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Ring cleavage of sulfur heterocycles: how does it happen?

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Abstract

Sulfur heterocycles are common constituents of petroleum and liquids derived from coal, and they are found in some secondary metabolites of microorganisms and plants. They exist primarily as saturated rings and thiophenes. There are two major objectives driving investigations of the microbial metabolism of organosulfur compounds. One is the quest to develop a process for biodesulfurization of fossil fuels, and the other is to understand the fates of organosulfur compounds in petroleum- or creosote-contaminated environments which is important in assessing bioremediation processes. For these processes to be successful, cleavage of different types of sulfur heterocyclic rings is paramount. This paper reviews the evidence for microbial ring cleavage of a variety of organosulfur compounds and discusses the few well-studied cases which have shown that the C–S bond is most susceptible to breakage leading to disruption of the ring. In most cases, the introduction of one or more oxygen atom(s) onto the adjacent C atom and/or onto the S atom weakens the C–S bond, facilitating its cleavage. Although much is known about the thiophene ring cleavage in dibenzothiophene, there is still a great deal to be learned about the cleavage of other sulfur heterocycles.

Introduction

Sulfur heterocycles found in the environment often originate from fossil fuels. Spills of petroleum or creosote can lead to contamination of terrestrial and aquatic environments, and evaporation or incomplete combustion of fossil fuels can lead to atmospheric contamination with subsequent deposition in soils and surface waters. Other sources of sulfur heterocycles are some secondary metabolites of microorganisms and plants. For example, the microbially-produced penicillins and cephalosporins have sulfur-containing rings. Some chemicals used in industrial processes also contain sulfur heterocycles. For instance, sulfolane (tetrahydrothiophene sulfone) is used in the Shell Sulfinol^{TM} process for removal of hydrogen sulfide from sour natural gas (Goar 1971, Taylor et al. 1991). Spills at sour gas plant sites have caused soil and groundwater contaminations (McLeod et al. 1992; Fedorak & Coy 1996).

Among the most commonly found sulfur heterocycles are thiophenes. These may have alkyl side chains or may be condensed with one or more benzene ring(s) to form benzothiophenes, dibenzothiophenes, naphthothiophenes or benzonaphthothiophenes. Indeed, sulfur is the third most abundant element in crude oils (Speight 1980), and the condensed thiophenes are the most common form in which sulfur is present.

Alkyl dibenzothiophenes have been shown to be quite persistent in petroleum-contaminated environments (Boehm et al. 1981; Hostettler & Kvenvolden 1994; Wang et al. 1994) and they concentrate in the tissues of aquatic species (Laseter et al. 1981; Ogata & Fujisawa 1985). Nonetheless, C_1- and C_2-dibenzothiophenes are susceptible to biodegradation (Atlas et al. 1981; Fedorak & Westlake 1982, 1984; Saftić et al. 1993; Hostettler & Kvenvolden 1994; Kropp et al. 1997a).

The focus of this paper is to review the literature on the ring cleavage of sulfur heterocycles. First, the types, sources and structures of various sulfur heterocycles are examined. Then, evidence for ring cleavage is presented. Often this has been reported as the release

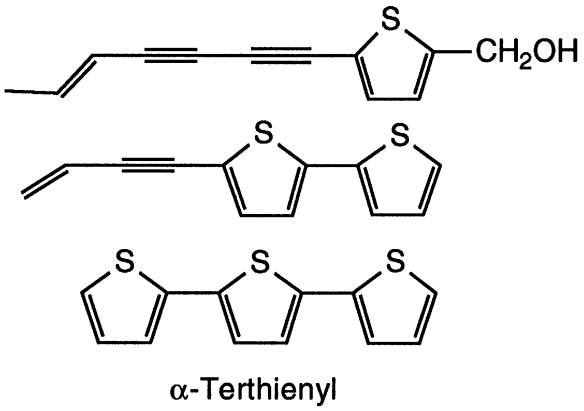

Figure 1. Examples of one-, two- and three-ring thiophenes synthesized by plants (after Christensen & Lam 1990).

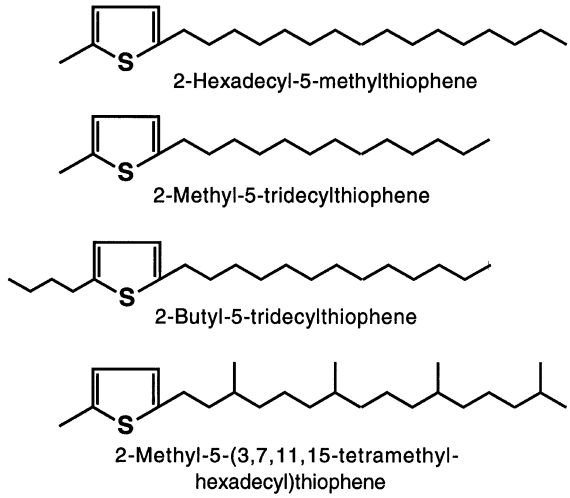

Figure 2. Examples of 2,5-dialkylthiophenes that have been identified in bitumens, crude oils and in pyrolysates of kerogens and asphaltenes (after Sinninghe Damsté & de Leeuw 1989).

of the sulfur atom from organosulfur compounds, and much of this information has come from investigations focussed on the application of microbial processes for the biodesulfurization of fossil fuels (Monticello & Finnerty 1985; Foght et al. 1990; Monticello 1994; Shennan 1996). Although there is a considerable amount of information on the biodegradation of benzothiophenes (Bohonos et al. 1977; Fedorak & Grbić-Galić 1991; Eaton & Nitterauer 1994; Kropp et al. 1994) and dibenzothiophenes (Kodama et al. 1970, 1973; Laborde & Gibson 1977; Kargi & Robinson 1984; Monticello et al. 1985; van Afferden et al. 1990; Saftić et al. 1993; Kropp et al. 1997a), our discussion will concentrate on those findings that indicate ring cleavage, with little attention given to the formation of other metabolites. Next, the surprisingly few studies that have clearly demonstrated sulfur heterocycle ring cleavage and described these processes in some detail, are reviewed. Finally, some investigations of ring cleavages catalyzed by transition metal complexes are summarized.

Types, structures and sources of sulfur heterocycles

Thiophenes

A large number of plants produce thiophene compounds as secondary metabolites, particularly members of the family Asteracea (Compositae) (Christensen & Lam 1990), and dozens of structures have been reported (Bohlmann et al. 1973; Christensen & Lam 1990). Plant thiophenes are biosynthesized by the addition of sulfide or an alkyl sulfide to a conjugated diyne (Schulte et al. 1965). The diyne is typically a C_{13} polyyne derived from a fatty acid, such as oleic acid (Christensen & Lam 1990), which allows the formation of thiophenes with one, two or three rings as well as alkyl substituents (Figure 1). The three-ring compound, α-terthienyl, was isolated from marigolds (Zechmeister & Sease 1947). Its abundance was 15 to 21 mg/kg of fresh marigold petals. Many of these compounds are toxic to other organisms, and may play a role in defense against predators or pathogens (Christensen & Lam 1990). No reports on the natural biodegradation of these plant-derived thiophenes could be found.

