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New microviridins from a water bloom of the cyanobacterium *Microcystis aeruginosa*

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Abstract—Three new microviridins namely, SD1684 (**1**), SD1634 (**2**), and SD1652 (**3**), were isolated from the hydrophilic extract of *Microcystis aeruginosa*. The planar structures of compounds **1–3** were determined by homonuclear and inverse-heteronuclear 2D-NMR techniques as well as by high-resolution mass spectrometry. The absolute configuration of the asymmetric centers was studied using Marfey's method for HPLC. Compounds **1–3** contain L-*threo*-β-hydroxy aspartic acid as a building block of the peptide chain. This is the first example where microviridins contain non-proteinogenic amino acid in their structure. Compound **2** is a mild serine protease inhibitor.
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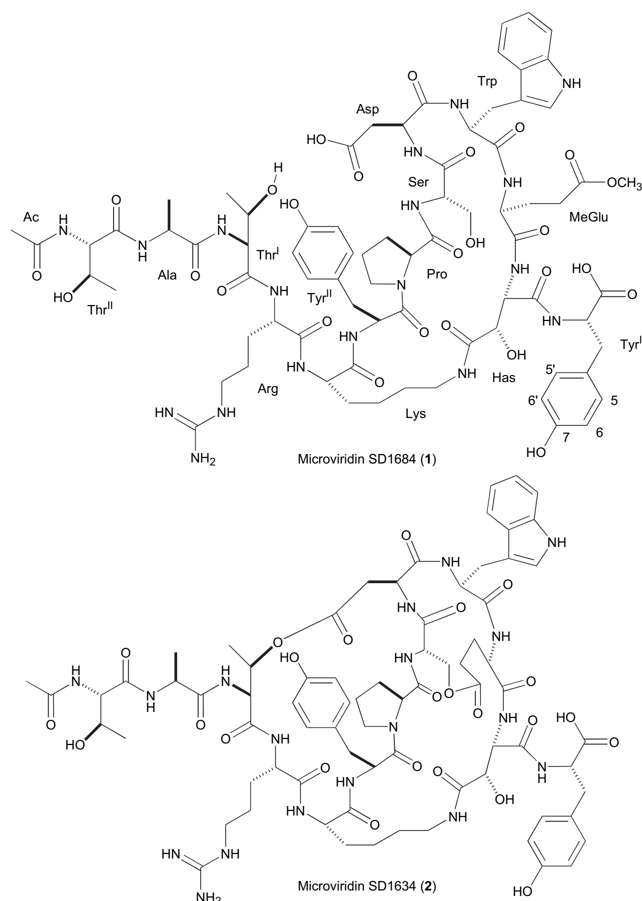
1. Introduction

Microviridins are cyclic peptides produced by strains of cyanobacteria (*Microcystis*, *Nostoc*, and *Oscillatoria* spp.) that produce massive water blooms and have the capacity to synthesize hepatotoxins of the microcystins family.[1] They contain either 13 or 14 L-amino acids, nine of which are conserved: Tyr$^{(1)}$, Glu$^{(3)}$, Trp$^{(4)}$, Asp$^{(5)}$, Ser$^{(6)}$, Pro$^{(7)}$, Tyr$^{(8)}$, Lys$^{(9)}$, and Thr$^{(11)}$. They consist of a monocyclic peptide structure with zero to two lactone bridges, which produce a rigid core structure between positions 2 and 11. Ten cyclic peptides of this type have thus far been characterized from extracts of water-bloom-forming cyanobacteria. Microviridin A was isolated from *Microcystis viridis* as tyrosinase inhibitor.[2] Microviridins B and C were isolated from *Microcystis aeruginosa* as elastase inhibitors.[3] Microviridins D, E, and F were isolated from *Oscillatoria* (*Planktothrix*) *agardhii* as an elastase and chymotrypsin inhibitors.[4] Microviridins G and H were isolated from *Nostoc minutum* as elastase inhibitors.[5] Microviridin I was isolated from *O. (P.) agardhii* as an elastase, chymotrypsin, and trypsin inhibitor.[6] Microviridin J was isolated from a cultured *M. aeruginosa* and possesses potent toxicity to *Daphnia* and inhibits serine proteases.[7] Here we report the isolation and structure elucidation of three new microviridins.



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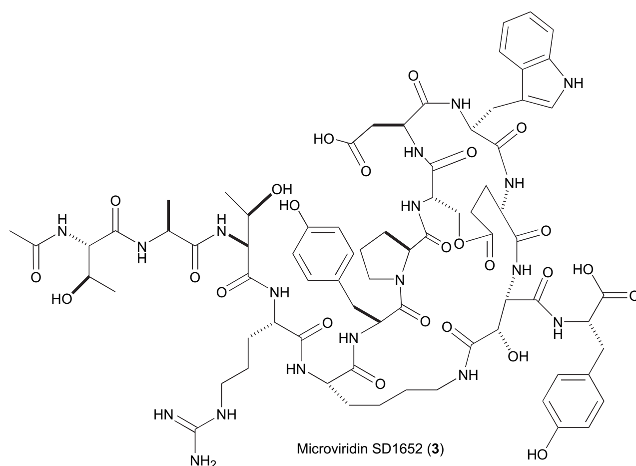

A non-toxic strain of the cyanobacterium *M. aeruginosa* (strain IL-215) was collected in the summer of 1998, from a pond in the Dan District sewage treatment plant, the Shofdan. The freeze-dried sample of the cyanobacterium was extracted with 70% MeOH in H_2O. The extract was found to inhibit several serine and amino proteases. The active extract was flash-chromatographed on an ODS column. Two fractions eluted from the column, with 60 and 70% MeOH in H_2O, exhibited protease inhibitory activity and were further purified on a reversed-phase HPLC column. Seven serine- and amino-protease inhibitors were isolated from these fractions and their structures were published.[8] The fractions eluted from an ODS flash-column with 30%–50% methanol, exhibit serine–protease inhibitory activity but were insoluble in chloroform/methanol solutions, like the less polar protease inhibitors. Repeated preparative reversed-phase HPLC afforded seven pure compounds with molecular weights between 1634 and 1715 mass units. The composition of the amino acids of all seven compounds was found to be identical by amino acid analysis of the hydrolyzed peptides. The structures of three of these compounds, microviridins SD1684 (**1**), SD1634 (**2**), and SD1652 (**3**), were determined by NMR and MS techniques. The other four compounds exist, in solution, as a complex mixture of conformers, and their structure could not be solved by NMR.[†] An attempt to solve the structure of the latter four compounds by MS–MS measurements, failed due to the uninterpretable fragmentation pattern of the cyclic portion, of either **1–3** or the four unsolved ones. The fragmentation pattern of the side chains of all compounds, on the other hand, was interpretable and consistent with the proposed structures of **1–3**.

