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Application of solid-phase extraction to the analysis of the isomers generated in biodesulfurization against methylated dibenzothiophenes

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Abstract

A solid-phase extraction (SPE) technique was applied to analyze and characterize the biodesulfurization reactions against asymmetrically methylated dibenzothiophenes (mDBTs) such as 1-, 2-, 3- and 4-methyldibenzothiophenes present in fossil fuels. Recently, we found that these mDBTs are efficiently degraded by the bacterial strain, *Rhodococcus erythropolis* KA2-5-1. Separation and concentration of the microbial desulfurization products from each of the mDBTs could be carried out with high efficiency and reproducibility by the SPE procedure. These desulfurization products were identified using the SPE technique combined with GC, GC–atomic emission detection, GC–MS and ^{1}H nuclear magnetic resonance spectroscopy. The analytical data obtained suggested that the desulfurization reactions against mDBTs by this bacterial strain may occur through specific carbon–sulfur bond-targeted cleavages that can be affected by the positions of methyl groups.

Keywords: Dibenzothiophenes; Sulfur compounds

1. Introduction

Organic sulfur compounds are contained in fossil fuels [1,2] and are thought to be a source of environmental pollutants. In petroleum fractions, e.g., gas oil, organosulfur compounds exist in the form of alkyl-substituted dibenzothiophenes (Cx-DBTs), which are very difficult to remove by the conventional hydrodesulfurization (HDS) process [3]. It has recently been noted that several microbial strains can remove sulfur from Cx-DBTs by sulfur-specific degradation [4–8]. These biocatalysts are expected to be useful for refining of petroleum safely and at lower cost [9,10].

The metabolic pathway for DBT desulfurization has been intensively investigated with *Rhodococcus* sp. IGTS8 [11–14] and ECRD-1 [15].

In *Rhodococcus* sp. IGTS8, DBT is degraded by four enzymes, Dsz A, B, C which are encoded by the plasmid-located *dsz* operon, and Dsz D, a flavin mononucleotide (FMN)-dependent reduced pyridine nucleotide (NADH) oxidoreductase which is considered to be located on the genome [14]. According to the mechanism proposed for the selective removal of sulfur from DBT, one of the C–S carbons of DBT-5,5-dioxide (DBT-sulfone), which is produced by the

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two-step S-oxygenation of DBT by Dsz C that requires NADH and reduced FMN, is attacked by FMNH 4a-hydroperoxide, leading to cleavage of the C–S bond and formation of 2-(2-hydroxyphenyl)benzenesulfinate (HPBS). This molecule (FMNH 4a-hydroperoxide) is given by $FMNH_2$, which would bind to Dsz A, reacting with molecular oxygen. Dsz D is absolutely required in the reaction catalyzed by Dsz C and A [12–14]. HPBS is then converted to 2-hydroxybiphenyl (2-HBP) by Dsz B, leading to the release of sulfite. The hydroxyl group in 2-HBP is derived from 4a-hydroxyperoxide and thus should be linked to the carbon in the benzene ring, which has been attacked by the molecule. In other words, the benzene ring with an OH group in each of the final products of the microbial desulfurization may have been originally linked to the sulfur atom by the C–S bond attacked first in the substrate. DBT has two C–S carbons. This raises the question of whether the FMNH 4a-hydroperoxide attacks these carbons randomly. To understand the manner of the attack on the DBT skeleton by microbial catalysts, it was necessary to use asymmetrically substituted DBTs as substrates. However, no detailed investigations on the desulfurization of Cx-DBTs except 4,6-diethylDBT with a symmetrical conformation [16] have been reported.

We have isolated a DBT-degrading bacterium, *Rhodococcus erythropolis* KA2-5-1, from the soil and found that this microbial strain has strong desulfurization activities against Cx-DBTs including asymmetrically substituted DBTs [17].

In the present investigation, we focused on the analysis of the microbial desulfurization products (metabolites) of four kinds of asymmetrically methylated DBTs (mDBTs), i.e., 1-, 2-, 3- and 4-methyl DBT (MDBT).

