Raocyclamides A and B, Novel Cyclic Hexapeptides Isolated from the Cyanobacterium *Oscillatoria raoi*

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Raocyclamides A and B, two novel oxazole- and thiazole-containing cyclic hexapeptides, were isolated from the cyanobacterium *Oscillatoria raoi* (TAU strain IL-76−1−2). The gross structures of raocyclamides A (**1**) and B (**2**) were elucidated by homonuclear and inverse-heteronuclear 2D-NMR techniques and HREIMS. The absolute stereochemistry of compounds **1** and **2** was determined by Marfey's HPLC Method. Raocyclamide A (**1**) was established as *cyclo*-thiazole-D-Ile-oxazole-L-Ala-D-oxazoline-D-Phe and raocyclamide B (**2**) as *cyclo*-thiazole-D-Ile-oxazole-L-Ala-D-Ser-D-Phe. Raocyclamide A (**1**) exhibits a moderate cytotoxicity against sea urchin embryos.

Cyanobacteria have become well known as prolific producers of biologically active peptides with highly modified amino acid residues (e.g., microcystins,[1] tantazoles,[2,3] schizotrins,[4] cryptophycins,[5,6] and mirabimide E[7]). Only two cyclically modified amino acids containing cyclic hexapeptide, westiellamide,[8] and nostocyclamide[9] have been isolated from cyanobacteria. Westiellamide is identical to trisoxazoline, which was isolated from the ascidian, *Lissoclinum bistratum*,[10] and it was suggested that it is produced by its obligate prokaryotic symbiont, *Prochloron* sp.[8]

Five other modified cyclic hexapeptides are known from marine organisms, bistratamides A−D (from *L. bistratum*[11,12]) and dolastatin E (from the sea hare *Dolabella auricularia*[13]). Dolastatin E is probably a cyanobacterial metabolite, as *Dolabella* spp. are known to feed upon marine cyanobacteria and algae.[14] As part of our continuing effort to isolate biologically active compounds from cultured cyanobacteria, we now report the isolation and structure elucidation of two novel cyclic hexapeptides, raocyclamides A (**1**) and B (**2**), from the cultured cyanobacterium *Oscillatoria raoi* De Toni (Nostocales, Oscillatoriaceae).[15]

Results and Discussion

The cyanobacterium was isolated from a soil sample collected at a greenhouse in Kfar Azar, Israel, and identified as *O. raoi*.[15] A pure clonal strain (TAU strain IL-76-1-2) was grown in mass culture in our laboratory.[16] The freeze-dried cells were extracted with 70% MeOH in H_2O, and the resulting extract was subjected to repeated reversed phase (C_{18}) chromatography, guided by cytotoxicity-assay. The active fraction was applied to a preparative C_{18} HPLC column using MeOH−H_2O (3:1) as the eluent to afford compound **1** (13.3 mg) and compound **2** (5.0 mg). Raocyclamide A (**1**) inhibits the division of embryos[17] of *Paracentrotus lividus* with an ED_{100} of 30 μg/mL, whereas raocyclamide B (**2**) was not active at a concentration of 250 μg/mL.

Raocyclamide A (**1**) was isolated as a glassy solid. The molecular formula of **1**, $C_{27}H_{30}N_6O_5S$ (HREIMS molecular ion: m/z 550.1991, Δ 0.7 mDa), indicated 16 double-bond equivalents. The hexapeptide nature was deduced from the six nitrogen atoms in the formula and six sp^2 carbon signals in the amide region of the ^{13}C NMR spectrum. The presence of only three amide protons and two singlet aromatic protons in the ^1H-NMR spectrum suggested three cyclically modified amino acids. Three standard amino acid residues—isoleucyl, alanyl, and phenylalanyl—and three modified amino acids—thiazole, oxazole, and oxazoline—were established based on the analysis of ^1H, ^{13}C, COSY, HMQC, and HMBC spectra (Table 1). Confirmation of the sequencing of the subunits of **1** was provided by EIMS fragmentations.

The first amino acid was determined as isoleucine. The amide proton [δ 8.19 (NH-1)] was correlated through COSY and HMQC experiments to the nitrogen-bearing methine [δ 5.55 (H-5), 55.6 (C-5)]. The latter proton was connected to another methine [δ 2.03 (H-6), 42.3 (C-6)] possessing methyl and methylene groups [δ 1.05 (H_3-9), 15.7 (C-9); 1.22, 1.38 (H_2-7), 24.2 (C-7)]. The chain was terminated by a methyl group [δ 0.94 (H_3-8)].

