Schizopeptin 791, a New Anabeanopeptin-like Cyclic Peptide from the Cyanobacterium Schizothrix sp.

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Schizopeptin 791, a New Anabeanopeptin-like Cyclic Peptide from the Cyanobacterium *Schizothrix* sp.

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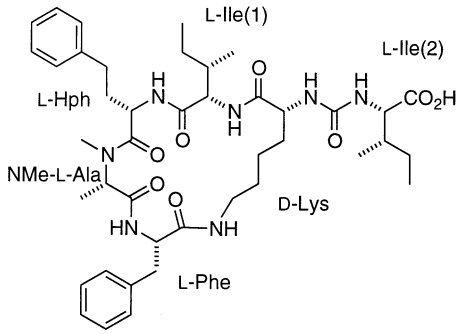
Schizopeptin 791 (**1**), a new trypsin inhibitor, related in structure to the anabeanopeptins, was isolated from the hydrophilic extract of the cultured terrestrial cyanobacterium *Schizothrix* sp. Homo- and heteronuclear 2D NMR techniques as well as HRFABMS determined the gross structure of **1**. The relative and absolute stereochemistry of **1** was deduced from a combination of spectral data and Marfey's method for HPLC. Prior to this finding, anabeanopeptins have been isolated only from water-bloom-forming strains of cyanobacteria.

Anabaenopeptins are a group of cyclic hexapeptides, which are characterized by a 19-membered peptidic ring derived from the cyclization of the C-terminal amino acid carboxyl to the primary *ω*-amine of the N-terminus lysine. The α-amine of the lysine is linked through an ureido bridge to the side chain amino acid. All 18 of the anabaenopeptins[1−7] described to date, from toxic-water-bloom-forming strains of cyanobacteria, contain D-lysine and have an L stereochemistry for the other amino acids. Two related metabolites that were isolated from sponges of the genus *Theonella*, konbamide and keramamide, contain L-lysine.[1] Anabaenopeptins possess inhibitory activity to several proteolytic enzymes[3,4] and protein phosphatases.[7] Proteolytic enzymes or proteases are distributed in all organisms, from bacteria to plants and mammals, where they are present intracellularly, on the surface of cell membranes, or secreted extracellularly. These enzymes are involved in a great variety of physiological processes in the human body including cell cycle progression, food digestion, angiogenesis, blood coagulation, blood pressure regulation, antigen presentation, inflammation, and apoptosis. Overexpression of proteolytic enzymes or poor regulation of the activity of these enzymes, due to depressed levels of their physyological protein inhibitors, may result in a variety of pathological situations. Low molecular weight compounds, capable of controlling the activity of these enzymes, thereby redressing the protease/antiprotease imbalance, may lead to the emergence of new therapeutic agents. Yet another aspect of using protease inhibitors for human disease relates to parasitic, bacterial, or viral infection. Inhibition of a key protease for the life cycle of the invasive microorganism could be beneficial for the therapy against the pathogen. As part of our ongoing research to isolate biologically active natural products from cultured cyanobacteria strains, we isolated, for the first time, an anabaenopeptin derivative from a terrestrial cyanobacterium, *Schizothrix* sp. (IL-208-2-2, Oscillatoriacea, Cyanophyceae). We hereby report the isolation and structure elucidation of a serine protease inhibitor, schizopeptin 791 (**1**).

The cyanobacterium *Schizothrix* sp. (IL-208-2-2) was collected in the Givat Brener nursery (Israel) in August 1996 and cultured in 20 L bottles containing BG-11 medium under constant illumination.[8] The freeze-dried cells were extracted with 70% MeOH in H_2O and 1:1 MeOH/dichloromethane. As part of our screening program, the extracts were screened for their capacity to inhibit several proteolylic enzymes. The MeOH/water extract was found to inhibit trypsin and chymotrypsin. The bioassay-guided separation of the active principal of the extract started with flash chromatography on an ODS column. The active fraction that eluted from the column with 50% MeOH in H_2O was further purified on a Sephadex LH-20 and reversed-phase HPLC columns to yield the active principal, schizopeptin 791 (**1**, 4.8 mg). The 75% MeOH in H_2O fraction from the ODS column contains the known schizothrins that were isolated from another *Schizothrix* sp. (IL-89-2).[9]



