

Spiroidesin, a Novel Lipopeptide from the Cyanobacterium *Anabaena spiroides* That Inhibits Cell Growth of the Cyanobacterium *Microcystis aeruginosa*

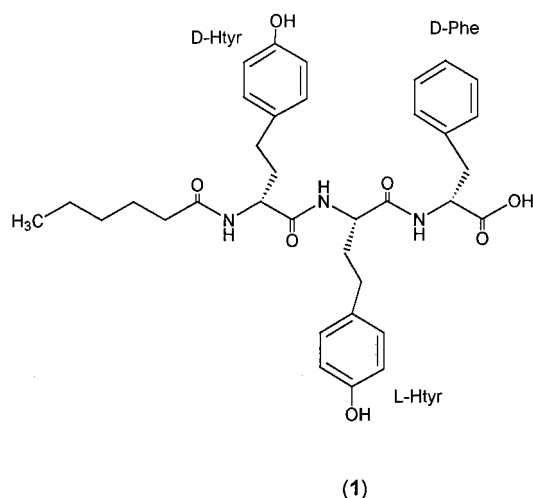
Kunimitsu Kaya,^{*,†} Aparat Mahakhant,[‡] Ladda Keovara,[§] Tomoharu Sano,[†] Takuya Kubo,[⊥] and Hiroo Takagi[†]

Laboratory of Intellectual Fundamentals for Environmental Studies, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan, TISTR Culture Collection, Thailand Institute of Scientific and Technological Research, 196 Phahonyothin Road, Chatuchak, Bangkok 10900, Thailand, Regional Office 9, Chiang Mai Provincial Waterwork Authority, 133 Wangsingkum, Muang, Chiang Mai 50300, Thailand, and Department of Polymer Science & Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

Received December 28, 2001

Spiroidesin (**1**), a novel D-amino acid-containing linear lipopeptide, was isolated from waterblooms of the cyanobacterium *Anabaena spiroides*. The structure was identified by 2D NMR and chemical degradation analyses. Spiroidesin inhibited cell growth of the toxic cyanobacterium *Microcystis aeruginosa* (IC₅₀, 1.6 × 10⁻⁶ M).

Recently, cyanobacteria have been reported to produce bioactive lipopeptides.¹⁻⁶ In many cases, amino acids found in these linear peptides possess only an L- or both D- and L-configurations, and the N-terminal amino acids are combined with fatty acids. These peptides have various kinds of biological activities such as protease inhibition,¹⁻³ neurotoxic,⁴ and ichthyotoxic⁵ properties. During investigations into toxins from waterblooms of *Anabaena spiroides*, we have found a novel D- and L-homotyrosine-containing linear lipopeptide. Herein we report the structure and biological activity of the novel linear lipopeptide, spiroidesin (**1**).



Spiroidesin (**1**) is a colorless amorphous solid: λ_{max} (H₂O) 276 nm (ε 2200); [α]_D²⁵ -62° (c 0.56, MeOH). In the positive HRFABMS using glycerol as the matrix, the [M + H]⁺ ion was observed at m/z 618.3134. From the results, the molecular formula of **1** was established to be C₃₅H₄₃N₃O₇ (calcd for [M + H] 618.3179, Δ -4.5 mmu). The spectral data (Table 1) of ¹H and ¹³C NMR of **1** suggested that spiroidesin is a fatty acid-containing peptide. In the nin-

Table 1. ¹H^a and ¹³C^b NMR Data for Spiroidesin (**1**) in Methanol-*d*₄

position	¹ H J (Hz)	¹³ C	HMBC ^d
Hex ^c			
1		176.4	2,3, D-Htyr-2
2	2.22 (t, 7.4)	36.8	
3	1.57 (q, 7.4)	26.6	
4	1.27 (m)	32.6	
5	1.25 (m)	23.4	
6	0.82 (t, 7.0)	14.2	
D-Htyr			
1		174.7	2,3, L-Htyr-2
2	4.24 (dd, 4.9, 9.5)	54.2	
3	1.86 (m)	35.3	
	1.71 (m)		
4	2.36 (m)	32.0	6, 10
	2.28 (m)		
5		132.5	4
6, 10	6.83 (d, 8.6)	130.3	
7, 9	6.62 (d, 8.6)	116.3	
8		157.0	
L-Htyr			
1		173.1	2,3, D-Phe-2
2	4.21 (dd, 5.2, 9.2)	55.2	
3	1.97 (m)	34.9	
	1.88 (m)		
4	2.60 (m)	32.4	6, 10
	2.53 (m)		
5		132.7	4
6, 10	6.98 (d, 8.6)	130.4	
7, 9	6.65 (d, 8.6)	116.5	
8		157.3	
D-Phe			
1		178.2	2,3
2	4.48 (dd, 4.3, 8.6)	57.3	
3	3.15 (dd, 4.3, 13.7)	39.3	
	2.88 (dd, 8.5, 13.7)		
4		139.6	2, 3
5,9	7.12 (d, 7.3)	132.5	3
6, 8	7.05 (t, 7.3)	129.1	
7	6.99 (t, 7.3)	127.2	