2,5-Dialkylthiophenes have been identified in bitumens, crude oils and in pyrolysates of kerogens and asphaltenes (Sinninghe Damsté & de Leeuw 1989). Sinninghe Damsté et al. (1987, 1989) identified several classes of these, including 2-alkyl-5-methyl-, 2-alkyl-5-ethyl-, 2-alkyl-5-propyl-, and the so called 'mid-chain' 2,5-dialkyl- thiophenes. The alkyl substituents may be linear or branched (Sinninghe Damsté et al. 1987, 1989) as shown in Figure 2. The biodegradation of some of these compounds is discussed later.

Condensed thiophenes

The monograph by Jacob (1990) is an excellent reference on structures, sources, synthesis and toxicity of condensed thiophenes. Chou (1990) reviewed

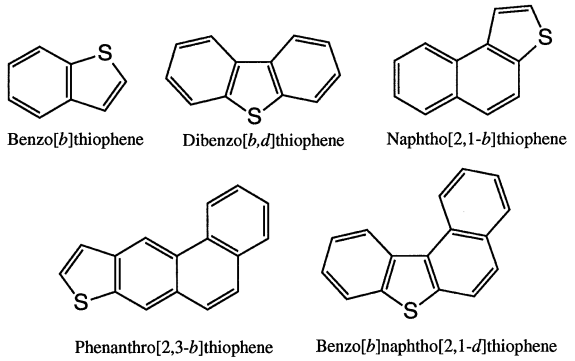

Figure 3. Examples of unsubstituted condensed thiophenes found in petroleum and coal derivatives (after Jacob 1990).

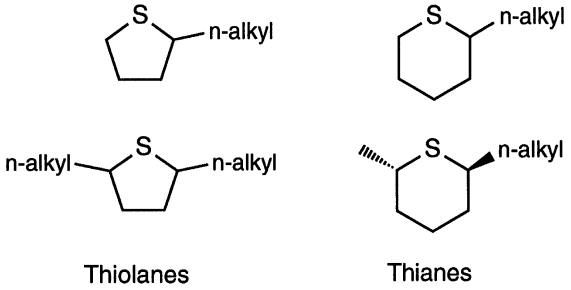

Figure 4. Examples of thiolanes and thianes found in non-biodegraded petroleums. The total number of carbon atoms in these compounds ranged from at least C_8 to C_{30} (after Payzant et al. 1989a).

the forms of sulfur in coal, and among these were condensed thiophenes. Often oxidations, pyrolysis or extractions are required to release these compounds from the coal. For example, benzothiophene, dibenzothiophene and benzonaphthothiophene (Figure 3) were found in bituminous coal and anthracite after oxidation with $Na_2Cr_2O_7$. In addition, C_2- and C_3-benzothiophenes, methyldibenzothiophenes and phenanthrothiophene were tentatively identified in the benzene extracts of a Kentucky high-volatile bituminous coal (Chou 1990). Czogalla & Boberg (1983) listed about 350 condensed thiophenes found in crude oils. These range in complexity from compounds containing two rings to compounds with nine rings, some of which contain other heteroatoms such as nitrogen and oxygen.

Thiacycloalkanes

Two more classes of sulfur heterocycles found in petroleum and crude oils are thiolanes and thianes (Figure 4). The five-member ring of the thiolanes (tetrahydrothiophenes or thiacyclopentanes) is the preferred cyclization product over the six-member ring of thianes (thiacyclohexanes) when synthetic sulfides with linear carbon frameworks are heated in the presence of calcium carbonate to simulate geological conditions (Payzant et al. 1989a). The 2,5-dialkylthiolanes occur in significant amounts in immature petroleums (Schmid et al. 1987). These cyclic sulfides are more prevalent in petroleums that have not been subjected to biodegradation (Payzant et al. 1989a,b; Grimalt et al. 1991).

Few biodegradation studies have been conducted with these non-aromatic sulfur heterocycles. Using aerobic laboratory cultures, Fedorak et al. (1988) demonstrated removal of the side chain of a monoalkylthiolane yielding 2-thiolanecarboxylic acid (2-tetrahydrothiophenecarboxylic acid).

Penicillins and cephalosporins

Penicillins and cephalosporins are important beta-lactam antibiotics synthesized by prokaryotic and eukaryotic microorganisms. Both groups of antibiotics contain two fused rings with one of these containing sulfur and nitrogen atoms. In *Cephalosporium* and *Streptomyces* spp., the five-member ring (Figure 5), undergoes an expansion to a six member ring (Baldwin et al. 1987). This enzyme-mediated ring cleavage and expansion has been studied in detail and it will be discussed later.

Evidence for ring cleavage

Investigators often look for the release of the sulfur atom from sulfur heterocycles, and this release provides convincing evidence of ring cleavage. The released sulfur atom has been found as sulfide, sulfite and sulfate. In other cases, growth of a culture on a sulfur heterocycle as its sole sulfur source is used as an indication of ring cleavage. A third line of evidence is the identification of some metabolites that contain organosulfur that is not part of a ring system, or the identification of organic metabolites that are devoid of sulfur.

Thiophenes

Despite an extensive literature search, only two reports of biodegradation of unsubstituted thiophene were

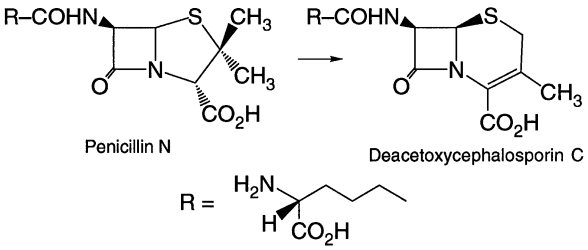

Figure 5. Penicillin N is converted deacetoxycephalosporin C by an enzyme found in cephalosoporin-producing microorganisms (after Baldwin et al. 1987).

found. Kurita et al. (1971) reported anaerobic degradation of thiophene by a bacterial culture isolated from oil-contaminated sludge. A mineral medium supplemented with polypeptone, lactic acid and thiophene was used to cultivate the bacterial culture which released hydrogen sulfide from thiophene. Moriya & Horikoshi (1993) reported a small amount of aerobic thiophene degradation by a *Bacillus* species isolated from a deep sea sediment. Neither study conclusively proved that thiophene could serve as a sole carbon source, nor did either study attempt to determine a pathway for thiophene degradation.

Mixed cultures of petroleum-degrading bacteria can attack the side chains of several 2,5-dialkyl thiophenes yielding various carboxylic acids, including 5-methyl-2-thiophenecarboxylic acid (Fedorak & Peakman 1992) and 2,5-thiophenedicarboxylic acid (Fedorak et al. 1996). The latter compound appeared to be a key intermediate, and some of the mixed cultures oxidized 5-methyl-2-thiophenecarboxylic acid to 2,5-thiophenedicarboxylic acid which served as a growth substrate. Approximately 50% of the sulfur in this substrate was detected as sulfate in the medium at the end of the 15-day incubation time (Fedorak et al. 1996). 2,5-Thiophenedicarboxylic acid was detected in a mixed culture grown on 2,5-diundecylthiophene, and 37% of the sulfur from this dialkylthiophene was detected as sulfate in the medium after 35 days of incubation (Fedorak et al. 1996).

