery in Sterile Control (%)	Difference[c]
W1[d]	1-MN	9.6	0.6	17	0.5	27.7	88	60.3
F[d]	1-MN	12	0.7	24	1.3	38.0	88	50.0
BT1[e]	1-MN & DBT	0.8	0.2	1.6	0.2	2.8	88	85.2
BT1[e]	DBT	1.3	0.2	9.4	0.3	11.2	86	74.8
BT1d[d]	DBT	5.9	52	8.9	0.8	67.6	86	18.4

[a]DBT=dibenzothiophene

[b]HFBT=3-hydroxy-2-formylbenzothiophene

[c]Difference between total recovery in culture extracts and the corresponding sterile control.

[d]21 µmol dibenzothiophene given.

[e]22 µmol dibenzothiophene given.

Table 6.2 Comparison of the recovery of organosulfur from dibenzothiophene as determined by GC-AED analyses and by total sulfur analyses of DCM extracts of acidified cultures of four *Pseudomonas* strains incubated with 22 μmol dibenzothiophene for 7 days.

		Percent recovery by	
Strain	Growth Substrate	GC-AED Analysis	Total Sulfur Analysis
W1	1-MN	22	31
F	1-MN	50	45
BT1	1-MN & DBT[a]	4	14
BT1	DBT	10	22
BT1d	DBT	64	58

[a]DBT=dibenzothiophene

Table 6.3 Comparison of the recovery of organosulfur from dibenzothiophene by DCM extraction of acidified cultures and by lyophilization. The four *Pseudomonas* strains were incubated with 22 µmol dibenzothiophene for 7 days. Recovery was detemined by total sulfur analysis of extracts obtained by the two methods.

		µmol organosulfur recovered by		Additional organosulfur found with lyophilization[b] (µmol)
Strain	Growth Substrate	DCM Extraction	Lyophilization[a]	
W1	1-MN	8.7	21.7 (5.6)	7.4
F	1-MN	12.5	18.2 (3.5)	2.2
BT1	1-MN & DBT[c]	4.6	17.0 (3.9)	8.5
BT1	DBT	6.8	19.9 (NA)	13.1
BT1d	DBT	16.8	19.6 (NA)	2.8

[a]The number in brackets represents the µmol organosulfur that was also observed by total sulfur analysis of controls that did not receive dibenzothiophene. An appropriate control was not available (NA) for the cultures of isolate BT1 and BT1d with dibenzothiophene as growth substrate, because omission of dibenzothiophene yielded no growth of the cultures.

[b]Additional sulfur found = amount found by lyophilization – (amount found in control + amount in DCM extract).

[c]DBT=dibenzothiophene

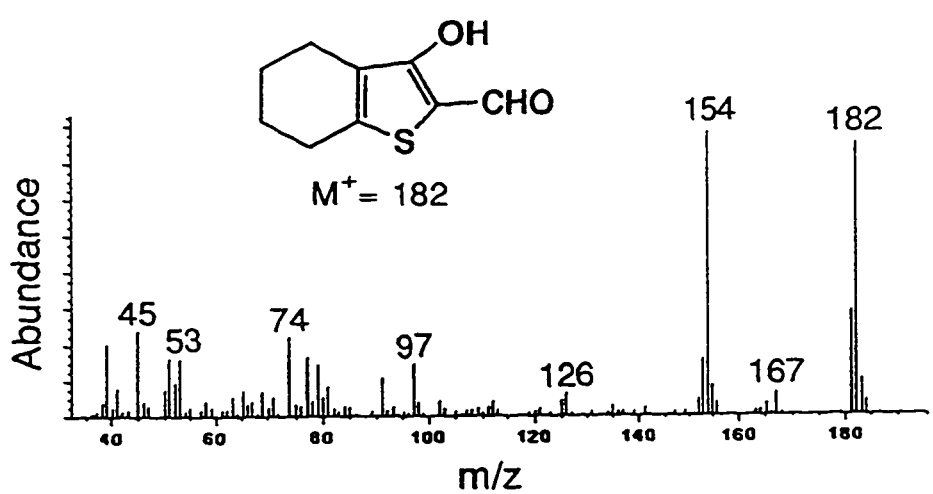

Figure 6.1 From GC-MS analysis, the mass spectrum of sulfur-containing metabolite A from a culture of isolate F grown on 1-MN in the presence of tetrahydrodibenzothiophene. These results indicate that metabolite A is 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene.

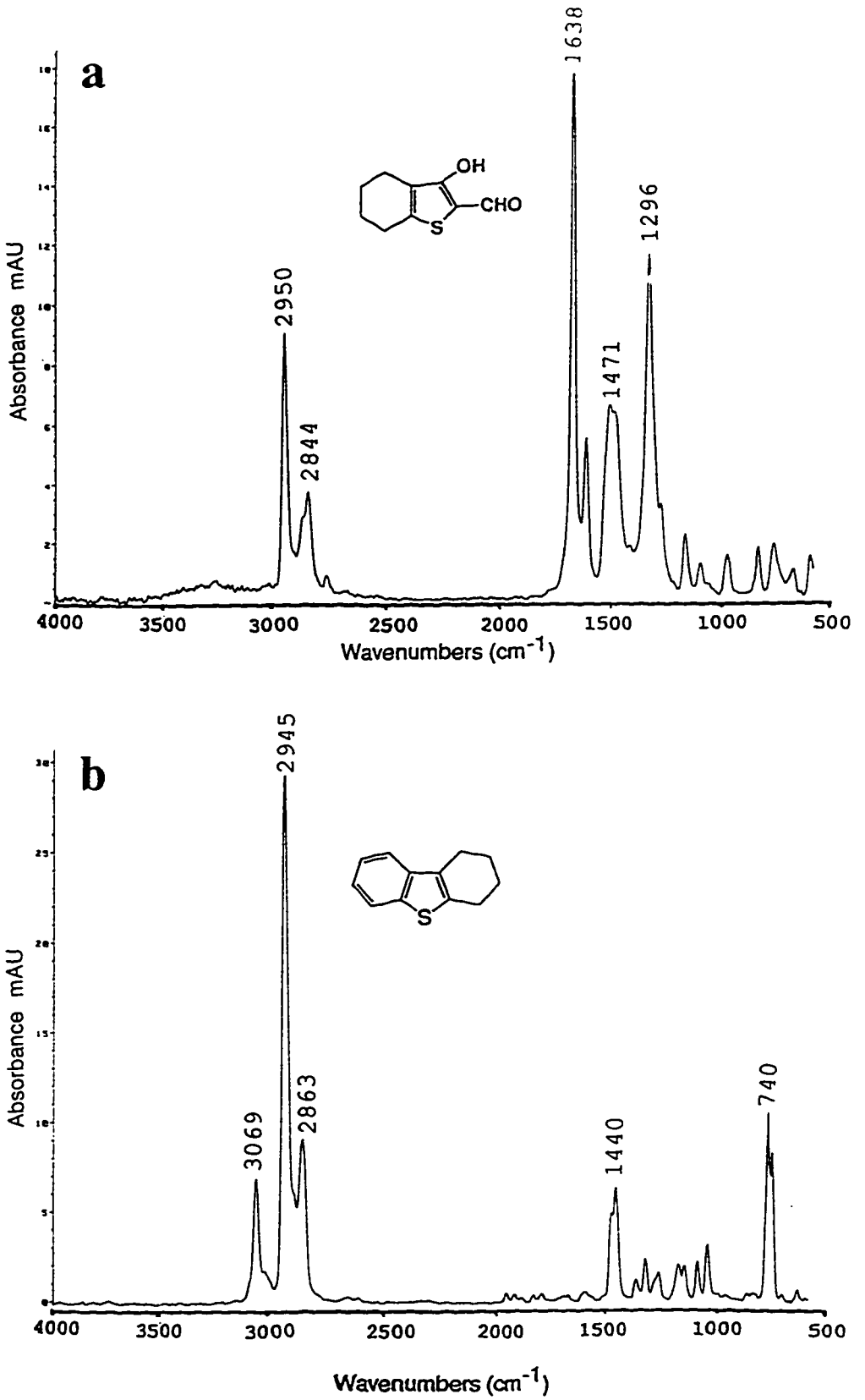

Figure 6.2 From GC-FTIR analyses, the FTIR spectra of sulfur-containing metabolite A from a culture of isolate F grown on 1-MN in the presence of tetrahydrodibenzothiophene (a) and of tetrahydrodibenzothiophene (b). These results indicate that metabolite A is 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene.

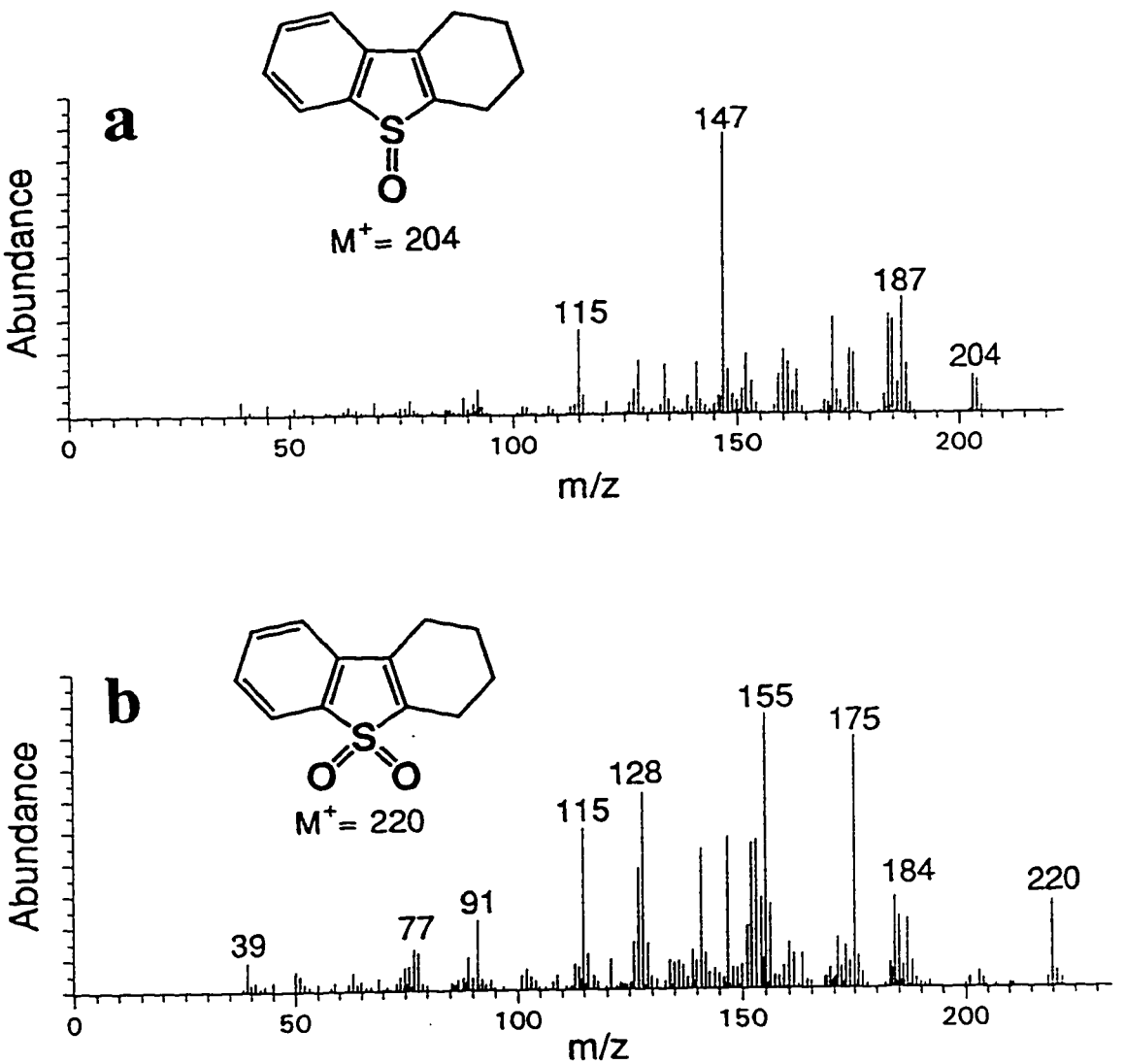

Figure 6.3 From GC-MS analyses, the mass spectra of sulfur-containing metabolite B (a) and metabolite C (b) from a culture of isolate F grown on 1-MN in the presence of tetrahydrodibenzothiophene. These results indicate that metabolites B and C are the sulfoxide and sulfone of tetrahydrodibenzothiophene, respectively.

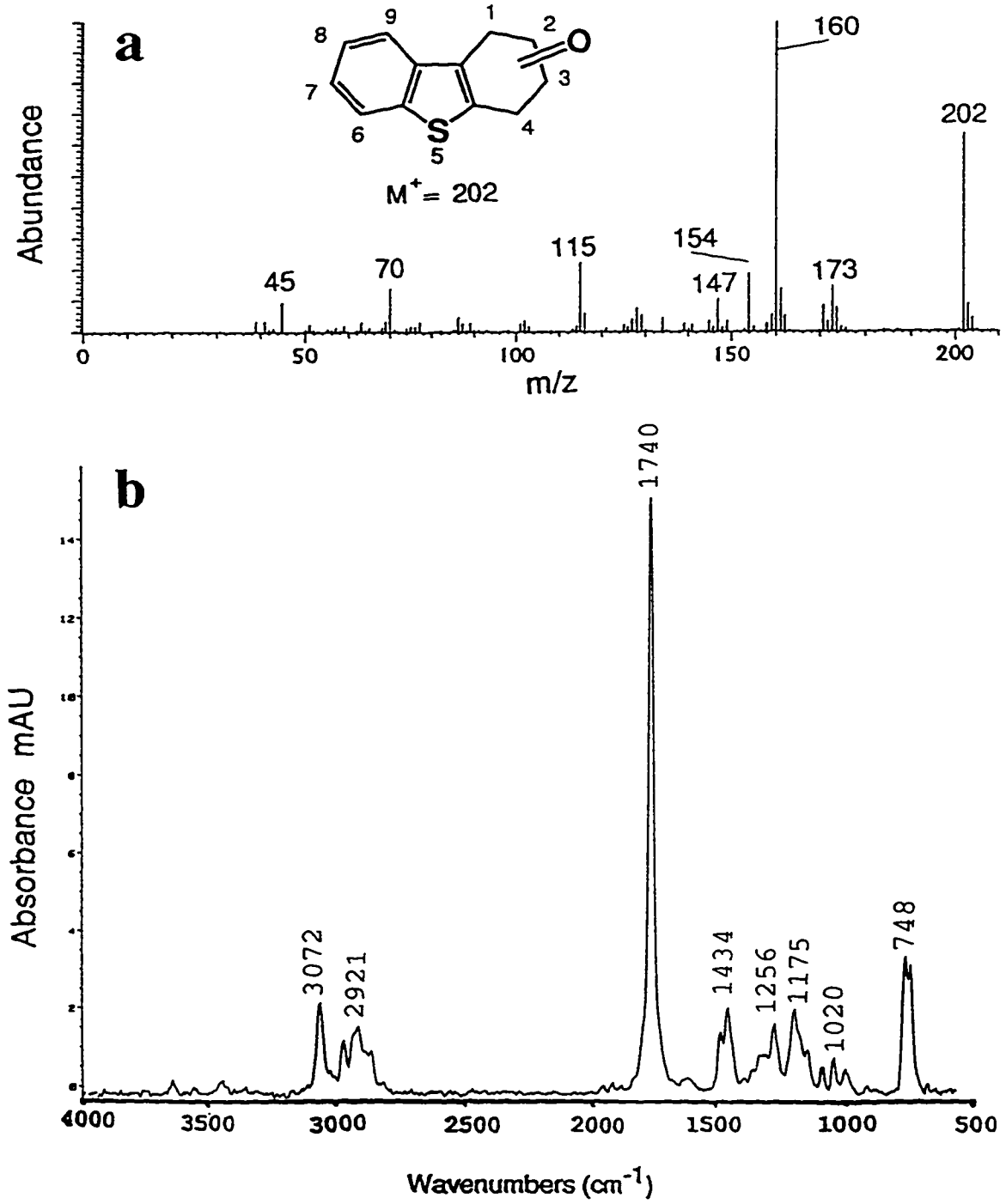

Figure 6.4 Mass spectrum (a) and FTIR spectrum (b) of sulfur-containing metabolite D from the analyses of the extract of the cell suspension of strain CB1323 incubated with tetrahydrodibenzothiophene. The results indicate that one of the methylene carbon atoms of tetrahydrodibenzothiophene has been oxidized to a carbonyl group, yielding a cyclic ketone.

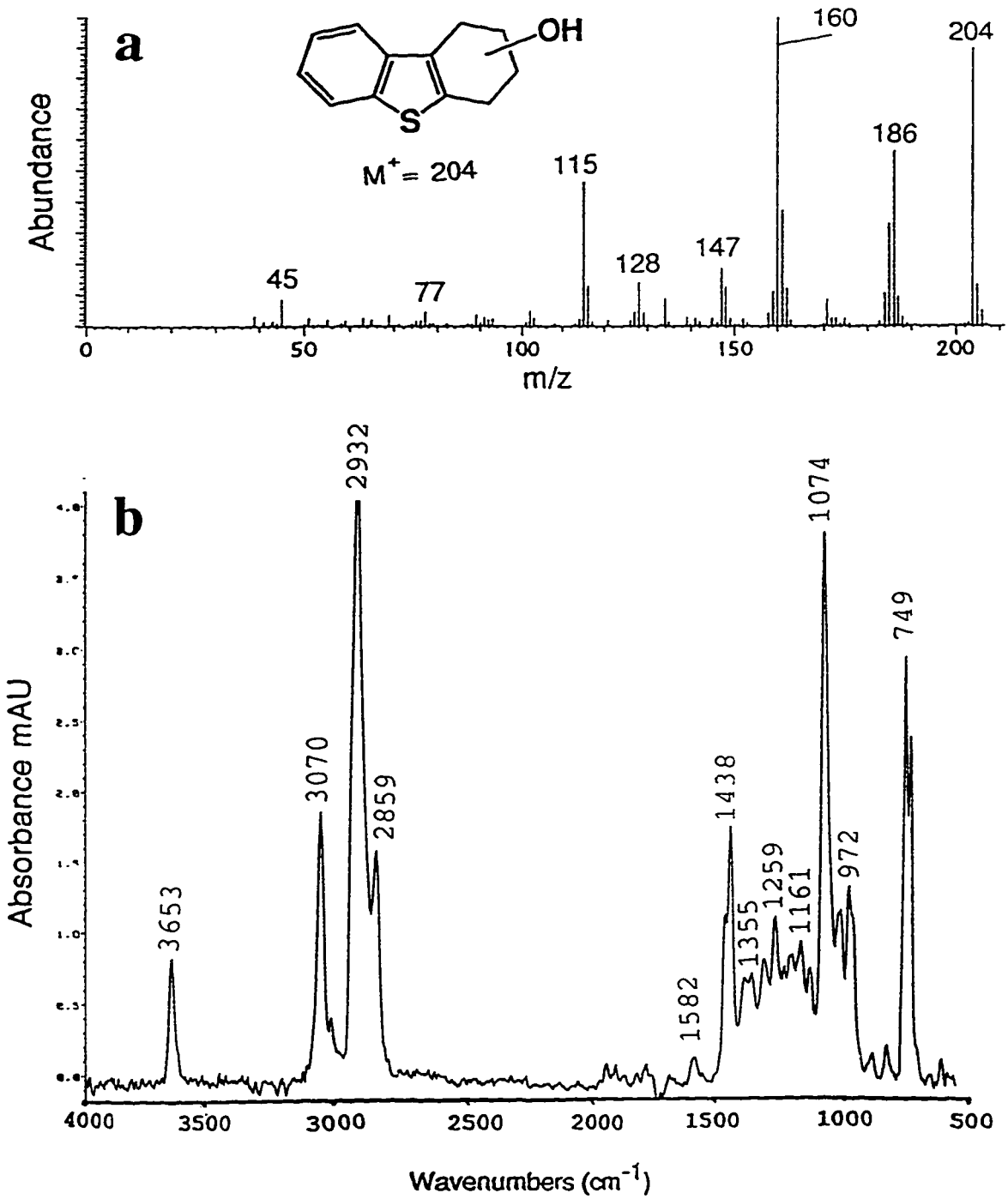

Figure 6.5 Mass spectrum (a) and FTIR spectrum (b) of sulfur-containing metabolite E from the analyses of the extract of the cell suspension of strain CB1323 incubated with tetrahydrodibenzothiophene. The results indicate that the metabolite is an isomer of hydroxy-substituted tetrahydrodibenzothiophene, bearing the hydroxy group on the saturated ring.