The second amino acid unit, alanine, was identified by the COSY and HMQC correlations of a nitrogen-bearing methine [δ 4.66 (H-14), 44.8 (C-14)] with an amide proton and a methyl [δ 7.33 (NH-2); 1.52 (H_3-15), 20.2 (C-15)]. The oxazoline ABX spin system was established by the COSY correlations of the nitrogen-bearing methine [δ 4.63 (H-17), 67.6 (C-17)] with the oxygen-bearing methylene [4.55 and 4.93 (H_2-18), 69.2 (C-18)].

The last amino acid was deduced to be phenylalanine. The nitrogen-bearing methine [δ 5.27 (H-20), 48.0 (C-20)] had correlations with an amide proton [δ 8.03 (NH-3)] and a methylene [δ 3.12 and 3.13 (H_2-21), 37.7 (C-21)]. The methylene protons were correlated through an HMBC experiment (Table 1) to a phenyl ring system [δ 6.77 (H-23 and H-27), 129.5 (C-23 and C-27); 6.82 (H-24 and H-26), 127.6 (C-24 and C-26); 6.64 (H-25), 125.9 (C-25)].

The HMBC spectrum was essential for sequencing the cyclic peptide (Table 1). Correlations from H-3 to C-1 and C-4 established the latter as thiazole carbons. H-5 and NH-1 exhibited correlations with C-4 and C-10, thus establishing the linkage of the thiazole through isoleu-

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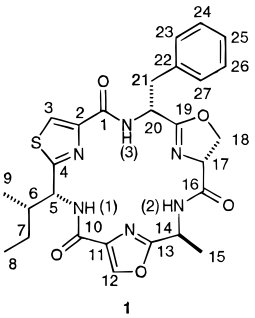
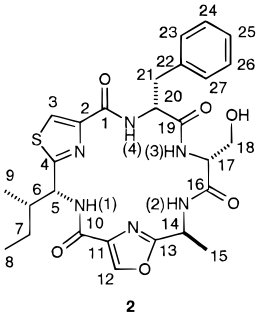
Table 1. NMR Data and Assignments for Raocyclamides A (**1**) and B (**2**) in $CDCl_3$[a]

raocyclamide A (**1**)				raocyclamide B (**2**)			
position	δ_C, mult[b]	δ_H, mult, *J* (Hz)	LR H-C corrs.[c]	position	δ_C, mult[b]	δ_H, mult, *J* (Hz)	LR H-C corrs.[c]
1	159.5 s		3, NH-3	1	159.8 s		NH-4
2	147.8 s		3	2	148.1 s		3
3	124.6 d	8.23 s		3	122.9 d	8.08 s	
4	169.5 s		3, 5, NH-1	4	168.5 s		3, 5
5	55.6 d	5.55 dd 8.9, 3.6	9	5	55.6 d	5.53 dd 4.0, 9.0	6, 9
6	42.3 d	2.03 m	5, 8, 9	6	41.5 d	1.99 ddq 4, 9.8, 6.7	8, 9
7	24.2 t	1.22 m	5, 8, 9	7	24.6 t	1.29 ddq 13.5, 9.8, 7.4;	5, 8, 9
		1.38 m				1.47 ddq 13.5, 6.7, 7.4	
8	11.9 q	0.94 t 7.4	7	8	11.7 q	0.95 d 7.4	
9	15.7 q	1.05 d 6.8	6	9	15.6 q	1.04 d 6.7	
NH-1		8.19 d 8.9		NH-1		8.05 d 9.0	
10	159.3 s		5, NH-1, 12	10	159.9 s		5, NH-1
11	135.5 s		12	11	136.2 s		12
12	141.1 d	8.24 s		12	141.4 d	8.18 s	
13	164.1 s		12, 15, NH-2	13	163.5 s		12, 14, 15, NH-2
14	44.8 d	4.66 dq 4.7, 6.7	15	14	44.3 d	4.93 dq 6.8, 6.8	15
15	20.2 q	1.52 d 6.7	14	15	20.9 q	1.47 d 6.8	14
NH-2		7.33 d 4.7		NH-2		8.01 d 6.8	
16	170.1 s		NH-2, 17, 18, 18′	16	170.2 s		NH-2, 17, 18′
17	67.6 d	4.63 dd 10.7, 6.2	18, 18′	17	53.5 d	4.42 ddd 2.2, 5.3, 7	18, 18′
18	69.2 t	4.55 dd 8.4, 10.7		18	61.3 t	3.55 dd 5.3, 11.9	
		4.93 dd 6.2, 8.4				4.23 dd 2.2, 11.9[d]	
				NH-3		7.09 d 7.0	
19	167.0 s		17, 18, 18′, 21, 21′	19	171.8 s		NH-3, 20, 21, 21′, NH-4
20	48.0 d	5.27 ddd 3.2, 4.1, 9.0	21, 21′	20	53.7 d	4.96 ddd 6.3, 6.2, 4.0	21, 21′, NH-4
21	37.7 t	3.12 dd 13.8, 4.1	23, 27	21	36.7 t	3.26 dd 4.0, 14.8	23, 27
		3.13 dd 13.8, 3.2				3.34 dd 6.2, 14.8	
22	134.9 s		21, 21′, 24, 26	22	135.2 s		21, 21′, 24, 26
23,27	129.5 d	6.77 dd 1.6, 6.6	21, 21′, 27, 23, 25	23,27	129.4 d	7.04 d 7.1	21, 21′, 27, 23, 25
24,26	127.6 d	6.82 dd 6.6, 7.0	26, 24	24,26	128.2 d	6.99 dd 7.0, 7.1	26, 24
25	125.9 d	6.64 tt 1.6, 7.0	23, 27	25	126.8 d	6.94 t 7.0	23, 27
NH-3		8.03 d 9.0		NH-4		8.99 d 6.3	

