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Porpoisamides A and B, two novel epimeric cyclic depsipeptides from a Florida Keys collection of *Lyngbya* sp

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Abstract

NMR-guided fractionation of a non-polar extract of a Florida Keys collection of *Lyngbya* sp. resulted in the isolation of two novel epimeric cyclic depsipeptides, porpoisamides A (**1**) and B (**2**). The planar structures of these compounds were determined using NMR spectroscopic techniques. The absolute configurations of amino and hydroxy acid subunits were assigned by enantioselective HPLC analysis. These compounds showed weak cytotoxicity towards HCT-116 colorectal carcinoma and U2OS osteosarcoma cells. The porpoisamides are a unique pair of cyclic depsipeptides that are epimeric at C-2 of the β -amino acid, 3-amino-2-methyloctanoic acid.

Keywords

Cyanobacteria; *Lyngbya majuscula*; depsipeptides; porpoisamide

1. Introduction

An ever increasing number of cyclic peptides and depsipeptides are being isolated from marine cyanobacteria of the genus *Lyngbya*.¹ The structures of these secondary metabolites are as diverse as the biological activities they produce. They can range in size from as many as ten or more amino acid units to as few as two units. Many of the peptides and depsipeptides produced by cyanobacteria contain modified amino acid units as well as polyketide portions. These modifications presumably confer resistance to degradation and produce a variety of biological activities, including antimicrobial, antiviral, and cytotoxic effects.²⁻⁴

One type of modification seen in cyclic peptides and depsipeptides produced by marine cyanobacteria are β -amino acid units.⁴ These β -amino acids often contain functionalized polyketide chains of varying lengths. The guineamides A-F are an example of a series of

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Supporting information available: ¹H, ¹³C, DQF COSY, HSQC, HMBC, and NOESY NMR spectra in CD₃CN for compounds **1** and **2**.

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cyclic depsipeptides that contain a variety of β -amino acid units as well as other modifications to the amino and hydroxy acid units found within the molecule.⁵ Other examples from cyanobacteria include homodolastatin 16,⁶ lobocyclamide B,⁷ obyamide,⁸ ulongamides A-F,⁹ grassypeptolides,^{10, 11} and ulongapeptin.¹² These secondary metabolites have shown biological activity in anti-fungal and cytotoxicity assays.⁶⁻¹² Reported here is the isolation and structure determination of two epimeric cyclodepsipeptides, porpoisamide A (**1**) and porpoisamide B (**2**).

2. Results and Discussion

A sample of the cyanobacterium *Lyngbya* sp. was collected from the seagrass bed adjacent to Porpoise Key in the Florida Keys in July 2008. The freeze-dried sample was extracted with a 1:1 mixture of MeOH and EtOAc and then partitioned between EtOAc and H₂O. The EtOAc-soluble portion was fractionated by silica gel column chromatography. The fraction eluting with EtOAc showed proton resonances indicative of two peptides in a 2:1 ratio and was further separated using normal phase column HPLC. All resulting fractions with similar ¹H NMR spectra were combined and purified using reversed-phase HPLC to yield two new cyclic depsipeptides, porpoisamide A (**1**) and porpoisamide B (**2**).

Porpoisamide A (**1**) was isolated as a white powder and its molecular formula was determined to be C₃₃H₅₀N₄O₆ based on the [M + H]⁺ ion peak at *m/z* 599.3795 in HRESIMS. Analysis of the ¹H, ¹³C, and edited HSQC NMR data indicated five carbonyl carbons and a phenyl ring to account for nine of the eleven degrees of unsaturation suggested by the molecular formula. The IR spectrum indicated the presence of phenyl, amide, ester, and NH groups with broad bands at 1032, 1622, 1744, and 3332 cm⁻¹, respectively. Based on the IR data and the ¹H and ¹³C NMR spectroscopic data, the four nitrogen atoms in the molecule could be accounted for by one tertiary amide, two secondary amides (δ_{H} 6.57, 8.93) and one *N*-Me amide (δ_{H} 2.96). The six oxygen atoms were found to make up one ester and four amide carbonyl groups, implying that porpoisamide A (**1**) was a depsipeptide consisting of one hydroxy and four amino acid units.

