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Microseiramide from the Freshwater Cyanobacterium *Microseira* sp. UIC 10445

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Abstract

Microseiramide (1), a cyclic heptapeptide, was isolated from a sample of the freshwater cyanobacterium *Microseira* sp. UIC 10445 collected in a shallow lake in Northern Indiana. Taxonomic identification of UIC 10445 was performed by a combination of morphological and phylogenetic characterization. Phylogenetic analysis revealed that UIC 10445 was a member of the recently described genus *Microseira*, which is phylogenetically distinct from the morphologically similar genera *Moorea* and *Lyngbya*. The planar structure of microseiramide (1) was determined by extensive 1D and 2D NMR experiments as well as HRESIMS analysis. The absolute configurations of amino acid residues were determined using acid hydrolysis followed by the advanced Marfey's analysis. microseiramide (1) is the first cyclic peptide reported from a *Microseira* sp., and the structure of microseiramide (1) is distinct from the previously known metabolites from cyanobacteria of the genera *Moorea* and *Lyngbya*.

Graphical abstract

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Appendix A. Supplementary data

Morphological and phylogenetic analysis of *Microseira* sp. UIC 10445; ¹H NMR, DEPTQ, COSY, HSQC, HMBC and ROESY spectra of **1**; Advanced Marfey's analysis of **1**; MS/MS spectrum of **1** can be found in the online version. This material is available free of charge via the Internet.

Keywords

Cyanobacteria; Microseira sp; cyclic heptapeptide; microseiramide

1 Introduction

Cyanobacteria, in particular marine members of the genus *Lyngbya* (now classified as *Moorea*), are known to be a rich source of biologically active natural products. Over 260 novel secondary metabolites had been discovered from marine strains of the genus *Lyngbya* up to 2010. (Engene et al., 2011) These metabolites account for over 40% of all the metabolites reported from marine cyanobacteria. These metabolites exhibit a broad spectrum of activities including antimicrobial, antiproliferative, anticancer, antifeedant, antifungal and anti-inflammatory activities. (Burja et al., 2001; Jones et al., 2011) The genus *Lyngbya* is considered a polyphyletic genus and cyanobacteria belong to the marine *Lyngbya* lineage have been reclassified and constituted a new genus named *Moorea*, in recognition of the extraordinary contributions of Dr. Richard E. Moore in the area of marine natural products research. (Engene et al., 2012) A SciFinder search of publications from January of 2009 to March of 2014 revealed that 84 novel metabolites were reported from marine cyanobacteria identified as *Lyngbya* and six novel metabolites were obtained from cyanobacteria of the

genus *Moorea*. Freshwater cyanobacteria strains identified as *Lyngbya* spp. do not form a monophyletic clade in phylogenetic analysis, and few secondary metabolites have been reported from freshwater *Lyngbya* spp. (Engene et al., 2010) To our knowledge, the only metabolites reported from freshwater *Lyngbya* strains are lyngbyazothrins A–D and lyngbyaureidamides A and B, all obtained from *Lyngbya* sp. (SAG 36.91). (Zainuddin et al., 2009; Zi et al., 2012) Compounds structurally related to lyngbyazothrins A – D have also been reported from strains that are morphologically similar to *Lyngbya*. These compounds include schizotrin A isolated from *Schizotrix* sp. (TAU strain IL-89-2) (Pergament and Carmeli, 1994), pahayokolides A and B isolated from a marine *Lyngbya* sp. (An et al., 2007), tychonamides A and B isolated from a *Tychonema* sp. (Mehner et al., 2008), and portoamides A – D isolated from *Oscillatoria* sp. LEGE 05292 (Leao et al., 2010).