[a] Assignments were carried out on an ARX-500 Bruker instrument. [b] Multiplicity and assignment are from HMQC experiment. [c] Values determined from HMBC experiment, $^nJ_{CH} = 8$ Hz, recycle time 1 s. [d] 4.46 brs, O**H**.



1



2

cine to the oxazole. Correlations of H-12 with C-10 and C-13, and H_3-15 and NH-2 with C-13 established the connection of the alanyl residue to the oxazole ring. Correlations of C-16 with NH-2, H-17, H-18, and H-18′ linked the oxazoline to the alanyl nitrogen. Correlations of H-17, H-18, H-18′, H-21, and H-21′ with C-19 established the connection of the Phe "carbonyl" to the "serine" residue through the oxazoline heterocycle. Finally, the correlation of the Phe amide proton to C-1 closed the macrocycle.

The EIMS of **1** exhibited only four strong fragment ions (Figure 1): *m*/*z* 493 (45%) derived from loss of 57 mass units (butyl from Ile); *m*/*z* 459 (100%) loss of 91 mass units (benzyl from Phe); *m*/*z* 336 (25%) loss of C-16 to NH(3) fragment (plus 2H); and *m*/*z* 180 (65%) C-1 to C-9 fragment. The latter two fragments confirm the Tzl-Ile-Ozl-Ala sequence and, thus, the proposed planar structure of compound **1**.

The absolute stereochemistries of the amino acids were analyzed, after ozonolysis, by Marfey's HPLC method.[18] The amino acids were determined as: L-alanine, D-serine, D-phenylalanine, and D-isoleucine, by comparison with authentic D and L standards of each amino acid.

Raocyclamide B (**2**) was isolated as a glassy solid. The molecular formula of **2** [$C_{27}H_{32}N_6O_6S$ (*m*/*z* 568.2099, Δ 0.5 mDa)] indicated 15 double-bond equivalents. The presence of four amide protons and two singlet aromatic protons in the ^{1}H NMR spectrum suggested two cyclically modified amino acids. Four standard amino acid residues—isoleucyl, alanyl, phenylalanyl, and serinyl—and two modified amino acids—thiazole and oxazole—were established based on the analysis of ^{1}H, ^{13}C, COSY, HMQC, and HMBC spectra (Table 1). The assignment of the amino acid residues was similar to that of **1** (see Table 1) except for the serine residue. The COSY and HMQC spectra revealed that the nitrogen-bearing methine [δ 4.42 (H-17), 53.5 (C-17)] was connected to an amide proton [δ 7.09 (NH-3)] and to the oxygen-bearing methylene [δ 3.55 and 4.23 (H_2-18), 61.3 (C-18)]. The latter methylene protons were, in turn, joined to the hydroxyl proton [δ 4.46] by COSY correlations.