Thiophene substituted in the 2-position with carboxylate, acetate, methanol or methylamine served as sole carbon sources for mutants of *Escherichia coli* (Abdulrashid & Clark 1987). Presumably, growth on these compounds required cleavage of the thiophene ring. In low-sulfate basal medium, thiophene-2-carboxylic, 5-methylthiophene-2-carboxylic, and thiophene-2-acetic acids served growth substrates for *Rhodococcus* strains isolated from sewage sludge (Kanagawa & Kelly 1987). One of these strains was studied in more detail with thiophene-2-carboxylic acid, and there was stoichiometric recovery of the sulfur as sulfate. Evans & Venables (1990) described *Vibrio* YC1 isolated from oil-contaminated mud. Thiophene-2-carboxylic and thiophene-2-acetic acids supported growth of this bacterium. Stoichiometric release of the sulfur atom as sulfate was observed with the former compound, but no data were reported for the latter compound. No biodegradation pathways were determined in any of these studies. Interestingly, thiophenes substituted in the 3-position would not support growth of the isolates described by Kanagawa & Kelly (1987) and Evans & Venables (1990).

The degradation pathway for substituted thiophenes appears to share some elements with the degradative pathways for homologous heterocycles, as mutants of *Vibrio* YC1 which could no longer degrade thiophene-2-carboxylic acid also lost their ability to degrade furan-2-carboxylic acid and pyrrole-2-carboxylic acid (Evans & Venables 1990).

An unclassified aerobic, Gram-positive, soil bacterium, designated FE-9, was isolated which used hexadecane as its sole carbon and energy source and dibenzothiophene or thianthrene as its sole sulfur source (Finnerty 1993). This isolate was suspended in dimethylformamide and used for biodesulfurization studies. When it was incubated with terthiophene (α-terthienyl, Figure 1) under a hydrogen atmosphere, the terthiophene was converted to hydrogen sulfide and a highly unsaturated product, tentatively identified as 1,3,5,7,9,11-dodecahexaene.

Chou & Swatloski (1983) studied the biodegradation of sulfolane (tetrahydrothiophene sulfone) in a laboratory-scale completely mixed activated sludge system. They found that sulfolane bio-oxidation generated acid, requiring pH control, and that there was a nearly stoichiometric release of the sulfur atom as sulfate. Recently, E.A. Greene & P.M. Fedorak (unpublished data) enriched mixed cultures from sulfolane-contaminated aquifer material on sulfate-free medium. These cultures used sulfolane as their sole source of sulfur, and sulfate was released from sulfolane. Thus, the heterocyclic ring of sulfolane can be broken by microorganisms.

Benzothiophenes

Eaton & Nitterauer (1994) studied the aerobic biotransformation of benzothiophene by isopropylbenzene-degrading bacteria, and detected several metabolites.

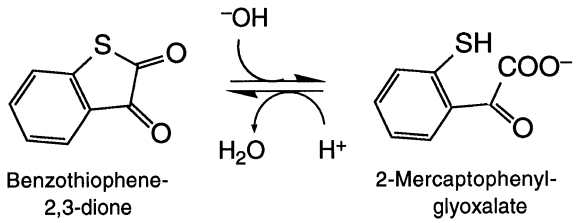

Figure 6. 2-Mercaptophenylglyoxalate cyclizes under acidic conditions to give benzothiophene-2-3-dione (after Eaton & Nitterauer 1994).

Among the metabolites that resulted from cleavage of the thiophene, they tentatively identified 2-mercaptophenylglyoxaldehyde, 2′-mercaptomandelate and 2′-mercaptomandelaldehyde, and they unequivocally identified 2-mercaptophenylglyoxalate. Eaton & Nitterauer (1994) observed that the latter compound existed at neutral pH of the culture medium, but it cyclized under acidic conditions to give benzothiophene-2,3-dione (Figure 6). This 2,3-dione has been observed in the extracts of acidified cultures incubated with benzothiophene (Bohonos et al. 1977; Fedorak & Grbić-Galić 1991; Kropp et al. 1994). Similarly, 2,3-diones were observed in the extracts of acidified cultures incubated with methyl-substituted benzothiophenes containing the alkyl substituent on the benzene ring (Saftić et al. 1992; Kropp et al. 1994). Based on the finding of Eaton & Nitterauer (1994), these 2,3-diones must actually result from cyclization of 2-mercaptophenylglyoxalates which are thiophene ring cleavage products.

Interestingly, photooxidation of benzo[*b*]thiophene gave benzothiophene-2,3-dione (Andersson & Bobinger 1992). 2-Mercaptophenylglyoxalate was postulated to form from the dione, and subsequent photooxidations of the former compound oxidized the sulfhydryl group to a sulfonic acid, and caused the loss of CO to give an 85% yield of 2-sulfobenzoic acid.

Saftić et al. (1992) identified *m*-tolyl methyl sulfoxide as an aerobic bacterial metabolite of 6-methylbenzothiophene, and Kropp et al. (1994) identified *o*-tolyl methyl sulfoxide as a metabolite of 7-methylbenzothiophene. These products can only be formed as a result of cleavage of the heterocyclic ring, but the mechanism of their formation is unknown.

Sulfones have been found as a bacterial metabolites of various benzothiophenes (Fedorak & Grbić-Galić 1991; Saftić et al. 1992; Selifonov et al. 1996). D.C. Bressler & P.M. Fedorak have enriched a bacterial culture that uses benzothiophene sulfone as its sole carbon and sulfur source (unpublished data). In addition, sulfate was observed to accumulate in this culture, providing evidence of thiophene ring cleavage in a sulfone.

There are some reports of anaerobic biodegradation of benzothiophene leading to cleavage of the thiophene ring. Using bacteria in oil sludge, Kurita et al. (1971) observed hydrogen sulfide release from benzothiophene, but no identification of the resulting organic compounds was given. Grbić-Galić (1989) described a study in which benzothiophene was degraded in methanogenic microcosms containing aquifer solids and water from a creosote-contaminated aquifer. Gas chromatography-mass spectrometry was used to identify some of the metabolites, and some of these were *o*-hydroxybenzenesulfonic acid, phenylacetic acid, benzoic acid and phenol.

Dibenzothiophenes

The first reported pathway for dibenzothiophene biodegradation was the so-called ‘Kodama pathway’ (Kodama et al. 1970, 1973). It involves oxidation of one of the benzene rings, yielding 3-hydroxy-2-formylbenzothiophene as the most commonly identified product. Further oxidation of this metabolite may yield benzothiophene-2,3-dione, which was found by Bohonos et al. (1977) in extracts of cultures incubated with dibenzothiophene.

