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New cyclic depsipeptides from the green alga *Bryopsis* species; application of a carboxypeptidase hydrolysis reaction to the structure determination

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Abstract—New cyclic depsipeptides, kahalalides P (**1**) and Q (**2**), were isolated from the Hawaiian green alga *Bryopsis* sp. The sequential positions of the DL anti-podal amino acids were determined by a carboxypeptidase hydrolysis reaction. This enzymatic method will be applicable to the structure determination of other non-ribosomal peptides. The absolute chemistry of 3-hydroxy-9-methyldecanoic acid in kahalalides P and Q were determined by the recently introduced convenient Mosher ester procedure.

1. Introduction

Kahalalides, cyclic, and acyclic peptides, have been previously characterized from the sacoglossan mollusk *Elysia rufescens* and its diet, the green alga *Bryopsis* sp.[1–6] Kahalalide F exhibits selective activity against solid tumors and is currently undergoing phase II clinical trials.[7] Kahalalide A exhibits anti-mycobacterium tuberculosis activity.[8,9] Re-investigation of a Hawaiian alga *Bryopsis* sp. extract led to the isolation of two new cyclic depsipeptides kahalalide P (**1**) and kahalalide Q (**2**). We report herein the determination of the absolute stereochemistry of these compounds. A carboxypeptidase hydrolysis reaction was utilized for determining the sequential position of the anti-podal DL amino acids.

Keywords: Marine natural product; Green alga; Depsipeptide; Carboxypeptidase.
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2. Results and discussion

2.1. Isolation of kahalalides P and Q

Bryopsis sp. (1.0 kg wet wt) was collected in Hawaii. The alga was lyophilized and stored at −30 °C. The freeze dried alga was extracted with methanol. After drying, the extract (31 g) was mixed with Celite powder and subjected to low-pressure flash chromatography on an ODS column. The MeOH/H_2O 9:1 fraction was further separated by reverse phase HPLC, which yield kahalalide P (**1**) [5.2 mg (0.0005%)], kahalalide Q (**2**) [1.8 mg (0.0002%)] as well as previously described kahalalides G and F.

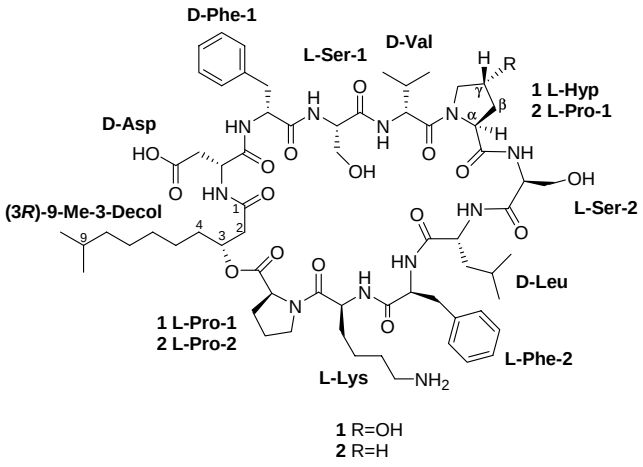
2.2. Structures of kahalalides P and Q

2.2.1. Planar structure of kahalalide P. The molecular formula of kahalalide P (**1**) was established as $C_{66}H_{99}N_{11}O_{17}$ on the basis of the HRFABMS data, *m/z* 1318.7319 $[M+H]^+$(Δ +2.1 mmu). It was corroborated by the ^{13}C NMR spectrum, which displayed signals for 66 carbons. NMR experiments were performed in DMSO-d_6 with addition of 0.05% of TFA, as peak broadening was observed in C_5D_5N and pure DMSO-d_6 solvents. Detailed analysis of the 2D NMR data enabled us to assign all the signals for kahalalide P and revealed a structural framework consisting of peptidal and fatty acid moieties. Examination of the ^1H NMR spectra

suggested **1** was a peptide with aromatic and aliphatic residues. The low field portion of the spectrum showed eight doublets of amide proton signals at 7.4–8.8 ppm, one broad peak (two protons from the NH_2 group of lysine) at 7.55 ppm and two sets of signals of protons of monosubstituted aromatic moieties, each of the five protons were between 7.0–7.4 ppm. Investigation of the 2D NMR data from TOCSY, DQF-COSY, HSQC, and HMBC experiments led to identification of the corresponding ten amino acid residues: Asp, Val, Lys, Leu, 4-*trans*-hydroxy-Pro (Hyp), Pro, two Ser, and two Phe. The Asp spin system could be traced by TOCSY cross peaks between signals 7.47 ppm (NH), 4.54 ppm (Hα), 2.52 and 2.25 ppm (Hβ); Val protons showed correlation in TOCSY spectra between 7.81 ppm (NH), 4.64 ppm (Hα), 1.98 ppm (Hβ) and two methyl doublets at 0.93 and 0.88 ppm; Leu protons were connected from TOCSY cross peaks of NH at 7.74 ppm, Hα at 4.54 ppm, two methylene protons of Hβ at 1.65 and 1.30 ppm, Hγ at 1.47 ppm and two methyl doublets at 0.83 and 0.79 ppm. TOCSY spectrum showed correlation for Ser1 protons at 8.75 ppm (NH), 5.54 ppm (Hα) and 3.58 (2Hβ); and for Ser2 at 8.13 ppm (NH), 4.24 ppm (Hα), 3.71 and 3.56 ppm (2Hβ). Two spin systems, 8.32 ppm (NH), 4.79 ppm (Hα), 2.91 and 2.59 ppm (2Hβ); and 8.67 ppm (NH), 4.99 ppm (Hα) and 2.81 ppm (2Hβ) were assigned to Phe1 and Phe2 residues. Lys residue was traced by TOCSY and COSY cross peaks between NH at 8.41 ppm, Hα at 4.52 ppm, two Hβ protons at 1.68 and 1.38 ppm, two Hγ at 1.20 ppm, two Hδ at 1.50 ppm, two Hε at 2.75 ppm and two NH_2 protons at 7.55 ppm.