The DQF COSY and HMBC experiments enabled the identification of three of the amino acid units and one α -hydroxy acid group. The COSY correlations observed from the proton resonance at δ_{H} 4.35 to both the methyl group at δ_{H} 1.25 and the NH group at δ_{H} 6.57 along with the HMBC correlation to the carbonyl carbon resonance at δ_{C} 172.7 identified the alanine unit. The HMBC and COSY correlations connected the phenyl ring to the methine at δ_{H} 5.52 through a methylene (δ_{H} 2.90, 3.26; δ_{C} 34.3). Further HMBC correlations linked the methine at δ_{H} 5.52 to the *N*-methyl group at δ_{H} 2.96 and to the carbonyl carbon resonance at δ_{C} 170.5 to form *N*-Me-phenylalanine. The chemical shifts of the methine at C-24 (δ_{H} 4.51; δ_{C} 59.6) indicated that it was adjacent to a nitrogen atom. HMBC correlations connected this methine to the carbonyl carbon resonance at δ_{C} 175.3, and the COSY correlations attached it to a methylene (δ_{H} 1.80, 1.57), which is part of three consecutive methylenes. The chemical shifts of the end methylene (δ_{H} 3.70, 3.44) of this chain and its HMBC correlation to the methine (δ_{H} 4.51) through the nitrogen established the proline unit. COSY correlations indicated that the oxymethine C-29 at δ_{H} 5.23 was connected to two methyl groups through a methine and a methylene to form 2-hydroxy-3-methylpentanoic acid. Therefore, four of the subunits of the peptide were identified as alanine, *N*-Me-phenylalanine, proline, and 2-hydroxy-3-methylpentanoic acid (Hmpa).

Based on the NMR data, the remaining subunit of this depsipeptide was identified as an unusual amino acid containing two methyl, four methylene, and two methine groups. HMBC correlations from the methine at δ_{H} 2.70 and the methyl doublet at δ_{H} 1.13 to the carbonyl carbon resonance at δ_{C} 174.8 showed the methine was alpha to the carbonyl carbon. The

COSY correlation from the methine at δ_{H} 2.70 to the methine at δ_{H} 3.97 showed the methine at δ_{H} 3.97 to be beta to the carbonyl carbon. The methine at δ_{H} 3.97 was shown to be connected to the NH group at δ_{H} 8.93 through COSY correlations. The four methylene groups were shown to be connected in a chain starting at the methine at δ_{H} 3.97 and ending with the methyl group at δ_{H} 0.86 to form the 3-amino-2-methyloctanoic acid (Amoa) as the final subunit of porpoisamide A(**1**).

The five subunits of porpoisamide A(**1**) were connected together through HMBC correlations. The 3-amino-2-methyloctanoic acid group had to be connected to alanine based on the correlation between the NH group at δ_{H} 8.93 and the carbonyl group at δ_{C} 172.7. Alanine was connected to the *N*-Me-phenylalanine because of a correlation between the NH group at δ_{H} 6.57 and the carbonyl carbon at δ_{C} 170.5. *N*-Me-phenylalanine was attached to the proline subunit based on the correlation between the *N*-Me group at δ_{H} 2.96 and the proline carbonyl carbon at δ_{C} 175.3. Finally, the proline group was connected to the Hmpa subunit through a correlation between the methylene proton H-27a at δ_{H} 3.70 and the carbonyl carbon C-28 at δ_{C} 170.0. To account for the remaining degree of unsaturation and the molecular formula the Hmpa subunit had to be attached to the Amoia group to form the cyclic depsipeptide structure depicted for **1**. This connection was confirmed by the HMBC correlation between the methine proton H-29 at δ_{H} 5.23 and the carbonyl carbon C-1 at δ_{C} 174.8.