The primary step in analysis of the metabolites involves their separation from non-aqueous solvent such as *n*-tetradecane. For this purpose, a solid-phase extraction (SPE) technique was used because of its rapid and simple procedure. Moreover, SPE treatment was capable of reducing the volume of organic solvents required for subsequent sample preparation, i.e., liquid–liquid extraction. We had already developed the SPE technique with silica packing for the separation and concentration of polar compounds such as 2-HBP, 2,2′-biphenol and DBT-sulfone from non-polar, non-aqueous matrices such as kerosene [18]. To identify the microbial metabolites of the mDBTs isolated by the SPE cartridges, we applied gas chromatography (GC), gas chromatography with atomic emission detection (GC–AED), gas chromatography–mass spectrometry (GC–MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy.

Here, we describe the successful application of the SPE technique combined with some analytical systems to the identification of microbial metabolites of asymmetrical mDBTs. This approach enabled us to characterize the cleavage pattern of the C–S bonds in these mDBTs.

2. Experimental

2.1. Materials

The two aromatic sulfur compounds used in this study, 1-MDBT and 4-MDBT, were purchased from Nard Institute (Hyogo, Japan). Purities of these compounds were greater than 93%.

2-MDBT and 3-MDBT were obtained from Kansai Research Institute International (Kyoto, Japan). Their purities were greater than 99.5%.

The analytical-grade solvents *n*-hexane, *n*-tetradecane, ethyl acetate and ethanol were purchased from Wako (Osaka, Japan), and used without further purification.

Each of the mDBTs was dissolved in *n*-tetradecane to give a sulfur concentration of approximately 100 μg/ml.

2.2. Resting cell reactions

DBT was dissolved in ethanol (5 g/l) and added to pre-sterilized medium A as the sole source of sulfur [19].

Resting cell reactions were carried out essentially as described previously [20] with some modifications to allow for large reaction volumes. Briefly, *R. erythropolis* KA2-5-1 cells were grown in 200 ml of medium A supplemented with 25 μg/ml of DBT by shaking (130 strokes per min) in 500-ml Sakaguchi flasks at 30°C for 2 days. The bacterial cells (approximately 12 g dry cell/l) were resuspended in 0.1 *M*

potassium phosphate buffer (pH 7.0) after washing twice with 0.85% NaCl. The resting cell reactions were allowed to proceed at 30°C in 500-ml Sakaguchi flasks containing 50 ml of the mDBT solution and an equal volume of the cell suspension with rotary shaking (130 rpm) for 1 day.

2.3. Solid-phase extraction of microbial metabolites from reaction solvents

The SPE cartridges used for the isolation and concentration of microbial metabolites from the reaction solvents contained 500 mg of silica packing with a column volume of 2.8 ml and were obtained from Varian (Harbor City, CA, USA).

The SPE procedure was performed as described previously [18] with slight modifications. Briefly, the cartridges were first conditioned with 3 ml of *n*-hexane. If the analytes were concentrated 10-fold, aliquots (10 ml) of the samples were loaded onto the columns and washed with 3 ml of *n*-hexane and then 500 μl of ethyl acetate–ethanol (6:4, v/v). Finally, elution of the absorbed substances was carried out with 1 ml of ethyl acetate–ethanol (6:4, v/v). To accelerate the elution rate, positive pressure was exerted on the column with a syringe.

2.4. Instrumentation

Total sulfur determination with the samples obtained before and after biodesulfurization was performed with a sulfur analyzer (7000 V, Antek Instruments). Aliquots of 6 ml of the samples (*n*-tetradecane solution) were injected into the sulfur analyzer with a high-temperature furnace (1100°C). Upon combustion of the sample, sulfur dioxide was generated and UV irradiated leading to the emission of fluorescence, which was detected with a photomultiplier tube. Aliquots of 250 μg/ml of DBT solution in *n*-tetradecane and various dilutions with the same solvent were used for sulfur calibration.

The microbial metabolites of each mDBT were detected by GC using a Shimadzu GC-17A capillary gas chromatograph with a flame ionization detection (FID) system and identified by GC–MS (Magnum, Finnigan Mat). Whether the microbial metabolites contained sulfur atoms was checked with GC–AED (5890II/5921A, Hewlett-Packard).

The experimental conditions for instrumental analysis are described in more detail in our previous paper [18].

^{1}H-NMR spectroscopy was carried out with a 500 MHz JEOL JNM-GSX500 (the spectra were recorded to obtain more detailed information on the structures of the microbial metabolites). After the eluate containing the metabolites concentrated by the SPE treatment was evaporated, the solid recovered was dissolved in deuteroacetone $(C^{2}H_{3})_{2}CO$ at room temperature to obtain the ^{1}H-NMR spectra. A small quantity of tetramethylsilane (TMS), $(CH_{3})_{4}Si$, was added as an internal standard.