^a Recorded at 500 MHz (δ values). ^b Recorded at 125 MHz (δ values). ^c Hex, hexanoic acid. ^d Proton showing HMBC correlation to indicated carbon.

hydrin spot test, spiroidesin was negative. Amino acid analysis of the acid hydrolysate (6 M HCl, 110 °C for 20 h) indicated the presence of homotyrosine (Htyr) and phenylalanine (Phe). The molar ratio of Htyr and Phe was 2:1. Htyr was shown to have both of the L- and D-configurations by HPLC analysis of the Marfey's derivatives of the acid

* To whom correspondence should be addressed. Tel: +81-298-50-2428. Fax: +81-298-50-2900. E-mail: kayakuni@nies.go.jp.

[†] National Institute for Environmental Studies.

[‡] Thailand Institute of Scientific and Technological Research.

[§] Chiang Mai Provincial Waterwork Authority.

[⊥] Kyoto Institute of Technology.

hydrolysate. In the case of Phe, it was shown to have the D-configuration by the HPLC analysis.

The sequence of residues in **1** was deduced by HMBC correlations (Table 1) from the α -H to the carbonyl carbon at adjacent residues and the configuration analysis of the partial methanolysis products. From the HMBC correlation, the sequence was deduced as hexanoic acid (Hex)-(D- or L-Htyr)-(L- or D-Htyr)-D-Phe, but the sequence of L- and D-Htyr could not be deduced from these data. To determine the sequence of L- and D-Htyr, spirioidin was partial methanolized. After the methanolysis in 2 M HCl–MeOH/*n*-hexane (1:4, v/v) at 74 °C for 12 h, three major peptides were isolated by HPTLC using chloroform–methanol (9:1, v/v) as a solvent. Peptide-1, -2, and -3 on the HPTLC plate showed R_f 0.67, 0.46, and 0.23, respectively. Peptide-1 was ninhydrin negative. In the positive HRFABMS using glycerol as the matrix, the $[M + H]^+$ ion was observed at m/z 308.1838. From this, the molecular formula of Peptide-1 was established to be $C_{17}H_{25}N_1O_4$ (calcd for $[M + H]^+$ 308.1862, Δ –2.4 mmu). This molecular formula indicated that Peptide-1 consisted of Htyr and hexanoic acid. After the hydrolysis of Peptide-1, the configuration of the amino acid was determined. Htyr in the peptide was shown to have the D-configuration. Peptide-2 was ninhydrin positive and consisted of D- and L-Htyr. Peptide-3 was ninhydrin positive. After the hydrolysis of Peptide-3, Htyr and Phe were detected. The configuration of the amino acid was determined. Htyr was shown to have the L-configuration, and Phe the D-configuration. From these data, the structure of spirioidesin was established as **1** [(Hex)-(D-Htyr)-(L-Htyr)-(D-Phe)].

Spiroidesin inhibited the growth of the toxic cyanobacterium *Microcystis aeruginosa* (NIES-88). When *M. aeruginosa* cells (2×10^5 cells/mL) in logarithmic growth were cultured in MA medium containing various concentrations of spirioidesin for 5 days, cell density was decreased to 50% of the control by sprodesin at a concentration of 1.6×10^{-6} M. Spiroidesin also inhibited chymotrypsin activity with an IC_{50} of 1.0×10^{-5} M.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a JEOL JMN-500 spectrometer (500 MHz). 1H and ^{13}C NMR chemical shifts are referenced to TMS. Homonuclear 1H connectivities were determined from the COSY and HOHAHA experiments, and heteronuclear 1H – ^{13}C connectivities were determined by HSQC and HMBC experiments. LRMS and HRMS were performed with a JEOL JMS-700 spectrometer.

Collection. Blooms of *Anabaena spiroides* were collected from a freshwater pond in Chaing Mai, Thailand. The blooms were immediately freeze-dried, then stored at –20 °C until use.

Extraction and Isolation. Methanol extract from 10 g of freeze-dried cells was evaporated under reduced pressure. The remaining residue was suspended in 5% (v/v) aqueous acetic acid solution. The suspension was centrifuged at 2000 rpm for 20 min and the supernatant retained. The lipopeptide **1** was fractionated by solid-phase extraction using ODS cartridges (Sep-pak ODS).² The fractionated lipopeptide was isolated by reversed-phase HPLC (Mightysil RP-18, 20 mm i.d. \times 25 cm) with methanol (60%) containing 0.05 M phosphate buffer (pH 3.0) at 10 mL·min^{–1}. The isolated lipopeptide was further purified by HPTLC (Merck, Si gel 60 containing fluorescent indicator) using chloroform–methanol–water (60:40:10, v/v) as the solvent. The yield of the lipopeptide **1**, spirioidesin, was 35 mg.

