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Pleofungins, Novel Inositol Phosphorylceramide Synthase Inhibitors, from *Phoma* sp. SANK 13899

II. Structural Elucidation

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Abstract Pleofungins (formerly called F-15078) A, B, C and D, novel depsipeptide antifungal antibiotics, were found in a mycelium extract of the producing fungus, *Phoma* sp. SANK 13899. The structures of pleofungins A, B, C and D were elucidated mainly by various NMR studies. The absolute configurations of the amino acids and *N*-methyl amino acids of pleofungin A constituents in the hydrolysate were determined by the application of advanced Marfey's method in combination with gas chromatography/mass spectrometry analysis of their silylation products with *N*-methyl-*N*-(*tert*-butylsilyl)-trifluoroacetamide. Two α-hydroxy acid constituents, α-hydroxyisocaproic acid and α-hydroxyisovaleric acid, were isolated from the hydrolysate and their stereochemistries were determined by their specific rotations.

Keywords pleofungin, F-15078, inositol phosphorylceramide synthase inhibitor, physico-chemical properties, structure

Introduction

Amphotericin B, fluconazole, itraconazole and flucytosine are common commercially available systemic antifungal agents useful for the therapy of deeply invasive mycoses [1, 2]. Although, amphotericin B, a polyene macrolide antibiotic, exhibits strong fungicidal activity against yeast and filamentous fungi including *Aspergillus fumigatus*, its severe cytotoxic side effects make chemotherapy difficult. Azoles such as fluconazole and itraconazole are widely used antifungal agents because of their strong anti-*Candida* activity with low toxicity and relatively wide antifungal spectrum. However, their activity is fungistatic and the emergence of azole resistant *Candida* species is widely recognized as becoming a clinically serious problem [3, 4]. Flucytosine, a thymidine synthase inhibitor, is known to have a narrow antifungal spectrum and resistant strains easily develop. For this reason, the usage is restricted to combination therapy with amphotericin B. Recently, 1,3-β-glucan synthesis inhibitors such as echinocandin and pneumocandin types have been paid much attention as fungal cell wall specific inhibitors [5, 6]. This class of antibiotics is known to have little inhibitory activity against *Cryptococcus neoformans* [7, 8].

The worldwide increase in patients with deeply invasive mycoses in response to that of the number of immunocompromised hosts [9] is commonly reported. According to such background and the shortcomings of commercially available antifungal agents, the discovery of fungicide with novel mode of action is strongly anticipated clinically and socially.

Inositol phosphorylceramide (IPC) is an essential structural sphingolipid in fungal cell membrane [10]. Hence, sphingomyelin instead of IPC is synthesized in mammalian cells, specific inhibitory activity against fungi

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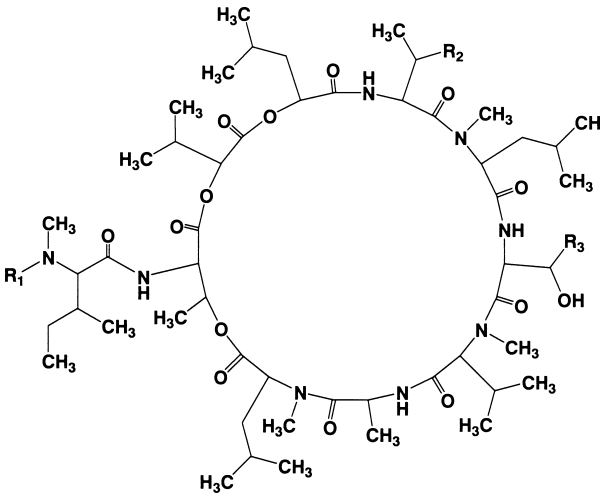

Pleofungin	R_1	R_2	R_3
A	-H	$-CH_2CH_3$	$-CH_3$
B	$-COCH_3$	$-CH_2CH_3$	$-CH_3$
C	-H	$-CH_3$	$-CH_3$
D	-H	$-CH_2CH_3$	-H

Fig. 1 Structures of pleofungins A, B, C and D.

being expected for the IPC synthase inhibitor [11].

Our preceding report describes the discovery and biology of novel IPC synthase inhibitors named pleofungins (formerly called F-15078) A, B, C and D (Fig. 1) [12]. Here, we report structural elucidation of these compounds, novel cyclic depsipeptide antibiotics, and the absolute configuration of pleofungin A.

Materials and Methods

General

^1H and ^{13}C NMR spectra were recorded at 298 K on an AVANCE 500 (Bruker Biospin) spectrometer operating at 500 MHz and 125 MHz, respectively. The samples for NMR characterization were dissolved in chloroform-*d* or methanol-d_4. Optical rotation and IR spectra were measured with a DIP-370 (JASCO) and FT-IR 8300 (Shimadzu), respectively. Mass spectra were measured with a JMS700QQ (JEOL) or LCT (Micromass). High-resolution mass spectra were measured with a JMS700 (JEOL) or LCT. Mass spectra for advanced Marfey's method [13] were recorded with an LCQ ion-trap mass spectrometer equipped with an electrospray ionization (ESI) probe (Thermo Electron Corporation) coupled on-line to an HP1100 system (Agilent Technology).

