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Aeruginazole A, a Novel Thiazole-Containing Cyclopeptide from the Cyanobacterium *Microcystis* sp.

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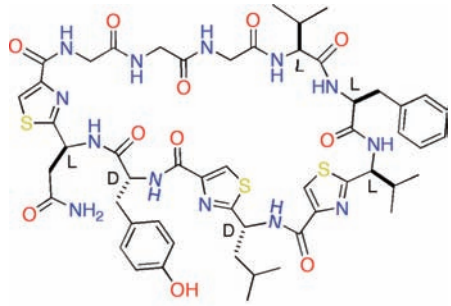
ABSTRACT



A novel thiazole-containing cyclic peptide, aeruginazole A (1), was isolated from the cyanobacterium *Microcystis* sp. strain (IL-323), which was collected from a water reservoir near Kfar-Yehoshua, Valley of Armageddon, Israel. The planar structure of aeruginazole A was established using homonuclear and inverse-heteronuclear 2D NMR techniques, as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers was determined using Marfey's method. Aeruginazole A potently inhibited *Bacillus subtilis*.

The concurrent isolation of the same cyclic hexapeptide dubbed westiellamide from the cyanobacterium *Westiellopsis prolifica*[1] and trioxazolline from the ascidian *Lissoclinum bistratum*[2] have marked the beginning of a research effort to reveal the biosynthesis of azoline- and azole-containing cyclic peptides in marine invertebrate and cyanobacteria. Prinsep et al.[1] suggested that these modified cyclic peptides are synthesized in the ascidian by its obligate prokaryotic symbiont, *Prochloron* sp. This suggestion was verified through the research of Donia et al., who have shown that indeed the patellamides and related cyclic peptides are synthesized by the symbiotic *Prochloron* spp. in ascicidians, unpredictably, through a ribosomal pathway.[3] Recently, the same authors have shown that the tenuecyclamides are synthesized by the free living cyanobacterium *Nostoc spongaeiforme* by a similar ribosomal pathway.[4] Whereas the cyclic octapeptides patellamides and patellins are produced by *Prochloron* spp. symbionts in ascidians and the cyclic hexapeptides are produced both by *Prochloron* spp. and free living strains of cyanobacteria, very few azoline- and azole-containing cyclic peptides with larger cycles have been isolated only from free living marine cyanobacteria. One of these is kororamide, a cyclic nonapeptide that was isolated from a Palauan collection of *Lyngbya majuscula*,[5] and the other is trichamide, whose structure was predicted from

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Table 1. NMR Data of Aeruginazole A (**1**) in DMSO-d_6