Porpoisamide B (**2**) was found to have the same molecular formula as porpoisamide A(**1**) ($\text{C}_{33}\text{H}_{50}\text{N}_4\text{O}_6$, m/z 599.3798, $[\text{M}+\text{H}]^+$). Analysis of the ^1H , ^{13}C , and 2D NMR spectra indicated that **2** was similar in structure to **1** as well. While the ^1H and ^{13}C NMR resonance values for the alanine, *N*-Me-phenylalanine, proline, and Hmpa were consistent with that of porpoisamide A (**1**), chemical shifts of the proton and carbon resonances of the β -amino acid portion indicated a difference in this segment of the molecule. The 1D TOCSY experiments suggested the only difference between these segments of porpoisamides A (**1**) and B (**2**) involved the stereoconfiguration of the molecule. Excitation of the methine proton at C-2 (δ_{H} 2.70 for **1**; δ_{H} 2.67 for **2**) in both molecules produced spectra with proton resonances for the methine at C-3, the methyl group at C-9, and the NH group. Excitation of the methine proton at C-3 (δ_{H} 3.97 for **1**; δ_{H} 4.02 for **2**) in both molecules produced spectra with proton resonances for the methyl group at C-9, the methine at C-2, the NH group, the methylene groups at C-4 through C-7, and the methyl group at C-8. This data in conjunction with COSY and HMBC correlations indicated that the planar structure of the unusual amino acid was the same in both porpoisamide A (**1**) and B (**2**).

Analysis of the NMR spectra of **1** and **2** showed them to have the same planar structure. Each contained three typical amino acids (alanine, *N*-Me-phenylalanine, and proline), one α -hydroxy acid (2-hydroxy-3-methylpentanoic acid), and the rare β -amino acid (3-amino-2-methyloctanoic acid). Therefore, it was necessary to investigate the configuration of the eight chiral centers found within the molecules in order to differentiate the structures of the two depsipeptides.

The absolute configurations of the alanine, *N*-Me-phenylalanine, proline, and Hmpa were determined by stereoselective HPLC. Porpoisamides A (**1**) and B (**2**) were subjected to acid hydrolysis and the retention times of the amino acids and the hydroxy acid were compared to those of authentic standards run under the same conditions. The results of this analysis showed the absolute configuration of these α -hydroxy and amino acids to be the same for both compounds. The absolute configuration of alanine and proline was found to be *L*, the configuration of *N*-Me-phenylalanine was *D*, and Hmpa was found to have 2*S*,3*S* configuration. This analysis determined that six of the eight stereogenic centers within the peptides were identical.

To determine the absolute configuration of the remaining two stereogenic centers within the 3-amino-2-methyloctanoic acid, advanced Marfey's method was used.¹³ The methods and retention times published by Williams *et al.* in 2003¹² were used for comparison with the derivatized hydrolysate of **1** and **2**. The published elution order for the α -FDLA derivatized Amoa units using reversed-phase HPLC is (2*R*,3*S*)-Amoa, (2*S*,3*S*)-Amoa, (2*R*,3*R*)-Amoa, and (2*S*,3*R*)-Amoa.^{12, 14} Performing the same analysis with porpoisamides A and B indicated that **1** contained (2*S*,3*R*)-Amoa and **2** contained (2*R*,3*R*)-Amoa. The results from this analysis were supported by NOE correlations from the NMR spectra. The 2D NOESY spectrum for porpoisamide A (**1**) showed strong correlations from H-2 to H-3 and H-9. This data indicated that H-2 and H-3 were on the same side of the ring structure. This configuration matches that seen in the 2*S*,3*R* isomer of 3-amino-2-methyloctanoic acid. The 2D NOESY spectrum of porpoisamide B (**2**) showed a strong correlation from H-3 to H-9 and no NOE correlation between H-2 and H-3, indicating that H-3 and H-9 were close in space and therefore on the same side of the ring structure. The resulting configuration is that seen in the 2*R*,3*R* isomer of 3-amino-2-methyloctanoic acid.

