A NEW ANTHELMINTIC CYCLODEPSIPEPTIDE, PF1022A

TORU SASAKI, MASAYUKI TAKAGI, TAKASI YAGUCHI, SHINJI MIYADOH, TADAAKI OKADA and MASAO KOYAMA

Pharmaceutical Research Laboratories, Meiji Seika Kaisha Ltd., Morooka-cho, Kohoku-ku, Yokohama 222, Japan

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The novel anthelmintic cyclodepsipeptide PF1022A was isolated from cultured mycelia of Mycelia Sterilia PF1022 (FERM BP-2671). It showed strong anthelmintic activities against *Ascaridia galli* in chickens. The structure of PF1022A was determined to be cyclo(D-lactyl-L-*N*-methylleucyl-D-3-phenyllactyl-L-*N*-methylleucyl-D-lactyl-L-*N*-methylleucyl-D-3-phenyllactyl-L-*N*-methylleucyl) by spectroscopic analyses and chemical studies.

In the course of screening for new anthelmintic antibiotics using *Ascaridia galli*[1] as a test organism, the new cyclodepsipeptide PF1022A was isolated from a mycelial cake of Mycelia Sterilia[2] PF1022. This paper describes the producing strain, isolation, physico-chemical properties, structure and biological activities of the cyclodepsipeptide.

Producing Organism

The strain PF1022 was isolated from a plant sample (*Camellia japonica*) collected in Ibaraki Prefecture, Japan. The strain grew abundantly with white fluffy hyphae covering all over the Petri dish (>85 mm) at 25°C in 7 days on the following four media; potato glucose agar, potato carrot agar, malt extract agar and oatmeal agar. The reverse side of the colony was initially white to light yellow. Soluble pigment formation was insignificant. The organism did not grow at 37°C. Any particular morphology such as conidia formation was not observed on various media after incubation at 25°C for 2 months.

Therefore, we tentatively assigned strain PF1022 to Mycelia Sterilia[2] (order Agonomycetales), until morphological characteristics become evident. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, which an accession number of FERM BP-2671.

Fermentation

Strain PF1022 on agar slant was inoculated into a 100-ml Erlenmeyer flask that contained 20 ml of a seed medium consisting of 1.0% starch, 1.0% glucose, 0.5% cotton seed meal, 0.5% wheat germ, 0.5% soybean meal, 0.5% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCO_3$, 0.2% NaCl and tap water (pH 7.0). The inoculated flask was shaken on a rotary shaker (200 rpm) at 26°C for 7 days. Four milliliters of the first seed culture was transferred into 80 ml of the same medium in a 500-ml Erlenmeyer flask. After shaking at 26°C for 2 days, the second seed culture was added to a 50-liter jar fermenter containing 35 liters of the following production medium; 3.0% maltose syrup, 1.0% soybean oil, 0.8% wheat germ, 1.0% soybean meal, 1.0% dry yeast, 0.3% $CaCO_3$, 0.2% $MgSO_4 \cdot 7H_2O$ and 0.2% NaCl in tap water (pH 7.0 before sterilization). Fermentation was carried out at 26°C for 5 days with an air-flow rate of 20 liters per minute and an agitation of 250 rpm initially and then 400 rpm after 65 hours.

Isolation

The fermentation broth was filtered with the aid of diatomaceous earth. The mycelial cake was extracted with 100 liters of methanol. The methanol extract was concentrated to 20 liters and the residue was extracted with 18 liters of ethyl acetate. The solvent layer was concentrated to give a brown oil (68.3 g) which was dissolved in chloroform and applied to a silica gel column (1 liter, Wakogel C-200). The column was successively developed with chloroform and a mixture of chloroform and methanol (100 : 1). The active fractions were combined and evaporated to give a pale yellow powder (1.2 g). It was crystallized from a mixture of acetone and hexane to give pure PF1022A (954 mg) as colorless needles.

