New Antibiotics Phthoxazolins B, C and D Produced by *Streptomyces* sp. KO-7888

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(Received for publication March, 8, 1995)

New antibiotics, phthoxazolins B, C and D were isolated from the fermentation broth of *Streptomyces* sp. KO-7888. They are geometrical isomers of 10-hydroxyphthoxazolin A. They showed selective antifungal activity against *Phytophthora parasitica in vitro* and modest herbicidal activity in a laboratory test, but the potencies were different among isomers.

In the course of screening for novel antibiotics produced by microorganisms, new oxazole-triene antibiotics called phthoxazolins B, C and D (**1**~**3**) have been isolated from the culture broth of *Streptomyces* sp. KO-7888. Several members of the oxazole-triene group of antibiotics have been reported. We have previously reported phthoxazolin A (**4**)[1~3] isolated from a separate strain, *Streptomyces* sp. OM-5714 as an herbicidal inhibitor of cellulose biosynthesis. Other groups described inthomycins B and C (**5** and **6**)[4], oxazolomycin[5] and saigenmycin[6]. The new phthoxazolins (**1**~**3**) presented here are additional members of this group, which were co-produced with **4** by *Streptomyces* sp. KO-7888.

In this paper, we describe taxonomy of the producing strain and fermentation, isolation, structure elucidation and biological activity of **1**~**3** (Fig. 1).

Taxonomy of Producing Strain KO-7888

The strain KO-7888 was isolated from a soil sample collected in Yokohama, Kanagawa Prefecture, Japan.

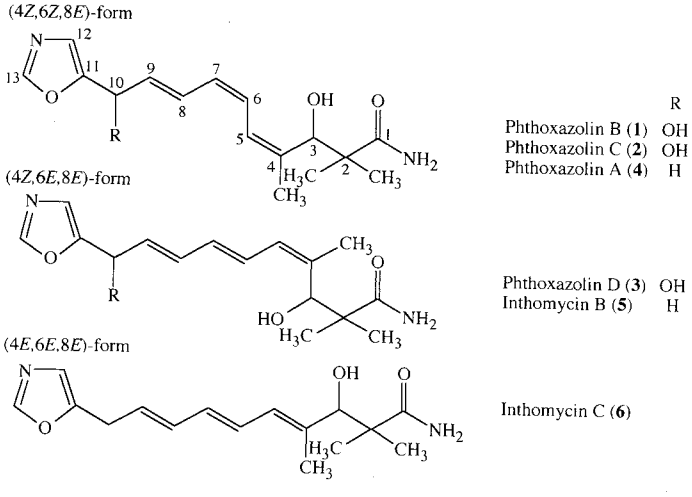
Morphological Properties

The vegetative mycelia grew abundantly on both synthetic and complex media, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on inorganic salts - starch agar and glucose - asparagine agar. The spore chains were *Spirales* type and each had more than 20 spores per chain. The spores were cylindrical in shape, 1.1 × 0.6 μm in size and had a smooth surface (Fig. 2). Whirls, sclerotic granules, sporangia and flagellated spores were not observed.

Chemical Composition

The DAP-isomer in whole - cells of strain KO-7888 was determined to be LL-type. The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed brown or red color on various media. The aerial mass color was white to red. Melanoid pigment and other soluble pigments were

Fig. 1. Structures of phthoxazolins and related compounds.

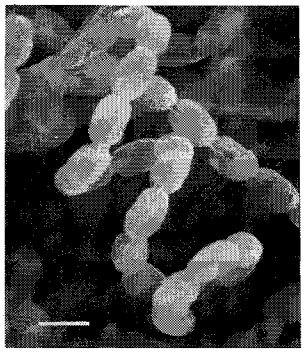


not produced. The utilization of carbon sources is shown in Table 3.

Based on the taxonomic properties described above, strain KO-7888 is considered to belong to the genus *Streptomyces* and to the gray or red series. The strain was deposited in National Institute of Bioscience and Human Technology, Japan, under the name *Streptomyces* sp. KO-7888 with an accession number FERM P-13339.

Fig. 2. Scanning electron micrograph of spore chain of strain KO-7888 grown on inorganic salts - starch agar for 14 days.

Bar represents 1 μm.