position		δ_C/δ_N, mult	δ_H, mult, (J in Hz)	$^1H-^1H$ COSY	$^1H-^{13}C/^{15}N$ HMBC	ROESY
Tzl-Asn	1	160.9, s			Gly(3)-2,2′NH, Tzl-Asn-3	
	2	149.0, s			Tzl-Asn-3	
	3	124.3, d	8.16, s			
	N	*307.7, s*			*Tzl-Asn-3*	
	4	173.5, s			Tzl-Asn-3,5,6,6′	
	5	49.0, d	5.50, ddd, (5.8, 7.0, 8.3)	Tzl-Asn-6,6′,NH	Tzl-Asn-6,6′	Tzl-Asn-6,6′,5-NH,7-NH′
	6	40.0, t	2.72, dd, (13.8, 8.3)	Tzl-Asn-5,6′	Tzl-Asn-5,7-NH	Tzl-Asn-5,6′,5-NH,7-NH′
			2.87, dd, (13.8, 5.8)	Tzl-Asn-5,6		Tzl-Asn-5,6,5-NH,7-NH′
	7	170.6, s			Tzl-Asn-5,6,6′,7-NH,7-NH′	
	5-NH	*122.4, d*	9.09, d, (7.0)	Tzl-Asn-5		Tzl-Asn-5,6,6′, Tyr-2,3′
	7-NH_2	*109.7, t*	7.00, brs	Tzl-Asn-7-NH′		
			7.47, brs	Tzl-Asn-7-NH		Tzl-Asn-5,6,6′
Tyr	1	170.9, s			Tzl-Asn-5-NH, Tyr-2,3,3′	
	2	53.7, d	4.79, ddd, (5.5, 8.8, 9.0)	Tyr-3,3′,NH	Tyr-3,3′	Tzl-Asn-5-NH, Tyr-3,3′,5,5′,NH
	3	37.4, t	2.91, m	Tyr-2,3′	Tyr-5,5′	Tyr-2,5,5′,NH
			3.06, dd, (13.0, 8.8)	Tyr-2,3		Tzl-Asn-5-NH, Tyr-2,5,5′
	4	127.2, s			Tyr-3,3′,6,6′	
	5,5′	130.4, d × 2	7.01, d × 2, (8.3)	Tyr-6,6′	Tyr-3,3′,5′,5	Tyr-2,3,3′,6,6′,NH
	6,6′	115.2, d × 2	6.59, d × 2, (8.3)	Tyr-5,5′	Tyr-5,5′,6′,6	Tyr-5,5′
	7/7-OH	156.1, s	9.19, brs		Tyr-5,5′,6,6′	
	2-NH	*114.6, d*	7.88, d, (9.0)	Tyr-2		Tyr-2,3,5,5′
Tzl-Leu	1	159.7, s			Tyr-NH, Tzl-Leu-3	
	2	148.8, s			Tzl-Leu-3	
	3	124.8, d	8.14, s			
	N	*307.3, s*			*Tzl-Leu-3*	
	4	171.2, s			Tzl-Leu-3,5	
	5	48.2, d	5.42, ddd, (6.4, 8.8, 9.0)	Tzl-Leu-6,6′,NH	Tzl-Leu-6,6′,5-NH	Tzl-Leu-6,6′,7,8,9,5-NH
	6	42.2, t	1.96, m	Tzl-Leu-5,6′,7	Tzl-Leu-5,8,9	Tzl-Leu-5,8,9,5-NH
			2.01, m	Tzl-Leu-5,6,7		Tzl-Leu-5,8,9,5-NH
	7	24.8, d	1.60, m	Tzl-Leu-6,6′,8,9	Tzl-Leu-6′,8,9	Tzl-Leu-NH
	8	22.9, q	0.94, d, (6.5)	Tzl-Leu-7	Tzl-Leu-6′,7,9	Tzl-Leu-5,6,6′
	9	22.0, q	0.93, d, (6.4)	Tzl-Leu-7	Tzl-Leu-6,6′,7,8	Tzl-Leu-5,6,6′,5-NH
	5-NH	*123.1, d*	8.38, d, (9.0)	Tzl-Leu-5		Tzl-Leu-5,6,6′,7,9
Tzl-Val	1	160.1, s			Tzl-Leu-5,5-NH, Tzl-Val-3	
	2	148.5, s			Tzl-Val-3	
	3	124.5, d	8.20, s			
	N	*309.2, s*			*Tzl-Val-3*	
	4	171.2, s			Tzl-Val-3,5,6	
	5	55.7, d	4.94, dd, (7.2, 9.2)	Tzl-Val-6,NH	Tzl-Val-6,7,8	Tzl-Val-6,7,8,5-NH
	6	32.7, d	2.20, dqq, (7.2, 6.7, 6.7)	Tzl-Val-5,7,8	Tzl-Val-5,7,8	Tzl-Val-5,7,8,5-NH
	7	19.5, q	0.78, d, (6.7)	Tzl-Val-6	Tzl-Val-5,6,8	Tzl-Val-5,6,8,5-NH
	8	18.0, q	0.83, d, (6.7)	Tzl-Val-6	Tzl-Val-5,6,7	Tzl-Val-5,6,7,5-NH
	5-NH	*121.6, d*	8.10, d, (9.2)	Tzl-Val-5		Tzl-Val-5,6,7,8, Phe-2
Phe	1	170.9, s			Tzl-Val-5-NH, Phe-2,3,3′	
	2	54.5, d	4.46, dt, (7.4, 7.8)	Phe-3,3′,NH	Phe-3,3′	Tzl-Val-5-NH, Phe-3,3′,5,5′,NH
	3	37.3, t	2.75, dd, (12.5, 7.8)	Phe-2,3′	Phe-2,5,5′	Phe-2,5,5′
			2.89, m	Phe-2,3		Phe-2
	4	137.4, s			Phe-2,3,3′,6,6′	
	5,5′	129.2, d × 2	7.07, d, (7.2)	Phe-6,6′	Phe-3,3′,5′,5,7	Phe-3,6,6′
	6,6′	128.0, d × 2	6.96, dd, (7.2, 7.5)	Phe-5,5′,7	Phe-6′,6	Phe-5,5′,7
	7	126.2, d	6.84, t, (7.5)	Phe-6,6′	Phe-5,5′	Phe-6,6′
	2-NH	*119.0, d*	8.08, d, (7.8)	Phe-2		Phe-2,3,3′,5,5′ Val-2,3,4,NH
Val	1	170.7, s			Phe-NH, Val-2	
	2	57.8, d	4.01, dd,(7.1, 8.8)	Val-3,NH	Val-3,4,5	Phe-NH, Val-3,4,5,NH
	3	30.6, d	1.81, dqq, (7.1, 6.5, 5.5)	Val-2,4,5	Val-2,4,5	Phe-NH, Val-2,4,5,NH
	4	17.6, q	0.60, d, (6.5)	Val-3	Val-2,3,5	Phe-NH, Val-2,3,NH
	5	18.9, q	0.62, d, (5.5)	Val-3	Val-3,4	Val-2,3,NH
	2-NH	*113.1, d*	7.45, d, (8.8)	Val-2		Phe-NH,Val-2,3,4, Gly(1)-2,2′,NH
Gly(1)	1	169.2, s			Val-NH, Gly(1)-2,2′	
	2	42.8, t	3.64, dd, (16.6, 5.9)	Gly(1)-2′,NH		Val-NH, Gly(1)-2′,NH
			3.76, dd, (16.6, 5.9)	Gly(1)-2,NH		Val-NH, Gly(1)-2,NH
	2-NH	*105.4, d*	8.24, t, (5.9)	Gly(1)-2,2′		Val-NH, Gly(1)-2,2′
Gly(2)	1	169.9, s			Gly(1)-NH, Gly(2)-2,2′	
	2	42.6, t	3.68, dd, (17.7, 5.6)	Gly(2)-2′,NH		Gly(2)-2′,NH
			3.72, dd, (17.7, 5.6)	Gly(2)-2,NH		Gly(2)-2,NH
	2-NH	*106.1, d*	8.43, t, (5.6)	Gly(2)-2,2′		Gly(2)-2,2′
Gly(3)	1	169.8, s			Gly(2)-NH, Gly(3)-2,2′	
	2	42.5, t	3.84, dd, (16.6, 5.5)	Gly(3)-2′,NH		Gly(3)-2′,NH
			4.07, dd, (16.6, 6.2)	Gly(3)-2,NH		Gly(3)-2,NH
	2-NH	*105.6, d*	8.48 t, (5.8)	Gly(3)-2,2′		Gly(3)-2,2′

