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New Antibiotics Produced by *Streptomyces melanosporofaciens* II. Antimicrobial Activities and Isolation, Purification, and Structure Determination of the Active Compound

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*Streptomyces melanosporofaciens*가 생산하는 새로운 항생물질 II. 물질의 항균활성과 활성물질의 분리·정제 및 구조결정

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Abstract — A phthalic acid derivative and basic macrolide antibiotics, with antimicrobial activity against Gram positive bacteria and phytopathogenic fungi, respectively, were found to be produced by a strain 88-GT-161 identified as being a variety of *Streptomyces melanosporofaciens*. This paper describes an isolation procedure of the active compounds produced by this strain, their *in vitro* and *in vivo* (pot test) antimicrobial activites, and structure determination of one of the compounds, bis (2-ethylhexyl) phthalate, a phthalic acid derivative antibiotic. This compounds, upon comparision with authentic bis (2-ethylhexyl) phthalate, dioctyl phthalate, revealed a difference in antimicrobial activity even though physico-chemical properties of these two compounds seemed indentical. This is the first report that dioctyl phthalate is biosynthetically produced by a *Streptomyces* sp. and shows antimicrobial activity.

In a previous report (1), we described taxonomical characteristics of this strain by means of numerical taxonomy (2) and ISP (3) methods. The strain was identified to be *S. melanosporofaciens* but differences were observed between the strain 88-GT-161 and the reference type strain *S. melanosporofaciens* in several unit characters, thus made us conclude that the strain was a variety of *S. melanosporofaciens*. In addition, active compouds produced by the isolate demonstrated clearly different properties from those antibiotics, such as chilaphylin (4), elaiophylin and melanosporin (5), reported to be produced by *S. melanosporofaciens* so far. Therefore, isolation and purification of the active compouds were done along with tests of antimicrobial activities, *in vitro* or *in vivo*, and structure determination of one of the active compounds as well.

Key words: *Streptomyces melanosporofaciens*, basic macrolide antibiotics, bis (2-ethylhexyl) phthalate, dioctyl phthalate (DOP)
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Material and Methods

Microbial strains

Test microorganisms employed for *in vitro* bioassay were obtained from the Institute of Applied Microbiology, Tokyo University (IAM), the Institute

for Fermentation, Osaka (IFO), and the American Type Culture Collection (ATCC). Other organisms without above-mentioned abbreviations were kindly supplied by the antibiotic laboratory in RIKEN, Japan, with the exception of *Valsa ceratosperma* which were given by the Sumitomo CO., Ltd. in Japan. Plant pathogens used for *in vivo* (pot test) assay were the stock strains of Korea Research Institute of Chemical Technology.

Fermentation conditions

Cultivation of the strain was made at 27℃, 250 rpm for 96 hours, in 500 m*l* Erlenmeyer flasks containing 100 m*l* of antibiotic production medium consisted of; 2% glucose, 1% soluble starch, 0.1% meat extract, 0.4% dried yeast extract, 2.5% soybean flour, 0.2% NaCl, and 0.005% K_2HPO_4.

Isolation and purification

As shown in Fig. 1, whole culture broth (6*l*) was centrifuged to separate broth filtrate and mycelial cake. Mycelial cake was extracted overnight with 60% aqueous acetone and filtered. Filtrate was concentrated under reduced pressure to eliminate acetone. The broth filtrate and mycelial acetone extract were combined and adsorbed on Amberlite IRC-50 (H^+ form, Sigma), washed with H_2O, and eluted with 1 N NH_4OH. The eluate was concentrated to about 3 liter volume and extracted 3 times with 2 liter portions of butanol. The extracts were combined and concentrated *in vacuo* to dryness. The resulting dark-brown gum (6.227 g) was applied to silica gel column chromatography and eluted with the stepwise solvent mixtures:' $CHCl_3$-MeOH-H_2O (14:5:→10:5:1→4:5:1). Active fractions were monitored by antimicrobial activity against *Staphylococcus aureus* and *Rhizoctonia solani*. They were pooled and divided into 2 parts on the basis of antimicrobial activity and TLC (silica gel) patterns with bioautography.

Fractions 1 (2.0 g) was further purified by silica gel column chromatography with solvent systems of EtAc-$CHCl_3$ (1:1), $CHCl_3$-MeOH (19:1→9:1) and $CHCl_3$-MeOH-H_2O (14:5:1). Active portions were collected and concentrated *in vacuo* to produce 2 fractions, Fr. I-1 (627 mg) and Fr. I-2 (187 mg). Fr. I-1 was rechromatographed on silica gel with $CHCl_3$-MeOH (19:1) to give Fr. I-11 (500 mg) and Fr.