The signal of the oxymethyne proton at 4.32 ppm, which correlated with the carbon at 68.2 ppm in the HSQC spectrum was considered as Hγ of the hydroxyproline residue. TOCSY and COSY correlations led to assignment of other hydroxyproline signals: two Hβ protons at 2.11 and 1.88 ppm, Hα at 4.20 ppm and two Hδ protons at 3.77 and 3.55 ppm. Relative stereochemistry of 4-hydroxy-Pro (Hyp) was determined from the NOESY spectrum. The cross peaks Hyp Hα (4.20)/Hyp Hβa (2.11) and Hyp Hγ (4.32)/Hyp Hβb (1.88) appeared stronger than the cross peaks Hyp Hα/Hyp Hβb and Hyp Hγ/Hyp Hβa, indicating that the relative stereochemistry between Hyp Hα and Hγ is trans. This result was further supported by Marfey's analysis. The last amino acid, proline was revealed from TOCSY correlations of Hα at 4.17 ppm, two Hβ at 2.01 and 1.83 ppm, two Hγ at 2.10 and 1.78 ppm and two Hδ protons at 3.56 and 3.13 ppm.

The presence of a 3-hydroxy-fatty acid residue in **1** was indicated by analysis of the NMR and MS data. Sequential COSY correlations were observed between the methylene signals at 2.38, 2.33 ppm (H-2), H-3 at 5.34 ppm, two H-4 protons at 1.58 ppm and methylene signals at 1.24 ppm, and between the signals of the two terminal methyl groups (0.85 ppm, 6H), H-9 (1.48 ppm), two H-8 (1.13 ppm) and methylenes at 1.24 ppm. Exact length of the fatty acid chain was confirmed by HRFAB MS and QTOF MS/MS analysis. Chemical shift of the oxymethyne proton H-3 (5.33 ppm) indicated an acyloxy nature of this bond and cyclic structure of the peptide (the chemical shifts of related protons of the fatty acid residues in the acyclic kahalalides H and J are 3.95 and 4.04 ppm,[3] whereas related protons in the cyclic peptide kahalalides E and K resonate at 5.11 and 5.16 ppm[2,4]).



The sequence of the amino acids was established utilizing NOESY and HMBC experiments and ESI QTOF MS/MS analysis. Sequential HMBC correlations from the NH proton to the neighboring carbonyls were seen between Asp NH/9-Me-3-Decol CO, Leu NH/Ser2 CO, Val NH/Ser1 CO, and Lys NH/Phe2 CO. Other HMBC cross peaks from the NH to the carbonyls were inconclusive due to overlaps in the spectra. Sequential NOESY correlations from the NH proton to the neighboring α proton were seen between 8.67 ppm (Phe2 NH) and 4.536 ppm (Leu Hα), 8.32 ppm (Phe1 NH) and 4.542 ppm (Asp Hα), 8.13 ppm (Ser2 NH) and 4.20 ppm (Hyp Hα), 8.75 ppm (Ser1 NH) and 4.79 ppm (Phe1 Hα), 8.41 ppm (Lys NH) and 4.99 ppm (Phe2 Hα), 7.81 ppm (Val NH) and 5.54 ppm (Ser1 Hα), and 7.74 ppm (Leu NH) and 4.24 ppm (Ser2 Hα). NOESY correlations were also observed between Val Hα at 4.64 ppm and proton Hδ of Hyp at 3.55 ppm, and between Lys Hα at 4.52 ppm and proton Hδ of Pro at 3.13 ppm. The NH proton of the Asp residue at 7.47 ppm showed NOESY peaks with H-2 protons (2.33, 2.38 ppm) and H-3 proton (5.34 ppm) of the 9-Me-3-Decol. However, no correlations were observed between H-3 proton of this fatty acid residue and the proline protons or carbons.

Peptide **1** was subjected to base hydrolysis, which yielded a linear product, **3**. Product **3** was analyzed by nanoelectrospray MS/MS measurement. MS/MS spectrum (Fig. 1), b- and y-type ions as well as several prominent peaks of internal ions clearly confirmed the sequence of amino acids and fatty acid of the acyclic kahalalide P (**3**).

2.2.2. Absolute stereochemistry of kahalalide P. The absolute stereochemistry of amino acids in **1** was determined by Marfey's method,[10] which showed Asp, Val, and Leu to be D, and Ser, Hyp, Pro, and Lys to be L. Both D- and L-Phe enantiomers were present in the peptide.