Microviridin SD1684 (**1**) was isolated as a glassy solid material that exhibited a negative-FAB quasi-molecular ion at *m/z* 1683.6 but failed to give a positive-FAB molecular ion. Its molecular formula, $C_{76}H_{104}N_{18}O_{26}$, is based on HR MALDI-TOF MS measurements. The structure elucidation of **1** was complicated due to the overlapping of several protons and carbons in the NMR spectra. The NMR spectra of **1** were examined in several deuterated solvents. The best resolved spectra were obtained in methanol-d_3 and pyridine-d_5. Even in these two solvents at 500 MHz, the overlapping of too many signals did not allow the unambiguous assignment of all of the amino acid subunits. To overcome this problem we measured the 1-D and 2-D NMR spectra of **1** in CD_3OH on an 800 MHz spectrometer. Sixteen exchangeable protons were observed in the proton NMR spectrum (δ_H 10.20–6.50 ppm). Two *para*-substituted phenol moieties (δ_H 7.10 d, 2H; 7.06 d, 2H; 6.79 d, 2H, and 6.71 d, 2H, ppm) and five vinylic protons (δ_H 7.61 d, 7.36 d, 7.12 t, 7.08 s, and 7.04 t, ppm) of the tryptophan moiety were observed in the aromatic region. Upfield from the aromatic region (δ_H 5.50–3.30 ppm), sixteen methine protons, six protons of three methylenes next to nitrogen and oxygen, and one *O*-methoxyl group, were observed. Protons of six methylenes located next to a nitrogen and a carbonyl resonate between δ_H 3.30 and 2.20 ppm. A singlet of acetyl protons and three doublet methyls along with protons of eight aliphatic methylenes appear at the high-field end of the spectrum. In the carbon NMR spectrum, 17 carboxyl-carbons, a guanidine carbon, two phenol-carbons, and 18 vinyl carbons (13 methines and five quaternary) were observed at the low field end of the spectrum. Sixteen methines, two methylenes, and a methyl were detected in the region between 75 and 45 ppm. Fourteen methylene and four methyl carbons appear in the aliphatic region of the ^{13}C NMR spectrum. H–H COSY and TOCSY experiments allowed the assignment of 17 fragments, which account for 87 of the 104 protons of **1**. The fragments are: two *para*-substituted phenol moieties of tyrosine, a 1,2-disubstituted phenyl moiety, a three substituted vinyl imide moiety, three moieties of α-NH to the β-methylene of aromatic amino acids, α-NH to the β-methine of an unidentified amino acid, α-NH to the γ-methylene of glutamic acid, α-NH to the β-methylene of aspartic acid, α-NH to the β-methylene of serine, α-methine to the δ-methylene of proline, α-NH to ε-NH of an ε-*N*-substituted lysine, α-NH to δ-NH of arginine, two moieties of α-NH to γ-methyl of threonine and α-NH to the β-methyl of alanine. The correlations from the HMQC spectrum (see Table 1) reinforced these assignments and confirmed the existence of the *O*-methyl and *N*-acetyl moieties, in **1**. HMBC correlations (see Table 1) allowed full assignment of the 14 acid units of **1**: two Tyr, β-hydroxy aspartic acid (Has), 5-*O*-methyl glutamic acid, Trp, Asp, Ser, Pro, ε-*N*-substituted lysine, Arg, two Thr units, Ala, and an acetyl amide. The amino acid sequence of microviridin SD1684 (**1**) was determined from HMBC correlations of the NH proton of an amino acid with the carbonyl of an adjacent amino acid (Tyr^{I}-Has, Glu-Trp, Trp-Asp, Asp-Ser, Ser-Pro, Tyr^{II}-Lys, Lys-Arg, Arg-Thr^{I}, Thr^{I}-Ala, Ala-Thr^{II}, and Thr^{II}-Ac) and *O*Me with a 5-carbonyl of Glu. These correlations established all but three amide connectivities of the cyclopeptide, the connectivity between Pro-N and Tyr^{II}-carbonyl, the connectivity between ε-NH of Lys and 4-carbonyl of Has, and the connectivity between Has-NH and the Glu 1-carbonyl. Has 4-carbonyl and Tyr^{II}-carbonyl (δ_C 172.8 ppm), as well as Pro-carbonyl and Glu 1-carbonyl (δ_C 174.0 ppm),

[†] The proton NMR spectrum of each of these compounds was measured in several deuterated solvents and solvent mixtures at room temperature and if possible in higher temperatures. All seven compounds that were homogenous by HPLC, presented a MALDI-TOF mass spectrum, which indicated that it contains a single component (only one set of MH^+, MNa^+, and MK^+ ions). Compounds **1–3**, which presented a set of two or three NMR signals for each chemical unit (easily observed on methyl groups and aromatic protons) gave one sharp set of signals in the solvent that were used for structure elucidation. For the other four compounds such a solvent could not be obtained.

Table 1. NMR data of microviridin SD1684 (**1**) in CD_3OH[a]

