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Inhibitors of Serine Proteases from a Waterbloom of the Cyanobacterium *Microcystis* sp.

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Abstract: Three new protease inhibitors, micropeptins SF909 (**1**) and SF995 (**2**) and microcin SF608 (**3**), were isolated from the hydrophilic extract of a *microcystis* sp. waterbloom. The planar structure of compounds **1-3** was determined by homonuclear and inverse-heteronuclear 2D-NMR techniques as well as high-resolution mass spectrometry. The absolute configuration of the asymmetric centers was studied using Marfey's method for HPLC. Micropeptin SF909 (**1**) inhibited chymotrypsin with IC_{50} of 4.0 μg/mL while micropeptin SF995 (**2**) and microcin SF608 (**3**) inhibited trypsin with IC_{50}'s of 0.2 and 0.5 μg/mL, respectively.

The vast majority of the toxic cyanobacterial waterblooms worldwide are formed by cyanobacteria of the genus *microcystis*. These waterblooms, found in eutrophic lakes, ponds and drinking water reservoirs, pose a risk to plant, animal and human health.[1-3] About 50% of the *microcystis* waterblooms show hepatotoxicity to mammals and other animals. The hepatotoxicity is induced by the well known cyclic heptapeptides, microcystins.[4] Recently, several groups of inhibitors of serine and amino proteases were isolated from toxic and non-toxic *microcystis* species.[5] The most abundant are depsipeptides, such as cyanopeptolin A,[6] which contain the modified amino acid, 3-amino-6-hydroxy-2-piperidone (Ahp). The modified linear peptides, aeruginosins (such as, aeruginosin 298-A[7]) and the linear peptides of the microginin type (such as, microginin[8]) are less abundant. The death of birds which was associated with *microcystis* sp. waterbloom, in the central pond of the Tel Aviv Safari, late in the summer of 1997 drew our attention and was the major driving force for the research work summarized below. A sample from this cyanobacterial waterbloom was collected and its extracts were subjected to biological-activity guided fractionation. None of the known hepatotoxins were present in the extracts of this waterbloom. Guided by protease inhibition assays, three new potent inhibitors of serine proteases, namely micropeptins SF909 (**1**) and SF995 (**2**) and microcin SF608 (**3**), were isolated.

The 70% methanol extract of the freeze-dried cyanobacterium (40 gr) was flesh-chromatographed on an ODS column. Fractions 4 and 5, eluted from the column with 75 and 85% MeOH in water, respectively, potently inhibited trypsin and chymotrypsin. The two fractions were farther purified on a reversed-phase HPLC column to yield micropeptins SF909 (**1**, 31.2 mg) and SF995 (**2**, 8.0 mg) and microcin SF608 (**3**, 20.0 mg).

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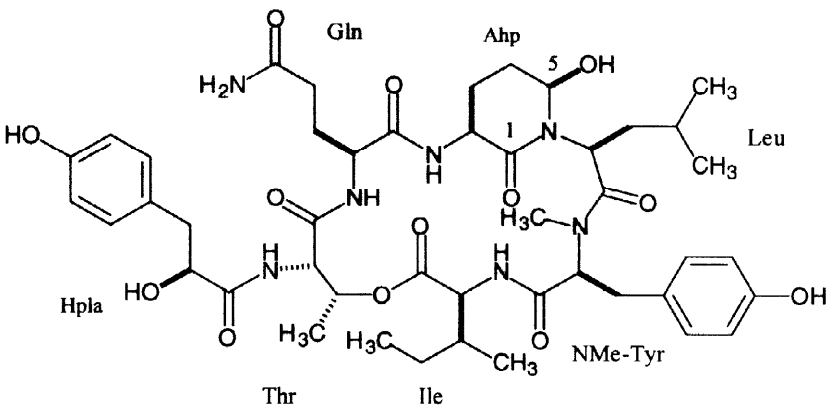
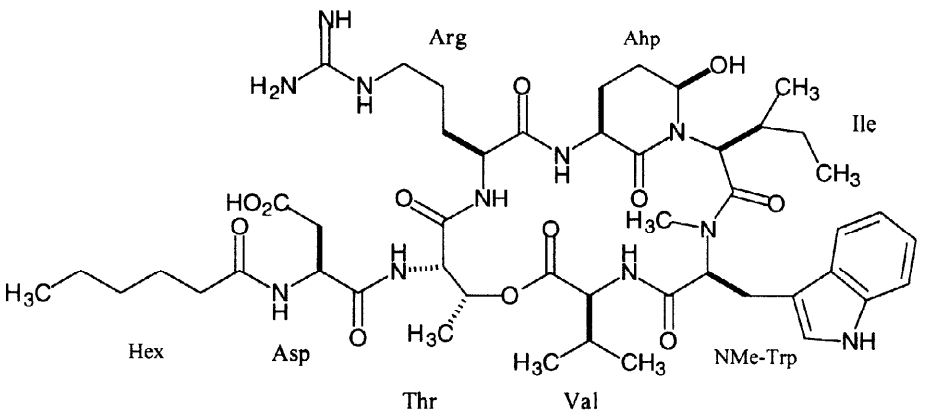
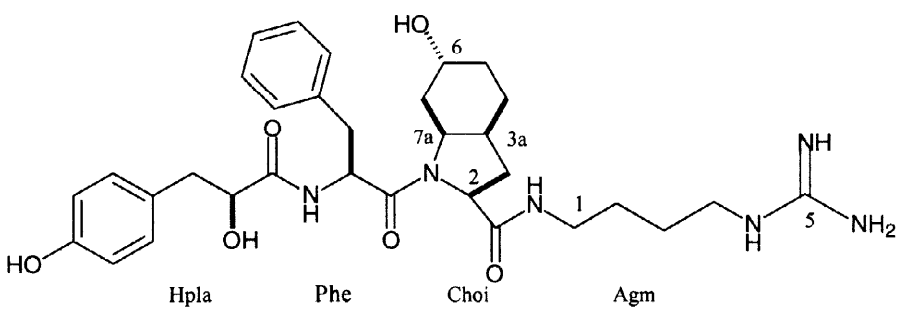