GC/MS Analysis of Acid Hydrolysate from Pleofungin A

The hydrolysate (6 M HCl, 105°C, 12 hours) from 1 mg of pleofungin A was concentrated to dryness under reduced pressure. The hydrolysate and each amino acid standard (100 μg) were dissolved in acetonitrile (0.1 ml) and *N*-methyl-*N*-(*tert*-butylsilyl)trifluoroacetamide (MTBSTFA, 0.1 ml), respectively. After 15 hours reaction at 66°C in a sealed ampoule, the reactant was added with 0.1 ml of acetonitrile and analyzed by GC/MS equipped with an HP-5MS column (Hewlett Packard, Cross-linked 5% PH ME Siloxane, 0.25 μm×0.25 mm×30 m) under the following conditions: flow rate of helium carrier gas, 1 ml/minute; ion source temperature, 230°C; inlet temperature, 250°C; column temperature, 50～300°C (35°C/minute) except for the detection to determine the stereochemistry of Ile and *N*-Me-Ile (3°C/minute).

The retention time and detected major ions of each silylated derivative are as follows.

1) Authentic samples: *N*-Me-Val: Rt (minutes), 6.23; *m*/*z*, 230, 202, 188, 160, 86. *N*-Me-Leu: Rt, 6.51; *m*/*z*, 259, 244, 202, 174, 100. *N*-Me-Ile: Rt; 6.59, *m*/*z*, 244, 202, 174, 100. α-Hydroxyisovaleric acid: Rt, 7.33; *m*/*z*, 331, 289, 261, 189, 147, 133, 115, 73. α-Hydroxyisocaproic acid: Rt, 7.48; *m*/*z*, 345, 303, 275, 245, 231, 219, 201, 189, 171, 147, 133, 73. L-Ile: Rt, 33.74; *m*/*z*, 302, 274, 200, 147, 133, 73. L-*allo*-Ile: Rt, 33.57; *m*/*z*, 302, 274, 200, 147, 133, 73. *N*-Me-L-Ile: Rt, 20.36; *m*/*z*, 202, 100, 75. *N*-Me-L-*allo*-Ile: Rt, 20.64; *m*/*z*, 202, 100, 75. *N*-Me-D-*allo*-Ile: Rt, 20.66; *m*/*z*, 202, 100, 75.

2) Pleofungin A: *N*-Me-Val: Rt, 6.23; *m*/*z*, 230, 202, 188, 160, 86. *N*-Me-Leu: Rt, 6.51; *m*/*z*, 259, 244, 202, 174, 100. *N*-Me-Ile: Rt, 6.59; *m*/*z*, 244, 202, 174, 100. α-Hydroxyisovaleric acid: Rt, 7.33; *m*/*z*, 331, 289, 261, 189, 147, 133, 115, 73. α-Hydroxyisocaproic acid: Rt, 7.48; *m*/*z*, 345, 303, 275, 245, 231, 219, 201, 189, 171, 147, 133, 73. L-Ile: Rt, 33.69; *m*/*z*, 302, 274, 200, 147, 133, 73. *N*-Me-L-Ile: Rt, 20.35; *m*/*z*, 202, 100, 75.

Derivatization of Constitutive Amino Acids of Pleofungin A with L-FDLA and LC/MS Analyses

An acid hydrolysate (6 M HCl, 105°C, 12 hours) of pleofungin A was derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-leucineamide (L-FDLA), and then analyzed by LC/MS using a negative mode electrospray ionization (ESI) method. Samples were processed as follows. The pH of 50 μl of 50 mM authentic amino acid solution or the hydrolysate in water was adjusted to approximately 9 with 1 M $NaHCO_3$. One hundred microliters of 1% L-FDLA in acetone was added. Then the mixture was incubated at 37°C for 1 hour followed by

quenching with the addition of 20 μl of 1 M HCl. The reaction mixture was diluted up to 1 ml with acetonitrile. One-microliter aliquots of the solution were subjected to LC/MS analysis. HPLC conditions: Column; Senshu pak PEGASIL ODS, 4.6 i.d.×150 mm. Flow rate; 1 ml/minute. Detection; 210 nm. Mobile phase 1; acetonitrile - water containing 0.02% TFA (3 : 7 to 1 : 1 from 0 to 17 minutes, 1 : 1 to 9 : 1 from 17 to 30 minutes). Mobile phase 2; acetonitrile - water containing 0.02% TFA (3 : 7 to 1 : 1 from 0 to 25 minutes, 1 : 1 to 9 : 1 from 25 to 40 minutes).

1) Authentic samples

Mobile phase 1: Rt (minutes), L-Ala: 13.51, D-Ala: 16.50, L-Thr: 9.70, L-*allo*-Thr: 10.41, D-Thr: 13.32, D-*allo*-Thr: 11.73, L-Ile: 18.19, D-Ile: 20.58, *N*-Me-L-Val: 18.10, *N*-Me-D-Val: 21.27, *N*-Me-L-Leu: 20.59, *N*-Me-D-Leu: 22.55, *N*-Me-L-Ile: 20.27, *N*-Me-L-Ile: 23.18.

Mobile phase 2: Rt (minutes), L-Ile: 22.35, L-allo-Ile: 22.17, D-allo-Ile: 30.01.

2) Pleofungin A

Mobile phase 1: Rt (minutes), L-Ala: 13.46, L-Thr: 9.62, L-Ile: 18.18, *N*-Me-L-Val: 18.18, *N*-Me-L-Leu: 20.29, *N*-Me-L-Ile: 20.64.

Mobile phase 2: Rt (minutes), L-Ile: 22.28.

Acetylation of Pleofungin in Methanol with Acetic Anhydride

Pleofungin A (20 mg) was dissolved in methanol (2 ml) and acetic anhydride (0.1 ml) was added. After 4 hours at room temperature, the reaction mixture was evaporated *in vacuo* to dryness and then re-dissolved in methanol. The acetylated product was purified by HPLC (column; Shodex Asahipak C8P-90 2F, 20 i.d.×250 mm, mobile phase; acetonitrile - water (3 : 2) containing 10 mM NH_4HCO_3, flow rate; 14 ml/minute, detection; 210 nm) to give 8 mg of pleofungin A acetate as a colorless powder. The physico-chemical properties were identical with those of pleofungin B.

