Tetrahedron 64 (2008) 6628–6634

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Three novel metabolites from a bloom of the cyanobacterium *Microcystis* sp.

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ARTICLE INFO

Article history:
Received 18 February 2008
Received in revised form 17 April 2008
Accepted 8 May 2008
Available online 13 May 2008

Keywords:
Cyanobacteria
Microcystis
Natural products

ABSTRACT

Three new metabolites, microphycin AL828, microguanidine AL772, and microginin AL584 and three known metabolites, anabaenopeptin F, oscillamide Y, and microcin SF608 were isolated from the extracts of a *Microcystis* sp. bloom collected in Alonim reservoir, Valley of Armagedon, Israel. The planar structure of the compounds was determined by homonuclear and inverse-heteronuclear 2D-NMR techniques as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers of the amino acids was studied using Marfey's method for HPLC.

1. Introduction

Natural blooms of cyanobacteria in water bodies frequently produce a large array of toxic and non-toxic metabolites. The toxic metabolites most frequently produced by certain genera of water-bloom forming cyanobacteria including the genus *Microcystis*, are the microcystins. Eighty different microcystins variants are known, exhibiting potent protein phosphatases (PP's) inhibitory activity[1] and mammal's hepatotoxicity.[2] The non-toxic metabolites are usually members of five discrete families of protease inhibitors: micropeptins,[3] anabaenopeptins,[4] aeruginosins,[5] microginins,[6] and microviridins.[7] Cyanobacteria blooms, toxic and non-toxic, usually contain one or several of these groups of protease inhibitors and in certain cases also other groups of non-toxic metabolites.[8] In most cases, the latter non-toxic secondary metabolites are cyclic and non-cyclic modified peptides. Modification of proteinogenic amino acids, by cyanobacteria, result in new amino acids mimics (i.e., Choi in aeruginosins, see microcin SF608 below). These amino acids mimics might be used as pharmacophores for new pharmaceutically useful drugs. As part of our continuous interest in the chemical ecology of cyanobacterial water-blooms and search for novel drugs for human diseases, we examined the extracts of a *Microcystis* sp. bloom collected in October 2002 from Alonim reservoir, Valley of Armagedon, Israel. The extract of this bloom (IL-306) afforded six non-toxic secondary metabolites. Three of the compounds are new natural products: microphycin AL828 (**1**), an octacyclic peptide composed of proteogenic amino acids; microguanidine AL772 (**2**), a unique zwitterionic metabolite; and microginin AL584 (**3**), an amino protease inhibitor; while the remaining three are known protease inhibitors: anabaenopeptin F (**4**),[9] oscillamide Y (**5**),[10] and microcin SF608 (**6**).[11]

2. Results and discussion

Microphycin AL828 (**1**) was isolated as a transparent glassy material with a FABMS protonated pseudo-molecular ion of 829 mass units. Its molecular formula was deduced from HRFABMS of the pseudo-molecular ion as $C_{40}H_{60}O_{11}N_8$, indicating 15 degrees of unsaturation. Its peptide nature was evident from its NMR data; i.e., nine carboxylic carbons, eight methine, and methylene carbons between 42 and 62 ppm in the ^{13}C NMR spectrum and seven amide protons in the ^{1}H NMR spectrum. In addition, a *para*-substituted phenol system, five doublet methyls, and a triplet methyl were evident from the ^{1}H NMR spectrum. The results from COSY, TOCSY, and HSQC 2D NMR experiments allowed the assignment of the side chains of two glycine units, a valine, a leucine, an isoleucine, a proline and partial structure of the side chains of tyrosine and glutamic acid. The structure of the side chains of the latter two amino acids and the assignment of the carboxyl carbons were achieved by analysis of the results of an ^{1}H–^{13}C HMBC experiment (see Table 1). The sequence of the amino acids in the peptide: Gly(I), Val, Tyr, Gly(II), Leu, Pro, Glu, and Ile was assigned on the basis of HMBC and ROESY correlations as follows: HMBC correlations between the carboxyl of Gly(I) and Val H-2 and NH, the carboxyl of Val and the NH of Tyr, the carboxyl of Tyr and H-2,2′ and NH of Gly(II), the carboxyl of Gly(II) and H-2 and NH of Leu, NOE correlation between H-5 and 5′ of the proline and Leu H-2, HMBC correlations between the carboxyl of Pro and Glu NH, between carboxyl-1 of Glu