Micropeptin SF909 (**1**)



Micropeptin SF995 (**2**)



Microcin SF608 (**3**)

Micropeptin SF909 (**1**) was isolated as two separable (t_R 29.3 and 36.6 min, see Experimental Section) conformers of the amino-hydroxy-piperidone (Ahp). Both conformers in DMSO solution at room temperature slowly inter-converted to a 3:1 mixture. The ratio of the conformers in DMSO changed to 2:1 upon heating to 330°K. The 3:1 conformer mixture of compound **1**, in DMSO-d_6, was used for the structure elucidation. High resolution FAB MS data furnished a molecular formula of $C_{45}H_{63}N_7O_{13}$ for micropeptin SF909 (**1**). Analysis of the 1D (^1H, ^{13}C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (see Table 1 for the NMR data of the major conformer and Experimental Section for the NMR data of the minor

Table 1. NMR Data of the Major Conformer of Micropeptin SF909 (**1**).[a]

Position		δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H-C Correlations[c]	NOE Correlations[d]
Ile	1	172.3 s		Thr-3, Ile-2	
	2	55.0 d	4.59 dd 9.4,4.0	Ile-4,4',6,NH	Ile-2,3,4,5
	3	37.4 d	1.74 m	Ile-2,4,4',5	Ile-4',5,6
	4	24.4 t	1.25 m	Ile-2,3,5,6	Ile-4', Tyr-NMe
			1.03 m		Ile-4, Tyr-NMe
	5	11.0 q	0.82 t 6.6	Ile-4,4'	Ile-2,3, Tyr-NMe
	6	15.7 q	0.77 d 6.0	Ile-2	Ile-3
	NH		7.61 d 9.4		Tyr-2,NMe, Ile-2,3,4,4',5
NMeTyr	1	168.9 s		Ile-NH, Tyr-2	
	2	60.7 d	4.88 dd 12.0,2.2	Tyr-3,3',NMe	Tyr-3,NMe
	3	32.7 t	3.09 brd 12.1	Tyr-2,5,5'	Tyr-NMe,3'
			2.66 dd 12.1,12.0		Tyr-3
	4	127.2 s		Tyr-2,3,3',6,6'	
	5,5'	129.8 (dx2)	6.89 d 8.6 (x2)	Tyr-3,3',5',5	Tyr-2,3,3',6, Leu-2,6
	6,6'	115.2 (dx2)	6.63 d 8.6 (x2)	Tyr-5,5',6',6	Tyr-5, Leu-2,6
	7	155.9 s		Tyr-5,5',6,6'	
	NMe	30.2 q	2.67 s	Tyr-2	Tyr-3, Ile-4,4',5
Leu	1	170.7 s		Tyr-2,NMe, Leu-2	
	2	47.7 d	4.58 dd 9.3,5.6	Leu-3, Ahp-5	Leu-3,3',4,6, Tyr-NMe
	3	38.4 t	1.53 m	Leu-2,5,6	Leu-3',4,5,6
			0.44 ddd		Leu-3
			13.8,9.3,3.7		
	4	23.7 d	0.97 m	Leu-3,5,6	Leu-2,3,5,6
	5	23.2 q	0.68 d 7.3	Leu-6	Leu-2,3,4,6
	6	22.0 q	0.49 d 6.5	Leu-3,5	Leu-2,3,4,5
Ahp	1	168.8 s		Ahp-2,5	
	2	49.0 d	4.36 ddd	Ahp-3,3',4,4',NH	Aph-3',4, Leu-4
			16.0,9.2,6.6		
	3	22.0 t	2.52 m	Ahp-2	
			1.71 m		
	4	29.7 t	1.71m		
	5	73.4 d	4.87 brs	Leu-2	Ahp-3,4,OH, Leu-4
	NH		7.25 d 9.2		Ahp-2,3, Thr-3,Gln-2,NH
	OH		6.12 brs		Ahp-5
Gln	1	169.9 s		Ahp-2,NH, Gln-2,3,3'	
	2	52.0 d	4.24 ddd	Gln-3,3',4,4',NH	Gln-2,3,3',4,4'
			11.6,8.4,4.1		
	3	26.4 t	2.18 m	Gln-2,3,3',NH	Gln-2
			1.67 m		
	4	31.4 t	2.05 m	Gln-2,NH_2	Aph-4,4' , Gln- NH_2
			2.06 m		
	5	173.6 s		Gln-4,4', NH_2	
	NH_2		7.29 s		Gln-4
			6.72 s		
	NH		8.66 d 8.4		Aph-NH, Thr-2,3, Gln-2,3,4,4'
Thr	1	168.8 s		Gln-2,NH, Thr-2,3,NH	
	2	54.2 d	4.67 d 9.4	Thr-4,NH	Thr-4
	3	71.9 d	5.49 q 6.3	Thr-4	Thr-4
	4	17.7 q	1.14 d 6.3	Thr-2,3	Thr-2,3
	NH		7.67 d 9.4		Thr-2,3,4, Hpla-2,3'
Hpla	1	174.0 s		Thr-2,NH, Hpla-2,3,3'	
	2	72.2 d	4.17 dd 8.8,3.8	Hpla-3,3'	Hpla-3,3', Thr-4
	3	39.5 t	2.89 dd 13.8,3.8	Hpla-2,5,5'	
			2.56 dd 13.8,8.8		
	4	128.9 s		Hpla-2,3,3'6,6'	
	5,5'	130.1 d (x2)	7.01 d 8.6 (x2)	Hpla-3,3',5',5	Hpla-2,3,3'6
	6,6'	114.7 d (x2)	6.64 d 8.6 (x2)	Hpla-5,5',6',6	Hpla-5
	7	156.0 s		Hpla-5,5',6,6'	