Enzymatic cleavage by carboxypeptidase to determine the positions of D- and L-Phe in **3** was performed (Fig. 2). Although peptides containing D-amino acid in the second position are resistant to hydrolysis by endopeptidases,[11–13] to the best of our knowledge, this property has never been utilized in the structure elucidation of natural products. The compound (**1**, 0.2 mg) was subjected to base hydrolysis to

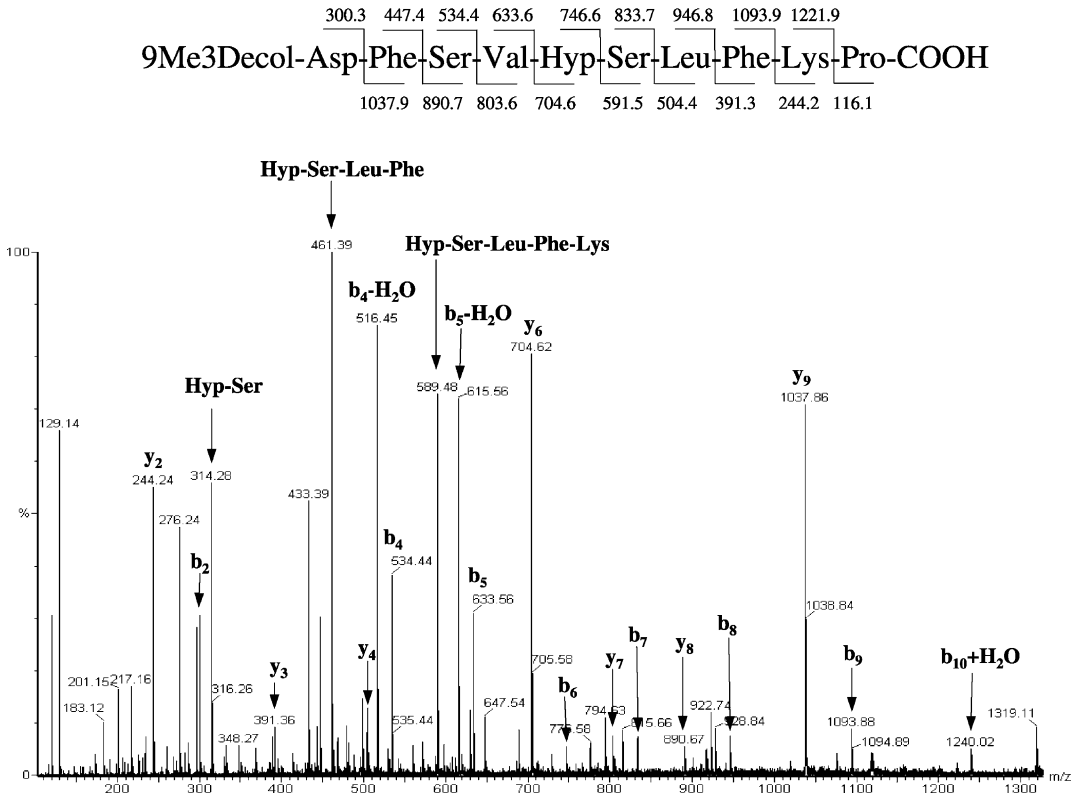


Figure 1. Positive ESI QTOF MS/MS spectrum of the acyclic kahalalide P.

yield the linear peptide **3**. After HPLC purifications, **3** was subjected to enzymatic cleavage with carboxypeptidase P. This enzyme non-specifically releases L-amino acids from the carboxy terminal of proteins and peptides. The reaction was monitored by MALDI TOF and QTOF MS and MS/MS measurements. The reaction completely terminates after two residues, Pro and Lys, are cleaved from the parent peptide, thus indicating a -D-Leu-L-Phe-L-Lys-L-Pro–COOH sequence at the C-terminus of the peptide. Sequencing of **3** with carboxypeptidase Y showed identical results as with carboxypeptidase P. Furthermore, we synthesized two linear model peptides with anti-podal positions of D- and L-Phe, **5a** (H_2N-D-Asp-**D-Phe**-L-Ser-D-Val-L-4-*trans*-hydroxy-Pro-L-Ser-D-Leu-**L-Phe**-L-Lys-L-Pro–COOH) and **5b** (H_2N-D-Asp-**L-Phe**-L-Ser-D-Val-L-4-*trans*-hydroxy-Pro-L-Ser-D-Leu-**D-Phe**-L-Lys-L-Pro–COOH), which were subjected to enzymatic sequencing in the same conditions as **3**. Two C-terminal residues, Pro and Lys, were cleaved off in the reaction with **5a** (mw of final product $[M+H]^+$ 927.27 Da, $[M+Na]^+$ 949.29 Da) similar to the reaction observed with **3**. However, only one C-terminal (Pro) residue was released in the reaction with anti-podal **5b** (mw of final