Examination of the ^1H and ^{13}C NMR spectra of schizopeptin 791 (**1**) reveal that it is related in structure to known anabaenopeptins. Several proton signals in the ^1H NMR spectrum are indicative of the anabaenopeptin structure: (i) The presence of six amino acids, identified through the six α-proton signals between 4.90 and 3.90 ppm; (ii) the presence of at least one aromatic amino acid; (iii) an upfield-shifted *N*-methyl at ∼1.77 ppm and a methyl at ∼1.06 ppm, characteristic of the *N*-methylalanine situated between two aromatic amino acids, and (iv) a diasterotopic methylene amide of the lysine side chain, indicative of the cyclization between lysine *ω*-amine and the C-terminal amino acid carboxyl. In the ^{13}C NMR spectrum, the presence of a ureido carbon at ∼157 ppm is indicative of an anabaenopeptin skeleton.

Schizopeptin 791 (**1**) was isolated as an amorphous white solid. The molecular formula of **1**, $C_{42}H_{61}N_7O_8$, was deduced from high-resolution FABMS measurements of its sodiated molecular cluster ion (*m*/*z* 814.4493). Analysis of the 1D (^1H, ^{13}C, and DEPT) and 2D (COSY, TOCSY, ROESY,

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Table 1. NMR Data of Schizopeptin 791 (**1**) in DMSO-d_6[a]

	position	δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H−C correlations[c]	NOE correlations[d]
Phe	1	171.0 s		Lys-ω-NH,Phe-2,3	
	2	55.1 d	4.38 ddd 12.3, 8.9, 3.3	Phe-3	Phe-5,5,NH
	3	37.7 t	2.78 m	Phe-2,5,5′,6,6′,NH	Phe-5,5′
			3.35 m		Phe-5,5′
	4	138.4 s		Phe-6,6′,7,3,3′	
	5,5′	129.0 d×2	7.05 d 7.3	Phe-3	Phe-2,3,3′,NH
	6,6′	128.3 d×2	7.18 t 7.3	Phe-7	Phe-NH
	7	126.2 d	7.14 t 7.3	Phe-5,5′	Phe-NH
	NH		8.64 d 8.9		Phe-2,5,5′,6,6′,7, NMeAla-2,NMe
NMeAla	1	169.9 s		Phe-NH, NMeAla-2,3	
	2	54.4 d	4.80 q 6.7	NMeAla-3,NMe	Phe-NH, NMeAla-3,NMe
	3	13.9 q	1.06 d 6.7	NMeAla-2	NMeAla-2,NMe, Hph-4,4′
	NMe	27.2 q	1.77s	NMeAla-2	NMeAla-2,3, Phe-NH
Hph	1	171.0 s		NMeAla-2,NMe, Hph-2	
	2	49.0 d	4.74 ddd 8.0, 5.2, 4.8	Hph-3,NH	Hph-NH,3,4′,7,7′
	3	33.0 t	1.76 m	Hph-2,4,4′,NH	Hph-NH
			1.92 m		Hph-4,4′
	4	31.5 t	2.55 dd 6.2, 11.3	Hph-6,6′	Hph-NH,3′,6,6′, NMeAla-3
			2.77 m		Hph-NH,3′,6,6′, NMeAla-3
	5	141.2 s		Hph-4,4′,7,7′	
	6,6′	128.4 d×2	7.22 d 7.3	Hph-7,7′	Hph-4,4′
	7,7′	128.5 d×2	7.28 t 7.3		Hph-2
	8	126.2 d	7.19 t 7.3	Hph-6,6′	
	NH		8.95 d 4.8		Ile(1)-2,6, Hph-2,3,4,4′
Ile(1)	1	172.9 s		Ile(1)-2, Hph-NH	
	2	56.8 d	3.98 dd 8.6, 6.9	Ile(1)-6	Hph-NH, Ile(1)-NH,3,4,5,6
	3	35.9 d	1.74 m	Ile(1)-2,4,5,6,NH	Ile(1)-NH,2,4′
	4	24.9 t	1.16 m	Ile(1)-2,5,6	Ile(1)-NH,2
			1.37 m		Ile(1)-3
	5	10.5 q	0.81 t 7.4	Ile(1)-4	Ile(1)-2
	6	15.1 q	1.00 d 6.8	Ile(1)-2	Ile(1)-2, Hph-NH
	NH		6.91 d 6.9		Ile(1)-2,3,4
Lys	1	172.4 s		Lys-2,3, Ile(1)-NH	
	2	54.9 d	3.92 dt 7.1, 5.7	Lys-3	Lys-α-NH, Ile(2)-NH
	3	31.7 t	1.62 q		Lys-α-NH
	4	20.5 t	1.12 m	Lys-2	Ile(2)-NH
			1.35 m		Ile(2)
	5	28.3 t	1.44 m	Lys-6′	Lys-ω-NH
	6	38.5 t	2.80 m		Lys-ω-NH
			3.57 m		Lys-ω-NH
	α-NH		6.49 d 7.1		Lys-2,3, Ile(2)-2,NH
	ω-NH		7.13 t 7.3		Lys-5,6,6′
Urea	1	157.4 s		Ile(2)-2,NH	
Ile(2)	1	173.9 s		Ile(2)-2,NH	
	2	56.8 d	4.07 dd 8.9, 5.1	Ile(2)-6	Ile-NH, Lys-α-NH
	3	37.3 d	1.70 m	Ile(2)-2,4,4′,5,6	Ile(2)-NH
	4	24.8 t	1.12 m	Ile(2)-2,5,6	Ile(2)-NH
			1.32 m		Ile(2)-NH
	5	11.6 q	0.84 t 7.3	Ile(2)-4,4′	Ile(2)-NH
	6	15.8 q	0.83 d 6.9	Ile(2)-2,4′	Ile(2)-NH
	NH	6.32 d	6.32 d 8.9		Ile(2)-2,3,4,4′,5,6, Lys-2,α-NH