Position		$\delta_{C/N}$, Mult.[b]	δ_H, Mult., J (Hz)	HMBC correlations[c]	ROESY correlations[d]
Tyr^I	1	174.9 s		Tyr^I-2,3,3′	
	2	55.9 d	4.65 dt 5.8, 7.5	Tyr^I-3,3′	
	3	37.7 t	2.98 dd 13.9, 7.5; 3.09, dd, 13.9, 5.8	Tyr^I-2,5,5′	
	4	128.7 s		Tyr^I-2,3,3′,6,6′	
	5,5′	131.3 d×2	7.10 d 8.4	Tyr^I-3,3′,5,5′	
	6,6′	116.3 d×2	6.71 d 8.4	Tyr^I-5,5′,6,6′	
	7	157.3 s		Tyr^I-5,5′,6,6′	
	NH	115.9 d	8.25 d 7.2		Has-3
Has	1	171.3 s		Has-2, Tyr^I-NH	
	2	56.8 d	5.03 br s		
	3	73.4 d	4.77 br s		Lys-ε-NH, Tyr^I-NH
	4	172.8 s		Has-3	
	NH	111.1 d	8.67 br s		Glu-2,4, Tyr^I-2
Glu	1	174.0 s		Glu-2,3,3′	
	2	52.2 d	5.29 ddd 8.2, 8.4, 7.8	Glu-3,3′,4,4′	Has-NH,3
	3	30.5 t	1.92 dq 14.8, 7.3; 1.88 dq 14.8, 7.3	Glu-4,4′	
	4	29.5 t	2.33 dt 16.9, 7.2; 2.49 dt 16.9, 7.2	Glu-3,3′	Glu-NH, Has-NH
	5	175.0 s		Glu-3,3′,4,4′,*O*Me	
	*O*Me	52.1 q	3.57 s		
	NH	123.8 d	8.32 d 7.8		Glu-4
Trp	1	173.4 s		Trp-2,3, Glu-NH	
	2	55.8 d	4.61 m	Trp-5, NH	Ser-NH, Glu-NH
	3	28.6 t	3.14 dd 14.7, 7.8; 3.09 dd 14.7, 5.4	Trp-2	Trp-NH,5
	4	110.5 s		Trp-3,5,9,NH(9)	
	4a	128.5 s		Trp-3,5,8,9,NH(9)	
	5	119.2 d	7.61 d 8.0	Trp-7	Trp-2,3
	6	119.7 d	7.12 t 7.5	Trp-8	
	7	122.3 d	7.04 t 7.5	Trp-5	Trp-NH(9)
	8	112.3 d	7.36 d 8.1	Trp-5,6	Trp-NH(9)
	8a	138.0 s		Trp-5,7,9,NH(9)	
	9	124.7 d	7.08 br s	Trp-3,NH(9)	
	NH(9)	127.1 d	10.12 br s		Trp-7,8
	NH	119.2 d	8.02 d 6.5		Trp-2,3, Asp-NH,2
Asp	1	173.1 s		Trp-NH, Asp-2,3,3′	
	2	51.8 d	4.68 dt 6.3, 8.0	Asp-3,NH	Asp-NH, Trp-NH
	3	36.1 t	2.70 dd 17.5, 7.9; 3.07 dd 17.5, 6.3	Asp-2	Asp-NH
	4	174.4 s		Asp-2,3,3′	
	NH	115.9 d	8.69 d 8.0		Asp-2,3, Ser-2, Trp-NH
Ser	1	171.8 s		Ser-3, Asp-NH	
	2	58.2 d	4.29 br s		Asp-NH
	3	63.0 t	3.85 br s (2H)		Ser-NH, Trp-NH
	NH	122.1 d	8.27 m		Ser-3, Trp-2
Pro	1	174.0 s		Pro-2,3, Ser-NH	
	2	62.1 d	3.78 br d 8.3	Pro-4′	Pro-5, Tyr^{II}-2,5,5′,6,6′
	3	32.1 t	1.52 m; 2.05 m	Pro-2,5′	Tyr^{II}-6,6′
	4	22.6 t	1.62 m; 1.80 m	Pro-5	Tyr^{II}-6,6′
	5	48.0 t	3.47 dt 9.6, 7.2; 3.57 m	Pro-2,3,3′	Pro-2, Tyr^{II}-5,5′
Tyr^{II}	1	172.8 s		Tyr^{II}-3,3′	
	2	55.4 d	4.62 m	Tyr^{II}-3	Tyr^{II}-NH, Pro-2
	3	38.6 t	2.89 dd 13.1, 6.1; 3.00 dd 13.1, 9.1	Tyr^{II}-5,5′	Tyr^{II}-NH
	4	127.0 s		Tyr^{II}-3,3′,6,6′	
	5,5′	131.5 d×2	7.06 d 8.4	Tyr^{II}-3,3′,5,5′	Pro-2,5
	6,6′	116.9 d×2	6.79 d 8.4	Tyr^{II}-5,5′,6,6′	Pro-2,3,4′
	7	158.1 s		Tyr^{II}-5,5′,6,6′	
	NH	118.8 d	8.29 m		Tyr^{II}-2,3
Lys	1	174.5 s		Lys-3′, Tyr^{II}-NH	
	2	53.3 d	4.54 m	Lys-3′	Lys-α-NH
	3	29.9 t	1.69 m; 1.79 m		Lys-α-NH; Lys-α-NH
	4	25.0 t	1.44 m; 1.42 m		Lys-α,ε-NH; Lys-α,ε-NH
	5	30.0 t	1.44 m; 1.42 m		Lys-α,ε-NH; Lys-α,ε-NH
	6	40.6 t	3.70 br m; 2.81 br m		Lys-ε-NH; Lys-ε-NH
	α-NH	127.0 d	8.83 d 7.7		Lys-2,3,3′,4,4′,5,5′, Arg-2
	ε-NH	118.6 d	8.08 t 6.6		Lys-4,4′,5,5′,6,6′, Has-3
Arg	1	174.7 s		Arg-2, Lys-α-NH	
	2	53.4 d	4.78 q 7.5	Arg-3,4	Lys-α-NH
	3	29.7 t	1.80 m (2H)		Arg-NH
	4	25.9 t	1.48 m; 1.57 m	Arg-2,5	Arg-6(NH)
	5	42.0 t	3.19 br m; 3.15 br m	Arg-4,4′	Arg-6(NH)

(*continued*)

Table 1. (*continued*)

Position		$\delta_{C/N}$, Mult.[b]	δ_H, Mult., J (Hz)	HMBC correlations[c]	ROESY correlations[d]
	6(NH)	83.7 d	7.34 t 4.1		Arg-4,5
	7	158.5 s		Arg-5	
	8,9	22.0 t; 74.0 d	7.25 br m; 6.55 br m		
	NH	121.1 d	8.54 br s		Arg-3, Thr^{I}-2,3
Thr^{I}	1	172.0 s		Arg-NH, Thr^{I}-2	
	2	58.8 d	4.59 m	Thr^{I}-4,NH	Arg-NH
	3	69.9 d	4.25 dq 4.4, 6.3	Thr^{I}-4	Arg-NH
	4	20.4 q	1.17 d 6.3		Thr^{I}-NH
	NH	110.9 d	8.07 d 7.9		Thr^{I}-4, Ala-NH
Ala	1	174.8 s		Ala-2,3, Thr^{I}-2,NH	
	2	51.0 d	4.43 dq 6.3, 6.8	Ala-3,NH	Thr^{I}-NH
	3	17.7 q	1.38 d 6.8	Ala-2,NH	Ala-NH
	NH	125.4 d	8.19 d 6.3		Ala-3, Thr^{II}-3, Thr^{I}-NH
Thr^{II}	1	172.5 s		Thr^{II}-2, Ala-NH	
	2	60.3 d	4.33 dd 4.7, 8.0	Thr^{II}-4,NH	Ala-NH
	3	68.5 d	4.17 dq 4.7, 6.3	Thr^{II}-2,4,NH	Thr^{II}-NH, Ala-NH
	4	19.9 q	1.20 d 6.3		Thr^{II}-NH
	NH	117.3 d	8.17 d 8.0		Thr^{II}-3,4, Ac-2
Ac	1	173.9 s		Ac-2, Thr^{II}-2,NH	
	2	22.5 q	2.06 s		Thr^{II}-NH

[a] Carried out on an DMX-800 Bruker instrument.
[b] Multiplicity and assignment from HMQC experiment.
[c] Determined from HMBC experiment, $^{n}J_{CH}$=8 Hz, recycle time 1 s, the HMBC correlations are reported as correlations of the protons printed in the column with the carbons in the rows.
[d] By ROESY experiment, mixing time 400 ms.