Many of the linear and cyclic peptides produced by cyanobacteria have potent activity in cytotoxicity assays.^{2, 4, 15} For this reason, both of the cyclic peptides were tested for their ability to inhibit the growth of two solid tumor cell types: HCT-116 colorectal carcinoma and U2OS osteosarcoma cells. The IC₅₀ values for compound **1** were 25 μ M and 28 μ M, respectively. Compound **2** gave similar IC₅₀ values of 21 μ M and 22 μ M, respectively. Thus, these cyclic depsipeptides produce only moderate antiproliferative activity against these cell lines.

3. Experimental

3.1 General experimental procedures

The optical rotation was measured on a Perkin Elmer model 343 polarimeter. IR spectroscopic data were obtained on a Perkin Elmer Spectrum 100 FT-IR spectrometer. ¹H, ¹³C and 2D NMR spectra for **1** and **2** were recorded in CD₃CN on a JEOL ECA-600 operating at 600 MHz using residual solvent resonances for reference (δ_{H} 1.94, δ_{C} 118.7 for acetonitrile). The HRMS data were obtained using an Agilent 6210 LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector at the Mass Spectrometer Facility at the University of California, Riverside, California.

3.2 Biological material, collection and identification

The marine cyanobacterium, *Lyngbya* sp., was collected by hand from the seagrass bed located adjacent to Porpoise Key of the Florida Keys in July 2008. The sample was drained of seawater, frozen at -20 °C, and subsequently freeze-dried. A voucher specimen (#VJP_PK_7_22_08) was preserved in 5% formalin/seawater and is retained at the Smithsonian Marine Station, Fort Pierce, FL.

3.3 Extraction and Isolation

The freeze-dried sample (298.1 g) was exhaustively extracted with 1:1 MeOH-EtOAc to yield 40.3 g of crude extract. This extract was then partitioned between EtOAc and H₂O. The EtOAc partition (3.1 g) was further separated on a silica gel column using a hexanes-EtOAc-MeOH step gradient system to give six fractions (90:10:0, 50:50:0, 0:100:0, 0:95:5, 0:90:10, 0:0:100; 200 mL of each). Fraction 3 (118.8 mg), 100% EtOAc, was separated by NP-HPLC [Alltima Silica, 10 μ m, 10 \times 250 mm, 3 mL/min, detection at 220 and 254 nm] with 50% EtOAc in hexanes. All resulting fractions were examined by NMR. Those with similar ¹H NMR spectra were combined into one fraction (26.8 mg). This fraction was then separated by RP-HPLC [Econosil C18, 10 μ m, 10 \times 250 mm, 3 mL/min, detection at 220

and 254 nm], first with 90% MeOH in H₂O with 0.1% acetic acid to give a mixture of porpoisamides (9.4 mg, *t_R* 7.2 min) and then with 70% CH₃CN in H₂O with 0.1% acetic acid to yield 6.0 mg of **1** (*t_R* 16.7 min, yield 0.002% dry wt) and 3.3 mg of **2** (*t_R* 14.7 min, yield 0.0011% dry wt). The NMR guided-fractionation of this sample provided no evidence that epimerization may have occurred during the isolation process. Both purified porpoisamides were stable and did not interconvert.

3.3.1 Porpoisamide A (1)—Yield 6.0 mg. white powder; $[\alpha]_D^{20} +77.6$ (*c* 0.45, CH₃OH); IR ν_{\max} 3332, 2940, 1744, 1622, 1456, 1032 cm⁻¹; UV (MeOH), λ_{\max} (log ϵ) 255 (2.45); ¹H NMR (CD₃CN, 600 MHz) and ¹³C NMR (CD₃CN, 150 MHz) data see Table 1; HRESI/APCIMS *m/z* 599.3795 [M + H]⁺ (calcd for C₃₃H₅₁N₄O₆, 599.3809).