genome mining.[6] Here we report the isolation and structure elucidation of a thiazole-containing cyclic dodecapeptide, aeruginazole A (**1**), which was isolated along with aeruginosins KY642, KY608, and 98A from a waterbloom material of the cyanobacterium *Microcystis* sp. collected in a water reservoir near Kfar-Yehoshua, Valley of Armageddon, Israel in August 2003.[7]

Aeruginazole A (**1**) was isolated from a 7:3 MeOH/water crude extract following separation on a reversed phase open

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column, gel filtration on a Sephadex LH-20 column, and repeated purification on a reversed phase HPLC column to produce a yellow glassy solid. Its positive HR ESI TOFMS quasi-molecular ion at *m*/*z* 1156.4196 matched the molecular formula $C_{53}H_{66}N_{13}O_{11}S_3$ with a 2.9 mDa error. The lower field ^{1}H NMR spectrum of **1** in DMSO-d_6 revealed three sharp singlets between 8.20 and 8.10 ppm, suggesting that **1** contains three thiazole moieties. Additional signals in this region were two exchangeable broad singlet protons, six exchangeable broad doublet protons, and three exchangeable broad triplet protons, as well as signals of a monosubstituted phenyl and a *para*-substituted phenol moiety. In the middle region of the ^{1}H NMR spectrum of **1**, 12 protons were observed, and in the higher field of the spectrum, six doublet methyls were present. The ^{13}C NMR spectrum exposed 49 absorption lines, suggesting that four of the signals are doubled as a result of symmetry. Of the 49 carbon signals, 13 resonate between 174 and 159 ppm, suggesting that they belong to carboxamide carbons. Three of these signals, resonating around 160 ppm, were of conjugated carboxamide groups characteristic of carboxazole moieties.[8] The ^{1}D (one dimensional) NMR data thus suggest that **1** is a peptide containing three thiazole moieties.