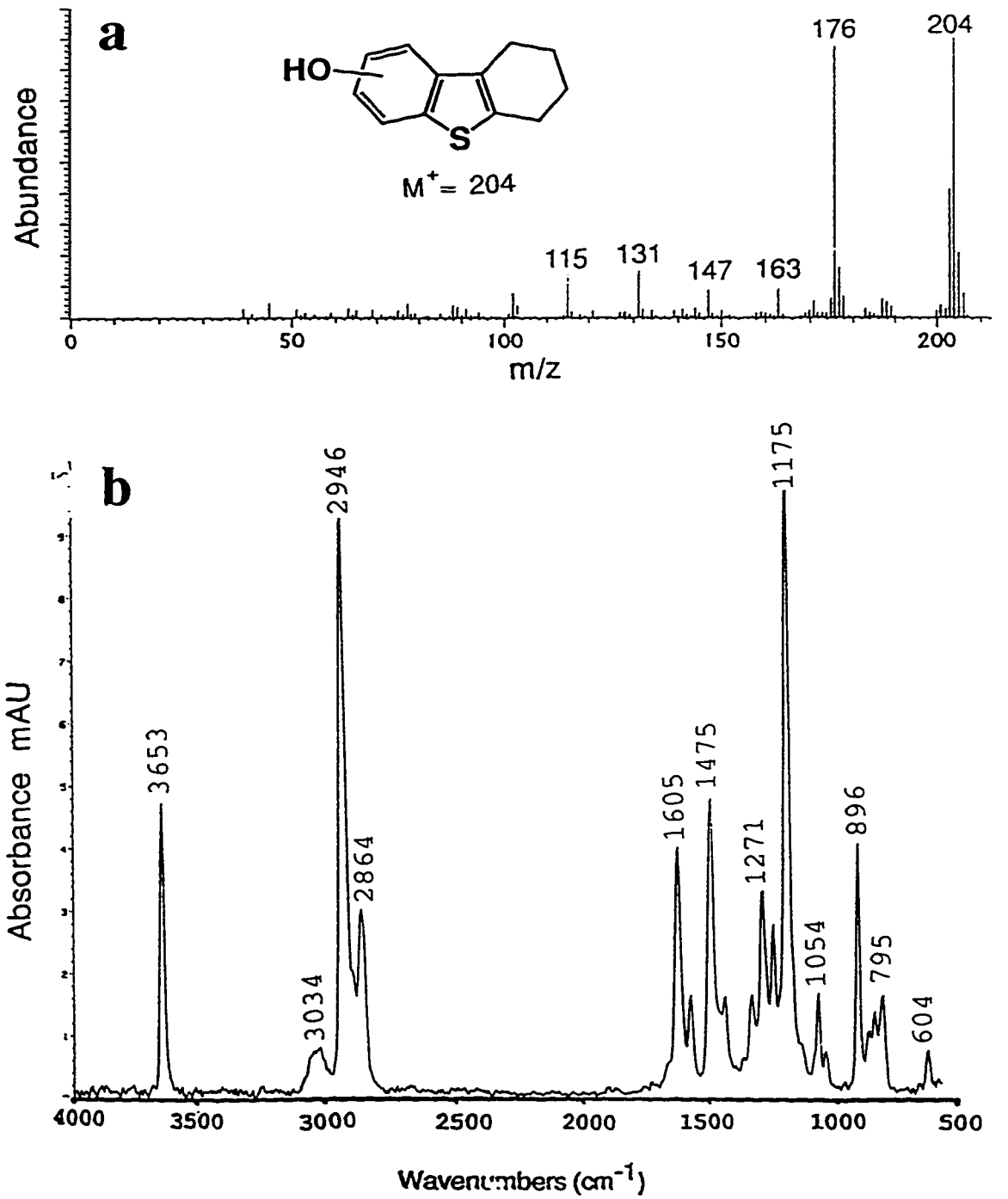

Figure 6.6 Mass spectrum (a) and FTIR spectrum (b) of sulfur-containing metabolite F from the analyses of the extract of the cell suspension of strain CB1323 incubated with tetrahydrodibenzothiophene. The results indicate that the metabolite is an isomer of hydroxy-substituted tetrahydrodibenzothiophene, bearing the hydroxy group on the benzene ring.

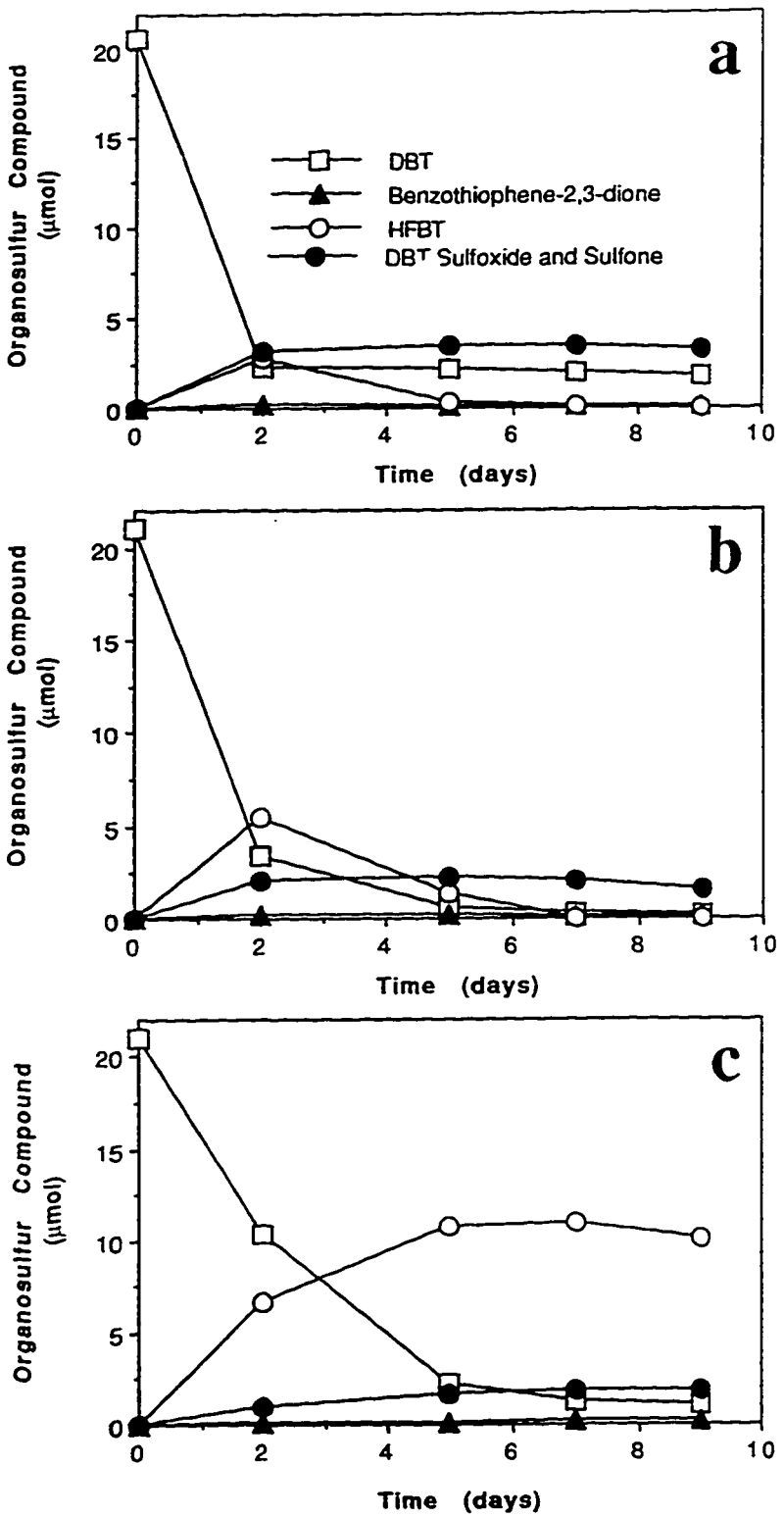

Figure 6.7 Transformation of dibenzothiophene to metabolites detected by GC-AED analysis of a culture of isolate W1 grown on 1-MN in the presence of dibenzothiophene (a), a culture of isolate BT1 grown on dibenzothiophene as sole source of carbon and energy (b), and a culture of isolate BT1d grown on dibenzothiophene as sole source of carbon and energy (c).

6.5 LITERATURE CITED

Allen, C. C. R., D. R. Boyd, H. Dalton, N. D. Sharma, S. A. Haughey, R. A. S. McMordie, B. T. McMurray, G. N. Sheldrake, and K. Sproule. 1994. Sulfoxides of high enantiopurity from bacterial dioxygenase-catalyzed oxidation. J. Chem. Soc. Chem. Commun. 1995: 119-120.

American Public Health Association. 1989. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC.

Andersson, J. T., and B. Schmid. 1993. The atomic emission detector in gas chromatographic trace analysis. Fresenius' J. Anal. Chem. 346: 403-409.

Berthou, F., Y. Gourmelun, Y. Dreano, and M. P. Friocourt. 1981. Application of gas chromatography on glass capillary columns to the analysis of hydrocarbon pollutants from the *Amoco Cadiz* oil spill. J. Chromatogr. 203: 279-292.

Boehm, P. D., J. E. Barak, D. L. Fiest, and A. Elskus. 1982. A chemical investigation of the transport and fate of petroleum hydrocarbons in littoral and benthic environments: The *Tsesis* oil spill. Mar. Environ. Res. 6: 157-188.

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. In: Amoco Cadiz: Fates and effects of the oil spill. Proceedings of the International Symposium. Centre Oceanologique de Bretagne. Brest. Le Centre National pour l'Exploration des Oceans: Paris; p. 159-173.

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.

Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. Current Microbiol. 21: 229-231.

Denome, S. A., D. C. Stanley, E. S. Olson, and K. D. Young. 1993. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. 175: 6890-6901.

Eaton, R. W., and P. J. Chapman. 1992. Bacterial metabolism of naphthalene: construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. J. Bacteriol. 174: 7542-7554.

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

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., and D. W. S. Westlake. 1986. Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. 51: 435-437.

Friocourt, M. P., F. Berthou, and D. Picart. 1982. Dibenzothiophene derivatives as organic markers of oil pollution. Toxicol. Environ. Chem. 5: 205-215.

Fritz, J. S., and S. S. Yamamura. 1955. Rapid microtitration of sulfate. Anal. Chem. 27: 1461-1464.

Gallagher, J. R., E. S. Olson, and D. C. Stanley. 1993. Microbial desulfurization of dibenzothiophene: A sulfur specific pathway. FEMS Microbiol. Lett. 107: 31-36.

Gieg, L. M., A. Otter, and P. M. Fedorak. 1996. Carbazole degradation by *Pseudomonas* sp. LD2: Metabolic characteristics and the identification of some metabolites. Environ. Sci. Technol. 30: 575-585.

Gonçalves, J. A., and P. M. Fedorak. 1996. Aerobic degradation of 2-sulphobenzoic acid by *Pseudomonas* strain SB(W). Lett. Appl. Microbiol. 23: 299-302.

Heller, S.R., and G.W.A. Milne. 1978. EPA/NIH Mass Spectral Data Base. Vol. 1. U.S. Department of Commerce: Washington, D.C.

Hou, C. T., and A. I. Laskin. 1976. Microbial conversion of dibenzothiophene. Dev. Ind. Microbiol. 17: 351-362.

Izumi, Y., T. Oshiro, H. Ogino, Y. Hine, and M. Shimao. 1994. Selective desulfurization of dibenzothiophene by *Rhodococcus erythropolis* D-1. Appl. Environ. Microbiol. 60: 223-226.

Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. Part III. Isolation and identification of products from dibenzothiophene. Agr. Biol. Chem. 34: 1320-1324.

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. Agr. Biol. Chem. 37: 45-50.

Kropp, K. G., J. T. Andersson, and P. M. Fedorak. 1997. Biotransformations of three dimethyldibenzothiophenes by pure and mixed bacterial cultures. Environ. Sci. Technol. 31: 1547-1554.

Kropp, K. G., J. A. Gonçalves, J.T. Andersson, and P. M. Fedorak. 1994a. Bacterial transformations of benzothiophene and methylbenzothiophenes. Environ. Sci. Technol. 28: 1348-1356.

Kropp, K. G., J. A. Gonçalves, J.T. Andersson, and P. M. Fedorak. 1994b. Microbially mediated formation of benzonaphthothiophenes from benzo[b]thiophenes. Appl. Environ. Microbiol. 60: 3624-3631.

Kropp, K. G., S. Saftic´, J. T. Andersson, and P. M. Fedorak. 1996. Transformations of six isomers of dimethylbenzothiophenes by three *Pseudomonas* strains. Biodegradation 7: 203-221.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* species. Appl. Environ. Microbiol. 34: 783-790.

Laseter, J. L., G. C. Lawler, E. B. Overton, J. R. Patel, J. P. Holmes, M. I. Shields, and M. Maberry. 1981. Characterization of aliphatic and aromatic hydrocarbons in flat

and Japanese type oysters and adjacent sediments collected from L'Aver Wrac'h following the Amoco Cadiz oil spill. In: Amoco Cadiz: Fates and effects of the oil spill. Proceedings of the International Symposium, Centre Oceanologique de Bretagne. Brest. Le Centre National pour l'Exploration des Oceans: Paris; p. 633-644.

Later, D. W., M. L. Lee, K. D. Bartle, R. C. Kong, and D. L. Vassilaros. 1981. Chemical class separation and characterization of organic compounds in synthetic fuels. Anal. Chem. 53: 1612-1620.

Lin-Vien, D., N. B. Colthup, W. G. Fateley, and J. G. Grasselli. 1991. The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules. Academic Press, Inc.: San Diego; p. 119.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Mormile, M. R., and R. M. Atlas. 1988. Mineralization of dibenzothiophene biodegradation products 3-hydroxy-2-formylbenzothiophene and dibenzothiophene sulfone. Appl. Environ. Microbiol. 54: 3183-3184.

Mormile, M. R., and R. M. Atlas. 1989. Biotransformation of dibenzothiophene to dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. 35: 603-605.

Nagai, M., T. Sato, and A. Aiba. 1986. Poisoning effect of nitrogen compounds on dibenzothiophene desulfurization on sulfided NiMo/Al_2O_3 catalysts and relation to gas phase basicity. J. Catal. 97: 52-58.

Nishioka, M. 1988. Aromatic sulfur compounds other than condensed thiophenes in fossil fuels: enrichment and identification. Energy Fuels 2: 214-219.

Ogata, M., and K. Fujisawa. 1985. Organic sulfur compounds and polycyclic hydrocarbons transferred to oyster and mussel from petroleum suspension. Identification by gas chromatography and capillary mass chromatography. Water Res. 19: 107-118.

Omori, T., L. Monna, Y. Saiki, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1. Appl. Environ. Microbiol. 58: 911-915.

Perry, J. J. 1984. Microbial metabolism of cyclic alkanes. In: Petroleum Microbiology. Atlas, R. M., Ed. Macmillan Publishing Co.: New York; p. 61-97.

Pierce, A. E. 1968. Silylation of Organic Compounds. Pierce Chemical Company: Rockford, IL.

Rankel, L. A. 1991. Effects of H_2S addition on the performance of fresh vs. used CoMo catalysts. Fuel Sci. Technol. Int. 9: 435-557.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1992. Diones, sufoxides and sulfones from the aerobic cometabolism of methylbenzothiophenes by *Pseudomonas* strain BT1. Environ. Sci. Technol. 26: 1759-1764.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Schreiber, A. F., and U. K. Winkler. 1983. Transformation of tetralin by whole cells of *Pseudomonas stutzeri* AS39. Eur. J. Appl. Microbiol. Biotechnol. 18: 6-10.

Selifonov, S. A., M. Grifoll, R. W. Eaton, and P J. Chapman. 1996. Oxidation of naphthenoaromatic and methyl-substituted aromatic compounds by naphthalene 1,2-dioxygenase. Appl. Environ. Microbiol. 62: 507-514.

Sikkema, J., and J. A. M. deBont. 1991a. Isolation and initial characterization of bacteria growing on tetralin. Biodegradation 2: 15-23.

Sikkema, J., and J. A. M. de Bont. 1991b. Biocatalytic production of hydroxylated aromatic and alicyclic compounds: Products derived from tetralin. Recl. Trav. Chim. Pays-Bas 110: 189-194.

Sikkema, J., and J. A. M. de Bont. 1993. Metabolism of tetralin (1,2,3,4-tetrahydronaphthalene) in *Corynebacterium* sp. Strain C125. Appl. Environ. Microbiol. 59: 567-572.

Silverstein, R. M., G. C. Bassler, and T. C. Morrill. 1991. Spectrometric Identification of Organic Compounds. John Wiley & Sons, Inc.: New York.

Speight, J. G. 1980. The Chemistry and Technology of Petroleum. Marcel Dekker, Inc.: New York; p. 65.

Teal, J. M., K. Burns, and J. Farrington. 1978. Analyses of aromatic hydrocarbons in intertidal sediments resulting from two spills of no. 2 fuel oil in Buzzards Bay, Massachusetts. J. Fish. Res. Board Can. 35: 510-520.

van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. Arch. Microbiol. 153: 324-328.

Wang, P., and S. Krawiec. 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in batch and fed-batch cultures. Appl. Environ. Microbiol. 62: 1670-1675.

Wilputte, R., and R. H. Martin. 1956. Synthèses dans le domaine des composés cancérogènes. XV. (Poly)benzo-9-thiafluorènes et anhydride thianaphtène-4,5-dicarboxylique. Bull. Soc. Chim. Belg. 65: 874-898.

