Microbial transformation of benzothiophenes, with carbazole as the auxiliary substrate, by *Sphingomonas* sp. strain XLDN2-5

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Benzothiophenes are a toxic and relatively recalcitrant fraction of coal-tar creosote. We investigated the co-metabolic transformation of benzothiophene (BT) and its derivatives by the carbazole (CA) degrader *Sphingomonas* sp. XLDN2-5, which is not able to grow on benzothiophenes as the sole carbon source. Among the benzothiophenes tested, BT, 2-methylbenzothiophene (2-MBT) and 5-methylbenzothiophene (5-MBT) were co-metabolically converted. For 3-methylbenzothiophene, there was complete inhibition of growth on CA. The common transformation products for BT, 2-MBT and 5-MBT are the corresponding sulfoxides and sulfones. For BT, several high-molecular-mass sulfur-containing aromatic compounds, including benzo[b]naphtho[1,2-d]thiophene, benzo[b]naphtho[1,2-d]thiophene-7-oxide, 6a,11b-dihydrobenzo[b]naphtho[1,2-d]thiophene, 6a,11b-dihydrobenzo[b]naphtho[1,2-d]thiophene-7-oxide, and a new product, 6,12-epithiobenzo[b]naphtho[1,2-d]thiophene, were detected by GC-MS. These high-molecular-mass products are thought to be generated from a Diels–Alder-type reaction. Investigations with a combination of GC and flame ionization detection showed that about 17 % of BT was transformed to benzo[b]naphtho[1,2-d]thiophene. Aerobic transformation of benzothiophenes to sulfoxides and sulfones can reduce their toxicity, and facilitate their biodegradation. However, the formation of the high-molecular-mass products, such as benzo[b]naphtho[1,2-d]thiophene, should be considered in the biodegradation of benzothiophenes.

INTRODUCTION

Coal-tar creosote is a by-product of the fractional distillation of crude coal tars. This liquid is a complex mixture that consists of polycyclic aromatic hydrocarbons, phenolic compounds and heterocyclic compounds (Gundlach et al., 1983; Mueller et al., 1989; Annweiler et al., 2001; Mundt & Hollender, 2005; Xu et al., 2006; Andersson et al., 2006). S-, N- and O-heterocyclic aromatic compounds constitute approximately 5–13 % of creosote (Mueller et al., 1989). Some heterocyclic aromatic compounds, such as carbazole (CA), quinoline, benzothiophene-1-oxide (BTO) and benzo[ghi]perylene, are mutagenic, or at least genotoxic (Eastmond et al., 1984; Seymour et al., 1997; Kropp & Fedorak, 1998). Moreover, because of their relatively high water solubility, S-, N- and O-heterocyclic aromatic compounds can amount to 35–40 % of the water-soluble fraction of creosote, and they are therefore of great environmental concern (Licht et al., 1996). Bioremediation is a potential treatment alternative for eliminating these contaminants in the environment. Fundamental research has been conducted to examine the biodegradation of these hazardous compounds (Fedorak & Gribić-Galić, 1991; Boyd et al., 1993; Kropp et al., 1994a; Boyd et al., 2003; Yu et al., 2006; Safinowski et al., 2006; Gai et al., 2007; Wang et al., 2007). However, it remains unclear whether benzothiophenes have an inhibitory effect on N-heterocyclic compounds such as CA, although other studies have shown that S-, N- and O-heterocyclic aromatic compounds can have a significant inhibitory effect on the biodegradation of polycyclic aromatic hydrocarbons (Dyreborg et al., 1996a, b, 1997; Meyer & Steinhart, 2000).

CA is the major nitrogen heteroaromatic compound in coal-tar creosote, and is one of the 13 most common heterocyclic compounds in creosote. Many bacterial strains...
have the ability to use CA as the sole carbon, nitrogen and energy source (Nojiri et al., 1999; Gai et al., 2007). Most isolates degrade CA by following the so-called angular pathway, in which CA is initially attacked at the 1 and 9a positions by CA dioxygenase, followed by the spontaneous conversion of the dihydroxylated intermediate to 2′-aminobiphenyl-2,3-diol (Fig. 1). An extradiol dioxygenase then attacks the hydroxylated ring at the meta position to give 2-hydroxy-6-(2′-aminophenyl)-6-oxo-2,4-hexadienoic acid, which is hydrolysed to produce anthranilic acid and 2-hydroxypenta-2,4-dienoic acid (Nojiri et al., 1999; Gai et al., 2007).