The structure elucidation of the amino acids that compose **1** was initiated with the analysis of the COSY and TOCSY correlation maps measured in DMSO-d_6. This resulted in the assignment of the α-amide and side chains as three glycines, two valines, a leucine, three ABMX spin systems where X is an amide proton, a primary amide (δ_H 7.47 brs and 7.00 brs ppm), a monosubstituted phenyl ring, and a *para*-substituted phenol moiety, accounting for 62 out of the 65 protons of **1**. Data from the ^{1}H−^{13}C HMQC experiment (see Table 1) assigned all of the protonated carbons to the fragments suggested by the COSY and TOCSY experiments, as well as the thiazoles singlet protons (δ_H 8.20, 8.16, and 8.14 ppm) to the corresponding sp^2 carbons (δ_C 124.5, 124.4, and 124.8 ppm, respectively). The ^{1}H−^{15}N HMQC experiment (see Table 1) assigned the nine secondary nitrogens and the primary amide to the amino acid fragments (see Table 1). The structure elucidation of the thiazole moieties was based on HMBC correlations. Each one of the thiazole singlet protons (δ_H 8.20, 8.16, and 8.14 ppm) presented, in the ^{1}H−^{13}C HMBC experiment, a ^2J correlation with C-2 of the thiazole moiety (resonating at δ_C 148.5, 149.0, and 148.8 ppm, respectively) and ^3J correlations with C-1 (resonating at δ_C 160.1, 160.9, and 159.7 ppm, respectively) and C-4 (resonating at δ_C 171.2, 173.5, and 171.2 ppm, respectively). The latter thiazole protons presented ^3J correlations in the ^{1}H−^{15}N HMBC experiment with the quaternary nitrogen atoms resonating at δ_N 309.2, 307.7, and 307.3 ppm, respectively, thus establishing the structure of the three thiazole rings. The structure elucidation of the thiazole moieties was completed with the assignment of the ^2J correlation of the α-protons of the amino acid with C-4 of the thiazole system (the carboxyl of the precursor amino acid) (see Table 1 and Figure 1). For the other six amino acids, a ^2J (and in some cases ^3J H−C correlation) correlation of the α-proton(s) and the corresponding carboxamide assigned the latter to the complete substructures (see Table 1 and Figure 1). The nine

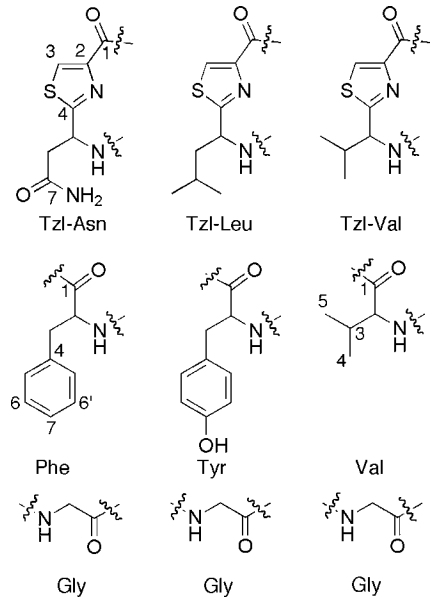

Figure 1. Substructures assigned for **1**.

fragments could be assembled to the complete structure by HMBC correlations of the carboxamide of a subunit with amide (^2J) or α-proton (^3J) of the adjacent subunit, namely, the carboxamide of Tyr with the 5-amide proton of Tzl-Asn, the carboxamide of Tzl-Leu with Tyr-α-amide proton, the carboxamide of Tzl-Val with the 5-proton and 5-amide proton of Tzl-Leu, the carboxamide of Phe with the 5-amide proton of Tzl-Val, the carboxamide of Val with Phe-α-amide proton, the carboxamide of Gly(1) with Val-α-amide proton, the carboxamide of Gly(2) with Gly(1)-α-amide proton, the carboxamide of Gly(3) with Gly(2)-α-amide proton, and the carboxamide of Tzl-Asn with Gly(3)-α-amide proton that assigned the closure of the macrocyclic ring (see Figure 2). The NOE correlations