resonate at the same chemical shift and thus introduce some ambiguity to the assignment and correlations of these amino acids. The connectivity between these amino acids was established on the basis of NOE correlations from ROESY experiments. The experiment in methanol-d_3 presents an NOE between Pro H-2 and Tyr^{II} H-2, suggesting a cis conformation of the Pro amide bond (reinforced by the ^{13}C chemical shifts difference between positions 3 and 4 of proline, >9 ppm[9]), between Has H-3 and ε-NH of Lys, confirming the cyclic structure of the peptide, and between Has NH and Glu H-2. The ROESY experiment in pyridine-d_5 (see Table 4) presents an additional NOE between Ser H-2 and Pro H-4 confirming the correlation between Ser-NH and Pro-carbonyl that was established from the HMBC experiment in methanol-d_3. The rest of the amino acid sequence could also be assembled from the ROESY data (see Table 1). A set of NOE's between Asp-NH and Trp-NH, Ser-NH and Trp-H-2 as well as Ser-H$_2$-3 and Trp-NH suggest that the peptide has a β-turn around the Asp residue. The ^{1}H–^{15}N HSQC experiment allowed the assignment of the ^{15}N signals of the various amino acids of compound **1** other than that of Pro (see Table 1). All the secondary amide nitrogen signals resonated in the expected region (δ_N 105–120 ppm, relative to ammonia).[10] Acid hydrolysis of microviridin SD1684 (**1**) and derivatization with Marfey's reagent,[11] followed by HPLC analysis, demonstrated the L-stereochemistry of all amino acids. The stereochemistry of the Has residue was established by this method as L-*threo*-β-hydroxy aspartic acid.

Microviridin SD1634 (**2**) was isolated as a glassy solid material that exhibits a negative-FAB quasi-molecular ion at *m*/*z* 1633.9. Its molecular formula, $C_{75}H_{98}N_{18}O_{24}$, is based on HR MALDI-TOF MS measurements. This molecular formula corresponds to a loss of a molecule of water and a molecule of methanol from compound **1**. The NMR spectra of **2** were examined in several deuterated solvents. The best resolved spectra were obtained in 10:1 methanol-d_3/aq TFA at pH 3.3. When the proton NMR spectrum of **2** is compared with that of **1** some differences are encountered: (i) the methoxyl signal is missing in the spectrum of **2**; (ii) one of the two threonine methyl groups is downfield shifted and a quartet methine proton appears at 5.54 ppm; (iii) there is a dramatic change in the chemical shifts of the amide protons that resonate between 8.85 and 8.00 ppm in the spectrum of **1** and between 9.11 and 6.30 ppm in the spectrum of **2**. Combining these observations with the mass-spectral data and the structure of previously known microviridins suggested that **2** contains two lactone bridges that cause conformational changes in the peptide backbone. The conformational changes were also reflected in ^{15}N chemical shifts of the amide nitrogen atoms (see Tables 1 and 2). Analyses of the NMR spectra and Marfey's analysis[11] revealed that **2** contains the same 13 amino acids (L-Ala, L-Arg, L-Asp, L-Glu, L-Has, L-Lys, L-Pro, L-Ser, 2×L-Thr, L-Trp, and 2×L-Tyr) and acetate as **1**. The assignment of the NMR signals further suggested that the serine methylene (δ_H 4.90 m and 2.77 br d in **2**, relative to δ_H 3.85 br s in **1**) and a methine next to oxygen of one of the two threonine moieties (δ_H H-3: 5.56 q and H$_3$-4 1.39 d in **2**, relative to δ_H H-3: 4.25 dq and H$_3$-4 1.17 d in **1**) are esterified. Seven of the carbonyl carbons overlapped in the ^{13}C NMR spectrum (Ser, Lys, and Thr^{II}, resonate at 172.9 ppm and Asp, Pro, Tyr^{II}, and Ac, at 173.7 ppm) and thus prevented the assignment of the amino acid sequence solely on the basis of HMBC correlation between the amide NH proton of an amino acid with the carbonyl of an adjacent amino acid. Based on the HMBC connectivity two fragments could be assigned (see Table 2): Tyr^{I}-Has-Glu-Trp and Lys-Arg-Thr^{I}-Ala. The following NOE's between: Trp-NH and Asp-2 and NH; Asp-NH and Ser-2; Ser-NH and Pro-4 and 5′; Pro-2 and Tyr^{II}-2, 5 and 5′; Tyr^{II}-NH and Lys-2; Ala-NH and Thr^{II}-2

Table 2. NMR data of microviridin SD1634 (**2**) in 10:1 CD_3OH/aq TFA pH 3.3[a]