7. BACTERIAL TRANSFORMATIONS OF NAPHTHOTHIOPHENES*

7.1 INTRODUCTION

Sulfur is usually the most abundant hetero-element in crude oils, contributing between 0.04 to 5% of the mass of conventional crudes (Speight, 1980). 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 (Finnerty and Robinson, 1986; Foght *et al.*, 1990). 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 (Finnerty and Robinson, 1986). 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 (Bohonos *et al.*, 1977; Eaton and Nitterauer, 1994; Fedorak and Grbic´-Galic´, 1991; Finnerty *et al.*, 1983; Kropp *et al.*, 1994a; Sagardía *et al.*, 1975), 3-methylbenzothiophene (Fedorak and Grbic´-Galic´, 1991; Kropp *et al.*, 1994a), and dibenzothiophene (Bohonos *et al.*, 1977; Crawford and Gupta, 1990; Gallagher *et al.*, 1993; Hou and Laskin, 1976; Izumi *et al.*, 1994; Kargi and Robinson, 1984; Kodama *et al.*, 1970, 1973; Kropp *et al.*, 1997b; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989; Omori *et al.*, 1992; van Afferden *et al.*, 1990; Wang and Krawiec, 1996). As well, it is not surprising that only a few studies have been done with alkyl-substituted benzothiophenes (Kropp *et al.*, 1994a, b, 1996; Saftic´ *et al.*, 1992) and dibenzothiophenes (Kropp *et al.*, 1997a; Lee *et al.*, 1995; Ohshiro *et al.*, 1996; Saftic´ *et al.*, 1993), 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 GC analysis of solvent refined coal liquids, coal tars, anthracene oils, shale oils, and higher boiling petroleum fractions using sulfur-selective FPD or AED detectors and GC-MS. The compounds identified include benzonaphthothiophenes, phenanthrothiophenes, dinaphthothiophenes, benzophenanthrothiophenes, and other compounds containing two to four benzene rings condensed with one or two

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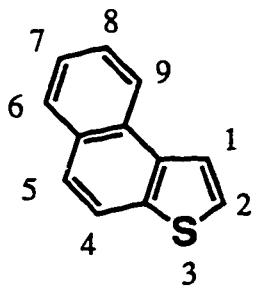
thiophene rings (Burchill *et al.*, 1982a, b; Drushel and Sommers, 1967; Later *et al.*, 1981; Nishioka, 1988; Willey *et al.*, 1981).

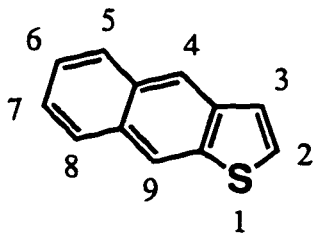
Using a very polar cyanopropyl stationary phase for GC analysis with an AED, Andersson and Schmid (1995) detected naphtho[1,2-*b*]thiophene ($C_{12}H_8S$) in an Austrian shale oil, and reported that this isomer co-eluted with dibenzothiophene ($C_{12}H_8S$) during GC analysis with the non-polar stationary phases typically used to analyze aromatic fractions. Naphtho[2,1-*b*]thiophene and naphtho[2,3-*b*]thiophene, the two other 3-ring polycyclic aromatic sulfur heterocycles with the chemical formula of $C_{12}H_8S$, were also detected. The co-elution of dibenzothiophene with naphtho[1,2-*b*]thiophene is consistent with earlier reports of Burchill *et al.* (1982a, b) that only three chromatographic peaks of chemical formula $C_{12}H_8S$ 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 analysis with sulfur-selective detection to assess the biodegradation of crude oil in microbial cultures (Fedorak and Westlake, 1983, 1984; Foght and Westlake, 1988), in contaminated environments (Fayad and Overton, 1995; Wang *et al.*, 1995), and within natural petroleum reservoirs (Westlake, 1983) 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 naphthothiophene are commercially available and none of them have been previously subjected to biodegradation studies.

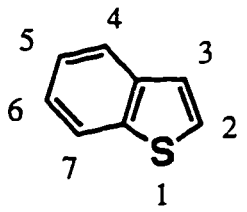
The objective of this work was to test the abilities of three 1-MN-degrading *Pseudomonas* strains W1, BT1, and F, which have been previously shown to metabolize dibenzothiophene (Kropp *et al.*, 1997b), to grow on and/or to cometabolize naphtho[2,1-*b*]thiophene and naphtho[2,3-*b*]thiophene. The ring numbering conventions for these chemically synthesized condensed thiophenes and for benzothiophene are shown below. A small amount of 1-methylnaphtho[2,1-*b*]thiophene was also synthesized and used in these studies.



Naphtho[2,1-*b*]thiophene



Naphtho[2,3-*b*]thiophene



Benzothiophene

7.2 MATERIALS AND METHODS

7.2.1 Chemicals

1-MN was purchased from Fluka (Buchs, Switzerland). Naphtho[2,1-*b*]thiophene (Banfield *et al.*, 1956), 1-methylnaphtho[2,1-*b*]thiophene (Dann and Kokorudz, 1958), and naphtho[2,3-*b*]thiophene (Carruthers *et al.*, 1962) were synthesized.

7.2.2 Bacterial cultures and culture methods

The isolation and characterization of the three 1-MN-degrading *Pseudomonas* strains BT1, W1, and F were described previously (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1993). The 10-mL inocula used for biotransformation experiments with each of these isolates was 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 (Kropp *et al.*, 1994b) and these were incubated at 28°C on a rotary shaker.

After inoculation, the cultures were given a naphthothiophene either as a solid, for qualitative studies, or as a solution in DCM (50 to 150 µL), 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 co-substrate 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 μL 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 sole carbon source, they were always capable of oxidation of at least a small portion of the substrate and some metabolites were observed to form. However, the isolates were only considered to be capable of growth on a naphthothiophene if they survived 14 days incubation of this 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 second transfer, was demonstrated by monitoring the increase in colony forming units (CFU) over the following 2-week incubation. At various time intervals, 1.0 mL of the culture was removed, serially diluted in 9 mL volumes of sterile phosphate buffer (pH 7.2, 10 mM), and then 0.1 mL of these dilutions were plated onto PCA (Difco; Detroit, MI). After 3 days of incubation of these plates at 28°C, the numbers of colonies present were counted.

Two metabolites of naphtho[2,1-*b*]thiophene were detected and these 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 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 non-acidic products in the DCM extract by back extraction into aqueous 5% $NaHCO_3$. After acidification to pH<2 with concentrated H_2SO_4, 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, v:v). Then 1.0-mL portions were added to each of five flasks of sterile medium together with 50 μL of 1-MN and incubated as follows: one of these 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.

7.2.3 Solvent extraction

Cultures and sterile controls were acidified with 2 M H_2SO_4 to pH<2 and extracted with DCM (4 times 20 mL) to recover substrates and products. The DCM extract was dried over anhydrous Na_2SO_4 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, WI) for cultures incubated with naphtho[2,1-*b*]thiophene; dibenzothiophene (98%, Fluka) for cultures incubated with 1-methyl-naphtho[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.

7.2.4 GC analyses

To screen for the presence of sulfur-containing metabolites, the extracts were analyzed by capillary GC using a 30-m DB-5 column in a HP (Mississauga, ON) model 5890 equipped with a FID and a sulfur-selective FPD. Details of the operating conditions were given previously (Fedorak and Grbic´-Galic´, 1991). The methods routinely used for GC-MS have also been described previously (Saftic´ *et al.*, 1992, 1993). To obtain high resolution GC-MS data, analyses were done in the Mass Spectrometry Laboratory, Chemistry Department, University of Alberta (Fedorak and Westlake, 1986). The operation of the GC-AED for quantitative analyses was outlined by Kropp *et al.* (1997a).

To facilitate GC-MS identification of some of the metabolites, TMS-derivatives of compounds in culture extracts were made by silylating with BSA in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, IL; method 5). As well, some metabolites were methyl-esterified by treating culture extracts with an ethereal solution of diazomethane generated using a Diazald kit (Aldrich).

7.2.5 ^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectroscopy

Metabolites were dissolved in deuterated-DCM and ^{1}H-NMR spectra were recorded on an AM 400 NMR spectrometer by personnel in the NMR Service Laboratory, Chemistry Department, University of Alberta. Development of the sample isolation and purification procedure is given in the Results section.

7.2.6 Total sulfur analysis

DCM extracts were diluted to 1.0 mL with DCM in volumetric flasks and 200-µL portions were applied to filter paper (Whatman #42). After evaporation of the DCM, the

filter paper samples were combusted in sealed Schöninger oxygen flasks and the SO_2 produced was trapped as H_2SO_4 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 $BaClO_4$ (0.01077 M) using Thorin as the endpoint indicator (Fritz and Yamamura, 1955).

7.2.7 Lyophilization and methyl-esterification

Cultures (200 mL) were made alkaline (pH>12) by the addition of 0.5 g 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 H_2SO_4 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.

7.3 RESULTS

The ability of the isolates to grow on the naphthothiophenes as 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 sole carbon source for isolate W1 and compares 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.

7.3.1 Metabolites from naphtho[2,1-*b*]thiophene

When a 13-day-old culture of isolate W1 incubated with naphtho[2,1-*b*]thiophene as 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 saturated 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 (Figure 7.1). 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 (Silverstein *et al.*, 1991).

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 products 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 Figure 7.2. The derivatives were not the expected TMS-ethers of the hydroxybenzothiophenes. The ion at m/z 147 is indicative of the addition of two TMS moieties onto the molecule (Pierce, 1986). TMS derivatives typically lose a methyl group to give a strong $(M-15)^+$ ion which was likely the m/z 323 ion in Figure 7.2. If this was true, then the molecular ion would be at m/z 338, which is not visible in Figure 7.2. 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 gave two isomers of hydroxybenzothiophene carboxylic acid which were present in the culture extract and would react with BSA to give the TMS-derivatives observed. When underivatized, these metabolites were too polar to be chromatographed under the conditions used, but 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. 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 over glass wool in a pasteur pipette to remove the precipitate. After the glass wool was rinsed with 0.5 mL 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 MS with the sample introduced into the source of the mass spectrometer 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 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 (Figure 7.1) 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 (Figure 7.1) 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, because it would be more volatile than hydroxybenzothiophene carboxylic acid. Thus, the hydroxybenzothiophenes (Figure 7.1) 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 was further aided by the use of diazomethane derivatization. Reports in the literature (i.e. March, 1985) suggest that diazomethane is capable of reacting with carboxylic acids and phenols to give methyl-esters and methyl-ethers, respectively. Although I have been able to demonstrate methyl-esterification of authentic standards of 1-naphthoic, 3-methyl-2-thiophenecarboxylic, and 5-methyl-2-thiophenecarboxylic acids, I 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 my 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 Figure 7.3. 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 $C_2H_4O_2$ $(M-60)^+$ from the molecular ion and a subsequent loss of CO (28 amu) would give the ion at m/z 120. These fragmentations are consistent with the structural assignments in Figure 7.3.

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 ^{1}H-NMR spectrum of this methyl-ester was recorded. The ^{1}H-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.

Figures 7.4a 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 Figure 7.4. The aromatic region (7 to 8 ppm) shows the aromatic benzene 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 (Silverstein *et al.*, 1991). 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 one 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 based on their smaller ortho coupling constants. Then, 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 ^{1}H-NMR spectrum of benzothiophene in benzene, acetone and carbon tetrachloride was reported by Balkau and Heffernan (1972) with unambiguous assignments for all 6 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 (Jackman and Sternhell, 1969). 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) (Jackman and Sternhell, 1969). Thus, for the methyl ester of 4-hydroxybenzothiophene-5-carboxylic acid, the carboxymethyl group at position 5 would be 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 (Balkau and Heffernan, 1972) to approximately 0.33 ppm. This is consistent with the difference of 0.37 ppm observed for the protons H-6 and H-7 in metabolite I (Figure 7.4a). Thus, metabolite I was identified as 4-hydroxybenzothiophene-5-carboxylic acid.

Using similar logic, it can be seen that the ^{1}H-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 at even higher field. 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 at even lower field. 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 (Balkau and Heffernan, 1972) to approximately 1.1 ppm. This is what is observed in the ^{1}H-NMR spectrum of metabolite II (Figure 7.4b), 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.

7.3.2 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 $\times 10^4$ CFU/ml to 1.5 $\times 10^7$ CFU/ml after 7 days of incubation in medium containing 52 µmol naphtho[2,1-*b*]thiophene as the sole carbon source. Replicate cultures were prepared for a time course experiment and Figure 7.5 shows the depletion of naphtho[2,1-*b*]thiophene over 2 weeks in cultures of isolate W1 grown on this substrate as sole carbon source. Each time point in Figure 7.5 represents the results from GC-AED analysis of a single culture extract. Figure 7.5 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 (Figure 7.5).

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 7.1 (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 µmol 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 percentage of the sulfur from naphtho[2,1-*b*]thiophene that was detected as metabolites I, II, and the unidentified products was similar in both trials.

Total sulfur analysis of DCM extracts gave a higher recovery of sulfur from naphtho[2,1-*b*]thiophene than was observed by GC-AED analysis (Table 7.1, lines 1 and 2), suggesting that there may be 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.

7.3.3 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 co-substrate, the naphtho[2,1-*b*]thiophene was depleted from the cultures within 7 days in both of the quantitative trials (Table 7.1, lines 3 and 4). As well, comparable amounts of metabolite II were produced (lines 3 and 4) as had been observed in the incubations with naphtho[2,1-*b*]thiophene as sole carbon source (lines 1 and 2). However, when 1-MN was included in the incubations (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 which included 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 accounting for the evaporative loss observed in the sterile control, 59% (line 3) and 56% (line 4) of the sulfur from naphtho[2,1-*b*]thiophene was 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 7.2, line 1). Total sulfur analysis of the extract obtained by this procedure showed an increased recovery of 17.1 μmol organosulfur (32% of the 54 μmol naphtho[2,1-*b*]thiophene given). This increase does not include the 5.8 μmol 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 resulted from microbial incorporation

of sulfate provided in the medium into organic compounds. The observed increase in total sulfur recovery (Table 7.2) suggests that there is a significant portion of sulfur from naphtho[2,1-*b*]thiophene that was present in cultures as organic metabolites that were too polar to recover by DCM extraction of acidified cultures.

Some of the metabolites that were too polar to recover 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 into four replicate cultures of isolate W1 and a sterile control. One culture was extracted immediately and analyzed by GC-AED to show 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 incubated for 98 h detected 4.0 μmol (103%) and 2.7 μmol (96%) of metabolites I and II. 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 sole carbon source. Metabolite II accumulated in cultures of isolate W1 regardless of whether or not 1-MN was present.

7.3.4 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 Figure 7.6. 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 CH_3OH and $C_2H_4O_2$, 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 one equivalent 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 ^{1}H-NMR spectrum of the methyl-esterified metabolite, that was purified from a culture extract by back extraction into 5% $NaHCO_3$ prior to diazomethane derivatization, was recorded, and part of the spectrum is shown in Figure 7.4c. 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 (Figure 7.4a), 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 incubation of the W1 culture is summarized in Table 7.1 (lines 5 and 6). In two separate trials the 1-methylnaphtho[2,1-*b*]thiophene was almost completely degraded, with only 2.5 µmol (5.1%, line 5) and 0.8 µmol (1.5%, line 6) remaining after 7 days incubation. A small amount of the sulfur from the depleted substrate was present as 6 or 7 unidentified minor products, but the vast majority of the organosulfur (34 and 35 µmol, 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 (lines 3 and 4) of 79%. A quantitative sterile control with approximately 50 µmol of 1-methylnaphtho[2,1-*b*]thiophene was not done 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 naphtho[2,1-*b*]thiophene (lines 3 and 4). A qualitative sterile control with 5 mg 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.

7.3.5 Cometabolism of naphtho[2,1-*b*]thiophene by strains BT1 and F

Neither isolate BT1 nor isolate F was capable of growth with naphtho[2,1-*b*]thiophene, but both isolates were able to oxidize this compound when provided with 1-

MN as growth substrate. After 7 days incubation with isolate BT1, the 54 μmol naphtho[2,1-*b*]thiophene was essentially gone (0.1 μmol remaining), and only trace amounts of 13 sulfur-containing metabolites, none of which were consistent with metabolites I and II, were detected (Table 7.1, 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% of the sulfur from naphtho[2,1-*b*]thiophene was observed by total sulfur analysis of the DCM extract (Table 7.1, line 7). Most of the sulfur from naphtho[2,1-*b*]thiophene remained 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 μmol of the original 54 μmol (46%) remaining. However, of the depleted substrate sulfur, only 0.1 and 0.8 μmol were detected as metabolites I and II, respectively, and there was only a small amount (0.3 μmol) of 6 minor unidentified products (Table 7.1, 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 7.1, line 8). Thus, the cometabolism of naphtho[2,1-*b*]thiophene by 1-MN grown cultures of isolates BT1 and F does 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 is 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 7.2, lines 2 and 3). With both isolates there was an increase in the amount of organosulfur detected in cultures that were lyophilized (19.5 μmol and 13.1 μmol 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 exists in the form of organic metabolites that are too polar to extract from acidified cultures with DCM.

7.3.6 Cometabolism of naphtho[2,3-*b*]thiophene by strains W1, BT1, and F

None of the three isolates was capable of growth with 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 14 days with 25 μmol 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 Figure 7.2 (after BSA treatment) or in Figure 7.3 (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 amount of these two metabolites detected in cultures of isolates W1 and F, respectively, corresponds 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, where no metabolites were observed to accumulate over the 14 day incubation.

7.4 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 naphtho[2,3-*b*]thiophene, the observed depletion of the naphthothiophene was 32%, 64%, and 30%, respectively. However, in only 7 days incubation with 1-MN and 54 μmol naphtho[2,1-*b*]thiophene, 97%, 99%, and 53% loss was observed in cultures of isolates W1, BT1, and F, respectively (Table 7.1). 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 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 incubation with little, if any, further degradation of these metabolites occurring (Figure 7.5a). 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 7.7 shows this proposed pathway. This is analagous to the upper pathway for naphthalene

catabolism that converts naphthalene to salicylic acid (Eaton and Chapman, 1992), 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 (Denome *et al.*, 1993), phenanthrene to 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid (Denome *et al.*, 1993; Kiyohara *et al.*, 1994; Sanseverino *et al.*, 1993), and anthracene to 2-hydroxy-3-naphthoic acid (Kiyohara *et al.*, 1994; Sanseverino *et al.*, 1993).

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 favoured for initial dioxygenation. This is consistent with the observation of Kiyohara *et al.* (1994) with *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 that 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 analagous 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 7.1). Total sulfur analysis gave slightly higher recoveries, suggesting that small amounts of DCM extactable organic sulfur may be present that was not detected by GC-AED analysis. 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 is not recovered by DCM extraction, that is not amenable to GC analysis, or even that which is present in numerous minor sulfur-containing peaks detected in the GC-AED chromatogram that were not identified could potentially exist as intermediates in the formation of metabolites I and II or as metabolites that result from 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 similar levels as were observed without 1-MN, but metabolite

I was not observed to accumulate (Table 7.1). 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 is not present in DCM extracts appears to exist, at least in part, in the form of organic metabolites that are too polar to extract with DCM, but were recovered by the lyophilization and methyl-esterification procedure (Table 7.2).

