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Hapalocyclamide: a novel phytotoxic hexapeptide of the cyanobacterium *Hapalosiphon* sp.

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ABSTRACT

Hapalocyclamide, a novel oxazole-, thiazole- and thiazoline-containing cyclic hexapeptide, was isolated from the terrestrial cyanobacterium *Hapalosiphon* sp., and which showed phytotoxic activity on lettuce seedling growth. The gross structure of hapalocyclamide was established from spectroscopic data and chemical degradation. The absolute stereochemistry was determined by Marfey's analysis. Hapalocyclamide was established as cyclo-thiazole-L-alanine-oxazole-D-alanine-D-thiazoline-D-phenylalanine.

Cyclic peptides are a class of cyanobacterial metabolites that incorporate peptides or possess peptide-substructures containing a range of proteinogenic and nonproteinogenic amino acids with a high level of structural variation.[1,2] Cyanobactin was proposed as a collective name for cyclic peptides that contain heterocyclized amino acids or isoprenoid amino acid derivatives. Cyanobactins were initially defined as containing oxazolines, thiazolines, or their oxidized derivatives oxazoles and thiazoles. To date, more than 100 cyanobactins have been identified from symbiotic associations between cyanobacteria and ascidians or from free-living cyanobacteria.[3] As part of our continuing effort to isolate phytotoxic compounds from cultured cyanobacteria, we report here the isolation and structural elucidation of a novel cyclic hexapeptide, hapalocyclamide[4] (**1**) (Fig. 1), from the terrestrial cyanobacterium *Hapalosiphon* sp., Stigonemataceae.

The *Hapalosiphon* sp., isolated from soil collected in Bangkok, Thailand, was cultured in controlled conditions.[5] Dried cells (20 g) were extracted with MeOH, and the resulting extract was fractionated using a silica gel column, an ODS column and preparative HPLC, guided by a phytotoxicity bioassay. The active fraction was applied to silica gel column and eluted with hexane–EtOAc (1:1) and EtOAc to afford hapalocyclamide (**1**) (44.0 mg, 0.220% yield based on dry cyanobacterial mass).

Hapalocyclamide (**1**) was isolated as a colorless needle-like crystal. Direct inlet electron impact mass spectrum showed the molecular ion at *m/z* 538 (M^+, 86%) and a characteristic fragment ion at *m/z* 447 (100%, M^+-91) indicating the loss of a benzyl group from the molecule. High resolution mass spectra of these ions demonstrated the molecular formulas as $C_{25}H_{26}N_6O_4S_2$ (observed 538.1471; calcd 538.1457) and $C_{18}H_{19}N_6O_4S_2$ (observed 447.0905; calcd 447.0909).

Intense absorptions in the IR spectrum at 3398 cm^{-1} (NH stretching vibration of secondary amide), 1675 cm^{-1} (amide-I band), and 1520 cm^{-1} (amide-II band) and its lipophilic nature suggested that **1** is a cyclic peptide. In addition, the ^1H NMR spectrum showed the presence of three broad doublets at δ 7.45, 8.39, and 8.66 ppm (Table 1) that were attributed to amide NH signals of amino acid, indicating that the compound was a peptide.

The products of acid hydrolysis,[6] which were converted into methyl esters and N-acetylated for GC–MS analysis, afforded phenylalanine, alanine, cysteine, and thiazole-containing amino acids (**2**, **3**) (Fig. 2). The first three amino acids were identified by comparison

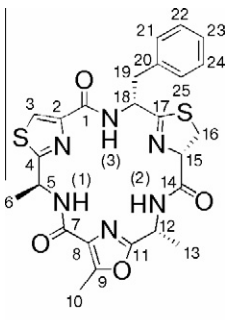

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Figure 1. Structure of hapalocyclamide (**1**).