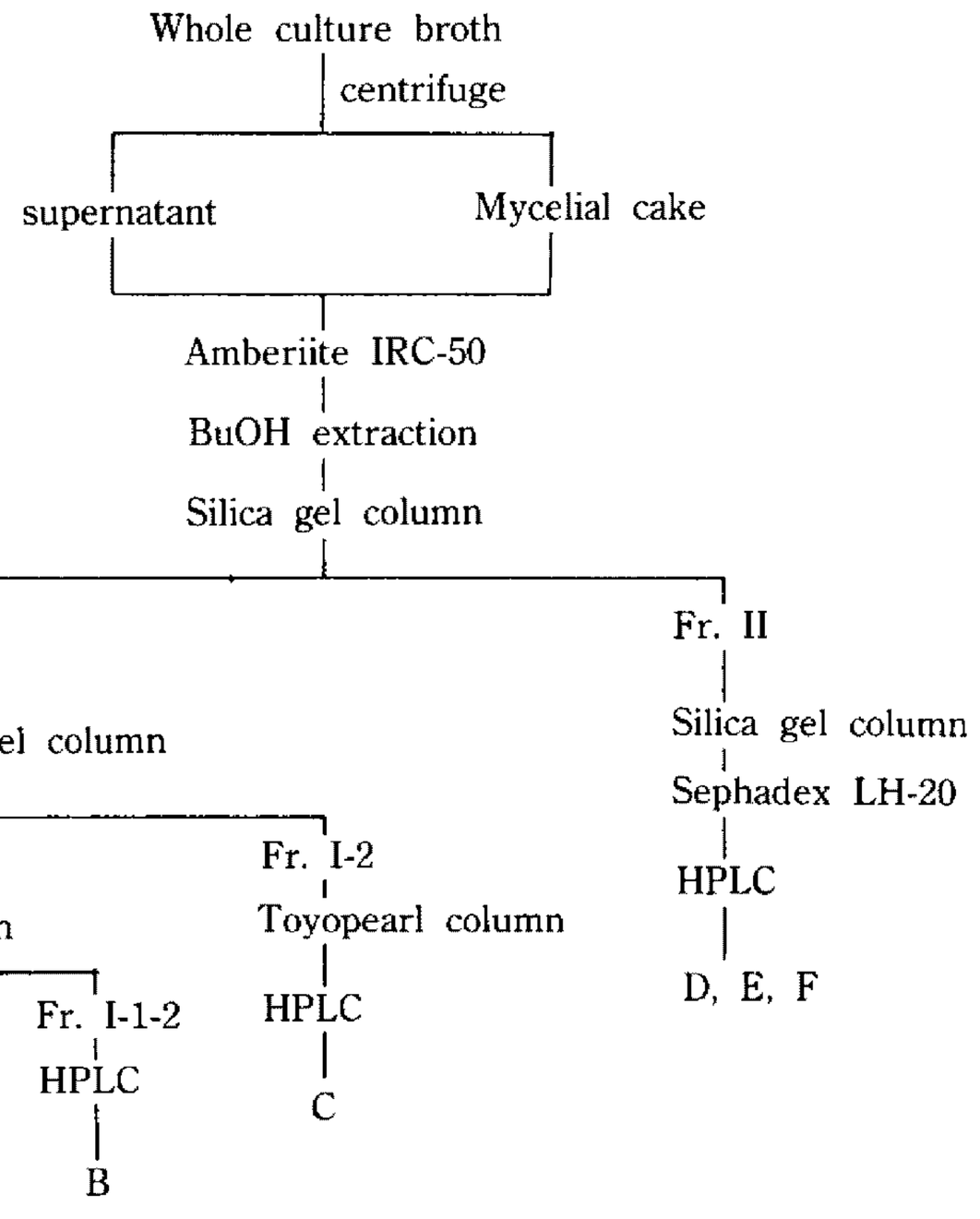

Fig. 1. Procedure for the purification of active compounds produced by *S. melanosporofaciens* isolate.