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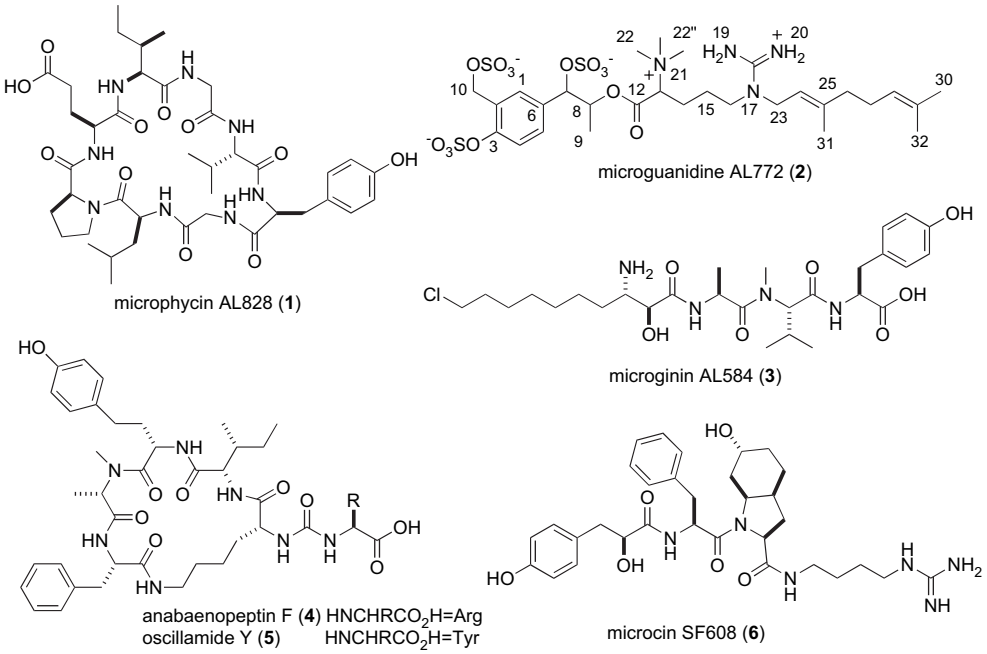
doi:10.1016/j.tet.2008.05.031



and Ile H-2 and NH, and between the carboxyl of Ile and H-2,2′ and NH of Gly(I). Acid hydrolysis of microphycin AL828 (**1**) and derivatization with Marfey's reagent,[12] followed by HPLC analysis, demonstrated the L-stereochemistry of all amino acids. This established the structure of microphycin AL828 (**1**) as *cyclo*-(Gly-L-Val-L-Tyr-Gly-L-Leu-L-Pro-L-Glu-L-Ile).

Microguanidine AL772 (**2**) was isolated as a glassy solid. Its molecular formula, $C_{29}H_{48}N_4O_{14}S_3$, was deduced from its negative mode HRESIMS quasi-molecular ion, $[M-H]^-$, at *m*/*z* 771.2256. An attempt to acquire the MS in a positive mode failed, most probably, due to the low stability of the sulfate moieties under these conditions. Its ^{1}H NMR spectrum revealed a tri-substituted aromatic moiety (δ_H 7.35 br s, 7.24 d, 7.16 br d), an AB quartet methylene (δ_H 4.91 and 4.82, J=14.0 Hz), three equivalent *N*-methyls (δ_H 3.04 br s), three vinylic methyls (δ_H 1.69 br s, 1.63 br s, and 1.57 br s), and a doublet methyl (δ_H 1.15 d). The ^{13}C NMR spectrum revealed 12 sp^2 carbons (between 167 and 119 ppm), 3 methines and a methylene next to electron-withdrawing groups (δ_C 78.0, 75.4, 73.4 and 63.1 ppm, respectively), a methyl signal at δ_C 51.8 ppm equivalent to 3 units by integration suggesting a tri-methyl ammonium moiety in **2** and 2 methylenes next to nitrogen (δ_C 47.0 and 45.9 ppm). The assignment of the structure of **2** started with the aromatic moiety. As stated above the chemical shifts and multiplicity of the three aromatic protons pointed to a three-substituted aromatic ring. COSY correlations established the *ortho*-relationship between H-4 and H-5 (J=8.4 Hz) and the *meta*-relationship between H-1 and H-5. Carbons 1, 4, and 5 were assigned from the correlation map of an HMQC experiment (see Table 2). Three-bond HMBC correlations of H-1 with C-5, H-5 with C-1 and both H-1 and H-5 with an oxygenated sp^2 carbon resonating at δ_C 149.5 ppm assigned the latter as C-3 of the aromatic system. H-4 presented two ^2J HMBC correlations with C-5 and C-3 and two ^3J correlations with the sp^2 carbons resonating at δ_C 133.9 and 129.8 ppm. Positioning of the latter two carbons in the aromatic ring was achieved with the aid of correlations from the two side-chains of the aromatic moiety. The methineoxy resonating at δ_H 5.16 ppm presented two long-range COSY correlations, as well as, NOE correlations with H-1 and H-5 and four HMBC correlations, two strong (^3J) with C-1 and C-5, a weaker with the carbon at δ_C 133.9 ppm and the weakest with the carbon at δ_C 129.8 ppm. Assuming that the weakest correlation will be the ^4J assigned the later two as C-6 and C-2, respectively. The methineoxy is thus situated on C-6 and was assigned as H-7. C-7 has the two expected ^3J HMBC correlations (see Table 2) with H-1 and H-5. COSY correlations allowed the extension of this side-chain to methineoxy-8 and methyl-9. C-2 didn't show any farther correlation in the HMBC map. C-1 and C-3, on the other hand, presented strong HMBC correlations with the AB quartet methylene at δ_H 4.91 and 4.82 ppm, assigning it as methyleneoxy-10, the C-2 substituent. This assignment is supported by the ^3J HMBC correlation of H-1 with C-10 (see Table 2). The relatively low chemical shifts of H-7, H-8, H-10, and H-10′ and the relatively high chemical shift of C-3 suggest that the oxygen atom next to them might be esterified. H-8, indeed presents an HMBC correlation to the carboxyl carbon at δ_C 166.6 ppm. The latter carbon (C-12) has additional correlation with a methine (H-13) resonating at δ_H 4.12 ppm. COSY and HMQC correlations allowed the extension of this methine to a 1,1,4-tri-substituted butane (see Table 2). HMQC correlation assigned the carbon resonating at δ_C 73.4 ppm to this methine. The chemical shift of this methine carbon usually points to an oxygenated carbon, but the HMBC correlations of H-13 with the carbon signal of the symmetrical tri-methyl ammonium moiety and of the proton-signal of this moiety with C-13 suggested that the tri-methyl ammonium (22, 22′, 22″) residue is attached to C-13. The chemical shift (47.0 ppm) of the methylene in the other termini of the butane unit suggested that a nitrogen atom substitutes it. This nitrogen (N-17) is tri-substituted since no COSY correlation was observed between methylene-16 and an acidic proton, while it posses NOE's with a four protons acidic signal at 7.40 ppm and another methylene next to a tri-substituted nitrogen (δ_H 3.86 m, 3.90 m, δ_C 45.9 ppm, CH_2-23). Methylene-16 exhibits three HMBC correlations with C-14, C-23 and an sp^2 carbon resonating at 156.2 ppm. The latter carbon (C-18) was assigned as a guanidine carbon on the basis of its HMBC correlation with methylene-16 and the latter NOE's with the four acidic protons singlet at δ_H 7.40 ppm. This assigned the segment 11 to 22″ as an ε-substituted-α-trimethyl ammonium arginine. Methylene-23 turned to be the head of a geranyl moiety. Like methylene-16, it exhibited HMBC correlations with C-16 and C-18 and NOE with the guanidine protons. Methylene-23 was connected to a vinylic methine through COSY correlation, but since the latter vinylic methine overlapped with a second vinylic methine COSY couldn't be used to farther extend this fragment. HMBC correlations of the vinylic methyl-31, on the