Mild Alkaline Hydrolysis Products 1 and 2 of Pleofungin A

To a methanol solution (2 ml) of pleofungin A (21 mg), 0.02 N NaOH (2 ml) was added, and the mixture was stirred for 20 hours at room temperature. After neutralization, the hydrolysis products were purified by HPLC (column; Pegasil ODS 20 i.d.×250 mm, mobile phase; acetonitrile - water containing 0.04% TFA [1 : 4 to 9 : 1 from 0 to 25 minutes, 9 : 1 from 25 to 35 minutes], flow rate; 14 ml/minute, detection; UV at 210 nm), to give products **1** (1.4 mg, Rt [minutes]; 10.26) and **2** (10 mg, Rt; 19.52) as colorless powders. The molecular formulae of the products **1** and **2** were determined to be $C_{11}H_{18}N_2O_2$ ($(M)^+$, *m/z* 210.1365, calcd 211.1381 for $C_{11}H_{18}N_2O_2$) by EI-MS and $C_{39}H_{72}N_6O_{10}$ ($(M+Na)^+$, *m/z* 807.5167, calcd 807.5208 for $C_{39}H_{72}N_6O_{10}Na$) by TOF-MS. Their structures were elucidated mainly by NMR chemical-shift assignments. **1**: ^{1}H NMR ($CDCl_3$) δ 0.89 (3H, d, J=6.8 Hz), 1.00 (3H, t,

Table 1 Physico-chemical properties of pleofungins A, B, C and D

	Pleofungin A	Pleofungin B	Pleofungin C	Pleofungin D
Appearance	Colorless powder	Colorless powder	Colorless powder	Colorless powder
$[\alpha]_D^{25}$	−120° (*c* 1.0, MeOH)	−131° (*c* 1.0, MeOH)	−116° (*c* 0.37, MeOH)	−116° (*c* 0.38, MeOH)
Molecular formula	$C_{55}H_{98}N_8O_{14}$	$C_{57}H_{100}N_8O_{15}$	$C_{54}H_{96}N_8O_{14}$	$C_{54}H_{96}N_8O_{14}$
FAB-MS (*m/z*)	1095 $(M+H)^+$	1137 $(M+H)^+$	1081 $(M+H)^+$	1081 $(M+H)^+$
HRFAB-MS (*m/z*)	for $C_{55}H_{99}N_8O_{14}$	for $C_{57}H_{101}N_8O_{15}$	for $C_{54}H_{97}N_8O_{14}$	for $C_{54}H_{97}N_8O_{14}$
Found:	1095.7365	1137.7410	1081.7126	1081.7122
Calcd.:	1095.7281	1137.7387	1081.7124	1081.7124
UV absorption	End absorption	End absorption	End absorption	End absorption
IR ν_{max}^{KBr} cm^{-1}	3434, 3335, 2962, 2937, 2875, 2806, 1750, 1684, 1641, 1509, 1469, 1412, 1371, 1314, 1294, 1271, 1204, 1156, 1128, 1074, 1020	3433, 3333, 2963, 2937, 2875, 1751, 1686, 1642, 1516, 1469, 1409, 1388, 1372, 1311, 1292, 1272, 1201, 1156, 1128, 1074, 1017	3434, 3338, 2961, 2936, 2873, 2805, 1749, 1684, 1641, 1508, 1470, 1412, 1385, 1371, 1312, 1270, 1203, 1155, 1127, 1086, 1073, 1020	3334, 2961, 2937, 2874, 2805, 1749, 1684, 1640, 1509, 1469, 1412, 1385, 1370, 1312, 1271, 1202, 1155, 1128, 1088, 1072, 1017
Solubility				
Soluble	Ethyl acetate, methanol, chloroform, DMSO	Ethyl acetate, methanol, chloroform, DMSO	Ethyl acetate, methanol, chloroform, DMSO	Ethyl acetate, methanol, chloroform, DMSO
Amino acid analysis	Ala, Ile, Thr	Ala, Ile, Thr	Ala, Thr, Val	Ala, Ile, Thr, Ser

Table 2 NMR spectral data of pleofungin A in $CDCl_3$

Assignment	δ_C[a] (m)	δ_H[b] (Hz)	Assignment	δ_C (m)	δ_H (Hz)
N-Me-Ile			*N*-Me-Leu		
CO	174.0 s[c]		CO	170.3 s	
α-CH	70.1 d	2.96	α-CH	63.9 d	3.56
β-CH	38.4 d	1.81	β-CH_2	37.7 t	1.49, 2.31
γ-CH_2	25.5 t	1.21, 1.55	γ-CH	25.4 d	1.65
γ-CH_3	16.0 q	1.01	δ-CH_3	21.4 q	1.02
δ-CH_3	11.9 q	0.94	δ-CH_3	23.54 q	0.98
N-CH_3	36.1 q	2.48	N-CH_3	40.9 q	3.29
Thr-1			Thr-2		
CO	169.8 s		CO	173.4 s	
α-CH	55.1 d	4.77	α-CH	51.8 d	4.91
β-CH	73.4 d	5.21	β-CH	68.1 d	4.14
γ-CH_3	17.4 q	1.41	γ-CH_3	18.7 q	1.06
NH		7.94	NH		6.39
α-Hydroxyisovaleric acid			*N*-Me-Val		
CO	169.0 s		CO	169.0 s	
α-CH	77.1 d	4.96	α-CH	64.7 d	4.06
β-CH	29.8 d	2.28	β-CH	27.7 d	2.37
γ-CH_3	16.6 q	0.96	γ-CH_3	18.3 q	0.93
γ-CH_3	18.6 q	1.03	γ-CH_3	19.1 q	0.87
α-Hydroxyisocaproic acid			N-CH_3	29.5 q	2.89
CO	169.8 s		Ala		
α-CH	74.3 d	5.25	CO	173.8 s	
β-CH_2	39.7 t	1.48, 1.87	α-CH	46.2 d	4.84
γ-CH	24.6 d	1.73	β-CH_3	15.0 q	1.52
δ-CH_3	22.1 q	0.82	NH		7.83
δ-CH_3	23.1 q	0.98	*N*-Me-Leu		
Ile			CO	169.6 s	
CO	172.0 s		α-CH	54.7 d	5.53
α-CH	53.1 d	4.78	β-CH_2	36.5 t	1.70
β-CH	38.3 d	1.66	γ-CH	24.8 d	1.48
γ-CH_2	24.2 t	1.41, 0.88	δ-CH_3	21.0 q	0.79
γ-CH_3	15.1 q	0.78	δ-CH_3	23.51 q	0.92
δ-CH_3	10.9 q	0.80	N-CH_3	30.2 q	2.94
NH		8.28			