Fermentation

A stock culture of strain KO-7888 was inoculated into a test tube (i.d. 2 × 20 cm) containing 10 ml of a seed medium consisting of glucose 2%, peptone 0.5%, dry yeast 0.3%, meat extract 0.5%, NaCl 0.5% and $CaCO_3$ 0.3% at pH 7.0 before sterilization. The culture was incubated at 27°c for 96 hours on a reciprocal shaker and 2 ml of this culture broth was transferred into each of four 500-ml Erlenmeyer flasks containing 100 ml of the same medium. The flasks were then shaken on a rotary shaker at 27° for 72 hours. Four ml of this culture broth was transferred into each of one hundred 500-ml Erlenmeyer flasks containing 180 ml of a production medium consisting of dry yeast 0.5%, glucose 0.2%, corn steep liquor 0.3%, NaCl 0.3%, $CaCO_3$ 0.3% and fructo-oligo saccharide (Meiji Seika, Tokyo) 1.0% at pH 7.0 before sterilization. The fermentation was carried out at 27°C for 96 hours on a rotary shaker (210 rpm).

Isolation

The culture filtrate (16 liters) from the above fermentation was extracted with an equal volume of ethyl acetate. The extract was concentrated *in vacuo* to give a

Table 1. Cultural characteristics of strain KO-7888.

Medium	Growth	Reverse color	Aerial mass color	Soluble pigment
Yeast extract - malt extract agar*	Good, bamboo (2gc)	Musterd brown (2pi)	Abundant, ashes (5fe)	None
Oatmeal agar*	Good, ivory tint ~ geranium rose	Lt. musterd tan ~ cherry (2ie ~ 7lc) (2cb ~ 71/2la)	Abandunt, alabaster tint ~ covert gray (13ba ~ 2fe)	None
Inorganic salts - starch agar*	Good, ivory ~ tomato red (2dc ~ 61/2pe)	Beige~tomato red (3ge ~ 61/2pe)	Abandunt, alabaster tint (13ba)	None
Glycerol - asparagine agar*	Good, biscuit (2ec)	Bamboo (2gc)	Abundunt, alabaster tint ~ silver gray (13ba ~ 3fe)	None
Glucose - asparagine agar**	Good, ivory (2db)	Bamboo (2gc)	Abundunt, alabaster tint ~ silver gray (13ba ~ 3fe)	None
Peptone - yeast extract iron agar*	Good, pearl pink (3ca)	Pearl pink (3ca)	Moderate, white ~ pearl gray (a ~ 13bc)	None
Tyrosine agar*	Good, peach tan (5gc)	Rosewood tan (5ie)	Abundunt, alabaster tint ~ ashes (13ba ~ 5fe)	None
Sucrose - nitrate agar**	Moderate, colorless	Cololess ~ shrimp (61/2ga)	Moderate, alabaster tint (13ba)	None
Glucose - nitrate agar**	Moderate, biscuit (2ec)	Pearl (2ba)	Poor, Lt.tan (3gc)	None
Glycerol - calcium malate agar**	Good, orange rust (4pe)	Biscque (4ec)	Abundunt, alabaster tint ~ dusk (13ba ~ 13fe)	None
Glucose - peptone agar**	Good, musterd ~ brick red (2le ~ 61/2ne)	Bamboo ~ cardinal (2gc ~ 71/2pc)	Abundant, alabaster tint (13ba)	None
Nutrient agar**	Good, gold (2lc)	Gold (2lc)	Moderate, alabaster tint ~ natural (13ba ~ 2dc)	None

* Medium recommended by International Streptomyces Project.

** Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain KO-7888.

Melanin formation	-
Tyrosinase reaction	-
H_2S production	-
Liquefaction of gelatin (21°C~23°C)	-
Peptonization of milk (27°C)	+
Coagulation of milk (27°C)	+
Cellulolytic activity	-
Hydrolysis of starch	+
Nitrate reduction	+
Temperature range for growth	10°C~37°C

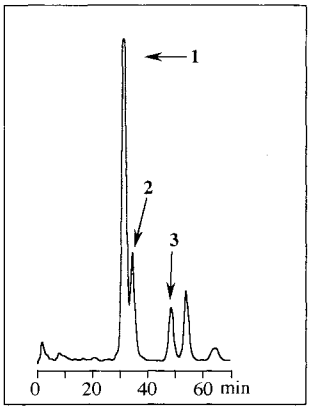
+, active; −, not active.