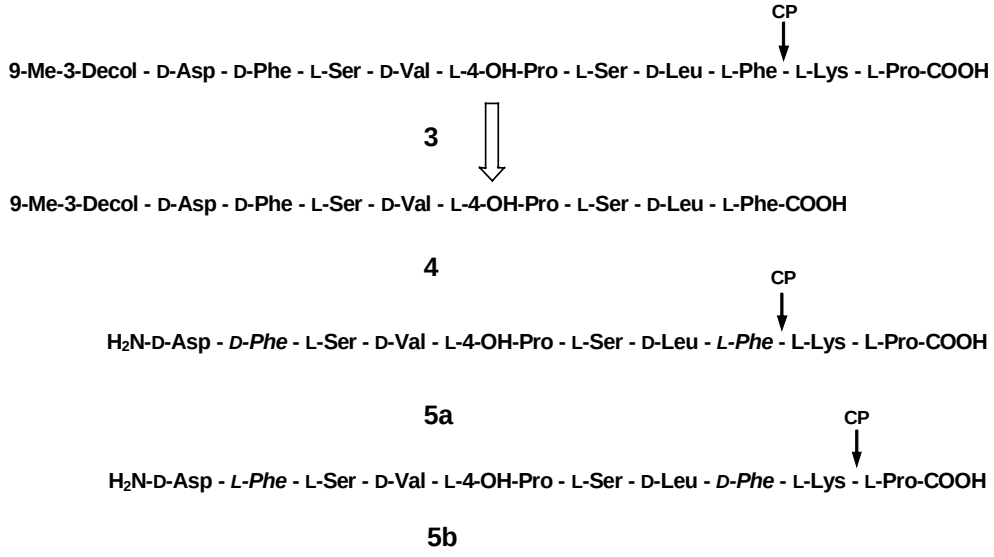
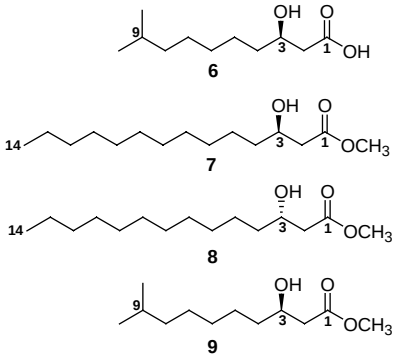


Figure 2. The carboxypeptidase (CP) hydrolysis reaction of acyclic kahalalide P (**3**) and synthesized model compounds.

product $[M+H]^+$ 1055.87 Da, $[M+Na]^+$ 1077.86 Da). These results verify the proposed position of D- and L-Phe in **1**. This enzymatic method will be applicable to the DL determination of anti-podal amino acids in other non-ribosomal peptides, including kahalalides E,[2] J,[3] and O[5] (stereochemistry of these compounds has not been elucidated).



Although the 3-hydroxy-9-methyldecanoic acid (9-Me-3-Decol) moiety in kahalalides E, J, H, and K, and 3-hydroxy-7-methyloctanoic acid moiety in kahalalide D have been reported, the absolute stereochemistries of these fragments were not determined due to the paucity of sample available.[2–4] We applied the Mosher ester procedure[14,15] to determine the absolute stereochemistry of 9-Me-3-Decol (**6**) in **1**. MTPA esters were obtained directly in NMR tubes with deuterated pyridine. The ^{1}H NMR spectra of the products of the reactions were measured without purification following the recently described convenient Mosher ester procedure.[16] We used model compounds, methyl (3*R*)-3-hydroxytetradecanoate (**7**) and methyl (3*R*+3*S*)-3-hydroxytetradecanoate (**7**+**8**, racemic body) to obtain MTPA derivatives. (*R*)- and (*S*)-MTPA esters of these compounds were obtained and the differences in proton chemical shifts between (*S*)-MTPA esters and (*R*)-MTPA esters were measured in C_5D_5N. MTPA esters of **7** have distinctive differences in ^{1}H NMR (Fig. 3). Furthermore, proton spectrum of the MTPA ester of the methyl 3(*R*+*S*)-hydroxytetradecanoate (**7**+**8**, racemic body) was almost the same as the sum of the spectra of (*R*)-MTPA ester of **7** and (*S*)-MTPA ester of **7**.

Two milligram of **1** was subjected to acid hydrolysis and the hydrolyzate was partitioned between $CHCl_3$ and H_2O. The organic layer was evaporated and the free fatty acid obtained was converted to methyl 3-hydroxy-9-methyldecanoate (**9**) with diazomethane. Products of the reaction were divided into two parts, and then converted to MTPA esters directly in NMR tubes as described above.[16] While the spectra of the MTPA derivatives of the fatty acid residue contains signals from impurities, multiplets of two H-2 protons and H-3 proton are clearly visible (Fig. 3). Difference in chemical shift of the protons in the (*S*)-MTPA ester of methyl 3-hydroxy-9-methyldecanoate and (*R*)-MTPA ester of methyl 3-hydroxy-9-methyldecanoate were found to be almost identical to the MTPA esters of **7** (Fig. 3). Thus, the absolute configuration of 9-Me-3-Decol from **1** was proved to be (3*R*) (**6**).