Position		$\delta_{C/N}$, Mult.[b]	δ_H, Mult., *J* (Hz)	HMBC correlations[c]	ROESY correlations[d]
Tyr^I	1	174.4 s		Tyr^I-3	
	2	55.7 d	4.58 m	Tyr^I-3	Tyr^I-3,NH
	3	37.4 t	3.09 m	Tyr^I-2,5,5′	Tyr^I-2,NH
	4	128.8 s		Tyr^I-3,5,5′,6,6′	
	5,5′	131.4 d×2	7.11 d 8.3	Tyr^I-3,5,5′	
	6,6′	116.3 d×2	6.73 d 8.3	Tyr^I-5,5′,6,6′	
	7	157.2 s		Tyr^I-5,5′	
	NH	118.6 d	7.67 d 8.0		Tyr^I-2,3, Has-2,3
Has	1	171.2 s		Has-2, Tyr^I-NH	
	2	57.2 d	4.74 br d 9.5	Has-NH	Tyr^I-NH, Has-NH
	3	72.1 d	4.54 br s		Lys-ε-NH, Tyr^I-NH
	4	173.3 s		Has-3, Lys-ε-NH	
	NH	104.2 d	7.02 d 8.6		Glu-2,3, Has-2
Glu	1	172.0 s		Glu-2, Has-NH	
	2	56.8 d	4.09 m	Glu-NH	Glu-4,4′,NH, Has-NH
	3	30.6 t	1.37 m	Glu-NH	Glu-4,4′,NH, Has-NH
	4	31.6 t	2.09 m; 1.07 m		Glu-2,3; Glu-2,3,NH
	5	172.6 s		Ser-3,3′, Glu-3,3′,4	
	NH	116.8 d	6.54 d 7.5		Glu-2,3,4′, Trp-2
Trp	1	174.3 s		Trp-2,3, Glu-NH	
	2	55.5 d	4.79 m	Trp-3,3′	Trp-3,3′,NH, Glu-NH
	3	26.6 t	3.23 m; 3.58 dd 4.5, 15.5		Trp-3′,NH; Trp-3,9
	4	109.8 s		Trp-3,3′,9,NH(9)	
	4a	128.8 s		Trp-3,3′,8,9,NH(9)	
	5	118.8 d	7.58 d 8.0	Trp-6,7,8	
	6	120.7 d	7.10 t 7.9	Trp-8	
	7	122.9 d	7.11 t 7.9	Trp-5	
	8	113.0 d	7.30 d 7.5	Trp-6	Trp-NH(9)
	8a	138.4 s		Trp-5,9,NH(9)	
	9	125.0 d	7.18 s	Trp-3,3′,NH(9)	
	NH(9)	129.5 d	10.62 s		Trp-8,9
	NH	111.3 d	6.90 d 7.5		Trp-2,3, Asp-NH,2
Asp	1	173.7 s		Asp-2, Trp-NH	
	2	51.4 d	4.47 br d 10.0	Asp-3,NH	Asp-3′,NH, Trp-NH
	3	35.4 t	3.04 m; 2.94 m	Asp-NH	Asp-NH; Asp-2
	4	172.1 s		Asp-2,3,3′, Thr(2)-3	
	NH	116.9 d	9.11 br s		Asp-2,3, Ser-2,3′, Trp-NH
Ser	1	172.9 s		Ser-2,3,3′, Asp-NH	
	2	55.0 d	4.30 br s		Ser-NH, Asp-NH
	3	62.5 t	4.90 m; 2.78 br d 11.5		Lys-ε-NH; Asp-NH
	NH	109.0 d	6.33 d 2.9		Ser-2, Pro-4,5′
Pro	1	173.7 s		Pro-2, Ser-NH	
	2	62.2 d	3.38 m		Pro-3′,4,4′, Tyr^{II}-2,5,5′
	3	31.7 t	1.63 m; 1.33 m	Pro-2	Pro-2
	4	22.6 t	1.33 m; 1.59 m	Pro-2	Pro-2,5′, Ser-NH; Pro-2,5′
	5	47.4 t	3.20 m; 3.38 m		Pro-4,4′, Ser-NH
Tyr^{II}	1	173.7 s		Tyr^{II}-2,3	
	2	54.1 d	4.35 m	Tyr^{II}-3,NH	Tyr^{II}-3,5,5′,NH, Pro-2
	3	38.6 t	2.84 d 8.2	Tyr^{II}-2,5,5′,NH	Tyr^{II}-2,5,5′,NH
	4	127.5 s		Tyr^{II}-3,6,6′	
	5,5′	131.3 d×2	6.96 d 8.2	Tyr^{II}-3,5,5′	Tyr^{II}-2,3, Pro-2
	6,6′	116.6 d×2	6.70 d 8.2	Tyr^{II}-5,5′,6,6′	
	7	157.9 s		Tyr^{II}-5,5′	
	NH	125.4 d	8.48 d 7.0		Tyr^{II}-2,3, Lys-2
Lys	1	172.9 s		Lys-2, Tyr^{II}-NH	
	2	55.4 d	3.95 m	Lys-α-NH	Lys-3,4,α-NH, Tyr^{II}-NH
	3	32.6 t	1.70 m; 1.57 m		Lys-2; Lys-α-NH,4
	4	23.0 t	1.18 m; 1.44 m		Lys-2,3′
	5	29.4 t	1.68 m; 1.24 m		
	6	39.9 t	2.97 m; 3.21 m		Lys-ε-NH
	α-NH	120.9 d	7.54 d 6.5		Lys-2,3′, Arg-NH, Asp-2, Thr^I-3
	ε-NH	118.6 d	7.61 t 5.7		Lys-6, Has-3, Ser-3
Arg	1	173.0 s		Arg-2, Lys-α-NH	
	2	53.3 d	4.46 m	Arg-NH	Arg-3,3′,4,4′,5,NH
	3	28.9 t	1.56 m; 2.11 m		Arg-2,NH,6(NH); Arg-2,6(NH)
	4	25.9 t	1.56 m; 1.67 m	Arg-5	Arg-2,6(NH); Arg-2,5
	5	41.9 t	3.17 m		Arg-2,4′,6(NH)
	6(NH)	83.3 d	7.34 t 5.0		Arg-3,3′,4,5

(*continued*)

Table 2. (*continued*)

Position		$\delta_{C/N}$, Mult.[b]	δ_H, Mult., J (Hz)	HMBC correlations[c]	ROESY correlations[d]
	7	158.7 s		Arg-6(NH)	
	8,9	22.1 t	7.23 br m		
	NH's	74.3 d	6.40 br m		
	NH	118.8 d	8.74 d 9.0		Arg-2,3, Lys-α-NH, Thr^{I}-2,3
Thr^{I}	1	172.8 s		Arg-NH, Thr^{I}-2	
	2	57.8 d	4.58 br d 11.5	Thr^{I}-4,NH	Thr^{I}-NH, Arg-NH
	3	73.2 d	5.56 q 6.5	Thr^{I}-4	Thr^{I}-2,4,NH, Arg-NH, Lys-α-NH
	4	18.3 q	1.39 d 6.6	Thr^{I}-3	Thr^{I}-3
	NH	106.0 d	7.95 d 7.8		Thr^{I}-2,3, Ala-2
Ala	1	175.6 s		Ala-2,3, Thr^{I}-NH	
	2	50.4 d	4.59 q 7.0	Ala-3,NH	Ala-NH, Thr^{I}-NH
	3	17.6 q	1.45 d 7.0	Ala-2,NH	Ala-2,NH
	NH	122.7 d	8.26 d 6.5		Ala-2,3, Thr^{II}-2,3
Thr^{II}	1	172.9 s		Thr^{II}-2, Ala-NH	
	2	60.3 d	4.43 dd 4.0, 8.5	Thr^{II}-4,NH	Thr^{II}-4,NH, Ala-NH
	3	68.3 d	4.24 dq 4.0, 6.5	Thr^{II}-4	Thr^{II}-4,NH, Ala-NH
	4	20.0 q	1.24 d 6.4		Thr^{II}-2,3
	NH	115.3 d	7.94 d 8.5		Thr^{II}-2,3, Ac-2
Ac	1	173.7 s		Ac-2, Thr^{II}-2,NH	
	2	22.6 q	2.08 s		Thr^{II}-2,NH

[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 1 s, the HMBC correlations are reported as correlations of the protons printed in the column with the carbons in the rows.
[d] By ROESY experiment, mixing time 400 ms.

and 3; Thr^{II}-NH and Ac-2, allowed the assignment of the whole molecule backbone, Tyr^{I}-Has-Glu-Trp-Asp-Ser-Pro-Tyr^{II}-Lys-Arg-Thr^{I}-Ala-Thr^{II}-Ac. The amide bond between Lys-ε-NH and Has 4-carbonyl could be assigned on the basis of an HMBC correlation and NOE between Has-3 and Lys-ε-NH. The ester bond between Ser-3 oxygen and Glu-5 carbonyl was assigned on the basis of the HMBC correlation of Ser-3 and 3′ protons with Glu-5 carbonyl. Finally, the ester bond between Thr^{I}-3 oxygen and Asp-4 carbonyl was assigned on the basis of the HMBC correlation of Thr^{I}-3 proton with Asp-4 carbonyl. The discussion above led to the assignment of structure **2** to microviridin SD1634.