We recently collected a cyanobacteria strain (UIC 10445) from the bottom of a shallow freshwater lake in Northern Indiana. This strain grew as a thick mat in its natural environment. Morphological and phylogenetic analysis revealed that UIC 10445 was closely related to strains previously identified as *Lyngbya wollei*, a known producer of the neurotoxic saxitoxins, as well as the odor compounds geosmin and 2-methylisoborneol. (Carmichael et al., 1997; Foss et al., 2012; Schrader and Blevins, 1993; Watson et al., 2008) Recently, strains belong to the group of *Lyngbya wollei* were considered to form a separate genus described as *Microseira*. (McGregor and Sendall, 2015) Thus, UIC 10445 was identified as a *Microseira* sp. ¹H NMR and LC-MS dereplication of the organic extract indicated the presence of a potentially new peptide. (Orjala et al., 2011) Herein we present the isolation, structure determination, and biological evaluation of the first new cyclic peptide named microseiramide (**1**), from a *Microseira* sp. UIC 10445.

2 Results and discussion

The biomass was manually cleaned to remove debris, followed by lyophilization and extraction. The extract was subjected to vacuum column chromatography using Diaion HP-20SS resin with a gradient of isopropyl alcohol (IPA) in H₂O to yield eight fractions. LC-MS and ¹H NMR dereplication of the fraction eluting with 40% IPA indicated the presence of a potentially new peptide with a molecular weight of 756 Da. (Orjala et al., 2011) Final purification by reversed-phase HPLC yielded microseiramide (**1**, 1.9 mg, 0.01% of dry biomass).

Microseiramide (**1**) was obtained as a white, amorphous powder. The molecular formula of **1** was determined as C₃₇H₅₆N₈O₉ (*m/z* 757.4258 [M + H]⁺) by HRESIMS analysis. The signal distribution pattern observed in the ¹H NMR and DEPTQ spectra (Table 1) indicated the peptidic nature of **1**. ¹H NMR of **1** exhibited a group of exchangeable amide NH signals (δ_{H} 7.2–8.7) as well as one primary amide moiety (δ_{H} 7.19; 7.91), amino acid α -proton signals (δ_{H} 3.6–4.7), and aliphatic methylene and methyl signals (δ_{H} 0.7–3.0). DEPTQ spectrum of **1** exhibited amide carbonyl signals (δ_{C} 169–173), signals typical of α -carbons (δ_{C} 50–64), and aliphatic methyl, methylene and methine signals (δ_{C} 12–39). ¹H NMR of **1** revealed the presence of several small peaks, presumably due to the presence of a minor conformer, which is a commonly observed for cyclic peptides with proline residues. (Brennan et al., 2008; Tripathi et al., 2009; Zhang et al., 2010) The structure elucidation of **1**

was performed using the major conformer. Combined analysis of the COSY and TOCSY spectra identified the presence of seven amino acid residues: serine (Ser), isoleucine (Ile), proline (Pro), asparagine (Asn), phenylalanine (Phe) and two valines (Val1 and Val2).

The complete sequence of the seven amino acid residues in **1** was established by analysis of the HMBC and ROESY spectra (Figure 1 and Table 1). HMBC correlations from Val2-NH (δ_{H} 8.24) to Ser C-1 (δ_{C} 168.9), from Ser-NH (δ_{H} 8.65) to Ile C-1 (δ_{C} 170.0), from Ile-NH (δ_{H} 8.61) to Asn C-1 (δ_{C} 170.9), from Asn-NH (δ_{H} 8.63) to Val1 C-1 (δ_{C} 171.4), from Val1-NH (δ_{H} 7.29) to Phe C-1 (δ_{C} 170.5), and from Phe-NH (δ_{H} 8.08) to Pro C-1 (δ_{C} 169.9) suggested a sequence of Val2-Ser-Ile-Asn-Val1-Phe-Pro (Table 1). ROESY correlations confirmed this linear sequence. ROESY correlations between Pro H₂-5 (δ_{H} 3.83) and Val2 H-2 (δ_{H} 4.02), and between Pro H₂-5 (δ_{H} 3.83) and Val2 H-3 (δ_{H} 1.70) closed the macrocycle and identified the peptide sequence as cyclo[Val2-Ser-Ile-Asn-Val1-Phe-Pro-]. This sequence assignment was confirmed by MS/MS analysis using a quadrupole-time-of-flight (qTOF) tandem mass spectrometer (Figure 2). The parent ion $[\text{M}+\text{H}]^+$ 757.5 was fragmented using a 35 eV collision energy and the macrocycle was opened between Pro and Val2 residues. Continuous fragmentation generated fragment ions as shown in Figure 1, and were in complete agreement with the sequence determined by NMR analysis.