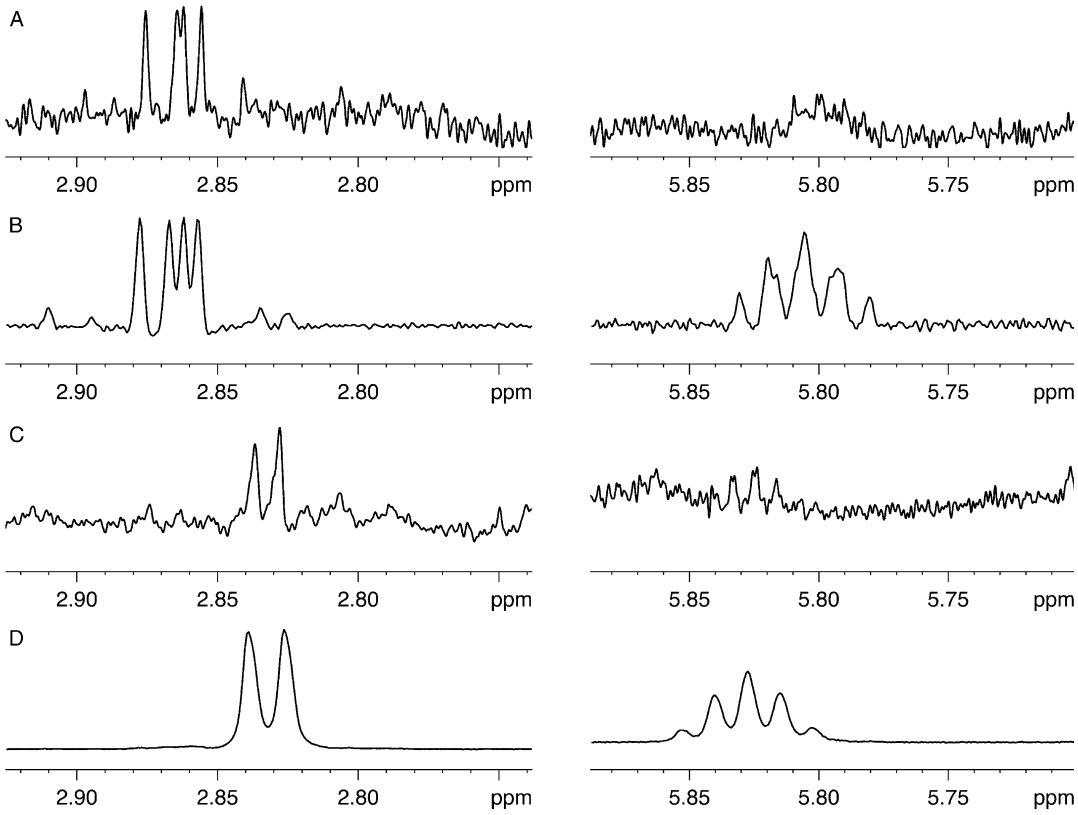


Figure 3. Partial ^{1}H NMR spectra of (*R*)-and (*S*)-MTPA esters of (3*R*)-methyl-3-hydroxytetradecanoate (**7**) and (3*R*)-methyl-3-hydroxy-9-methyldecanoate (**9**). (A) (*R*)-MTPA ester of **9**; (B) (*R*)-MTPA ester of **7**; (C) (*S*)-MTPA ester of **9**; (D) (*S*)-MTPA ester of **7**. (A) and (C) were measured with 750 MHz spectrometer. (B) and (D) were measured with 500 MHz spectrometer.

2.2.3. Structure of kalahalide Q. Kahalalide Q (**2**) showed $[M+H]^+$ ion 1302.7336 ($C_{66}H_{99}N_{11}O_{16}$, Δ −1.3 mmu) from HR FAB MS data, which differ by one oxygen atom from kahalalide P (**1**). ^{1}H HMR showed similar data for all moieties (Table 2) except for the signals of the hydroxyproline residue. Careful analysis of the NMR data showed **2** possessed a proline instead of a hydroxyproline residue. This result was further supported by QTOF MS/MS analysis of **2**, and its linear analog obtained from **2** by base hydrolysis. Determination of absolute stereochemistry of amino acid residues of **2** by Marfey's method showed D- and L-Phe, D-Asp, D-Val, D-Leu, L-Lys, two L-Ser, and two L-Pro. Since the NMR data of the peptides from **1** and **2** are almost indistinguishable, the stereochemistry of the fatty acid residue and positions of the D- and L-Phe in **2** are identical to **1**.

3. Bioactivity

At a concentration of 100 μg/mL, kahalalides P (**1**) and Q (**2**) showed 40 and 30% inhibition of HL-60 cancer cell lines, respectively. At a concentration of 40 μg (using a 8 mm diameter paper disk), both compounds showed no anti-microbial activity toward the marine bacterium *Ruegeria atlantica* TUF-D.[17] No hemolytic activity was observed (0.8% sheep red blood cell suspension assay)[18] with either compound at a concentration of 100 μg/mL.

4. Conclusion

New cyclic depsipeptides, kahalalides P (**1**) and Q (**2**), were isolated from the Hawaiian green alga *Bryopsis* sp. The sequential positions of DL anti-podal amino acids were determined by a carboxypeptidase hydrolysis reaction. This enzymatic method will be applicable to the structure determination of other non-ribosomal peptides. The absolute chemistry of 3-hydroxy-9-methyldecanoic acid in kahalalides P and Q were determined by the recently introduced convenient Mosher ester procedure. Interestingly, the structure of the acyclic kahalalide P (**1**) compared to kahalalides H and J[3] suggest that kahalalide P may possible be an intermediate in the metabolic pathway or end product of these kahalalides. Kahalalide P without the C-terminal Pro and Lys would be kahalalide H and without the C-terminal Pro might be kahalalide J (DL determination of Phe in kahalalide J has not been elucidated).

5. Experimental

5.1. General experimental procedures

Optical rotations were measured on a digital spectropolarimeter JASCO DP-100. ^{1}H and two-dimensional NMR spectra were recorded on Bruker DMX-750 spectrometer; ^{13}C NMR spectra were obtained on Bruker DRX-500 spectrometer. MALDI TOF measurements were performed on Perceptive Biosystem Voyager Elite MALDI TOF mass spectrometer. Nanoflow electrospray ionization time of flight MS/MS and MS were run on Micromass Q-TOF mass spectrometer. HRFAB MS data were obtained on JEOL JMX HX/HX-110A mass spectrometer.

