ISOLATION AND STRUCTURE DETERMINATION OF NOVEL PHOSPHATIDYLINOSITOL TURNOVER INHIBITORS, PIERICIDIN B_5 AND B_5 *N*-OXIDE, FROM *Streptomyces* sp.

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In the course of a screening program for inhibitors of epidermal growth factor (EGF)-induced phosphatidylinositol turnover in human epidermoid carcinoma cell line A431, we discovered two novel compounds of the piericidin family from the culture broth and mycelia of a *Streptomyces* strain MJ288-OF3. We named them piericidin B_5 and B_5 *N*-oxide. By NMR and mass spectrometric analyses, the molecular formulas of piericidin B_5 and B_5 *N*-oxide were determined to be $C_{27}H_{41}NO_4$ (MW 443) and $C_{27}H_{41}NO_5$ (MW 459), respectively. Piericidin B_5 and B_5 *N*-oxide inhibited phosphatidylinositol turnover of A431 cells with IC_{50}s of 10.0 μg/ml and 1.1 μg/ml, respectively.

Phosphatidylinositol turnover is considered to be correlated with transformation by some types of oncogenes[1,2] and cellular response to growth factors such as EGF[3] and platelet-derived growth factor[4]. Enzymatic breakdown of phosphatidylinositol 4,5-bisphosphate by phospholipase C generates inositol 1,4,5-triphosphate and 1,2-diacylglycerol. These products both act as intracellular second messengers[5], the former mobilizing Ca^{2+} from intracellular stores and the latter stimulating protein kinase C activity. We have screened inhibitors of phosphatidylinositol turnover from microbial secondary metabolites, and previously isolated psi-tectorigenin[6], inostamycin[7] and piericidin B_1 *N*-oxide[8]. Piericidins are insecticidal substances found from *Streptomyces mobaraensis*[9,10] and *Streptomyces pactum*[11]. They are systematically named piericidins A_n, B_n, C_n and D_n (n=1, 2, 3, 4)[12]. We have isolated novel and potent inhibitors of phosphatidylinositol turnover produced by a *Streptomyces* strain and named them piericidin B_5 and B_5 *N*-oxide because of their structural relationship to the piericidin B group.

Materials and Methods

Materials

The A431 cell line was a gift from Prof. S. KAWAI, Institute of Medical Science, University of Tokyo. Piericidin B_1 was kindly supplied by Dr. S. YOSHIDA, the Institute of Physical and Chemical Research, Wako. *Myo*-[^{3}H]inositol was purchased from DuPont-New England Nuclear.

In Situ Phosphatidylinositol Turnover Assay

The phosphatidylinositol turnover assay was carried out as described previously[6,7]. In brief, A431

cells (3×10^5 cells/well) plated on 22-mm plastic wells were grown in DULBECO's modified EAGLE's medium supplemented with 5% calf serum for 16~18 hours. The cells were pre-incubated in 1 ml of HEPES-buffered saline (HBS) containing *myo*-[^{3}H]inositol (1 μCi/ml) at 37°C for 30 minutes. Then, the inhibitor and EGF (400 ng/ml) were added and the incubation was continued at 37°C for 60 minutes. The cells were washed with 1.0 ml of 10% TCA containing 0.01 M sodium pyrophosphate and the acid-insoluble fraction was solubilized using 1.0 ml of 0.25 N NaOH. The radioactivity of labeled phosphoinositides was measured by liquid scintillation counting.

Fermentation

The *Streptomyces* strain MJ288-OF3 was inoculated into 110 ml of seed medium consisting with 4.0% sucrose, 2.5% soybean meal, 0.25% NaCl, 0.32% $CaCO_3$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$ and 0.0005% $ZnSO_4 \cdot 5H_2O$ (adjusted to pH 7.4 before sterilization). The seed culture was incubated for 3 days at 28°C on a rotary shaker (180 rpm). Two ml of the culture was then transferred to 110 ml of fermentation medium whose composition was equivalent to that of the seed medium. The fermentation was carried out for 4 days at 28°C on a rotary shaker (180 rpm).

Isolation

The fermentation broth (12 liters) was filtered, and the mycelia were extracted with acetone (1 liter). After removal of the acetone, the extract was combined with the filtrate (10.8 liters). The mixture was extracted with EtOAc (14 liters) and the EtOAc layer was dried over anhydrous Na_2SO_4. The EtOAc extract was then concentrated *in vacuo* to give an oily matter (4.65 g), which was subjected to a silica gel column chromatography (200 ml; column I).