3.3.2 Porpoisamide B (2)—Yield 3.3 mg. white powder; $[\alpha]_D^{20} +22.7$ (*c* 0.24, CH₃OH); IR ν_{\max} 3437, 2962, 2344, 1576, 1415, 1046 cm⁻¹; UV (MeOH), λ_{\max} (log ϵ) 253 (2.38); ¹H NMR (CD₃CN, 600 MHz) and ¹³C NMR (CD₃CN, 150 MHz) data see Table 2; HRESI/APCIMS *m/z* 599.3798 [M + H]⁺ (calcd C₃₃H₅₁N₄O₆, 599.3809).

3.4 Enantioselective HPLC analysis

2-hydroxy-3-methylpentanoic acid standards were synthesized according to literature methods.¹⁶ Compounds **1** and **2** were hydrolyzed (~0.2 mg each) in 6 N HCl at 115 °C for 18 h in a sealed tube. The hydrolysis products were then concentrated to dryness and re-dissolved in 2.0 mM CuSO₄ in H₂O. Analysis by enantioselective HPLC was carried out using three separate conditions and the retention times were compared to those of authentic standards. Condition 1: 5% CH₃CN in 2.0 mM CuSO₄ in H₂O, 1.0 mL/min, Phenomenex Chirex 3126 (4.6 × 250 mm) column; elution times (*t_R*, min) for standards: L-alanine (6.2), D-alanine (7.3), L-proline (9.2), and D-proline (17.9). Condition 2: 15% CH₃CN in 2.0 mM CuSO₄ in H₂O, 1.0 mL/min, Phenomenex Chirex 3126 (4.6 × 250 mm) column; elution times (*t_R*, min) for standards: L-N-Me-phenylalanine (29.2), D-N-Me-phenylalanine (32.8). Condition 3: 15% CH₃CN in 2.0 mM CuSO₄ in H₂O, 1.0 mL/min, Diacel Chemical Laboratories, Ltd. Chiralpak MA (+) (4.6 × 50 mm) column; elution times (*t_R*, min) for standards: (2*R*,3*S*)-Hmpa (16.8), (2*R*,3*R*)-Hmpa (19.7), (2*S*,3*R*)-Hmpa (25.9), (2*S*,3*S*)-Hmpa (31.7). The hydrolysate for **1** was analyzed by the above methods to reveal the presence of L-alanine (6.1 min), L-proline (8.9 min), D-N-Me-phenylalanine (32.5 min), and (2*S*,3*S*)-Hmpa (31.8 min). The hydrolysate for **2** was analyzed by the above methods to reveal the presence of L-alanine (6.2 min), L-proline (8.9 min), D-N-Me-phenylalanine (32.0 min), and (2*S*,3*S*)-Hmpa (31.8 min). The presence of D-N-Me-phenylalanine in both compounds was confirmed by co-injection with the standard.

3.5 Analysis of 3-amino-2-methyloctanoic acid by advanced Marfey's method

Compounds **1** and **2** were hydrolyzed (~0.8 mg each) in 6 N HCl at 115 °C for 18 h in a sealed tube. The hydrolysates were dried under air and then re-dissolved in 400 μL of 50:50 acetonitrile:water. Then 100 μL of each hydrolysate product was derivatized with L-FDLA and 100 μL of each was derivatized with DL-FDLA using standard procedures.^{13, 17} Standard samples of L-valine, L-alanine, L-proline, and N-Me-D-phenylalanine were also derivatized with L-FDLA. The analysis was carried out by RP-HPLC using the same column as Williams *et al.*¹² (YMC-Pack AQ-ODS, 10 × 250 mm, 57% acetonitrile in water with 0.01 N TFA, flow rate 2.5 mL/min, absorbance detection at 340 nm). The published retention times of L-FDLA derivatized Amino standards were 25.7 min (2*R*,3*S*), 26.7 min (2*S*,3*S*), 45.6 min (2*R*,3*R*), and 54.1 min (2*S*,3*R*).^{12, 14} The retention time of the Amino portion for **1** was 48.5 and 23.5 min for L-FDLA and D-FDLA, respectively. The retention time of the Amino portion for **2** was 40.5 and 24.5 min for L-FDLA and D-FDLA, respectively. The previously

identified amino acids appeared at 10.1 min (*L*-Ala), 9.6 min (*L*-Pro), and 12.6 min (*N*-Me-*D*-Phe).

