IT-143-A and B, Novel Piericidin-group Antibiotics Produced by *Streptomyces* sp.

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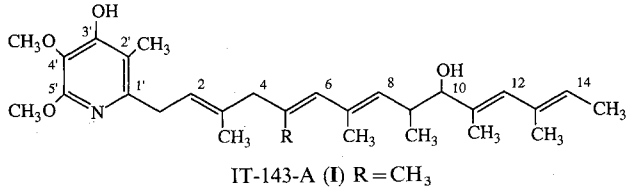
During the course of our screening program for new potent cytotoxic compounds, we found four piericidin group antibiotics, designated as IT-143-A, B, C, and D, in the culture mycelia of *Streptomyces* sp. IT-143. Of these isolated antibiotics, IT-143-A (**I**) and B (**II**) were shown to be new piericidin antibiotics, whereas IT-143-C (**III**) and D (**IV**) were identical to the known antibiotics, piericidin A_2 and A_1[1,2], respectively. In this communication, we describe the taxonomical characteristics of the producing strain and the fermentation procedure as well as the isolation, structure, and biological properties of these two new antibiotics in comparison with those of **III** (piericidin A_2) and **IV** (piericidin A_1).

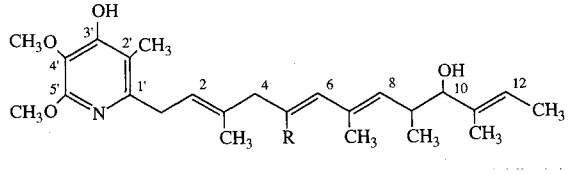
The producing strain IT-143 was isolated from a soil sample collected at Hejiang in Sichuan Province, China. It has been deposited at the National Institute of Bioscience and Human Technology, Ibaraki Prefecture, Japan, with the accession number of FERM BP-4957. Strain IT-143 produced aerial mycelia with straight or flexibile spore chains, each comprising 10 or fewer smooth-surfaced spores. Neither sporangia, zoospores, nor scleotia were observed. The spores were cylindrical in shape with dimensions of $0.4 \sim 0.5 \times 0.5 \sim 0.6\ \mu m$. The color of the aerial mycelium and spore mass was white to yellowish white. The reverse side of the colonial growth was pale yellow to dull yellow or yellowish brown. Melanoid pigments were produced in tyrosine-yeast extract agar and peptone-yeast extract-iron agar, but diffusible pigments 3 were not produced in various agar media. Strain IT-143 showed good growth on Pridham-Gottlieb medium by utilizing carbon sources such as glucose, inositol, D-galactose, maltose, and glycerol. LL-diaminopimelic acid was detected in whole-cell hydrolysates of the culture. According to this taxonomic study, strain IT-143 was considered to belong to the genus *Streptomyces*, but the species was not determined.

One loopful of the growth of *Streptomyces* sp. IT-143 was cultivated at 27°C for 3 days in a 500-ml Erlenmeyer flask containing 100 ml of medium on a rotary shaker (220 rpm). The seed medium consisted of glucose 0.5%, soluble starch 2.4%, meat extract 0.3%, yeast extract 0.5%, peptone 0.5%, corn steep liquor 0.4%, $CoCl_2$ 0.002%, and $CaCO_3$ 0.4% (pH 7.2 before sterilization). The resultant vegetative growth was inoculated at a concentration of 1.5% (v/v) into a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium consisting of glycerol 2.0%, dextrin 2.0%, yeast extract 0.3%, soytone 1.0%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2% (pH 7.0 before sterilization). The fermentation was carried out on the same rotary shaker at 27°C for 5 days, and the harvest day was determined by the cytotoxic activity of the broth filtrate against KB carcinoma cells, which was assessed each day in a preliminary fermentation. After fermentation the mycelial cake was separated by centrifugation at 2800 rpm for 10 minutes from the culture broth and extracted three times with acetone. The organic solvent was removed from the extract by evaporation, and the concentrate was then extracted three times with ethylacetate. The solvent layer was concentrated *in vacuo* to yield an oily residue (*ca.* 11 g). The crude extract was chromatographed on a silica gel column (47 × 7.5 cm, i.d., Merck) with chloroform as the eluent. The active fractions were combined and concentrated *in vacuo*. Further purification was performed by reversed-phase silica gel column (33.0 × 5.0 cm, i.d., Yamazen) chromatography. The active eluate was applied onto an ODS column, and then eluted stepwise with a series of three acetonitrile-water mixtures consisting of (75:25), (80:20) and (90:10). The active fractions containing **III** and **IV** were eluted with acetonitrile-water (75:25). Compound **IV** eluted earlier than compound **III**, and these two compounds could be easily separated. Compounds **I** and **II** were eluted with acetonitrile-water (90:10) and (80:20), respectively. The active eluates containing the respective compounds were evaporated to remove the organic solvent, and then the concentrate was extracted three times with

Fig. 1. IT-143-A (**I**), B (**II**), C (**III**), and D (**IV**.)