When compared to isolate W1 grown on naphtho[2,1-*b*]thiophene as 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 cosubstrate 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. Since the amount of metabolite II accumulated is nearly that 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 3-methyl-metabolite I, unoxidized substrate, and numerous minor sulfur-containing products as determined by GC-AED analysis (76% and 81%, Table 7.1, 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 7.1). Assuming 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.* (1997b), 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 (Izumi *et al.*, 1994; Lee *et al.*, 1994; Ohshiro *et al.*, 1996; Omori *et al.*, 1992; Wang and Krawiec, 1996; Wang *et al.*, 1996). In studies with the same three *Pseudomonas* strains (Kropp *et al.*, 1997b), 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 7.1 that shows 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 analogue of metabolite I as the major product. In fact, the 3-methyl analogue of metabolite II was not detected (Table 7.1, lines 5 and 6), so the dioxygenase attack at positions 6 and 7 was not significant. As well, 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 extract 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 (Eaton and Nitterauer, 1994; Fedorak and Grbic´-Galic´, 1991; Kropp *et al.*, 1994a, 1996; Saftic´ *et al.*, 1992). Those investigations showed that the thiophene ring is oxidized and cleaved to give 2-mercaptophenylglyoxalates which are often detected as the benzothiophene-2,3-diones formed by acid-catalyzed dehydration during culture extraction (Eaton and Nitterauer, 1994). Also, Eaton and Nitterauer (1994) showed that both the homocyclic and 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 my 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 prevented its further degradation.

Isolate W1 is known to oxidize the methyl groups of some methyl- and dimethylbenzothiophenes to methanols and carboxylic acids (Kropp *et al.*, 1994a, 1996) and of some methyldibenzothiophenes to methanols (Saftic´ *et al.* 1993). 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 7.1), so if the methanol and/or carboxylic acid were formed, they were minor products.

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 7.1, line 8), but were not even detected in extracts of BT1 cultures (Table 7.1, 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 7.2).

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 and 6-hydroxybenzothiophene-5-carboxylic acids. These metabolites would result from dioxygenase attack at position 5 and 6, and position 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 (a) the effect of 1-MN on the further degradation of 4-hydroxybenzothiophene-5-carboxylic acid (metabolite I) in cultures of isolate W1 and (b) the effect of the methyl group of 1-methylnaphtho[2,1-*b*]thiophene on its metabolism. The effect of 1-MN on the subsequent degradation of metabolite I from bacterial metabolism of naphtho[2,1-*b*]thiophene suggests that co-contaminants 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 condensed thiophenes. The methyl group of 1-methylnaphtho[2,1-*b*]thiophene influenced the location of dioxygenase attack of the naphtho[2,1-*b*]thiophene nucleus and the resistance of the 4-hydroxybenzothiophene-5-carboxylic acid that was thus formed to further degradation. As has been observed previously (Bayona *et al.*, 1986; Kropp *et al.*, 1994a, 1997a), the methyl-substitution pattern of condensed thiophenes can also influence the microbial metabolism of these compounds. In addition, the availability of the 1-methylnaphtho[2,1-*b*]thiophene provided some clues into the mechanism for further degradation of metabolite I, but these have not been elucidated.

Table 7.1 Quantification of naphtho[2,1-*b*]thiophene (NT), 1-methylnaphtho[2,1-*b*]thiophene (1-MeNT), and their sulfur-containing metabolites as determined by GC-AED analyses of DCM extracts of acidified cultures of three *Pseudomonas* strains 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.

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

[a] 4-Hydroxybenzothiophene-5-carboxylic acid
[b] 5-Hydroxybenzothiophene-4-carboxylic acid
[c] The µmol organosulfur present in other minor sulfur peaks which were not identified and the number of peaks [in brackets] which are represented by this total.
[d] With isolate W1 the results of two separate trials are shown for each set of substrates.
[e] 52 µmol naphtho[2,1-*b*]thiophene or 1-methylnaphtho[2,1-*b*]thiophene initially in the culture.
[f] 54 µmol naphtho[2,1-*b*]thiophene initially in the culture.
[g] 49 µmol 1-methylnaphtho[2,1-*b*]thiophene initially in the culture.
[h] The 3-methyl-analogue of metabolite I.
[i] The 3-methyl-analogue of metabolite II.
[j] ND=not determined.

Table 7.2 Comparison of the recovery of organosulfur from naphtho[2,1-*b*]thiophene (NT) by DCM extraction of acidified cultures and by lyophilization. The three *Pseudomonas* strains were incubated with 1-MN and 54 µmol naphtho[2,1-*b*]thiophene for 7 days. Recovery was detemined by total sulfur analysis of extracts obtained by the two methods.

		µmol organosulfur recovered by			Additional organosulfur found with lyophilization[b]
Strain	Substrates	DCM Extraction	Lyophilization[a]		(µmol)
W1	1-MN & NT	9.1	32	(5.8)	17.1
BT1	1-MN & NT	11	34	(3.5)	19.5
F	1-MN & NT	17	34	(3.9)	13.1

[a]The number in brackets represents the µmol organosulfur that was also observed by total sulfur analysis of controls that did not receive naphtho[2,1-*b*]thiophene.

[b]Additional sulfur found = amount found by lyophilization – (amount found in control + amount in DCM extract).

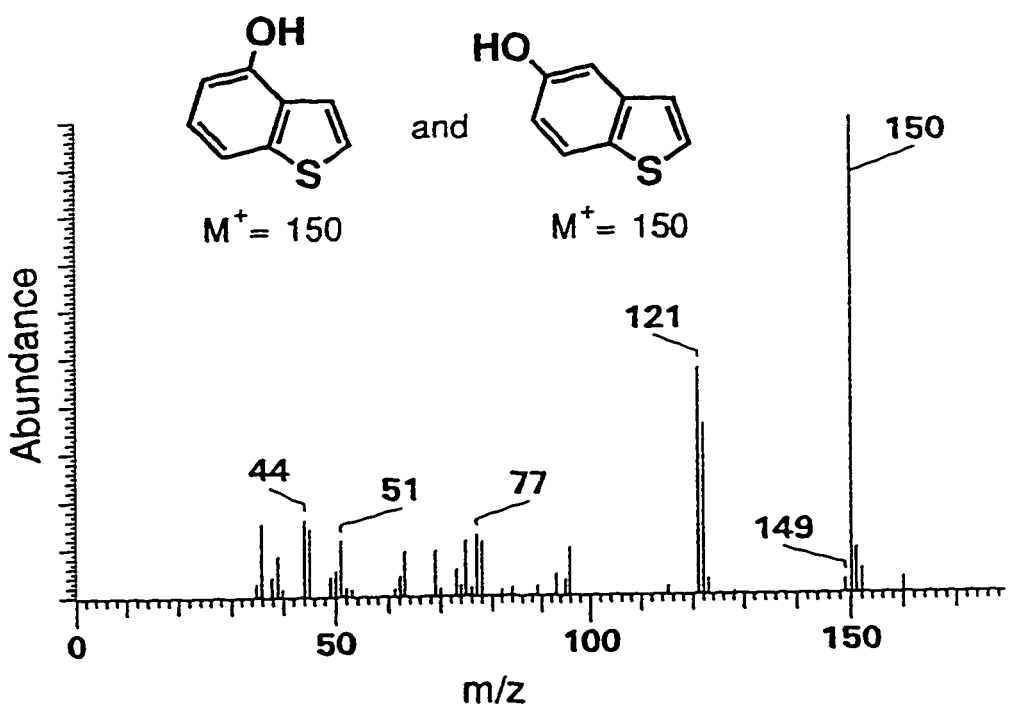


Figure 7.1 From GC-MS analysis, the mass spectrum observed for the two abundant sulfur-containing products detected during analysis of underivatized extracts of cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene. These products were identified as 4- and 5-hydroxybenzothiophene, which formed by thermal decarboxylation in the GC injection port liner of the metabolites 4-hydroxybenzothiophene-5-carboxylic acid and 5-hydroxybenzothiophene-4-carboxylic acid.

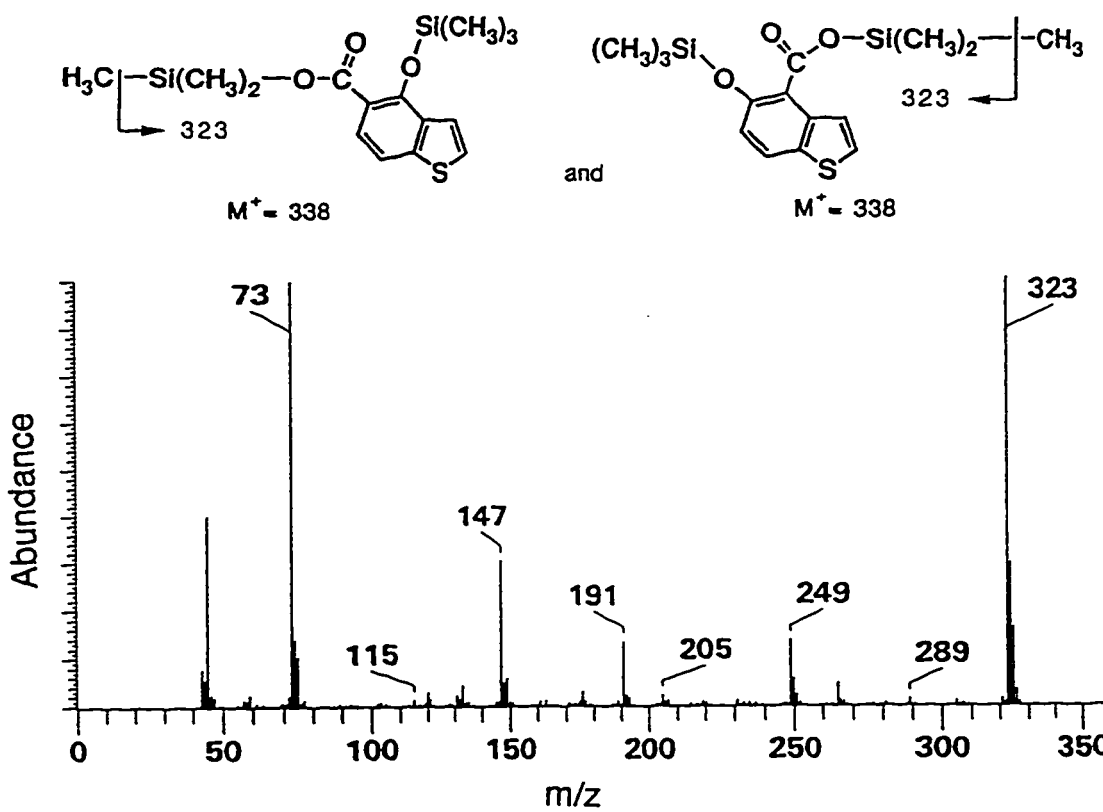


Figure 7.2 From GC-MS analysis, the mass spectrum observed for the two abundant sulfur-containing metabolites detected in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene after treatment with BSA. These metabolites were identified as 4-hydroxybenzothiophene-5-carboxylic and 5-hydroxybenzothiophene-4-carboxylic acids.

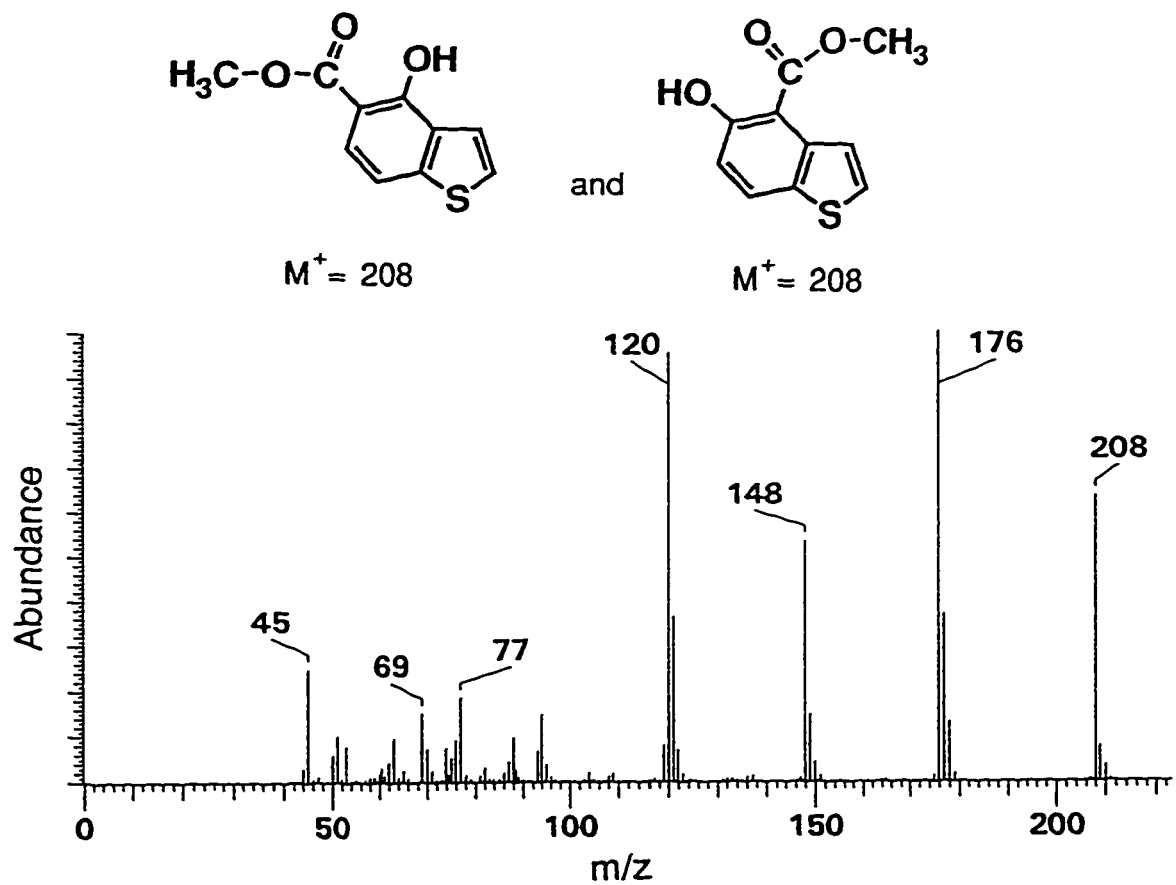


Figure 7.3 From GC-MS analysis, the mass spectrum observed for the two abundant sulfur-containing metabolites detected in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene after treatment with diazomethane. These metabolites were identified as 4-hydroxybenzothiophene-5-carboxylic and 5-hydroxybenzothiophene-4-carboxylic acids.

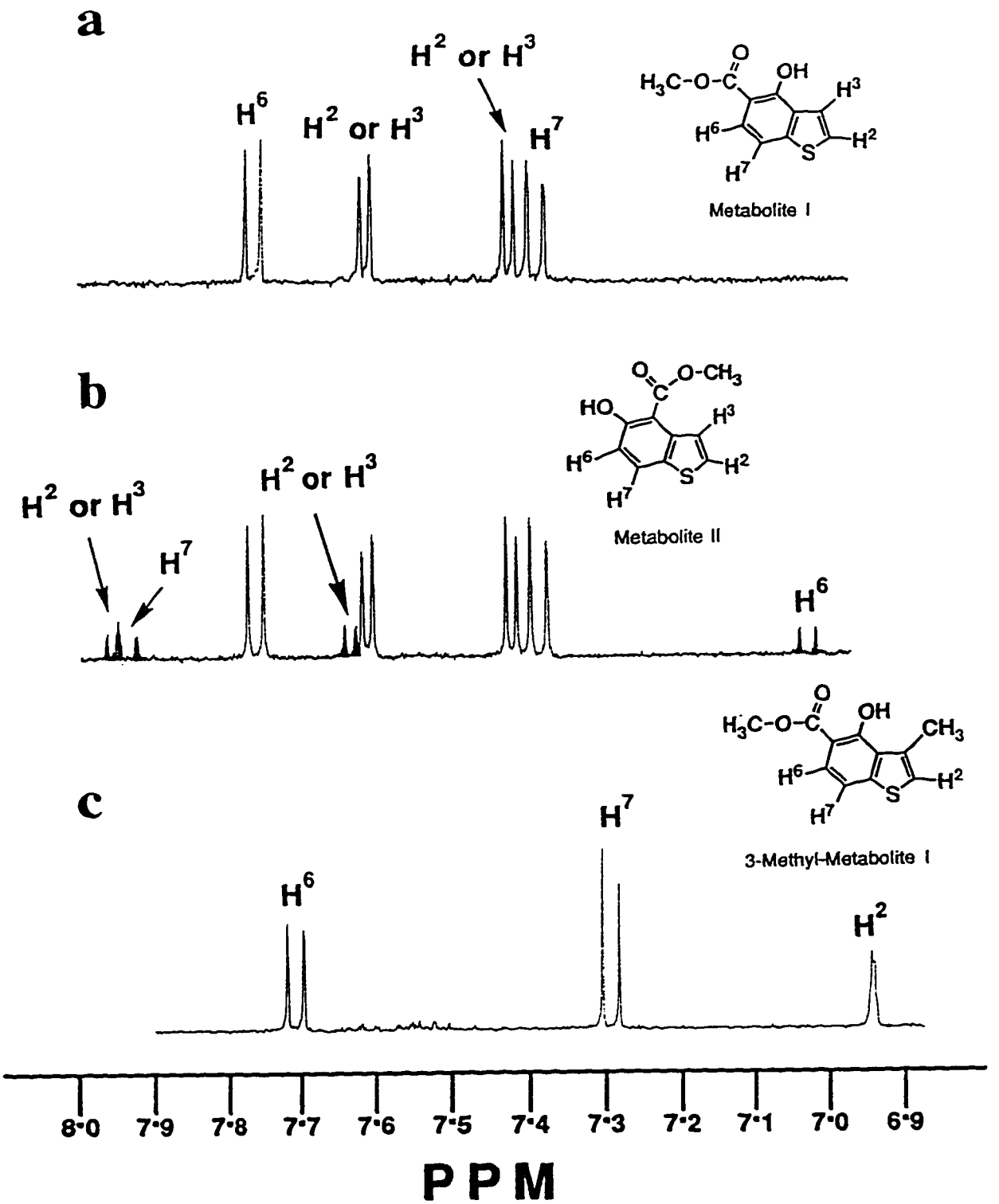


Figure 7.4 400 MHz ^{1}H-NMR spectra of methyl-esters of metabolite I (a) and a mixture of metabolites I and II (b) detected in cultures of isolate W1 grown on naphtho[2,1-*b*]thiophene, and of the only abundant metabolite (c) detected in cultures of isolate W1 grown on 1-MN in the presence of 1-methylnaphtho[2,1-*b*]thiophene. These metabolites were derivatized with diazomethane prior to analysis. The shaded signals in panel (b) are from metabolite II.