Benzothiophene (BT) and its derivatives are the major sulfur heteroaromatic compounds. However, no bacterial strain has been found that is able to grow on benzothiophenes as the sole carbon source, and all reported biotransformations of benzothiophenes are based on co-metabolism. Attacks on the thiophene ring of benzothiophenes lead to the formation of sulfoxides and sulfones, or to ring opening and the formation of 2-mercaptomandelaldehyde and 2-mercaptophenylglyoxalate (Saftic´ et al., 1992; Eaton & Nitterauer, 1994; Gilbert et al., 1998).

Interestingly, it has been reported that BTO is trapped as a Diels–Alder-type disulfoxide cycloadduct, which yields benzo[b]naphtho[1,2-d]thiophene (BN12T) (Kropp et al., 1994b). However, the mechanism for BN12T formation is not well known, and very little information is available. From a bioremediation point of view, it would be interesting to investigate the transformation products of benzothiophenes that are produced by CA-degrading bacteria, because of the ubiquitous co-occurrence of CA and benzothiophenes in coal-tar- and creosote-polluted sites. A better understanding of the metabolic products of contaminants is also necessary to evaluate and improve the bioremediation process.

The aim of this study was to investigate the transformation of benzothiophenes by Sphingomonas sp. XLDN2-5, employing CA as an auxiliary substrate. We selected BT, 2-methylbenzothiophene (2-MBT), 3-methylbenzothiophene (3-MBT) and 5-methylbenzothiophene (5-MBT) as typical S heterocycles. Co-metabolic conversion was assessed by identification of metabolites originating from the co-substrates by investigation of the culture extract by GC-MS.

**METHODS**

**Bacterial strain and growth conditions.** Sphingomonas sp. XLDN2-5 utilizes CA as the sole carbon and nitrogen source (Gai et al., 2007; Wang et al., 2007). The strain was grown in Luria–Bertani medium, or in mineral salt medium (MSM) containing the appropriate carbon source. The carbon sources used in this study were CA (0.5 g l⁻¹), glucose (10 g l⁻¹) and peptone (5 g l⁻¹). When glucose served as the carbon source, NH₄Cl (0.8 g l⁻¹) was added to serve as a nitrogen source. All cultures were incubated at 30 ºC on a reciprocal shaker at 180 r.p.m.

**Chemicals.** CA, BT, 2-MBT and DMSO were purchased from Sigma-Aldrich. Benzothiophene-1,1-dioxide (BTO₂) was purchased from Acros Organics. 3-MBT and 5-MBT were purchased from Lancaster Synthesis. BN12T was purchased from Fluka. Diazomethane, synthesized from N-nitroso-N-methyl urea (purchased from Sigma-Aldrich), was used for the derivatization. Stock solutions of CA, benzothiophenes and BTO₂ were 200 mM in DMSO, and were added to MSM to the required concentrations, as described below. All other commercially available chemicals were of analytical grade.

**Biotransformation of benzothiophenes by Sphingomonas sp. strain XLDN2-5.** In order to investigate the transformation of benzothiophenes by cells of XLDN2-5 growing in CA, each test flask was inoculated with bacterial biomass to an OD620 of 0.1. Degradation studies were carried out in 250 ml seal-capped flasks. CA and benzothiophenes (dissolved in DMSO) were added to each

![Fig. 1. Biodegradation of CA via the angular pathway by various CA-utilizing bacteria.](http://mic.sgmjournals.org)
flask, containing 20 ml MSM, to final concentrations of 2 and 0.3 mM, respectively. In all experiments, controls containing bacteria and no benzothiophenes (bacterial control), and controls containing sterile medium with benzothiophenes (sterile control) were included. The sterile controls were inoculated with heat-killed cells (autoclaved at 115 °C for 20 min). For controls for BT and its derivatives, MSM was spiked with 0.3 mM of each benzothiophene, and inoculated in the same way as the test flasks. Each test flask was prepared in triplicate.