In light of the results of Eaton & Nitterauer (1994) (Figure 6), the 2,3-dione must result from cyclization of the ring cleavage product, 2-mercaptophenylglyoxalate. In our laboratory studies, there have been numerous 2,3-diones found in extracts from acidified cultures incubated with dibenzothiophenes. For example, benzothiophene-2,3-dione was found in the extracts of acidified cultures of four stains of *Pseudomonas* incubated with dibenzothiophene (Kropp et al. 1997b). Similarly, the corresponding isomers of methylbenzothiophene-2,3-dione were found as metabolites of each of the four isomers of methyldibenzothiophene (Saftić et al. 1993). Also, 4,6-dimethylbenzothiophene-2,3-dione and 4,7-dimethylbenzothiophene-2,3-dione were identified in cultures incubated in the presence of 4,6-, and 4,7-dimethylbenzothiophene, respectively (Kropp et al. 1996). In addition, 6,7-dimethylbenzothiophene-2,3-dione was identified as a metabolite of 3,4-dimethyldibenzothiophene, and 5-methylbenzothiophene-2,3-dione was a metabo-

lite of 2,8-dimethyldibenzothiophene (Kropp et al. 1997a). Thus, the metabolism of several dibenzothiophenes yield 2,3-diones that exist as 2-mercaptophenylglyoxalates at the neutral pH of the culture medium (Figure 6).

Kim et al. (1990a, 1990b) investigated the potential of anaerobic biodesulfurization by sulfate-reducing bacteria. Under a hydrogen atmosphere, their cultures transformed dibenzothiophene to biphenyl and released hydrogen sulfide.

Pioneering work by Isbister et al. (1988) showed that the sulfur atom from dibenzothiophene could be selectively removed from this molecule. Using radiolabeled dibenzothiophene and a genetically modified bacterium 'CB1' they demonstrated the liberation ^{35}S-sulfate from ^{35}S-dibenzothiophene. Using ^{14}C-dibenzothiophene, Isbister et al. (1988) found no $^{14}CO_2$ liberation and no incorporation of ^{14}C into biomass of CB1. The bacterium produced 2,2′-dihydroxybiphenyl as an unassimilated organic residue.

This mode of enzymatic attack became known as the '4S pathway' (Krawiec 1990), in which bacteria selectively oxidize the S atom in dibenzothiophene to the **s**ulfoxide, the **s**ulfone, **s**ulfonate, and release it as **s**ulfate, with no cleavage of C–C bonds, thereby maintaining the caloric value of the hydrocarbon moiety of dibenzothiophene. Other investigators have reported that the carbon atoms in dibenzothiophene are released as biphenyl or monohydroxybiphenyl, and Krawiec (1990) refers to the pathway that yields these products as the 'modified 4S' or 'extended 4S' pathway.

The isolation and characterization of *Rhodococcus rhodochrous* IGTS8 (Kilbane & Jackowski 1992; Kayser et al. 1993) led to major advancements in the investigations of biodesulfurization. Work with this bacterium has provided insight into the mechanism of the thiophene ring cleavage. Indeed, Energy BioSystems Corporation was reported to be using a 5-barrel/day pilot plant to study the biodesulfurization of diesel fuel (Rhodes 1995) by *R. rhodochrous* IGTS8, which was recently shown to be a strain of *Rhodococcus erythropolis* by 16S rRNA and physiological studies (Monticello et al. 1995). Figure 7 shows desulfurization of dibenzothiophene by strain IGTS8 using the modified 4S pathway in which a sulfinate, rather than a sulfonate, was an intermediate.

The versatility of organisms using the modified 4S pathway has been examined in several studies. *Rhodococcus* spp. can desulfurize many condensed thiophenes (Monticello 1994), including benzo[*b*]naphtho[1,2-*d*]thiophene (Ohshiro 1996). Lee et al. (1995) demonstrated that an *Arthrobacter* species could desulfurize the sterically hindered compound 4,6-diethyldibenzothiophene, yielding 2-hydroxy-3,3′-diethylbiphenyl as the sulfur-free product. Similarly, Ohshiro et al. (1996) showed that *R. erythropolis* H-2 removed the sulfur atom from 2,8-dimethyldibenzothiophene, 4,6-dimethyldibenzothiophene and benzo[*b*]naphtho[2,1-*d*]thiophene. The product from the desulfurization

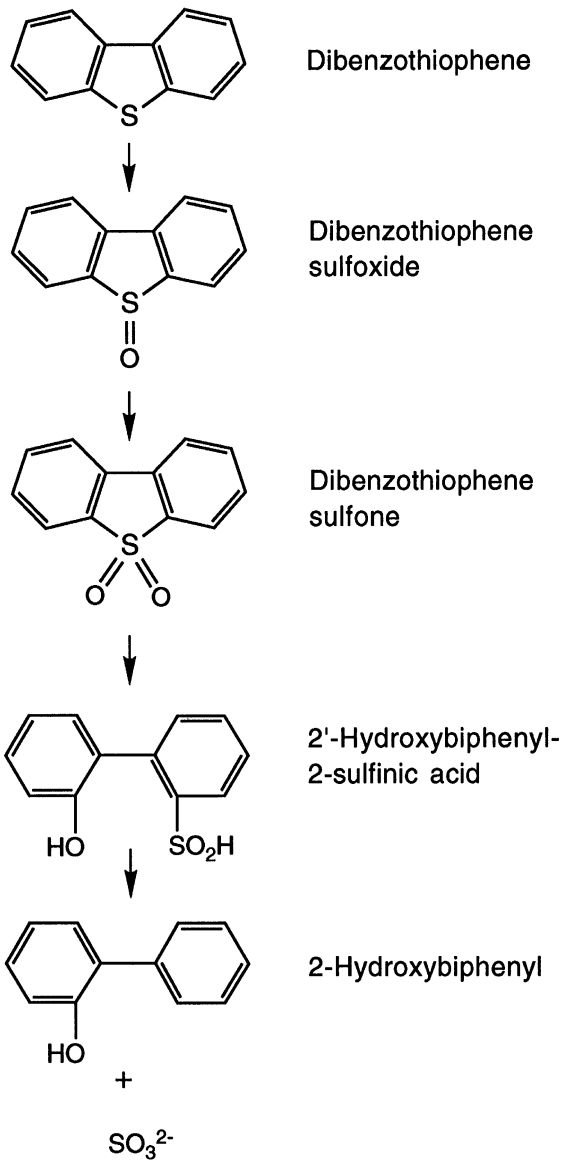

Figure 7. The desulfurization of dibenzothiophene by strain IGTS8 using the modified 4S pathway (after Gray et al. 1996).