I-1-2 (100 mg). Final purification of Fr. I-1-1 was done by HPLC (Waters associate, RP, Econosil $10C_{18}$, 227 nm) using 95% aq. MeOH as developing solvent to obtain an active fraction (A) with antimicrobial activity only against Gram positive bacteria. Fr. I-1-2 was also subjected to HPLC (248 nm, 90% aq. MeOH) for final purification of active agent (B) with the same activity.

Fr. I-1-1 (187 mg) was applied to Toyopearl (Toyo Soda Mfg. Co., Inc.) column chromatography and developed with a solvent mixture of $CHCl_3$-MeOH (3:7). Active portions were collected and concentrated to dryness (70 mg). This was finally purified by HPLC with 85% aq. MeOH to get an active fraction (C).

Fr. II (1.67 g), on the other hand, was rechromatographed on silica gel column stepwise with solvent systems of $CHCl_3$-MeOH-H_2O (14:5:1→10:5:1 →4:5:1). Active portions were pooled and concentrated (1.062 g), and subsequently applied to Sephadex LH-20 (Sigma) and developed wih $CHCl_3$MeOH (3:7) to get an active fraction (750 mg). Final purification was achieved by HPLC using Econosil $10C_{18}$ column with a solvent system of MeOH-H_2O-Tetrahydrofuran (70:28:2) to give 3 active fractions, D, E, and F, in the order of decreasing Rf value. The six final active fractions were more than 95% pure as determined by HPLC and designated as compounds hereafter.

Minimum inhibitory concentration (MIC)

MICs against bacteria were investigated on Mueller Hinton agar (Difco) and those against fungi were done on potato dextrose agar (Difco), against yeast on Sabouraud (Difco), and against *Chlorella* on Armon's A_5 agar media by conventional agar dilution method.

***In vivo* bioassay**

Pot tests for the assessment of plant protection activity of the Frs. I and II, obtained from first silica gel column chromatography, were done using six important plant pathogens, such as *Pyricularia oryzae, Rhizoctonia solani, Botrytis cinerea, Phytophthora infestans, Puccinia recondita, Erysiphe graminis* causing rice cavara blast, rice sheath blight, cucumber gray mold, tomato late blight, wheat leaf rust, and barley powdery mildew, respectively. The bioassay was conducted by the methods developed at Korea Research Institute of Chemial Technology (6).

Structure determination

Proton NMR spectrum was recorded on Jeol GSX FT NMR spectrometer (270 MHz). ^{13}C-NMR and DEPT (distortionless enhanced by polarization transfer) spectra were obtained by Bruker FT spectrometer (75.5 MHz). IR spectrum was investigated by Analect FX-6160. Sample was dissolved in CD_3OD for NMR and $CHCl_3$ for IR, and TMS was used for internal reference in NMR spectrum. Authenic dioctyl phthalate was purchased from Aldrich Chem. Co.

Results

Physico-chemical properties

Compounds, A and B were soluble in $CHCl_3$ and methanol, but insoluble in ether, acetone, ethanol, and H_2O. They showed UV absorption maxima at 227 (A) and 248 nm (B), respectively (Fig. 2). Retention time on HPLC (RP, Econosil $10C_{18}$, ID 10 mm, 3 m*l*/min) was 19 minutes in 95% aq. methanol for compound A and 8 minutes in 90% aq. methanol for compound B. Compound C, thought to be similar to melanosporin isolated by Arcamone (5) showed UV absorption maximum at 234 nm and was soluble in methanol but not in $CHCl_3$, ether, acetone, benzene, ethanol, propanol, and butanol. Compounds D, E, and F contained in Fr. II were thought to be basic macrolides for that they were adsorbed on weakly cationic ion exchange resin and showed UV absorption maxima at 232 nm as is common to basic macrolide antibiotics with chromogenic moiety I, such as platenomycin (6) and nystatin (7). They showed the same solubility properties as of compound C but UV absorption patterns were slightly different from that of compound C. These 3 compounds showed exactly the same UV absorption patterns. And they were found to be highly unstable

in acidic condition and so even at pH 5.

Antimicrobial activity

Compound A and B showed antimicrobial activity only against Gram positive bacteria except *B. subtilis*. Compound C showed the strongest activity among the 4 compounds with antifungal activity (Table 1). But these compounds showed different antimicrobial activities regardless of similarities in physico-chemical properties they shared.