Table 1
NMR data of microphycin AL828 (**1**) in DMSO-d_6[a]

Position	δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H–C correlations[c]	NOE correlations[d]
Gly(I)-1	168.2, s		Gly(I)-2,2′, Val-2, NH	
2	43.2, t	3.33, dd, 16.8, 5.5; 3.85, dd, 16.8, 5.8		Ile-2, Val–NH
NH		7.70, t, 5.9		Ile-2, NH, Val–NH
Val-1	172.1, s		Val-2, Tyr–NH	
2	55.2, d	4.45, dd, 9.7, 3.5	Val-3,4,5	Tyr–NH
3	32.0, d	1.84, m	Val-2,4,5	Tyr–NH
4	17.7, q	0.69, d, 6.7	Val-5	
5	18.6, q	0.81, d, 3.5	Val-4	
NH		6.71, d, 9.7		Gly(I)-2′, NH, Pro-2
Tyr-1	171.4, s		Tyr-2,3,3′, Gly(II)–NH,2,2′	
2	57.0, d	3.94, m	Tyr-3,3′	Gly(II)–NH
3	35.0, t	2.76, d, 7.5	Tyr-2,5,5′	Gly(II)–NH
4	126.7, s		Tyr-3,3′,5,5′,6,6′	
5,5′	129.8, d×2	6.98, d×2, 8.4	Tyr-3,3′,5′,5	
6,6′	114.9, d×2	6.65, d×2, 8.4	Tyr-6′,6, 7-OH	
7	155.9, s		Tyr-5,5′,6,6′, 7-OH	
7-OH		9.21, s		
NH		8.64, br d, 2.6		Val-2,3
Gly(II)-1	168.3, s		Gly-2,2′, Leu-2,NH	
2	42.2, t	3.16, dd, 16.8, 3.8; 4.00, dd, 16.8, 8.2		
NH		8.44, dd, 8.2, 3.8		Leu–NH, Tyr-2,3
Leu-1	171.1, s		Leu-2, NH	
2	48.9, d	4.63, q, 6.3		Glu–NH, Pro-5,5′
3	39.4, t	1.38, m	Leu-4,5,6	
4	23.6, d	1.47, m	Leu-5,6	
5	22.2, q	0.89, d, 6.5	Leu-3,3′,6	
6	23.3, q	0.83, d, 6.5	Leu-3,3′,5	
NH		7.64, d, 8.5		Gly(II)–NH, Pro-5
Pro-1	174.1, s		Pro-2,3,3′, Glu–NH	
2	62.0, d	4.03, m	Pro-4,4′	Glu–NH, Ile–NH, Val–NH
3	28.7, t	2.13, m; 1.83, m	Pro-2,4,4′,5,5′	
4	24.5, t	1.85, m; 1.85, m	Pro-3,5	Glu–NH
5	46.9, t	3.44, m; 3.59, m		Leu-2, NH, Leu-2
Glu-1	171.6, s		Glu-2,3,3′, Ile-2,NH	
2	55.2, d	3.94, m	Glu-3,3′,4,4′	
3	25.4, t	1.86, m	Glu-2,4,4′	
4	30.2, t	2.35, m	Glu-2,3,3′	
5	174.0, s		Glu-3,3′,4,4′	
NH		8.33, br, d 4.4		Ile–NH, Leu-2, Pro-2,5′
Ile-1	171.3, s		Ile-2, Gly(I)-2,2′,NH	
2	56.6, d	4.42, dd, 10.0, 4.3	Ile-6	Gly(I)–NH
3	35.2, d	2.13, m	Ile-2	
4	24.1, t	1.17, m; 1.40, m	Ile-5,6	
5	11.5, q	0.78, t, 6.4		
6	15.3, q	0.78 d 6.3		
NH		7.45, d, 10.0		Glu–NH, Gly(I)–2′, NH, Pro-2