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Table 1
^{1}H and ^{13}C NMR data for hapalocyclamide (**1**)[a]

No.	δ_C	δ_H M[b], J[c]	HMBC
1	159.3 qC		C-3, NH-3
2	148.0 qC		C-3
3	124.7 CH	8.23 s	NH-3
4	171.7 qC		C-3, 5, 6, NH-1
5	47.7 CH	5.41 qui, 6.61	C-3, 6, NH-1
6	24.3 CH_3	1.70 d, 6.70	C-5
NH-1		8.66 d, 6.15	
7	160.6 qC		C-5, 10, NH-1
8	153.8 qC		C-10
9	128.5 qC		C-10
10	11.6 CH_3	2.67 s	
11	161.0 qC		C-12, 13, NH-2
12	44.0 CH	4.97 qui, 6.83	C-13, NH-1
13	19.9 CH_3	1.45 d, 6.80	C-12
NH-2		7.45 d, 6.85	
14	169.7 qC		C-15, 16, NH-2
15	77.8 CH	4.91 ddd, 12.14, 9.46, 2.46	C-16, NH-2
16	36.8 CH_2	3.38 dd, 12.28, 11.28	C-15
		3.83 dd, 11.20, 9.25	
17	172.5 qC		C-16, 18, 19, NH-3
18	52.5 CH	5.30 m	C-19, NH-3
19	39.1 CH_2	3.28 dd, 13.93, 3.68	C-21
		3.44 dd, 13.93, 4.85	
20	135.3 qC		C-18, 19, 21, 22
21, 24	128.1 CH	7.10 m	C-19, 22, 23
22, 25	129.7 CH	7.11m	C-21, 23
23	126.9 CH	7.05m	C-21, 22
NH-3		8.39 d 7.55	

[a] Recorded in $CDCl_3$ at 150 MHz for ^{13}C and 600 MHz for ^{1}H using TMS as internal standard.
[b] M = Multiplicity.
[c] J in Hz.

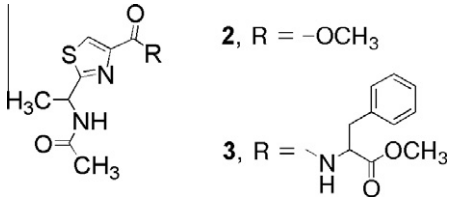

Figure 2. Thiazole containing amino acid fragments, Ala-Tzl (**2**), Ala-Tzl-Phe (**3**) after hydrolysis of **1**.

with derivatized authentic amino acids. Compound **2** was identified by MS and NMR analyses as an alanine-thiazole residue (Ala-Tzl residue) (*m/z* 228 [M^+]). This finding was confirmed by spectral comparison with the reported compound.[7] Compound **3** (*m/z* 375 [M^+]) contained a prominent alanine-thiazole-containing fragment at *m/z* 197 (100%) as the base peak; *m/z* 284 (24%) derived from the loss of 91 mass units (benzyl from Phe) and *m/z* 213 (38%) from the loss of 162 mass units. These fragmentation patterns were considered to be an alanine-thiazole-phenylalanine substituent (Ala-Tzl-Phe) (**3**).

NMR data (Table 1), including detailed proton decoupling experiments, confirmed the presence of phenylalanine, two alanine residues as well as a three modified amino acids: oxazole, thiazole, and thiazoline. The latter two modified amino acids were derived from cysteine, indicating that there were six amino acids in the compound. Confirmation of the sequence of the subunits of **1** was provided by HMBC experiments as described below.

The first and second amino acids were identified as alanine. The amide protons [δ 8.66 (NH-1); δ 7.45 (NH-2)] were correlated through COSY and HMBC experiments to the nitrogen-bearing methine [δ 5.41 (H-5), 47.7 (C-5); δ 4.97 (H-12), 44.0 (C-12)] and methyl [δ 1.70 (3H-6), 24.3 (C-6); δ 1.45 (3H-13), 19.9 (C-13)] groups. The third amino acid was deduced to be phenylalanine. The nitrogen-bearing methine [δ 5.30 (H-18), 52.5 (C-18)] had correlations with an amide proton [δ 8.39 (NH-3)] and methylene [δ 3.28, 3.44 (2H-19), 39.1 (C-19)]. The methylene protons were correlated through an HMBC experiment to a phenyl ring system [δ 7.10 (H-21, 24), 128.1 (C-21, 24); δ 7.11 (H-22, 25), 129.7 (C-22, 25); δ7.05 (H-23), 126.9 (C-23)].