[a]Carried out on an ARX-500 Bruker instrument. [b]Multiplicity and assignment from HMQC experiment. [c]Determined from HMBC experiment, $^nJ_{CH}$ = 8 Hz, recycle time 1s. [d]By ROESY experiment.

conformer) revealed the seven acid units that build compound **1**: isoleucine, NMe-tyrosine, N,N-disubstituted-leucine, Aph, glutamine, threonine and p-hydroxy-phenyl-lactic acid (Hpla). The proton and carbon NMR chemical shifts of these building units were found to be similar to the corresponding units in microcystilide A[9] and micropeptin A.[10] Despite the doubling of the signals, the proton and carbon signals were well resolved allowing the amino acid sequence to be determined independently by HMBC or ROESY experiments. The following inter-residual HMBC connectivities allowed the construction of the planar structure of **1**, Ile-NMeTyr-Leu-Ahp-Gln-Thr-Hpla, with an ester linkage between the hydroxyl of Thr and the carboxyl of Ile. The NH of isoleucine is coupled with the carbonyl of NMe-tyrosine. H-2 and the NMe of NMe-tyrosine are coupled with the carbonyl of leucine. H-2 of the leucine is coupled with C-5 of the Ahp unit and the Ahp's H-5 is coupled with the C-2 of leucine. H-2 and the NH of the Ahp are coupled with the carbonyl of glutamine. H-2 and the NH of the glutamine are coupled with the carbonyl of threonine. H-3 of the threonine is coupled with the carboxyl of the isoleucine, thus establishing the ester linkage of this cyclic depsipeptide. H-2 and the NH of threonine are coupled with the carbonyl of the Hpla. The data from the ROESY experiment (see Table 1) corroborate the acid sequence proposed above. The ROESY data and proton-proton coupling constants allowed the identification of the two conformers of the Ahp ring system (see Figure 1). Moreover, these conformers are associated with totally different conformations of the lactone ring, which were not studied in detail. The major conformer of the Ahp in **1**, is a chair conformer similar to that found in other Ahp containing depsipeptides (*i.e.* micropeptin 103[11] and micropeptin 478A[12]). The minor conformer is a twisted boat conformer in which the axial Ahp's H-2 interacts with both the pseudoaxial and pseudoeqatorial protons at position 3 and the 5-hydroxyl is pseudo-axial. The latter change in the conformation of the Ahp ring system (relative to the major conformer) influenced the orientation of the leucine residue in such a way that H-3 is upfield shifted due to a diamagnetic shielding by the current of the neighboring aromatic ring (see Figure 1). Acid hydrolysis of **1** and derivatization with Marfey's reagent,[13] followed by HPLC analysis, demonstrated the L-stereochemistry of the isoleucine, NMe-tyrosine, leucine, glutamine and threonine residues. Jones oxidation of **1**, followed by a similar

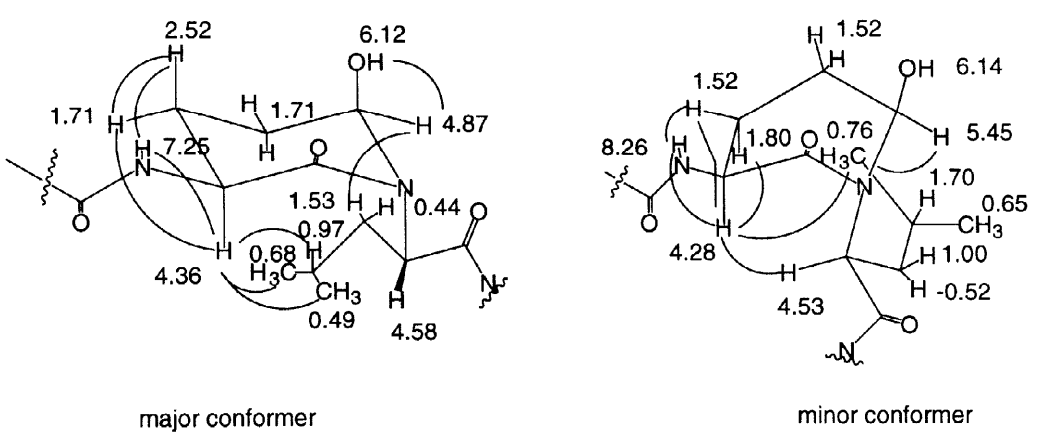


Figure 1. NOE's of the Ahp and Leu units in the two conformers of **1**.

Table 2. NMR Data of the Major Conformer of Micropeptin SF995 (**2**).