Protective effect against fungal phytopathogens

Plant protective effect of Frs. I and II obtained from the first silica gel column chromatography, of which purity is thought to be lower than 5%, is shown in Table 2. Both fractions showed 100% protective activity against rice cavara blast (*P. oryzae*) and rice sheath blight (*R. solani*). Fr. I was also effective against wheat left rust (*P. recondita*) and barley powdery mildew (*E. graminis*). But Fr. II was relatively much lower in its activity than Fr. I. However, phytotoxicity was observed on monocot host plants in which 100% protection was demonstrated.

Structure of the compound A

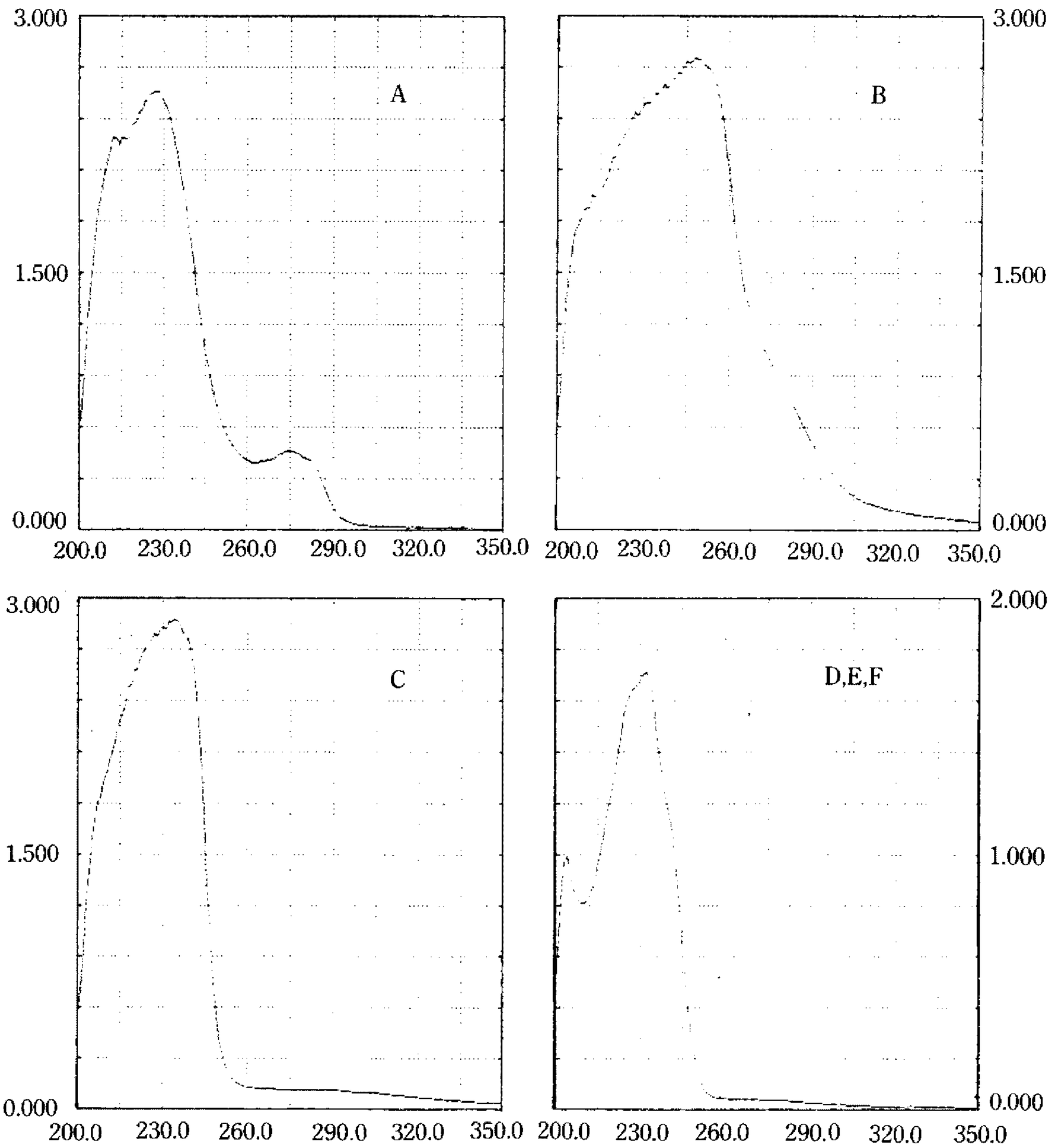

Fig. 2. UV spectra of active compounds produced by *S. melanosporofaciens* isolate.