TMS was used as the internal reference. [a] 125 MHz, [b] 500 MHz, [c] Multiplicity by DEPT experiment.

J=7.6 Hz), 1.44 (1H, m), 1.64 (1H, m), 1.73 (3H, d, J=7.2 Hz), 1.96 (1H, m), 3.03 (3H, s), 3.97 (1H, d, J=3.2 Hz), 6.15 (1H, q, J=7.2 Hz), 7.40 (1H, br, s). ^{13}C NMR ($CDCl_3$) δ 10.9 (q), 12.1 (q), 14.1 (q), 26.1 (t), 33.8 (q), 38.9 (d), 66.7 (d), 112.8 (d), 127.4 (s), 150.9 (s), 164.5 (s).

NMR signals of the product **2** were heavily overlapped that the proton and carbon signals capable of listing were as follows. **2**: ^{1}H NMR (CD_3OD) δ 0.80 (3H), 0.87 (3H), 0.92 (3H), 0.92 (3H), 0.93 (3H), 0.94 (3H), 0.94 (3H), 0.95 (3H), 0.95 (3H), 0.97 (3H), 1.15 (3H), 1.17 (1H), 1.30 (3H), 1.44 (1H), 1.50 (1H), 1.50 (2H), 1.60 (1H), 1.66 (2H), 1.73 (2H), 1.84 (1H), 2.22 (1H), 3.03 (3H), 3.14 (3H), 3.15 (3H), 4.01 (1H), 4.04 (1H), 4.10 (1H), 4.66 (1H), 4.74 (1H), 4.76 (1H), 4.81 (1H), 5.19 (1H). ^{13}C NMR (CD_3OD) δ 11.3 (q), 16.0 (q), 17.1 (q), 19.0 (q), 19.8 (q), 20.0 (q), 21.8 (q), 21.8 (q), 22.2 (q), 23.5 (q), 24.0 (q), 24.0 (q), 25.5 (t), 25.6 (d), 26.0 (d), 26.0 (d), 27.7 (d), 31.6 (q), 31.7(q), 32.2 (q), 38.3 (t), 38.7 (t), 40.1 (d), 45.1 (t), 46.8 (d), 54.4 (d), 56.3 (d), 56.3 (d), 63.4 (d), 68.5 (d), 71.3 (d), 71.3 (d), 171.1 (s), 172.8 (s), 173.6 (s), 174.8 (s), 175.0 (s),177.5 (s).

Isolation of (*S*)-α-Hydroxyisovaleric Acid and (*S*)-α-Hydroxyisocaproic Acid from Pleofungin A Hydrolysate

Two-hundred milligrams of the hydrolysate (6 M HCl, 105°C, 12 hours) from pleofungin A was subjected to HPLC (column, Senshu pak PEGASIL ODS, 20 i.d.×250 mm; mobile phase, acetonitorile - water (1 : 9) containing 0.05% TFA; flow rate, 14 ml/minute; detection,

Table 3 NMR spectral data of pleofungin B in $CDCl_3$

Assignment	δ_C[a] (m)	δ_H[b] (Hz)	Assignment	δ_C (m)	δ_H (Hz)
N-Me-Ile			*N*-Me-Leu		
CO	171.7 s[c]		CO	170.3 s	
CH_3	22.0 q	2.12	α-CH	63.9 d	3.56
CO	170.6 s		β-CH_2	37.6 t	1.49, 2.31
α-CH	61.2 d	4.73	γ-CH	25.4 d	1.64
β-CH	31.6 d	2.13	δ-CH_3	21.4 q	1.01
γ-CH_2	24.6 t	1.03, 1.37	δ-CH_3	23.6 q	0.98
γ-CH_3	15.6 q	0.95	N-CH_3	40.9 q	3.28
δ-CH_3	10.5 q	0.90	Thr-2		
N-CH_3	31.8 q	2.97	CO	173.3 s	
Thr-1			α-CH	51.8 d	4.91
CO	169.1 s		β-CH	68.1 d	4.15
α-CH	54.7 d	4.82	γ-CH_3	18.7 q	1.05
β-CH	73.1 d	5.15	NH		6.37
γ-CH_3	16.7 q	1.28	*N*-Me-Val		
NH		6.87	CO	168.9 s	
α-Hydroxyisovaleric acid			α-CH	64.6 d	4.03
CO	168.9 s		β-CH	27.7 d	2.37
α-CH	77.0 d	4.97	γ-CH_3	18.3 q	0.93
β-CH	29.8 d	2.26	γ-CH_3	19.0 q	0.87
γ-CH_3	16.6 q	0.95	N-CH_3	29.5 q	2.88
γ-CH_3	18.6 q	1.01	Ala		
α-Hydroxyisocaproic acid			CO	173.8 s	
CO	169.9 s		α-CH	46.1 d	4.83
α-CH	74.3 d	5.28	β-CH_3	14.9 q	1.51
β-CH_2	39.6 t	1.47, 1.86	NH		7.86
γ-CH	24.7 d	1.72	*N*-Me-Leu		
δ-CH_3	22.1 q	0.83	CO	169.9 s	
δ-CH_3	23.0 q	0.92	α-CH	54.7 d	5.50
Ile			β-CH_2	36.1 t	1.66, 1.78
CO	172.0 s		γ-CH	24.8 d	1.46
α-CH	53.1 d	4.78	δ-CH_3	20.8 q	0.80
β-CH	38.4 d	1.65	δ-CH_3	23.6 q	0.98
γ-CH_2	24.1 t	0.87, 1.40	N-CH_3	30.2 q	2.93
γ-CH_3	15.1 q	0.78			
δ-CH_3	10.9 q	0.79			
NH		8.29			