[a] Frequency: 500 MHz for ^{1}H, 125 MHz for ^{13}C.
[b] Multiplicity and assignment from HSQC experiment.
[c] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s, presented as a correlation of a proton with carbon in the row.
[d] Selected NOE's from a ROESY experiment.

other hand, with the vinylic (C-24 and C-25) and allylic (C-26) carbons established the C-23 to C-26 segment (see Table 2). Methylene-26 and -27 almost overlapped in the ^{1}H NMR spectrum and thus couldn't be correlated unequivocally, by neither COSY nor TOCSY experiments. The mutual ^2J HMBC correlations between those two methylenes and of methyl-30-protons with C-27, C-28, C-29, and C-32 (see Table 2) established the structure of the rest of the geranyl moiety. The elucidated structure counts to the molecular formula $C_{29}H_{47}N_4O_5$. Taking into consideration, the three oxygen atoms that are supposed to be esteric due to the chemical shifts of their protons and carbons, and the molecular formula of **2**, that was calculated from the HRMS molecular ion, we suggest that the later three oxygen atoms are sulfated. The structure of **2** is thus containing three sulfate anions and two ammonium ions in a unique structure that contains a double zwitterionic portion and a liphophylic portion in the same structure. The most intense fragment ions found in the negative ion HR ESI mass spectrum of **2** (see Scheme 1), absolutely support the proposed structure. Microguanidine AL772 (**2**) is related in structure to a group of similar compounds, aeruginamides A–C, that contain a nitrogen instead of oxygen at position 11 and were recently isolated from a strain of *Microcystis aeruginosa* collected in Japan.[13]

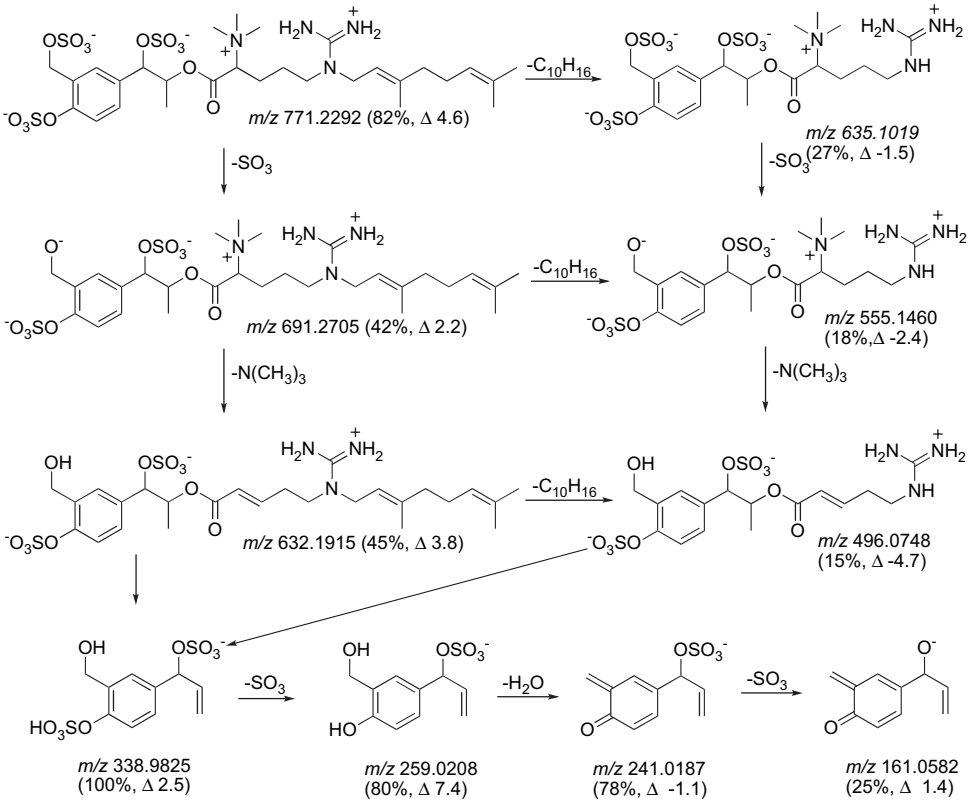
The third new compound, microginin AL584 (**3**), was isolated as a transparent solid with a HR MALDI TOF MS quasi-molecular ion peak of m/z 585.3032/587.3045 (3:1). The molecular formula calculated for the latter molecular ion is $C_{28}H_{45}ClN_4O_7$ (with a −2.3 mDa error). The ^{1}H and ^{13}C NMR spectra of **3** presented doublings of most of the proton and carbon signals at a ratio of ca. 4:1. The ^{1}H NMR data suggested a *para*-substituted phenol moiety, two amide protons, four protons next to electron-withdrawing groups (δ_H 4–5 ppm), an *N*Me group, and three aliphatic doublet methyl groups. The ^{13}C NMR data revealed four carboxyl carbon signals (δ_C 174.6, 172.5, 170.1, 169.4 ppm), a *para*-substituted phenol moiety, and four methine carbons next to nitrogen and one next to oxygen (δ_C 48.8, 53.3, 53.9, 62.0, and 71.3 ppm, respectively). This NMR data (see Table 3) suggested that **3** is a tetrapeptide. The structure of both rotamers was elucidated simultaneously, but for the clarity of the discussion only the structural elucidation of the major rotamer is presented below. The results from COSY, TOCSY, and HSQC 2D NMR experiments allowed the assignment of the side