Nuclear Overhauser effect (NOE) analysis was also performed to confirm the positions of functional groups in the metabolites.

3. Results and discussion

3.1. GC and GC–AED analysis of microbial metabolites

Desulfurization efficiencies of *R. erythropolis* KA2-5-1 against mDBTs were evaluated by determining the decreases in total sulfur concentration in the oil phase during the resting cell reactions. As shown in Table 1, desulfurization against four kinds of mDBTs occurred with efficiencies between approximately 68% and 95%.

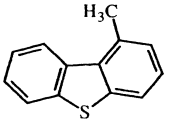
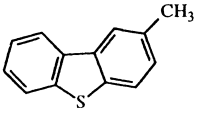
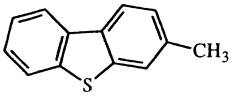
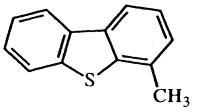
To detect the microbial metabolites of each of the mDBTs, GC analysis of the oil phase obtained after the resting cell reaction was carried out (Fig. 1).

As shown in Fig. 1c, the desulfurization of 3-MDBT by KA2-5-1 gave only one unknown peak (indicated by an arrow) in addition to the peak of the substrate. On the other hand, two additional peaks (indicated by arrows) were detected in the case of microbial desulfurization against the other substrates, i.e., 1-MDBT, 2-MDBT and 4-MDBT (Fig. 1a, b and d, respectively).

GC–AED is a powerful method to simultaneously detect multiple elements of which a given compound is composed. In this study, simultaneous detection of sulfur (at 181 nm) and carbon (at 193 nm) in the microbial metabolites of each mDBT was carried out with GC–AED.

Representative GC–AED chromatograms are illus-

Table 1
Substrates and their percentages of desulfurization

Substrate	Structure	Desulfurization percentage
1-Methyldibenzothiophene (1-MDBT)		95%
2-Methyldibenzothiophene (2-MDBT)		72%
3-Methyldibenzothiophene (3-MDBT)		68%
4-Methyldibenzothiophene (4-MDBT)		69%

trated in Fig. 2. After incubation of 2-MDBT with the bacterial cells, a few new carbon peaks appeared, but none contained any sulfur. The SPE procedure with a silica cartridge resulted in a fourfold concentration of three major peaks, and thus it was expected that they had polar functional groups. In addition, none of the mDBT metabolites detected by GC contained sulfur suggesting that they were desulfurization products.

3.2. Identification of microbial metabolites

GC–MS was used to separate and identify the microbial metabolites of the four kinds of mDBTs.

Fig. 3 shows the GC–MS chromatogram and the mass spectra of the metabolites of 2-MDBT. Both peaks 1 and 2 had a molecular ion at m/z 184. By analogy of the microbial conversion of DBT to 2-HBP, 2-MDBT was expected to be transformed into the hydroxylated desulfurization product, 5-methyl-2-hydroxybiphenyl or 3′-methyl-2-hydroxybiphenyl in the course of biodesulfurization. The ion at m/z 184 corresponded to the molecular mass of these methyl hydroxybiphenyls. It should be noted that the peak intensities of some of their fragment ions, especially m/z 183 and 169 ions corresponding to each of the H ($[M-1]^+$) and CH_3 ($[M-15]^+$) eliminations from the molecular ions, were different from each other. The difference between peak intensities of these fragment ions (m/z 183 and 169) was estimated to determine the differences in their chemical structures, although both fragment ion peaks in the low-mass ion series less than m/z 141 exhibited the same spectral pattern. The mass spectrum of peak 1 indicated a very similar fragment pattern to that of 2-methoxybiphenyl. Therefore, fragmentation of this product may have occurred through the same pathway as 2-methoxybiphenyl fragmentation. The main pathway was considered to be as follows. Elimination of CH_3 from the m/z 184 ion initiating the 1,6-H shift might yield the ion at m/z 169 $[C_6H_5C_6H_4O^+]$ as well as the fragment ion of 2-methoxybiphenyl. Subsequent loss of CO from the m/z 169 ion led to the ion at m/z 141 $[C_6H_5C_5H_4^+]$. Furthermore, the ion at m/z 141 mainly lost C_2H_2 to form the ion at m/z 115 $[C_6H_5C_3H_2^+]$. The 3′-