Microviridin SD1652 (**3**) is a glassy solid material that exhibits a negative-FAB quasi-molecular ion at *m/z* 1651.7. Its molecular formula, $C_{75}H_{100}N_{18}O_{25}$, is based on HR MALDI-TOF MS measurements. This molecular formula corresponds to the loss of a molecule of methanol from compound **1**. The NMR spectra of **3** were examined in several deuterated solvents. The best resolved spectra were obtained in DMSO-d_6 at 330 K. Examination of the proton NMR spectrum of **3** (see Table 3) revealed that: (i) the methoxyl signal is missing in the spectrum of **3**; (ii) the two threonine methyls resonate at a similar chemical shift; (iii) the serine methyleneoxy protons resonate in a relatively low field suggesting the involvement of the oxygen in an ester bond. Combining this data with the mass spectral data suggested that compound **3** contains only one lactone bridge, most probably between the Glu carbonyl and the serine methyleneoxy. Analyses of the COSY, TOCSY, HMQC, and HMBC revealed the structures of the same 13 amino acids (Ala, Arg, Asp, Glu, Has, Lys, Pro, Ser, 2×Thr, Trp, and 2×Tyr) and acetyl residues while Marfey's analysis[11] revealed that they posses the same absolute stereochemistry as that of **1** and **2**. Four of the amide carbonyl signals were not assigned because they did not show any correlation in the HMBC spectrum, probably due to the relatively small amount of material available. The amide backbone of the peptide was determined on the basis of HMBC and NOE correlations. Tyr^{I} was connected to Has on the basis of the HMBC correlation of Tyr^{I}-NH and Has-CO. An NOE between Has-NH and Glu-2 proton connected the two acids. Glu-NH correlates in the HMBC map with Trp-carbonyl. The sequence Trp-Asp-Ser-Pro-Tyr^{II} was connected on the basis of an NOE between Trp-NH and Asp-NH, Asp-NH and Ser-2, Ser-NH and Pro-2, and Pro-5,5′ and Tyr^{II}-2. An HMBC correlation between the amide NH proton of an amino acid and the carbonyl of an adjacent amino acid established the sequence: Tyr^{II}-Lys-Arg-Thr^{I}-Ala. An NOE between Ala-NH and Thr^{II}-2 and an HMBC correlation between Thr^{II}-NH and the acetyl carbonyl established the sequence Ala-Thr^{II}-Ac. An NOE between Has-3 and Lys-ε-NH established the cyclization of the peptide through the Has and Lys side chains. The lactone bridge between Glu-5 carbonyl and Ser-methyleneoxy was established by the HMBC correlation of Ser-3′ proton and Glu-5 carbonyl. On the basis of the arguments discussed above, the structure **3** was assigned to microviridin SD1652.

Compounds **1**–**3** were isolated through a serine protease (chymotrypsin and trypsin) inhibition-guided separation. The final fractions that yielded compounds **1**–**3**, inhibited both chymotrypsin and trypsin. Pure **1** and **3** were found to be inactive in the assay, while **2** completely inhibited the proteolytic activity of chymotrypsin and trypsin at a concentration of 45 μg/mL. The IC_{50} values were determined only for compound **2**, against the serine proteases trypsin and chymotrypsin. Microviridin SD1634 (**2**) inhibited trypsin activity with an IC_{50} value of 13.4 μg/mL (8.2 μM) and chymotrypsin with an IC_{50} value of 25.7 μg/mL (15.7 μM). These IC_{50}

Table 3. NMR data of microviridin SD1652 (**3**) in DMSO-d_6 at 330 K[a]