The singlet aromatic proton [δ 8.23 (H-3), 124.7 (C-3)] exhibited a signal for thiazole. The thiazoline ABX spin system was established by COSY correlations of nitrogen-bearing methine [δ 4.91 (H-15), 77.8 (C-15)] with sulfur-bearing methylene [δ 3.38, 3.83 (2H-16), 36.8 (C-16)]. HMBC experiments demonstrated the correlation of C-16 with C-15 and C-17 to confirm the thiazoline system. The last modified amino acid was identified as methyloxazole. The oxygen-bearing carbon [δ 128.5 (C-9)] had a correlation with a methyl group [δ 2.67 (3H-10), 11.6 (C-10)] and a quaternary carbon [δ 153.8 (C-8)]in HMBC findings. In addition, the chemical shifts of the thiazole amino acid unit (Ala-Tzl) and methyloxazole amino acid unit (Ala-mOzl) were in good agreement with those reported for dendroamide[8] and venturamide.[11]

HMBC data (Table 1 and Fig. 3) provided information on the amino acid sequence, thus constructing the cyclic hexapeptide structure of hapalocyclamide (**1**). Correlation from C-7 to H-10 established the linkage of the first alanyl residue of **3** through the oxazole ring. C-11 of oxazole exhibited correlations with H-12, H-13 and NH-2, thus establishing the connection of the oxazole ring to the second alanyl residue. The second alanyl residue was linked to the thiazoline ring through HMBC correlation of C-14 with H-15 and H-16. Finally, the correlation of C-17 with H-18, H-19 and NH-3 established the connection of thiazoline through the benzyl residue of **3** to close the macrocyclic ring.

The absolute configurations of the chiral centers of amino acids in **1** were analyzed by the Marfey's method[9,10] after hydrolysis (1 h and 19 h). This procedure established the presence of D-cysteine from the thiazoline ring. Only the oxazole ring was cleaved by direct hydrolysis to generate D-alanine. After hydrolysis for 1 h, D-phenylalanine was detected; however, racemization of phenylalanine was observed after hydrolysis for 19 h. It was reported that for peptides containing both oxazole and thiazole heterocycles direct hydrolysis under acidic conditions results in the exclusive cleavage of the oxazole ring.[8,11] In order to liberate the amino acid adjacent to thiazole ring, it is necessary to cleave the ring by ozonolysis prior to hydrolysis.[8,11] Ozonolysis[12] of **1** led to degradation of alanylthiazole to yield L-alanine. Based on these findings the structure of hapalocyclamide (**1**) was established as *cyclo*-thiazole-L-alanine-oxazole-D-alanine-D-thiazoline-D-phenylalanine.

The hapalocyclamide (**1**) had phytotoxic activity to suppress lettuce (*Lactuca sativa* L. cv. Great Lake no. 366) seedling growth in a concentration-dependent manner. Growth of root and shoot was inhibited to 44.0 ± 1.4% and 47.8 ± 3.1% against controls,

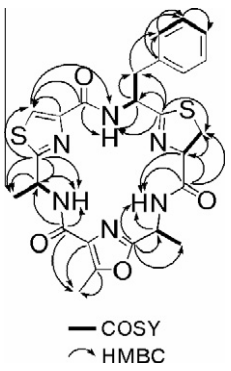

Figure 3. Key COSY and HMBC correlations establishing the spiral fused ring.

respectively after 48 h exposure to hapalocyclamide at 4.2 μg/cm^2 on a filter paper. Swelling of the root tips could also be observed. I_{50} (dose required to cause a 50% reduction in plant growth) values for root and shoot growth were 1.08 and 1.36 mM, respectively. Our current research on the mode of action of hapalocyclamide suggests that it acts on the process of mitosis.

Acknowledgments

We express our gratitude to Assistant Professor Dr. Kaori Yokotani-Tomita, University of Tsukuba for her help in analyzing the absolute stereochemistry by LC–MS. The authors are grateful to the Chemical Analysis Center, University of Tsukuba, for NMR experiments.

Supplementary data

Color photomicrograph of *Hapalosiphon* sp., MS and NMR data of compound **2** and **3**, ^{1}H and ^{13}C NMR spectra of **1**, LC–MS analysis of Marfey's derivatized hydrolysate of **1**, details of phytotoxic assay.

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2011.12.048.