Table 3. Utilization of carbon sources by strain KO-7888.

D-Glucose	+
D-Fructose	+
L-Rhamnose	+
D-Mannitol	+
L-Arabinose	+
i-Inositol	+
Raffinose	+
D-Xylose	+
Sucrose	+/−
Melibiose	+

+, utilized; +/−, weakly utilized.

Fig. 3. An elution profile of phthoxazolins on HPLC.



Column; Capcell pak C18 SG-120 i.d. 20×250 mm (Shiseido Co.), solvent: 17% CH_3CN, flow rate: 8 ml/minute, UV: 254 nm.

brown oil (*ca.* 500 mg). The oily material was applied on a silica gel column (20 g, i.d. 0.7×30 cm) and the column was developed stepwise with chloroform-methanol (96:4) and chloroform-methanol (92:8). A white powder of **4** (120 mg) was obtained from the eluate of chloroform-methanol (96:4). The eluate of chloroform-methanol (92:8) was further purified by reverse phase HPLC (Capcell pak C_{18} SG-120, i.d. 20×250 mm, Shiseido Co.) with 17% aqueous acetonitrile as mobile phase to yield white powders of **1** (6.5 mg), **2** (2.9 mg) and **3** (1.8 mg). The HPLC chromatogram is shown in Fig. 3.

Physico-chemical Properties

The physico-chemical properties of **1**, **2** and **3** are summarized in Table 4. Antibiotics **1**, **2** and **3** are soluble in methanol, ethanol and ethyl acetate, but practically insoluble in water. They gave positive color reactions to iodine and sulfuric acid. The UV spectra of **1**~**3** (Fig. 4) were similar to each other, which showed absorption

Fig. 4. UV spectra of **1**~**3**.

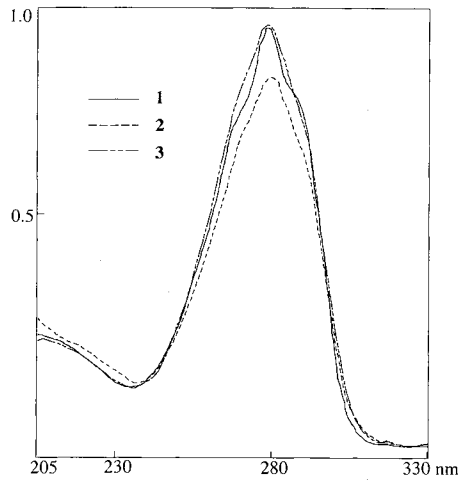


Table 4. Physico-chemical properties of **1**~**3**.

		1	**2**	**3**
Appearance		White powder	White powder	White powder
HR FAB-MS *m/z* (M+H)	found	307.1679	307.1638	307.1706
	calcd.	307.1658	307.1658	307.1658
Molecular formula		$C_{16}H_{22}N_2O_4$	$C_{16}H_{22}N_2O_4$	$C_{16}H_{22}N_2O_4$
Optical rotation $[\alpha]_D^{25}$ (MeOH)		+141°(*c* 0.6)	+30°(*c* 0.2)	+51°(*c* 0.19)
Melting point (°C)		63~65	64~67	63~69
Solubility	soluble	MeOH, EtOH, acetone		
	insoluble	H_2O, $CHCl_3$		
IR ν max (KBr) cm^{-1}		3340, 2950, 2910, 2850, 1650 (amide carbonyl), 1590, 1500, 1460, 1440, 1370.		

Fig. 5. ^{1}H NMR spectra of **1**~**3**.