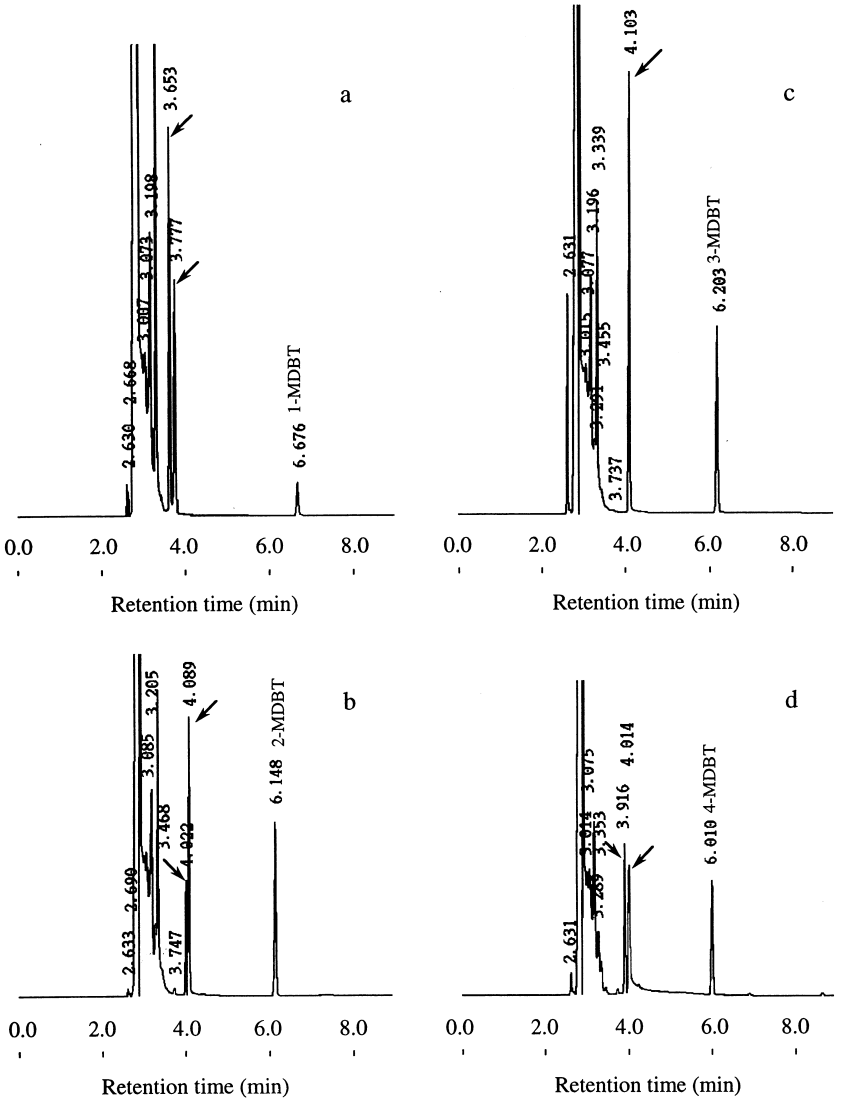


Fig. 1. GC–FID chromatograms of each of the methylated DBTs and their desulfurization products (indicated by arrows). (a) 1-MDBT; (b) 2-MDBT; (c) 3-MDBT; (d) 4-MDBT.

methyl-2-hydroxybiphenyl structure was expected to permit this fragmentation. The main fragmentation pathway of another product (peak 2) was also estimated as follows. The molecular ion at m/z 184 underwent dehydrogenation in its methyl group to form an ion at m/z 183. The m/z 141 ion seemed to be due to fragmentation of the m/z 183 ion. That is, this fragment ion at m/z 183 dissociated to an ion at m/z 141 through loss of m/z 42 [CH=COH] followed by loss of C_2H_2 to form an ion at m/z 115. This fragmentation pathway could explain the mass spectrum of 5-methyl-2-hydroxybiphenyl obtained.

The mass spectrum of peak 3 was typical of paraffin-related compounds. Since this component could be trapped with the SPE column, it may have some polar functional group (e.g., OH).