The absolute configurations of the amino acids were assigned using the advanced Marfey's method after acid hydrolysis (Supporting information), and assigned L configurations for all the amino acid residues. (Fujii et al., 1997a; Fujii et al., 1997b) Thus, the final structure of **1** was established as cyclo[L-Val2-L-Ser-L-Ile-L-Asn-L-Val1-L-Phe-L-Pro-].

Microseiramide (**1**) is a cyclic peptide containing seven proteinogenic amino acids with L configurations. The structural features of **1** matched with the definition of cyanobactins, which are structurally variant and ribosomally synthesized cyclic peptides composed of seven to 20 amino acids, including a conserved proline residue. (Leikoski et al., 2010; Sivonen et al., 2010) Recently, genome sequencing revealed the broad distribution of ribosome-dependent biosynthetic pathways across the multiple genera in the phylum *Cyanobacteria*. (Shih et al., 2013) Further investigations of UIC 10445 are necessary to determine whether microseiramide (**1**) is biosynthesized by ribosome through the posttranslational modifications of short precursor proteins, or through a nonribosomal peptide synthetase (NRPS) pathway.

Microseiramide (**1**) was evaluated in the brine shrimp lethality assay (132 μM), cytotoxicity assays against HT-29 and MDA-MB-435 human cancer cell lines (33 μM), protease (elastase, trypsin and chymotrypsin) inhibition assays (13 μM), as well as growth inhibition assays against *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (50 $\mu\text{g/mL}$), however no activity was observed in any of the assays at the highest concentration tested.

Although the ecological functions of most natural products produced by cyanobacteria are still undetermined, it is broadly accepted that these compounds are produced to affect the surrounding environment and act as defense against predators and competitors. (Burja et al., 2001) Microseiramide (**1**) may contribute to the extremely high population density of UIC

10445 observed in its wild habitat. However, the limited supply prevented evaluation in further assays to explore its potential ecological function.

The initial taxonomic designation of UIC 10445 was performed using traditional morphological analysis, and indicated that this strain was morphologically similar to cyanobacteria of the genus *Lyngbya* (Supporting Information). (Castenholz, 2001; Komárek et al., 2003) A phylogenetic analysis, using a 1.2 kb partial sequence of 16S rDNA, was performed to assist the taxonomic identification and indicated that UIC 10445 was a member of the *Lyngbya wollei* clade. A very recently published report revealed that cyanobacteria previously identified as *L. wollei* were distinct from the other freshwater species of the genus *Lyngbya* and that these cyanobacteria should be considered a separate genus described as *Microseira*. (McGregor and Sendall, 2015) Our data also showed that the clade containing UIC 10445 also contains *M. wollei* references strains. That clade was phylogenetically distinct from the other *Lyngbya* clades, as well as the *Moorea* clade (previously known as marine *Lyngbya* clade) (Figure 3). Thus, we identify UIC 10445 as a *Microseira* sp.