5.2. Isolation

Bryopsis sp. (1.0 kg wet wt) was collected at Kewalo Basin, Oahu, Hawaii (1998). The alga was lyophilized and stored at −30 °C. Freeze dried alga was extracted with methanol. The combined extracts were evaporated to dryness to give 31 g of green powder. The residue was mixed with Celite and purified by two-step ODS flash chromatography (column 2×25 cm) (first elution with 20–50–70–90–100% aqueous MeOH, second with 50–70–80–90–95–100% aqueous MeOH). The MeOH/H_2O 9:1 fraction was further purified by repeated reverse phase HPLC, (Cosmosil 5C_{18}-MS-II, 20×250 mm with aqueous CH_3CN gradient 80–100% and then Cosmosil 5C_{18}-AR-II, 4.6×250 mm with aqueous CH_3CN gradient 60–70%) to yield kahalalide P (5.2 mg, 0.0005%) and kahalalide Q (1.8 mg, 0.0002%) as well as previously described kahalalides G and F.

5.2.1. Kahalalides P (1). White powder; $[\alpha]_D^{25}$ +4.5 (*c* 0.22, MeOH); ^{1}H and ^{13}C NMR data were shown in Table 1; HRFABMS $C_{66}H_{100}N_{11}O_{17}$ as $[M+H]^+$ *m/z* 1318.7319 (Δ +2.1 mmu).

5.2.2. Kahalalides Q (2). White powder; $[\alpha]_D^{25}$ +10.5 (*c* 0.033, MeOH); ^{1}H and ^{13}C NMR data were shown in Table 2; HRFABMS $C_{66}H_{100}N_{11}O_{16}$ as $[M+H]^+$ *m/z* 1302.7336 (Δ −1.3 mmu).

5.3. Acid hydrolysis of kahalalides

Peptides were hydrolyzed with 6 N HCl at 100 °C for 12 h. The hydrolyzate was dried with centrifugation in vacuo.

5.4. Base hydrolysis of kahalalides

Peptides (0.2 mg) were hydrolyzed with 0.1 N NaOH in aqueous methanol (50 μL) at 37 °C for 90 min. After neutralization with 0.1 N HCl, ODS HPLC of the hydrolyzate was performed (Cosmosil 5C18-AR-II, 4.6×250 mm, aqueous CH_3CN 0–60%) to yield the acyclic peptides.

5.5. Marfey analysis of 1 and 2

Part of the acid hydrolyzate was added to a 1% 1-fluoro-2,4-bis(nitrophenyl)-5-L-alanine amide (FDAA) solution in acetone (10 μL) and 1 M $NaHCO_3$ (20 μL). The sample was incubated at 40 °C for 1 h. The reaction mixture was neutralized with 2 N HCl (20 μL) after cooling to room temperature. The mixture was dried with centrifugation in vacuo. The residue was dissolved in DMSO and subjected to HPLC analysis. The FDAA derivatives of standard amino acids were prepared by the same procedure. HPLC analysis was carried out on a Cosmosil 5C_{18}-AR-II, 4.6×250 mm column with two different solvent system (system I: solvent A (0.1 M ammonium acetate, pH 3.0) and solvent B (CH_3CN), samples were eluted as follows: A, 85–55% in 45 min, system II: solvent A (0.1 M ammonium acetate, pH 3.0), 10% and solvent B (CH_3CN), 90%). The retention times (min) of the hydrolyzate FDAA derivatives and the standard derivatives in the solvent system I were as follows: L-Ser (8.00–8.04), D-Asp (10.76–10.81), L-Pro (14.24–14.30), L-Phe (25.72–25.74), D-Val (28.05–28.10),

Table 1. ^{1}H[a] and ^{13}C NMR[b] data for kahalalide P (**1**) in 0.05% TFA/DMSO-d_6