Position		$\delta_{C/N}$, Mult.[b]	δ_H, Mult., J (Hz)	HMBC correlations[c]	ROESY correlations[d]
Tyr^{I}	1	171.9 s		Tyr^{I}-2,3	
	2	54.3 d	4.37 m	Tyr^{I}-3,3′,NH	Tyr^{I}-3,5,5′,NH, Has-NH
	3	36.3 t	2.95 dd 7.1, 13.7; 3.00 dd 6.3, 13.8	Tyr^{I}-2,5,5′	Tyr^{I}-2,5,5′,NH; Tyr^{I}-NH
	4	127.3 s		Tyr^{I}-2,3,6,6′	
	5,5′	129.8 d×2	7.01 d 8.1	Tyr^{I}-3,3′,5,5′	Tyr^{I}-2,3, Has-2,3,NH
	6,6′	114.9 d×2	6.64 d 8.2	Tyr^{I}-5,5′,6,6′	
	7	155.7 s		Tyr^{I}-5,5′,6,6′	
	NH	116.8 d	7.13 d 7.4		Tyr^{I}-2,3,3′, Has-2,3
Has	1	168.2 s		Has-2, Tyr^{I}-NH	
	2	55.5 d	4.73 m		Has-NH, Tyr^{I}-5,5′,NH
	3	70.5 d	4.48 br s	Has-2	Has-NH, Lys-ε-NH, Tyr^{I}-5,5′NH
	4	170.3 s[e]			
	NH	100.0 d	7.00 d 7.9		Has-2,3, Tyr^{I}-2
Glu	1	171.0 s		Has-NH	
	2	55.5 d	3.99 m		Has-NH
	3	25.6 t	1.87 m; 2.09 m	Glu-4′	Glu-NH
	4	30.7 t	1.47 m; 2.68 m	Glu-3′	Glu-NH
	5	170.6 s		Glu-4,4′, Ser-3′	
	NH	112.3 d	6.89 d 7.9		Glu-3′,4, Ser-3
Trp	1	172.0 s		Trp-2,3,3′, Glu-NH	
	2	53.8 d	4.56 m	Trp-3,3′,NH	Trp-3,3′,NH
	3	26.2 t	3.15 m; 3.17 m	Trp-2	Trp-2,9,NH; Trp-2,9,NH
	4	109.3 s		Trp-3,3′,5,9,NH(9)	
	4a	127.1 s		Trp-3,3′,5,6,8,9,NH(9)	
	5	117.9 d	7.45 d 7.9	Trp-7	
	6	118.3 d	6.95 t 7.5	Trp-8	
	7	121.0 d	7.06 t 7.7	Trp-5	
	8	111.6 d	7.30 d 8.1	Trp-5,6	Trp-NH(9)
	8a	136.2 s		Trp-5,7,9,NH(9)	
	9	123.1 d	7.17 s	Trp-3,3′,NH(9)	Trp-3,3′,NH(9)
	NH(9)	131.5 d	10.69 s		Trp-8,9
	NH	111.5 d	7.50 d 6.8		Trp-2,3,3′, Asp-3,3′,NH, Ser-3
Asp	1	171.1 s[e]			
	2	53.0 d	4.31 m	Asp-3,3′	Asp-3,3′
	3	34.5 t	2.60 m; 2.67 m		Asp-2,NH, Trp-NH
					Asp-2,NH, Trp-NH
	4	171.5 s[e]			
	NH	122.6 d	9.12 s		Asp-2,3,3′, Ser-2,3,NH, Trp-NH
Ser	1	170.6 s		Ser-2,3′	
	2	51.1 d	4.72 br s	Ser-3′,NH	Ser-3, Asp-NH
	3	63.4 t	4.11 br d 10.7; 4.57 br d 10.7		Ser-2, Asp-NH, Trp-NH, Glu-NH
	NH	109.8 d	8.09 d 7.3		Asp-NH, Pro-2,5′
Pro	1	171.2 s		Ser-NH	
	2	59.1 d	4.59 m		Pro-3,3′,5, Ser-NH
	3	28.0 t	1.80 m; 1.87 m	Pro-5′	Pro-2; Pro-2
	4	24.8 t	1.85 m; 1.91 m		Pro-5′; Pro-5′
	5	46.5 t	3.00 m; 3.68 m		Tyr^{II}-2; Pro-4,4′, Tyr^{II}-2, Ser-NH
Tyr^{II}	1	170.8 s[e]			
	2	52.4 d	4.49 m	Tyr^{II}-3,3′	Tyr^{II}-3,3′, Pro-5,5′, Lys-NH
	3	35.8 t	2.62 m; 2.76 m	Tyr^{II}-2,5,5′	Tyr^{II}-2,NH; Tyr^{II}-2,NH
	4	127.5 s		Tyr^{II}-2,3,3′,6,6′	
	5,5′	129.8 d×2	7.01 d 8.1	Tyr^{II}-3,3′,5,5′	Tyr^{II}-3,3′
	6,6′	115.0 d×2	6.65 d 8.2	Tyr^{II}-5,5′,6,6′	
	7	155.8 s		Tyr^{II}-5,5′,6,6′	
	NH	114.2 d	8.06 d 8.1		Tyr^{II}-3,3′
Lys	1	170.7 s		Lys-2, Tyr^{II}-NH	
	2	51.3 d	4.38 m	Lys-α-NH	Lys-3,4,4′
	3	31.9 t	1.56 m	Lys-4	Lys-2,α-NH,ε-NH
	4	21.4 t	0.80 m; 1.17 m		Lys-2; Lys-2,α-NH,ε-NH
	5	28.9 t	1.26 m; 1.17 m		Lys-ε-NH
	6	39.5 t	2.85 m; 3.00 m		Lys-ε-NH; Lys-ε-NH
	α-NH	114.6 d	7.48 d 8.0		Lys-3,4,4′, Arg-3, Tyr^{II}-2
	ε-NH	121.0 d	8.05 t 6.2		Lys-3,4′,5′,6,6′, Has-2
Arg	1	170.2 s		Arg-2, Lys-α-NH	
	2	51.8 d	4.32 m	Arg-NH	Arg-3,3′,4,4′,5,NH
	3	29.0 t	1.50 m; 1.71 m	Arg-2,4,4′,5	Arg-2,NH,6(NH), Lys-α-NH; Arg-2,NH,6(NH)
	4	24.7 t	1.48 m; 1.69 m	Arg-2,3,3′,5	Arg-2,6(NH); Arg-2,NH,6(NH)
	5	40.5 t	3.09 m	Arg-4	Arg-2,6(NH)

(*continued*)

Table 3. (*continued*)

Position		$\delta_{C/N}$, Mult.[b]	δ_H, Mult., J (Hz)	HMBC correlations[c]	ROESY correlations[d]
	6(NH)	85.0 d	7.33 t 5.5		Arg-3,3′,4,4′,5
	7	156.8 s		Arg-6(NH)	
	8,9	22.0 d	7.10 m		
	NH's	74.0 t	6.95 m (2H)		
	NH	118.6 d	7.69 d 8.0		Arg-2,3,4,4′, Thr^{I}-2,3
Thr^{I}	1	169.7 s		Arg-NH, Thr^{I}-2	
	2	58.2 d	4.20 dd 4.2, 8.2	Thr^{I}-4,NH	Thr^{I}-NH, Arg-NH
	3	66.5 d	3.98 m	Thr^{I}-2,4,NH	Thr^{I}-4,NH, Arg-NH
	4	19.3 q	1.04 d 6.5	Thr^{I}-2	Thr^{I}-3,NH, Ala-3
	NH	109.2 d	7.53 d 8.2		Thr^{I}-2,3,4
Ala	1	172.1 s		Ala-3, Thr^{I}-NH	
	2	48.3 d	4.35 m	Ala-3,NH	Ala-3,NH
	3	17.6 q	1.25 d 6.9	Ala-2,NH	Ala-2,NH, Thr^{I}-NH
	NH	121.5 d	7.83 d 7.0		Ala-2,3, Thr^{II}-2,3
Thr^{II}	1	170.0 s		Ala-NH	
	2	58.4 d	4.21 dd 4.2, 8.1	Thr^{II}-4,NH	Thr^{II}-NH, Ala-NH
	3	66.5 d	3.98 m	Thr^{II}-2,4,NH	Thr^{II}-4,NH, Ala-NH
	4	19.5 q	1.05 d 6.4	Thr^{II}-2	Thr^{II}-3
	NH	115.4 d	7.56 d 8.1		Thr^{II}-2,3, Ac-2
Ac	1	169.6 s		Ac-2, Thr^{II}-2,NH	
	2	22.4 q	1.91 s		Thr^{II}-NH

[a] Carried out on an ARX-500 Bruker instrument.
[b] Multiplicity and assignment from HMQC experiment.
[c] Determined from HMBC experiment, $^{n}J_{CH}$=8 Hz, recycle time 1 s, the HMBC correlations are reported as correlations of the protons printed in the column with the carbons in the rows.
[d] By ROESY experiment, mixing time 400 ms.
[e] The assignment of these carbon signals may be interchange.

values are mild relative to the standard inhibitors used for comparison: antipain,[12] for trypsin (IC_{50} 0.39 μM) and nostopeptin BN920[13] for chymotrypsin (IC_{50} 0.11 μM).