For washed-cell experiments, cells grown on different carbon sources were harvested by centrifugation (6000 g for 5 min) when they were in exponential phase, and then washed twice in MSM. The pellets were resuspended in MSM to an OD_{600} of 5.0. Degradation studies were carried out in 250 ml sealed-capped flasks. Benzothiophenes were added to 20 ml medium to a final concentration of 2 mM. The inactive controls were inoculated with heat-killed cells (autoclaved at 115 °C for 20 min). All test flasks were prepared in triplicate, and incubated for 24 h. The presence of residual benzothiophenes was determined by HPLC, as described previously (Gai et al., 2007). The mobile phase was a 70:30 mixture of methanol and MilliQ water, at a flow rate of 0.5 ml min⁻¹.

UV/visible spectrophotometric scans. UV/visible spectrophotometry scans were performed on a 3100 spectrophotometer (Shimadzu). The supernatants of the cultures exposed to the benzothiophenes were obtained by centrifugation (13 500 g for 10 min), and analysed spectrophotometrically to identify new peaks formed as a result of the accumulation of specific metabolites.

Identification and analysis of the metabolites. Cells from 5 l exponential-phase overnight cultures of XLDN2-5, grown in MSM supplemented with 3 mM CA, were harvested by centrifugation (6000 g for 5 min). Cells were resuspended in 500 ml MSM (OD_{600} 5.0), 3 mM of one of the benzothiophenes to be tested was added, and the cultures were incubated for 24 h. After the cells were removed by centrifugation (13 500 g for 20 min), metabolites were extracted from the supernatant of the culture medium with ethyl acetate (neutral fraction) and after acidification with HCl to pH 2 (acidic fraction). The extract containing metabolites was concentrated, and used for metabolite identification by GC-MS. Metabolites in the acidic fraction were also derivatized with diazomethane to enhance the detection of polar metabolites. Metabolites were identified by comparison with published mass spectra, and identification was confirmed by co-injection with commercially available authentic compounds. For metabolites for which there were no authentic metabolites or reference spectra available, the metabolites were identified according to the following criteria: (i) the compound did not appear when strain XLDN2-5 grew on CA only, and was also not present in the substrate used, (ii) the mass spectrum was highly analogous to the mass spectrum of the defined compound.

GC-MS analysis was carried out with Hewlett Packard 1800C GCD equipped with a J&W PTE-5MS column (30 m × 0.25 mm × 0.25 μm). The temperature programme was as follows: initial oven temperature 60 °C for 6 min, 10 °C min⁻¹ to 150 °C, then 15 °C min⁻¹ to 280 °C, and this temperature was held for 6 min. High-resolution mass spectra were recorded on a GC-MS system (Waters GCT mass spectrometer, coupled to an Agilent HP6890 gas chromatograph), which was equipped with a J&W DB-5MS column. The oven temperature programme used for high-resolution mass spectra was 60 °C for 3 min, followed by an increase of 12 °C min⁻¹ to 280 °C, and this temperature was maintained for 3 min.

Quantification of BN12T. Cultures of XLDN2-5 grown with CA and BT were used to determine the amount of BN12T produced. Metabolites were extracted from the supernatant culture medium with ethyl acetate; extractions were performed in triplicate. The extracts were combined and dried with anhydrous magnesium sulfate. After evaporation of the solvent, residues were dissolved in 100 μl methanol, and 2 μl of the solution was used for analysis by GC equipped with a flame-ionization detector (FID). Peak areas were compared with those of a known amount of BN12T.

RESULTS AND DISCUSSION

Benzothiophenes are toxic and relatively recalcitrant compounds of creosote, and are thus important from the perspective of bioremediation processes. Spingomonas sp. XLDN2-5 is able to grow on CA as the sole carbon, nitrogen and energy source, and it is able to degrade dibenzofuran and dibenzothiophene simultaneously (Gai et al., 2007). Here, we showed that strain XLDN2-5 was able to co-metabolize BT, 2-MBT or 5-MBT, with CA as the auxiliary substrate. Co-metabolic conversion was assessed by GC-MS analysis to identify the metabolites originating from benzothiophenes. Although numerous studies have demonstrated the co-metabolism of benzothiophenes, this is believed to be the first report of CA supporting co-metabolism.