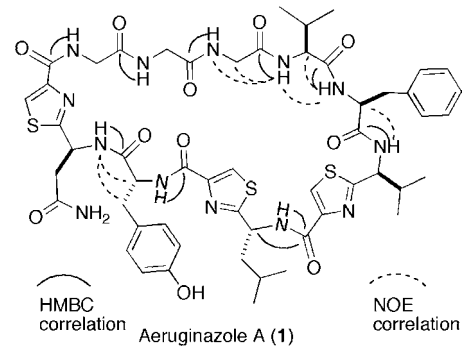

Figure 2. HMBC and NOE correlations between the subunits of aeruginazole A (**1**).

from the ROESY experiment, on the other hand, could assign only small fragments because the thiazole protons did not show correlations to the neighboring amino acids (see Table 1 and Figure 2).

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The absolute configuration of the stereocenters in **1** was established using Marfey's method, which was performed twice. The first one included hydrolysis of **1**, to elaborate Gly, Phe, Tyr, Val, Tzl-Asp, Tzl-Leu, and Tzl-Val, followed by derivatization with Marfey's reagent (FDAA), and the second, which included an initial step of ozonolysis of **1**, followed by hydrolysis to elaborate Asp, Gly, Leu, Phe, Tyr, and Val, and then derivatization with FDAA. The chromatograms of the derivatized amino acids that resulted from these procedures were spiked separately with D,L-mixtures of authentic FDAA-derivatized amino acids. The first procedure revealed the presence of L-Phe, D-Tyr, and L-Val, and the second procedure established the presence of L-Asp, D-Leu, L-Phe, D-Tyr, and L-Val. On the basis of these results the structure of aeruginazole A was established as *cyclo*-[Tzl-L-Asn-D-Tyr-Tzl-D-Leu-Tzl-L-Val-L-Phe-L-Val-Gly-Gly-Gly]. The structure of **1** contains four regions: an achiral region in the northeren sphere, two regions with L-amino acids in the eastern and western spheres, and a region with D-amino acids in the southern sphere.

The biological activity of **1** was assessed in several bioassays, including cytotoxicity against MOLT-4 human leukemia cell line and peripheral blood lymphocytes (PBL); induction of growth arrest of *Saccharomyces cerevisiae* expressing (and not expressing) the proteins that induce p53-independent, protein phosphatase 2A-dependent apoptosis in transformed mammalian cells;[9] inhibition of trypsin and chymotrypsin; and antibacterial activity (against *Escherichia coli*, *Staphyloccocus albus*, and *Bacillus subtilis*). Aeruginazole A exhibited moderate cytotoxicity against MOLT-4 cell line with IC_{50} of 41 μM but higher cytotoxicity (IC_{50} of 22.5 μM) against PBL.[10] It did not induce PP2A-dependent apoptosis in engineered *S. cerevisiae* and did not inhibit trypsin or chymotrypsin at a concentration of 40 μM. Aeruginazole A inhibited the growth of *B. subtilis* and *S. cerevisiae* with MICs of 2.2 and 43.3 μM, respectively, but not *E. coli* and *S. albus* at 8.7 μM.

Aeruginazole A is the first example of a polythiazole-containing cyclic peptide isolated from a fresh water cyanobacterium in general and *Microcystis* sp. in particular. It adds a new skeleton to the diverse groups of linear and cyclic peptides isolated from bloom-forming cyanobacteria. This new group, which is most probably ribosomally synthesized, differs from most of the other groups that are nonribosomally synthesized. The ecological role of this new group of cyanobacteria metabolites is yet unknown.

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Supporting Information Available: Full details of collection, extraction and isolation, 1D and 2D NMR spectra in DMSO-d_6, and positive and negative HR ESI mass spectra of aeruginazole A. This material is available free of charge via the Internet at http://pubs.acs.org.

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