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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.

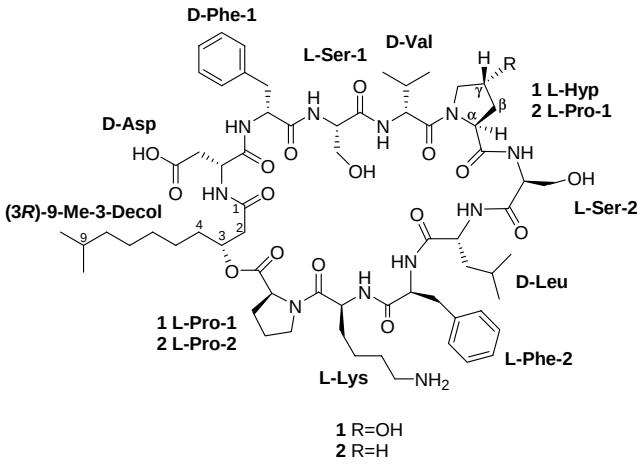
2.2. Structures of kahalalides P and Q

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