The HMBC correlations (Table 1) allowed us to construct two fragments: C-2 to C-10 and C-11 to C-1, which account for all of the atoms in the molecule. The connection of C-1 to C-2 and C-10 to C-11 is proposed on the basis of comparing the carbon atoms' chemical shifts (Table 1) and EIMS fragmentations of compounds **1** and **2**. Only carbons 17−20 show significant changes in their chemical shifts in accordance with their changed structure. All of the other carbons show minute differences, which might be attributed to conformational changes.

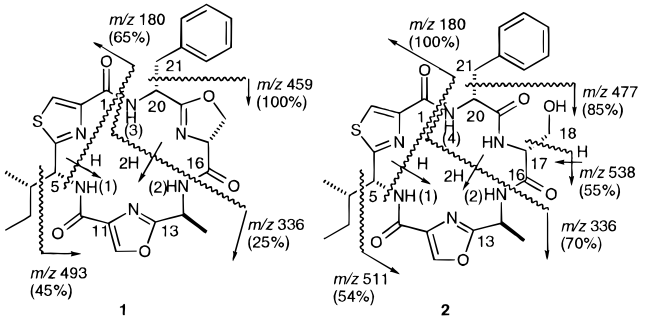


Figure 1. Fragmention patteren of the EIMS of raocyclamides A (**1**) and B (**2**).

The mass spectrum of **2** presents fragments derived from the loss of the Phe [*m*/*z* 477 (85%)], Ile [*m*/*z* 511 (54%)], and Ser [*m*/*z* 538 (55%)] side chains and loss of H_2O [*m*/*z* 550 (30%)]. The other two intense fragments in the mass spectrum of **2** at *m*/*z* 336 (70%) and 180 (100%) are identical to those found for **1**, thus implying that the C-1 to NH-2 fragment in both compounds is the same and suggesting that the difference between them is in the C-16 to C-20 fragment.

The absolute stereochemistries of the amino acids were analyzed, after ozonolysis, by Marfey's HPLC method.[18] The amino acids were determined to be: L-alanine, D-serine, D-phenylalanine, and D-isoleucine, by comparison with authentic D and L standards of each amino acid. On the basis of these arguments structure **2** was assigned to raocyclamide B.

Experimental Section

Instrumentation. IR spectra were recorded on a Nicolet FTIR in $CHCl_3$ or neat. HRMS were recorded on a Fisons VG AutoSpecQ M 250 instrument. 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 ^1H and 125.76 MHz for ^{13}C. ^1H, ^{13}C, DEPT, COSY-45, HMQC, and HMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were performed with an Applied Biosystem, Inc., instrument equipped with two model 150 pumps and a 893 programmable UV detector.

Culture Conditions. An edaphic form of *Oscillatoria raoi* De Toni (Nostocales, Oscillatoriaceae),[15] designated Tel Aviv University (TAU) strain number IL-76-1-2, was isolated from a soil sample collected at a greenhouse in Kfar Azar, Israel. A clonal strain was purified on BG-11 agar medium.[16] The cyanobacterium was cultured in 20-L glass bottles containing a BG-11 medium.[16] Cultures were illuminated continuously at an intensity of 100 μeinstein m^{-2} s^{-1} 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 0.4 to 0.6 g/L of culture.

Isolation. Freeze-dried bacterium (30.6 g) was extracted with 7:3 MeOH−H_2O (3 × 1 L) overnight. The filtered extracts were combined and concentrated under reduced pressure to a brown gum (7.5 g).

The extract (7.5 g) was applied to a reversed-phase column (ODS-YMC-gel, 120A; 4 × 10 cm), successively using 250-mL amounts of water, MeOH−H_2O (1:9), MeOH−H_2O (3:7), MeOH−H_2O (1:1), MeOH−H_2O (3:1), MeOH−H_2O (9:1), MeOH, and EA. Fraction 5 (140 mg) was found to be active in the sea urchin embryos inhibition assay. The active fraction was applied to a preparative C_{18} HPLC column (Allthec Econosil C_{18}, 10 μm, 250 × 20 mm), eluted with 3:1 MeOH−H_2O (5 mL/min) and UV-detection at 254 nm. Two related compounds were obtained from the latter chromatography, compound **1** (13.3 mg, t_R 35.5 min) and compound **2** (5.0 mg, t_R 18.5 min).