Table 2
NMR data of microguanidine AL772 (**2**) in DMSO-d_6[a]

Position	δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H–C correlations[c]	NOE correlations[d]
1	125.9, d	7.35, br s	H-5,7,10a,10b	H-7,10a,10b, H_3-22,22′,22″
2	129.8, s		H-4,7	
3	149.5, s		H-1,4,5,10a,10b	
4	120.6, d	7.24, d, 8.3		H-5
5	126.0, d	7.16, br d, 8.3	H-1,4,7	H-4,7
6	133.9, s		H-4,7	
7	78.0, d	5.16, br d, 5.2	H-1,5, H_3-9	H-1,5
8	75.4, d	5.21, dq, 5.2, 6.4	H-7, H_3-9	
9	16.1, q	1.15, d, 6.4	H-7,8	
10	63.1, t	4.82, d, 14.0; 4.92, d, 14.0	H-1	H-1, H-1
12	166.6, s		H_3-9, H-13	
13	73.4, d	4.12, br d, 10.5	H_3-22,22′,22″	
14	23.4, t	2.06, m; 1.81, m	H-15a, H_2-16	
15	23.6, t	1.50, m; 1.35, m	H-13,14b	
16	47.0, t	3.26, m	H-23a	H_2-19
18	156.2, s		H_2-16, H-23a,23b	
19		7.40, br s		H_2-16
20		7.40, br s		H-23a,23b,24
22,22′,22″	51.8, q	3.04, s	H-13, H_3-22′,22″,22	H-1
23	45.9, t	3.90, m; 3.86, m	H_2-16, H-24	H_2-20, H_2-20
24	119.3, d	5.07, m	H-23a,23b, H_3-31	H_2-20
25	140.4, s		H-23a,23b, H_3-31	
26	31.7, t	2.03, m	H-24, H_2-27, H_3-31	
27	26.1, t	2.00, m	H_2-26, H-28	
28	123.9, d	5.07, m	H_2-26, H_3-30,32	
29	131.5, s		H_2-27, H_3-30,32	
30	17.8, q	1.57, br s	H_3-32	
31	23.2, q	1.69, br s	H-24	
32	25.7, q	1.63, br s	H_3-30	

[a] Frequency: 500 MHz for ^{1}H, 125 MHz for ^{13}C.
[b] Multiplicity and assignment from HSQC experiment.
[c] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s, presented as a correlation of a proton with carbon in the row.
[d] Selected NOE's from a ROESY experiment.

chains of Ala and Val and partial structure of the side chain of tyrosine. The structure of the side chain of tyrosine, the attachment of *N*Me to the Val and the assignment of the carboxyl carbons of Tyr, Ala and Val were achieved by analysis of the results of an ^{1}H–^{13}C HMBC experiment (see Table 3). The absolute stereochemistry of these amino acids was established by Marfey's procedure for



Scheme 1. Fragmentation pattern of the molecular ion of the negative HRESIMS of microguanidine AL772 (**2**).