2. Experimental

2.1. General

High-resolution MS were recorded on a Fisons VG Auto-SpecQ M 250 instrument and an Applied Biosystems Voyager System 4312 instrument. 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 Avance 800 Spectrometer at 800.13 MHz for ^{1}H and 201.19 MHz for ^{13}C, a Bruker ARX-500 spectrometer at 500.136 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 and 40.55 MHz for ^{15}N. ^{1}H, ^{13}C, DEPT, gCOSY, gTOCSY, gROESY, gHMQC, and gHMBC spectra were recorded using standard Bruker pulse sequences. HPLC separations were performed on an ISCO HPLC system (model 2350 pump and model 2360 gradient programer) equipped with an Applied Biosystems Inc. diode-array detector and Merck–Hitachi HPLC system (model L-4200 UV–VIS detector and model L-6200A Intelligent pump).

2.1.1. Water bloom material. *M. aeruginosa*, TAU strain IL-215, was collected, in July 1998, from a pond in the Dan District sewage treatment plant, the Shofdan, in Israel. Morphological classification of the preserved field sample was done under a microscope using the morphological criteria proposed by Komárek and Anagnostidis.[14] A preserved sample of the bloom material is kept in our laboratory labeled as IL-215.

2.1.2. Isolation procedure. The freeze-dried cells (131 g) were extracted with 7:3 MeOH/H_2O. The crude extract (21.2 g) was evaporated and separated on an ODS (YMC-GEL, 120A, 4.4×6.4 cm) flash-column with increasing amounts of MeOH in water. Fractions 4–6 (3:7, 6:4, and 1:1 MeOH/H_2O, 353 mg) were subjected to a reversed-phase HPLC (YMC-ODS-A 5 mm, 250 mm×20.0 mm, DAD at 238 nm, flow rate 5.0 mL/min) in 6:4 water/methanol to obtain three fractions: fraction 1 (71.9 mg, retention time of 20.0 min), fraction 2 (42.8 mg, retention time of 23 min), and fraction 3 (74.5 mg, retention time of 35 min). Fraction 3 was subjected to a reversed-phase HPLC (YMC-ODS-A 5 mm, 250 mm×20.0 mm, DAD at 238 nm, 3:2 0.1% TFA in water/acetonitrile, flow rate 5.0 mL/min). Compound **1** (26.1 mg, 0.02% yield based on the dry weight of the bacteria) was eluted from the column with a retention time of 36.5 min, while compound **2** (13.1 mg, 0.01% yield based on the dry weight of the bacteria) was eluted from the column with a retention time of 29.3 min. Fraction 2 was subjected to the same column and conditions to afford semi-pure **3** (22.3 mg, retention time 28.3 min), which was further purified on the same column with 7:3 0.1% TFA in water/acetonitrile as eluent, to afford pure **3** (10.2 mg, 0.008% yield based on the dry weight of the bacteria), which was eluted from the column with a retention time of 40.6 min.

2.1.2.1. Microviridin SD1684 (1). $[\alpha]_D^{21}$ −21.0 (c 16.0, MeOH); UV λ_{max} (MeOH) 224 nm (ε 14,300), 281 nm

(ε 2500). For NMR data see Table 1. Negative FABMS *m/z* 1683.6 [M−H]$^-$; HR MALDI-TOF MS *m/z* 1685.7370 (MH$^+$, calcd for $C_{76}H_{105}N_{1826}$ *m/z* 1685.7441).

2.1.2.2. Microviridin SD1634 (2). $[\alpha]_D^{21}$ −4.9 (*c* 6.7, MeOH); UV λ_{max} (MeOH) 224 nm (ε 13,500), 281 nm (ε 2600). For NMR data see Table 2. Negative FABMS *m/z* 1633.9 [M−H]$^-$; HR MALDI-TOF MS *m/z* 1635.7208 (MH$^+$, calcd for $C_{75}H_{99}N_{18}O_{24}$ *m/z* 1635.7074).

2.1.2.3. Microviridin SD1652 (3). $[\alpha]_D^{21}$ −7.3 (*c* 6.8, MeOH); UV λ_{max} (MeOH) 227 nm (ε 22,900), 280 nm (ε 6800). For NMR data see Table 3. Negative FABMS *m/z* 1651.7 [M−H]$^-$; HR MALDI-TOF MS *m/z* 1653.7134 (MH$^+$, calcd for $C_{75}H_{101}N_{18}O_{25}$ *m/z* 1653.7179).

2.1.3. Determination of the absolute configuration of the amino acids. Portions of compounds **1–3** (0.5 mg) were dissolved in 6 M 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 hydrolysate 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 M $NaHCO_3$ (20 mL) was added to each reaction vessel and the reaction mixture was stirred at 40 °C for 1 h. A 2 M 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 hydrolysates, were compared with similar derivatized standard amino acids by HPLC analysis: Knauer GmbH Eurospher 100 C18, 10 μm, 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 hydrolysates with the derivatized authentic amino acids. Retention times of the derivatized amino acids were: L-Ala, 36.1 min; D-Ala, 41.3 min; 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; D-*threo*-β-Has, 21.7 min; L-*threo*-β-Has, 22.4 min; L-Lys, 53.9 min; D-Lys, 56.7 min; L-Pro, 38.5 min; D-Pro, 41.5 min; L-Ser, 29.0 min; D-Ser, 29.0 min; L-Thr, 29.7 min; D-Thr, 35.0 min; L-Trp, 54.0 min; D-Trp, 57.1 min; L-Tyr, 63.0 min; and D-Tyr, 67.6 min. HPLC analysis of Marfey's derivatives of **1**, **2**, and **3** established: L-*threo*-β-Has, 22.4 min; L-Arg, 24.5 min; L-Ser, 29.0 min; L-Thr, 29.7 min; L-Asp, 30.6 min; L-Glu, 32.0 min; L-Ala, 36.1 min; L-Pro, 38.5 min; L-Trp, 54.0 min; L-Lys, 53.9 min; and L-Tyr, 63.0 min, for the three compounds.

2.1.4. Protease inhibition assays. Trypsin and chymotrypsin were purchased from Sigma Chemical Co. 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. A 2 mM solution of *N*-benzoyl-D,L-arginine-*p*-nitroanilide (for trypsin) and Suc-Gly-Gly-*p*-nitroanilide (for chymotrypsin) in the appropriate buffer solution was used as substrate solution. The test sample was dissolved in ethanol and diluted with the same buffer solution used for the enzyme and substrate. A 100 mL buffer solution, 10 mL enzyme solution, and 10 mL of test solution were added to each microtiter plate well and pre-incubated at 37 °C for 5 min. Then 100 mL 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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Supplementary data

^1H, ^{13}C NMR, COSY, TOCSY, ROESY, HMQC, and HMBC spectra of microviridins SD1684, SD1634, and SD1652 and Table 4, presenting NMR data of microviridin SD1684 in pyridine-d_5, are available. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2006.05.028.

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