Growth of XLDN2-5 with different benzothiophenes

Among the benzothiophenes tested, BT, 2-MBT, and 5-MBT were co-metabolically converted (Fig. 2a, b and d); however, 2-MBT and 5-MBT produced a reduction in bacterial growth. Total inhibition of growth on CA was observed for 3-MBT (Fig. 2c). The transformation of BT, 2-MBT and 5-MBT accompanied a decrease in the level of CA, while no prominent decrease of BT, 2-MBT and 5-MBT was observed in the controls without inocula, or in those in which CA was absent. The results suggested that the presence and the positions of the methyl groups in the benzothiophenes have a significant influence on the biodegradation of benzothiophenes. An inhibitory effect of creosote compounds on the biodegradation of toluene and benzene has also been observed (Dyreborg et al., 1996a, b). It has been reported that a 1-methylnaphthalene-degrading enrichment culture was able to use glucose or peptone as the primary substrate to produce sulfoxide and sulfone from 3-MBT (Fedorak & Grbić-Galic, 1991). Strain XLDN2-5 showed the ability to transform benzothiophenes, but only when grown on CA as a carbon source (data not shown). These results strongly suggest that the CA-metabolizing enzymic system is responsible for the transformation process in XLDN2-5. The results are consistent with our previous report, in which the dibenzothiophene and dibenzofuran degradation activity shown by strain XLDN2-5 was controlled by the CA-degrading enzymes (Gai et al., 2007).

Co-metabolic transformation products of benzothiophenes

Some compounds that were identified in the course of our studies by GC-MS analysis are presented in Table 1. The common transformation products for BT, 2-MBT and 5-
Microbial transformation of benzothiophenes

**Fig. 2.** Microbial transformation of BT (a), 2-MBT (b), 3-MBT (c) and 5-MBT (d) by *Sphingomonas* sp. XLDN2-5 cells in CA. BT, 2-MBT, and 5-MBT were co-metabolically converted, and 3-MBT totally inhibited the growth on CA. ▲, CA; ○, BT control; ●, BT; □, 2-MBT control; ■, 2-MBT; △, 3-MBT control; ▲, 3-MBT; ★, 5-MBT control; ★, 5-MBT. Values are means of three replicates (±SD).

**Table 1.** GC-MS data for the intermediates formed during the transformation of benzothiophenes by *Sphingomonas* sp. XLDN2-5

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>Extract</th>
<th>Retention time (min)</th>
<th>m/z of fragment ions (proposed composition of ions, percentage relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT</td>
<td>3-Hydroxybenzothiophene*</td>
<td>Neutral</td>
<td>14.65</td>
<td>150 (M+, 100), 122 (M+ -CO, 45), 121 (M+ -CHO, 78), 94 (7), 77 (24), 69 (15)</td>
</tr>
<tr>
<td></td>
<td>2-Hydroxybenzothiophene*</td>
<td>Neutral</td>
<td>14.91</td>
<td>150 (M+, 100), 122 (M+ -CO, 45), 121 (M+ -CHO, 73), 105 (7), 78 (19), 76 (16), 50 (22)</td>
</tr>
<tr>
<td></td>
<td>Benzo thiophene-2,3-dione*</td>
<td>Neutral</td>
<td>15.88</td>
<td>164 (M+, 4), 136 (M+ -CO, 100), 108 (M+ -CO-CO, 42), 92 (5), 82 (15), 69 (20)</td>
</tr>
<tr>
<td></td>
<td>BTO*</td>
<td>Neutral</td>
<td>16.79</td>
<td>150 (M+, 32), 134 (M+ -O, 62), 121 (M+ -CHO, 100), 105 (7), 89 (25), 78 (20), 63 (19)</td>
</tr>
<tr>
<td></td>
<td>Benzo thiophene-1,1-dioxide†</td>
<td>Neutral</td>
<td>16.94</td>
<td>166 (M+, 30), 137 (M+ -CHO, 100), 109 (M+ -CHO-CO, 45), 89 (20), 65 (19), 50 (19)</td>
</tr>
<tr>
<td>2-MBT</td>
<td>2-Mercaptobenzoic acid (methyl ester)†</td>
<td>Acidic</td>
<td>14.80</td>
<td>168 (M+, 51), 152 (4), 136 (100), 108 (32), 69 (7)</td>
</tr>
<tr>
<td>5-MBT</td>
<td>2-Methylbenzothiophene-1,1-dioxide*</td>
<td>Neutral</td>
<td>17.94</td>
<td>180 (M+, 30), 137 (100), 89 (20), 63 (22)</td>
</tr>
<tr>
<td></td>
<td>Monohydroxy-5-methylbenzothiophene</td>
<td>Neutral</td>
<td>16.83</td>
<td>164 (M+, 100), 147 (M+ -OH, 75), 135 (M+ -CHO, 52), 131 (12), 91 (30), 69 (7), 63 (6)</td>
</tr>
<tr>
<td></td>
<td>Monohydroxy-5-methylbenzothiophene</td>
<td>Neutral</td>
<td>17.06</td>
<td>164 (M+, 77), 147 (M+ -OH, 28), 135 (M+ -CHO, 100), 131 (13), 91 (38), 69 (5), 63 (7)</td>
</tr>
<tr>
<td></td>
<td>5-Methylbenzothiophene-1-oxide*</td>
<td>Neutral</td>
<td>17.27</td>
<td>164 (M+, 7), 150 (M+ -O, 100), 121 (49), 78 (15)</td>
</tr>
</tbody>
</table>