TMS was used as the internal reference. [a] 125 MHz, [b] 500 MHz, [c] Multiplicity by DEPT experiment.

UV at 210 nm) to give two fractions containing α-hydroxyisovaleric acid (Rt [minutes], 10.94) and α-hydroxyisocaproic acid (Rt, 26.90). These α-hydroxy acids were identified by the comparison studies of retention time with those of the authentic materials. Ten preparations of each fraction were combined separately and evaporated under reduced pressure to give pure a-hydroxyisovaleric acid (10.9 mg) and α-hydroxyisocaproic acid (3.5 mg) as a colorless powder.

Results

Physico-chemical Properties

The physico-chemical properties of pleofungins A, B, C and D are summarized in Table 1. These compounds obtained as colorless powders were soluble in most organic solvents including ethyl acetate, methanol, chloroform, acetonitrile and acetone, and slightly soluble in hexane. The UV spectra showed end absorption. The IR spectra of each compound showed the presence of esters (pleofungin A, 1750 cm^{-1}; B, 1751 cm^{-1}; C, 1749 cm^{-1}; D, 1749 cm^{-1}) and amide carbonyls (pleofungin A, 1641 cm^{-1}; B,

Table 4 NMR spectral data of pleofungin C in $CDCl_3$

Assignment	δ_C[a] (m)	δ_H[b] (Hz)	Assignment	δ_C (m)	δ_H (Hz)
N-Me-Ile			*N*-Me-Leu		
CO	173.9 s[c]		CO	170.3 s	
α-CH	70.0 d	2.96	α-CH	63.9 d	3.56
β-CH	38.4 d	1.82	β-CH_2	37.7 t	1.48, 2.31
γ-CH_2	25.4 t	1.22, 1.64	γ-CH	25.4 d	1.61
γ-CH_3	15.9 q	1.00	δ-CH_3	21.4 q	1.00
δ-CH_3	11.9 q	0.93	δ-CH_3	23.6 q	0.96
N-CH_3	36.1 q	2.49	N-CH_3	40.8 q	3.28
Thr-1			Thr-2		
CO	169.9 s		CO	173.3 s	
α-CH	55.1 d	4.77	α-CH	51.8 d	4.90
β-CH	73.4 d	5.24	β-CH	68.1 d	4.15
γ-CH_3	17.4 q	1.40	γ-CH_3	18.7 q	1.05
NH		7.95	NH		6.38
α-Hydroxyisovaleric acid			*N*-Me-Val		
CO	169.1 s		CO	169.0 s	
α-CH	77.0 d	4.97	α-CH	64.7 d	4.04
β-CH	29.8 d	2.27	β-CH	27.6 d	2.36
γ-CH_3	16.7 q	0.94	γ-CH_3	18.3 q	0.92
γ-CH_3	18.7 q	1.01	γ-CH_3	19.1 q	0.86
α-Hydroxyisocaproic acid			N-CH_3	29.5 q	2.88
CO	169.9 s		Ala		
α-CH	74.3 d	5.27	CO	173.7 s	
β-CH_2	39.7 t	1.45, 1.88	α-CH	46.2 d	4.82
γ-CH	24.6 d	1.73	β-CH_3	15.0 q	1.51
δ-CH_3	22.1 q	0.81	NH		7.85
δ-CH_3	23.1 q	0.91	*N*-Me-Leu		
Val			CO	169.7 s	
CO	171.9 s		α-CH	54.7 d	5.55
α-CH	54.4 d	4.72	β-CH_2	36.4 t	1.69
β-CH	32.1 d	1.89	γ-CH	24.8 d	1.48
γ-CH_3	18.0 q	0.78	δ-CH_3	20.9 q	0.78
γ-CH_3	19.3 q	0.82	δ-CH_3	23.5 q	0.92
NH		8.27	N-CH_3	30.2 q	2.93

TMS was used as the internal reference. [a] 125 MHz, [b] 500 MHz, [c] Multiplicity by DEPT experiment.

