Identification of Microbial Products from Dibenzothiophene and Its Proposed Oxidation Pathway[†]

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One of the microbial products produced from dibenzothiophene by *Pseudomonas abiko-nensis* or *P. jianii* was identified as a new substance, *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid, and the other as its hemiacetal form. From the structures of the products, an oxidation pathway of dibenzothiophene was suggested. Moreover, the culture broth and the isolated products were examined for plant growth activities on rice plants.

In the previous reports[1~3] the authors indicated that dibenzothiophene utilizing bacteria were isolated from soils, and the activities of these bacteria were enhanced under the adequate culture conditions, and three of the microbial products from DBT were identified as 3-hydroxy-2-formyl-benzothiophene (product A), dibenzothiophene-5-oxide (product D) and 3-oxo-2[3′-hydroxy-thianaphthenyl-(2)-methylene]-dihydrothianaphthene (product E).

This report deals with the structures of the remaining two substances (products B and C) produced by the bacteria, and physiological effects of the broth and the isolated products, and then an assumed pathway of DBT oxidation by the bacteria.

MATERIALS AND METHODS

Microorganisms and cultivation. In this studies, *Pseudomonas jianii* DDC 279 was used as a representative of 8 strains in DBT utilizing bacteria. The medium had the following composition (g/liter): $MgCl_2 \cdot 6H_2O$, 0.2; KH_2PO_4, 1.4; $Na_2HPO_4 \cdot 12H_2O$, 9.5; meat extract, 4.0; and DBT, 1.0 (pH 7.3). Cultivation was carried out in the same procedures as described in the previous paper.[3]

Isolation procedures of products. Extraction procedure and silicagel column chromatography for

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  Abbreviation used in this paper; DBT, dibenzothiophene.

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separation of the products from the culture broth were the same as reported in the previous paper.[3]

Analytical procedures. Ultraviolet spectra, infrared spectra and nuclear magnetic resonance spectra were obtained with following instruments: UV spectra, a Hitachi EPS–3; IR spectra, a Hitachi EPI–S2; NMR spectra, a VARIAN A60.

Color reaction with 2,4-dinitrophenylhydrazine, ferric chloride and bromine were employed for detection of functional groups as reported in the previous paper.[3]

Methylation of product. One hundred and seventy-five milligrams of product B were dissolved in 30 ml of tetrahydrofuran, and 5 ml of ether solution of diazomethane (*ca.* 140 mg) was added gradually and agitated. After agitation, the reaction mixture was allowed to stand overnight in an ice box, and evaporated to remove the solvents, and the residue was extracted with ethyl acetate. The ethyl acetate solution was washed with 5% sodium hydrogen carbonate and then with water, dried on anhydrous sodium sulfate and evaporated to remove the ethyl acetate. After removal of solvent, the remaining residue was purified by crystallization from ethyl alcohol. Thus, methylated product B was obtained as brown needle crystals.

Synthesis of product B. Product B was synthesized in the following procedures. One point two grams of 3-hydroxy-2-formyl-benzothiophene was added into 10 ml of cooled 10% the sodium hydroxide solution containing 1 ml of pyruvic acid and agitated for an hour in an ice bath, then 5 ml of piperidine was dripped gradually for 30 min. The reaction mixture was allowed to stand for 2 days at a room temperature, acidified with 2 N hydrochloric acid and extracted with

ether. The ether extract was well washed with water and dried on anhydrous magnesium sulfate. After removal of the solvent, the synthesized substance was purified through a silica gel column with the procedure reported in the previous paper.[3]

Enzymatic reaction. Crude enzyme was prepared by the following method. Two grams of wet packed cells cultured for 6 days were dehydrated and defatted with cold acetone, suspended in 40 ml of phosphate buffer consisting of potassium phosphate monobasic 0.14%, sodium phosphate 0.95% and magnesium chloride 0.02% at pH 7.3. The cell suspension was treated with an ultrasonic oscillator at 19.5 kc/sec for 15 min. The supernatant was obtained by centrifugation at $10,000 \times g$ for 20 min.

