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Two new microcyclamides from a water bloom of the cyanobacterium *Microcystis* sp.

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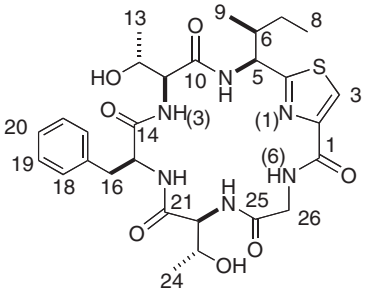
ABSTRACT

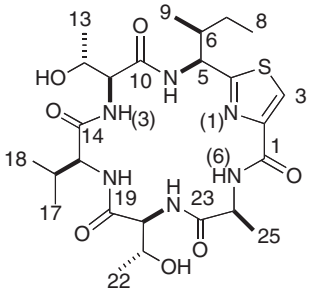
Two new thiazole-containing cyclic hexapeptides, microcyclamides MZ602 and MZ568, were isolated from the hydrophilic extract of the cyanobacterium *Microcystis* sp. The structures of the pure natural products were elucidated using spectroscopic methods including UV, 1D and 2D NMR, and MS techniques. The absolute configuration of the chiral centres of the modified cyclic peptides was determined using Marfey's method for HPLC. The microcyclamides have been evaluated for their cytotoxicity against leukemia cell lines and inhibition of serine proteases.

The recent findings that cyanobacterial cyclic hexapeptides, known as cyclamides, are biosynthesized via a ribosomal pathway,[1] have redrawn attention to this group of natural products that usually exhibit mild cyctotoxicity to cancer cell lines and whose ecological role in cyanobacteria is still unknown. The cyclamides are biosynthesized by a ribosomal pathway using a set of processing enzymes closely resembling those involved in the biosynthesis of the patellamides in the cyanobacterial symbionts of ascidians.[2] The cyclamides are divided into two subgroups: those derived from cyclization of a hexapeptide containing two cysteines and one threonine or serine, and those derived from one cysteine and two threonine/serine moieties. Most of the known cyclamides, for example, tenuecyclamides,[3] nostocyclamides,[4,5] banyascyclamides,[6] dendroamides,[7] microcyclamides,[8,9] venturamides[10] and aerucyclamides A and B,[11] belong to the first group formed from two cysteines. Fewer members of the second subgroup are known, for example, raocyclamides,[12] microcyclamide GL616,[9] aerucyclamide C,[13] and microcyclamides 7806A and 7806B.[1] In this letter we describe the isolation, structure elucidation and biological activity of two new members of the latter subgroup of cyclamides, microcyclamides MZ602 (**1**) and MZ568 (**2**).

As part of our continuing interest in the chemical ecology of cyanobacteria water blooms and the search for novel drugs for treatment of human diseases, we investigated the extracts of a *Microcystis* sp. bloom material (sample IL-361), collected from a fishpond in Kibbutz Ma'ayan-Tzvi, Israel, on July 2006.[14] The sam-



microcyclamide MZ602 (**1**)



microcyclamide MZ568 (**2**)

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ple of the cyanobacterium was freeze-dried and extracted with 70% MeOH in water. The extract was flash-chromatographed on an ODS column. The fractions eluted from the column with 2:3 and 1:1 MeOH:water were further separated on a Sephadex LH-20 column (7:3 MeOH:water). The late-eluting fractions from this column were separated on a C-8 HPLC column eluting with acetonitrile/water solutions to afford pure **1** (0.5 mg, 0.0003% yield based on the dry weight of the bacteria) and **2** (0.5 mg, 0.0003% yield based on the dry weight of the bacteria).

Microcyclamide MZ602 (**1**)[15] was isolated as a glassy material exhibiting a HR-MALDI-TOF pseudo-molecular ion cluster ($[M+Na]^+$) at *m/z* 625.2457, consistent with the molecular formula $C_{28}H_{38}N_6O_7S$. Examination of the NMR spectrum of **1** in DMSO-d_6 revealed its peptide nature; that is, six sp^2 carbons between 160 and 172 ppm in the ^{13}C NMR spectrum and five amide protons in the ^1H NMR spectrum, along with a singlet at 8.22 ppm, characteristic of a thiazole moiety. The NMR data thus suggested that **1** was a thiazole-containing hexapeptide. Analysis of the COSY, TOCSY, ROESY, HSQC and HMBC 2D NMR experiments allowed the structure elucidation of **1** as discussed below. Correlations from the ^1H-^1H COSY and TOCSY experiments allowed the assignment of the proton spin system of an isoleucine, two threonine moieties, a glycine, an ABMX spin system characteristic of an aromatic amino acid and a mono-substituted phenyl moiety. The latter two subunits could be assembled into a phenylalanyl moiety on the basis of the correlations from HSQC and HMBC experiments (see Table 1). The carbon signals of the above fragments and the thiazole singlet proton were assigned through correlations in the HSQC experiment (see Table 1). The thiazole-carboxamide moiety was established through HMBC correlations of H-3 with C-1, C-2 and C-4. The isoleucine moiety was linked to the thiazole through the HMBC correlation of H-5 with C-4. The first threonine unit was attached to the isoleucine by the HMBC correlations of C-10 with N(2)H and H-11. HMBC correlations of C-14 with H-11, N(3)H, H-15 and H-16 assigned the phenylalanine as the next amino acid in the sequence. The second threonine moiety was linked to the phenylalanine α-amide [N(4)H] through HMBC correlations of C-21 with N(4)H and H-23. Correlations of C-25 with N(5)H, H-26 and H-26′ allowed the connection of the glycine to the threonine and finally correlation of N(6)H with C-1 established the closure of the macrocyclic ring. Methyl-24 was found to resonate at a higher field than expected for a threonine methyl, most probably due to the anisotropic affect of the neighboring phenyl moiety. The absolute configurations of the chiral centers in **1** were established using Marfey's method.[16] The procedure included an initial ozonolysis of **1** in the presence of triphenylphosphine, in order to oxidize the double bonds of the thiazole ring, followed by hydrolysis to elaborate Gly, Ile, Phe and Thr, and then derivatization with FDAA.[17] The chromatogram of the derivatized amino acids that resulted from this procedure was spiked separately with D,L-mixtures of authentic FDAA-derivatized amino acids. This procedure revealed the presence of L-Ile, L-Phe, and L-Thr. Based on these arguments the structure of microcyclamide MZ602 (**1**) was established as cyclo-(Tzl-L-Ile-L-Thr-L-Phe-L-Thr-Gly).