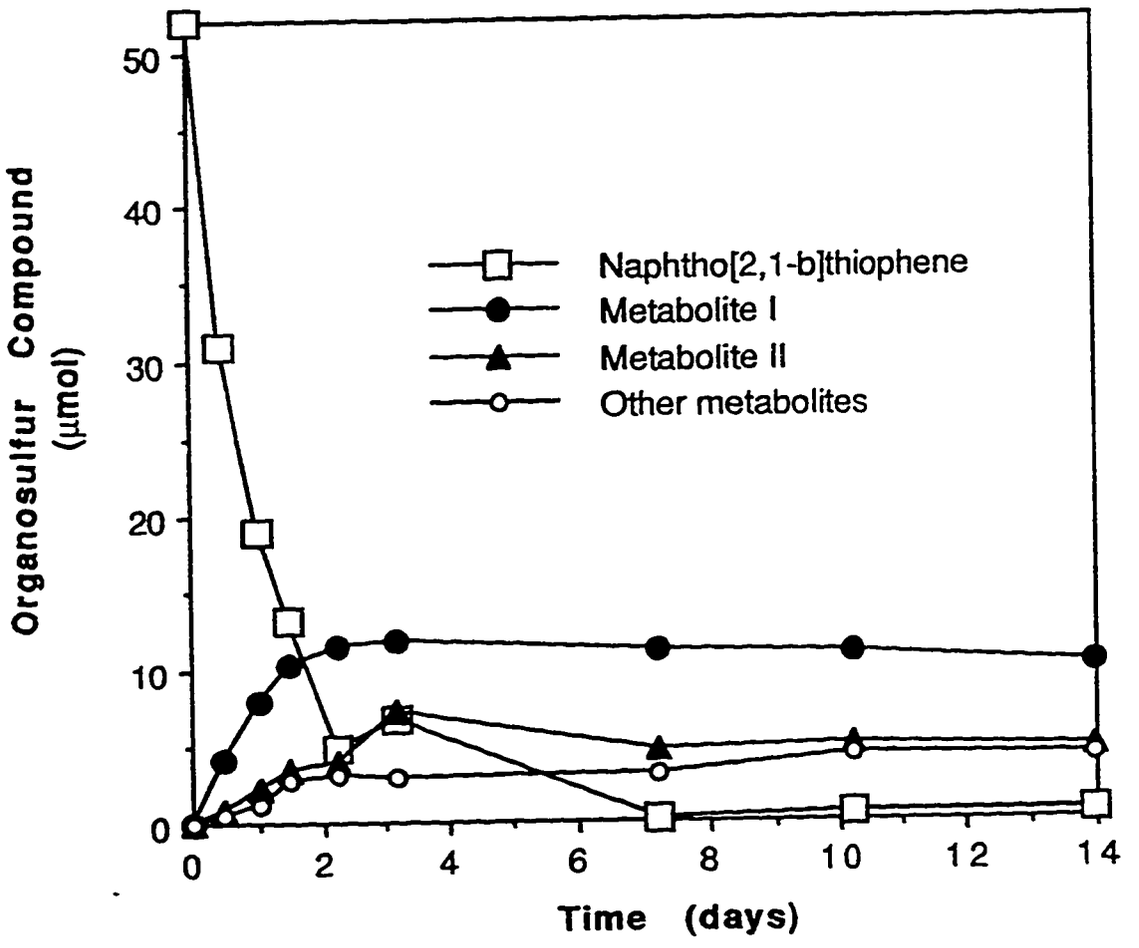


Figure 7.5 From GC-AED analysis, the oxidation of naphtho[2,1-*b*]thiophene to metabolites I, II, and other minor, unidentified sulfur-containing products in cultures of isolate W1 incubated with naphtho[2,1-*b*]thiophene as sole carbon source.

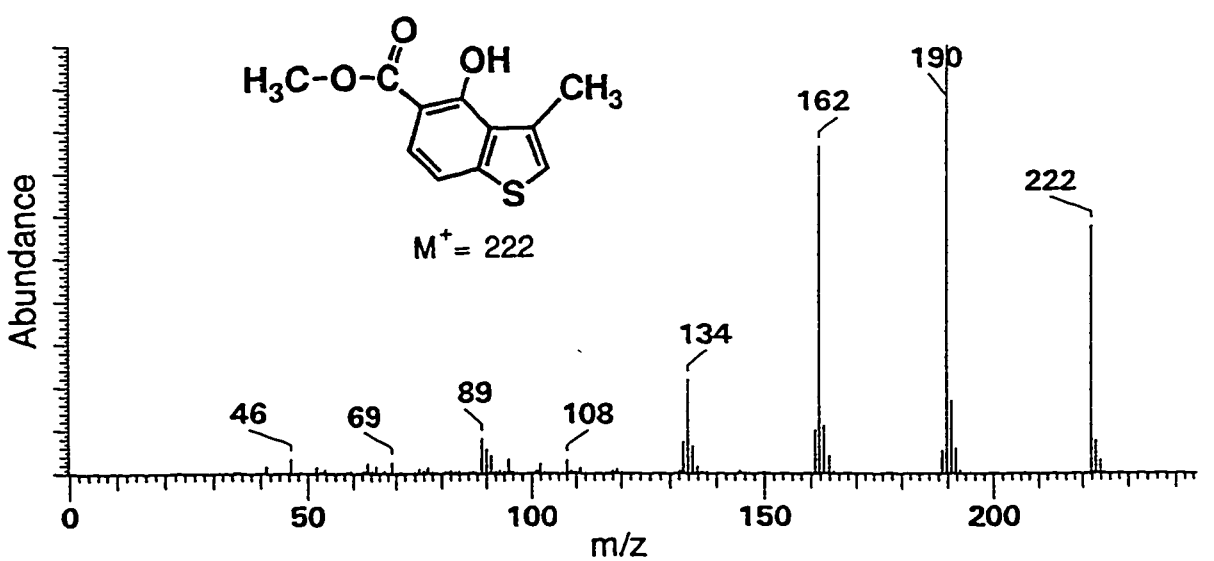


Figure 7.6 From GC-MS analysis, the mass spectrum of the only abundant metabolite detected in cultures of isolate W1 grown on 1-MN in the presence of 1-methylnaphtho[2,1-*b*]thiophene after treatment with diazomethane. The metabolite was identified as 4-hydroxy-3-methylbenzothiophene-5-carboxylic acid.

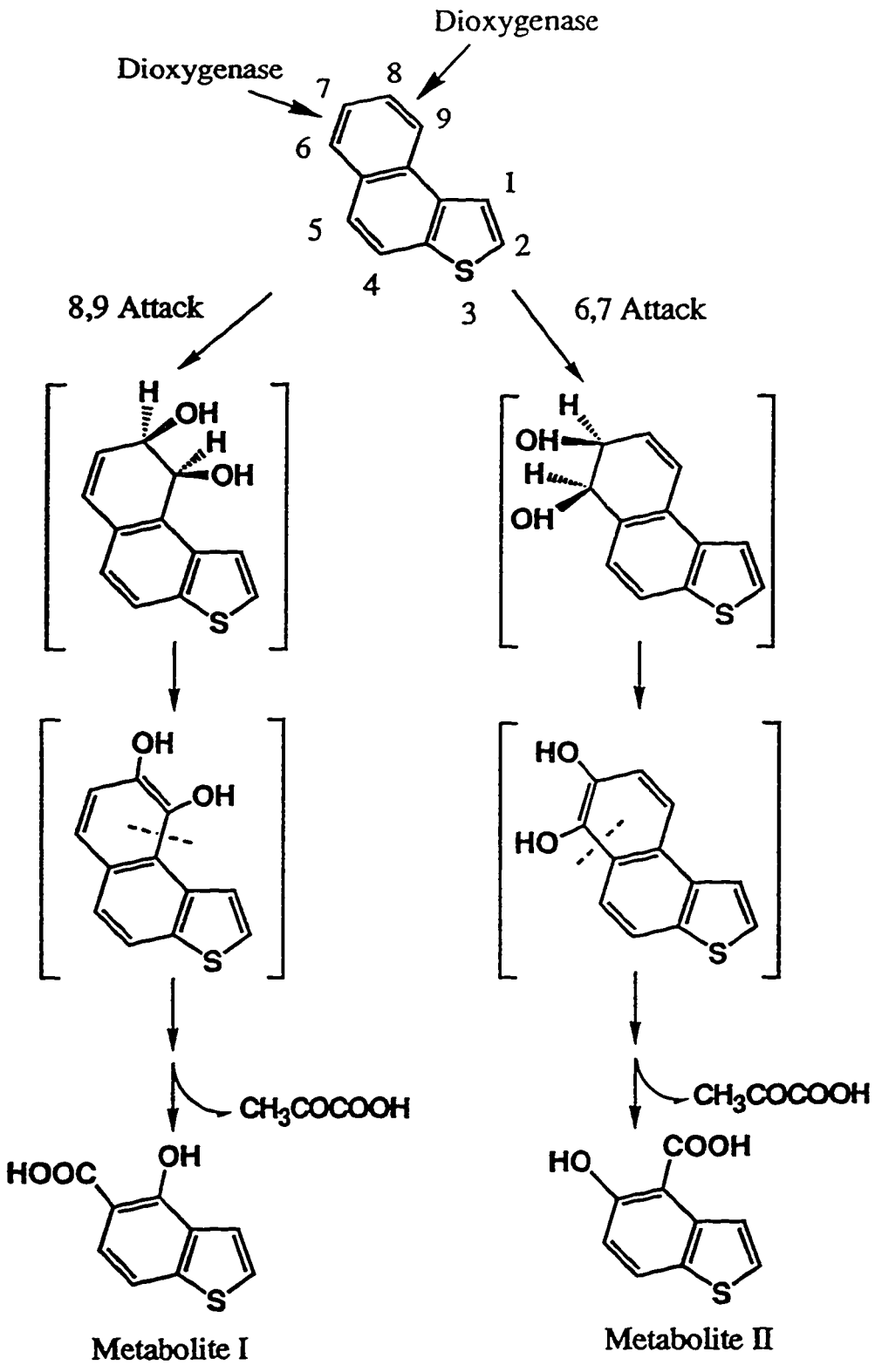


Figure 7.7 Proposed pathways for the formation of metabolites I and II. The compounds in brackets are hypothesized intermediates in the formation of metabolites I and II that were not detected. The dashed line (----) indicates the proposed location of meta-cleavage of dihydroxy-substituted naphtho[2,1-*b*]thiophenes.

7.5 LITERATURE CITED

Andersson, J. T., and B. Schmid. 1995. Polycyclic aromatic sulfur heterocycles. IV. Determination of polycyclic aromatic compounds in a shale oil with the atomic emission detector. J. Chromatogr. A. 693: 325-338.

Balkau, F., and M. L. Heffernan. 1972. Deceptive simplicity in ABMX N.M.R. spectra. III. The spectrum of benzothiophen. Aust. J. Chem. 25: 327-333.

Banfield, J. E., W. Davies, B. C. Ennis, S. Middleton, and Q. N. Porter. 1956. The synthesis of thionaphthen derivatives. Part I. The cyclisation of arylthioacetaldehyde diethyl acetals. J. Chem. Soc. 1956: 2603-2608.

Bayona, J. M., J. Albaigés, A. M. Solanas, R. Parés, P. Garrigues, and M. Ewald. 1986. Selective aerobic degradation of methyl-substituted polycyclic aromatic hydrocarbons in petroleum by pure microbial cultures. Intern. J. Environ. Anal. Chem. 23: 289-303.

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.

Burchill, P., A. A. Herod, and E. Pritchard. 1982a. Identification of sulphur heterocycles in coal tar and pitch. J. Chromatogr. 242: 51-64.

Burchill, P., A. A. Herod, and E. Pritchard. 1982b. Determination of nitrogen-sulphur mixed heteroaromatic compounds and sulfur heterocycles in an anthracene oil. J. Chromatogr. 242: 65-76.

Carruthers, W., A. G. Douglas, and J. Hill. 1962. Synthesis of naphtho[2,3-*b*]thiophen. J. Chem. Soc. 1962: 704-708.

Crawford, D. L., and R. K. Gupta. 1990. Oxidation of dibenzothiophene by *Cunninghamella elegans*. Current Microbiol. 21: 229-231.

Dann, O., and M. Kokorudz. 1958. Ringschlüsse von arylketosulfiden zu thionaphthenen. Chem. Ber. 91: 172-180.

Denome, S. A., D. C. Stanley, E. S. Olson, and K. D. Young. 1993. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: Complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. 175: 6890-6901.

Drushel, H. V., and A. L. Sommers. 1967. Isolation and characterization of sulfur compounds in high-boiling petroleum fraction. Anal. Chem. 39: 1819-1829.

Eaton, R. W., and P. J. Chapman. 1992. Bacterial metabolism of naphthalene: Construction and use of recombinant bacteria to study ring cleavage reactions of 1,2-dihydroxynaphthalene and subsequent reactions. J. Bacteriol. 174: 7542-7554.

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

Fayad, N. M., and E. Overton. 1995. A unique biodegradation pattern of the oil spilled during the 1991 Gulf war. Mar. Pollut. Bull. 30: 239-246.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Fedorak, P. M., and D. W. S. Westlake. 1983. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Can. J. Microbiol. 29: 291-296.

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.

Fedorak, P. M., and D. W. S. Westlake. 1986. Fungal metabolism of *n*-alkylbenzenes. Appl. Environ. Microbiol. 51: 435-437.

Finnerty, W. R., and M. Robinson. 1986. Microbial desulfurization of fossil fuels: A review. Biotechnol. Bioeng. Symp. 16: 205-221.

Finnerty, W. R., K. Shockley, and H. Attaway. 1983. Microbial desulfurization and denitrogenation of hydrocarbons. In: Microbial Enhanced Oil Recovery. Zajic, J. E., D. G. Cooper, T. R. Jack, and N. Kosaric, Eds. Pennwell Books: Oklahoma; p. 83-91.

Foght, J. M., P. M. Fedorak, M. R. Gray, and D. W. S. Westlake. 1990. Microbial desulfurization of petroleum. In: Microbial Mineral Recovery. Ehrlich, H. L., and C. L. Brierley, Eds. McGraw-Hill Publishing Co.: New York; p. 379-407.

Foght, J. M., and D. W. S. Westlake. 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. Can. J. Microbiol. 34: 1135-1141.

Fritz, J. S., and S. S. Yamamura. 1955. Rapid microtitration of sulfate. Anal. Chem. 27: 1461-1464.

Gallagher, J. R., E. S. Olson, and D. C. Stanley. 1993. Microbial desulfurization of dibenzothiophene: A sulfur specific pathway. FEMS Microbiol. Lett. 107: 31-36.

Hou, C. T., and A. I. Laskin. 1976. Microbial conversion of dibenzothiophene. Dev. Ind. Microbiol. 17: 351-362.

Izumi, Y., T. Oshiro, H. Ogino, Y. Hine, and M. Shimao. 1994. Selective desulfurization of dibenzothiophene by *Rhodococcus erythropolis* D-1. Appl. Environ. Microbiol. 60: 223-226.

Jackman, L. M., and S. Sternhell. 1969. Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry. Pergamon Press: London; p. 202.

Kargi, F., and J. M. Robinson. 1984. Microbial oxidation of dibenzothiophene by the thermophilic organism *Sulfolobus acidocaldarius*. Biotechnol. Bioeng. 26: 687-690.

Kiyohara, H., S. Torigoe, N. Kaida, T. Asaki, T. Iida, H. Hayashi, and N. Takizawa. 1994. Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. J. Bacteriol. 176: 2439-2443.

Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada. 1970. Microbial conversion of petro-sulfur compounds. Part III. Isolation and identification of products from dibenzothiophene. Agr. Biol. Chem. 34: 1320-1324.

Kodama, K., K. Umehara, K. Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. 1973. Identification of microbial oxidation products from dibenzothiophene and its proposed oxidation pathway. Agr. Biol. Chem. 37: 45-50.

Kropp, K. G., J. T. Andersson, and P. M. Fedorak. 1997a. Biotransformations of three dimethyldibenzothiophenes by pure and mixed bacterial cultures. Environ. Sci. Technol. 31: 1547-1554.

Kropp, K. G., J. T. Andersson, and P. M. Fedorak. 1997b. Bacterial transformations of 1,2,3,4-tetrahydrodibenzothiophene and dibenzothiophene. Appl. Environ. Microbiol., in press.

Kropp, K. G., J. A. Gonçalves, J.T. Andersson, and P. M. Fedorak. 1994a. Bacterial transformations of benzothiophene and methylbenzothiophenes. Environ. Sci. Technol. 28: 1348-1356.

Kropp, K. G., J. A. Gonçalves, J.T. Andersson, and P. M. Fedorak. 1994b. Microbially mediated formation of benzonaphthothiophenes from benzo[b]thiophenes. Appl. Environ. Microbiol. 60: 3624-3631.

Kropp, K. G., S. Saftic´, J. T. Andersson, and P. M. Fedorak. 1996. Transformations of six isomers of dimethylbenzothiophenes by three *Pseudomonas* strains. Biodegradation 7: 203-221.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* species. Appl. Environ. Microbiol. 34: 783-790.

Later, D. W., M. L. Lee, K. D. Bartle, R. C. Kong, and D. L. Vassilaros. 1981. Chemical class separation and characterization of organic compounds in synthetic fuels. Anal. Chem. 53: 1612-1620.

Lee, M. K., J. D. Senius, and M. J. Grossman. 1995. Sulfur-specific microbial desulfurization of sterically hindered analogs of dibenzothiophene. Appl. Environ. Microbiol. 61: 4362-4366.

March, J. 1985. Advanced Organic Chemistry: Reactions, Mechanisms and Structures, 3rd edition. John Wiley & Sons, Inc.: New York; p. 344.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Mormile, M. R., and R. M. Atlas. 1989. Biotransformation of dibenzothiophene to dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. 35: 603-605.

Nishioka, M. 1988. Aromatic sulfur compounds other than condensed thiophenes in fossil fuels: enrichment and identification. Energy Fuels 2: 214-219.

Ohshiro, T., T. Hirata, and Y. Izumi. 1996. Desulfurization of dibenzothiophene derivatives by whole cells of *Rhodococcus erythropolis* H-2. FEMS Microbiol. Lett. 142: 65-70.

Omori, T., L. Monna, Y. Saiki, and T. Kodama. 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. SY1. Appl. Environ. Microbiol. 58: 911-915.

Pierce, A. E. 1986. Silylation of Organic Compounds. Pierce Chemical Company: Rockford, IL; p. 33-39.

Saftic´, 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.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Sagardía, F., J. J. Rigau, A. Martínez-Lahoz, F. Fuentes, C. López, and W. Flores. 1975. Degradation of benzothiophene and related compounds by a soil *Pseudomonas* in an oil-aqueous environment. Appl. Microbiol. 29: 722-725.

Sanseverino, J., B. M. Applegate, J. M. Henry King, and G. S. Sayler. 1993. Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene. Appl. Environ. Microbiol. 59: 1931-1937.

Silverstein, R. M., G. C. Bassler, and T. C. Morrill. 1991. Spectrometric Identification of Organic Compounds. John Wiley & Sons, Inc.: New York.

Speight, J. G. 1980. The Chemistry and Technology of Petroleum. Marcel Dekker Inc.: New York.

van Afferden, M., S. Schacht, J. Klein, and H. G. Trüper. 1990. Degradation of dibenzothiophene by *Brevibacterium* sp. DO. Arch. Microbiol. 153: 324-328.