Piericidin B_5 was isolated as follows. The fractions containing piericidin B_5, eluted with $CHCl_3$ from the column I, were concentrated *in vacuo* to yield 716.0 mg of oily material. The oily material was further applied to a silica gel column (30 ml). The column was successively washed with toluene, toluene - EtOAc (50 : 1) and toluene - EtOAc (30 : 1), and piericidin B_5 was eluted with toluene - EtOAc (10 : 1). After evaporation to dryness, the residue (340.5 mg) was chromatographed on Sephadex LH-20 (150 ml) with EtOAc. The combined active fraction (228.6 mg) was partitioned in a solvent system *n*-hexane - CH_3CN (1 : 1) by centrifugal partition chromatography (CPC, Sanki Engineering Co. Ltd.), in which the lower portion was stationary. The fractions containing piericidin B_5 were collected and further purified with *n*-hexane - CH_3CN - 28%NH_4OH (125 : 125 : 1) in CPC. Thus, 6.3 mg of piericidin B_5 was obtained.

Piericidin B_5 *N*-oxide was isolated as follows. The fractions containing piericidin B_5 *N*-oxide eluted with $CHCl_3$ - MeOH (10 : 1) from column I were concentrated *in vacuo* to give 976.4 mg of oily material. It was rechromatographed on a silica gel column (40 ml) and eluted with $CHCl_3$ - MeOH (30 : 1). After evaporation to dryness, the residue (523.0 mg) was partitioned in the solvent system $CHCl_3$ - MeOH - H_2O (5 : 6 : 4) in CPC. The combined active fraction (496.8 mg) was further purified by preparative HPLC using a reverse phase silica gel column (Nucleosil $_5C_{18}$ 30 × 250 mm) with a solvent system of 85% MeOH. The eluate was concentrated to yield 39.9 mg of purified piericidin B_5 *N*-oxide.

Physico-chemical Properties

Piericidin B_5 and B_5 *N*-oxide are pale yellow oils, soluble in common organic solvents such as $CHCl_3$, EtOAc and MeOH, but insoluble in water. Piericidin B_5 ($C_{27}H_{41}NO_4$): $[\alpha]_D^{24}$ −12.5° (*c* 0.2, MeOH); FAB-MS *m/z* 444 $(M+H)^+$; UV λ_{max}^{MeOH} nm (ε) 232 (31,270), 237 (31,890), 267 (5,010), $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε) 210 (34,960), 237 (30,970), 273 (9,380), $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε) 238 (37,200); IR ν_{max} ($CHCl_3$) cm^{-1} 3530, 2980, 2950, 2890, 2840 sh, 1630, 1600, 1480, 1450, 1430, 1400, 1390, 1370, 1340, 1260, 1230 sh, 1200, 1140, 1110, 1090, 1060, 1010, 980, 960, 910, 890, 870. Piericidin B_5 *N*-oxide ($C_{27}H_{41}NO_5$): $[\alpha]_D^{24}$ −8.0° (*c* 0.2, MeOH); FAB-MS *m/z* 460 $(M+H)^+$; UV λ_{max}^{MeOH} nm (ε) 227 (39,760), 238 sh (34,250), 246 sh (23,850), 267 (8,930), $\lambda_{max}^{0.1\,N\,HCl\text{-}MeOH}$ nm (ε) 214 (36,150), 238 (30,310), 246 sh (23,850), 275 (6,060), $\lambda_{max}^{0.1\,N\,NaOH\text{-}MeOH}$ nm (ε) 230 sh (44,280), 238 sh (39,480), 246 sh (25,840), 276 (12,870); IR ν_{max} ($CHCl_3$) cm^{-1} 3530, 2980, 2950, 2890, 2840 sh, 1610, 1510, 1470, 1430, 1390, 1350, 1310, 1240, 1190, 1160, 1140, 1120, 1100, 1090, 1070, 1020, 970, 950, 920, 890, 870.