Table 1. Minimum inhibitory concentrations (μg/m*l*) of the active compounds produced by *S. melanosporofaciens* isolate

Test microorganisms	Compounds					
	A	B	C	D	E	F
Gram positive bacteria						
S. aureus FDA 209P	8	15	125	250	–	–
S. aureus resistant R-209P	16	31	250	500	–	–
S. lutea	16	31	500	–	–	–
M. phlei II DIPH IFO 3158	8	16	125	1,000	500	500
B. subtilis IAM 1069	–	–	–	–	–	–
Yeast						
G. albicans IAM 4905	–	–	125	250	500	–
S. cerevisiae IFO 1008	–	–	–	–	–	–
Fungi						
A. mali IFO 8594	–	–	125	–	–	62
B. cinerea IFO 5365	–	–	31	250	250	62
P. oryzae IFO 5994	–	–	15	125	125	62
G. miyabeanus IFO 5277	–	–	31	31	125	31
F. oxysporium IFO 5277	–	–	62	125	500	125
G. cingulata IFO 9767	–	–	62	125	500	125
R. Solani IFO 6258	–	–	31	62	125	62
M. ramannianus IAM 6219	–	–	125	500	–	250
A. niger ATCC 9642	–	–	250	–	–	–
V. ceratosperma	–	–	31	62	125	62
Algae						
Ch. vulgaris	–	–	31	125	250	125

MIC was investigated by the conventional agar dilution method on Mueller Hinton agar for bacteria, Sabouraud agar for yeast, patato dextrose agar for fungi, and Armon's A_5 agar for algae.

Table 2. Preventive effect (%) of the active compounds produced by *S. melanosporofaciens* isolate against fungal plant pathogens

Fr. No.	Phytopathogenic fungi					
	PO	RS	BC	PI	PR	EG
I	100*	100*	95	62	100*	100*
II	100*	100*	93	89	67	89

Fractions obtained from the first silica gel column chromatography were dissolved in methanol and diluted to the final concentration of 1 mg/m*l* prior to spraying. PO: *Pyricularia oryzae* (Rice Cavara Blast), *RS*; *Rhizoctonia solani* (Rice Sheath Blight), BC; *Bolrytis cinerea* (Cuccumber Gray Mold), PI; *Phytophthora infestans* (Tomato Late Blight), PR; *Puccinia recondita* (Wheat Leaf Rust), EG; *Erysiphe graminis* (Barley Powdery Mildew). Asterisks denote phytotoxity.