Enzymatic reactions of products B and C were conducted as follows. One milliliter of the supernatant was mixed in 4 ml of substrate solution consisting of 1 μmol of product B or C dissolved in 5 ml of the phosphate buffer, respectively. Reaction was carried out at 30°C under shaking condition. After the reaction, amounts of product B (485 mμ), product C (440 mμ) and product A (400 mμ) were measured spectrophotometrically at each wave length as shown in the above parentheses.

Tests for physiological effects. Auxin or antiauxin assay was examined by the lamina joint test,[4] in which the angle between lamina and sheath of rice plant was measured after the treatment. Gibberellin assay was examined by the seedling test,[5] in which the plant height and the root length were measured after the treatment of 7 days.

Antibiotic effects of the products were assayed with the pulp disc method.[6] The substances were used at the concentration of 0.1%.

RESULTS AND DISCUSSION

Analysis and identification of product B

Product B, a red substance, was found in a trace amount in the culture broth. It had a value of *Rf* 0.75 on paper chromatogram (EtOH–NH$_4$OH–H$_2$O, 20: 1: 15) and an absorption peak at 475 mμ in phosphate buffer at pH 7.3, mp 269~273°C (decomp). *Anal.* Found: C, 57.81; H, 3.46; S, 12.95. Calcd. for $C_{12}H_8O_4S$: C, 58.06; H, 3.25; S, 12.92%. This substance was positive to the alcoholic ferric chloride test, the 2,4-dinitrophenylhydrazine test and the bromine test. From the results of the color reactions, the presence of phenolic hydroxyl group and carbonyl
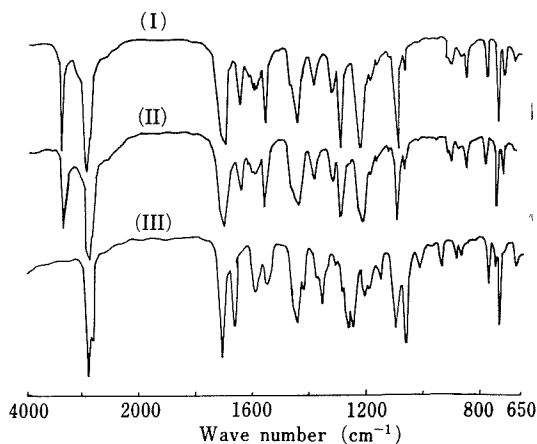

FIG. 1. IR Spectra of Product B (I), Synthetic *trans*-4[2-(3-Hydroxy)-thianaphthenyl]-2-oxo-3-butenoic Acid (II) and Methylated Product B (III) in Nujol.
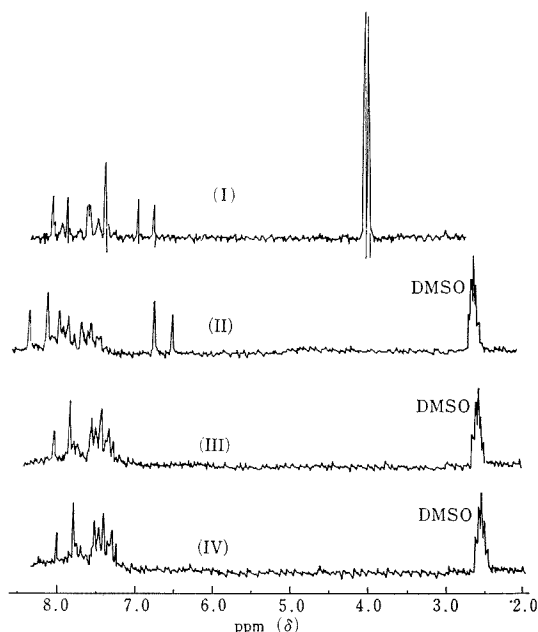

FIG. 2. NMR Spectra of Products.

(I): Methylated Product B in CDCl$_3$, 60 Mc.
(II): Product B in d$_6$-DMSO, 60 Mc.
(III): Product C in d$_6$-DMSO, 60 Mc.
(IV): Product C' in d$_6$-DMSO, 60 Mc.

group were presumed. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1700, 1640, 1600, 1500, 1430, 730 (Fig. 1–I). NMR (DMSO): δ 8.10 (1H, d, $J=14$ cps, –CHCHCO–), 6.50 (1H, d, $J=14$ cps, –CHCHCO–), 6.33~7.89 (4H, m) (Fig. 2–II).