Physico-chemical Properties

Physico-chemical properties of PF1022A (**1**) are summarized in Table 1. PF1022A is a neutral substance which melts at 104~106°C and soluble in methanol, acetone, ethyl acetate, chloroform and dimethyl sulfoxide and insoluble in water. It showed positive color reactions to Na_2MoO_4, I_2 and Dragendorff reagents, but is negative to ninhydrin reagent. The molecular formula was determined to be $C_{52}H_{76}N_4O_{12}$ by elemental analysis and EI-MS (M^+) (*m/z* 948). Dominant absorptions at 1740 and 1660 cm^{-1} in the IR spectrum (Fig. 1) indicated the presence of ester and amide functions in **1**.

The ^1H and ^{13}C NMR spectra of **1** in CD_3OD are shown in Figs. 2 and 3, respectively.

Table 1. Physico-chemical properties of PF1022A.

Appearance	Colorless prisms
MP	104~106°C
$[\alpha]_D^{22}$	−102° (*c* 0.1, MeOH)
Molecular formula	$C_{52}H_{76}N_4O_{12}$
Anal calcd:	C 65.80, H 8.07, N 5.90
found:	C 65.46, H 8.25, N 6.10
EI-MS (*m/z*)	948 (M^+)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	257 (3.9), 263 (2.9)
IR ν_{max}^{KBr} cm^{-1}	1740 (ester), 1660 (amide)

Structural Elucidation

The IR and NMR spectral data (Table 2) suggested a depsipeptide structure for **1**. Acid hydrolysis of **1** with 6 N HCl at 105°C for 18 hours gave one amino acid and two hydroxy acids. An ether extract of the hydrolysate solely yielded

Fig. 1. IR spectrum of PF1022A (**1**) (KBr).

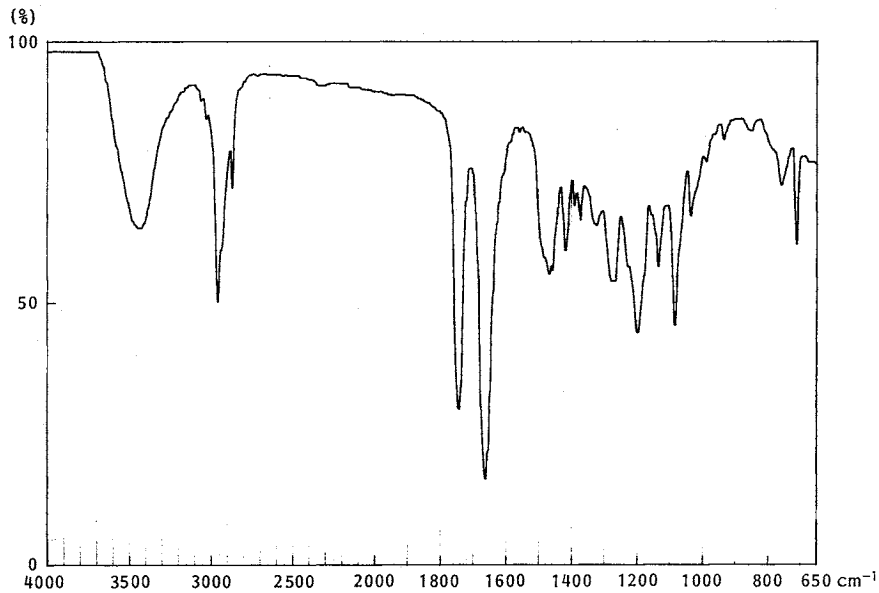


Fig. 2. ^{1}H NMR spectrum of PF1022A (**1**) (400 MHz in CD_3OD).

