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

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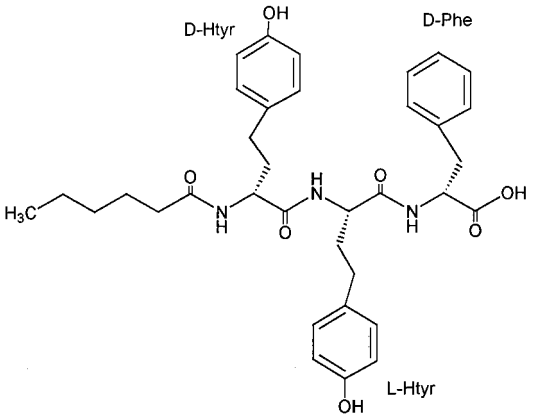
Received December 28, 2001

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* (IC_{50}, 1.6 $\times$ 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 ichthyotoxic[5] properities. 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**).



(**1**)

Spiroidesin (**1**) is a colorless amorphous solid: λ_{max} (H_2O) 276 nm (ϵ 2200); $[\alpha]^{25}_D$ −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 $C_{35}H_{43}N_3O_7$ (calcd for [M + H] 618.3179, Δ −4.5 mmu). The spectral data (Table 1) of ^1H and ^{13}C NMR of **1** suggested that spiroidesin is a fatty acid-containing peptide. In the ninhydrin spot test, sprodesin 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

Table 1. ^1H[a] and ^{13}C[b] NMR Data for Spiroidesin (**1**) in Methanol-d_4

position	^1H *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.

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10.1021/np010660x CCC: $22.00
Published on Web 05/09/2002

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, spriroidin was partial methanolyzed. 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 spiroidesin 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 spiroidesin 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 JMNA-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. × 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**, spirodesin, was 35 mg.

Acid Hydrolysis. Approximately 1−0.1 mg of spiriodesin 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 × 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 spiroidesin 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 medium[10] 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 spiroidesin 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); $[\alpha]^{25}_D$ −62° (*c* 0.56, MeOH); UV λ_{max} (H_2O) 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.

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NP010660X