IT-143-A (**I**) R = CH_3
IT-143-B (**II**) R = H



IT-143-C (**III**) R = CH_3 (Piericidin A_2)
IT-143-D (**IV**) R = H (Piericidin A_1)

Table 1. Physico-chemical properties of IT-143-A, B, C, and D.

	IT-143-A (I)	IT-143-B (II)	IT-143-C (III, piericidin A_2)	IT-143-D (IV, piericidin A_1)
Appearance	Pale yellow oil	Pale yellow oil	Pale yellow oil	Pale yellow oil
$[\alpha]_D^{25}$ (MeOH)	−13.9° (*c* 0.18)	−26.3° (*c* 0.11)	10.0° (*c* 0.20)	1.0° (*c* 0.10)
UV λ_{max} (MeOH) nm (ε)	204 (45,600) 235 (34,300)	204 (47,600) 239 (48,100)	204 (42,400) 229 (23,200)	205 (40,800) 237 (41,800) 267 (6,600)
IR ν_{max} (KBr) cm^{-1}	3401, 2927, 1588, 1473, 1413	3401, 2932, 1588, 1474, 1413	3446, 2974, 1588, 1474, 1413	3401, 2937, 1588, 1474, 1413
Rf value[a]				
$CHCl_3$-acetone (20:1)	0.67	0.63	0.58	0.55
Benzene-AcOEt (10:1)	0.39	0.37	0.34	0.31
Retention time (HPLC) minute[b]	38.0	26.9	19.6	13.9
Molecular formula	$C_{29}H_{43}NO_4$	$C_{28}H_{41}NO_4$	$C_{26}H_{39}NO_4$	$C_{25}H_{37}NO_4$
Molecular weight	469	455	429	415

[a] TLC on silica gel $60F_{254}$ (Merck Art. 5715). [b] HPLC conditions; Column: Inertsil ODS-2 (150×4.6 mm, i.d., 5 μm, GL Sciences), Mobile phase: CH_3CN-H_2O (70:30), Flow rate; 1.0 ml/minute, Detection: UV-210 nm.

ethylacetate. All extracts were concentrated *in vacuo* to yield compounds: **I** (35.9 mg), **II** (37.3 mg), **III** (142.8 mg), and **IV** (241.0 mg). The retention times of the respective compounds by high-performance liquid chromatography (HPLC) on an Inertsil ODS-2 column are shown in Table 1. The progress of purification of the respective compounds as described above was monitored by measurement of cytotoxicity against KB cells, detection with UV absorbance at 254 nm on silica gel thin-layer chromatography (TLC), and detection of each peak obtained by high-performance liquid chromatography (HPLC) under the conditions indicated in Table 1.

The physico-chemical properties of **I** and **II** in comparison with those of **III** and **IV** are summarized in Table 1. From these data, a similarity in structure among these compounds was revealed; especially, the difference in the molecular weight between **I** and **III**, *i.e.*, 40, was the same as that between **II** and **IV**. This indicated that **I** and **III**, and **II** and **IV**, possessed relatively the same structural relationship with each other. The ^1H NMR spectrum (400 MHz, in $CDCl_3$) of **I** was as follows: 6.15 (1H, br s, 3′-OH), 5.85 (1H, s, 12-H), 5.70 (1H, s, 6-H), 5.42 (1H, q, J=7 Hz,14-H), 5.41 (1H, t, J=7 Hz, 2-H), 5.10 (1H, d, J=10 Hz, 8-H), 3.96 (3H, s, 5′-OCH_3), 3.86 (3H, s, 4′-OCH_3), 3.63 (1H, d, J=9 Hz, 10-H), 3.40 (2H, d, J=7 Hz, 1-H), 2.71 (2H, s, 4-H), 2.66 (1H, m, 9-H), 2.10 (3H, s, 2′-CH_3), 1.79 (3H, d, J=1 Hz, 7-CH_3), 1.79 (3H, d, J=1 Hz, 11-CH_3), 1.74 (3H, t, J=1 Hz, 13-CH_3), 1.69 (3H, d, J=7 Hz, 15-H), 1.69 (3H, d, J=1 Hz, 3-CH_3), 1.68 (3H, d, J=1 Hz, 5-CH_3), 0.86 (3H, d, J=6.5 Hz, 9-CH_3). The ^1H NMR spectrum (400 MHz, in $CDCl_3$) of **II** was as follows: 6.09 (1H, dq, J=15, 1 Hz, 6-H), 5.84 (1H, br s, 12-H), 5.61 (1H, dt, J=15, 7 Hz, 5-H), 5.42 (1H, overlapped, 14-H), 5.41 (1H, overlapped, 2-H), 5.22 (1H, d, J=9 Hz, 8-H), 3.95 (3H, s, 5′-OCH_3), 3.86 (3H, s, 4′-OCH_3), 3.64 (1H, d, J=9 Hz, 10-H), 3.37 (2H, d, J=7 Hz, 1-H), 2.79 (2H, d, J=7 Hz, 4-H), 2.71 (1H, m, 9-H), 2.09 (3H, s, 2′-CH_3), 1.80 (3H, d, J=1 Hz, 7-CH_3), 1.78 (3H, d, J=1 Hz, 11-CH_3), 1.74 (3H, d, J=1 Hz, 3-CH_3), 1.74 (3H, d, J=1 Hz, 13-CH_3), 1.69 (3H, d, J=7 Hz, 15-H), 0.85 (3H, d, J=7 Hz, 9-CH_3).