In a similar manner, the microbial metabolites of the other substrates were also analyzed by GC–MS. The substrates used in this study and the metabolites whose structures were suggested by GC–MS are

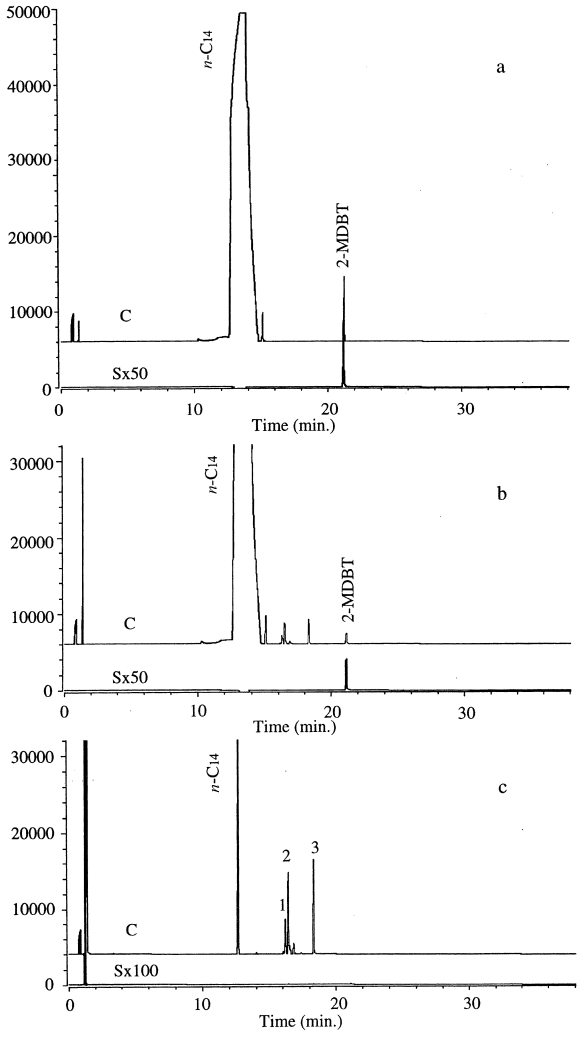


Fig. 2. GC–AED chromatograms of 2-MDBT and its desulfurization products. (a) *n*-Tetradecane (n-C_{14}) phase containing 2-MDBT; (b) *n*-tetradecane (n-C_{14}) phase after biodesulfurization; (c) eluate concentrated fourfold by SPE. Peaks 1, 2 and 3: main metabolites in the eluate.

listed in Table 2. Although it was suggested by GC–MS analysis that the products of desulfurization by strain KA2-5-1 existed in two isomeric forms, the structures of these isomers were not determined. As GC–MS alone could not determine the structures of the metabolites corresponding to each of the peaks except those of the substrates with symmetrical conformations (e.g., 4,6-DMDBT), ^{1}H-NMR analysis was used for decisive identification of the microbial metabolites of each of the mDBTs with asymmetrical conformations. The microbial metabolites concentrated more than 30-fold by the SPE procedure were measured by ^{1}H-NMR and also by using NOE analysis.

The NOE difference spectra of the 2-MDBT main metabolite and ^{1}H-NMR spectrum of 2-MDBT metabolites are shown in Fig. 4. The following relationship was clarified from the NOE experiments of the main metabolite. That is, it was found that each of proton NMR signals (δ 7.09 and 6.98: corresponding to positions d and e in Fig. 5, respectively) were adjacent to the CH_3 group by irradiation of the methyl substituent (δ 2.27) and proton signal (δ 6.87: corresponding to position f in Fig. 5) was also adjacent to the OH group by irradiation of the hydroxyl substituent (δ 7.97). Moreover, the signals of δ 6.98 and 6.87 showed *ortho* coupling (J=8.2 Hz). These data, together with those from ^{1}H-NMR normal spectrum, were consistent with the view that this main metabolite was 5-methyl-2-hydroxybiphenyl (see assignment in Fig. 5). Another minor metabolite was also assigned to 3′-methyl-2-hydroxybiphenyl by analysis of the ^{1}H-NMR spectrum and the NOE data which were measured by irradiation of the CH_3 group (δ 2.36) and the OH group (δ 8.13) (data not shown). The molar ratio of these two microbial metabolites of 2-MDBT was calculated as 2.6 to 1 on the basis of the OH signal intensities (*h* and *J*: corresponding to the OH groups of 5-methyl-2-hydroxybiphenyl and 3′-methyl-2-hydroxybiphenyl, respectively). On the other hand, the ratio was estimated as 2.5 to 1 on the basis of GC peak areas. Thus, we concluded that the main peak (the peak at 4.09 min in Fig. 1b and peak 2 in Fig. 3) observed in GC and GC–MS chromatograms of the 2-MDBT metabolites corresponded to 5-methyl-2-hydroxybiphenyl, while the minor peak (the peak at 4.02 min in Fig. 1b and peak 1 in Fig. 3) corresponded to 3′-methyl-2-hydroxybiphenyl. The structures of the microbial metabolites of the other mDBTs were also assigned according to the same analytical methods (these metabolite structures are summarized in Fig. 6).