Position		δ_C, mult.	δ_H, mult., J (Hz)	LR H-C Correlations	NOE Correlations
Val	1	172.2 s		Val-2, Thr-3	
	2	56.2 d	4.70 dd 8.4,4.3	Val-3,4,5	Val-3,4,5,NH, Trp-3'
	3	30.8 d	2.06 m	Val-2,4,5	Val-2,4,5,Nh
	4	17.6 q	0.74 d 6.6	Val-2,3,5	Val-2,3, Aph-OH, Thr-3
	5	19.3 q	0.87 d 6.3	Val-3,4	Val-2,3
	NH		7.60 d 8.4		Val-2,3, Trp-2,NMe
NMeTrp	1	169.9 s		Trp-2, Val-2,NH	
	2	59.8 d	5.23 dd 11.9,2.1	Trp-3,NMe	Val-NH, Trp-3',9,NMe,Ile-2
	3	24.2 t	3.00 dd 12.9,11.9		Val-2,Trp-3',5,9
			3.09 brd 12.9		Trp-2,3,5,9
	4	109.2 s		Trp-3,3',5,9,NH	
	4a	127.6 s		Trp-3,5,6,8,9,NH	
	5	118.2 d	7.59 d 7.4	Trp-7	Trp-6, Ile-2,6
	6	118.5 d	6.93 t 7.4	Trp-8	Trp-5
	7	120.9 d	7.02 t 7.4	Trp-5	Ile-4',6
	8	111.4 d	7.28 d 8.0	Trp-6	Trp-NH, Ile-6
	8a	136.5 s		Trp-5,7,9,NH	
	9	124.5 d	7.12 s	Trp-3,3',8,NH	Trp-2,3,3',NH, Ile-2
	NH		10.83 s		Trp-8,9, Ile-6
	NMe	30.1 q	2.73 brs	Trp-2	Trp-2, Val-NH, Aph-5
Ile	1	170.1 s		Ile-2, Trp-NMe	
	2	53.9 d	4.38 d 10.3	Ile-6	Trp-2,5,9,Ile-3,4,4',5,6
	3	33.1 d	1.61 m	Ile-2,5,6	Ile-2,5,6, Aph-2
	4	23.6 t	0.88 m	Ile-5,6	Ile-2, Aph-5
			0.38 m		Ile-2, Trp-7
	5	10.2 q	0.38 t 6.3		Ile-2,3
	6	12.9 q	-0.72 d 6.3	Ile-2	Ile-2,3, Trp-5,7,8,9,NH, Aph-OH
Aph	1	169.4 s		Aph-5	
	2	49.2 d	4.35 m		Aph-3,3',NH
	3	21.9 t	2.54 m	Aph-5	Aph-3',NH
			1.17 m		Aph-2,3,5
	4	29.9 t	1.70 m (2H)		Aph-5
	5	73.2 d	4.86 brs	Ile-2, Aph-3	Aph-3',4,OH, Trp-NMe, Ile-3,4
	NH		7.35 d 8.8		Aph-2,3, Arg-2,NH, Thr-3
	OH		5.97 brs		Aph-5, Ile-6, Val-5
Arg	1	170.1 s		Aph-NH, Arg-2	
	2	52.1 d	4.32 brd 9.0		Arg-3,3',5,Nh, Aph-NH
	3	27.5 t	2.01 m	Arg-5	Arg-2,3',5,5'
			1.48 m		Arg-2,3,5,5',NH
	4	25.3 t	1.46 m	Arg-2	Arg-5,5'
			1.17 m		Arg-6(NH)
	5	40.1 t	3.09 m (2H)		Arg-2,6(NH)
	6(NH)		7.60 m		Arg-4,5
	7	156.8 s		Arg-5	
	NH		8.59 d 9.0		Arg-2,3, Aph-NH, Thr-2,3
Thr	1	169.1 s		Thr-2	
	2	54.8 d	4.61 brd 8.8	Thr-4	Arg-NH, Thr-NH
	3	72.1 d	5.48 brq 6.2	Thr-4	Thr-4, Arg-NH, Aph-NH, Val-4
	4	17.9 q	1.18 d 6.2	Thr-3	Thr-3,NH
	NH		7.67 d 8.8		Asp-2,NH, Thr-2,4
Asp	1	171.8 s		Thr-2,NH, Asp-3,3'	
	2	49.6 d	4.65 ddd	Asp-3,3'	Asp-3,3',NH, Thr-NH, Hex-2
			8.5,7.5,5.7		
	3	35.5 t	2.77 dd 17.3,5.7		Asp-2,NH
			2.51 dd 17.3,8.5		Asp-2,NH
	4	171.6 s		Asp-2,3,3'	
	NH		8.28 d 7.5		Thr-NH, Asp-2,3,3', Hex-2
Hex	1	172.9 s		Asp-NH, Hex-2,3	
	2	35.3 t	2.13 t 7.4	Hex-4	Asp-2
	3	24.9 t	1.52 tt 7.4,7.4	Hex-2	
	4	29.1 t	1.21 m	Hex-2,6	
	5	21.9 t	1.26 m	Hex-3,4,6	
	6	13.9 q	0.84 t 6.4	Hex-4	

hydrolysis, derivatization and HPLC analysis, demonstrated an L-stereochemistry for the Ahp residue (the oxidation and subsequent hydrolysis liberated glutamic acid from Ahp). The Hpla residue was determined as the L-form by HPLC comparison of the *l*-mentyl ester with the authentic diastereomers.[9]

Micropeptin SF995 (**2**) was isolated as an amorphous white solid. The molecular formula of **2**, $C_{48}H_{73}N_{11}O_{12}$, was deduced from high-resolution FAB MS measurements of its protonated molecular cluster ion (*m/z* 996.5527). It appears as a single isomer both in DMSO and 3:1 $CHCl_3$/MeOH solutions. Micropeptin SF995 (**2**) contains three modified amino acid residues (Ahp, NMe-Trp and N,N-disubstituted Ile), four normal amino acid residues (Val, Arg, Thr and Asp) and a fatty acid residue (hexanoic acid). Its ester linkage arises from the carbonyl of valine and the hydroxyl of threonine. The structures of the acid residues were determined by 1D (^1H, ^{13}C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (see Table 2). The amino acid sequence of micropeptin SF995 (**2**) was assembled by HMBC correlations (see Table 2) of the NH protons and vicinal carbonyl carbons (Val-NMe-Trp, Ahp-Arg, Thr-Asp and Asp-Hex) of the NMe protons and Ile-carbonyl and of the Ile-H-2 and C-5 of the Ahp residue. The ester bond was assigned by an HMBC correlation between H-3 of threonine and the carbonyl of valine, leaving one connection to be made by arginine and threonine. A ROESY experiment provided the Arg-Thr connection by correlating the NH of arginine to the α– and β-protons of threonine. The rest of the amino acid sequence can also be assembled from the ROESY data (see Table 2). The absolute configuration of the amino acids, of **2**, was determined by a similar procedure, to that described above for **1**. All amino acid residues have the L configuration.