A comparison of ^{13}C NMR spectral data of **I** and **II** with those of **III** and **IV** is made in Table 2. The spectral data were similar to each other. Two signals for olefinic carbons at δ_C 133.1 (13-C) and δ_C 124.5 (14-C) and one signal for a methyl carbon at δ_C 13.6 (15-C) were observed in the spectrum of **I**, along with the other signals that were similar to those of **III**. These former signals accounted for the difference in the molecular weight between **I** and **III**. Furthermore, in the ^1H-^1H DQF COSY spectrum of **I**, ^1H-^1H long-range coupling correlations were observed among 12-H and 11-CH_3, 13-CH_3, 15-H. In the HMBC spectrum of **I**, long-range ^1H-^{13}C couplings were observed between 12-H and 14-C; 14-H and 12-C, 15-C; 15-H and 14-C, 13-C; 13-CH_3 and 12-C, 13-C, 14-C. These results indicate that **I** is a new member of the piericidin-group of antibiotics possessing the structure shown in Fig. 1. In the same way, two signals for olefinic carbons at δ_C 133.0 (13-C) and δ_C 124.6 (14-C) and one signal for a methyl carbon at δ_C 13.6 (15-C) appeared in the spectrum of **II**, together with signals similar to those of **IV**. Furthermore, in the ^1H-^1H DQF COSY spectrum of **II**, ^1H-^1H long-range coupling correlations were observed among 12-H and 11-CH_3, 15-H; 14-H and 13-CH_3. In the HMBC spectrum of **II**, long-range ^1H-^{13}C couplings were observed between 12-H and 14-C; 14-H and 15-C, 13-C; 15-H and 14-C, 12-C; 13-CH_3 and 13-C. Thus, compound **II** was shown to be another new member of the piericidin-group of antibiotics having the structure shown in Fig. 1. As IT-143-A and B have one more carbon atom on their side chain than piericidin A_4 and A_3[1,2], these two compounds have the longest side chain among the known piericidin-group of antibiotics. In addition, both compounds contain a new conjugated

Table 2. ^{13}C NMR spectral data of IT-143-A (**I**), B (**II**), C (**III**), and (**IV**).