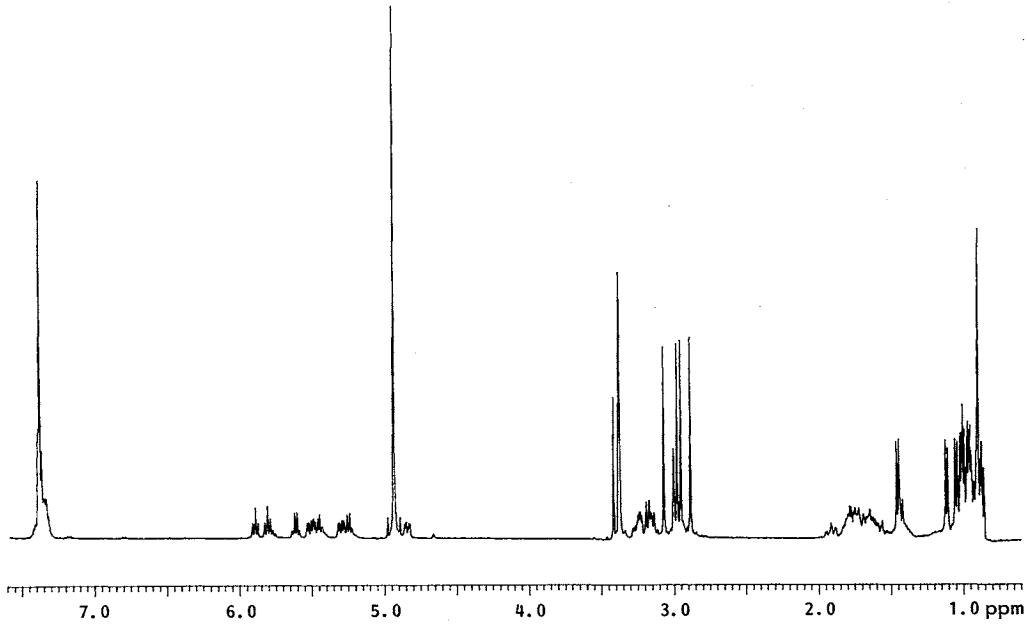
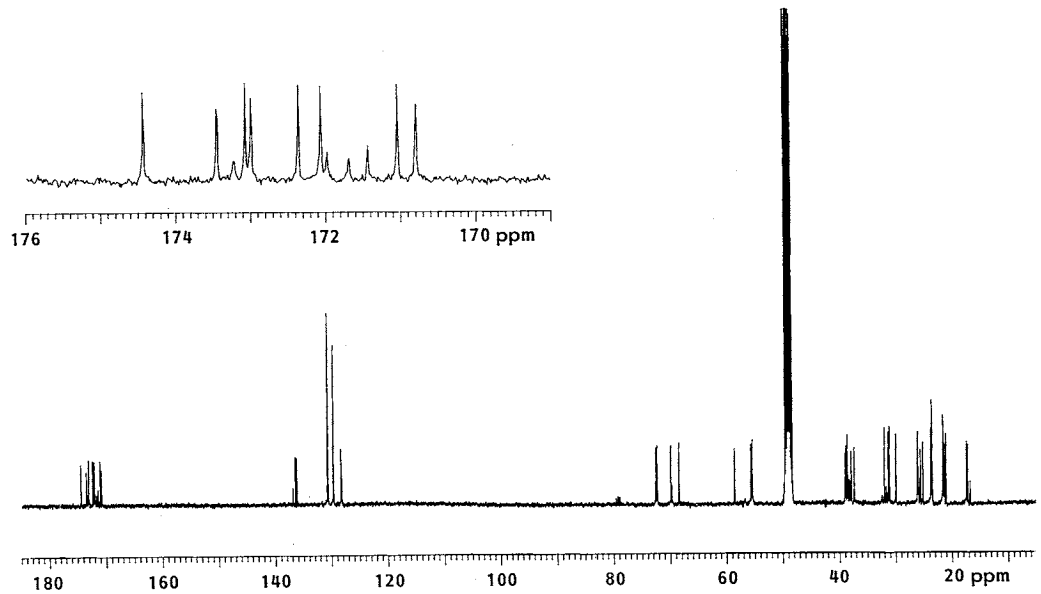


Fig. 3. ^{13}C NMR spectrum of PF1022A (**1**) (100 MHz in CD_3OD).