Table 3
NMR data of microginin AL584 (**3**) in DMSO-d_6[a]

Position	δ_C, mult.[b]	δ_H, mult., J(Hz)	LR H–C correlations[c]	NOE correlations[d]
ClAhda 1	170.0, s		ClAhda-2, Ala-NH	
2	71.3, d	4.19, br s		ClAhda-3, Ala-NH
3	53.3, d	3.30, m		ClAhda-2,4,5
4	27.3, t	1.32, m	ClAhda-2	ClAhda-3
5	27.3, t	1.24, m		ClAhda-3
6	28.8, t	1.26, m	ClAhda-7	
7	28.8, t	1.26, m	ClAhda-5	ClAhda-8,10
8	26.3, t	1.32, m	ClAhda-9,10	ClAhda-7,9,10
9	32.1, t	1.67, m	ClAhda-10	ClAhda-8,10
10	45.5, t	3.60, t, 6.5	ClAhda-9	ClAhda-7,8,9
Ala 1	172.0, s		Ala-2,3, *N*MeVal-*N*Me	
2	44.8, d	4.64, dq, 7.4, 6.8	Ala-3	Ala-3, *N*MeVal-*N*Me
3	17.4, q	1.15, d, 6.8	Ala-2	Ala-2,NH, *N*MeVal-*N*Me
NH		7.94, d, 7.4		Ala-2,3, ClAhda-2
*N*MeVal 1	169.0, s		*N*MeVal-2	
2	62.0, d	4.54, d, 10.8	*N*MeVal-*N*Me	*N*MeVal-3,4,5,*N*Me, Tyr-2
3	26.3, d	2.06, m	*N*MeVal-2,4,5	*N*MeVal-2,4,5,*N*Me
4	18.6, q	0.66, d, 6.5	*N*MeVal-5	*N*MeVal-2,3,5,*N*Me
5	19.6 q	0.85, d, 6.1	*N*MeVal-4	*N*MeVal-2,3,4,*N*Me
*N*Me	30.2, q	2.79, s	*N*MeVal-2	*N*MeVal-2,3,4,5, Ala-2,3
Tyr 1	174.5, s		Tyr-3a	
2	53.4, d	4.24, m		Tyr-3a,3b,5,5′, *N*MeVal-*N*Me
3	35.7, t	2.78, m; 2.93, dd, 9.9, 3.4		Tyr-2,3b, Tyr-2,3a
4	128.2, s		Tyr-3a,3b,6,6′	
5,5′	130.0, d×2	6.92, d, 8.0	Tyr-3a,3b,5′,5,6,6′	Tyr-2,3a,3b,6,6′
6,6′	114.9, d×2	6.60, d, 8.0	Tyr-5,5′,6′,6	Tyr-5,5′
7	155.9, s		Tyr-5,5′,6,6′	
7-OH		9.13, s		
NH		7.94, br d, 7.0		

[a] Frequency: 500 MHz for ^{1}H, 125 MHz for ^{13}C.
[b] Multiplicity and assignment from HSQC experiment.
[c] Determined from HMBC experiment, $^nJ_{CH}$=8 Hz, recycle time 1 s, presented as a correlation of a proton with carbon in the row.
[d] Selected NOE's from a ROESY experiment.

HPLC,[12] which demonstrated the L-stereochemistry of all three amino acids. The structure of the 3-amino-10-chloro-2-hydroxy-decanoic acid (Achda) was elucidated as follows. The amidic carboxyl resonating at 170.1 ppm presents an HMBC correlation (^2J) with a methineoxy proton resonating at δ_H 4.19 ppm (δ_C 71.3 ppm). The latter is connected to an amino-methine (δ_H 3.30, δ_C 53.3 ppm) through a COSY correlation, which is in turn connected, through COSY and TOCSY to an aliphatic chain terminated by a chloromethylene (see Table 3). Methylene-4, -5, -8, and -9 were established unequivocally by the NMR data (COSY, TOCSY, HSQC, and HMBC) analysis while methylenes-6 and 7 were established by combination of the NMR data and the mass weight of **3** (see Table 3). The assignment of the (2*S*,3*S*) stereochemistry to the stereogenic centers of Achda, in **3**, is based on the comparison of the NMR data of the protons and carbons at these centers (2-CH–OH: δ_C 71.3 d, δ_H 4.19 br s; 3-CH–NH_2: δ_C 53.3 d, δ_H 3.30 m) with those of the following four synthetic stereoisomers of the natural product microginin 299-A:[14] (2*S*,3*R*)-Ahda (2-CH–OH: δ_H 4.05 br s; 3-CH–NH_2: δ_H 3.22 m), (2*S*,3*S*)-(Ahda) (2-CH–OH: δ_H 4.17 br s; 3-CH–NH_2: δ_H 3.22 m), (2*R*,3*R*)-(Ahda) (2-CH–OH: δ_H 4.17 br s; 3-CH–NH_2: δ_H 3.07 m), and (2*R*,3*S*)-Ahda (2-CH–OH: δ_H 4.07 br s; 3-CH–NH_2: δ_H 3.18 m). The similarity of the chemical shifts of the carbons and protons of the 2*S*,3*S* centers in natural microginin 299-A (2-CH–OH: δ_C 70.6 d, δ_H 4.23 br s; 3-CH–NH_2: δ_C 53.0 d, δ_H 3.37 m) and in compound **3** (see above) suggest the same 2*S*,3*S* stereochemistry in **3**. The sequence of the amino acids in the peptide was assigned on the basis of HMBC and ROESY correlations as follows: HMBC correlations between the carboxyl of Achda and the amide proton of Ala, Ala carboxyl and NMe of NMeVal; NOE's of Achda-2 with Ala-2 and NH, Ala-2 and 3 with the NMe of NMeVal and NMeVal-2 with Tyr-2. These HMBC and ROESY correlations established the sequence of the peptide as: *2S,3S*-Achda-L-Ala-L-NMeVal-L-Tyr.