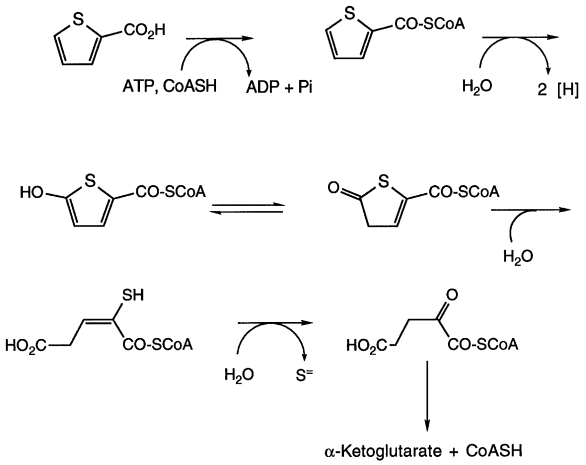

Figure 8. Hypothesized pathway of thiophene-2-carboxylic acid degradation to α-ketoglutarate (after Cripps 1973).

of the latter compound was identified as α-hydroxy-β-phenylnaphthalene by gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy. The paper by Ohshiro et al. (1996) appears to be the first evidence of microbial attack of a four-ring condensed thiophene.

There have been reports of bacterial oxidation of the sulfur in dibenzothiophene and subsequent degradation of the homocyclic rings. For example, van Afferden et al. (1990, 1993) described *Brevibacterium* sp. DO that uses dibenzothiophene as its sole source of sulfur, carbon and energy. Similarly, an aerobic microorganism isolated from a deep sea thermal vent utilized dibenzothiophene as its sole sulfur and carbon source, but grew better in medium supplemented with yeast extract (Kitchell et al. 1991).

Using the bacterial isolate FE-9 suspended in dimethylformamide, Finnerty (1993) demonstrated that dibenzothiophene was desulfurized to biphenyl and hydrogen sulfide under an atmosphere of nitrogen or hydrogen. The highest conversion rates were under a hydrogen atmosphere. When incubated with air in the headspace of the reaction tube, isolate FE-9 produced biphenyl, 2-hydroxybiphenyl, 2,2′-hydroxybiphenyl and sulfate from dibenzothiophene.

Detailed studies of microbially-mediated ring cleavage

2-Substituted thiophenes

In studies with radioactively labeled thiophene-2-carboxylic acid and resting cells of a bacterium designated R1, Cripps (1973) observed the formation of α-ketoglutarate (2-oxoglutarate) with the same specific activity as the substrate. He provided evidence that the first step in the metabolism of thiophene-2-carboxylic acid was the formation of a CoA ester as shown in Figure 8, however none of the subsequent steps shown in the pathway were fully characterized. Because the overall conversion shown in Figure 8 occurred under anaerobic conditions, Cripps (1973) hypothesized that water was the source of the oxygen atom in the hydroxylation step. Although the end product of the metabolism of the sulfur atom was sulfate, Cripps (1973) detected sulfide in cultures of organism R1 under certain growth conditions. The release of sulfide is shown in Figure 8. The point in the pathway at which CoA is released is unknown, and Cripps (1973) wrote that it is conceivable that it might be released at any stage after the introduction of the hydroxyl group. However, Figure 8 shows the release to occur at the time α-ketoglutarate is formed. Organism R1 also degraded thiophene-2,5-dicarboxylic acid, but a pathway was not determined.

Dibenzothiophene

van Afferden et al. (1993) studied the biodegradation of dibenzothiophene by *Brevibacterium* sp. DO. They found that the first metabolic steps involved the oxidation of the sulfur atom to the sulfoxide then to the sulfone (Figure 9). Because strain DO cometabolized fluoren-9-one (the carbon analogue of dibenzothiophene sulfoxide) to 1,1a-dihydrodihydroxyfluoren-9-one, van Afferden et al. (1993) proposed that dibenzothiophene sulfone was oxidized at the angular position in a similar manner as fluoren-9-one, yielding 4,4a-dihydroxy-4-hydrodibenzothiophene sulfone (Figure 9), which could not be isolated. This compound is an unstable hemimercaptal (S-oxidized form) and is expected to spontaneously decay to form 2′,3′-dihydroxybiphenyl-2-sulfinic acid (van Afferden et al. 1993). Strong evidence for the formation of the sulfinated dihydroxybiphenyl was provided, but this metabolite could not be isolated. The mechanism for the cleavage of the C–S bond in a hemimercaptal (S-

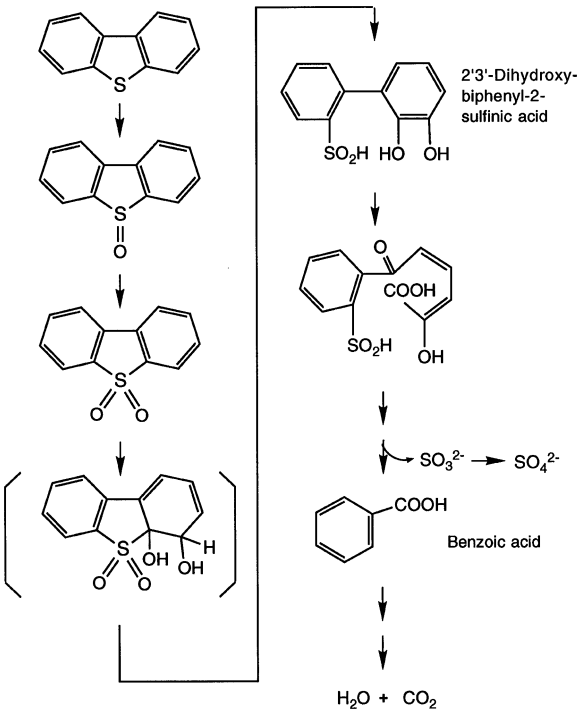

Figure 9. The pathway for dibenzothiophene metabolism by *Brevibacterium* sp. DO. The hypothesized product of angular dioxygenation is shown in brackets and this is the sulfoxidized hemimercaptal shown in Figure 10 (after van Afferden et al. 1993).

oxidized form) is analogous to the cleavage of the C–O bond in a hemiacetal (van Afferden et al. 1993), and the former mechanism is shown in Figure 10. Using a similar approach with dibenzothiophene and fluoren-9-one oxidation, Dahlberg et al. (1993) also suggested the angular oxidation of the former compound by an *Arthobacter* sp. resulting in C–S bond cleavage.

The mechanism for removal of the sulfur atom from dibenzothiophene by *R. erythropolis* strain IGTS8 is the most extensively studied example of microbially-mediated thiophene ring cleavage. To a large part, this is because of the efforts of Energy BioSystems Corporation to develop a commercial-scale, biodesulfurization process (Monticello 1994). Gray et al. (1996) have summarized much of the recent work on the molecular mechanisms of this ring cleavage.