Microcyclamide MZ568 (**2**)[18] was isolated as a glassy transparent material. Its molecular formula, $C_{25}H_{40}N_6O_7S$, was deduced

Table 1
^1H and ^{13}C NMR data of compounds **1** and **2** in DMSO-d_6

Compound	**1**[a]			**2**[b]			
Position	δ_C, (M)[c]	δ_H, (M,[c] J[d])	HMBC	Position	δ_C, (M)[c]	δ_H, (M,[c] J[d])	HMBC
1	161.1 (s)		3, 26, N(6)H	1	160.7 (s)		N(6)H
2	148.8 (s)		3	2	148.4 (s)		3
3	124.0 (d)	8.22 (s)		3	124.9 (d)	8.24 (s)	
4	168.8 (s)		3, 5	4	169.9 (s)		3, 5, N(2)H
5	55.1 (d)	5.16 (dd, 8.8, 5.7)	8, 9, N(2)H	5	55.7 (d)	5.09 (dd, 8.5, 4.5)	8, 9
6	39.2 (d)	2.00 (m)	5, 7, 7′, 8, 9	6	38.9 (d)	2.03 (m)	8, 9
7	24.6 (t)	1.41 (m), 1.08 (m)	5, 8, 9	7	23.9 (t)	1.29 (m), 1.05 (m)	8, 9
8	11.8 (q)	0.86 (t, 7.7)	7, 7′	8	11.7 (q)	0.87 (t, 7.3)	
9	15.7 (q)	0.87 (d, 7.0)	5, 7, 7′	9	16.0 (q)	0.91 (d, 6.6)	8
N(2)H		7.74 (d, 8.2)		N(2)H		7.19 (d, 8.6)	
10	169.9 (s)		11, N(2)H	10	170.3 (s)		11, N(2)H
11	61.0 (d)	3.95 (m)	13, N(3)H	11	61.9 (d)	3.88 (t, 4.5)	13
12	65.3 (d)	4.16 (m)	11, 13, N(3)H	12	65.1 (d)	4.14 (ddq 4.5, 5.6, 6.6)	13
13	20.5 (q)	1.09 (d, 6.2)	12	13	20.8 (q)	1.17 (d, 6.6)	
N(3)H		8.15 (d, 7.0)		N(3)H		8.12 (d, 4.8)	
12-OH		5.16 (br s)		12-OH		4.96 (d, 5.6)	
14	172.0 (s)		11, N(3)H, 15, 16	14	172.5 (s)		N(3)H, 15
15	54.4 (d)	4.49 (m)	16, N(4)H	15	55.9 (d)	4.44 (dd, 9.6, 2.2)	17, 18
16	37.2 (t)	3.32 (m), 2.87 (dd, 14.5, 11.4)	15, 18, 18′	16	31.0 (d)	2.29 (m)	17, 18
17	138.0 (s)		16, 16′, 19, 19′	17	20.0 (q)	0.84 (d, 6.6)	18
18,18′	129.2 (d)	7.27 (m)	16, 20	18	15.6 (q)	0.48 (d, 6.7)	17
19,19′	128.2 (d)	7.24 (m)		N(4)H		6.73 (d, 9.6)	
20	126.4 (d)	7.17 (m)	18, 18′, 19, 19′	19	170.3 (s)		N(4)H, 20
N(4)H		8.09 (d, 7.9)		20	61.2 (d)	3.96 (dd, 7.7, 3.8)	22
21	170.6 (s)		N(4)H, 23	21	65.5 (d)	4.11 (m)	22
22	60.7 (d)	3.90 (m)	24, N(5)H	22	20.8 (q)	1.14 (d, 6.4)	
23	66.1 (d)	3.86 (m)	22, 24	N(5)H		8.45 (d, 7.6)	
24	20.2 (q)	0.82 (d, 6.1)	23	21-OH		4.79 (d, 6.1)	
N(5)H		8.09 (d, 7.9)		23	171.2 (s)		N(5)H, 24, 25
23-OH		4.84 (br s)		24	48.4 (d)	4.82 (m)	25
25	169.1 (s)	—	N(5)H, 26, 26′	25	16.8 (q)	1.22 (d, 6.6)	
26,26′	43.6 (t)	3.94 (m)	N(5)H	N(6)H		8.53 (d, 7.9)	
N(6)H		8.53 (t, 5.7)					