Methylated product B was found to have

following characteristics: positive to the 2,4-dinitrophenylhydrazine test and the bromine test, negative to the alcoholic ferric chloride test; brown needle crystals; mp 155~156°C. *Anal.* Found: C, 60.96; H, 4.26; S, 11.17. Calcd. for $C_{14}H_{12}O_4S$; C, 60.86; H, 4.38; S, 11.61%. The IR spectrum of methylated product B showed the absence of phenolic hydroxyl group at 3350 cm^{-1} (Fig. 1-III). NMR (CDCl$_3$): δ 7.80 (1H, d,J=12 cps, –CHCHCO–), 6.75 (1H, d, J=12 cps, –CH-CHCO–), 3.93 (3H, s, –OCH$_3$), 3.90 (3H, s, –COOCH$_3$) (Fig. 2–I).

The NMR coupling constants (J=14 cps) show *trans* form of this product comparing with those of product C (J=13 cps) as mentioned later. Therefore, product B(I) and its methyl ester (II) were considered to have following structures, respectively.

OH
S CH:CHCOCOOH
(I)

OCH₃
S CH:CHCOCOOCH₃
(II)
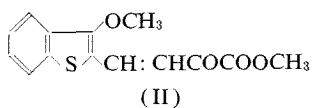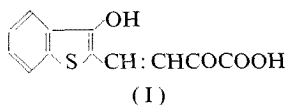
Product B had the IR spectrum identical with that of the synthesized *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid as shown in Fig. 1. Therefore, product B was identified as the above compound.

Analysis of product C

Product C, orange needle crystals, had a value of Rf 0.77 on paper chromatogram (EtOH–NH$_4$OH–H$_2$O, 20:1:15), and an absorption peak at 437 mμ in the phosphate buffer, mp 189~191°C (decomp). *Anal.* Found: C, 57.01; H, 3.20; S, 12.72. Calcd. for $C_{12}H_8O_4S$: C, 58.06; H, 3.25; S, 12.92%. This substance was positive to the alcoholic ferric chloride test, the 2,4-dinitrophenylhydrazine test. IR ν_{max}^{Nujol} cm^{-1}: 3200, 1690, 1640, 1620, 1560, 1440, 710 (Fig. 3–I). Broad spectra at the vicinity of 3200 cm^{-1} suggest that the hydroxyl group is not phenolic, but alcoholic one. NMR (DMSO): δ 7.9 (1H,
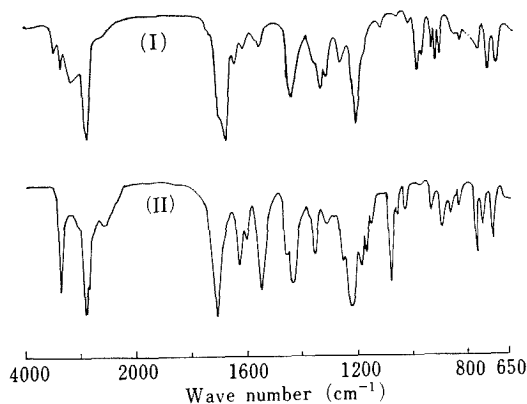

FIG. 3. IR Spectra of Product C (I) and Product C' (II) in Nujol.

d, J=13 cps), 7.4 (1H, d, J=13 cps) (Fig. 2–III). Thus, product C had an empirical formula identical with that of product B and the functional groups similar to those of it without phenolic hydroxyl group, but had a lower melting point, λ_{max} at a shorter wave length and smaller coupling constants of NMR spectrum than those of product B. From the above reasons, product C was presumed to be hemiacetal form (III) of product B.