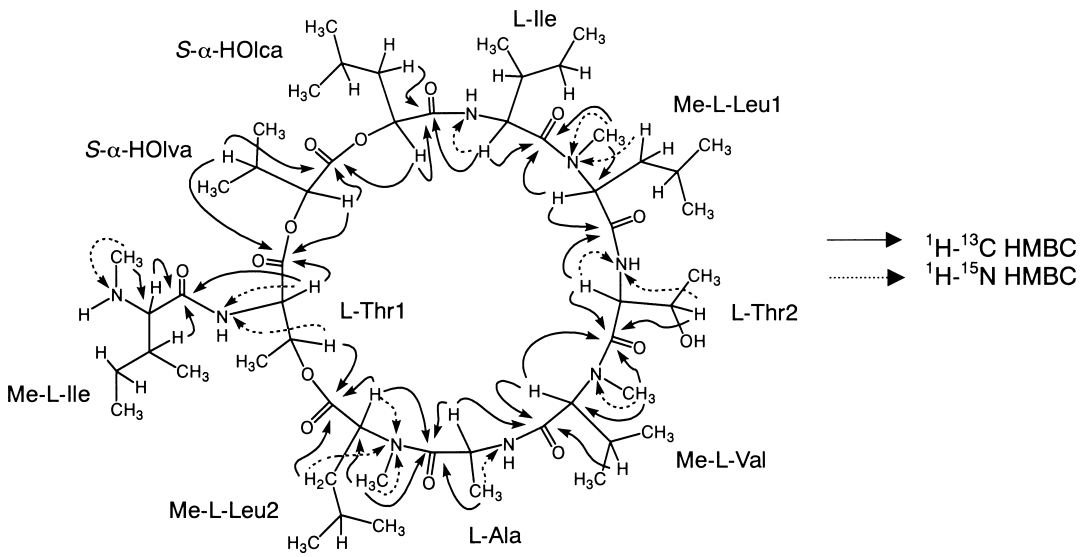

Fig. 2 ^1H–^{13}C and ^1H–^{15}N long-range couplings of pleofungin A.

Table 5 NMR spectral data of pleofungin D in $CDCl_3$

Assignment	δ_C[a] (m)	δ_H[b] (Hz)	Assignment	δ_C (m)	δ_H (Hz)
N-Me-Ile			*N*-Me-Leu		
CO	174.0 s[c]		CO	169.7 s	
α-CH	70.1 d	2.95	α-CH	64.1 d	3.53
β-CH	38.4 d	1.80	β-CH_2	37.3 t	1.43, 2.29
γ-CH_2	25.4 t	1.21, 1.54	γ-CH	25.3 d	1.61
γ-CH_3	16.0 q	1.00	δ-CH_3	21.4 q	1.00
δ-CH_3	11.9 q	0.92	δ-CH_3	23.6 q	0.95
N-CH_3	36.1 q	2.49	N-CH_3	40.9 q	3.28
Thr			Ser		
CO	169.8 s		CO	172.4 s	
α-CH	55.1 d	4.77	α-CH	49.3 d	5.02
β-CH	73.3 d	5.22	β-CH_2	63.9 t	3.57, 3.94
γ-CH_3	17.4 q	1.40	NH		6.46
NH		7.95	*N*-Me-Val		
α-Hydroxyisovaleric acid			CO	169.0 s	
CO	169.0 s		α-CH	65.1 d	3.88
α-CH	77.0 d	4.97	β-CH	27.9 d	2.33
β-CH	29.8 d	2.27	γ-CH_3	18.4 q	0.92
γ-CH_3	16.6 q	0.95	γ-CH_3	19.1 q	0.84
γ-CH_3	18.7 q	1.02	N-CH_3	29.5 q	2.92
α-Hydroxyisocaproic acid			Ala		
CO	169.7 s		CO	173.6 s	
α-CH	74.3 d	5.28	α-CH	45.9 d	4.87
β-CH_2	39.6 t	1.47, 1.85	β-CH_3	15.2 q	1.48
γ-CH	24.6 d	1.71	NH		8.10
δ-CH_3	22.2 q	0.82	*N*-Me-Leu		
δ-CH_3	23.1 q	0.92	CO	169.6 s	
Ile			α-CH	54.7 d	5.53
CO	171.9 s		β-CH_2	36.4 t	1.68
α-CH	53.3 d	4.84	γ-CH	24.8 d	1.47
β-CH	38.8 d	1.63	δ-CH_3	21.0 q	0.78
γ-CH_2	24.1 t	0.90, 1.36	δ-CH_3	23.5 q	0.92
γ-CH_3	15.4 q	0.80	N-CH_3	30.2 q	2.94
δ-CH_3	11.1 q	0.78			
NH		8.20			

TMS was used as the internal reference. [a] 125 MHz, [b] 500 MHz, [c] Multiplicity by DEPT experiment.

1642 cm^{-1}; C, 1641 cm^{-1}; D, 1640 cm^{-1}).

Structure Elucidation

1) Pleofungin A

The ^{13}C and ^{1}H NMR data (CD_3OD, $CDCl_3$) of pleofungin A are shown in Table 2, respectively. The molecular weight of pleofungin A was determined to be 1,094 by FAB-MS. The molecular formula was established as $C_{55}H_{98}N_8O_{14}$ from the result of HRFAB-MS as well as with the assistance of the NMR studies. Amino acid analysis of its acid hydrolysate (6 M HCl, 105°C, 15 hours) showed the presence of Ala, Ile and Thr as the constitutive amino acids. The ^{13}C NMR data and DEPT experiment revealed the presence of 21 methyl carbons, 5 methylene carbons, 19 methine carbons and 10 carbonyl carbons. The ^{1}H NMR spectrum of pleofungin A indicated the presence of 4 amide NH protons ($CDCl_3$, δ 6.39~8.28) and 4 *N*-methyl protons ($CDCl_3$, δ 2.48~3.29). In the ^{1}H NMR spectrum, the signals around 3~6 ppm due to the α-methine protons of several amino acid residues were observed.