Cyanobacteria of the genus *Lyngbya* are thought to be some of the most prolific producers of secondary metabolites. (Burja et al., 2001; Engene et al., 2011; Jones et al., 2011) However, the traditional taxonomic identification of cyanobacteria has been based on morphological characterization, and cyanobacteria previously identified as members of the genus *Lyngbya* have been found to be phylogenetically distinct from each other. A comprehensive study of the phylogeny of the genus *Lyngbya* revealed that *Lyngbya* is a polyphyletic genus containing at least three distinct lineages: a marine lineage, a lineage closely related to the genus *Oscillatoria*, and a halophilic/brackish/freshwater lineage. (Engene et al., 2010) Cyanobacteria belong to the marine lineage, recently reclassified to be members of the genus *Moorea*, are responsible for more than 40% of all reported marine cyanobacterial secondary metabolites. (Engene et al., 2011; Engene et al., 2012) Freshwater *Lyngbya* strains either fall into the clade of halophilic/brackish/freshwater lineage, or belong to a larger polyphyletic group. (Engene et al., 2010) Although the freshwater *Lyngbya wollei* cluster was recently redefined to form a new genus *Microseira*, other cyanobacteria identified as freshwater *Lyngbya* spp. are still polyphyletic, and the taxonomy of *Lyngbya* genus may need further revision (Figure 3).

In summary, we have isolated a new cyclic heptapeptide from a field collected sample of the freshwater cyanobacterium *Microseira* sp. UIC 10445. This is the first report of cyclic peptide produced by a *Microseira* sp. (previously known as *L. wollei*). microseiramide (**1**) consists of seven standard amino acid residues with $\pm$ configurations and displayed no significant activity in the brine shrimp lethality assay, cytotoxicity assays, protease inhibition assays, anti-microbial and anti-fungal assays.

3 Experimental

3.1 General Experimental Procedures

The optical rotation was measured using a Perkin-Elmer 241 polarimeter at 22 °C MeOH. The UV spectrum was recorded using a Shimadzu UV spectrophotometer UV2401. The IR

spectrum was acquired on a Perkin-Elmer 577 IR spectrophotometer. The 1D and 2D NMR spectra including ^1H NMR, COSY, TOCSY, HSQC, HMBC, ROESY were acquired on a Bruker Avance DRX 600 MHz spectrometer, whereas a Bruker Avance AVII 900 MHz NMR spectrometer was used to acquire the DEPTQ spectrum. ^1H and ^{13}C NMR chemical shifts were referenced to the DMSO- d_5 residual solvent signals (δ_{H} 2.50 and δ_{C} 39.51, respectively). The TOCSY experiment was conducted using a 60 ms mixing time. The ROESY spectrum was acquired with a 200 ms mixing time. The HSQC spectrum was recorded with the average $^1J_{\text{CH}}$ of 145 Hz and the HMBC spectrum was recorded with the average $^3J_{\text{CH}}$ of 8 Hz. The high-resolution ESI mass spectrum and LC-MS data were acquired using a Shimadzu HPLC-IT-TOF spectrometer. The HPLC separations were performed using an Agilent 1100 series instrumentation and a Waters instrumentation with Waters Delta 600 pump and Waters 2487 UV detector.

3.2 Biological Material and Morphological Identification

Microseira sp. UIC 10445 was collected from the bottom of a shallow freshwater lake in Northern Indiana in July 2013 (N 41° 18' 24", W 85° 44' 6"). The biomass was manually cleaned to remove debris and freeze-dried. The initial taxonomic identification was performed by morphological observation according to the system by Komárek *et al* using a Zeiss Axiostar Plus light microscope equipped with a Canon PowerShot A620 camera. (Komárek *et al.*, 2003) The morphological characteristics can be found in Supporting Information.