Wang, Z., M. Fingas, and G. Sergy. 1995. Chemical characterization of crude oil residues from an arctic beach by GC/MS and GC/FID. Environ. Sci. Technol. 29: 2622-2631.

Wang, P., A. E. Humphrey, and S. Krawiec. 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in continuous cultures. Appl. Environ. Microbiol. 62: 3066-3068.

Wang, P., and S. Krawiec. 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in batch and fed-batch cultures. Appl. Environ. Microbiol. 62: 1670-1675.

Westlake, D. W. S. 1983. Microbial activities and changes in the chemical and physical properties of oil. In: Proceedings of the 1982 International Conference on Microbial Enhancement of Oil Recovery. Donaldson E. C. and J. B. Clark, Eds. Technology Transfer Branch, Bartlesville Energy Technology Centre: Bartlesville, Oklahoma; p. 102-111.

Willey, C., M. Iwao, R. N. Castle, and M. L. Lee. 1981. Determination of sulfur heterocycles in coal liquids and shale oils. Anal. Chem. 53: 400-407.

8. OVERALL DISCUSSION AND SUGGESTIONS FOR FURTHER RESEARCH

8.1 OVERALL DISCUSSION

The biodegradation of petroleum leads to a decrease in the content of saturate and aromatic fractions coupled with a corresponding increase in the content of polar N-, S-, and O-containing materials, which is greater than would result simply from enrichment caused by the loss of the saturate and aromatic fractions (Jobson *et al.*, 1972). This is likely due to the accumulation of oxygenated metabolites from the degradative processes that deplete the saturate and aromatic compounds, which are not completely mineralized. The nature of the compounds in this polar fraction will influence the ability of bioremediation to reduce the toxicity of a petroleum-contaminated environment, which is the aim of bioremediation as an engineered cleanup technology. However, the identification of these metabolites within the complex mixture that constitutes the polar fraction of biodegraded petroleum is very difficult due to the lack of suitable analytical methods. This hinders the assessment of the toxicological properties of these compounds.

The research described in this dissertation has identified more than 80 metabolites of 21 pure condensed thiophenes, that accumulate at least transiently and, in some cases, as dead-end products, in pure cultures of the 1-MN-degrading *Pseudomonas* spp. W1, BT1, and F. As well, in some cases, mixed cultures of petroleum-degrading bacteria and mixed cultures inoculated with an environmental sample (i.e. river water) were used to investigate the biodegradation of condensed thiophenes in the presence of petroleum or the aromatic fraction of petroleum. Metabolites were also identified in these more complex systems which had been supplemented with condensed thiophenes to concentrations much higher than naturally present in crude oil. These metabolites, which have been identified in culture systems that are simplified relative to contaminated environments, may also form during the biodegradation of condensed thiophene-containing crude oil in contaminated environments. If so, they would contribute to the increase in the content of polar materials that is observed to result from the biodegradation of crude oils and therefore may influence the toxicity of residual biodegraded oil.

In previous studies, *Pseudomonas* sp. BT1 was shown to oxidize the thiophene ring of benzothiophene and methylbenzothiophenes that were substituted on the homocyclic ring leading to the formation of benzothiophene-2,3-diones (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992). In this investigation, isolates W1 and F also produced benzothiophene-2,3-diones from oxidation of benzothiophene and homocyclic ring-

substituted methylbenzothiophenes (Chapter 2), and together with isolate BT1, from oxidation of 4,6- and 4,7-dimethylbenzothiophene (Chapter 4).

Isolates W1, BT1, and F were also previously shown to oxidize, cleave, and degrade the unsubstituted homocyclic ring of methyldibenzothiophenes with the resulting formation of methyl-substituted benzothiophene-2,3-diones (Saftic´ *et al.*, 1993). In this investigation, attack and cleavage of the unsubstituted ring of 3,4-dimethyldibenzothiophene and of a methyl-substituted ring of 2,8-dimethyldibenzothiophene yielded 6,7-dimethyl- and 5-methylbenzothiophene-2,3-dione, respectively, in pure and mixed bacterial cultures (Chapter 5). Benzothiophene-2,3-dione was also detected as a metabolite from oxidation of dibenzothiophene by isolates W1, BT1, BT1d, and F (Chapter 6). However, the analogous product 4,5,6,7-tetrahydrobenzothiophene-2,3-dione was not detected from the observed attack and degradation of the aromatic ring of 1,2,3,4-tetrahydrodibenzothiophene by 1-MN-grown cultures of isolates W1, BT1, and F (Chapter 6). As well, naphtho[2,1-*b*]- and -[2,3-*b*]thiophene, which have a terminal thiophene ring analogous to that of benzothiophene, were not observed to yield naphtho[2,1-*b*]thiophene-1,2-dione or naphtho[2,3-*b*]thiophene-2,3-dione, respectively, in cultures of isolates W1, BT1, or F (Chapter 7), as might be expected from studies with benzothiophenes (Chapter 2; Chapter 4; Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992; Eaton and Nitterauer, 1994).

Eaton and Nitterauer (1994) showed that benzothiophene-2,3-dione, detected in culture extracts of an isopropylbenzene-degrading bacterium incubated with benzothiophene, resulted from acid-catalyzed dehydration of 2-mercaptophenylglyoxalate during culture extraction. Thus, the numerous benzothiophene-2,3-diones which have been detected during this investigation in extracts of acidified cultures incubated with benzo- and dibenzothiophenes indicate that degradation of these compounds includes thiophene ring cleavage. It also suggests that the pathways for biodegradation of dibenzothiophenes converge with those of benzothiophenes, since mercaptophenylglyoxalates, detected as benzothiophene-2,3-diones, are products common to cultures incubated with both of these classes of condensed thiophenes.

Previous reports also demonstrated that 1-MN-grown cultures of isolate BT1 catalyzed oxidation of the sulfur atom of 2-methyl-, 3-methyl-, 7-methyl- and 2,3-dimethylbenzothiophene to yield sulfoxides and sulfones (Fedorak and Grbic´-Galic´, 1991; Saftic´ *et al.*, 1992). In this investigation, sulfoxides and sulfones were detected in extracts of cultures of isolates W1 and F incubated with benzothiophene and 2-, 3-, and 7-methylbenzothiophene (Chapter 2), and in extracts of cultures of isolates W1, BT1, and F incubated with 2,3-, 2,7-, and 3,7-dimethylbenzothiophene (Chapter 4).

Furthermore, benzonaphthothiophenes were observed (Chapters 2 and 3) to form in cultures incubated with benzothiophene, 4-, 5-, and 7-methylbenzothiophene, a mixture of 4- and 6-methylbenzothiophene, and with 4,6- and 4,7-dimethylbenzothiophene (Chapter 4). The formation of these benzonaphthothiophenes was shown to be catalyzed by microbial oxidation of benzothiophenes that are unsubstituted at positions 2 and 3 to their corresponding sulfoxides (Chapter 3). Thus, the detection of these high-molecular-weight compounds suggests that sulfoxidation did occur, even though the corresponding benzothiophene sulfoxides were not always detected because they underwent further abiotic condensation reactions. The sulfoxide and sulfone of naphtho[2,1-*b*]- and -[2,3-*b*]thiophene were not detected in extracts of cultures of isolates W1, BT1, and F, nor were the corresponding high-molecular-weight products (Chapter 7), which might be expected to form after oxidation to the sulfoxide because the unsubstituted thiophene ring would be amenable to a Diels-Alder-type condensation reaction (Chapter 3).

Isolates W1, BT1, and F were also previously shown to oxidize 2- and 4-methyldibenzothiophene to their corresponding sulfones (Saftic´ *et al.*, 1993). In studies with 2,8-, 3,4-, and 4,6-dimethyldibenzothiophenes (Chapter 5), sulfoxides and sulfones were not detected in pure cultures of these three isolates, nor were they detected as products in mixed cultures of petroleum-degrading bacteria. However, the oxidation of the sulfur atom of 1,2,3,4-tetrahydrodibenzothiophene and dibenzothiophene in pure cultures of isolates W1 and F yielded the sulfoxide and sulfone as the most abundant products detected (Chapter 6). Recent reports suggest that the observed sulfoxidation of benzo- and dibenzothiophenes is likely a fortuitous oxidation catalyzed by the aryl dioxygenase enzymes that allow growth of these isolates on 1-MN (Allen *et al.*, 1995; Selifonov *et al.*, 1996). However, the sulfoxidation of dibenzothiophene to its sulfone was not a dead-end pathway for isolate BT1, which was shown to further metabolize dibenzothiophene sulfone when provided with 1-MN as a growth substrate (Chapter 6). The resulting metabolites were not detected by the extraction and analytical methods used.

Previous studies with isolate BT1 and methyl-substituted benzo- (Saftic´ *et al.*, 1992) and dibenzothiophenes (Saftic´ *et al.*, 1993) did not detect metabolites of these condensed thiophenes that resulted from oxidation of the methyl groups to the corresponding methanols and carboxylic acids. However, oxidation of the methyl groups of methyldibenzothiophenes to give dibenzothiophene methanols was observed with isolates W1 and F (Saftic´ *et al.*, 1993). This is consistent with the ability of these isolates to oxidize the methyl group of 1-MN to form 1-naphthalene methanol and 1-naphthoic acid, which are dead-end products that accumulate in cultures and do not support the growth of these isolates (Saftic´ *et al.*, 1993; Chapter 2).

In this investigation, methanols and/or carboxylic acids were produced by isolates W1 and F from all methylbenzothiophenes (Chapter 2) and nearly all dimethylbenzothiophenes tested (Chapter 4). Furthermore, isolate BT1 was shown to oxidize the methyl groups of 2,3-, 2,7-, and 3,7-dimethylbenzothiophene yielding methanols and/or carboxylic acids as minor products after 7 days incubation (Chapter 4). After 1 or 2 days incubation, methanols and carboxylic acids were the only major products detected in cultures of isolate BT1 incubated with 3,5-dimethylbenzothiophene. These were only transiently accumulating metabolites which were subsequently degraded within 3 days incubation, resulting in the formation of other metabolites that were not detected by the extraction and analytical methods used (Chapter 4). This further degradation of methanol and carboxylic acid metabolites of 3,5-dimethylbenzothiophene by isolate BT1 is consistent with the ability of isolate BT1 to oxidize the methyl group of 1-MN to 1-naphthalene methanol and 1-naphthoic acid, and subsequently degrade these methyl group oxidation products (Chapter 4). Indeed, isolate BT1 was shown to be capable of growth on 1-naphthalene methanol and 1-naphthoic acid as sole carbon sources (Chapter 4).

The monooxygenation of benzylic methyl and methylene groups in naphthenoaromatic and methyl-substituted aromatic compounds has been reported to be a fortuitous activity of the naphthalene dioxygenase enzyme whose normal physiological function is the dioxygenation of aromatic rings as a precursor to ring cleavage (Selifonov *et al.*, 1996). If this mechanism is responsible for the oxidation of methyl groups of substituted benzo- and dibenzothiophenes by isolates W1, BT1, and F, it is not a dead-end pathway for isolate BT1 which is able to further metabolize the carboxy-substituted products (Chapter 4).

Metabolites consistent with the oxidation of the methyl groups of dimethyldibenzothiophenes (Chapter 5) and 1-methylnaphtho[2,1-*b*]thiophene (Chapter 7) were not detected. It is conceivable that both of the methyl groups of a dimethyldibenzothiophene could be oxidized to give a dicarboxylic acid which would be too polar to extract and analyze by the methods used (Chapter 5). However, in the studies with 1-methylnaphtho[2,1-*b*]thiophene and 1-MN-grown cultures of isolate W1, a sulfur mass balance was obtained in the quantitative studies, but methyl group oxidation products were not detected (Chapter 7).

The formation of 2-mercaptophenylglyoxalates, detected in extracts of acidified cultures as benzothiophene-2,3-diones, the sulfoxidation to form sulfoxides and sulfones, and the oxidation of methyl groups to methanols and carboxylic acids have all been observed to some extent as general oxidation pathways with both benzo- and dibenzothiophenes. A final class of metabolites observed as products from both benzo- and

dibenzothiophenes are the benzothiophene-2(3*H*)- and -3(2*H*)-ones. These metabolites of 4,6- and 4,7-dimethylbenzothiophene were detected in cultures of isolates W1, BT1, and F and presumed to form by acid-catalyzed dehydration of 2,3-dihydrodiols of 4,6- and 4,7-dimethylbenzothiophene during culture extraction (Chapter 4). 6,7-Dimethylbenzothiophene-2(3*H*)- and -3(2*H*)-one were detected as metabolites that resulted from degradation of the unsubstituted homocyclic ring of 3,4-dimethyldibenzothiophene in the mixed petroleum-degrading culture ERN BIO (Chapter 5). Degradation of the methyl-substituted ring of 2,8-dimethyldibenzothiophene by 1-MN-grown cultures of isolate BT1 led to the formation of 5-methylbenzothiophene-3(2*H*)-one (Chapter 5). These 3(2*H*)- and 2(3*H*)-ones are the keto tautomers of 3-hydroxy- and 2-hydroxy-substituted benzothiophenes, respectively. In the studies of 3,4-dimethyldibenzothiophene degradation by ERN BIO and 2,8-dimethyldibenzothiophene degradation by isolate BT1, the products 3-hydroxy-6,7-dimethylbenzothiophene-2(3*H*)-one and 3-hydroxy-5-methylbenzothiophene-2(3*H*)-one that were detected are the keto tautomers of benzothiophene-2,3-diols (Chapter 5). These keto tautomers of benzothiophene-2,3-diols were only observed in the studies with dimethyldibenzothiophenes (Chapter 5), and not with any of the benzothiophenes studied.

Other metabolites that were observed as products of dibenzothiophenes included analogues of 3-hydroxy-2-formylbenzothiophene. 3-Hydroxy-2-formylbenzothiophene was a metabolite of dibenzothiophene produced in cultures of isolates W1, BT1, BT1d, and F, which was presumably further oxidized leading to the formation of 2-mercaptophenylglyoxalate that was detected as benzothiophene-2,3-dione (Chapter 6). The transformation of dibenzothiophene by other isolates has been frequently reported to yield 3-hydroxy-2-formylbenzothiophene which accumulates without further metabolism (Kodama *et al.*, 1973; Laborde and Gibson, 1977; Monticello *et al.*, 1985; Mormile and Atlas, 1989). However, there is one previous report of oxidation of dibenzothiophene to 3-hydroxy-2-formylbenzothiophene and benzothiophene-2,3-dione (Bohonos *et al.*, 1977). As well, the oxidation of methyldibenzothiophenes to methyl-3-hydroxy-2-formylbenzothiophenes and methylbenzothiophene-2,3-diones has been previously reported for isolates W1, BT1, and F (Saftić *et al.*, 1993).

Attack of the unsubstituted ring of 3,4-dimethyldibenzothiophene by isolates W1, BT1, and F, and by the mixed cultures SLPB and ERN BIO led to the formation of 6,7-dimethyl-3-hydroxy-2-formylbenzothiophene and 6,7-dimethylbenzothiophene-2,3-dione (Chapter 5). 5-Methyl-3-hydroxy-2-formylbenzothiophene was not detected from degradation of the methyl-substituted ring of 2,8-dimethyldibenzothiophene in pure and mixed cultures, although the presumed further oxidized product 5-methylbenzothiophene-2,3-dione was detected (Chapter 5). Isolates W1, BT1, and F attacked the aromatic ring of

1,2,3,4-tetrahydrodibenzothiophene leading to the formation of 3-hydroxy-2-formyl-4,5,6,7-tetrahydrobenzothiophene (Chapter 6).

The hydroxybenzothiophene carboxylic acid metabolites detected, that resulted from oxidation of the external homocyclic ring of naphtho[2,1-*b*]thiophene, naphtho[2,3-*b*]thiophene, and 1-methylnaphtho[2,1-*b*]thiophene by isolate W1, were products unique to the naphthothiophenes (Chapter 7). These metabolites form by a mechanism analogous to that which yields 3-hydroxy-2-formylbenzothiophene from dibenzothiophene, with initial dioxygenase attack taking place at either of two locations on the external homocyclic ring of the naphthothiophenes, and further oxidation of the formyl group in the products responsible for its oxidation to a carboxyl group. The further degradation of these hydroxybenzothiophene carboxylic acids was affected by the presence of 1-MN as a cosubstrate and by the presence of a methyl-substituent on the thiophene ring of the metabolites (Chapter 7).

8.2 SUGGESTIONS FOR FURTHER RESEARCH

A literature review by Fedorak (1990) showed that of the hundreds of organosulfur compounds found in petroleum, fewer than 20 of these had been subjected to biodegradation studies. The discussion above summarizes the types of organosulfur-containing metabolites that were observed from microbial metabolism of 21 different benzo-, dibenzo-, and naphthothiophenes. Over 80 metabolites were recovered from cultures by extraction with DCM and were analyzed and identified primarily by GC methods, sometimes after derivatization in an organic solvent. In some cases, purification of metabolites and analysis by NMR was required for identification. In other cases, synthesized authentic standards were used for identifications. These methods were amenable to the rapid screening and identification of metabolites from a large number of condensed thiophenes as was dictated by the contract which funded this research project for more than two-thirds of its duration (Environment Canada, Groundwater and Soil Remediation Program, Contract KA168-2-2191).

Most of these studies were done before the GC-AED was available to quantify the metabolites observed by the GC-FPD method used. Thus, while the metabolites of benzothiophenes identified in Chapters 2 through 4 were those that were detected during GC-FPD analyses, it is not known that they existed in a sulfur mass balance with the depleted substrate, after accounting for substrate loss due to evaporation. Thus, there may be other metabolites formed that were not detected by the DCM extraction and GC methods used. The supply of synthesized benzothiophenes also limited the controls that could be done to accurately determine evaporative losses. Further research aimed at quantitative

studies of the metabolism of benzothiophenes, especially those which are now commercially available, namely, benzothiophene, 3-methylbenzothiophene, and 5-methylbenzothiophene, so that adequate sterile controls can be done to account for evaporative losses, would be valuable. This would indicate whether the metabolites detected in Chapters 2 through 4 were the only metabolites formed and whether they were dead-end products or transiently accumulating intermediates in the biodegradative processes catalyzed by the bacterial cultures tested. To date, no quantitative study of the bacterial metabolism of any isomer of benzothiophene has been reported in the literature.