Considering eleven degrees of unsaturation, the number of carbonyl groups and the absence of aromatic moiety, pleofungin A is a highly methylated cyclic peptide or cyclic depsipeptide antibiotic. To identify the constitutive units, GC-MS analysis was applied to the hydrolysate.

The comparison study with authentic compounds confirmed the presence of *N*-methylvaline (MeVal), *N*-methylleucine (MeLeu), *N*-methylisoleucine (MeIle), α-hydroxyisovaleric acid (α-HOIva) and α-hydroxyisocaproic acid (α-HOIca) in addition to the previously identified

amino acids.

By the detailed analysis of the DQF-COSY, HSQC, ^{1}H-^{13}C and ^{1}H-^{15}N HMBC, HOHAHA and HSQC-HOHAHA spectra in $CDCl_3$ proved intra-residual structures assigned as Ala×1, Ile×1, Thr×2, MeIle×1, MeLeu×2, MeVal×1, α-HOIca×1 and α-HOIva×1. Although the NOE information between the α-methine proton and the amide proton of the neighboring residue was very poor, two partial structures, MeIle-Thr1 and α-HOIca-Ile-MeLeu1-Thr2-MeVal-Ala-MeLeu2, were revealed by the ^{1}H-^{13}C HMBC correlation between the NH proton and the α-carbon of the neighboring residue, as well as the ^{1}H-^{15}N HMBC correlations between the *N*-methyl proton and amide nitrogen, α-methine proton and amide nitrogen.

The connectivity of these two partial structures and remaining α-HOIva was not clarified because of the absence of H–C long-range coupling information observed in $CDCl_3$ as a solvent.

As shown in Fig. 2, the planar structure of pleofungin A was finally elucidated by the observation of the newly-appeared three H–C long-range couplings between the α-methine proton of α-HOIca and the α-HOIva carbonyl, α-methine proton of α-HOIva and Thr1 carbonyl, and the β-methine proton of Thr1 and the MeLeu2 carbonyl in the HMBC spectrum in CD_3OD.

2) Pleofungin B

The ^{13}C and ^{1}H NMR data ($CDCl_3$) of pleofungin B are shown in Table 3. The molecular weight of pleofungin B was determined to be 1,136 by FAB-MS. The molecular formula was established as $C_{57}H_{100}N_8O_{15}$ from the result of HRFAB-MS as well as the assistance of the NMR studies. Amino acid analysis of its acid hydrolysate (6 M HCl, 105°C, 12 hours) showed the existence of Ala, Ile and Thr, which was the same as that of pleofungin A. The ^{1}H NMR data were quite similar to those for pleofungin A except for the presence of the signal assigned to the *N*-acetyl moiety. These data agreed well with the difference in the molecular formulae, suggesting that pleofungin B is the monoacetate of pleofungin A.

Finally, the HMBC correlation between the *N*-methyl proton of MeIle and the acetyl carbonyl carbons, and the acetyl methyl proton and the carbonyl carbon revealed the structure of pleofungin B as shown in Fig. 1.

3) Pleofungin C

The ^{13}C and ^{1}H NMR data ($CDCl_3$) of pleofungin C are shown in Table 4. The molecular weight of pleofungin C was determined to be 1,080 and the molecular formula was established as $C_{54}H_{96}N_8O_{14}$ from the result of HRTOF-MS. Amino acid analysis of its acid hydrolysate (6 M HCl, 105°C, 12 hours) showed the existence of Ala, Thr and Val. The existence of Val was a major difference from pleofungin A. The ^{1}H NMR data and ^{13}C NMR data were quite similar to those for pleofungin A except for the presence of the signal assigned to Val moiety. These data agreed well with the difference of the molecular formulae and suggested that Val replaced the Ile residue of pleofungin A. With the support of the HMBC correlations (data not shown), the structure of pleofungin C was determined as shown in Fig. 1.

4) Pleofungin D

The ^{13}C and ^{1}H NMR data ($CDCl_3$) of pleofungin C are shown in Table 5. The molecular weight of pleofungin C was determined to be 1,080 and the molecular formula was established as $C_{54}H_{96}N_8O_{14}$ from the result of HRTOF-MS. The presence of Ala, Ile, Thr and Ser was clarified by amino acid analysis of its acid hydrolysate (6 M HCl, 105°C, 12 hours). The existence of Ser was a major difference compared to pleofungin A. The ^{1}H NMR data and ^{13}C NMR data were quite similar to those for pleofungin A except for the presence of the signal assigned to the Ser moiety. These data agreed well with the difference of the molecular formulae, suggesting that Ser replaced one of the threonines of pleofungin A. Taken together with the results of the HMBC correlations (data not shown), the structure of pleofungin D was determined as shown in Fig. 1.

Stereochemistry of Pleofungin A

1) Constitutive Amino Acids and *N*-Methylated Amino Acids

The pleofungin A molecule contains two moles of threonine residue but the yield of the threonine by the normal acid hydrolysis was very low and not quantitative because of the dehydration of Thr1 shown in Fig. 2. Therefore, the acid hydrolysis was performed by using its *N*-acetyl derivative. The amino acid analysis of the hydrolysate clarified that the molar ratio of each amino acid was as follows: Ala : Ile : Thr = 1 : 1 : 2. Advanced Marfey's method [12] was applied to determine the absolute configurations of these constitutive amino acids and *N*-methylated amino acids from the acid hydrolysate of the acetylated pleofungin A. The stereochemistry of the Ile and MeIle was also confirmed by GC-MS analysis of their silylation products with MTBSTFA. As shown in Materials and Methods, the retention times of the derivatized amino acids and *N*-methylated amino acids in the hydrolysate were identical to those of the authentic L-Ala, L-Ile, L-Thr, Me-L-Val, Me-L-Leu and Me-L-Ile. Therefore, from these