[a] 500 MHz for ^1H and 125 MHz for ^{13}C.
[b] 400 MHz for ^1H and 100 MHz for ^{13}C.
[c] M = multiplicity.
[d] J in Hz.

from a high-resolution ESI mass measurement of its sodiated molecular cluster ion at *m/z* 591.2571 (Δ, −0.6 mDa). Similar to **1**, the ^{1}H NMR spectrum of **2** in DMSO-d_6 revealed five amide protons 6.7–8.6 ppm and a singlet at 8.24 ppm, thus suggesting that **2** is a thiazole-containing cyclic hexapeptide. Correlations from the ^{1}H-^{1}H COSY and TOCSY experiments allowed the assignment of the proton spin system of an alanine, an isoleucine, two threonines and a valine. The carbon signals of the above fragments and the thiazole singlet proton were assigned through correlations in the HSQC experiment (see Table 1). The thiazole moiety was established through HMBC correlations of H-3 with C-2 and C-4. The isoleucine moiety was linked to the thiazole through the HMBC correlation of H-5 with C-4. The first threonine unit was attached to the isoleucine α-amide through C-10 based on the HMBC correlations of C-10 with N(2)H and H-11 (the threonine α-proton). The threonine was linked through its α-amide to the carbonyl (C-14) of the valine moiety based on the HMBC correlations of C-14 with N(3)H and H-15. The second threonine moiety was assigned as the next amino acid in the sequence through the HMBC correlations of C-19 with N(4)H and H-20. The alanine moiety was attached to the α-amide of the threonine through carbonyl-23, based on the HMBC correlations of the latter carbon with N(5)H, H-24 and H_3-25. The α-amide of the alanyl moiety exhibited an HMBC correlation with the last and unpaired conjugated carboxamide carbon that resonated at 160.7 ppm. Based on its chemical shift, the eight degrees of unsaturation inferred from the molecular formula of **2** and the unpaired valency of C-2, carboxamide-1 must be connected to C-2. The similar chemical shifts of C-1, -2 and -3 and H-3 in **1** and **2** (see Table 1) further support this suggestion. An identical Marfey's procedure, as applied for **1**, (see above), established the absolute configuration of all the amino acid residues as L. The structure of microcyclamide MZ568 (**2**) was thus established as cyclo-(Tzl-L-Ile-L-Thr-L-Val-L-Thr-L-Ala). Microcyclamide MZ568 (**2**) is closely related to microcyclamides 7806A and B.[1]

The biological activity of **1** and **2** was determined against the serine proteases trypsin, thrombin, chymotrypsin and elastase, and against the Molt4 (leukemia) cell line. Microcyclamide MZ602 (**1**) exhibited very mild cytotoxicity against the Molt4 cell line (20% cell growth inhibition at 83 μM) and mild inhibition of chymotrypsin (IC_{50} = 75 μM). Microcyclamide MZ568 (**2**), on the other hand, exhibited a more potent cytotoxicity against the Molt4 cell line (36% cell growth inhibition in 1.8 μM) but did not inhibit the tested serine proteases.

Microcyclamide MZ602 (**1**), likely derived from the precursor peptide Cys-Ile-Thr-Phe-Thr-**Gly**, is closely related to microcyclamide GL616,[9] possibly derived from the precursor peptide: Cys-Ile-Thr-Phe-Thr-**Ala**. Microcyclamide MZ568 (**2**), for which the precursor peptide is assumed to be Cys-Ile-**Thr**-Val-Thr-Ala, is closely related to microcyclamides 7806A and B[1] and aerucyclamide C,[13] all sharing the same amino acid sequence of the precursor peptide: Cys-Ile-**Ser**-Val-Thr-Ala. The ecological function of these compounds is as yet unknown. In the past, it was proposed that these cyclic hexapeptides might be involved in metal ion binding, and indeed, some examples have shown the propensity to chelate metal ions.[19] Although their activity is usually mild, their association with cyanobacterial water blooms suggests that they might have a particular biological role. It is possible that they have a key role in protecting the oxygen sensitive nitrogenase in the cyanobacteria cells from oxidation, since the thiazoline and oxazoline moieties react readily with oxygen.[20] We continue to investigate the chemistry, biological activity and ecological role of these cyanobacteria secondary metabolites, as some may prove useful for medical applications, whereas others could give insight into the functional role of the peptides.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.10.051.

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