Microcin SF608 (**3**) is the smallest of the protease inhibitors isolated from the extracts of this *microcystis* sp. waterbloom. High-resolution FAB MS measurements furnished a molecular formula of $C_{32}H_{44}N_6O_6$ for microcin SF608 (**3**). This peptide is made up of two amino acids, L-phenylalanine and 2-carboxy-6-hydroxyoctahydroindole (Choi), one α-hydroxyacid, p-hydroxy-phenyl-lactic acid (Hpla) and agmatine (or decarboxylated arginine). In DMSO solution, compound **3** appears as a 3:1 mixture of *trans*- and *cis*-rotamers of the Choi-Phe peptidic bond. The structures of the acid residues, as well as agmatine, were determined by 1D (^1H, ^{13}C and DEPT) and 2D (COSY, TOCSY, ROESY, HMQC and HMBC) NMR data (see Table 3 for the NMR data of the major rotamer and Experimental Section for the NMR data of the minor rotamer). HPLC experiments were used to determine that the absolute configuration of Phe[13] and Hpla[9] are of the L-form. The absolute configuration of Choi was not determined. The NMR data, of Choi and Hpla, is in accordance with the data published for both residues in aeruginosin 298-A.[7] The relative stereochemistry and conformation of the Choi residue, as well as the nature of its two rotamers, were elucidated by analysis of the ROESY spectrum (see Figure 2). Because of the modest size of microcin SF608, the two sets of proton and carbon NMR signals, of the two rotamers, were well resolved and hence the acid sequence could be determined by HMBC and ROESY experiments. The Agm-Choi sequence (for both isomers) was independently determined by the HMBC

Table 3. NMR Data of the Major Rotamer of Microcin SF608 (**3**).[a]

Position		δ_C, mult.[b]	δ_H, mult., J (Hz)	LR H-C Correlations[c]	NOE Correlations[d]
Agm	1	38.0 t	3.09 q 5.5 (2H)	Agm-3,1-NH	
	2	25.9 t	1.47 m (2H)	Agm-4	
	3	26.3 t	1.36 m (2H)	Agm-1	
	4	40.5 t	3.11 q 5.5 (2H)	Agm-2,4-NH	
	5	157.0 s		Agm-4,4-NH	
	1-NH		7.88 t 5.5		Agm-1,2, Choi-2
	4-NH		7.67 t 5.5		Agm-3,4
	5-NH,NH_2		9.13 brs		
Choi	-1	171.3 s		Agm-1,1-NH, Choi-2,3'	
	2	59.9 d	4.23 dd 9.2,8.6	Choi-3',7a	Choi-3,3a
	3	30.4 t	1.95 m	Choi-2,3a,7a	Choi-3'
			1.82 m		Choi-3,4
	3a	36.5 d	2.19 m	Choi-3,3'5,7a	Choi-3,4
	4	26.1 t	1.45 m (2H)	Choi-3a	
	5	19.1 t	1.40 m		Choi-5'
			2.03 m		Choi-5
	6	64.1 d	3.87 brs	Choi-5	Choi-5,7,7'
	7	34.3 t	1.69 brd 8.0	Choi-3a	Choi-6,7a, Phe-2,3,3'
	7a	54.6 d	4.41 dt 9.1,7.3	Choi-3,3a,5,6,7	Choi-5',7peq
Phe	1	169.6 s		Phe-2,3,3'	
	2	50.5 d	4.69 dt 5.5,7.3	Phe-3,3',NH	Phe-3,3', Choi-3a,7peq,7a
	3	38.2 t	2.78 dd 13.8,8.0	Phe-2,5,5'	Choi-7peq, Phe-3'
			2.89 dd 13.8,4.8		Choi-7peq, Phe-3
	4	137.0 s		Phe-2,3,3',6,6'	
	5,5'	129.5 d	7.15 d 7.2	Phe-3,3',5',(5),7	Phe-2,3,3', Choi-7peq
	6,6'	128.1 d	7.23 t 7.3	Phe-6'(6)	
	7	126.4 d	7.19 t 6.5	Phe-5,5'	
	NH		7.59 d 8.2		Phe-2,3,3', Hpla-2,3
Hpla	1	172.8 s		Phe-2,NH,Hpla-2,3,3'	
	2	72.3 d	3.91 brd 7.3	Hpla-3,3'	Hpla-3
	3	30.7 t	2.42 dd 13.9,8.3	Hpla-2,5,5'	Hpla-3'
			2.70 dd 13.9,3.4		Hpla-3
	4	128.4 s		Hpla-2,3,3',6,6'	
	5,5'	130.3 d	6.90 d 8.4	Hpla-3,3'5'(5)	
	6,6'	114.8 d	6.61 d 8.4	Hpla-5,5',6'(6)	
	7	155.7 s		Hpla-5,5'6,6'	
	2-OH		5.48 brs		

[a]Carried out on an ARX-500 Bruker instrument. [b]Multiplicity and assignment from HMQC experiment. [c]Determined from HMBC experiment, $^nJ_{CH}$ = 8 Hz, recycle time 1s. [d]Selected correlations from ROESY experiment, spin lock delay of 200 ms.

connectivity between Agm-1-NH and the Choi carbonyl and the ROESY correlation between Agm-1-NH and Choi-H-2. The Phe-Hpla sequence was determined in a similar way (see Table 3). The Choi-Phe connectivity was determined by the ROESY correlation between Choi H-7a and Phe H-2, for the major isomer, and between Choi H-2 and Phe H-2, for the minor isomer.