Reduction of Piericidin B_5 *N*-Oxide to Piericidin B_5

A mixture of piericidin B_5 *N*-oxide (17.1 mg) and zinc powder (174.8 mg) in CH_3COOH (1.5 ml) was stirred at 40°C for 2 hours. The reaction mixture was diluted with distilled water (50 ml) and extracted with EtOAc (25 ml × 2 times). The organic layer was successively washed with saturated $NaHCO_3$ (50 ml) and water (25 ml × 2 times), and dried over anhydrous Na_2SO_4. The organic extract was concentrated *in vacuo* and subjected to the CPC system with a solvent system of *n*-hexane-CH_3CN (1:1). After concentration to dryness, 9.9 mg of the reduced material was obtained. The reduced substance was identical to piericidin B_5 by FAB-MS and ^1H NMR analyses.

Results and Discussion

We have found two novel phosphatidylinositol turnover inhibitors, piericidin B_5 and B_5 *N*-oxide, produced by a *Streptomyces* strain MJ288-OF3. Morphological and physiological studies indicated that the strain is similar to *Streptomyces aburaviensis*. The producing strain also gave piericidin B_1 and B_1 *N*-oxide previously. The FAB-MS, ^1H and ^{13}C NMR spectral analyses revealed that the molecular formulas of piericidin B_5 and B_5 *N*-oxide were $C_{27}H_{41}NO_4$ [*m/z* 444 $(M+H)^+$] and $C_{27}H_{41}NO_5$ [*m/z* 460 $(M+H)^+$], respectively. The ^1H and ^{13}C NMR spectra of piericidin B_5 and B_5 *N*-oxide are shown in Tables 1 and 2.

The UV spectrum of piericidin B_5 very closely resembled that of piericidin B_1[10], indicating that they have the same chromophore. By comparison of FAB-MS, ^1H and ^{13}C NMR spectra between piericidin B_5 and B_1[10], it was inferred that the side chain piericidin B_5 possessed one more methylene group ($-CH_2-$) than piericidin B_1. The side chain structure of piericidin B_5 was deduced by heteronuclear

Table 1. ^1H[a] NMR data of piericidin B_5 and B_5 *N*-oxide in $CDCl_3$.

Position	B_5	B_5 *N*-oxide
1	3.37 d ($J_{1,2}$=7.2 Hz)	3.67 d ($J_{1,2}$=6.8 Hz)
2	5.39 t	5.19 t
4	2.77 d ($J_{4,5}$=6.8 Hz)	2.73 d ($J_{4,5}$=6.8 Hz)
5	5.51 m ($J_{5,6}$=15.6 Hz)	5.46 m ($J_{5,6}$=15.6 Hz)
6	6.08 d	6.06 d
8	5.29 d ($J_{8,9}$=8.8 Hz)	5.29 d ($J_{8,9}$=8.8 Hz)
9	2.65 m ($J_{9,10}$=8.4 Hz)	2.64 m ($J_{9,10}$=8.6 Hz)
10	3.16 d	3.15 d
12	5.34 t ($J_{12,13}$=7.2 Hz)	5.34 t ($J_{12,13}$=7.2 Hz)
13	2.06 m ($J_{13,14}$=7.8 Hz)	2.07 m ($J_{13,14}$=7.8 Hz)
14	0.98 t	0.97 t
15	1.74 s	1.75 s
16	1.74 s	1.71 s
17	0.78 d ($J_{9,17}$=6.4 Hz)	0.78 d ($J_{9,17}$=6.4 Hz)
18	1.52 s	1.52 s
19	3.13 s	3.12 s
6′	2.09 s	2.16 s
7′	3.86 s	3.69 s
8′	3.96 s	3.96 s

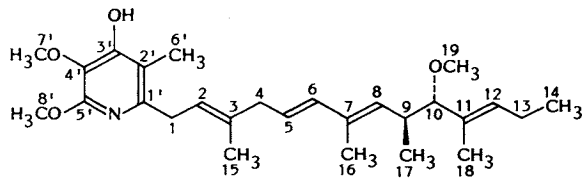
[a] ^1H chemical shifts (ppm), signal multiplicities and coupling constants (*J* in Hz) in parentheses at 400 MHz.

Table 2. ^{13}C[a] NMR data of piericidin B_5 and B_5 *N*-oxide in $CDCl_3$.