[a] Carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment obtained from HMQC experiment. [c] Determined from HMBC experiment, $^nJ_{CH}$ = 8 Hz, recycle time 1 s. The HMBC correlations are reported as correlations of the protons printed in the column with the carbon in the row. [d] Assigned from ROESY experiment.

HMQC, and HMBC) NMR data (see Table 1) revealed the six amino acids of **1**: isoleucine (×2), NMe-alanine, phenylalanine, homophenylalanine, and lysine. The amino acid sequence of **1** was assembled from HMBC correlations (see Table 1) of the α-NH of an amino acid with the carboxyl carbon of a vicinal amino acid: Phe-NMeAla, Hph-Ile(1) and Ile(1)-Lys, NMeAla-NMe with Hph-carboxyl and Lys ω-NH with the carboxyl of Phe. The connection between the ureido carbon and Ile(2) was assigned from an HMBC correlation between H-2 and the NH of Ile(2) and the ureido carbon. No correlation was observed between Lys and the ureido carbon in the HMBC map. The connection between Lys and Ile(2) was assigned from NOE correlations between Lys-α-NH and Ile(2)-H-2, Lys-2 and Ile(2)-NH, and Lys-α-NH and Ile(2)-NH. Acid hydrolysis of **1** and derivatization with Marfey's reagents, followed by HPLC analysis, demonstrated the L-stereochemistry of the isoleucine, NMe-alanine, phenylalanine, and homophenylalanine and the D-stereochemistry of lysine.[10] The inhibitory activity of **1** was determined for two enzymes, the serine proteases trypsin and chymotrypsin. Schizopeptin 791 (**1**) inhibited trypsin with an IC_{50} of 45.0 μg/mL but not chymotrypsin at 45.0 μg/mL.

Experimental Section

Instrumentation. UV spectra were recorded on a Kontron 931 plus spectrophotometer. NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.136 MHz for ^{1}H and 125.76 MHz for ^{13}C; DEPT, COSY-45, HMQC, and HMBC spectra were recorded using standard Bruker pulse sequences. Chemical shifts are reported relative to the DMSO signal at δ_H 2.49 ppm and δ_C 39.7 ppm. High-resolution MS were recorded on a Fisons VG AutoSpecQ M 250 instrument. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem Inc. diode-array detector.

Culture Conditions. An edaphic form of *Schizothrix* sp., designated Tel Aviv University (TAU) strain number IL-208-2-2, was isolated from a soil sample collected in the Givat Brener nursery, Israel, in August 1996. A clonal strain was purified on BG-11 agar medium.[11] The isolate is currently maintained in the culture collection at Tel Aviv University. The cyanobacterium was cultured in 20 L glass bottles containing a BG-11 medium.[11] Cultures were continuously illuminated at an intensity of 100 μein/M^2/s from fluorescent tubes and aerated with 0.5% CO_2 in air (1 L/min) at an incubation temperature of 25 °C for 30−35 days. Yields of lyophilized cells typically ranged from 0.5 to 1.0 g/L of culture.