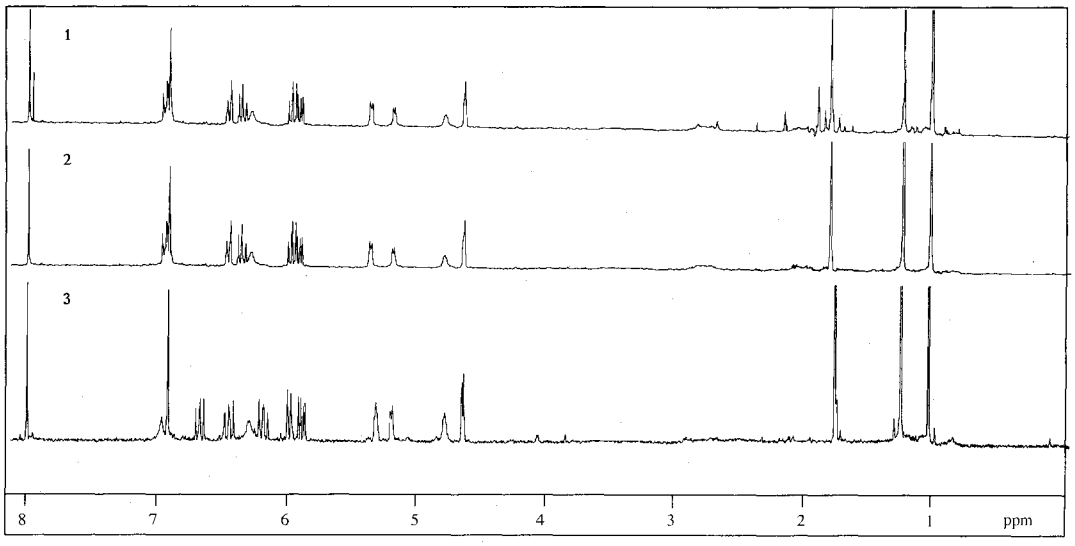


Table 5. ^{1}H NMR data of **1**~**4**.

	1	2	3
2-CH_3	1.07 (s, 3H)	1.07 (s, 3H)	1.07 (s, 3H)
2-CH_3	1.28 (s, 3H)	1.28 (s, 3H)	1.29 (s, 3H)
3	4.68 (d, 1H, *J*=5.6)	4.68 (d, 1H, *J*=5.6)	4.67 (d, 1H, *J*=5.6)
3-OH	5.22 (d, 1H, *J*=5.6)	5.22 (d, 1H, *J*=5.6)	5.22 (d, 1H, *J*=5.6)
4-CH_3	1.85 (s, 3H)	1.85 (s, 3H)	1.79 (s, 3H)
5	6.50 (br.d, 1H, *J*=12.0)	6.50 (br.d, 1H, *J*=12.0)	6.02 (br.d, 1H, *J*=11.6)
6	6.40 (dd, 1H, *J*=11.0,12.0)	6.40 (dd, 1H, *J*=11.0,12.0)	6.71 (dd, 1H, *J*=11.6,14.4)
7	6.01 (dd, 1H, *J*=11.0,11.3)	6.01 (dd, 1H, *J*=11.0,11.3)	6.22 (dd, 1H, *J*=10.8,14.4)
8	6.98 (dd, 1H, *J*=11.3,11.5)	6.98 (dd, 1H, *J*=11.3,11.5)	6.48 (dd, 1H, *J*=10.8,15.2)
9	5.96 (dd, 1H, *J*=6.5,15.0)	5.96 (dd, 1H, *J*=6.5,15.0)	5.92 (dd, 1H, *J*=6.3,15.2)
10	5.40 (d, 1H, *J*=6.5)	5.40 (d, 1H, *J*=6.5)	5.34 (dd, 1H, *J*=5.5,6.3)
10-OH	4.83 (d, 1H, *J*=6.5)	4.83 (d, 1H, *J*=6.5)	4.81 (d, 1H, *J*=5.5)
12	6.96 (s, 1H)	6.96 (s, 1H)	6.96 (s, 1H)
13	8.04 (s, 1H)	8.04 (s, 1H)	8.04 (s, 1H)
NH_2	6.33 (br.s, 1H), 6.99 (br.s, 1H)	6.33 (br.s, 1H), 6.99 (br.s, 1H)	6.33 (br.s, 1H), 6.99 (br.s, 1H)

maxima at 278 nm with two shoulders at 267 and 288 nm. It suggested that **1**~**3** had a conjugated triene moiety in their structure. The IR spectra of **1**~**3** showed amide carbonyl absorptions at 1650 cm^{-1}.