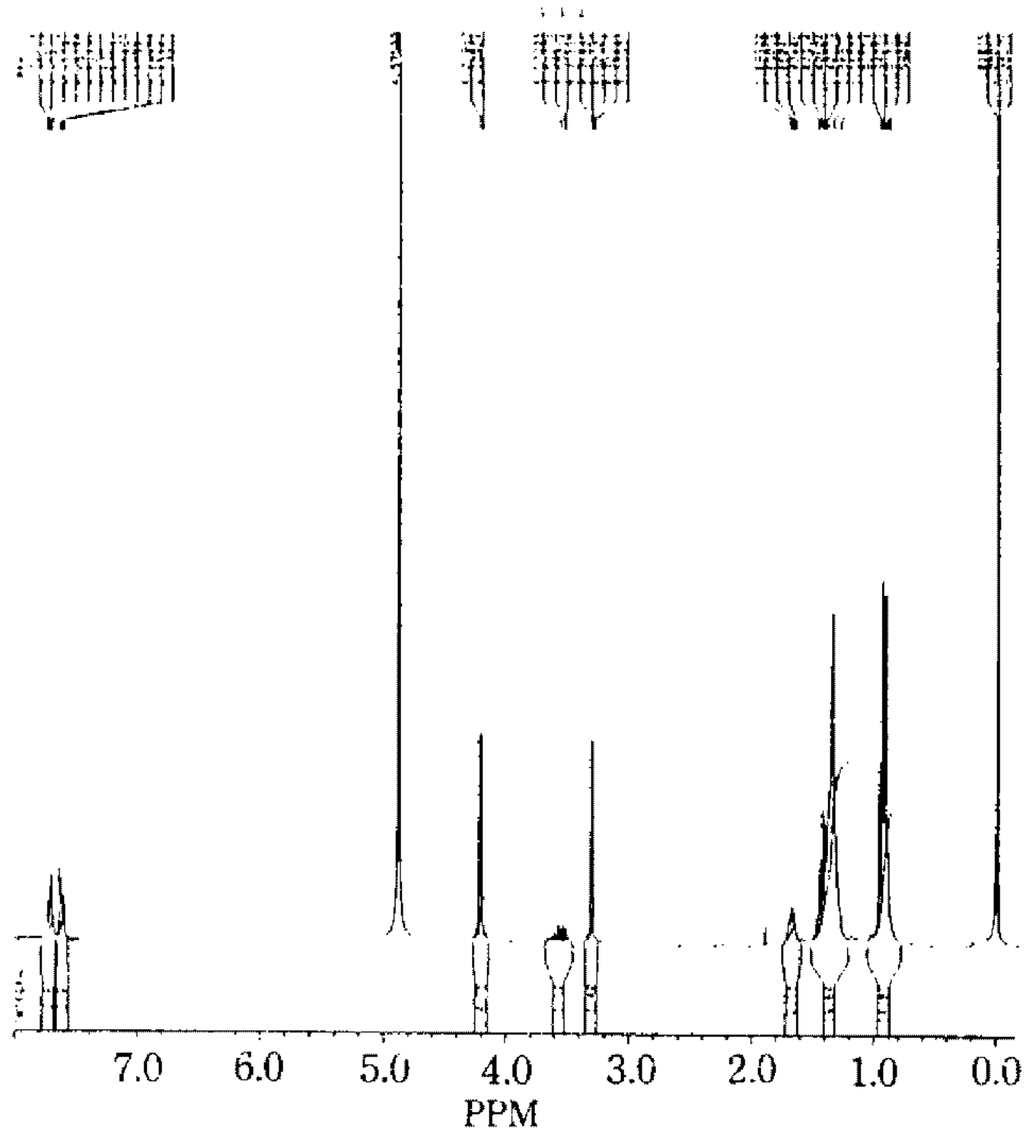

Fig. 3. ^{1}H-NMR (270 MHz) spectrum of the compound A produced by *S. melanosporofaciens* isolate.

Structure of the compound A was determined by the data, ^{1}H-NMR spectrum (Fig. 3. Signals at

Table 3. Chemical shift assignment of proton and carbon in compound A

Assignment	C-Shift	DEPT	H-shift
1	134	=C-	–
2	132	HC=	7.73
3	130	HC=	7.57
4	130	HC=	7.57
5	132	HC=	7.73
6	134	=C-	–
1′	169	C-O-	–
3′	69	-O-CH_2-	4.23
4′	40	HC-	1.62
5′	30	-CH_2-	1.34
6′	25	-CH_2-	1.34
7′	24	-CH_2-	1.34
8′	14	-CH_3-	0.91
9′	31	-CH_2-	1.34
10′	11	-CH_3	0.91

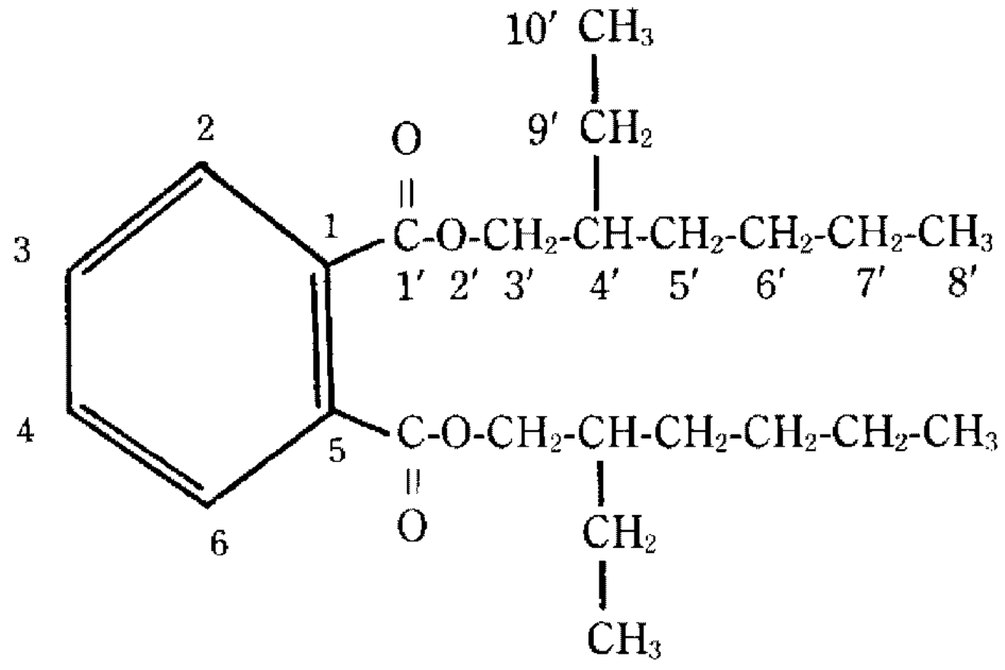

Fig. 4. Chemical structure of the active compound A.