Acid Hydrolysis. Approximately 1–0.1 mg of spirioidesin or partial methanolysis products was hydrolyzed in 6 M HCl at 110 °C for 20 h.⁷

Partial Methanolysis. Approximately 1 mg of sprodesin was dissolved with 0.2 mL of 2 M HCl–methanol and 0.8 mL of *n*-hexane. The tube was sealed with a screw cap and was heated at 74 °C for 12 h. After heating, the hydrolysate was dried under a nitrogen stream, and the remaining residue was dissolved with a small amount of chloroform. The chloroform solution was applied to a HPTLC plate (Si gel 60 F254 (Merck), thickness 0.25 mm) and developed with chloroform–methanol (9:1, v/v) as the solvent. Three major peptides were observed at R_f 0.67, 0.46, and 0.23, respectively. The peptides were collected from the plate and hydrolyzed in 6 M HCl.

Amino Acid Analysis. Authentic D- and L-Htyr were gifts from Dr. Mark Bradley of University of Southampton, UK. Other amino acids including D- and L-Phe were purchased from Sigma. The amino acids in the acid hydrolysate of sprodesin and partial methanolysis products were derivatized with Marfey reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) and analyzed by reversed-phase HPLC on a C-18 column (NOVA-PAK C18, 4.6 \times 150 mm).⁸ A linear gradient of 10 to 40% acetonitrile in 0.05 M triethylamine phosphate buffered at pH 3.0 (flow rate 2.0 mL/min; UV detection at 340 nm) was used to separate the amino acid derivatives.⁹ The absolute stereochemistry of each compound was determined by comparing the retention time with those for authentic L and D amino acid derivatives. The retention times (min) and configurations of the amino acids in the acid hydrolysate of spirioidesin were found to be 42.8 (L-Phe), 48.6 (D-Phe), 60.4 (L-Htyr), and 66.9 (D-Htyr).

Biological Activity. Spiroidesin was assayed for cell growth inhibition using cells of the toxic cyanobacterium *Microcystis aeruginosa* (NIES-88). *M. aeruginosa* cells (10^5 cells/mL) in logarithmic growth were cultured in MA medium¹⁰ containing various concentrations of sprodesin (0, 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M) for 5 days. Cell number was counted using microscopy and expressed as an average of three replicate experiments. Spiroidesin was also assayed for chymotrypsin inhibition using α -chymotrypsin type II (Sigma C-4129) and *N*-benzoyl-L-tyrosine ethyl ester as a substrate.¹¹ When spirioidesin at the concentration of 10^{-5} M was added in the chymotrypsin assay system using substrate at the concentration of 2×10^{-5} M, the activity was decreased to 50% of the original activity.

Spiroidesin:¹ colorless solid (35 mg, 0.35% dry wt); $[\alpha]_D^{25}$ –62° (*c* 0.56, MeOH); UV λ_{max} (H₂O) 276 nm (ϵ 2200); 1H and ^{13}C NMR, see Table 1; HRFABMS (pos. glycerol) m/z 618.3134 $[M + 1]^+$ (calcd for $C_{35}H_{43}N_3O_7$, 618.3179).

Acknowledgment. The authors thank Ms. C. Suzuki for measurements of NMR spectra, Ms. K. Nagano for measurements of HRFABMS spectra, and Ms. H. Tsukumo for assistance of amino acid analyses.

References and Notes

- (1) Okino, T.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *Tetrahedron Lett.* **1993**, *34*, 501–504.
- (2) Sano, T.; Kaya, K. *Phytochemistry* **1997**, *44*, 1503–2209.
- (3) Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2000**, *63*, 1106–1112.
- (4) Jiménez, J. I.; Scheuer, P. J. *J. Nat. Prod.* **2001**, *64*, 200–203.
- (5) Nogle, L. M.; Okino, T.; Gerwick, W. H. *J. Nat. Prod.* **2001**, *64*, 983–985.
- (6) Gerwick, W. H.; Tan, L. T.; Sitachitta, N. In *Alkaloids*; Cordell, G., Ed.; Academic Press: San Diego, 2001; pp 75–184.
- (7) Sano, T.; Kaya, K. *Tetrahedron Lett.* **1995**, *36*, 8603–8606.
- (8) Ishida, K.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Nat. Prod.* **1997**, *60*, 184–187.
- (9) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
- (10) Watanabe, M. M.; Hiroki, M. *NIES–Collection List of Strains fifth Edition Microalgal and Protozoa*; Microbial Culture Collection, Natl. Inst. Environ. Stud.: Japan, 1997; p 32.
- (11) Perlmann, G. E.; Lorrain, L. In *Methods in Enzymology*; Academic Press: New York, 1970; Vol. 19, pp 64–108.

NP010660X