Position	IT-143-A*	IT-143-B**	IT-143-C (piericidin A_2)	IT-143-D (piericidin A_1)
1-C	34.5 (t)	34.4 (t)	34.5 (t)	34.4 (t)
2-C	123.6 (d)	122.2 (d)	123.3 (d)	122.2 (d)
3-C	133.9 (s)	134.8 (s)	133.9 (s)[b]	134.8 (s)[e]
4-C	51.1 (t)	43.1 (t)	51.1 (t)	43.1 (t)
5-C	134.5 (s)	126.8 (d)	134.5 (s)[b]	126.7 (d)
6-C	130.1 (d)	135.7 (d)	130.1 (d)[c]	135.7 (d)
7-C	135.7 (s)	136.0 (s)	135.7 (s)[b]	135.6 (s)[e]
8-C	131.5 (d)	133.0 (d)	131.5 (d)[c]	133.0 (d)
9-C	37.2 (d)	37.1 (d)	37.0 (d)	36.9 (d)
10-C	83.2 (d)	83.3 (d)	82.7 (d)	82.8 (d)
11-C	134.2 (s)	134.1 (s)	135.7 (s)[b]	136.0 (s)
12-C	132.5 (d)	132.6 (d)	123.6 (d)	123.5 (d)
13-C	133.1 (s)	133.0 (s)	13.1 (q)	13.1 (q)[f]
14-C	124.5 (d)	124.6 (d)		
15-C	13.6 (q)	13.6 (q)		
3-CH_3	15.8 (q)	16.6 (q)[a]	15.8 (q)	13.1 (q)[f]
5-CH_3	17.3 (q)		17.3 (q)[d]	
7-CH_3	17.6 (q)	13.2 (q)	17.6 (q)[d]	16.6 (q)
9-CH_3	17.5 (q)	17.5 (q)	17.4 (q)[d]	17.4 (q)
11-CH_3	12.6 (q)	12.5 (q)	10.6 (q)	10.5 (q)
13-CH_3	16.6 (q)	16.6 (q)[a]		
1′-C	150.9 (s)	150.8 (s)	150.9 (s)	150.8 (s)
2′-C	111.9 (s)	111.9 (s)	111.9 (s)	111.9 (s)
3′-C	154.0 (s)	154.0 (s)	153.9 (s)	154.0 (s)
4′-C	127.8 (s)	127.8 (s)	127.8 (s)	127.8 (s)
5′-C	153.5 (s)	153.5 (s)	153.5 (s)	153.5 (s)
2′-CH_3	10.4 (q)	10.4 (q)	10.4 (q)	10.4 (q)
4′-OCH_3	60.6 (q)	60.6 (q)	60.6 (q)	60.6 (q)
5′-OCH_3	53.1 (q)	53.0 (q)	53.0 (q)	53.0 (q)

*,**: Assignments were based on HMBC and HMQC experiments.
[a~f]: Assignments may be interchanged.

Table 3. Antimicrobial spectra of IT-143-A, B, C, and D.

	MIC (μg/ml)			
	IT-143-A	IT-143-B	IT-143-C	IT-143-D
Staphylococcus aureus FDA 209P	>100	>100	>100	>100
Micrococcus luteus ATCC 10240	6.25	6.25	6.25	6.25
Bacillus subtilis ATCC 6633	>100	>100	>100	>100
Escherichia coli NIHJ	100	>100	>100	>100
Klebsiella pneumoniae ATCC 29665	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 13275	>100	>100	>100	>100
Aspergillus fumigatus H11-20	12.5	>100	25	>100
Aspergillus fumigatus BCL 41	12.5	>100	50	>100
Aspergillus fumigatus BCL55	25	>100	100	>100
Aspergillus fumigatus BCL70	12.5	>100	25	>100
Aspergillus fumigatus CI-2	25	>100	100	>100
Aspergillus oryzae IFO 4221	>100	>100	>100	>100
Aspergillus niger CI-6	>100	>100	>100	>100
Trichophyton rubrum IFO 5807	25	100	100	100

diene system from 11-C to 14-C as well as that from 5-C to 8-C. These characteristics set these two compounds apart from other members of the piericidin-group of antibiotics.

The *in vitro* antimicrobial activities of **I**, **II**, **III**, and **IV** were determined by a serial agar dilution method using Mueller-Hinton agar (Difco) for bacteria and Sabouraud agar (Nissui) for fungi. The minimum inhibitory concentrations (MIC) were observed after overnight incubation at 37°C for bacteria, and after incubation for 42 hours at 30°C for fungi. The antimicrobial spectra of **I**, **II**, **III**, and **IV** are shown in Table

3. These antibiotics had inhibitory activity against *Micrococcus luteus* among the Gram-positive bacteria tested, and against *Aspergillus fumigatus* among the fungi tested. Compound **I** had the strongest activities of the four compounds. The cytotoxic activities *in vitro* of **I**, **II**, **III**, and **IV** were examined by a dye-uptake method against KB carcinoma cells (2000 cells/well) in EAGLE's minimal essential medium supplemented with 10% calf serum. When the cells were exposed to these antibiotics for 3 days, the concentrations of **I**, **II**, **III**, and **IV** required to inhibit growth of the cells by 50% (IC_{50} value) were 0.36, 1.1, 2.0, 2.6 ng/ml, respectively.

Recent reports have suggested that piericidin-group antibiotics, *e.g.*, piericidin B_1 *N*-oxide[3,4], piericidin B_5, and B_5 *N*-oxide[5], exhibit phosphatidylinositol (PI) turnover-inhibiting activities. PI turnover has been shown to play a significant role in intracellular pathways elicited by a variety of cellular stimuei such as some types of oncogenes[6,7] and growth factors[8,9]. In view of the above reports, these novel antibiotics, IT-143-A and B, also may be expected to be inhibitors of PI turnover.

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