Compounds **1**–**3** were isolated through a serine protease (chymotrypsin and trypsin) inhibition-guided separation. Pure **2** was found to be inactive in the assay, while **1** and **3** partially inhibited the proteolytic activity of trypsin at a concentration of 45 μg/mL.[15] Compounds **1**–**3** didn't inhibit chemotrypsin,[15] papain[15] or Bovine amino peptidase N[16] at a concentration of 45 μg/mL. Compounds **2** and **3** selectively induce an irreversible growth arrest of *Saccharomyces cerevisiae* expressing the multifunctional adenovirus regulator, E4orf4, with MIC of 12.5 μg/mL, but not wild type or E4orf4-*cdc55Δ* mutant of *S. cerevisiae*. This assay was designed to simulate the induction of p53-independent, protein phosphatase 2A-depenent apoptosis in transformed mammalian cells.[17] To investigate the anticancer potential of compounds **1**–**3** they were assayed against four solid tumor (MCF7— breast, HCT116—colon, A549—lung, PC3—prostate) and one leukemia (Molt-4) cell lines and found inactive at a concentration of 1 μg/mL.[18]

3. Experimental

3.1. Instrumentation

Mass spectra were recorded on an Applied Biosystems Voyager System 4312 instrument. MS/MS data was recorded on an Applied Biosystems Inc. QSTAR Pulsar I mass spectrometer. IR spectra were obtained with a Bruker FTIR Vector 22 spectrometer. UV spectra were recorded on a Kontron 931 plus spectrophotometer. Optical rotation values were obtained on a Jasco P-1010 polarimeter at the sodium D line (589 nm). NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C and a Bruker Avance 400 Spectrometer at 400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C. DEPT, COSY-45, gTOCSY, gROESY, gHSQC, gHMQC, gHMBC, gNHHSQC, and gNHHMBC spectra were recorded using standard Bruker pulse sequences. 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.

3.2. Biological material

Microcystis sp., TAU strain IL-306, was collected in October 2002 from a water reservoir next to Kibbutz Alonim, Valley of Armageddon, Israel.

3.3. Isolation procedure

The freeze-dried cells (149.8 g) were extracted with 7:3 MeOH/H_2O (3×3 L). The crude extract (8.2 g) was evaporated to dryness and separated on an ODS (YMC-GEL, 120A, 4.4×6.4 cm) flash column with increasing amounts of MeOH in water. Fraction 9 (4:1 MeOH/H_2O, 303.3 mg) was subjected to a Sephadex LH-20 column in 1:1 chloroform/methanol to obtain 13 fractions. Fractions 6–8 (86.9 mg) were subjected to a reversed-phase HPLC (YMC-Pack C-8 250 mm×20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 1:1 water/acetonitrile to obtain five fractions: fraction 2 (27.2 mg, retention time of 16.8 min) was subjected to a reversed-phase HPLC (YMC-Pack, 250 mm×20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 9:11 acetonitrile/0.1% TFA in water to obtain five fractions. Compound **1** (4.7 mg, 0.003% yield based on the dry weight of the bacteria) was eluted from the column with retention time of 15.1 min. Fraction 5 (3:2 MeOH/H_2O, 151.1 mg) from the flash column was subjected to a Sephadex LH-20 column in 1:1 water/methanol to obtain fourteen fractions. Compound **2** (fractions 12–14, 12.4 mg, 0.008% yield based on the dry weight of the bacteria) was eluted from the column in fraction 12. Fraction 6 from the flash column (1:1 MeOH/H_2O, 178.0 mg) was subjected to a Sephadex LH-20 column in 1:1 chloroform/methanol to obtain 12 fractions. Fractions 8–11 (64.0 mg) were subjected to reversed-phase HPLC (YMC-Pack C-8 250 mm×20.0 mm, DAD at 210 nm, flow rate 5.0 mL/min) in 7:13 acetonitrile/0.1% TFA in water to obtain nine fractions. Compound **3** (7.2 mg, 0.005% yield based on the dry weight of the bacteria) was eluted from the column with a retention time of 37.3 min. Compound **4** (7.4 mg, 0.005% yield based on the dry weight of the bacteria) was eluted from the column with a retention time of 27.4 min. Compound **5** (3.3 mg, 0.002% yield based on the dry weight of the bacteria) was eluted from the column with a retention time of 46.0 min. Compound **6** (13.5 mg, 0.009% yield based on the dry weight of the bacteria) was eluted from the column with a retention time of 18.9 min.

3.3.1. *Microphycin AL828 (**1**)*

$[\alpha]_D^{22}$ +16.0 (*c* 1.0, MeOH); IR ($CHCl_3$) ν_{max} 3665, 3455, 2970, 1672, 1655, 1580, 1260 cm^{-1}; UV λ_{max} (MeOH) 220 nm (ε 15,900), 274 nm (ε 290); for NMR data see Table 1; positive MALDI TOF MS *m*/*z* 828.6 $[M+Na]^+$; HR MALDI TOF MS *m*/*z* 851.4272 (MNa^+, calcd for $C_{40}H_{60}N_8O_{11}$ *m*/*z* 851.4275).