Preliminary quantitative studies of the type suggested above for benzothiophene metabolism were carried out during completion of the studies with dimethyldibenzothiophenes (Chapter 5) and tetrahydrodibenzothiophene (Chapter 6) when a GC-AED became available. These results showed that the organosulfur present as the detected metabolites was only a small portion of the depleted substrate organosulfur. To follow up on this observation, further quantitative studies were done with commercially available dibenzothiophene (Chapter 6). Again, a large portion of the sulfur from the depleted substrate was not detected by the DCM extraction and GC methods used. The same observation was made with cultures of isolates W1, BT1, and F that were incubated with 1-MN and naphtho[2,1-*b*]thiophene or naphtho[2,3-*b*]thiophene (Chapter 7). However, since sulfate release from dibenzothiophene was not observed to occur (Chapter 6), the sulfur from these depleted substrates that was not detected as the identified metabolites is presumed to exist as organosulfur-containing metabolites that are too polar to extract from aqueous cultures with DCM. This argument is supported by the observed increases in total sulfur recovered from dibenzothiophene (Chapter 6) and naphtho[2,1-*b*]thiophene (Chapter 7) when cultures were lyophilized and methyl-esterified prior to DCM extraction over cultures that were acidified and extracted. Further research aimed at the characterization and quantification of the organic sulfur that is recovered by this lyophilization and methyl-esterification procedure would provide valuable insight into the nature of these highly polar organosulfur-containing metabolites. Alternatively, the application of new methods, such as high performance liquid chromatography (HPLC), to analyze these highly polar metabolites in aqueous cultures would be valuable. Such studies would likely be most easily developed with a commercially available organosulfur compound like dibenzothiophene. To date, studies of dibenzothiophene metabolism by bacteria that utilize the Kodama pathway have not established a sulfur mass balance.

Further quantitative investigations into the metabolism of condensed thiophenes by the bacterial cultures studied in this dissertation would give a better indication of the significance of the observed metabolites. These studies would help address the question of

whether the observed metabolites are dead-end products in the metabolism of condensed thiophenes or transiently accumulating intermediates in these processes. If the metabolites are only transiently accumulating intermediates, then future studies might lead to the identification of further oxidized, highly polar metabolites which are dead-end products that are of greater environmental relevance.

The results of the extensive qualitative studies described in this dissertation and the quantification that was done during completion of the project lead to numerous possibilities for further qualitative studies. All of these areas of further research would be facilitated by the application of methods such as HPLC for the analysis of highly polar metabolites that were not amenable to DCM extraction and GC analysis. Among the areas of further research are elucidation of the mechanism whereby isolate BT1 was able to further degrade the carboxylic acid metabolites that resulted from oxidation of the methyl groups of 3,5-dimethylbenzothiophene in the presence of 1-MN (Chapter 4). As well, isolate BT1 was shown to oxidize dibenzothiophene to its sulfoxide and sulfone, and to subsequently degrade dibenzothiophene sulfone in the presence of 1-MN (Chapter 6). The mechanism of further oxidation of dibenzothiophene sulfone by isolate BT1 is not yet known, since further oxidation products were not detected by extraction and GC analysis.

The mechanism used by isolates W1, BT1, and F to further degrade 3-hydroxy-2-formylbenzothiophene presumably resulting in the formation of 2-mercaptophenyl-glyoxalate, which is detected as benzothiophene-2,3-dione (Chapter 6), awaits elucidation. Further study of 3-hydroxy-2-formylbenzothiophene degradation may be facilitated by the use of isolate BT1d to produce 3-hydroxy-2-formylbenzothiophene from dibenzothiophene, which can be subseqently purified and fed to cultures of isolates W1, BT1, and F with 1-MN. Furthermore, the amount of benzothiophene-2,3-dione detected did not increase in cultures of isolates W1, BT1, and F, where 3-hydroxy-2-formylbenzothiophene degradation occurred, relative to cultures of isolate BT1d, where 3-hydroxy-2-formylbenzothiophene accumulated with little further degradation occurring over 9 days incubation (Chapter 6). This suggests that 2-mercaptophenylglyoxalate is also susceptible to further transformations which are not yet known.

Another suggestion for future research involves study of the mechanism of further degradation of 4-hydroxybenzothiophene-5-carboxylic acid, a metabolite of naphtho[2,1-*b*]thiophene produced by isolate W1, that is stimulated by the presence of 1-MN (Chapter 7). As well, it is interesting that degradation of the external homocyclic ring of dibenzothiophene leads to the formation of 3-hydroxy-2-formyl-substituted benzothiophene (Chapter 6) while naphthothiophenes yield hydroxybenzothiophene carboxylic acids (Chapter 7). The reason that the formyl group of the metabolite from dibenzothiophene is

not further oxidized to the carboxylic acid group or is not detected after it is formed is not yet known, but may provide insight into the mechanism of further degradation of 3-hydroxy-2-formylbenzothiophenes.

These suggestions for further research with the bacterial cultures and condensed thiophenes described in this dissertation would likely lead to the identification of other metabolites of condensed thiophenes that have avoided detection and identification thus far, because their high polarity meant that they were not amenable to the extraction and GC methods used in this investigation.

The metabolites identified in this study were produced primarily in pure cultures of 1-MN-degrading *Pseudomonas* spp. In some cases, the formation of these metabolites was studied in mixed cultures of petroleum-degrading bacteria or cultures inoculated with river water and given crude oil or the aromatic fraction of crude oil as a growth substrate. These latter studies more closely approximate an actual oil-contaminated environment and give a more reliable indication of whether or not the identified metabolites will be produced in actual oil-contaminated environments. However, the identification of metabolites is most easily accomplished in pure culture-pure compound systems. Thus, a trade-off exists in the design of experimental systems that closely resemble contaminated environments with those that give reasonable chance of successful identification of products. This is due to the limited analytical techniques available for the analysis of the polar fraction of petroleum. Future research should attempt to gain further insight into the question of whether or not the metabolites produced in pure culture-pure compound studies are actually formed in systems that more closely resemble oil-contaminated environments and whether or not the metabolites are further degraded in these systems.

If these metabolites are formed in contaminated environments, they may contribute to the toxicity and genotoxicity of the residual polar material that remains after petroleum is biodegraded. Few studies of the toxicity and genotoxicity of condensed thiophenes and their metabolites have been done. Eastmond *et al.* (1984) tested a variety of condensed thiophenes for their toxicity to the zooplankton *Daphnia magna*. They observed LC_{50} values of 0.22 mg/L for benzo[*b*]naphtho[1,2-*d*]thiophene and 63.7 mg/L for benzothiophene. Thus, the product of the microbially-mediated condensation reaction described in Chapter 3 is more toxic than the parent compound benzothiophene. However, benzo[*b*]naphtho[1,2-*d*]thiophene showed no mutagenicity in the standard or pre-incubation Ames test (Pelroy *et al.*, 1983). Jacob (1990) reviewed a study in which the mutagenicity of dibenzothiophene and three isomers of benzonaphthothiophene were compared with some of their sulfoxides and sulfones using *Salmonella typhimurium* strains TA 98 and TA 100. No mutagenicity was observed for the parent compounds, but all

sulfoxides and dibenzothiophene sulfone were weakly mutagenic, and benzo[*b*]naphtho[1,2-*d*]thiophene sulfone was a potent mutagen.

When administered orally to rats and mice, benzothiophene sulfone was less toxic than benzothiophene (Lagno and Sviridov, 1975). As well, Seymour *et al.* (1997) determined the acute toxicities of benzothiophene, 3- and 5- methylbenzothiophene, and dibenzothiophene, as well as of their metabolites benzothiophene sulfone, benzothiophene-2,3-dione, 3- and 5-methylbenzothiophene sulfone, 5- and 7-methylbenzothiophene-2,3-dione, and dibenzothiophene sulfoxide and sulfone. The toxicities of these compounds were determined by the Microtox® and *Daphnia magna* bioassays. In nearly every case, the oxidized compounds were less toxic than their parent thiophenes. These studies suggests that as a general rule microbial oxidation of 2- and 3- ring condensed thiophenes decreases their acute toxicity, except when microbially-produced benzothiophene sulfoxides condense to form benzonaphthothiophenes. However, sulfoxidation of dibenzo- and benzonaphthothiophenes may increase their genotoxicity. However, these conclusions are based on a limited body of literature and further studies of the toxicity of condensed thiophenes and their metabolites should be done.

The pursuit of these areas of further research will provide valuable insight into the fate of condensed thiophenes in petroleum-contaminated environments and the implications that bacterial metabolism of these compounds will have on the bioremediation of contaminated sites. Although much remains to be learned, the investigations of the bacterial metabolism of benzo-, dibenzo-, and naphthothiophenes described in this dissertation will serve as a strong foundation on which to build further studies.

8.3 LITERATURE CITED

Allen, C. C. R., D. R. Boyd, H. Dalton, N. D. Sharma, S. A. Haughey, R. A. S. McMordie, B. T. McMurray, G. N. Sheldrake, and K. Sproule. 1995. Sulfoxides of high enantiopurity from bacterial dioxygenase-catalyzed oxidation. J. Chem. Soc. Chem. Commun. 1995: 119-120.

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.

Eastmond, D. A., G. M. Booth, and M. L. Lee. 1984. Toxicity, accumulation, and elimination of polycyclic aromatic sulfur heterocycles in *Daphnia magna*. Arch. Environ. Contam. Toxicol. 13: 105-111.

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

Fedorak, P. M. 1990. Microbial metabolism of organosulfur compounds in petroleum. In: Geochemistry of Sulfur in Fossil Fuels. Orr, W. L., and C. M. White, Eds. ACS Symposium Series 429; American Chemical Society: Washington, DC; p. 93-112.

Fedorak, P. M., and D. Grbic´-Galic´. 1991. Aerobic microbial cometabolism of benzothiophene and 3-methylbenzothiophene. Appl. Environ. Microbiol. 57: 932-940.

Jacob, J. Sulfur Analogues of Polycyclic Aromatic Hydrocarbons (Thiaarenes). 1990. Cambridge University Press: Cambridge.

Jobson, A., F. D. Cook, and D. W. S. Westlake. 1972. Microbial utilization of crude oil. Appl. Microbiol. 23: 1082-1089.

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. Agr. Biol. Chem. 37: 45-50.

Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinckia* sp. Appl. Environ. Microbiol. 34: 783-790.

Lagno, Z. Y., and S. M. Sviridov. 1972. Toxicity of benzothiophene and benzothiophene sulfone. Khim. Seraorg. Soedin., Soderzh. Neftyakh Neftprod. 9: 580-583; Chem. Abstr. 1975. 82: 119738e.

Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. 49: 756-760.

Mormile, M. R., and R. M. Atlas. 1989. Biotransformation of dibenzothiophene to dibenzothiophene sulfone by *Pseudomonas putida*. Can. J. Microbiol. 35: 603-605.

Pelroy, R. A., D. L. Stewart, Y. Tominaga, M. Iwao, R. N. Castle, and M. L. Lee. 1983. Microbial mutagenicity of 3- and 4-ring polycyclic aromatic sulfur heterocycles. Mutat. Res. 117: 31-40.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1992. Diones, sufoxides and sulfones from the aerobic cometabolism of methylbenzothiophenes by *Pseudomonas* strain BT1. Environ. Sci. Technol. 26: 1759-1764.

Saftic´, S., P. M. Fedorak, and J. T. Andersson. 1993. Transformations of methyldibenzothiophenes by three *Pseudomonas* isolates. Environ. Sci. Technol. 27: 2577-2584.

Selifonov, S. A., M. Grifoll, R. W. Eaton, and P J. Chapman. 1996. Oxidation of naphthenoaromatic and methyl-substituted aromatic compounds by naphthalene 1,2-dioxygenase. Appl. Environ. Microbiol. 62: 507-514.

Seymour, D. T., A. G. Verbeek, S. E. Hrudey, and P. M. Fedorak. 1997. Acute toxicity and aqueous solubility of some condensed thiophenes and their microbial metabolites. Environ. Toxicol. & Chem., In press.

APPENDIX A. SCREENING ISOLATES W1, BT1, BT1d, AND F FOR PLASMIDS

A.1 INTRODUCTION

The upper pathway for naphthalene catabolism, which converts naphthalene to salicylate, has been shown to be biochemically and genetically similar to the pathway responsible for the oxidation of dibenzothiophene to 3-hydroxy-2-formylbenzothiophene (Denome *et al.*, 1993), and for the oxidation of phenanthrene and anthracene to isomers of hydroxynaphthoic acid (Denome *et al.*, 1993; Kiyohara *et al.*, 1994; Sanseverino *et al.*, 1993). Because the archetypal naphthalene-degradative genes are frequently plasmid encoded (Yen and Serdar, 1988), *Pseudomonas* spp. W1, BT1, BT1d, and F were screened for the presence of plasmids by a method that detected the TOL (or pWW0) plasmid (115 kb) in *Pseudomonas putida* strain ATCC 33015 (Williams and Murray, 1974), the pWW60 (or NAH2) plasmid (87 kb) and the pWW61 plasmid (65 kb) in *P. putida* strain NCIB 9816 (Cane and Williams, 1982), the NAH7 plasmid (83 kb) in *P. putida* PpG 1064 (Yen and Gunsalus, 1982), and the LP plasmid (63 kb) in *Pseudomonas fluorescens* LP6a (Foght and Westlake, 1991).

A.2 MATERIALS AND METHODS

Two-hundred-millilitre maintenance cultures of isolates W1, BT1, and F grown on 1-MN (50 µL); of isolates W1 and F grown on 1-MN with dibenzothiophene (4 mg) present; of isolate BT1d grown on dibenzothiophene (4 mg); and of isolates W1, BT1, and F grown with glucose (50 mg) as sole carbon source were streaked onto PCA (Difco, Detroit, MI). All of these maintenance cultures had been maintained with these carbon sources by weekly transfers for over 2 years prior to this plasmid screening experiment. After 4 days of incubation at 28°C, single colonies from the PCA plates were used to inoculate tubes containing 5 mL Trypticase Soy Broth (TSB; Difco).

Pseudomonas strains ATCC 33015, NCIB 9816, PpG 1064, and LP6a were gifts from Dr. J. M. Foght (Department of Biological Sciences, University of Alberta), and these were resuscitated from glycerol stocks stored at -70°C by streaking onto PCA. After incubation at 28°C for 2 days, a colony of strain ATCC 33015 was streaked onto a plate of mineral medium (Fedorak and Westlake, 1984) that had been supplemented with 2.5 mM *m*-toluate and solidified with purified agar (15 g/L; Difco). The other three strains were streaked onto solidified mineral medium and provided with naphthalene vapors as a growth substrate by placing crystals of this compound on the lid of the plate. After the colonies on

these plates were well grown, single colonies of each strain were transferred into tubes containing 5 mL of TSB.

After 24 h incubation at 28°C, 1.5-mL portions of the cultures grown in TSB were used for the small scale rapid plasmid preparation method of Kieser (1984). The DNA isolated by this procedure was analyzed by electrophoresis in 0.7% agarose gels and visualized under UV light after treatment with ethidium bromide.

A.3 RESULTS AND DISCUSSION

Figure A.1 is a photograph of the gel that resulted from electrophoresis of the DNA isolated by the procedure described above. Lane 1, on the far left, contained *lambda* DNA digested with *Hin*dIII as a standard. Lanes 2 through 14, from left to right, show the plasmid profiles of the various *Pseudomonas* strains. The intense band that is located 40 to 45 mm from the top of the gel in lanes 2 through 14 represents the chromosomal DNA that was recovered by the procedure used. The 115 kb TOL (or pWW0) plasmid of strain ATCC 33015 is observed in lane 2 and is the largest plasmid observed. In lane 3, two plasmids are observed for strain NCIB 9816 and these are consistent with the plasmids pWW60 (or NAH2) and pWW61 of 87 and 65 kb, respectively. In lane 4, the 83 kb NAH7 plasmid of strain PpG 1064 is observed to move slightly further than the 87 kb pWW60 (or NAH2) plasmid of strain NCIB 9816 (lane 3). Finally, strain LP6a shows a plasmid of approximately 63 kb in lane 5. Thus, this method was able to detect the plasmids bearing archetypal aromatic ring-degradative genes from strains that have been used in previous studies.

With isolates W1, BT1, BT1d, and F, the only strains that were shown to contain plasmids that could be detected by the procedure used were isolates BT1 and BT1d (lanes 7, 10, and 13). This plasmid was smaller than that observed in strain LP6a of 63 kb and was present in isolate BT1 regardless of whether the isolate was grown on 1-MN (lane 7) or glucose (lane 13). Isolate BT1d, which came from the 1-MN-grown maintenance culture of isolate BT1, but has been maintained for over 2 years with dibenzothiophene as sole carbon source, also contained the same plasmid (lane 10).

Although plasmids were not detected for isolates W1 and F, it is possible that these isolates contain plasmids which were not recovered by the method used. Furthermore, the fact that a plasmid was detected in strains BT1 and BT1d does not mean that the aromatic hydrocarbon-degrading capabilities of these strains are plasmid encoded. The genes for aromatic hydrocarbon degradation could also be chromosomally encoded in these two strains. The fact that the plasmid detected in isolate BT1 was still present in the isolate after 2 years of weekly transfers with glucose as sole carbon source indicate that the plasmid is

stable. Thus, curing the plasmid from strain BT1 by transfer with glucose as sole carbon source could not be accomplished and so it is not known if curing the strain of the plasmid would lead to the loss of aromatic hydrocarbon-degrading capabilities. This would have given indication of whether or not the genes for aromatic hydrocarbon degradation are actually located on the plasmid that is present in strain BT1.

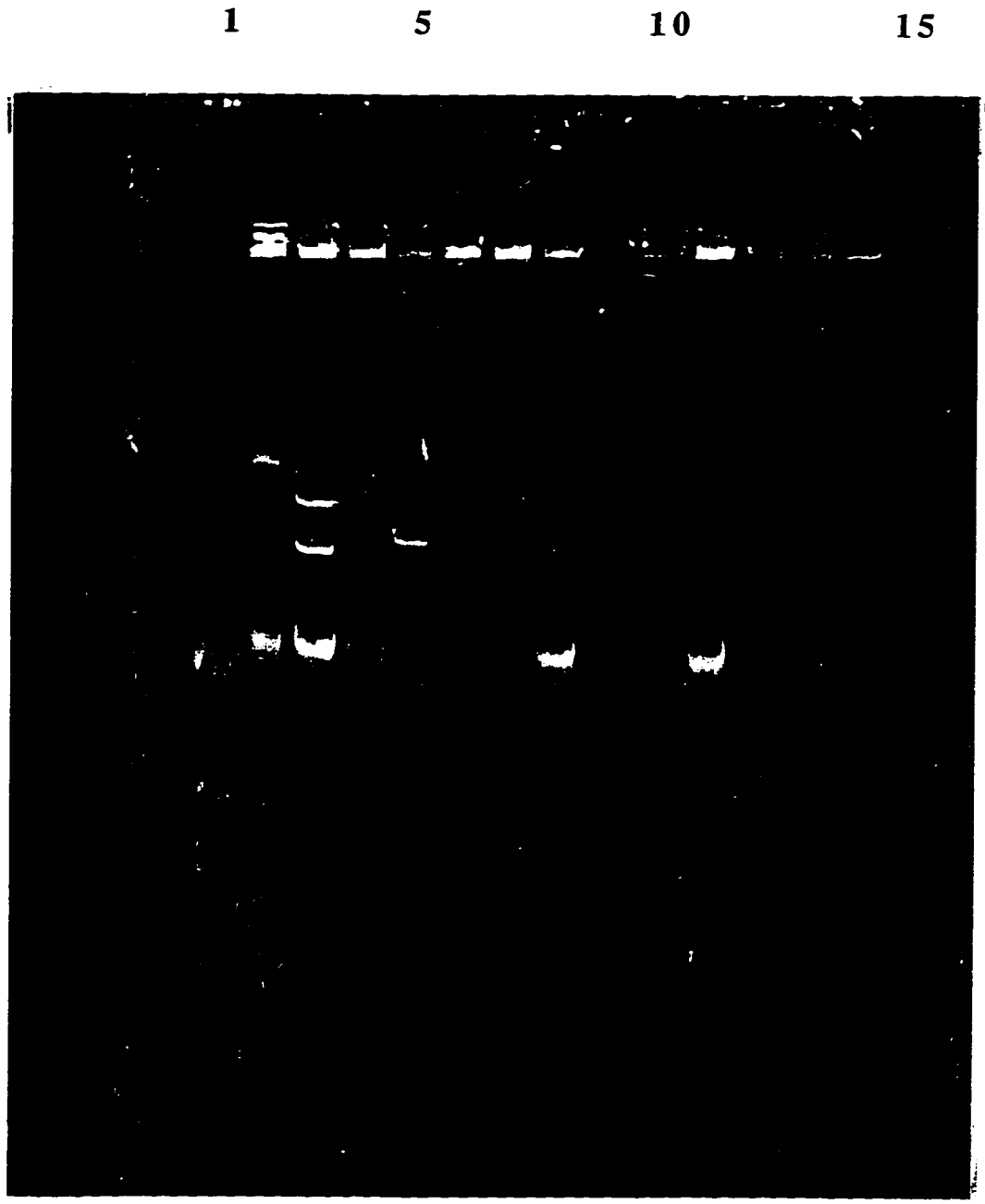


Figure A.1 Photograph of agarose gel showing plasmid profiles of various bacterial strains. From left to right: lane 1, *lambda* DNA digested with *Hin*dIII; lane 2, ATCC 33015; lane 3, NCIB 9816; lane 4, PpG 1064; lane 5, LP6a; lanes 6 through 8, 1-MN-grown isolates W1, BT1, and F, respectively; lane 9, isolate W1 grown on 1-MN in the presence of dibenzothiophene; lane 10, isolate BT1d grown on dibenzothiophene; lane 11, isolate F grown on 1-MN in the presence of dibenzothiophene; lanes 12 through 14, glucose-grown isolates W1, BT1, and F, respectively; lane 15, empty.