The inhibitory activity of **1-3** was determined for three enzymes, the serine protases trypsin and chymotrypsin and the metalloendopeptidase neprolysin. Micropeptin SF909 (**1**) inhibited chymotrypsin with IC_{50} of 4.0 μg/mL but not trypsin and neprolysine at 20.0 μg/mL. Micropeptin SF995 (**2**) and microcin SF608 (**3**) inhibited trypsin with IC_{50}'s of 0.2 and 0.5 μg/mL, respectively, but not chymotrypsin and neprolysine at 20.0 μg/mL.

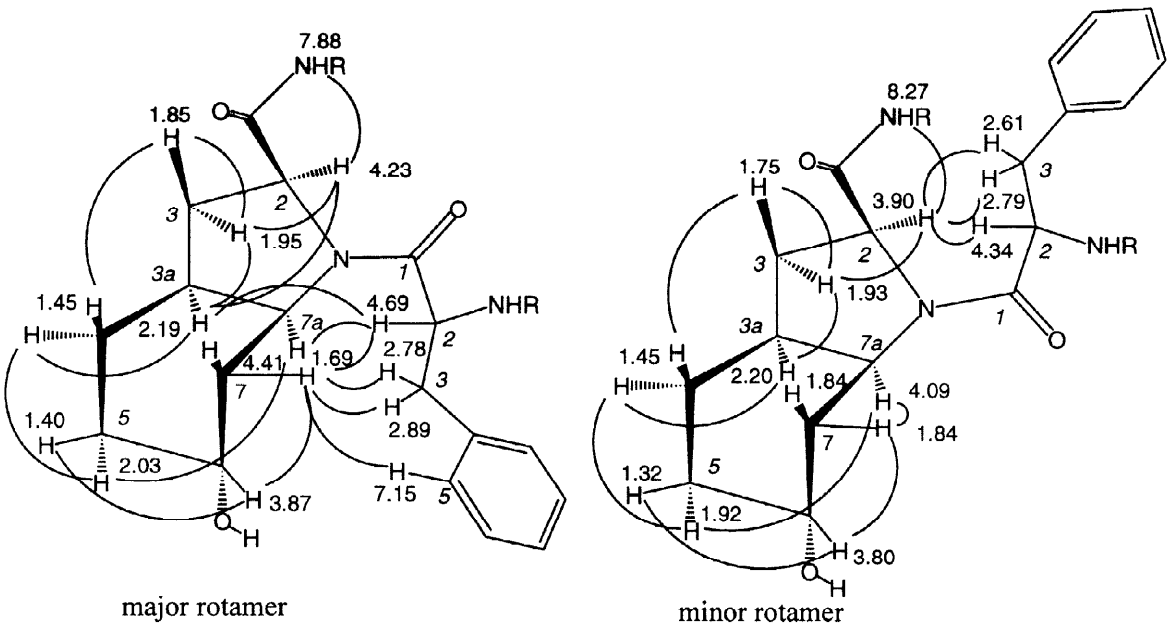


Figure 2. NOE's of the rotamers of **3**.