3.3.2. *Microguanidine AL772 (**2**)*

$[\alpha]_D^{22}$ +8.5 (*c* 2.0, MeOH); IR ($CHCl_3$) ν_{max} 3663, 3460, 2930, 1660, 1630, 1570, 1250, 1050, 1000 cm^{-1}; UV λ_{max} (MeOH) 269 nm (ε 1217); for NMR data see Table 2; HR ESI MS *m*/*z* 771.2292 (MH^-, calcd for $C_{29}H_{47}N_4O_{14}S_3$ *m*/*z* 771.2256).

3.3.3. *Microginin AL584 (**3**)*

$[\alpha]_D^{20}$ −18.1 (*c* 1.5, MeOH); IR ($CHCl_3$) ν_{max} 3669, 3460, 2940, 2840, 1657, 1633, 1490, 1245, 1020, 1000 cm^{-1}; UV λ_{max} (MeOH) 217 nm (ε 9920), 270 nm (ε 250); for NMR data see Table 3; HR MALDI TOF MS *m*/*z* 607.2838 (MNa^+, calcd for $C_{28}H_{45}ClN_4NaO_7$ *m*/*z* 607.2869).

3.3.4. *Anabaenopeptin F (**4**)*

$[\alpha]_D^{20}$ −15.0 (*c* 1.5, MeOH); UV λ_{max} (MeOH) 215 (ε 11,900), 279 nm (ε 1770); NMR data was found to identical with reported data.[9] HR positive MALDI TOF MS *m*/*z* 851.5114 (MH^+, calcd for $C_{42}H_{63}N_{10}O_9$ *m*/*z* 851.5111).

3.3.5. *Oscillamide Y (**5**)*

$[\alpha]_D^{20}$ −21.3 (*c* 1.3, MeOH); λ_{max} (MeOH) 210 (ε 15,437), 279 nm (ε 916); positive MALDI TOF MS *m*/*z* 857.4 $[M+K]^+$; NMR data was found to identical with reported data;[10] HR MALDI TOF MS *m*/*z* 896.3964 (MK^+, calcd for $C_{45}H_{59}KN_7O_{10}$ *m*/*z* 896.3955).

3.3.6. *Microcin SF608 (**6**)*

$[\alpha]_D^{20}$ −24.1 (*c* 1.5, MeOH); λ_{max} (MeOH) 225 (ε 5476), 278 nm (ε 1114); positive MALDI TOF MS *m*/*z* 608.4 $[M+H]^+$; NMR data was found to identical with reported data;[11] HR MALDI TOF *m*/*z* 609.3586 (MH^+, calcd for $C_{32}H_{45}N_6O_6$ *m*/*z* 609.3582).

3.4. Determination of the absolute configuration of the amino acids

Portions of compounds **1**–**3** (0.5 mg) were dissolved in 6 N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 110 °C for 20 h. After removal of HCl, by repeated evaporation in vacuo, the hydrolyzate was resuspended in water (40 mL). A solution of (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA) (4.2 mmol) in acetone (150 mL) and 1 N $NaHCO_3$ (20 mL) were added to each reaction vessel and the reaction mixture was stirred at 40 °C for 2 h. A 2 N HCl solution (10 mL) was added to each reaction vessel and the solution was evaporated in vacuo. The *N*-[(-dinitrophenyl)-5-L-alanine amide]-amino acid derivatives, from hydrolyzates, were compared with similarly derivatized standard amino acids by HPLC analysis: Knauer GmbH Eurospher 100 C18, 10 m, 4.6×300 mm, flow rate: 1 mL/min, 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 hydrolyzates with the derivatized authentic amino acids. HPLC analysis of Marfey's derivatives of **1** and **3** established the following retention times of the derivatized amino acids: L-Glu, 26.8 min; L-Ala, 30.2 min L-Pro, 31.3 min; L-Val, 38.1 min; L-Ile, 43.8 min; L-Leu, 44.4 min; L-NMeVal, 46.0 min L-Tyr, 53.4 min; for the two compounds.

Acknowledgements

We thank Ella Zafrir and Smadar Moshe for the bioassays and Ayelet Sacher and Ran Rosen, The Mass Spectrometry Laboratory of The Maiman Institute for Proteome Research of Tel Aviv University, for the MALDI and ESI mass spectra. Part of the research was conducted within the framework of the National High Throughput Screening Center for novel bioactive compounds located at Tel Aviv University and supported by the Israel Ministry of Science grant 432-201. This study was supported by the Israeli Science Foundation grant 037/02 to S.C.

Supplementary data

^1H, ^{13}C, COSY, TOCSY, ROSEY, HMQC or HSQC and HMBC spectra of microphycin AL828, microguanidine AL772 and microginin AL584 and ^1H and ^{13}C NMR spectra of anabaenopeptin F, oscillamide Y and microcin SF608 are available. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.05.031.

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