*Identification in agreement with results of other studies.
†Identified by comparison (of retention time and mass spectrum) with authentic material.
MBT were the corresponding sulfoxides and sulfones. Six metabolites of BT were detected by GC-MS: BTO₂ and 2-mercaptobenzoic acid were identified by comparison of their retention times and mass spectra with those of authentic compounds; 2-hydroxybenzothiophene, 3-hydroxybenzothiophene, benzothiophene-2,3-dione and BTO were identified by comparison of their mass spectra with published data (Safić et al., 1992; Eaton & Nitterauer, 1994; Kropf et al., 1994b; Gilbert et al., 1998). These results indicate that strain XLNDN2-5 is able to catalyse lateral dioxygenation and S oxidation on the thiophene ring of BT. For 2-MBT, only 2-methylbenzothiophene-1,1-dioxide was identified as a metabolite, by comparison of its mass spectrum with published data (Kirkwood et al., 2007). For 5-MBT, 5-methylbenzothiophene-5-oxide and two mono-hydroxy-5-methylbenzothiophenes were detected according to their mass spectra. These results indicate that, in addition to aromatic compounds such as isopropylbenzene (Eaton & Nitterauer, 1994) and 1-methylnaphthalene (Fedorak & Gribić-Galic, 1991), heteroaromatic compounds, such as CA, can also serve as a primary substrate in the concomitant metabolism of benzothiophenes.

Sulfoxides and sulfones were formed by two consecutive oxygenations of the sulfur atom of benzothiophenes by XLNDN2-5. Enzyme-catalysed sulfur oxidation is a common biotransformation process, such as that catalysed by CA 1,9α-dioxygenase from Pseudomonas sp. strain CA10 (Norji et al., 1999), dibenzofuran 4,4α-dioxygenase from Sphingomonas sp. strain RW1 (Bünz & Cook, 1993), naphthalene dioxygenase from Pseudomonas sp. strain NCIB 9816-4 (Resnick & Gibson, 1996), and toluene dioxygenase from Pseudomonas putida UV4 (Boyd et al., 1996). However, all of these strains lack the ability to cleave the sulfur-containing ring of sulfones, and this results in the accumulation of sulfoxides and sulfones. Sulfoxides and sulfones have higher water solubility, and are less toxic than their parent compounds (Seymour et al., 1997). An earlier study reported that a filamentous bacterium was able to grow on BTO₂, and 3- and 5-methylbenzothiophene-1,1-dioxide, as the sole carbon, sulfur and energy source (Bressler et al., 1999). Those results suggest that microbial co-metabolic transformation can reduce the toxicity and facilitate the biodegradation of BT. When CA-grown washed cells (OD620 5) were incubated with 1.0 mM BT, the culture became a yellow colour that had a maximal absorbance at 318 nm (A318) (Fig. 3). The peak at 318 nm was attributed to 2-mercaptomandelaldehyde, in analogy to a report by others (Eaton et al., 1994). The detection of 2-mercaptomandelaldehyde and monohydroxybenzothiophenes suggests that XLNDN2-5 is able to catalyse the thiophene ring of benzothiophenes to produce dihydrodiol. The formation of 2- and 3-hydroxybenzothiophene could be explained by the spontaneous conversion of the corresponding unstable 2,3-dihydroxy-2,3-hydrobenzothiophene, or by a mono-oxygenase catalysed reaction (Eaton et al., 1994; Corvini et al., 2006; Gai et al., 2007). Being a thiohemiacetal, 2,3-dihydroxy-2,3-hydrobenzothiophene appears to undergo spontaneous ring cleavage of the thiohemiacetal to generate 2-mercaptomandelaldehyde (Eaton et al., 1996). Subsequently, the oxidation of 2,3-dihydroxy-2,3-hydro-