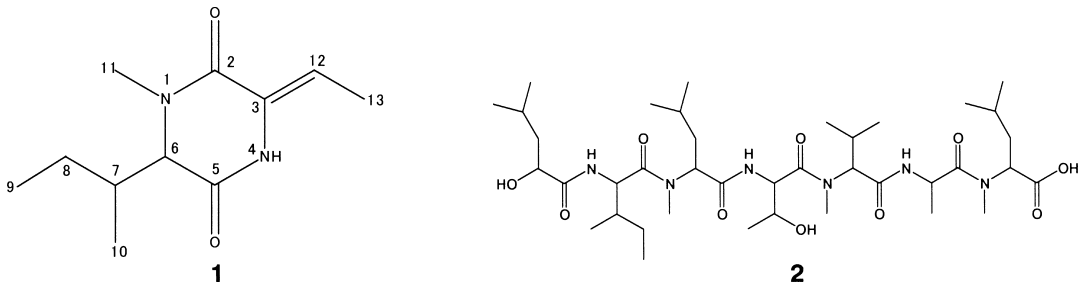

Fig. 3 Structures of **1** and **2**.

results, the configurations of all of the amino acids and *N*-methylated amino acids in pleofungin A were determined to be the L form (Fig. 2).

To re-confirm the absolute configuration of Thr2, mild alkaline hydrolysis was performed to obtain **1** and **2** mainly (Fig. 3). The molecular formulae of **1** and **2** were 210 and 784, respectively, from the results of high resolution TOF-MS. ^{1}H NMR, ^{13}C NMR, GS-DQF-COSY, GS-HSQC and GS-HMBC spectral analysis elucidated the structure of **1**. Because of the fact that the molecular weight of **2** was 784, the structure was deduced as shown in Fig. 3, and the structure **2** was further confirmed by ^{1}H NMR, ^{13}C NMR, GS-DQF-COSY, GS-HSQC and GS-HMBC spectral analysis. The degradation product **2**, which contained Thr2, was acid-hydrolyzed and then advanced Marfey's method was applied to determine the stereochemistry of the Thr2 as the L form.

2) α-Hydroxyisovaleric Acid and α-Hydroxyisocaproic Acid

To clarify the configurations of the asymmetric carbons in α-HOIva and HOIca, comparative studies of their specific rotations were performed. The absolute configuration of the a position of α-HOIva was revealed to be the *S* form, because the specific rotation was $[\alpha]_D^{20}$ +15° (*c* 0.68, $CHCl_3$), which was almost the same as that of the authentic one ($[\alpha]_D^{20}$ +19° (*c* 0.97, $CHCl_3$)) [14]. The asymmetric site of HOIca was also assigned to be the *S* form from the comparative study to the reported one, *i.e.* the specific rotation was $[\alpha]_D^{20}$ −24° (*c* 0.95, 1 M NaOH), which was almost the same as that of the reported value $[\alpha]_D$ −25.9° (*c* 1.0, 1 M NaOH) [15].

Therefore, the absolute structure of pleofungin A was elucidated as shown in Fig. 2.

Discussion

A large variety of cyclic depsipeptide antibiotics have been reported. Among this class of antibiotics, pleofungin A is unique in that it possesses characteristic Leu-Thr1-α-HOIva-α-HOIca moiety and all residues in the moiety are connected by ester bonds. To the best of our knowledge, it is a rare case that a cyclic depsipeptide antibiotic possesses three ester bonds linked sequentially in the molecule. Montanastatin [16] has been reported to have four ester bonds in its structure and valinomycin [17] six, however these ester bonds are not in sequence.

In the NMR studies, we succeeded in assigning all proton signals and carbon signals. In assignment of the aliphatic methyl region, gradient-selected band-selective HSQC spectrum [18] was very helpful, because numbers of signals were observed in a complicated overlapping manner within a narrow region on the ^{13}C NMR spectrum. Similarly, gradient-selected band-selective HMBC spectrum [18] gave us fruitful information for assigning carbonyl signals even though the sensitivity of this method is lower than that of the normal gradient-selected HMBC spectrum.

Mild alkaline hydrolysis revealed an unexpected degradation product **1** (Fig. 3). However, this is understandable because dipeptides are easily dehydrated to make very stable diketopiperazine derivatives. During the reaction, the β-hydroxyl group of Thr1, which took part in the ester linkage to the neighboring Leu, was dehydrated to form a double bond at position 3 of **1**. It often occurs that alkaline dehydration facilitates carboxylic acid as a leaving group because of the strength of the leaving-group activity. These are the probable reasons why **1** was yielded quantitatively.

The molecular target of aureobasidin A [19, 20], galbonolide B [21] and rustmicin [22] was specified to IPC synthase [10, 23] and khafrefungin was found as its inhibitor [24]. However, their activities were restricted only to yeast-type fungi or phytopathogenic fungi. Furthermore, none of them were reported to possess good fungicidal activity against *Aspergillus* species. Authough pleofungins and aureobasidin A are categorized in the same cyclic depsipeptide type antibiotic, their antifungal spectra are

distinct and pleofungin A shows a wide range of antifungal activity including clinically important filamentous fungi [12]. Further structure activity relationship study is needed to clarify the difference in the antifungal spectrum. The potent and broad spectrum antifungal activity including *Aspergillus* species as reported in the preceding paper [12] suggest pleofungin A could be a candidate as an antifungal agents that for clinical use.

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