D-3-phenyllactic acid (Phl)[3]. The residual aqueous solution was applied to a cation exchange resin (Diaion PK-208) and the effluent was freeze dried to give D-lactic acid (Lac) which was identified by HPLC on a chiral column (Chiralpak WH). The resin was eluted with 1 N NH_4OH and removal of the aqueous ammonia gave L-*N*-methylleucine (MeLeu)[4]. Considering the molecular formula of **1**, it was deduced that

Table 2. ^{13}C and ^{1}H NMR data for the major conformer of PF1022A.

Position		δ_C	δ_H	Position		δ_C	δ_H
Lac[1]	1	174.4		MeLeu[2]	1	172.4	
	2	69.9	5.16		2	58.6	4.78
	3	17.2	0.88		3	38.9	1.50~1.87
Lac[5]	1	173.4			4	26.2	1.40
	2	68.4	5.54		5	23.6*	0.75~1.02
	3	17.5	1.38		6	21.7**	0.75~1.02
Phl[3]	1	172.4			N-Me	29.9	2.80
	2	72.5	5.80	MeLeu[4]	1	170.8	
	3	38.6	3.15~3.22		2	55.5	5.42
	4	136.2			3	37.9	1.50~1.87
	5	130.7	7.20~7.30		4	25.6	1.40
	6	129.7	7.20~7.30		5	23.6*	0.75~1.02
	7	128.2	7.20~7.30		6	21.6**	0.75~1.02
	8	129.7	7.20~7.30		N-Me	31.3	2.88
	9	130.7	7.20~7.30	MeLeu[6]	1	171.0	
Phl[7]	1	172.1			2	55.7	5.43
	2	72.3	5.75		3	38.6	1.50~1.87
	3	39.0	3.15~3.22		4	26.1	1.40
	4	136.5			5	23.5*	0.75~1.02
	5	130.7	7.20~7.30		6	21.4**	0.75~1.02
	6	129.7	7.20~7.30		N-Me	31.1	2.90
	7	128.3	7.20~7.30	MeLeu[8]	1	172.1	
	8	129.7	7.20~7.30		2	55.4	5.22
	9	130.7	7.20~7.30		3	37.4	1.50~1.87
					4	25.2	1.40
					5	23.5*	0.75~1.02
					6	21.0**	0.75~1.02
					N-Me	32.0	3.00

In CD_3OD, ^{13}C NMR at 100 MHz, ^{1}H NMR at 400 MHz. * and **: Assignments may be interchanged.
Lac: lactic acid, Phl: 3-phenyllactic acid, MeLeu: *N*-methylleucine.
Structure: -O-Lac[1]-MeLeu[2]-Phl[3]-MeLeu[4]-Lac[5]-MeLeu[6]-Phl[7]-MeLeu[8]-CO.

Fig. 4. The structure of PF1022A (**1**).

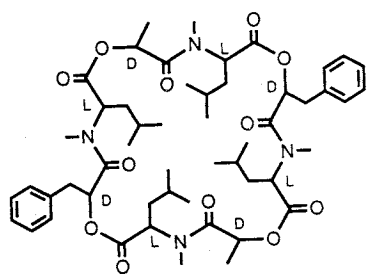
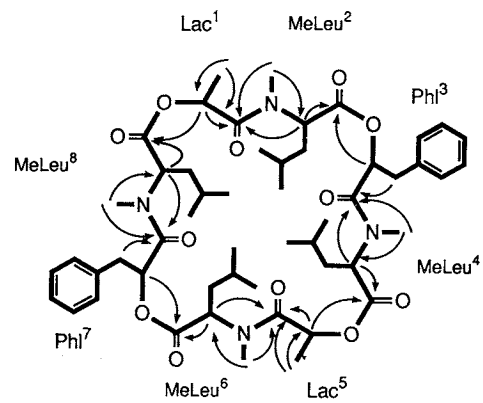


Fig. 5. The summary of HMBC experiment of PF1022A (**1**).