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3. Sivonen, K.; Leikoski, N.; Fewer, D. P. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 1213–1225.
4. *Hapalocyclamide*: A colorless needle-like crystals. $[\alpha]_D^{25.2}$ −0.071° (*c* 0.47, MeOH); DI-EIMS (70 eV) *m*/*z* (rel. int.) 538 (M^+, 86), 447 (100), 252 (31), 188 (25), 138 (33); HRMS *m*/*z* 538.1471 (M^+, $C_{25}H_{26}N_6O_4S_2$, Δ 1.4 mmu), *m*/*z* 447.0905 ($C_{18}H_{19}N_6O_4S_2$, Δ −0.4 mmu); UV (MeOH) λ_{max} nm (log ε): 247 (3.91), IR (CCl_4, NaCl) υ_{max} cm^{-1}: 3398 (m), 1675 (s), 1520 (s). For ^{1}H and ^{13}C NMR data, see Table 1.
5. *Hapalosiphon* sp. was cultured in BG-11 liquid medium (pH 9.0) with constant aeration in a incubator at 25 °C with continuous illumination (66–69 μmol photon m^{-2} s^{-1}) from a cool-white fluorescent lamp (FL20SS-W/18; National, FL40SS-W/37; Toshiba, Japan) in a growth chamber. After 21 days, the algae were harvested by filtration and oven-dried at 60 °C for 48 h. The dried algae were ground into a powder and kept in a desiccator until extraction and analysis.
6. *Acid hydrolysis*: A solution of 44.6 mg of **1** (obtained from 200 g of dry cyanobacterium) in 1 ml of MeOH and 3 ml of 3 N HCl was heated at 100 °C overnight. The solvent was removed under reduced pressure. The acid hydrolysate was treated with 5% HCl in MeOH (Wako Pure Chemical Industries, Japan). After being heated at 100 °C for 1 h, the solvent was evaporated. The product was then reacted with Ac_2O and pyridine for 24 h to produce N-acetylated methyl ester products for GC–MS analysis. The residue was subjected to sequential fractionation using a SiO_2 column, a reversed phase column, and preparative TLC to afford thiazole containing amino acid residues (**2**, **3**).
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10. *Marfey's analysis*: Two batches of 1 (0.2 mg each) were treated with 6 N HCl (200 μL) in sealed vials at 100 °C for 1 h and 19 h, respectively. After removal of HCl in vacuo, the hydrolyzate was resuspended in 40 μL of H_2O and treated with a solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent, 2.8 μmol) in acetone (80 μL) and a solution of 0.1 M $NaHCO_3$ (20 μL) in a sealed vial at 40 °C for 1 h. The reaction mixture was quenched with 2 N HCl (10 μL). The derivatized amino acids from hydrolysis products were compared with similarly derivatized standard amino acids (2.0 μmol) by LC–MS analysis. LC separation was performed on a C_{18} column (Waters, Symmetry® C_{18} column, 3.5 μm, 2.1 × 150 mm) and monitored with both UV detection at λ 340 nm and ESI-MS. A linear gradient elution was programed using a mixture of aqueous 0.01% formic acid (solvent A) and CH_3CN (solvent B) as follows; 0–30 min, 70–40% (A in B); 30–40 min, 40–20% (A in B); 40–50 min, 20% (A in B) at a flow rate of 0.2 mL/min. One microliter of the above prepared samples was injected and each of the amino acid FDAA derivatives was checked by ESI-MS; alanine-FDAA [*m*/*z* 342 (M^++H, 100%)]; phenylalanine-FDAA [*m*/*z* 418 (M^++H, 100%)]; cysteine-bis-FDAA [*m*/*z* 626 (M^++H, 32%), *m*/*z* 251 (35%), *m*/*z* 101 (100%)]. Retention times for the derivatized amino acid standards were as follows: L-alanine, 9.36 min; D-alanine, 11.19 min; L-phenylalanine, 18.36 min; D-phenylalanine, 22.31 min; L-cysteine, 30.26 min; D-cysteine, 34.57 min. In all runs, a peak at 8.17 min was observed as excess FDAA.
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12. *Ozonolysis*: The sample of **1** (2.0 mg) was dissolved in 4.0 mL of dichloromethane (CH_2Cl_2) in a reaction vial and cooled with an ice-NaCl bath. A stream of ozone was bubbled into the sample solution for 10 min. Afterwards a stream of O_2 was bubbled followed by N_2 stream to remove excess ozone. After the reaction was completed, CH_2Cl_2 was removed under a stream of N_2.