3.3 DNA Extraction, 16S rDNA PCR Amplification and Sequencing

A cleaned sample of *Microseira* sp. UIC 10445 was combined with 1.5 mL of lysozyme buffer and 0.5 mL of 20 mg/mL lysozyme stock solution. The cell mass was centrifuged and transferred to a 2 mL microcentrifuge tube after incubation at 35 °C for 1 h. The genomic DNA was extracted using a Wizard Genomic DNA purification kit from Promega. A partial sequence of the 16S rDNA gene was amplified by PCR using the cyanobacteria-specific primers 109F and 1509R. (Nubel *et al.*, 1997) The PCR reaction mixture contained DNA (1 μL , approximately 50 ng), 5 $\times$ GoTaq Reaction Buffer (5 μL), dNTP mix (0.5 μL , 10 mM), 109F and 1509R primer (1 μL each, 10 μM), GoTaq DNA Polymerase (0.25 μL , 2.5 u/ μL), and sufficient nuclease free water to make the total volume 25 μL . The reaction was performed in a Bio-Rad C1000 thermal cycler using the following reaction program: initial denaturation for 2 min at 95 °C, 35 amplification cycles of 50 s at 95 °C, 50 s at 49 °C and 2 min at 72 °C, and a final extension for 5 min at 72 °C. PCR products were purified using a MinElute PCR purification kit from Qiagen and subjected to Sanger sequencing using the cyanobacteria-specific primers 109F, 359F and 1509R. (Nubel *et al.*, 1997) The resulting 16S rDNA gene sequence was deposited in the NCBI GenBank under the Accession No. KJ813000.

3.4 Phylogenetic Analysis of *Microseira* sp. UIC 10445

MEGA 5.05 was used to perform the phylogenetic analysis. The resulting Sanger sequencing chromatograms were visually inspected, and the total sequence of 1234 nucleotides (GenBank accession number KJ813000) was aligned with 31 cyanobacterial

species obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). Multiple sequence alignment was performed via the ClustalW interface. The aligned sequences were used to construct phylogenetic trees in MEGA 5.05. The calculated optimum nucleotide substitution model was Kimura 2-parameter with a discrete Gamma distribution parameter of 0.19. The reference strains *Oscillatoria acuminata* PCC6304, *Oscillatoria sancta* PCC7515 and *Lyngbya aestuarii* PCC7419 were selected according to *Bergey's Manual of Systematic Bacteriology*. (Castenholz, 2001) The reference strains *Moorea producens* 3L and *Moorea bouillonii* PNG5-198 were chosen according to the literature by Engene et al. (Engene et al., 2012) The reference strains of *Microseira wollei* were selected according to the publication by McGregor et al. (McGregor and Sendall, 2015) All the other strains were selected based upon a BLAST search of the UIC 10445 16S rDNA partial sequence. Phylogenetic trees were constructed using neighbor joining, maximum parsimony and minimum deviation methods, and all three trees showed similar topology. The evolutionary history was inferred using the neighbor-joining, minimum evolution and maximum parsimony methods. For each method, the bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Phylogenetic analysis revealed that UIC 10445 was a member of the *Microseira* cluster. Combining the morphological characters of UIC 10445, we identify UIC 10445 as *Microseira* sp.

3.5 Extraction and Isolation

The lyophilized biomass (13.9 g) was extracted with a mixture of CH_2Cl_2 and MeOH (1:1) and dried *in vacuo* to yield 1.38 g organic extract. The extract was fractionated using Diaion HP-20SS vacuum liquid chromatography with an IPA/water step gradient (0%, 20%, 40%, 60%, 70%, 80%, 90% and 100%) to yield eight fractions. LC-MS and ^1H NMR dereplication indicated the presence of a potentially new peptide with a molecular weight of 756 Da in the fraction eluted at 40% IPA. (Orjala et al., 2011) Subsequent reversed-phase semi-preparative HPLC (Varian C₁₈ semi-preparative column, 10 × 250 mm, 5 μm , 3 mL/min, 65%~100% MeOH in water over 25 min) yielded microseiramide (**1**, 7.8 min, 1.9 mg, 0.01% of dry biomass).

3.6 Microseiramide (1)

White, amorphous powder; $[\alpha]_{\text{D}}^{22}$ -30.7 (c 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 214 (4.01), 265 (2.72) nm; IR (neat) ν_{max} 3303 (br), 2967, 2877, 1661, 1524, 1439, 1204, 1135 cm^{-1} ; ^1H and ^{13}C NMR see Table 1; HR-ESI-TOF-MS (+) m/z 757.4258 [$\text{M} + \text{H}$]⁺ (calcd for C₃₇H₅₇N₈O₉, 757.4249).