1 was a cyclic depsipeptide consisting of two mol each of Lac and Phl and four mol of MeLeu. This conclusion was confirmed by the ^{1}H and ^{13}C NMR spectral analyses of **1**. The NMRspectra revealed that **1** existed as a mixture of two conformers in CD_3OD (*ca.* 4 : 1). The major conformer should be spatially asymmetric and eight carbonyl carbon signals were observed as separated signals. On the other hand, another minor conformer should have a symmetry, since only four carbonyl carbon signals were observed.

The NMR analyses were carried out with the signals due to the major conformer in CD_3OD and their assignments were summarized as shown in Table 2. The sequence of the components was determined by analyses of the HMBC[5] spectrum. Long range couplings were observed between *N*-methyl protons of MeLeu and the carbonyl carbon of Lac or Phl. These observations indicated the presence of two pairs of the Lac-MeLeu and Phl-MeLeu units in **1**. These two units are attached alternately as revealed by the observed long range couplings between α-methine protons and carbonyl carbons. All the α-methine proton signals have correlation peaks with two carbonyl carbons except those of Phl. The summary of the HMBC spectral analysis is shown in Fig. 5. From the above mentioned results, the structure of **1** was established to be cyclo(D-lactyl-L-*N*-methylleucyl-D-3-phenyllactyl-L-*N*-methylleucyl-D-lactyl-L-*N*-methylleucyl-D-3-phenyllactyl-L-*N*-methylleucyl).

Table 3. Effect of PF1022A against *Ascaridia galli* in chickens.

Dose (mg/kg)	No. of worms excreted	No. of worms remaining	Efficacy rate (%)	Weight gain rate (%)
0.5	28	88	24.1	11.0
	16	51	23.9	4.9
	11	102	9.7	−7.4
1.0	95	30	76.0	9.7
	16	32	33.3	13.0
	76	68	52.8	17.2
2.0	146	0	100	14.5
	38	0	100	13.7
	77	7	91.7	15.6

$$\text{Efficacy rate (\%)} = \frac{\text{No. of worms excreted}}{\text{No. of total worms}} \times 100$$

One week old chickens were given 200 eggs of *Ascaridia galli* orally. After 35 days, the infected chickens were given PF1022A orally. The number of excreted worms were counted from their feces every day for the next two weeks. Two weeks after the administration, the chickens were sacrificed and the number of remaining worms were counted. The experiment was conducted in triplicate at each concentration of PF1022A administered.

Biological Activities

PF1022A exhibited potent anthelmintic activities against *Ascaridia galli* by oral administration in chickens at the dose of 2 mg/kg (Table 3). The activity was dose dependent and no toxic effect was observed to the host animals. Tested so far, PF1022A was inactive against Gram-positive and Gram-negative bacteria, yeasts and other fungi at the dose of 100 μg/ml. No acute toxicity was observed when PF1022A was administered at the dose of 1 g/kg (ip) and 2 g/kg (po) to mice.

Discussion

A new anthelmintic cyclodepsipeptide, PF1022A was isolated from a fungal strain and its structure was elucidated by spectroscopic analyses and chemical studies. The proposed structure was confirmed by the X-ray crystallographic analysis and the result will be reported in a separate paper. Among the known microbial products, PF1022A resembles bassianolide[6] which is an insecticidal cyclodepsipeptide consisting of four mol each of L-*N*-methylleucine and D-2-hydroxyisovaleric acid. In a solution, bassianolide exists as a mixture of two conformers which are interconverting very slowly on the NMR time scale[7,8]. This phenomenon was also observed with PF1022A. PF1022A showed potent anthelmintic activity against *Ascaridia galli* in chickens without toxic effect to the host animals. PF1022A is now under investigation for further evaluation of its anthelmintic effect.