Unit	Position	^{13}C NMR (ppm)[c]	^{1}H NMR (ppm)[d]	Mult	J (Hz)
9Me3Decol	1	168.2	—		
	2	40.4	Hα 2.38	dd	10.1, 14.7
			Hβ 2.33	dd	3.4, 14.7
	3	71.0	5.34	m	
	4	33.6	1.58, 2H	m	
	5	[e]	1.24, 2H	m	
	6	[e]	1.24, 2H	m	
	7	28.8	1.24, 2H	m	
	8	38.3	1.13, 2H	m	
	9	27.3	1.48	m	
	10, 11	22.5, 2C	0.85, 6H	d	6.6
Asp	NH	—	7.47	d	7.7
	α	49.3	4.54	m	
	β	35.0	Hα 2.52	dd	10.6, 16.2
			Hβ 2.25	dd	4.0, 16.2
	C=O	169.3	—		
	COOH	171.9	—		
Phe1	NH	—	8.32	d	8.9
	α	53.9	4.79	dt	9.5, 4.5
	β	39.3	Hα 2.91	dd	4.5, 13.0
			Hβ 2.59	dd	10.2, 13.0
	1′	137.8	—		
	2′, 6′	129.8, 2C	7.37, 2H	d	7.4
	3′, 5′	128.0, 2C	7.29, 2H	t	7.4
	4′	126.3	7.25	t	7.4
	C=O	171.7	—		
Ser1	NH	—	8.75	d	8.3
	α	53.9	5.54	m	
	β	63.7	3.58, 2H	d	5.5
	C=O	169.9	—		—
Val	NH	—	7.81	d	7.1
	α	54.8	4.64	t	7.1
	β	32.0	1.98	m	
	γ	19.5	0.93, 3H	d	6.8
	γ′	17.7	0.88, 3H	d	6.8
	C=O	170.9	—		
Hyp	α	60.5	4.20	t	8.5
	β	37.8	Hα 2.11	m	
			Hβ 1.88	ddd	4.4, 9.4, 13.4
	γ	68.2	4.32	m	
	δ	55.6	Hα 3.77	dd	4.0, 10.5
			Hβ 3.55	m	
	C=O	171.8	—		
Ser2	NH	—	8.13	d	8.5
	α	55.7	4.24	dt	4.0, 8.5
	β	60.9	Hα 3.71	dd	4.0, 11.3
			Hβ 3.56	m	
	C=O	169.0	—		
Leu	NH	—	7.74	d	8.9
	α	50.3	4.54	m	
	β	40.7	Hα 1.65	m	
			Hβ 1.30	m	
	γ	23.8	1.47	m	
	δ	23.2	Hα 0.83, 3H	d	6.7
	δ′	21.8	Hβ 0.79, 3H	d	6.7
	C=O	171.9	—		
Phe2	NH	—	8.67	d	9.0
	α	52.9	4.99	dd	8.0, 9.0
	β	38.3	2.81, 2H	d	8.0
	1″	137.1	—		
	2″, 6″	129.2, 2C	7.14, 2H	d	7.4
	3″, 5″	127.7, 2C	7.10, 2H	t	7.4
	4″	126.1	7.07	t	7.4
	C=O	169.8	—		
Lys	NH	—	8.41	d	8.8
	α	48.8	4.52	m	
	β	31.9	Hα 1.68	m	
			Hβ 1.38	m	
	γ	21.2	1.20, 2H	m	
	δ	26.4	1.50, 2H	m	
	ε	38.7	2.75, 2H	m	
	NH_2	—	7.55, 2H	br s	
	C=O	171.9	—		
Pro	α	58.5	4.17	dd	4.2, 8.5
	β	24.4	Hα 2.01	m	
			Hβ 1.83	m	
	γ	28.7	Hα 2.10	m	
			Hβ 1.78	m	
	δ	46.6	Hα 3.56	m	
			Hβ 3.13	m	
	C=O	171.9	—		

[a] At 750 MHz.
[b] At 188 MHz.
[c] Reference of chemical shift was DMSO-d_6 as 39.5 ppm.
[d] Reference of chemical shift was DMSO-d_6 as 2.49 ppm.
[e] δ ^{13}C 26.6 or 24.8 ppm.

Table 2. $^{1}H^{a}$ and ^{13}C NMR[b] data for kahalalide Q (**2**) in 0.05% TFA/DMSO-d_6

Unit	Position	^{13}C NMR (ppm)[c]	^{1}H NMR (ppm)[d]	Mult	J (Hz)
9Me-3decol	1	168.2	—		
	2	40.0	Hα 2.38	dd	9.8, 14.5
			Hβ 2.33	dd	3.6, 14.5
	3	71.0	5.33	m	
	4	33.6	1.58, 2H	m	
	5	[e]	1.24, 2H	m	
	6	[e]	1.24, 2H	m	
	7	28.7	1.24, 2H	m	
	8	38.3	1.13, 2H	m	
	9	27.4	1.48	m	
	10,11	22.5, 2C	0.85, 6H	d	6.6
Asp	NH	—	7.48	d	7.4
	α	49.2	4.54	m	
	β	35.0	Hα 2.52	m	
			Hβ 2.25	dd	4.0, 16.4
	C=0	169.2	—		
	COOH	[f]	—		
Phe1	NH	—	8.34	d	8.8
	α	53.4	4.78	dt	8.8, 4.5
	β	38.9	Hα 2.92	dd	4.5, 13.0
			Hβ 2.59	dd	11.0, 13.0
	1′	137.8	—		
	2′,6′	129.7, 2C	7.38, 2H	d	7.4
	3′,5′	128.0, 2C	7.29, 2H	t	7.4
	4′	126.3	7.25	t	7.4
	C=O	[f]	—		
Ser1	NH	—	8.77	d	9.0
	α	53.9	5.54	m	
	β	63.7	3.59, 2H	m	
	C=O	169.9	—		—
Val	NH	—	7.82	d	7.0
	α	55.0	4.68	t	7.0
	β	32.1	1.99	m	
	γ	19.0	0.91, 3H	d	6.8
		17.9	0.89, 3H	d	6.8
	C=O	170.7	—		
Pro1	α	61.5	4.13	dd	4.1, 8.6
	β	24.4	1.88, 2H	m	
	γ	29.5	Hα 2.16	m	
			Hβ 1.79	m	
	δ	47.7	Hα 3.72	m	
			Hβ 3.61	m	
	C=O	[f]	—		
Ser2	NH	—	8.08	d	8.5
	α	55.6	4.28	dt	4.0, 8.5
	β	60.9	Hα 3.70	m	
			Hβ 3.56	m	
	C=O	169.0	—		
Leu	NH	—	7.66	d	8.9
	α	50.3	4.54	m	
	β	40.3	Hα 1.66	m	
			Hβ 1.30	m	
	γ	23.8	1.44	m	
	δ	23.2	0.83, 3H	d	6.7
	δ′	21.8	0.79, 3H	d	6.7
	C=O	[f]	—		
Phe2	NH	—	8.66	d	9.1
	α	52.8	5.01	q	9.0
	β	38.6	2.81, 2H	m	
	1″	137.1	—		
	2″,6″	129.2, 2C	7.14, 2H	d	7.4
	3″,5″	127.7, 2C	7.10, 2H	t	7.4
	4″	126.0	7.07	t	7.4
	C=O	169.8	—		
Lys	NH	—	8.43	d	8.3
	α	48.7	4.52	m	
	β	31.9	Hα 1.65	m	
			Hβ 1.38	m	
	γ	21.1	1.19, 2H	m	
	δ	26.4	1.50, 2H	m	
	ε	38.5	2.75, 2H	m	
	NH_2	—	7.53, 2H	br s	
	C=O	169.2	—		
Pro 2	α	58.5	4.18	dd	4.0, 8.5
	β	24.3	Hα 2.01	m	
			Hβ 1.84	m	
	γ	28.7	Hα 2.10	m	
			Hβ 1.77	m	
	δ	46.5	Hα 3.54	m	
			Hβ 3.12	m	
	C=O	[f]	—		