**Table 2. HR-MS data for some high-molecular-mass products from cultures of BT exposed to Sphingomonas sp. XLNDN2-5**

<table>
<thead>
<tr>
<th>Metabolite*</th>
<th>Molecular formula</th>
<th>Experimental result</th>
<th>Calculated mass</th>
<th>Difference (p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6a,11b-Dihydrobenzo[b]naphtho[1,2-d]thiophene (I)</td>
<td>C₁₆H₁₂S</td>
<td>236.0645</td>
<td>236.0660</td>
<td>−6.2</td>
</tr>
<tr>
<td>6a,11b-Dihydrobenzo[b]naphtho[1,2-d]thiophene-7-oxide (II)</td>
<td>C₁₆H₁₂OS</td>
<td>252.0603</td>
<td>252.0609</td>
<td>−2.3</td>
</tr>
<tr>
<td>6,12-Epithiobenzo[b]naphtho[1,2-d]thiophene (III)</td>
<td>C₁₆H₁₀S₂</td>
<td>266.0224</td>
<td>266.0222</td>
<td>0.8</td>
</tr>
<tr>
<td>Benzo[b]naphtho[1,2-d]thiophene-7-oxide (IV)</td>
<td>C₁₆H₁₀OS</td>
<td>250.0468</td>
<td>250.0452</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*Roman numerals correspond to metabolites in Fig. 5.*
benzothiophene or 2-mercaptomandelaldehyde yields 2-mercaptophenylglyoxalate, which has a pH-dependent equilibrium with benzothiophene-2,3-dione (Bressler & Fedorak, 2001). Benzothiophene-2,3-dione has also been detected in the extracts of cultures containing dibenzothiophene (Kropp et al., 1997). 2-Mercaptophenylglyoxalate contains an α-keto-acid moiety that is susceptible to decarbonylation and decarboxylation to produce non-toxic 2-mercaptobenzoic acid (Siegel & Lanphear, 1979). These results suggest that XLDN2-5 is able to catalyse the lateral dioxygenation of BT, followed by spontaneous cleavage of the heterocyclic ring upon re-aromatization, in analogy to angular dioxygenation of CA and dibenzofuran, oxidation of the aldehyde, and oxidative decarboxylation. The proposed metabolic pathways of BT are summarized in Fig. 4.

Diels–Alder reaction of BTO, and quantification of BN12T

In addition to the metabolites identified above, some high-molecular-mass compounds of benzothiophenes were detected (Fig. 5 and Table 2). The dominating product detected by GC-MS was identified as BN12T, according to its mass spectrum, and this was confirmed by co-injection with authentic BN12T. The molecular ion of compound IV (Fig. 5) appeared to be m/z 250. We propose this to be benzo[b]naphtho[1,2-d]thiophene-7-oxide (BN12TO). The fragments at m/z 234 and 221 correspond to the loss of -O and -CHO, respectively. The fragment at m/z 202 corresponds to the loss of -OS. These losses have been observed for other sulfoxides, such as dibenzothiophene-5-oxide and BTO (Gai et al., 2007; Gilbert et al., 1998). Two pathways may explain the formation of BN12TO in the transformation of benzothiophene by strain XLDN2-5: one pathway involves a Diels–Alder dimerization of BTO, followed by the loss of two hydrogen atoms, two oxygen atoms, and one sulphur atom (Kropp et al., 1994b); the other pathway implies a subsequent oxidation of BN12T to BN12TO. In order to distinguish between these two pathways, transformation of BN12T with XLDN2-5 was performed. However, BN12TO was not detected, making it unlikely that BN12TO was formed from BN12T by strain XLDN2-5.