O OH
COOH
S
(III)
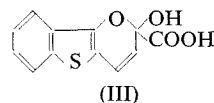
Properties and structure of product C

Product C changed to a reddish substance similar to product B when it was left a silica gel column with chloroform under dried condition at a room temperature for a week. The substance was purified with ether extraction and by chromatograph on a silica gel column. The purified substance was designated as product C'. The melting point and absorption spectrum (Fig. 4) were identical with those of product C. IR ν_{max}^{Nujol} cm^{-1}: 3350, 1710, 1630, 1600, 1550, 1440, 720 (Fig. 3–II). NMR (DMSO): δ 7.9 (1H, d, J=13 cps), 7.4 (1H, d, J=13 cps) (Fig. 2–IV). Thus, the functional groups of product C' should be identical with those of product B, but the coupling constants of two olefinic protons in the side chain, the λ_{max} of absorption spectrum and the melting
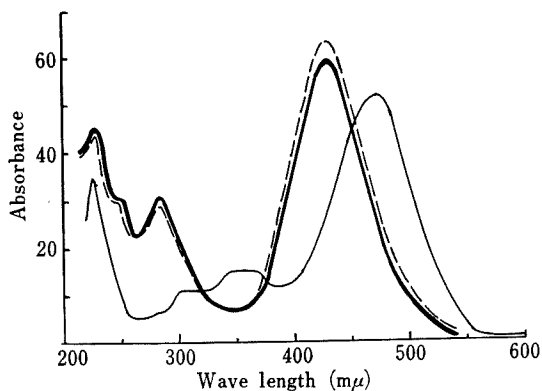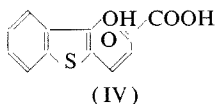
Fig. 4. Absorption Spectra of Products.
—— Product B, —— Product C, - - - Product C′
Solvent: pH 7.0, 0.05 M Phosphate buffer.

Fig. 5. Conversion of Product B and Product C by Crude Enzyme.
—— incubated with Product B
------ incubated with Product C

point were identical with those of product C. Product C′ (IV) was presumed to be *cis* form of product B on the basis of the smaller coupling constants, λ_{max} of absorption at a shorter wave length and a lower melting point than those of product B.

(IV)

Enzymatic degradation of product

As Fig. 5 shows, when product B was incubated with the enzyme solution, the amount of product B decreased and concomitantly product A appeared. In the case of product C, the amount of product C did not change, and no increase in the amount of product A was found. The results of the enzymatic reaction indicated that the enzyme converted product B to product A, not converting product C to Product A.

Proposed pathway of DBT metabolism

As reported in the previous paper, DBT was oxidatively converted to the five substances, *i.e.*, dibenzothiophene-5-oxide, 3-hydroxy-2-formyl-benzothiophene, 3-oxo-[3′-hydroxy-thianaphthenyl-(2)-methylene)-dihydrothianaphthene, products B and C. In this study, products B and C were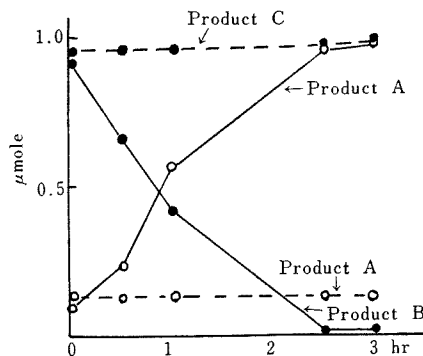 identified as *trans* and hemiacetal form of 4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid, respectively. And, *cis* form of product B was easily produced from product C by artificial procedures. As judged from the structures of the products and the results of the enzymatic reaction, DBT was assumed to be metabolized oxidatively to 3-hydroxy-2-formyl-benzothiophene by the DBT utilizing bacteria according to the pathway shown in Fig. 6, which is analogous to the pathway of naphthalene decomposition by *Pseudomonas*,[7~9] though the cleavage of the thiophene ring was not found in this experiment. On the assumption that *cis*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid was rapidly converted to the *trans* form by the action of *cis-trans* isomerase, the *cis* form was considered to be an immediate ring-fission product of DBT. The hemiacetal form was found in the broth, but it was not converted to 3-hydroxy-2-formyl-benzothiophene by the crude enzyme. The hemiacetal form is presumably not a member of the main pathway.