Structure Elucidation

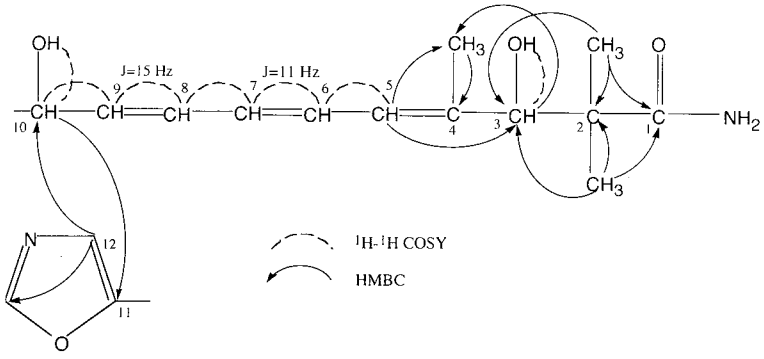
Phthoxazolin B (1)

The molecular formula of **1**~**3** were identical, $C_{16}H_{22}N_2O_4$ established by HR FAB-MS. ^{1}H NMR spectra of **1**~**3** are shown in Fig. 5 and chemical shifts of ^{1}H and ^{13}C NMR spectra of **1**~**3** are shown in Tables 5 and 6, respectively. Similar NMR signal patterns were observed among **1**, **2** and **3**. All 16 carbons of **1**~**3** were visible in ^{13}C NMR spectra. HMQC experiments revealed four quaternary carbons, seven sp^2 methines, two oxymethines, one methylene, three methyls and three active hydrogens in each of **1**~**3**.

Table 6. ^{13}C NMR data of **1**~**4**.

No.	1	2	3
1	180.4 s	180.4 s	180.4 s
2	44.8 s	44.8 s	44.8 s
3	74.9 d	74.9 d	75.3 d
4	140.7 s	140.6 s	140.0 s
5	124.0 d	124.0 d	129.2 d
6	125.7 d	125.7 d	129.7 d
7	127.4 d	127.4 d	131.2 d
8	126.8 d	126.8 d	132.2 d
9	133.1 d	133.1 d	131.7 d
10	66.1 d	66.1 d	66.1 d
11	153.9 s	153.9 s	151.3 s
12	122.8 d	122.8 d	122.7 d
13	150.4 d	150.4 d	151.3 d
2-CH_3	21.6 q	21.6 q	21.6 q
2-CH_3	25.7 q	25.7 q	25.7 q
4-CH_3	19.2 q	19.2 q	19.0 q

Fig. 6. HMBC and ^1H-^1H COSY experiments for **1**.



The structure of **1** was elucidated based on the results of ^1H-^1H COSY and HMBC experiments as shown in Fig. 6. ^1H-^1H COSY revealed the spin system of five olefinic protons (5-H to 9-H), one hydroxymethine (10-H, δ 5.40) and one hydroxy proton (10-OH, δ 4.83). A hydroxy proton (3-OH, δ 5.22) had a coupling with 3-H (δ 4.68). In HMBC experiment two methyl protons (2-$CH_3 \times 2$, δ 1.07 and δ 1.28) had the long-range couplings with C-1, C-2 and C-3, which suggested the arrangement of these carbons. The long-range couplings of C-3 (δ 74.9)/5-H (δ 6.50), C-4 (δ 140.7)/4-CH_3 (δ 1.85), 4-CH_3 (δ 19.2)/3-H (δ 4.68) and 4-CH_3/5-H revealed the arrangement of C-3 to C-5. The IR spectrum indicated the presence of an amide carbonyl group (1650 cm^{-1}) and the amino residue (δ_H 6.33, 6.99) was suggested to connect to C-1.

The long-range couplings of C-10 (δ 66.1)/H-12 (δ 6.96), C-11 (δ 153.9)/H-10 (δ 5.40) and C-13 (δ 151.3)/H-12 (δ 6.96) suggested that the remaining atoms (C_3H_2NO) should construct a monosubstituted oxazole that connected to C-10 as in **4**.