3.7 Determination of Absolute Configurations of Amino Acids by the Advanced Marfey's Analysis

Approximately 0.2 mg of **1** was hydrolyzed in 1 mL 6N HCl for 20 h at 110 °C in pressure tubes sealed with Teflon tape. The cooled hydrolysate mixtures were dried *in vacuo*, and traces of HCl were removed by repeated evaporation. The resulting hydrolysate was separated into two equal portions for derivatization with either L- or D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide, from TCI America). Each hydrolysate portion was dissolved in 110 μL of acetone followed by 50 μL of de-ionized water, and then mixed with 20 μL of 1 N

NaHCO₃. Finally, 20 µL of L-FDLA or D-FDLA (10 mg/mL in acetone) was added, and the mixtures were heated to 40 °C for 1 h. The reaction mixtures were cooled to room temperature, and 20 µL of 1 N HCl was added to quench the reaction. The cooled reaction mixtures were dried *in vacuo*, and re-suspended in 300 µL acetonitrile. LC-MS analysis was performed using a reversed-phase column (Phenomenex Kinetex C₁₈, 250 × 4.6 mm, 5 µm, 1.0 mL/min) with a linear gradient from 25% to 65% aqueous acetonitrile containing 0.1% formic acid over 50 min. The selective ion chromatograms of L-FDLA and DL-FDLA derivatives (DL-FDLA derivative was the mixture of L- and D-FDLA derivatives) of Marfey's derivative of each amino acid were compared for the assignment of amino acid configurations. The absolute configuration of isoleucine residue was assigned by comparing the retention time of hydrolysate-L-FDLA derivative with the retention times of appropriate isoleucine stereo isomers derivatized with L-FDLA or D-FDLA. The table for retention times of each amino acid can be found in Supporting Information.

3.8 Brine Shrimp Lethality assay

A modification of the previously described method was used to assess lethality to the brine shrimp (*Artemia salina*). (Singh et al., 1999) Approximately 10 hatched brine shrimps in 80 µL artificial seawater (made from Sigma Sea Salts, S9883, 40 g/L) were added to each well containing varying concentrations of microseiramide in 1 µL DMSO and 19 µL of artificial seawater to make a total volume of 100 µL. Each concentration of sample was run in duplicate. The number of dead and alive brine shrimps was counted under a dissecting microscope after 24 h incubation at room temperature.

3.9 Cytotoxicity assays

Cytotoxicity assays against the MDA-MB-435 and HT-29 cancer cell line were performed according to established protocols. (Ayers et al., 2011)

3.10 Protease Inhibition assays

The inhibition assays against elastase, trypsin and chymotrypsin were conducted according to established protocols. (Kang et al., 2012)

3.11 Anti-bacterial and anti-fungal assays

The inhibition assays against *M. tuberculosis*, *M. smegmatis*, *S. aureus*, *E. coli* and *C. albicans* were performed according to established protocols. (Luo et al., 2014)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We isolated a cyclic heptapeptide, named microseiramide, from a freshwater cyanobacterium UIC 10445.
- UIC 10445 was identified as a member of the recently described genus *Microseira*.
- Microseiramide is the first cyclic peptide reported from a *Microseira* sp.

Figure 1.
Key 2D NMR correlations of microseiramide (**1**).

Figure 2.
Q-TOF MS/MS fragmentation analysis of microseiramide (1).

Figure 3.

Neighbor-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequence. Species and strain numbers are given with accession numbers in parentheses. Reference strains obtained from *Bergey's Manual* were denoted with an asterisk (*). (Castenholz, 2001) Reference strains characterized by Engene *et al* were denoted with two asterisks (**). (Engene et al., 2012) Only bootstrap values greater than or equal to 75% are displayed.