The gene cluster responsible for desulfurization has been cloned and sequenced (Denome et al. 1993; 1994; Piddington et al. 1995) and the promoter and regulatory regions have been studied (Li et al. 1996). The cluster contains three open reading frames designated *dszA*, *dszB*, and *dszC* (Gray et al. 1996) (called *soxA*, *soxB*, and *soxC* by Denome et al. 1994). All three enzymes

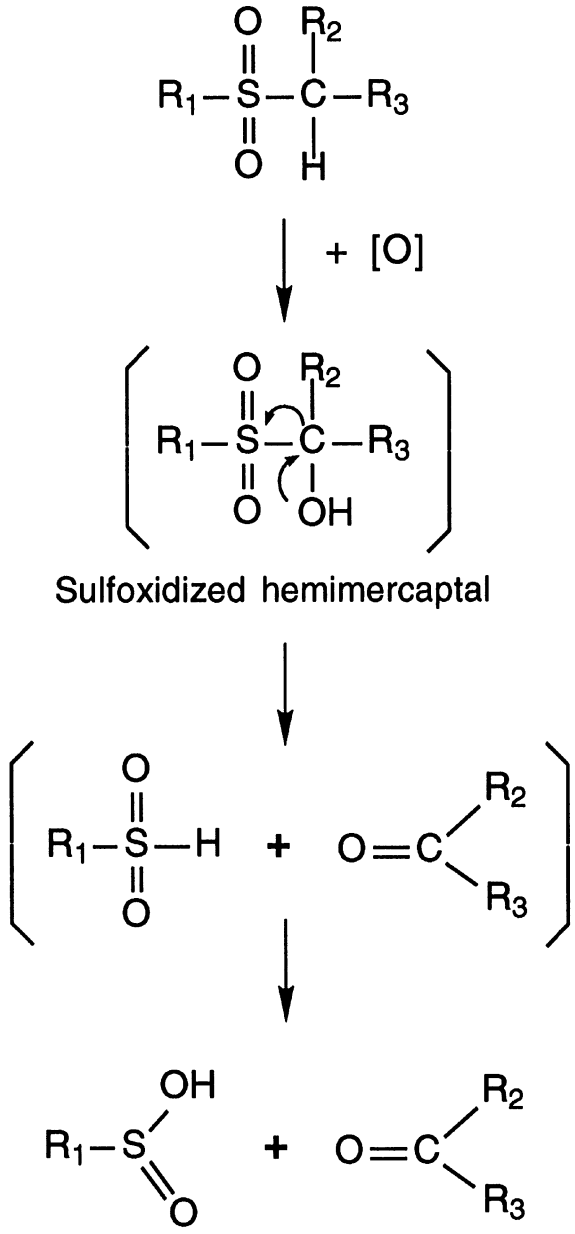

Figure 10. The C–S bond cleavage mechanism for a sulfoxidized hemimercaptal (after van Afferden et al. 1993).

have been purified and their activities studied (Gray et al. 1996).

The *dszC* gene encodes for a monooxygenase that, in a two-step process, oxidizes dibenzothiophene to the sulfoxide and then to the sulfone. The second oxidation step is about ten times as fast as the first (Gray et al. 1996). This enzyme appears to be specific for sulfoxidation because no other oxi-

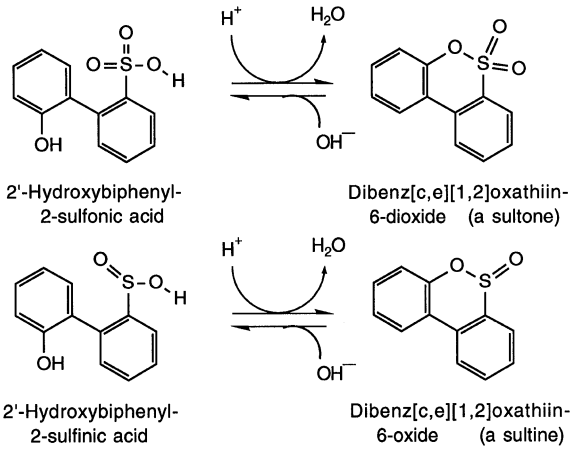

Figure 11. Acid catalyzed cyclization of 2'-hydroxybiphenyl-2-sulfonic acid and 2'-hydroxybiphenyl-2-sulfinic acid to form the sultone and sultine, respectively (after Olsen et al. 1993).

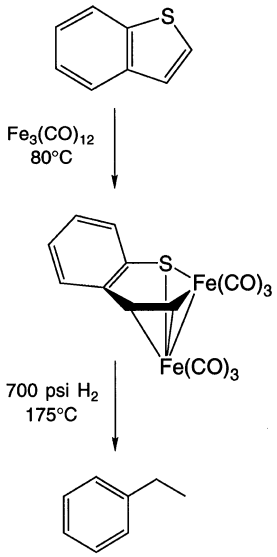

Figure 12. Example of insertion of a metal into the C–S bond of thiophene and subsequent hydrodesulfurization. The major product is ethylbenzene and the sulfur is removed as iron sulfide (after Myers et al. 1995).

dized products of dibenzothiophene were detected. The sulfone is oxidized by another monooxygenase which is the product of the *dszA* gene. This oxidation yields 2-(2′-hydroxyphenyl)benzenesulfinate (2′-hydroxybiphenyl-2-sulfinic acid, in Figure 11). The two monooxygenases require NADH and FMN for their catalytic activities.

Olsen et al. (1993) identified two compounds that were present in trace amounts in extracts of strain IGTS8 incubated with dibenzothiophene. These were a sultone and a sultine (Figure 11). They were postulated to be intermediates in the desulfurization pathway or compounds that could not be metabolized further. However, Olsen et al. (1993) cautioned that the sulfonic and sulfinic acids shown in Figure 11 will cyclize under the acidic conditions used in the workup of the culture supernatant. Sultones readily hydrolyze yielding an open ring compound containing a sulfonate and a hydroxyl group (Roberts & Williams 1987).

The product of the *dszB* gene is a novel desulfinase that converts 2-(2′-hydroxyphenyl)benzenesulfinate to 2-hydroxybiphenyl, and releases sulfite (Gray et al. 1996). The desulfinase is the slowest of the three enzymes, thus it controls the rate of the desulfurization of dibenzothiophene in strain IGTS8.

All three enzymes are colorless, indicating that they contain no tightly associated chromophores (Gray et al. 1996). None of the enzymes were inhibited by the addition of EDTA, indicating that they did not require metal ions as cofactors. The addition of Fe^{3+}, Fe^{2+} or Cu^{+} to reaction mixtures did not enhance the rates of the reactions of the monooxygenases (Gray et al. 1996).

Penicillins and cephalosporins

Deacetoxycephalosporin C synthase (or expandase) catalyzes the conversion of the five-member thiazolidine ring in penicillins to a six-member cephem ring in deacetoxycephalosporin C (Figure 5). This reaction involves the oxidative expansions of the thiazolidine ring by inserting one of the C2-methyl substituents to give the dihydrothiazine ring of cephalosoporins (Jensen & Demain 1995). In this process, a C–S bond is broken and another C–S bond is formed. In vitro, this enzyme requires α-ketoglutarate, Fe^{2+} and O_2 as cofactors, and ascorbate and dithiothreitol for maximum activity (Baldwin et al. 1987, Jensen & Demain 1995). Baldwin et al. (1991) suggested a mechanism for this expandase activity involving a bridged species intermediate with a sulfur radical or cation which decomposes, with the loss of hydrogen, or introduction of the hydroxyl group derived from α-ketoglutarate-penicillin coupled reduction of dioxygen.