3.3. Quantitative recovery of the microbial metabolites of mDBTs by SPE

Quantitative recovery of the isomeric desulfuriza-

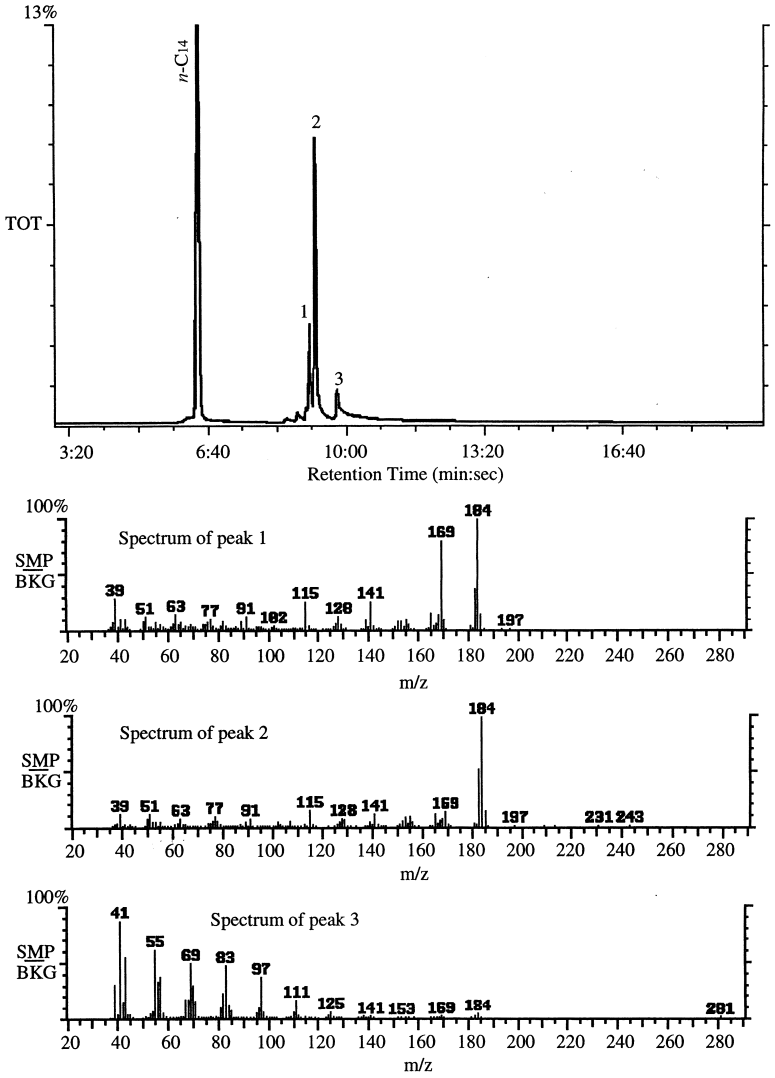


Fig. 3. GC–MS chromatogram of 2-MDBT desulfurization products extracted by SPE and their mass spectra. n-C_{14}=n-tetradecane.