EXPERIMENTAL

Instrumentation. IR spectra were recorded on a Nicolet FTIR in $CHCl_3$ or neat. High resolution MS 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 on an ISCO HPLC system (model 2350 pump and model 2360 gradient programmer) equipped with an Applied Biosystem Inc. diode-array detector.
Isolation Procedure. The freeze-dried cells (40 gr) were extracted with 7:3 MeOH:H_2O. The crude extract (5 gr) was evaporated and separated on an ODS (YMC-GEL, 120Å, 4.4 x 6.4 cm) flash column with increasing amounts of MeOH in water. An attempt to dissolve fraction 4 (3:1 MeOH:H_2O) in MeOH yielded a white precipitation that was separated from the supernatant by decantation. The MeOH solution was subjected to a reversed-phase HPLC (Econosil C18 10 μm, 250 mm x 22.5 mm, DAD at 238 nm, flow rate 5.0 mL/min) to obtain **1** in 0.078% yield, based on the dry weight of the bacteria. The retention time of the major and minor conformers of **1** in 49:26:25 water/methanol/acetonitrile was 29.3 and 36.6 min, respectively. Fraction 5 (85:15 methanol/water) was separated on a reversed-phase HPLC (Econosil C18 10 μm, 250 mm x 22.5 mm, DAD at 238 nm, flow rate 5.0 mL/min) to obtain **2** in 0.022 % yield, based on the dry weight of the bacteria. The retention time of **2** in 35:65 0.1% TFA in water/acetonitrile was 17.7 min. The white precipitation that was separated from fraction 4 was dissolved with 1:1 methanol/water and separated on reversed-phase HPLC (Econosil C18 5 μm, 250 mm x 22.5 mm, DAD at 238 nm, flow rate 5.0 mL/min) to obtain **3** in 0.05 % yield, based on the dry weight of the bacteria. The retention time of **3** in 20:80 0.1% TFA in water/acetonitrile: was 29.6 min.
Micropeptin SF909 (1). $[\alpha]_D^{25}$ -38.5 (*c* 0.17, MeOH); UV λ_{max} (MeOH) 227 nm (ε 10388), 278 nm (ε 2376); For NMR data of the major conformer see Table 1; Minor conformer: δ_H (500 MHz, DMSO-d_6) Ile: 4.60 (dd, *J* 9.4, 4.0 Hz, 2-H), 1.96 (m, 3-H), 1.70 (m, 4-H) 1.00 (m, 4-H'), 0.84 (t, *J* 6.6 Hz, 5-H_3), 0.76 (d, *J* 6.0 Hz, 6-H_3), 8.68 (d, *J* 9.4 Hz, NH), NMeTyr: 4.75 (dd, *J* 11.9, 1.9 Hz, 2-H), 3.09 (brd, *J* 14.2 Hz, 3-H), 2.80 (dd, *J* 14.2, 11.9 Hz, 3'-H) 7.00 (d, *J* 8.4 Hz, 5,5'-H), 6.73 (d, *J* 8.4 Hz, 6,6'-H), 2.67 (brs, NMe-H_3), Leu: 4.53 (m, 2-H), 1.00 (m, 3-H), -0.52 (brt, *J* 12.1 Hz, 3'-H), 1.70 (m, 4-H), 0.76 (d, *J* 7.3 Hz, 5-H_3), 0.65 (d, *J* 6.6 Hz, 6-H_3) Ahp: 4.28 (ddd, *J* 5.1, 9.2, 9.6 Hz, 2-H), 1.80 (m, 3-H), 1.52 (m, 3-H'), 1.52 (m, 4-H_2), 5.45 (brs, 5-H), 8.26 (d, *J* 9.6 Hz, NH), 6.14 (brs, 6-**OH**), Gln: 4.62 (m, 2-H) 1.98 (m, 3-H), 2.44 (m, 3-H'), 1.93 (m, 4-H_2), 8.68 (d, *J* 9.4 Hz, NH), 6.64 and 7.34 (2 s, NH_2), Thr: 4.51 (dd, *J* 9.8, 1.4 Hz, 2-H), 5.12 (dq, *J* 1.4, 6.2 Hz, 3-H), 1.09 (d, *J* 6.2 Hz, 4-H_3), 7.51 (d, *J* 9.8 Hz, NH), Hpla: 4.06 (brd, *J* 10.0 Hz, 2-H), 2.91 (brd, *J* 14.0 Hz, 3-H), 2.45 (dd, *J* 10.0, 14.0 Hz, 3'-H), 7.06 (d, *J* 8.5 Hz, 5,5'-H), 6.67 (d, *J* 8.5 Hz, 6,6'-H); δ_C (125

MHz, DMSO-d_6) Ile: 169.3 (s, C-1), 55.8 (d, C-2), 34.8 (d, C-3), 23.5 (t, C-4), 11.8 (q, C-5), 15.6 (q, C-6), NMeTyr: 169.7 (s, C-1), 62.2 (d, C-2), 32.6 (t, C-3), 127.9 (s, C-4), 129.9 (d, C-5,5'), 114.8 (d, C-6,6'), 155.5 (s, C-7), 30.2 (q, C-NMe), Leu: 173.0 (s, C-1), 46.8 (d, C-2), 37.0 (t, C-3), 23.5 (d, C-4) 19.0 (q, C-5), 23.5 (q, C-6), Ahp: 171.3 (s, C-1), 49.0 (d, C-2), 21.5 (t, C-3), 30.8 (t, C-4), 79.7 (d, C-5), Gln: 166.4 (s, C-1), 52.0 (d, C-2), 28.6 (t, C-3), 30.9 (t C-4), 173.0 (s, C-5) Thr: 166.4 (s, C-1), 53.3 (d, C-2), 72.7 (d, C-3), 16.0 (q, C-4) Hpla: 173.6 (s, C-1), 72.5 (d, C-2), 39.6 (t, C-3), 129.1 (s, C-4), 130.8 (d, C-5,5'), 114.7 (d, C-6,6'), 156.0 (s, C-7); HRFABMS *m/z* 932.4377 (MNa^+, calcd. for $C_{45}H_{63}N_7NaO_{13}$, 932.4381).

Micropeptin SF995 (2). $[\alpha]_D^{25}$ -37.6 (*c* 0.42, MeOH); UV: λ_{max} (MeOH) 206 nm (ε 19263), 280 nm (ε 1434); For NMR data see Table 2; HRFABMS *m/z* 996.5527 (MH^+, calcd. for $C_{48}H_{73}N_{11}O_{12}$, 996.5518).