Ring cleavages catalyzed by transition metal complexes

The development of the biodesulfurization process is aimed to replace the currently used hydrodesulfurization process which uses various transition metal catalysts (Gary & Handwerk 1984), high temperatures (500 ° to 825 °C) and H_2 under high pressure (150 to 3000 psi) (Jones & Chin 1994). These metals specifically react with and break the C–S bonds of the organosulfur compounds including mercaptans, sulfides, disulfides and thiophenes. Typically, the sulfur atom is released as H_2S (Gary & Handwerk 1984). Figure 12 illustrates iron insertion into the C–S bond of benzothiophene which leads to the desulfurization of this molecule.

Conclusions and challenges

A common feature of those sulfur heterocycle ring cleavages that have been studied in some detail is that the C–S bond is the most susceptible to cleavage. The specificity of this reaction is illustrated by the expansion of the ring in penicillins to form cephalosporins (Figure 5) and the modified 4S pathway (Figure 7). The metal-catalyzed hydrodesulfurization (Figure 12) also shows this selectivity. Sabbah (1979) calculated the strength of the C–S bonds in thiophene, benzothiophene and dibenzothiophene to be near 340 kJ/mol (Table 1). This value is slightly greater than the thiol bond in methylmercaptan (CH_3–SH) and the sulfide bond in dimethylsulfide (CH_3–SCH_3) (Table 1). Sabbah (1979) also calculated the average strength of the C–C bond in benzene to be 505 kJ/mol, and the bond strengths for the single and double carbon bonds are also shown in Table 1. Comparing the C–C bond strengths to the C–S bond strengths in thiophenes (Table 1) suggests that the heteroatomic bonds are the weakest in these molecules. Thus, one would predict the C–S bond would be the most susceptible to cleavage.

Another common feature of the aerobic microbial attack of sulfur heterocycles is the addition of one or more oxygen atoms to the molecule (Figures 7–9). Oxygen is typically added to the sulfur atom and/or to the carbon atom adjacent to the sulfur atom. Of all the elements, oxygen has the second highest electronegativity, and the presence of oxygen can weaken nearby bonds. Some examples of this phenomenon are given in Table 1. The strength of the H–C bond in methane is 438 kJ/mol. The addition of an OH group weakens this bond strength to 410 kJ/mol in methanol. Further oxidation to formaldehyde decreases the H–C bond strength to 364 kJ/mol. C–C bonds can also be weakened be the addition of oxygen. The C–C bond strength in propane (330 kJ/mol) is decreased to 290 kJ/mole in acetone (Table 1). In addition, the oxidation of the sulfur atom in dimethyl sulfide to give dimethyl sulfone decreases the C–S bond strength from 308 to 280 kJ/mol. Similarly, oxidation of methyl tolyl sulfide to the corresponding sulfone decreases the C–S bond strength from 257 to 221 kJ/mol. Given that the C–S bond in a sulfur heterocycle is likely the weakest bond in the molecule, the addition of oxygen to the sulfur atom or to a carbon atom adjacent to the sulfur atom, would further weaken the C–S bond, thereby increasing the possibility of ring cleavage between these two atoms. Figure 10 illustrates the spontaneous cleavage of C–S bond in a sulfoxidized hemimercaptal, which is the mechanism proposed by van Afferden et al. (1993) leading to the thiophene ring cleavage by their bacterial isolate. Also, the addition of an oxygen atom to a carbon atom adjacent to a sulfur atom facilitates cleavage by thioester hydrolysis.

Table 1. Bond strengths in selected compounds

Bond	Bond strength (kJ/mol)	Reference
C–C bonds		
H_3C–CH_3	376	Lide (1995)
H_2C=CH_2	733	Lide (1995)
C–C in benzene	505	Sabbah (1979)
H_3C–CH_2CH_3	330	Vedeneyev et al. (1966)
H_3C–$COCH_3$	290	Vedeneyev et al. (1966)
C–S bonds		
C–S in thiophene	341	Sabbah (1979)
C–S in benzothiophene	339	Sabbah (1979)
C–S in dibenzothiophene	338	Sabbah (1979)
HS–CH_3	312	Lide (1995)
H_3C–SCH_3	308	Lide (1995)
H_3C–SO_2CH_3	280	Lide (1995)
H_3C–$SCH_2C_6H_5$	257	Lide (1995)
H_3C–$SO_2CH_2C_6H_5$	221	Lide (1995)
H–C bonds		
H–CH_3	438	Lide (1995)
H–CH_2OH	410	Lide (1995)
H–CHO	364	Lide (1995)

This review has not cited all of the papers written about the biodegradation of sulfur heterocycles. Indeed, there are scores of publications on biodesulfurization and the microbial metabolism of dibenzothiophene which have not been cited, but none of these provide additional insight into how ring cleavage occurs. The title for this paper, 'Ring cleavage of sulfur heterocycles: how does it happen?' was chosen to ask a question, because there is still a large void in our knowledge of the mechanisms of these reactions. Among the many examples of ring cleavage, few have been studied in detail.

There is irrefutable evidence that aerobic microbial metabolism can result in the releases of the sulfur atom from dibenzothiophene and related compounds. This has been demonstrated by several research groups (Isbister et al. 1988; Kayser et al. 1993; van Afferden et al. 1993; Lee et al. 1995; Gray et al. 1996; Ohshiro et al. 1996) However, the evidence for the release sulfur from condensed thiophenes under anaerobic conditions is much weaker. Only a few groups have reported this phenomenon (Kurita et al. 1971; Kim et al. 1990a, 1990b; Lizama 1995), and this slow process appears to be difficult to demonstrate.

There are two major objectives driving the studies of the microbial metabolism of organosulfur compounds. One is the quest to develop a process for biodesulfurization of fossil fuels. The other is to understand the fates of organosulfur compounds in petroleum- or creosote-contaminated environments, which is important in the assessment of bioremediation activities. Of course, the goal of the biodesulfurization process is to leave the carbon skeletons of the organosulfur compounds intact, whereas the aim of any bioremediation process is to mineralize all of the contaminant compounds, including the organosulfur compounds.

Table 2 summarizes the aerobic microbial metabolism of many types of organosulfur compounds. Some common themes are evident from the data in Table 2. For example, in all cases, oxidation of the organosulfur compound occurs before ring cleavage.