3.6 Activity

Cell viability was assessed using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cancer cells were plated in 96-well plates (HCT-116, 10,000; U2OS, 4,000), and after 24 h were treated with various concentrations of compounds **1**, **2** (purity >95%) or solvent control (1% EtOH). After 48 h of incubation, cell viability was measured using MTT according to the manufacturer's instructions (Promega). Assays were run in quadruplicate and dose-response curves were generated using XLfit Excel (ID Business Solutions Ltd.).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of Porpoisamides A (1) and B (2)

Table 1

NMR data for found compound (1 in CD₃CN (600 MHz))

	δ_H (J in Hz)	δ_C , mult.	COSY ^a	HMBC ^b
Amox				
1		174.8, C		
2	2.70, qd (7.5, 4.1)	44.9, CH	3, 9	1, 3, 4, 9
3	3.97, m	52.8, CH	2, 4b, 9, NH	1, 2, 4
4a	1.60, m	29.7, CH ₂	4b, 5a, 5b	3, 5, 6
4b	1.11, m		4a, 5a	3, 5, 6
5a	1.28, m	32.9, CH ₂	4a, 4b, 5b	4, 7
5b	1.13, m		4a, 5a	4, 7
6a	1.28, m	27.1, CH ₂	6b	
6b	1.12, m		6a	
7	1.27, m	23.8, CH ₂	8	5, 6
8	0.86, d (6.2)	14.8, CH ₃	7	
9	1.13, d (6.9)	15.7, CH ₃	2	1, 2
NH	8.93, d (8.9)		3	10
Ala				
10		172.7, C		
11	4.35, dq (8.3, 6.9)	52.4, CH	12, NH	10, 12, 13
12	1.25, d (6.8)	20.8, CH ₃	11	11, 10
NH2	6.57, d (8.3)		11	11, 12, 13
N-Me-Phe				
13		170.5, C		
14	5.52, dd (10.3, 5.5)	58.4, CH	15a, 15b	13, 15, 16, 22, 23
15a	3.26, dd (-15.0, 5.5)	34.3, CH ₂	14, 15b	13, 14, 16, 17, 21
15b	2.90, dd (-15.0, 10.3)		14, 15a	13, 14, 16, 17, 21
16		139.6, C		
17	7.18, d (6.9)	130.2, CH	19, 21	15, 18, 19
18	7.24, m	129.5, CH	17, 19, 21	16, 17
19	7.18, t (6.9)	127.6, CH	18, 20	17, 18, 20, 21
20	7.24, m	129.5, CH	17, 19, 21	16, 21
21	7.18, d (6.9)	130.2, CH	18, 20	15, 19, 20
22	2.96, s	32.4, CH ₃		14, 23

	$\delta_{\text{H}}(J \text{ in Hz})$	δ_{C} , mult.	COSY ^a	HMBC ^b
Pro				
23		175.3, C		
24	4.51, dd (6.9, 3.4)	59.6, CH	25a, 25b	23, 25
25a	1.80, m	28.8, CH ₂	24, 25b, 26b	26, 27
25b	1.57, m		24, 25a, 26a, 26b	23, 24, 26
26a	2.06, m	27.6, CH ₂	25b, 26b, 27a, 27b	24, 25
26b	1.87, m		25a, 25b, 26a, 27a, 27b	25, 27
27a	3.70, dd (9.0, 9.0)	48.2, CH ₂	26a, 26b, 27b	24, 25, 28
27b	3.44, m		26a, 26b, 27a	26
Hmpa				
28		170.0, C		
29	5.23, d (2.7)	78.3, CH	30	1, 28, 30, 31, 33
30	2.15, m	35.4, CH	29, 31a, 31b, 33	31, 32, 33
31a	1.38, m	24.3, CH ₂	31b, 32	29, 30, 32, 33
31b	1.17, m		30, 31a, 32	30, 32, 33
32	0.87, t (6.9)	12.5, CH ₃	31a, 31b	30, 31
33	1.01, d (6.9)	16.4, CH ₃	30	29, 30, 31

^a COSY correlations are from proton stated to indicated proton.