[a] At 750 MHz.
[b] At 188 MHz.
[c] Reference of chemical shift was DMSO-d_6 as 39.5 ppm.
[d] Reference of chemical shift was DMSO-d_6 as 2.49 ppm.
[e] δ ^{13}C 26.6 or 24.8 ppm.
[f] δ ^{13}C 171.9 or 171.8 ppm.

L-Lys (28.97–29.02), D-Phe (31.97–32.03) and D-Leu (33.99–34.04). The configuration of Hyp could not be determined in this system due to poor peak resolution. Further analysis with solvent system II revealed L-Hyp in the samples. (retention time: authentic 4-*trans*-hydroxy-D-Pro 20.85 min, authentic 4-*trans*-hydroxy-L-Pro 22.73 min, the sample 22.90 min).

5.6. Enzymatic hydrolysis

C-terminus of peptide **3** was hydrolyzed with carboxypeptidase P (Takara Bio Inc., Japan) in 30 mmol ammonium acetate buffer (pH 5.0) and carboxypeptidase Y (Oriental Yeast Co., Osaka, Japan) in 30 mmol ammonium acetate buffer (pH 6.0). The carboxy end of peptides **4**, **5** were hydrolyzed with carboxypeptidase P in 30 mmol ammonium acetate buffer (pH 5.0). Each sample (1 nM in 10 μL buffer) was mixed with carboxypeptidase P or carboxypeptidase Y, 1 μL (1 unit), in a capped 0.5 mL polypropylene tube and kept at 37 °C. Reactions were monitored by MALDI MS measurement. Reactions completed approximately in 10 min for **3** and **4** and in 15 min for **5**.

5.7. Synthesis of peptides 5a and 5b

The peptides were synthesized using a FastMOC™ chemistry with a solid-phase peptide synthesizer (Model 433A, Applied Biosystems) and purified by reverse phase HPLC (Cosmosil 5C_{18}-AR-II, 4.6×250 mm) with 70% aqueous CH_3CN.

5.8. Preparation of the (*R*)- and (*S*)-MTPA ester derivatives

(*R*)- and (*S*)-MTPA esters of the compounds were obtained following the reported procedure.[16] Each model compound, methyl (3*R*)-3-hydroxytetradecanoate (**7**) and methyl (3*R*+3*S*)-3-hydroxytetradecanoate (**7**+**8**, racemic body), was divided into two parts, then transferred into a clean NMR tubes and dried under the stream of N_2 gas. Deuterated pyridine (0.6 mL) and (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (6 μL) or (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (6 μL) were added to NMR tube immediately under a N_2 gas stream, and then NMR tube was shaken carefully to mix the sample and MTPA chloride evenly. The reaction NMR tubes were permitted to stand at room temperature and were monitored by ^{1}H NMR. The reaction was completed in approximately 4 h. Selected signals of the (*S*)-MTPA ester of **7**: ^{1}H NMR (C_5D_5N, 500 MHz) δ 2.83 (2H, d, J=6.4 Hz, H-2a and H-2b), 5.83 (m, H-3), 1.83 (m, H-4a), 1.75 (m, H-4b) and 1.39 (m, H-5). Selected signals of the (*R*)-MTPA ester of **7**: ^{1}H NMR (C_5D_5N, 500 MHz) δ 2.89 (dd, J=8.5, 16.5 Hz, H-2a), 2.85 (dd, J=5.0, 16.5 Hz, H-2b), 5.81 (m, H-3), 1.70 (2H, m, H-4) and 1.20 (m, H-5, data from TOCSY spectrum).

Proton spectrum of the MTPA ester of the methyl (3*R*+3*S*)-hydroxytetradecanoate (**7**+**8**, racemic body) was almost the same as the sum of the spectra of (*R*)-MTPA ester of **7** and (*S*)-MTPA ester of **7**.

Two milligram of the peptide (**1**) was hydrolyzed by heating the sample in 6 N HCl for 12 h. The hydrolyzate was dried with centrifugation in vacuo. Hydrolyzate was partitioned between $CHCl_3$ and H_2O. Organic layer was evaporated and obtained free fatty acid (**6**) was converted to methyl-3-hydroxy-9-methyldecanoate (**9**) by diazomethane. (*R*)- and (*S*)-MTPA esters of **9** were obtained in the same manner as for the model compounds. Selected signals of the (*S*)-MTPA ester of **9**: ^{1}H NMR (C_5D_5N, 750 MHz) δ 2.83 (2H, d, H-2a and H-2b) and 5.82 (m, H-3). Selected signals of the (*R*)-MTPA ester of **9**: ^{1}H NMR (C_5D_5N, 750 MHz) δ 2.89 (dd, H-2a), 2.85 (dd, H-2b) and 5.80 (m, H-3).

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