Isolation Procedure. The freeze-dried cells (84.5 g from 200 L of culture) were extracted with 7:3 MeOH/H_2O and 1:1 MeOH/dichloromethane. The extracts were screened for their capacity to inhibit several proteolylic enzymes, trypsin, chymptrypsin, aminoproteinase N, and carboxypeptidase A. The MeOH/water extract was found to inhibit trypsin and chymotrypsin. The crude extract (7.8 g) was separated on an ODS (YMC-GEL, 120A, 4.4 × 6.4 cm) flash column with increasing amounts of MeOH in water. The bioassay-guided separation of the active fraction, 4, followed. Fraction 4 (1:1 MeOH/H_2O) was separated on a Sephadex LH-20 gel-filtration column with 1:1 $CHCl_3$/MeOH. The combined active fractions from the Sephadex LH-20 column were subjected to a reversed-phase HPLC (C-18, 5 μm, 250 mm × 20.0 mm at 21 °C, DAD at 238 nm, flow rate 5.0 mL/min) in 7:3 methanol/water to obtain compound **1**. Compound **1** (2.6 mg) was eluted from the column with a retention time of 26.0 min. Semipure samples of **1** were eluted from the column as a tail of the peak at t_R 26.0 min and when the column was washed with methanol. The fraction from the methanol wash was subjected to a reversed-phase HPLC (C-18, 5 μm, 250 mm × 20 mm at 21 °C, DAD at 238 nm, 8:2 methanol/water, flow rate 5.0 mL/min). Compound **1** (1.1 mg) was eluted from the column with a retention time of 32.7 min. The semipure fraction obtained from the tailing of **1** was subjected to a reversed-phase HPLC (C-18, 5 μm, 250 mm × 20.0 mm at 21 °C, DAD at 238 nm, 7:3 acetonitrile/water, flow rate 5.0 mL/min). Compound **1** (1.1 mg) was eluted from the column with a retention time of 16.7 min. Compound **1** was eluted three times, total of 4.8 mg, 0.0057% yield based on the dry weight of the cyanobacteria.

Schizopeptin 791 (1): $[\alpha]^{25}_D$ −43.7° (*c* 2.9, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (4.24) nm; for NMR data, see Table 1; FABMS *m*/*z* 831 $[MK]^+$ (45), 814 $[MNa]^+$ (100), 792 $[MH]^+$ (25), 683 (55); HRFABMS *m*/*z* 814.4493 (calcd for $C_{42}H_{61}N_7NaO_8$, 814.4479).

Determination of Absolute Configuration of the Amino Acids. Portions of **1** (0.5 mg) were dissolved in 6 N HCl (1 mL). The reaction mixture was placed in a sealed glass bomb at 110 °C for 20 h. After removal of the HCl, by repeated evaporation in vacuo, the hydrolysate was resuspended in water (40 μL) and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA). The N-[(dinitrophenyl)-5-L-alanine amide]amino acid (AA) derivatives, from the hydrolysates, were compared with similarly derivatized standard AA by HPLC analysis: Knauer GmbH Eurospher 100, 10 μm, 4.6 × 300 mm, flow rate 1 mL/min, at 21 °C, UV detection at 340 nm, linear gradient elution from 9:1 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/acetonitrile to 1:1 TEAP/acetonitrile within 60 min. The determination of the absolute configuration of each amino acid was confirmed by spiking the derivatized hydrolysates with the derivatized authentic amino acids. HPLC analysis of derivatized hydrolysates of **1** established L-Ile (×2), L-Phe, L-Hph, L-NMe-Ala, and D-Lys.

Protease Inhibition Assays. Trypsin and chymotrypsin were purchased from Sigma Chemical Co. Trypsin was dissolved in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$ to prepare a 1 mg/mL solution. Chymotrypsin was dissolved in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$/1 mM HCl to prepare a 1 mg/mL solution. A 2 mM solution of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (for trypsin) and Suc-Gly-Gly-*p*-nitroanilide (for chymotrypsin) in the appropriate buffer solution was used as a substrate solution. The test sample was dissolved in ethanol and diluted with the same buffer solution that was used for the enzyme and substrate. A 100 μL buffer solution, 10 μL enzyme solution, and 10 μL test solution were added to each microtiter plate well and preincubated at 37 °C for 5 min. Then, 100 μL of substrate solution was added to begin the reaction. The absorbance of the well was immediately measured at 405 nm. The developed color was measured after incubation at 37 °C for 30 min.

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