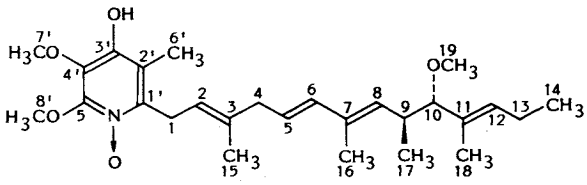
Position	B_5	B_5 *N*-oxide
1	34.4 t	27.1 t
2	121.8 d	118.7 d
3	135.2 s	136.9 s
4	43.1 t	43.2 t
5	125.1 d	124.7 d
6	136.4 d	136.6 d
7	133.3 s	133.3 s
8	135.0 d	135.2 d
9	35.4 d	35.3 d
10	92.7 d	92.6 d
11	132.3 s	132.2 s
12	132.2 d	132.2 d
13	20.9 t	20.8 t
14	14.1 q	14.1 q
15	16.6 q	16.4 q
16	12.9 q	12.9 q
17	17.7 q	17.6 q
18	10.4 q	10.5 q
19	56.1 q	56.1 q
1′	150.9 s	145.6 s
2′	112.0 s	117.6 s
3′	154.0 s	158.7 s
4′	127.8 s	135.2 s
5′	153.5 s	151.8 s
6′	10.5 q	11.3 q
7′	60.6 q	60.9 q
8′	53.1 q	60.9 q

[a] ^{13}C chemical shift (ppm) and signal multiplicities at 100 MHz.

Fig. 1. Structures of Piericidin B_5 and B_5 *N*-oxide.



Piericidin B_5



Piericidin B_5 *N*-oxide

multiple bond correlation (HMBC) *via* long range coupling spectra. Thus, we determined that the terminal methyl group of piericidin B_1 was changed to an ethyl group in B_5. The structure of piericidin B_5 *N*-oxide was also determined in comparison with piericidin B_1 *N*-oxide[8] by UV, FAB-MS, ^1H and ^{13}C NMR analyses. Further, we chemically reduced piericidin B_5 *N*-oxide to piericidin B_5 and confirmed that the reduced substance was identical to piericidin B_5 by FAB-MS and ^1H NMR analyses. Absolute configurations of C-9 and C-10 in both piericidin B_5 and B_5 *N*-oxide were determined to be *S*-*S* since the optical rotations of piericidin B_5 ($-12.5°$) and B_5 *N*-oxide ($-8.0°$) are similar to those of piericidin B_1 ($-6.5°$)[10] and B_1 *N*-oxide ($-4.5°$)[8], respectively. Thus, we have concluded that the structures of piericidin B_5 and B_5 *N*-oxide are as shown in Fig. 1.

Piericidin B_5 and B_5 *N*-oxide inhibited EGF-stimulated [^{3}H]inositol incorporation into phospholipids with IC_{50}s of 10.0 μg/ml and 1.1 μg/ml, respectively, in the A431 cells assay system. As shown in Table 3, piericidin B_5 *N*-oxide showed antimicrobial activity against Gram-positive and part of Gram-negative bacteria and fungi, although piericidin B_5 did not. In the case of piericidin B_1, again only the *N*-oxide shows antimicrobial activity[8].

Table 3. Antimicrobial activities of piericidin B_5 and B_5 *N*-oxide in agar dilution assay.

Test organisms	MIC (μg/ml)	
	B_5	B_5 *N*-oxide
Staphylococcus aureus Smith	>100	25
Micrococcus luteus FDA16	>100	0.8
Bacillus anthracis	>100	12.5
Corynebacterium bovis 1810	>100	6.3
Escherichia coli NIHJ	>100	12.5
Shigella dysenteriae JS11910	>100	6.3
Salmonella typhi T-63	>100	>100
Proteus vulgaris OX19	>100	>100
Serratia marcescens	>100	>100
Pseudomonas aeruginosa A3	>100	100
Klebsiella pneumoniae PCT602	>100	>100
Mycobacterium smegmatis ATCC 607	>100	>100
Candida albicans 3147	>100	>100
Saccharomyces cerevisiae F-7	>100	>100
Cryptococcus neoformans F-10	>100	50
Cochliobolus miyabeanus	>100	25
Pyricularia oryzae	>100	50
Pellicularia sasakii	>100	1.6
Xanthomonas citri	>100	3.1
Trichophyton asteroides 429	>100	6.3
Aspergillus niger F-16	>100	>100

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