The geometrical isomerism of the conjugated triene moiety was elucidated as 6*Z*,8*E* from the coupling constants ($J_{6,7}$ = 15 Hz, $J_{8,9}$ = 11 Hz). Though the ^{13}C chemical shifts of 4-CH_3 of **4** and **5** are δ 19.8 and δ 19.6, respectively, that of **6** is δ 13.4[3,4]. The geometrical isomerism of C-4 is *Z* in **4** and **5** and *E* in **6**. Thus 4*Z* was suggested for **1** ($\delta_{4\text{-}CH_3}$ 19.2) and the structure of **1** named phthoxazolin B was elucidated as shown in Fig. 1.

Phthoxazolin C (2)

Antibiotic **2** has the same molecular formula $C_{16}H_{22}N_2O_4$ as that of **1**, as established by HR FAB-MS. In ^1H and ^{13}C NMR spectra of **2**, the same signals were observed as those of **1**. Comparison of the $[\alpha]_D$ value between **1** and **2**, **2** named phthoxazolin C was elucidated as a diastereomer of **1**.

Phthoxazolin D (3)

Antibiotic **3** has also the same molecular formula as that of **1**. The chemical shifts of ^1H and ^{13}C NMR were differed between **1** and **3** at the triene moiety (C-5 ~ C-9). Comparing the proton chemical shifts of **3** with those of **1**, 5-H, 8-H, 9-H and 10-H shifted to lower field and 6-H and 7-H shifted to higher field. Since $J_{6,7}$ and $J_{8,9}$ were 14.4 and 15.2 Hz, respectively, **3** was suggested to be 6*E*,8*E*. The ^{13}C chemical shift of 4-CH_3 showed 19.0 ppm, which suggested 4*E* for **3**. Therefore the structure of **3** named phthoxazolin D was elucidated as shown in Fig. 1.

Biological Activity

Antimicrobial Activities

Antibiotics **1** and **2** at 1 mg/ml showed growth inhibition against *Phytophthora parasitica*, but were inactive against *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Micrococcus luteus*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Bacillus subtilis*, *Bacteroides fragilis*, *Acholeplasma laidrawii*, *Candida albicans*, *Pyricularia oryzae*, *Mucor racemosus* and *Saccharomyces sake*.

Herbicidal Activities

Antibiotic **1** was herbicidal; 90% inhibition against seedlings of radish (*Raphanus sativus* L.) and 70% inhibition against seedlings of sorgham (*Sorgham bicolor*) at 63 μg/tube. Antibiotics **2** and **3** were herbicidally less active, inhibiting both plants by 40% at 63 μg/tube and 250 μg/tube, respectively.

Experimental

General

NMR spectra were recorded on Varian XL-400 (400 MHz) NMR spectrometer in acetone-d_6. Mass spectra were obtained on JEOL model JMS-AX505 mass spectrometer. UV-visible spectra were measured on Shimadzu UV-200S spectrometer in methanol. IR spectra were recorded on Horiba FT-210 diffraction infrared spectrometer.

Taxonomic Studies

The isomer of diaminopimelic acid (DAP) was elucidated by the method of TAKAHASHI *et al.*[7] To investigate the cultural characteristics and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEV[8] and media recommended by WAKSMAN[9] were used. Cultures were observed after incubation at 27°C for two weeks. Color Harmony Manual, 4th *Ed.*, 1958 (Container Corporation of America, Chicago) were used for color names and hue numbers. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEV's medium[10] containing 1% carbon sources at 27°C. The morphological properties were observed with a scanning electron microscope (model S-430, Hitachi Co., Ltd.).

Herbicidal Activities

Herbicidal activity was evaluated by the method described previously[2] with radish and sorgham seedlings as test plants. Briefly, an appropriate amount of a test samples, dissolved in methanol, was absorbed on a small cotton piece in the test tubes. The cotton piece was made wet, then each five seeds of radish and sorgham were laid on the wet cotton. The test tubes with each top covered with a metal cap were incubated at 27°C for 5 days under lighting. Reduction in the hight of the test plant was measured and compared with the hights of control plant without samples. Mean values of duplicate tubes were taken.

Acknowledgement

We express our thanks to Ms. A. HATANO for NMR experiment, Ms. T. SAKABE and Ms. A. NAKAGAWA for mass spectra, and Ms. Y. IIDA for her assistance throughout this work.

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