Table 2
Substrates and their presumed metabolites by GC–MS

Substrate	Metabolite	
	Molecular ion ($M^{+\cdot}$)	Presumed metabolite
1-MDBT	184	6-Methyl-2-hydroxybiphenyl and 2′-methyl-2-hydroxybiphenyl
2-MDBT	184	5-Methyl-2-hydroxybiphenyl and 3′-methyl-2-hydroxybiphenyl
3-MDBT	184	4-Methyl-2-hydroxybiphenyl or 4′-methyl-2-hydroxybiphenyl
4-MDBT	184	3-Methyl-2-hydroxybiphenyl and 3′-methyl-2-hydroxybiphenyl

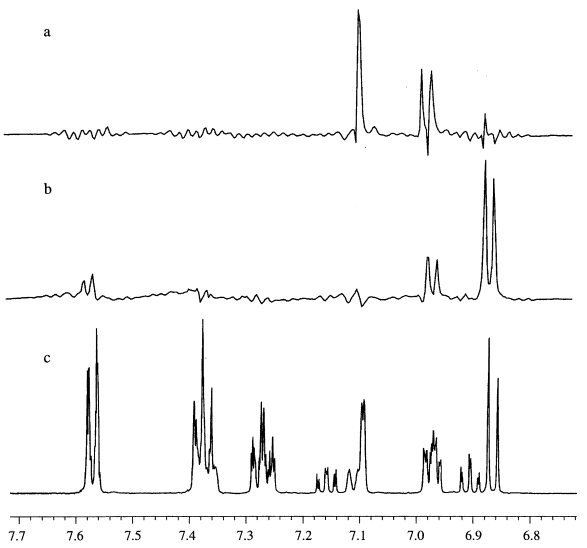


Fig. 4. NOE difference spectra (a and b) and the ^{1}H-NMR spectrum (c) of 2-MDBT desulfurization products extracted by SPE. (a) Saturation of the CH_3 at 2.27; (b) saturation of the OH at 7.97; (c) normal spectrum [500 MHz, $(C^2H_3)_2CO$].

tion products by SPE treatment is a prerequisite for precise evaluation of their molar ratios.

We then examined the recovery of the microbial metabolites by SPE as follows. 2-MDBT and its degradation products were chosen as model compounds. After resting cell reaction with approximately 100 ppm of 2-MDBT for 24 h in six 10-ml screw-cap test tubes, the *n*-tetradecane (*n*-C_{14}) phase was recovered from each of the reaction mixtures by centrifugation and applied to each of the SPE cartridge columns followed by elution of polar substances with organic solvent. Fourfold concentrates from two of the six test tubes were analyzed by GC. The eluates obtained from the other four test tubes were separately evaporated and resolved in *n*-C_{14}. These eluates were concentrated fourfold and extracted by each of the fresh SPE columns from the *n*-C_{14} solvent. The fourfold concentrates from two of the four test tubes were analyzed by GC. The remaining eluates were evaporated and resolved in *n*-C_{14} followed by fourfold concentration and extraction by each of the fresh SPE columns. The eluates obtained were measured by GC. The ratios of the contents of the two isomers, 5-methyl-2-hydroxybiphenyl and 3′-methyl-2-hydroxybiphenyl, were compared in each of the eluates obtained by SPE. At all rounds of SPE, the ratio was constant (0.730:0.270). These results indicated that the final products of the two isomers of 2-MDBT were recovered quantitatively through a series of SPE procedures. The same high reproducibility in the recovery by SPE was observed with the metabolites of other monomethyl DBTs. These observations implied that SPE and subsequent GC analysis allowed evaluation of the ratio of contents of degradation products of mDBTs. To determine whether the two C–S bonds in the DBT basic structure are equivalent in their cleavage in the microbial desulfurization reaction, we focused on the microbial reactions against the monomethyl DBTs. Desulfurization of 3-MDBT by KA2-5-1 resulted in the production of 4-methyl-2-hydroxybiphenyl. This

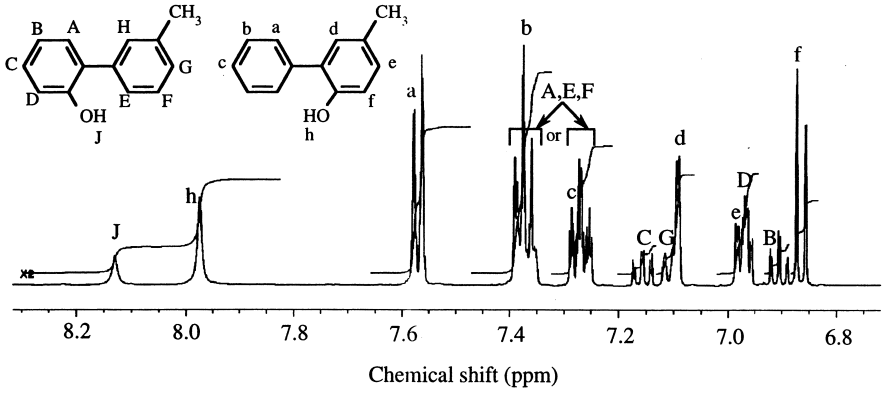


Fig. 5. ^{1}H-NMR spectrum of 2-MDBT desulfurization products extracted by SPE.