Table 1

NMR Spectroscopic Data of microseiramide (1) in DMSO-*d*₆

	position	δ_C^a , mult	δ_H^b , mult. (<i>J</i> in Hz)	COSY ^d	HMBC ^d	ROESY ^d
Ile	1	170.0, C				
	2	59.7, CH	3.70, d (6.1)	NH, 3	NH	4, 3-Me, NH _{Ser}
	3	35.2, CH	1.64, m	2, 3-Me, 4		
	3-Me	14.6, CH ₃	0.92, d (6.9)	3	2, 3, 4	
	4	25.7, CH ₂	1.17, m	3, 5		
Asn			1.64, m			
	5	11.4, CH ₃	0.89, t (7.2)	4	4	
	NH		8.61, s	2	2, 1 _{Asn}	2, 2 _{Asn}
	1	170.9, C				
	2	49.6, CH	4.67, m	NH, 3		3, NH _{Ile}
Val	3	34.2, CH ₂	3.03, dd (17.3, 3.5)	2	4	5 _{Pro}
			3.07, dd (17.3, 3.5)			
	4	172.6, C				
	NH		8.63, s	2	1 _{Val}	2 _{Val}
	NH ₂		7.19, s		3, 4	3
Phe			7.91, s			
	1	171.4, C				
	2	57.5, CH	4.36, overlapped	NH, 3	3, 3-Me, NH	4, NH _{Asn}
	3	32.4, CH	1.72, m	2, 3-Me, 4	NH	
	3-Me	19.3, CH ₃	0.90, d (6.7)	3	2, 3	
Phe	4	18.7, CH ₃	0.84, d (6.7)	3	2, 3	
	NH		7.29, overlapped	2	1 _{Phe}	2 _{Phe}
	1	170.5, C				
	2	54.2, CH	4.33, m	NH, 3	3, NH	NH _{Val}
	3	37.9, CH ₂	2.89, t (13.2)	2	2, 4, 5/9	
			3.20, dd (13.2, 3.2)			

position	δ_C^a , mult	δ_H^b , mult. (J in Hz)	COSY ^d	HMBC ^d	ROESY ^d
4	138.4, C				
5/9	126.2, CH	7.18, m	6/8, 7	3, 5/9, 6/8, 7	
6/8	128.0, CH	7.25, m	5/9, 7	5/9, 6/8, 7	
7	129.0, CH	7.13, m	5/9, 6/8		
NH		8.08, d (10.4)	2	1 _{Pro}	3, 2 _{Pro}
Pro					
1	169.9, C				
2	62.9, CH	3.91, dd (7.5, 2.6)	3	3	4, NH _{Pro}
3	28.8, CH ₂	1.23, m	2, 4		
		1.95, m			
4	25.3, CH ₂	1.65, m	3, 5	2, 3	
		1.77, m			
5	47.6, CH ₂	3.33, m	4		3 _{Asn} , 2 _{Val2} , 3 _{Val2}
		3.83, m			
Val2					
1	169.7, C				
2	61.4, CH	4.02, overlapped	NH, 3	3, 4, NH	4, 5 _{Pro}
3	31.8, CH	1.70, m	2, 3-Me, 4		5 _{Pro}
3-Me	19.5, CH ₃	0.85, d (6.7)	3	2, 3	
4	19.4, CH ₃	0.77, d (6.7)	3	2, 3	
NH		8.24, d (10.3)	2	1 _{Ser}	2 _{Ser}
Ser					
1	168.9, C				
2	58.9, CH	3.62, m	NH, 3	3	NH _{Val2}
3	60.0, CH ₂	3.82, overlapped	2	2	
		4.04, overlapped			
OH		nd ^c			
NH		8.65, s	2	1 _{Ile}	2 _{Ile}

^aCarbon chemical shifts were assigned from DEPTQ spectrum recorded at 226 MHz.^bRecorded at 600 MHz.^cnd: not detected.

p Correlations without a subscript are within the amino acid residue.

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