^b HMBC correlations are from proton stated to the indicated carbon.

Table 2

NMR data for compound(2) in CD₃CN (600 MHz)

	δ_{H} (J in Hz)	δ_{C} , mult.	COSY ^a	HMBC ^b
Amox				
1		173.4, C		
2	2.67, qd (6.9, 3.5)	45.9, CH	3, 9	1, 3, 9
3	4.02, m	53.1, CH	2, 4a, 4b, NH	
4a	1.47, m	35.3, CH ₂	3, 4b, 5a	3, 5, 6
4b	1.39, m		3, 4a, 5a, 5b	3, 5, 6
5a	1.26, m	32.8, CH ₂	4a, 4b, 5b	6, 7
5b	1.18, m		4a, 5a	4, 6, 7
6	1.26, m	27.2, CH ₂		5, 7
7	1.26, m	23.7, CH ₂	8	5, 6
8	0.86, t (6.9)	14.8, CH ₃	7	7
9	1.13, d (7.5)	14.8, CH ₃	2	1, 2, 3
NH	8.74, d (9.6)		3.00	10
Ala				
10		173.8, C		
11	4.37, dq (8.3, 7.3)	52.8, CH	12, NH	10, 12, 13
12	1.28, d (7.3)	21.3, CH ₃	11	10, 11
NH	6.67, d (8.3)		11	13
N-Me-Phe				
13		170.4, C		
14	5.52, dd (10.9, 5.2)	58.6, CH	15a, 15b	13, 15, 16, 22, 23
15a	3.27, dd (-14.4, 5.5)	34.3, CH ₂	14, 15b	14, 16, 17
15b	2.89, dd (-14.4, 10.9)		14, 15a	13, 14, 16, 17
16		139.6, C		
17	7.18, d (7.2)	130.2, CH	18	15, 19
18	7.24, m	129.5, CH	17	16, 17, 19
19	7.18, t (6.8)	127.6, CH	18, 20	17, 21
20	7.24, m	129.5, CH	19, 21	16, 19, 21
21	7.18, d (7.2)	130.2, CH	20	15, 19
22	2.95, s	32.3, CH ₃		14, 23
Pro				
23		174.6, C		

	$\delta_{\text{H}}(\text{J in Hz})$	$\delta_{\text{C, mult.}}$	COSY ^a	HMBC ^b
24	4.49, dd (6.8, 3.5)	59.5, CH	25a, 25b	23, 25
25a	1.80, m	28.8, CH ₂	24, 25b, 26b	26, 27
25b	1.56, m		24, 25a, 26a, 26b	24, 26
26a	2.06, m	27.5, CH ₂	25b, 26b, 27a, 27b	24, 25
26b	1.88, m		25a, 25b, 26a, 27a, 27b	25, 27
27a	3.70, t (9.6)	48.2, CH ₂	26a, 26b, 27b	24, 25
27b	3.44, dt (10.4, 6.1)		26a, 26b, 27a	26
Hmpa		169.9, C		
29	5.20, d (2.1)	78.1, CH	30	1, 28, 30, 31, 33
30	2.15, m	35.5, CH	29, 31a, 31b, 33	31
31a	1.41, m	24.5, CH ₂	30, 32	30, 32, 33
31b	1.20, m		30	32, 33
32	0.88, t (7.6)	12.5, CH ₃	31a	30, 31
33	1.01, d (6.8)	16.5, CH ₃	30	29, 30, 31

^aCOSY correlations are from proton stated to indicated proton.

^bHMBC correlations are from proton stated to the indicated carbon.