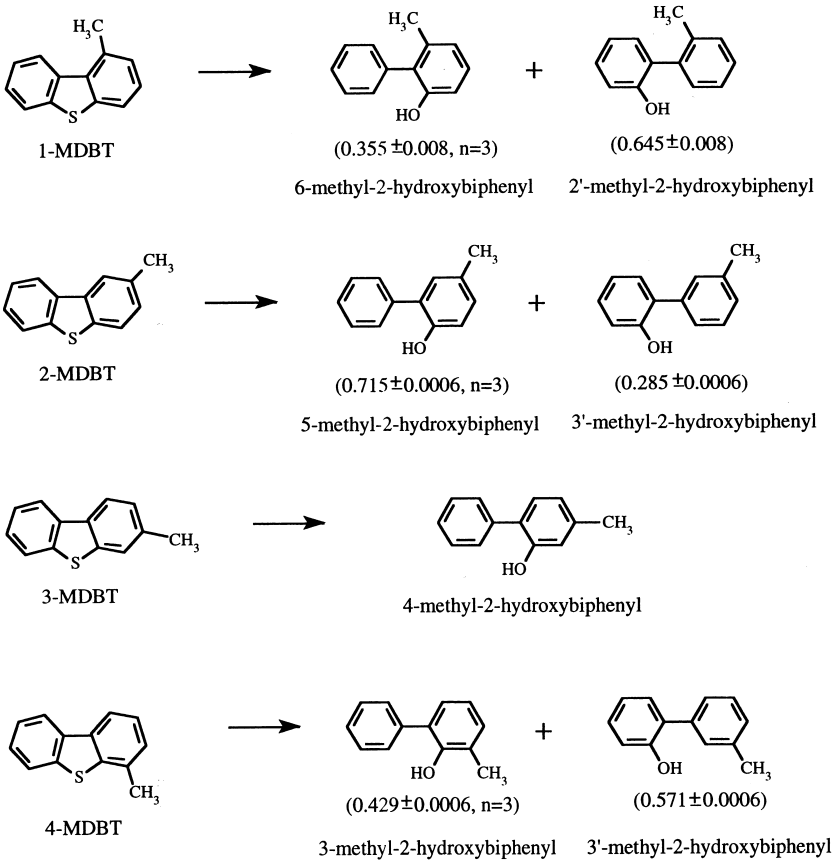


Fig. 6. Desulfurization reactions against mDBTs by *R. erythropolis* KA2-5-1.

indicated that the microbial enzymic system can recognize either of the two C–S bonds in the DBT basic structure highly selectively for sulfur-releasing reactions. On the other hand, in the microbial desulfurization of the other substrates, 1-MDBT, 2-MDBT and 4-MDBT, the first C–S bond cleavage is not specific but exhibits a bias toward either of the two C–S bonds.

Ratios of the desulfurization products of each mDBT substrate with sufficient reproducibilities (mean standard deviation, run number=3; in parentheses) are summarized in Fig. 6. The mDBT substrate desulfurized specifically by KA2-5-1 strain was 3-MDBT. The present quantitative analysis of the desulfurization products clearly indicated that the 3-methyl position in the DBT skeleton affects the selective enzymic attack leading to the first C–S bond cleavage. Such steric effects of methyl groups on selection of the C–S carbon to be attacked in the first step of cleavage of the two C–S bonds in the DBT skeleton can be determined by the structural analysis procedure described in this paper.

4. Conclusions

The combination of concentration of microbial metabolites by SPE and instrumental analyses such as GC–AED, GC–MS and ^{1}H-NMR was found to be useful for identification of small amounts of desulfurization products. This combined approach to the characterization of the desulfurization products indicated that enzymatic recognition of the C–S carbons can be biased by the positions of methyl groups in degradation of the asymmetrical mDBTs.

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