Microcin SF608 (3). $[\alpha]_D^{25}$ -19.1 (*c* 1.0, MeOH); UV λ_{max} (MeOH) 229 nm (ε 4360), 278 nm (ε 1038); For NMR data of the major rotamer see Table 3; Minor isomer: δ_H (500 MHz, DMSO-d_6) Agm: 8.27 (t, *J* 5.5 Hz, 1-NH), 2.94 and 3.19 (2 m, 2-H_2), 1.36 (m, 3-H_2), 3.11 (q, *J* 5.5 Hz, 4-H_2), 7.67 (t, *J* 5.5 Hz, 4-NH), 9.13 (brs, 5-NH and NH_2), Choi: 3.90 (m, 2-H), 1.75 and 1.93 (2 m, 3-H_2), 2.20 (m, 3a-H), 1.45 (m, 4-H_2), 1.32 and 1.92 (2 m, 5-H_2), 3.80 (brs, 6-H), 1.84 (m, 7-H_2), 4.09 (dt, *J* 9.1,7.3 Hz, 7a-H), Phe: 4.34 (dt, *J* 5.5 Hz, 2-H), 2.61 (dd, *J* 13.8, 4.9 Hz, 3-H), 2.79 (dd, *J* 13.8, 8.0 Hz, 3-H'), 6.97 (d x 2, *J* 7.2 Hz, 5,5'-H), 7.23 (dd, x 2, *J* 7.2, 6.5 Hz, 6,6'-H), 7.19 (t, *J* 6.5 Hz, 7-H), 7.51 (d, *J* 8.1 Hz, 2-NH), Hpla: 3.98 (dd, *J* 8.3, 3.4 Hz, 2-H), 5.80 (brs, 2-OH), 2.56 (dd, *J* 13.9, 8.3 Hz, 3-H), 2.77 (dd, *J* 13.9, 3.4 Hz, 3-H'), 6.98 (d x 2, *J* 8.4 Hz, 5,5'-H), 6.64 (d x 2, *J* 8.4 Hz, 6,6'-H); δ_C (125 MHz, DMSO-d6) Agm: 38.3 (t, C-1), 25.9 (t, C-2), 26.3 (t, C-3), 40.5 (t, C-4), 157.0 (s, C-5), Choi: 171.1 (s, C-1), 59.4 (d, C-2), 29.9 (t, C-3), 36.5 (d, C-3a), 26.2 (t, C-4), 19.1 (t, C-5), 64.1 (d, C-6), 34.6 (t, C-7), 54.4 (d, C-7a), Phe: 168.4 (s, C-1), 50.9 (d, C-2), 38.9 (t, C-3), 136.2 (s, C-4), 129.4 (d x 2, C-5,5'), 128.1 (d x 2, C-6,6'), 126.6 (d, C-7), Hpla: 172.3 (s, C-1), 72.0 (d, C-2), 30.5 (t, C-3), 128.4 (s, C-4), 130.5 (d x 2, C-5,5'), 114.8 (d x 2, C-6,6'), 155.8 (s, C-7); HRFABMS *m/z* 609.3405 (MH^+, calcd. for $C_{32}H_{44}N_6O_6$, 609.3400).

Determination of Absolute Configuration of the Amino Acids. 0.25-mg portions of **1**, **2** or **3** were dissolved in 6 N HCl (1 mL). The reaction mixture was then placed in a sealed glass bomb at 104°C for 20 h. In another experiment, 0.25-mg portions of **1** or **2** were first oxidized with Jones reagent[14] (1 drop) in acetone (1 mL) at 0°C for 10 min. Following the usual workup, the residue was dissolved in 6 N HCl (1 mL) and placed in a sealed glass bomb at 104°C for 20 h. After removal of HCl, by repeated evaporation *in vacuo*, the hydrolysate was resuspended in water (200μL) and derivatized with (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide (FDAA).[13] The N-[(2,4-dinitrophenyl)-5-L-alanine amide]-amino acid (AA) derivatives, from hydrolysates, were compared with similarly derivatized standard AA by HPLC analysis: Alltech C_{18} Econosil, 10μ, 4.6 x 250 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 hydrolysates with the derivatized authentic amino acids. Retention times of the derivatized amino acids were L-Arg, 24.5 min; D-Arg, 26.0 min; L-Asp, 30.6 min; D-Asp, 32.3 min; L-Glu, 32.0 min; D-Glu, 34.5 min; L-Ile, 51.9 min; D-Ile, 58.1 min; L-Leu, 53.5 min; D-Leu, 58.0 min; L-Phe, 50.0 min, D-Phe, 53.3 min; L-Thr, 29.7 min, D-Thr, 35.0 min; L-Val, 45.0 min; D-Val, 51.6 min; L-NMe-Trp, 46.3 min; D-NMe-Trp, 47.0 min; L-NMe-Tyr, 62.5 min; D-NMe-Tyr, 63.2 min. HPLC analysis of derivatized hydrolysates of **1** established L-Glu, L-Ile, L-Leu, L-Thr and L-NMe-Tyr; that of **2**, established L-Arg, L-Asp, L-Ile, L-Thr, L-Val and L-NMe-Trp and that of **3**, established L-Phe. HPLC analysis of the FDAA derivatives of oxidized **1** and **2** hydrolysates established L-Glu for both compounds **1** and **2**, and thus confirmed the L configuration of the Ahp units in these compounds.

Determination of Absolute Configuration of the Hydroxy-Acids. 0.25-mg portions of **1** and **3** were dissolved in 6 N HCl (1 mL) and the reaction mixture was then placed in a sealed glass bomb at 104°C for 20 h. The ethereal extract of the acid hydrolysate was dried and the residue was treated with *l*-menthol in toluene with *p*-toluenesulfonic acid under reflux for 72 h. The reaction mixture was washed with $NaHCO_3$ solution and water, dried and analyzed on Saulentechnik, Eurosphere 100-C18 (5μm, 300 x 4.6 mm) HPLC column using 65:35 water/acetonitrile as eluent at a flow rate of 1 mL/min. The D-isomer showed a retention time of 29.3 min and the L-isomer a retention time of 31.8 min. The Hpla residue, from both **1** and **2**, were determined as L-form.

Protease Inhibition Assays. Trypsin and chymotrypsin were purchased from Sigma Chemical Co. Neprilysin was purified from bovine kidney membranes.[15] 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. Neprilysin was dissolved in 50 mM Tris-HCl/100 mM NaCl/1 mM $CaCl_2$/0.25 mM Hepes/0.005% Triton X-100 to prepare a 1 mg/mL solution. A 2 mM solution of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (for trypsin), Suc-Gly-Gly-*p*-nitroanilide (for chymotrypsin) and Suc-Pro-Ala-Leu-*p*-nitroanilide (for neprilysin) in the appropriate buffer solution was used as 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 of 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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