The long side chains of the alkylthiophenes and alkylthiolanes are removed via beta-oxidation leaving thiophene carboxylic acids and thiolane carboxylic acids, respectively. The cleavage of thiophene-2-carboxylic acid (Cripps 1973) and thiophene-2,5-dicarboxylic acid (Fedorak et al. 1996) have been demonstrated, but the only pathway described is for the former compound (Figure 7). However, it must be emphasized that most of the steps in that pathway were only hypothesized by Cripps (1973). Ring cleavage of thiolane carboxylic acids has not been conclusively demonstrated. Thiolane-2-carboxylic acid and thiolane-2,5-dicarboxylic acid would be ideal model compounds for these investigations. Payzant et al. (1989a) showed that alkylthianes are present in non-biodegraded petroleums, including Bellshill crude oil, and Fedorak et al. (1988) demonstrated that these compounds could be removed from that oil by microbial activity. However, no information is available on the metabolites produced or the fate of six-member ring of alkylthianes.

The biodegradation of sulfolane has been observed (Chou & Swatloski 1983; Fedorak & Coy 1996) but no metabolites, other than sulfate, have been detected. Thus, there is no hint of the metabolic pathway which allows sulfolane to serve as a sole source of carbon and sulfur for bacterial cultures (A.E. Greene & P.M. Fedorak, unpublished data).

The most conclusive evidence demonstrating thiophene ring cleavage of benzothiophene is from the work of Eaton & Nitterauer (1994) who showed the formation of 2-mercaptophenylglyoxalate (Figure 6), which cyclized to benzothiophene-2,3-dione upon acidification of the culture medium (Table 2). Oxidation of the thiophene ring by a dioxygenase produces *cis*-2,3-dihydroxy-2,3-dihydrobenzothiophene, which is a transformed spontaneously and/or enzymatically to 2-mercaptophenylglyoxalate (Eaton & Nitterauer 1994). The net result of the oxidation of C2 and C3 in benzothiophene is the weakening of the bond between C2 and the sulfur atom, which leads to ring cleavage. Others have observed 2,3-diones from methyl- (Saftić et al. 1992; Kropp et al. 1994) and dimethyl- (Kropp et al. 1996) benzothiophenes. Thus, this mode of oxidation leading to thiophene ring cleavage appears to be common.

2,3-Diones cannot be formed in this manner if there is a methyl group on C2 and/or C3 (Fedorak & Grbić-Galić 1991; Saftić et al. 1992). In studies with 3-methylbenzothiophene, Selifonov et al. (1996) demonstrated that naphthalene 1,2-dioxygenase behaves as a sulfoxygenase oxidizing this compound to 3-methylbenzothiophene sulfoxide. Fedorak & Grbić-Galić (1991) provided evidence that this sulfoxide was an intermediate in the formation of 3-methylbenzothiophene sulfone, much the same as dibenzothiophene sulfoxide is an intermediate in the formation of dibenzothiophene sulfone the 4S pathway. D.C. Bressler & P.M. Fedorak have isolated a bacterial culture that grows on 3-methylbenzothiophene sulfone

Table 2. Summary of aerobic studies of microbial metabolism of organosulfur compounds.

Organosulfur compounds	Microbial transformation process and/or metabolites detected	Cleavage of sulfur-containing ring demonstrated
Alkylthiolanes	Side chain degraded to thiolane carboxylic acid (1)[a]	No
Alkylthiophenes	Side chain degraded to thiophene carboxylic acids (2, 3)	Yes, from some thiophene carboxylic acids (3, 4, 5)
Sulfolane	Process unknown, but sulfate detected (6)	Yes (6)
Benzothiophenes	Oxidized to 2,3-diones which exist as mercaptophenylglyoxalates at neutral pH (7, 8, 9, 10)	Yes (7). No, but the 2,3-diones detected (8, 9, 10)
	Oxidized to sulfones (9, 10, 11)	Yes, from sulfone (12)
Dibenzothiophenes	Oxidation of the sulfur atom leading to desulfurization by 4S pathway producing substituted biphenyl (13, 14, 15, 16, 17)	Yes (13, 14, 15, 16, 17)
	Degradation of homocyclic rings to give 2,3-diones which exist as mercaptophenylglyoxalates at neutral pH (18, 19, 20)	No, but the 2,3-diones detected (18, 19, 20)
	Oxidation of the sulfur atom, followed by oxidation of the homocyclic ring (21, 22)	Yes (21, 22)
Benzonaphthothiophenes	Oxidation of the sulfur atom leading to desulfurized by 4S pathway leaving α-hydroxy-β-phenylnaphthalene (17)	Yes (17)

[a] Reference number

1 Fedorak et al. (1988) 2 Fedorak & Peakman (1992) 3 Fedorak et al. (1996)
4 Cripps (1973) 5 Kanagawa & Kelly (1987) 6 Chou & Swatloski (1983)
7 Eaton & Nitterauer (1994) 8 Bohonos et al. (1977) 9 Saftić et al. (1992)
10 Kropp et al. (1994) 11 Selifonov et al. (1996) 12 Bressler & Fedorak (unpublished)
13 Isbister et al. (1988) 14 Kayser et al. (1993) 15 Lee et al. (1995)
16 Gray et al. (1996) 17 Ohshiro et al. (1996) 18 Saftić et al. (1993)
19 Kropp et al. (1996) 20 Kropp et al. (1997) 21 van Afferden et al. (1990)
22 van Afferden et al. (1993)

and releases sulfate from this compound (unpublished data). Hence, the sulfone is susceptible to ring cleavage, but the mechanism for this is unknown.

Studies with dibenzothiophene and related compounds show that the initial oxidation of the sulfur atom via the 4S pathway leads to cleavage of the thiophene ring prior to the selective removal of the sulfur atom (Table 2). The only reported biodegradation of a benzonaphthothiophene occurs via the modified 4S pathway (Ohshiro et al. 1996). van Afferden et al. (1993) demonstrated that some bacteria initially oxidize the sulfur atom in dibenzothiophene and then they oxidize the homocyclic ring, which leads to cleavage of the thiophene ring.

It appears that the thiophene ring of dibenzothiophene can also be broken by a mechanism analogous to that of benzothiophene via the same mechanism that yields 2-mercaptophenylglyoxalates, because 2,3-diones have been found in extracts of acidified culture supernatants (Table 2). The 2-mercaptophenylglyoxalates likely arise from the metabolism of 3-hydroxy-2-formylbenzothiophene, but the mechanism of transformation is unknown.

Although the modified 4S pathway is well-understood, there is clearly much more research needed to elucidate how ring cleavage in sulfur heterocycles happens. The lacking information is of special interest to those concerned with the biodegradation of sulfur heterocycles in contaminated environments. These investigation are hampered by the lack of commercially available compounds of interest. For example, although a few methylbenzothiophenes are commercially available, none of the methylated dibenzothiophenes or the known metabolites such as thiolane-2-carboxylic acid, 3-hydroxy-2-formylbenzothiophene and benzothiophene-2,3-dione are available from chemical companies. Indeed, these metabolic studies would be much easier if radiolabeled

sulfur heterocycles were readily available. Also, the cleavage of the sulfur heterocycles leads to very polar organosulfur compounds (Figures 6–9), which are not amenable to extraction into organic solvents for analysis by gas chromatography-mass spectrometry. In spite of these obstacles, the literature reviewed for this paper indicates that our understanding of how ring cleavage in sulfur heterocycles is slowly increasing.

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