Raocyclamide A (1): A glassy solid; $[\alpha]_D^{25}$ 25.5° (*c* 4.4, MeOH); EIMS (70eV) *m*/*z* (rel int) 550 (M^+, 90), 493 (45), 459 (100), 336 (25) 180 (65); HREIMS *m*/*z* 550.1991 (M^+, $C_{27}H_{30}N_6O_5S$, Δ 0.7 mDa), 493.1297 ($C_{23}H_{21}N_6O_5S$, Δ −0.3 mDa), 459.1448 ($C_{20}H_{23}N_6O_5S$, Δ 0.2 mDa), 336.1254 ($C_{15}H_{20}N_4O_3S$, Δ 0.1 mDa), 180.0479 ($C_9H_{10}NOS$, Δ 0.3 mDa); UV (MeOH) λ_{max} (ϵ) 242 (17,750) nm; IR (neat) ν_{max} 3692, 3612, 3027, 2400, 1676, 1663, 1509, 1490, and 1230 cm^{-1}; For ^1H- and ^{13}C-NMR data, see Table 1.

Raocyclamide B (2): A glassy solid, $[\alpha]_D^{25}$ 5.1° (*c* 4.1, MeOH); EIMS (70eV) *m*/*z* (rel int) 568 (M^+, 50), 550 (32), 538 (55), 525 (39), 511 (54), 477 (85), 336 (70) 180 (100); HREIMS (70eV) *m*/*z* 568.2099 (M^+, $C_{27}H_{32}N_6O_6S$, Δ 0.5 mDa), 550.2012 ($C_{27}H_{30}N_6O_5S$, Δ −1.4 mDa), 538.1993 ($C_{26}H_{30}N_6O_5S$, Δ 0.5 mDa), 525.1918 ($C_{25}H_{29}N_6O_5S$, Δ 0.2 mDa), 336.1264 ($C_{15}H_{20}N_4O_3S$, Δ −0.8 mDa), 180.0484 ($C_9H_{10}NOS$, Δ −0.2 mDa); UV (MeOH) λ_{max} (ϵ) 241 (17,166) nm; IR (neat) ν_{max} 3606, 3026, 2406, 1662, 1509, 1428, and 1206 cm^{-1}; For ^1H- and ^{13}C-NMR data, see Table 1.

Cytotoxicity Assay. Freshly collected specimens of the sea urchin *Paracentrotus lividus* were used for this assay. To collect eggs and sperm, a 0.5-M KCl solution (1 mL) is injected into each specimen. The collected sperm and eggs are mixed prior to transferring them into the wells by pipette. Samples are dissolved in EtOH and added to seawater to give an adequate concentration at 1 mL of seawater in a 24-multiwell plate. The final concentration of EtOH is less than 1%. About 100 fertilized eggs are pipetted into each well, which contains sample solution, and the plate is kept at 24 °C. At appropriate periods embryos are examined under microscope. The dosage that inhibits embryonic division (ED_{100}) is determined.

Stereochemical Analysis. A 0.25-mg portion of **1** and **2** was ozonized in CH_2Cl_2 for 10 min. The solvent was removed, and the residue was dissolved in 6 N HCl and placed in a sealed glass bomb at 104 °C for 20 h. In another experiment, a 0.25-mg portion of **1** and **2** was dissolved in 6 N HCl and placed in a sealed glass bomb at 104 °C for 20 h. After removal of HCl by repeated evaporation *in vacuo*, the hydrolyzate was resuspended in 200 μL of H_2O and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA).[18] The derivatized amino acids (AA) from hydrolyzates were compared with similarly derivatized standard AA by HPLC analysis: Alltech C_{18}-Econosphere, 5 μ, 4 × 250 mm, R_f 1 mL/min; UV detection at 340 nm; linear gradient elution from 50 mM triethylammonium phosphate (TEAP) buffer (pH 3)/AcCN (9:1) to TEAP/AcCN (1:1) within 40 min. Retention times of the derivatized AA were: L-Ser, 23.14 min; D-Ser, 23.74 min; L-Ala, 27.75 min; D-Ala, 31.51 min; L-Phe, 37.78 min; D-Phe, 41.30 min; L-Ile, 37.58 min, and D-Ile, 42.00 min. HPLC analysis of derivatized hydrolyzates of **1** and **2** estab-

lished L-Ala, D-Ser, and D-Phe, whereas that of derivatized hydrolyzates of ozonized **1** and **2** established L-Ala, D-Ser, D-Phe, and D-Ile.

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