Experimental

General

UV and IR spectra were recorded on a Shimadzu UV-260 spectrophotometer and a Hitachi 260-10 IR spectrophotometer, respectively. MS spectra were measured on a Hitachi M-80B mass spectrometer. Optical rotation was measured with a Jasco DIP-370 digital polarimeter. MP was determined with a

Yanaco MP-30 micro melting point apparatus and was uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Jeol JNM-GX400 spectrometer.

Acid Hydrolysis

PF1022A (60 mg) was hydrolyzed with 6 N HCl (10 ml) at 105°C in a sealed tube for 18 hours. The hydrolysate was diluted with water (50 ml) and extracted with Et_2O. The ether solution was dried over Na_2SO_4 and was concentrated to afford D-3-phenyllactic acid (13.2 mg); $[\alpha]_D^{22}$ +20.5° (*c* 0.31, EtOH), literature $[\alpha]_D^{12}$ +18.5° (*c* 3.53, EtOH)[3]; ^1H NMR (400 MHz, $CDCl_3$) δ 7.15 (5H, m), 4.50 (1H, dd, J=4.4 and 7.2 Hz), 3.21 (1H, dd, J=14.1 and 4.36 Hz), 2.99 (1H, dd, J=14.1 and 7.2 Hz). The water solution of the hydrolysate was introduced to Diaion PK208 resin (H^+ form, 10 ml) and was eluted with 1 N NH_4OH. The eluate was concentrated and crystallized from Me_2CO-H_2O to give MeLeu as white needles (20.4 mg) $[\alpha]_D^{22}$ +32° (*c* 1, 6 N HCl), literature $[\alpha]_D$ +31.3° (*c* 0.9, 5 N HCl)[4]; ^1H NMR (D_2O) δ 3.58 (1H, t, J=6.8 Hz), 2.70 (3H, s), 1.70 (3H, m), 1.00 (6H, d, J=6.5 Hz). The effluent of the resin was freeze dried to give D-lactic acid (Lac) as colorless solids; ^1H NMR (D_2O) δ 4.35 (1H, q, J=6.2 Hz), 1.43 (3H, d, J=6.2 Hz). The absolute configuration of lactic acid was analyzed by HPLC on a chiral column (Chiralpak WH; Daicel Chemical Industries, Ltd., solvent, 0.25 mM $CuSO_4$, flow rate, 1.0 ml/minute, detection, UV 230 nm). Rt's were as follows: L-Lac; 13.0 minutes, D-Lac; 15.1 minutes, Lac of PF1022A (D-Lac); 15.3 minutes.

References

1) LEVINE, N. D.: Genus ASCARIDIA dujardin, 1845. *In* Nematoda Parasites of Domestic Animals and of Man. pp. 323~325, Burgress Publishing Co., 1968
2) BESSEY, E. A.: Order *Mycelia sterilia*. *In* Morphology and Taxonomy of Fungi. pp. 601~602, The Blakiston Co., 1950
3) MCKENZIE, A. & H. WREN: Experiments on the Walden inversion. Part V. The interconversion of the optically active α-hydroxy-β-phenylpropionic acids. J. Chem. Soc. 97: 1355~1359, 1910
4) BUCKINGHAM, J. (*Ed.*): Dictionary of Organic Compounds. 4, p. 3909, Chapman & Hall Ltd., 1982
5) BAX, A. & M. F. SUMMERS: ^1H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J. Am. Chem. Soc. 108: 2093~2094, 1986
6) SUZUKI, A.; M. KANAOKA, A. ISOGAI, S. MURAKOSHI, M. ICHINOE & S. TAMURA: Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. Tetrahedron Lett. 1977: 2167~2170, 1977
7) KANAOKA, M.; A. ISOGAI, S. MURAKOSHI, M. ICHINOE, A. SUZUKI & S. TAMURA: Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. Agric. Biol. Chem. 42: 629~635, 1978
8) KANAOKA, M.; A. ISOGAI & A. SUZUKI: Syntheses of bassianolide and its two homologs, enniatin C and decabassianolide. Agric. Biol. Chem. 43: 1079~1083, 1979