A.4 LITERATURE CITED

Cane, P. A., and P. A. Williams. 1982. The plasmid-coded metabolism of naphthalene and 2-methylnaphthalene in *Pseudomonas* strains: phenotypic changes correlated with structural modification of the plasmid pWW60-1. J. Gen. Microbiol. 128: 2281-2290.

Denome, S. A., D. C. Stanley, E. S. Olson, and K. D. Young. 1993. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: Complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. 175: 6890-6901.

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.

Foght, J. M., and D. W. S. Westlake. 1991. Cross-hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. Can. J. Microbiol. 37: 924-932.

Kieser, T. 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12: 19-36.

Kiyohara, H., S. Torigoe, N. Kaida, T. Asaki, T. Iida, H. Hayashi, and N. Takizawa. 1994. Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. J. Bacteriol. 176: 2439-2443.

Sanseverino, J., B. M. Applegate, J. M. Henry King, and G. S. Sayler. 1993. Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene. Appl. Environ. Microbiol. 59: 1931-1937.

Williams, P. A., and K. Murray. 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existance of a TOL plasmid. J. Bacteriol. 120: 416-423.

Yen, K.-M., and I. C. Gunsalus. 1982. Plasmid gene organization: naphthalene / salicylate oxidation. Proc. Natl. Acad. Sci. USA 79: 874-878.

Yen, K.-M., and C. M. Serdar. 1988. Genetics of naphthalene catabolism in Pseudomonads. CRC Crit. Rev. Microbiol. 15: 247-268.

APPENDIX B. GROWTH OF STRAINS W1, BT1, AND F UNDER ANAEROBIC CONDITIONS

B.1 INTRODUCTION

Although *Pseudomonas* spp. undergo a strictly respiratory type of metabolism, some species are capable of anaerobic growth with nitrate as terminal electron acceptor (Krieg and Holt, 1984). As well, there have been several reports of isolates, identified as *Pseudomonas* spp., that are capable of anaerobic growth with the reduction of ferric iron (Lovely, 1987; Obuekwe *et al.*, 1981; Obuekwe and Westlake, 1982a, b; Ottow and Glathe, 1971). Other aerobic Gram negative bacteria that were capable of reducing ferric iron under anaerobic conditions, and some strains that had been previously identified as *Pseudomonas* spp., were shown to be more appropriately classified as strains of *Alteromonas putrefaciens* (Semple and Westlake, 1987). One of these strains, which was originally identified as a *Pseudomonas sp.* (strain 200, Obuekwe *et al.*, 1981), and shown to be more appropriately classified as a member of the genus *Alteromonas*, has since been reclassified as *Shewanella putrefaciens* (Semple *et al.*, 1989). The reclassification of this and other strains reflects the increase in knowledge about these facultatively anaerobic iron-reducing strains. Because it is evident that strains which are similar to and have been incorrectly classified as Pseudomonads in the past are capable of anaerobic growth with the reduction of ferric iron, the isolates W1, BT1, and F were tested for the ability to grow under anaerobic conditions with ferric iron and nitrate as terminal electron acceptors.

B.2 MATERIALS AND METHODS

B.2.1 Ferric iron reduction

The ability of the isolates to reduce ferric iron was tested using tubes of B10 broth and B10 soft agar (0.25% agar added to B10 broth) as described by Semple and Westlake (1987). These media turn from a brownish gold colour to green as ferrous ions are produced. The tubes were inoculated with 0.1 mL of the 1-MN-grown maintenance culture of each isolate, hand-mixed, and incubated at 28°C together with tubes that were inoculated with strains of *S. putrefaciens* obtained from K. M. Semple (Department of Biological Sciences, University of Alberta).

B.2.2 Nitrate reduction

As a preliminary test of the nitrate-reducing ability of isolates W1, BT1, and F, tubes of the semi-solid nitrate reduction medium of Stanier *et al.* (1966) were used. Tubes

of the semi-solid medium were inoculated with 1.0 mL of an isolate from the 1-MN-grown maintenance culture, hand-mixed, overlaid with the same medium, and incubated at 28°C. Nitrite production was monitored with alpha-naphthylamine and sulfanilic acid reagents in 5 M acetic acid and the formation of N_2 was observed by the presence of gas bubbles in the semi-solid medium.

Subsequent growth tests with individual carbon sources and isolates W1 and F used a mineral medium modified from Fedorak and Westlake (1984) by increasing the KNO_3 concentration from 2.0 to 6.0 g per L and by adding 10 mL of Wolfe's vitamin solution (Wolin *et al.*, 1963) per L of medium. For aerobic experiments with this medium, 100-mL portions were sterilized by autoclaving in 250-mL Erlenmeyer flasks stoppered with foam plugs. For anaerobic experiments, the medium was boiled on a hot plate for 2 min, cooled under a headspace of anoxic helium gas, and then 100-mL portions were dispensed into 158-mL serum bottles which had also been flushed with helium. These were then sealed with Teflon-lined serum stoppers that were held on with aluminum caps and autoclaved to sterilize.

The growth substrates were given so that 4.4 mmol of carbon would be present in each 100-mL culture. The substrates sodium acetate trihydrate, trisodium citrate dihydrate, and glucose were added in 2-mL volumes of aqueous solutions prepared using boiled water that had been cooled under helium. These solutions were prepared in serum bottles under helium, capped, and autoclaved for sterility. These solutions were added to culture bottles using sterile disposable syringes that had been rinsed with helium. When 1-MN was given as growth substrate, 70 µL was added directly to the culture with a glass syringe together with 1.9 mL of helium to give the same positive pressure in the culture as in those which received 2 mL of the aqueous solutions of the other substrates. In the same way, cultures which received no growth substrate received 2 mL of helium. All cultures were inoculated by syringe with 5-mL portions of aerobic 1-MN-grown maintenance cultures of isolates W1 or F. The cultures were incubated at 28°C. Appropriate sterile controls which received growth substrates and 5 mL helium, but no inoculum, were incubated with the cultures, but they never showed any activity so the data are not shown.

Dibenzothiophene (4 mg) was given to cultures grown on acetate, citrate, and glucose after they had incubated for 1 day to ensure that anaerobic conditions were reached in the cultures. The dibenzothiophene was added with a glass syringe as a solution in 150 µL hexadecane and the cultures were incubated with shaking to facilitate dispersion of the hexadecane as droplets into the aqueous phase.

After 15 days incubation, the culture headspace gas was analyzed for N_2 and CO_2 by triplicate injections of 0.1 mL headspace gas into a Varian Aerograph model 700 GC

fitted with a 3 m by 0.5 cm column packed with Poropak R. The thermal conductivity detector was operated at 25°C and 150 mV. Helium was used as the carrier gas at a flow rate of 107 mL/min. GC oven and injector temperatures were 60°C and 24°C, respectively. A HP model 3390A integrator was used for peak area measurements. Culture headspace gas sampling and GC injections were made with gas tight Lo-dose 1/2 cc u-100 insulin syringes with 28G1/2 needles (Becton Dickinson, Rutherford, NJ). The syringe was rinsed with helium between samples.The percent (by volume) N_2 and CO_2 in the headspace gas was determined by comparison with standard curves prepared in serum bottles that had been flushed with helium. The N_2 standard came from a gas cylinder whereas the CO_2 standard came from dry ice placed in a sealed flask with a sampling port. Culture headspaces were analyzed for N_2 before 1.5 mL samples of the aqueous phase were removed for NO_2^- analysis (described below). Then the cultures were acidified with 1.5 mL of 2M H_2SO_4, shaken, and the headspaces were analyzed for CO_2.

The 1.5 mL aqueous phase that was removed from the cultures for NO_2^- analysis was centrifuged at 13,500 RPM for 4 min and 1.0 mL of the supernatant was removed and serially diluted in 9 mL volumes of twice distilled water. Then the various dilutions were assayed for NO_2^- by a spectrophotometric method (American Public Health Association, 1989). This method was scaled down (1/50) so that 1.0 mL of each dilution was mixed with 40 µL of colour reagent. A calibration curve over the linear region of the assay was prepared using $NaNO_2$. Using the reading from the diluted samples, the standard curve, and the appropriate dilution factor, the concentration of NO_2^- in each culture was calculated.

Cultures that received dibenzothiophene in hexadecane were spiked with thianthrene as an internal standard prior to extraction with 3 × 20 mL DCM. The extracts were dried over anhydrous Na_2SO_4, concentrated on a rotary evaporator, and analyzed by GC-AED as described previously (Kropp *et al.*, 1997).

B.3 RESULTS AND DISCUSSION

B.3.1 Ferric iron reduction

The two strains of *S. putrefaciens* that were used as positive controls both grew throughout the tubes of broth and soft agar within 3 days incubation and turned the medium to a greenish-black colour, indicating that ferrous ions were produced. However, after 45 days incubation, isolates W1, BT1, and F had only grown in the top aerobic portion of the tubes of B10 broth and soft agar and the medium remained a brownish gold colour,

indicating that ferrous ions had not been formed. Thus, none of isolates W1, BT1, and F was able to reduce ferric iron.

B.3.2 Nitrate reduction

Using the nitrate reduction medium of Stanier *et al.* (1966) isolate W1 was observed to reduce nitrate to N_2 within 3 days incubation, while isolate BT1 was unable to reduce nitrate even after 45 days. Isolate F produced NO_2^- within 3 days incubation and reduced this further to N_2 which was first observed as bubbles in the soft agar after 2 weeks incubation.

After demonstrating that isolates W1 and F could grow under nitrate-reducing conditions with yeast extract and glycerol provided as carbon and energy sources, numerous experiments were done to demonstrate growth in mineral medium prepared using standard anaerobic techniques in serum bottles. In these conditions, growth was indicated by CO_2 and N_2 production, as measured by GC analysis of headspace gases, and NO_2^- production, as measured by spectrophotometric assay of culture supernatants, in cultures that received acetate, citrate, and glucose, and was compared with cultures that were inoculated but received no growth substrate. 1-MN was also tested, but none of the isolates were capable of growth on this carbon source under anaerobic conditions.

Table B.1 shows the levels of NO_2^-, N_2, and CO_2 detected in cultures of isolates W1 and F grown on acetate, citrate, and glucose in the presence of hexadecane and dibenzothiophene after 15 days of incubation. These levels are compared with inoculated cultures that were incubated with hexadecane and dibenzothiophene, but did not receive a growth substrate. As well, the recovery of dibenzothiophene in these anaerobic cultures is compared with the recovery from identical cultures that were incubated aerobically. From Table B.1 it can be seen that isolates W1 and F were capable of growth under nitrate-reducing conditions with acetate, citrate, and glucose in the presence of hexadecane and dibenzothiophene. However, significant depletion of dibenzothiophene was not observed in the anaerobic cultures. The depletion of dibenzothiophene in the identical cultures that were incubated aerobically suggests that the inability to oxidize dibenzothiophene is due to the lack of oxygen in the anaerobic incubations and not due to repression of the dibenzothiophene oxidative genes by the presence of acetate, citrate, or glucose or due to physical inaccessibility of the dibenzothiophene presented to the isolates in hexadecane. Thus, the oxidation of 1-methylnaphthalene and dibenzothiophene by isolates W1 and F requires O_2, even though these isolates can grow with simple carbon sources under nitrate-reducing conditions.

The anaerobic biodegradation of monocyclic aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylenes has been demonstrated under nitrate-reducing conditions (Colberg and Young, 1995). The anaerobic biodegradation of benzene and toluene under ferric iron-reducing conditions has also been documented (Colberg and Young, 1995; Lovley *et al.*, 1994). There are even pure cultures capable of anaerobic biodegradation of toluene under ferric iron-reducing and nitrate-reducing conditions, including anaerobic nitrate-reducing *Pseudomonas* spp. (Colberg and Young, 1995). The anaerobic biodegradation of naphthalene, which is the simplest polycyclic aromatic hydrocarbon, under nitrate-reducing conditions has been demonstrated for mixed microbial populations in soil-water systems (Al-Bashir *et al.*, 1990; Mihelcic and Luthy, 1988a, b), but there are no reports of pure cultures with this ability. There is no evidence for the biodegradation of any polycyclic aromatic hydrocarbon under ferric iron-reducing conditions (Coates *et al.*, 1996). Thus, since there are no reports in the literature of pure cultures capable of anaerobic biodegradation of polycyclic aromatic hydrocarbons, it is not surprising that none of the pure cultures of *Pseudomonas* spp. W1, BT1, and F was capable of oxidation of 1-MN or dibenzothiophene under anaerobic conditions.

Table B.1 Production of NO_2^-, N_2, and CO_2 in cultures of isolates W1 and F grown for 15 days on acetate, citrate, and glucose in the presence of hexadecane and dibenzothiophene as compared with inoculated cultures that did not receive a simple carbon source as growth substrate. The recovery of dibenzothiophene from the anaerobic cultures is also compared with identical cultures that were incubated aerobically.

Isolate	Growth Substrate	NO_2^- (mM)	% N_2 in headspace gas	% CO_2 in headspace gas	% DBT[a] recovered (anaerobic)	% DBT recovered (aerobic)
W1	None	< 0.1	1.6	< 0.16	ND[b]	ND
	Acetate	40	11	35	99	19
	Citrate	10	2.0	27	96	35
	Glucose	18	6.2	28	94	54
F	None	< 0.1	1.5	< 0.16	ND	ND
	Acetate	51	1.4	26	102	30
	Citrate	22	1.2	19	99	7.3
	Glucose	5	1.0	4	101	71

[a]DBT=dibenzothiophene

[b]ND=not determined

B.4 LITERATURE CITED

Al-Bashir, B., T. Cseh, R. Leduc, and R. Samson. 1990. Effect of soil/contaminant interactions on the biodegradation of naphthalene in flooded soil under denitrifying conditions. Appl. Microbiol. Biotechnol. 34: 414-419.

American Public Health Association. 1989. Standard methods for the examination of water and wastewater. American Public Health Association, Washington, DC.

Coates, J. D., R. T. Anderson, J. C. Woodward, E. J. P. Phillips, and D. R. Lovley. 1996. Anaerobic hydrocarbon degradation in petroleum-contaminated harbour sediments under sulfate-reducing and artificially imposed iron-reducing conditions. Environ. Sci. Technol. 30: 2784-2789.

Colberg, P. J. S., and L. Y. Young. 1995. Anaerobic degradation of nonhalogenated homocyclic aromatic compounds coupled with nitrate, iron, or sulfate reduction. In: Microbial Transformation and Degradation of Toxic Organic Chemicals. Young, L. Y., and C. E. Cerniglia, Eds. Wiley-Liss, Inc.: New York; p. 307-330.

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.

Krieg, N. R., and J. G. Holt. 1984. Bergery's Manual of Systematic Bacteriology, Volume 1. Williams & Wilkins Co.: Maryland.

Kropp, K. G., J. T. Andersson, and P. M. Fedorak. 1997. Biotransformations of three dimethyldibenzothiophenes by pure and mixed bacterial cultures. Environ. Sci. Technol. 31: 1547-1554.

Lovely, D. R. 1987. Organic matter mineralization with the reduction of ferric iron: A review. Geomicrobiol. J. 5: 375-399.

Lovely, D. R., J. C. Woodward, and F. H. Chapelle. 1994. Stimulated anoxic biodegradation of aromatic hydrocarbons using Fe (III) ligands. Nature 370: 128-131.

Mihelcic, J. R., and R. G. Luthy. 1988a. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Appl. Environ. Microbiol. 54: 1182-1187.

Mihelcic, J. R., and R. G. Luthy. 1988b. Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. Appl. Environ. Microbiol. 54: 1188-1198.

Obuekwe, C. O., and D. W. S. Westlake. 1982a. Effects of medium composition on cell pigmentation, cytochrome content, and ferric iron reduction in a *Pseudomonas* sp. isolated from crude oil. Can. J. Microbiol. 28: 989-992.

Obuekwe, C. O., and D. W. S. Westlake. 1982b. Effect of reducible compounds (potential electron acceptors) on reduction of ferric iron by *Pseudomonas* species. Microbios Lett. 19: 57-62.

Obuekwe, C. O., D. W. S. Westlake, and F. D. Cook. 1981. Effect of nitrate on reduction of ferric iron by a bacterium isolated from crude oil. Can. J. Microbiol. 27: 692-697.

Ottow, J. C. G., and H. Glathe. 1971. Isolation and identification of iron-reducing bacteria from gley soils. Soil. Biol. Biochem. 3: 43-55.

Semple, K. M., J. L. Doran, and D. W. S. Westlake. 1989. DNA relatedness of oil-field isolates of *Shewanella putrefaciens*. Can. J. Microbiol. 35: 925-931.

Semple, K. M., and D. W. S. Westlake. 1987. Characterization of iron-reducing *Alteromonas putrefaciens* strains from oil field fluids. Can. J. Microbiol. 33: 366-371.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic Pseudomonads: a taxonomic study. J. Gen. Microbiol. 43: 159-271.

Wolin, E. A., M. J. Wolin, and R. S. Wolfe. 1963. Formation of methane by bacterial extracts. J. Biol. Chem. 238: 2882-2886.