7.57 and 7.73 ppm were thought to be methine-methinecoupling in benzene ring, and 4.23 ppm was -O-CH_2-), ^{13}C-NMR spectrum, DEPT, IR spectrum ($\nu CHCl_3$, cm^{-1}; 2958.8, 2931.8, 2873.9, 2662.4, 1720.2, 1462.0, 1288.4, 1279.0), and Mass spectrum (EI/MS, m/z (%); 390 (M^+, 1), 279 (44), 167 (48), 149 (100), 113 (14), 57 (17). From these results, assignment of each signals on ^{1}H- and ^{13}C-NMR spectra was attempted as shown in Table 3 and the chemical structure, as shown in Fig. 4, of the compound was constructed.

It was later found from the chemical database that molecular weight of dioctyl phthalate is 390. Therefore, comparison of the compound with authentic dioctyl phthalate in biological and physico-chemical properties was attempted, but authentic sample showed no antimicrobial activity against *S. aureus* (data not shown).

Discussions

Thus far, chilaphylin (4), elaiophylin and melanosporin (5) were known to be produced by *S. melanosporofaciens*. Upon comparison with the compounds A and B, chilaphylin is similar in UV absorption maximum (225 nm in MeOH) but different in absorption pattern and antimicrobial spectrum. Chilaphylin was known to show activity against *C. albicans* and *B. subtilis* at MICs of 100 μg/m*l* and 1 μg/m*l*, respectively. But compounds A and B produced by our *Streptomyces* isolate were not active against those 2 organisms. Elaiophylin, on the other hand, showed similarity to compound A in antimicrobial spectrum but difference in UV spectrum. UV absorption maximum of elaiophylin is 252 nm but that of compound A was 227 nm. Compound B was different from any of the above-mentioned known compounds but less active than compound A and showed low yield (1.5 mg/*l*).

Compound C, thought to be melanosporin, was most potent among the 4 polar antibiotics produced by the isolate but fraction II which includes this compound together with compounds D, E, and F showed phytotoxicity in monocot plants. Therefore, these antibiotics do not seem to be good for practical use in disease control of monocot crops. Application for other use, such as herbicide or insecticide is now under study.

Chemically synthesized dioctyl phthalate (bis (2-ethylhexyl) phthalate) was found to be a patented material (9, 10) produced by organic synthesis for vacuum oil. Considering physico-chemical data, such as ^{1}H-and ^{13}C-NMR spectra, impurity in our sample which could possibly lead to antimicrobial activity, even in extreme trace, is not thought to be probable. Therfore, a slight stereochemical difference in the direction of two-ethyl branch can be taken into consideration and further corroboration is to be needed. So far, phthalic acid has been known to be produced by *Gibberella fujikuroi* (11),

but this is the first report that dioctoyl phthalate is produced by a *Streptomyces* sp. and naturally occurring dioctyl phthalate shows antimicrobial activity against Gram positive bacteria.

요 약

*Streptomyces melanosporofaciens*로 동정된 strain 88-GT-161 균주는, 그람양성세균 및 식물병원성 곰팡이에 각각 항균활성을 나타내는 phthalate 유도체 및 염기성 마크로라이드의 새로운 항생물질을 생산하는 것으로 밝혀졌다. 이들 활성물질들의 분리·정제과정에서 *in vitro* 및 *in vivo*(포트 시험) 항균활성을 조사하였으며, phthalate 유도체 항생물질은 IR, NMR, 질량분석 스펙트럼 조사를 통하여 bis (2-ethylhexyl) phthalate(dioctyl phthalate)로 동정하였다. Dioctyl phthalate가 *Streptomyces* sp.에 의하여 생산되며 생합성된 이 화합물이 항균활성을 가진다는 사실이 보고된 것은 이것이 처음이다.

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