![Fig. 4. Proposed BT transformation pathways by Sphingomonas sp. XLDN2-5. The compounds in the dashed box were tentatively identified. The compound in brackets was not detected. Dashed arrows indicate transformations that occur during the extraction of products.](http://mic.sgmjournals.org)
Compounds I and II (Fig. 5) were two mass units higher than compounds BN12T and BN12TO, respectively. Compound I had a similar mass fragment pattern to compound BN12T, and it was identified as 6a,11b-dihydrobenzo[b]-naphtho[1,2-d]thiophene. Signals at \( m/z \) 235 and 234 indicated the net loss of hydrogen. High-resolution MS gave compound II an empirical formula of \( C_{16}H_{12}OS \) (Table 2). Compound II had a molecular ion at \( m/z \) 252 (\( M^+ \), 100) and fragments at \( m/z \) 234 (\( M^+ - \text{H}_2\text{O} \), 8), 223 (\( M^+ - \text{CHO} \), 11), 219 (\( M^+ - \text{HS} \), 38) and 189 (15),...
which were similar to those of compound BN12TO, indicating their structural similarity. Based on these data, compound II was identified as 6a,11b-dihydrobenzo[h]naphtho[1,2-d]thiophene-7-oxide. The mass spectrum of compound III [m/z (relative intensity): 266 (M^+ 1, 100), 234 (15), 221 (4.5), 202 (4), 189 (5)] (Fig. 5) showed a molecular ion of m/z 266 which was 32 Da greater than the mass of BN12T, and high-resolution MS gave an empirical formula of C_{14}H_{16}S (Table 2). The mass spectrum and high-resolution MS results indicated that the 32 mass units were one sulfur atom rather than two oxygen atoms. Based on these data, compound III was identified as 6,12-epithiobenzo[h]naphtho[1,2-d]thiophene.

BTO generated from BT by Sphingomonas sp. XLDN2-5 resulted in the formation of some high-molecular-mass products (Fig. 5 and Table 2). Kropp et al. (1994b) were the first to propose the abiotic mechanism of BN12T formation from BTO, i.e. a Diels–Alder-type condensation of two molecules of BTO, with the subsequent loss of two hydrogen atoms, two oxygen atoms, and one sulphur atom. However, the detailed mechanism is unclear, including the pathway(s) by which oxygen, sulfur and hydrogen are lost. In another study, the biotransformation of BT by Pseudomonas putida UV4 also gave tetracyclic products, in addition to vicinal dihydrodiols (Boyd et al., 1996). Boyd et al. (1996) identified two new tetracyclic products: 6a,11b-dihydrobenzo[h]naphtho[1,2-d]thiophene-7-oxide and BN12TO, in addition to BN12T. Based on these results, Boyd et al. (1996) suggested that the extrusion of sulfur monoxide, followed by dehydrogenation during the transformation process, could account for the formation of BN12TO and BN12T. Therefore, the new product 6,12-epithiobenzo[h]naphtho[1,2-d]thiophene (Fig. 5) identified in our study provides further insight to this process, and more information concerning the pathway(s) by which BN12T is formed. The detection of 6,12-epithiobenzo[h]-naphtho[1,2-d]thiophene suggests that the loss of sulfur monoxide might involve a deoxygenation step rather than a direct loss of sulfur monoxide. Deoxygenation is a relatively common process in the photochemistry of aromatic sulfoxide (Gregory et al., 1997). It has been reported that the direct photolysis of dibenzothiophene-1-oxide produced a potent oxidant O(3P) (Gregory et al., 1997). We suggest that during the process of formation of BN12T from BTO, O(3P) or other oxidant species are also produced. These species might be toxic to bacteria, and contribute to the recalcitrance and persistence of BT in the environment.

GC-FID results showed that XLDN2-5 produced about 22 μM BN12T when 85% 0.3 mM BT was transformed (Fig. 6). Quantification of other high-molecular-mass products was not feasible because of the lack of authentic materials. Considering that two BTO molecules condense to give one molecule of condensation products, the yield of BTO by strain XLDN2-5 was more than 17%. In another study, it was reported that about 7% of the initial amount of BT was transformed to BN12T (Kropp et al., 1994b). The widespread occurrence of water contaminated with petroleum and creosote has led to development of treatment methods, including aerobic biodegradation, for their removal. Assessing biodegradation in contaminated aquifers is a very demanding task, especially for S-heterocyclic compounds for which there is scant knowledge available regarding their biological fate in groundwater. Thus, the aerobic co-metabolic conversion of benzothiophenes must be considered in bioremediation. Moreover, the toxicological consequences of these high-molecular-mass compounds, for which very little is known about the chemistry, remain to be determined.

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