Since 3-hydroxy-2-formyl-benzothiophene showed the greatest yield of the five products and being rapidly produced from *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid, DBT was mainly metabolized through *cis*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid and its *trans* form to 3-hydroxy-2-formyl-benzothiophene.

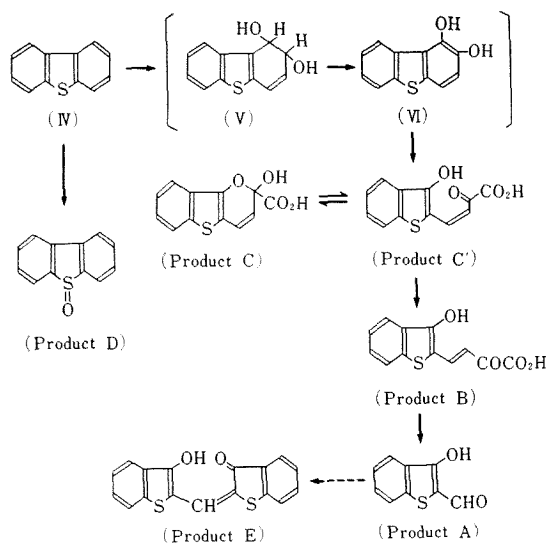Dibenzothiophene-5-oxide was an interest-

FIG. 6. Proposed Pathway of Dibenzothiophene Metabolism by *Pseudomonas abikonensis* or *P. jianii*.

(IV): Dibenzothiophene, (V): D-*trans*-1,2-Dihydro-1,2-dihydroxydibenzothiophene, (VI): 1,2-Dihydroxy-dibenzothiophene. (Product A): 3-Hydroxy-2-formyl-benzothiophene. (Product B): *trans*-4[2-(3-Hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid. (Product C): Hemiacetal form of Product C'. (Product C'): *cis*-4[2-(3-Hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid. (Product D): Dibenzothiophene-5-oxide. (Product E): 3-Oxo-[3'-hydroxy-thianaphthenyl-(2)-methylene]-dihydrothianaphthene.

ing substance, which was not presented by enzymatic evidence. The isolation procedures converted 3-hydroxy-2-formyl-benzothiophene to 3-oxo-2[3'-hydroxy-thianaphthenyl-(2)-methylene]-dihydrothianaphthene.

Physiological effects of the broth and the products

Antiauxin and gibberellin like activities were found in the cultured broth. As Table I shows, the angle of lamina joint was reduced to 50% against the control when the samples were treated with the broth diluted 5 times plus 10 ppm of indole-3-acetic acid. The presence of an antiauxin substance was shown by the reduction of the angle degree, and a 50% reduction was equivalent to the presence of 50 ppm of 2,3,5-triiodobenzoic acid. The culture broth diluted 5 times caused the elongation of the height of plants and the

TABLE I. EFFECTS OF THE CULTURE BROTH ON THE GROWTH OF RICE PLANTS

control: 100

Method	Concentration		
	1/5	1/10	1/20
Lamina joint test			
Broth only	79	96	93
Plus IAA 10 ppm	50	70	70
Seedling test			
Plant height	150	120	90
Length of 2nd leaf sheath	134	132	87

TABLE II. EFFECTS OF THE PRODUCTS ON THE ROOT GROWTH OF RICE PLANT SEEDLINGS

control: 100; 5 cm

Substance	Concentration (ppm)					
	100	50	25	10	5	2
Product A	90	132	146	145	122	110
B	96	88	111	119	103	103
C	74	103	113	123	101	104
D	58	62	80	81	86	93
E	109	109	103	104	103	96

length of the second leaf sheath of rice seedlings. Therefore, the presence of a gibberellin like substance was presumed to be at the concentration of 0.1 ppm of gibberellin A.

Among the separated products, as shown in Table II, product A was found to have gibberellin like effect; elongation of the root length of rice seedlings was observed with 10~50 ppm. Products D and E were found to have antibiotic effects. The growth of *Xanthomonas citri* which induces canker in citrus fruits was inhibited by product D, and that of *Piricularia orizae* which induces blast in rice plants was inhibited by product E.

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