JOURNAL OF NATURAL PRODUCTS

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Gombamide A, a Cyclic Thiopeptide from the Sponge *Clathria gombawuiensis*

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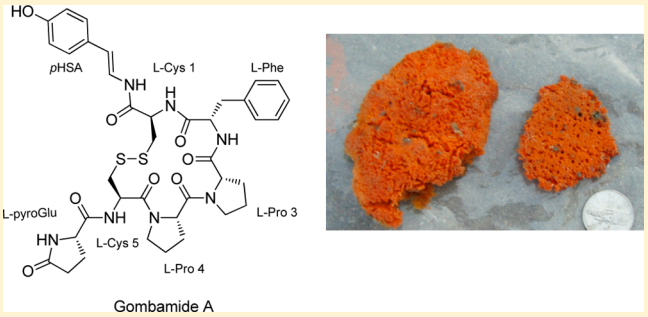
Supporting Information

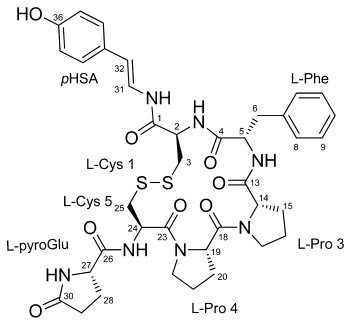
ABSTRACT: A new peptide, gombamide A (**1**), was isolated from the marine sponge *Clathria gombawuiensis*, collected from Korean waters. On the basis of the results of combined spectroscopic analyses, the structure of this compound was determined to be a cyclic C-terminally modified thiohexapeptide containing the unusual amino acid residues *para*-hydroxystyrylamide (*p*HSA) and pyroglutamic acid (pyroGlu). The absolute configurations of all amino acid residues were determined to be L by advanced Marfey's analysis. The new compound exhibited weak cytotoxicity against A549 and K562 cell lines as well as moderate inhibitory activity against Na^+/K^+-ATPase.



Sponges are widely recognized to be the most prolific sources of marine natural products, with diverse biogenetic origins and bioactivities.[1] Although peptides account for a relatively minor portion of the sponge-derived metabolites, several of these peptides possess highly unique chemical structures and potent bioactivities. The most notable examples include the cytotoxic jaspamides (=jasplakinolides) from *Jaspis* sp.,[2] the cytotoxic and antifungal theonellamide F and the thrombin-inhibiting cyclotheonamide A from *Theonella* sp.,[3,4] the HIV-inhibitory callipeltin A from *Callipelta* sp.,[5] and the HIV-inhibitory and cytotoxic papuamides from *Theonella mirabilis*.[6] More recently, highly modified peptides such as koshikamide B,[7] mutremdamide A,[8] and yaku'amides[9] have been isolated from these animals.

In our search for bioactive metabolites from Korean marine invertebrates, we encountered the red encrusting sponge *Clathria gombawuiensis* (order Poecillosclerida, family Microcionidae).[10] The organic extract of *C. gombawuiensis* exhibited moderate lethality against brine shrimp larvae (LC_{50} 225 ppm). The solvent partitioning of the extract followed by diverse chromatographic separation yielded a novel peptide. Here, we report the structure and bioactivity of gombamide A (**1**), a cyclic C-terminally modified thiohexapeptide.

The molecular formula of gombamide A (**1**) was deduced to be $C_{38}H_{45}N_7O_8S_2$ by HRFABMS analysis. The presence of seven carbonyl signals (ca. δ_C 170) in the ^{13}C NMR spectrum, in conjunction with the strong absorption band at 1635 cm^{-1} in the IR data, revealed the peptide nature of this compound. This interpretation was supported by the presence of several exchangeable protons in the downfield region ($<\delta_H$ 7.6) of the ^1H NMR spectrum.



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The planar structure of compound **1** was determined through a combination of COSY, TOCSY, gHSQC, and gHMBC experiments. First, starting with an exchangeable proton at δ_H 7.67, a four-proton spin system was found to contain a methine proton at δ_H 4.62 and methylene protons at δ_H 3.15 and 2.91 (Figure 1). The same type of coupling was

Received: April 24, 2013
Published: June 25, 2013

dx.doi.org/10.1021/np4003367 | *J. Nat. Prod.* 2013, 76, 1380–1383

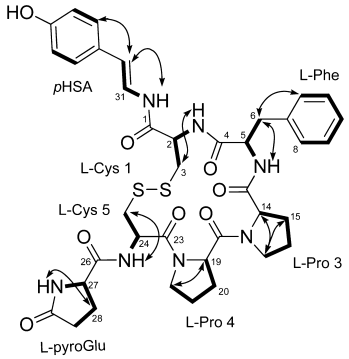

Figure 1. Key correlations of COSY (bold line) and TOCSY (arrow) experiments for compound **1**.

also found among protons at δ_H 8.64, 4.59, 3.19, and 2.62. Of several possible amino acid residues accommodating these spin systems, the chemical shifts of the methylene carbons at δ_C 41.8 and 37.6, in conjunction with the presence of two sulfur atoms in the molecular formula, showed that these were two cysteine (Cys) residues (Table 1). The presence of a phenyl moiety was readily identified by six aromatic carbons at δ_C 139−126 and their attached protons. This moiety was extended to accommodate benzylic methylene protons at δ_H 3.07 and 2.96, a methine proton at δ_H 4.88, and an exchangeable proton at δ_H 9.14, revealing the presence of a phenylalanine (Phe) residue. Two sets of proton couplings consisting of three methylenes and a methine each identified two proline (Pro) residues. All of these common amino acid residues were confirmed by gHMBC experiments.

In addition, a linear spin system consisting of an exchangeable proton at δ_H 7.93, a methine proton at δ_H 4.12, and two methylenes at δ_H 2.36, 2.24, 2.09, and 2.06 was found by 2-D NMR experiments (Figure 1). A γ-lactam moiety accommodating all of these protons was constructed on the basis of the long-range correlations of the carbonyl carbon at δ_C 177.9 with protons at δ_H 7.93, 4.12, and 2.24 in the gHMBC data (Figure 2). Further extension of this moiety by the gHMBC correlation between the carbonyl carbon at δ_C 172.0 and the proton at δ_H 2.24 revealed a pyroglutamic acid (pyroGlu) residue. Among the remaining carbons, six carbons at δ_C 156.4, 126.9, 126.5 (2 C), and 115.6 (2 C) and the protons attached to the latter two carbon signals were defined as a *para*-hydroxyphenyl moiety. Extension of this moiety to incorporate a double bond at δ_C 120.4 and 113.6 was revealed by allylic proton−proton and long-range carbon−proton couplings between these groups. The *E* configuration was assigned for the double bond due to the large coupling constant (J = 14.8 Hz) between the olefinic protons. The direct connection of this moiety to an amide proton at δ_H 10.21 was also determined by the large proton−proton coupling (J = 10.0 Hz). Thus, the last residue of compound **1** was identified as *para*-hydroxystyrylamide (*p*HSA), an uncommon amino acid-derived unit.

The assignment of the amino acid residues and the construction of the planar structure of gombamide A (**1**) were accomplished by a combination of gHMBC and NOESY experiments (Figure 2). The connection between Cys-1 and *p*HSA was revealed by the long-range correlations at 31-NH/C-2 and 31-NH/C-31, supported by NOESY cross-peaks at 31-NH/2-NH and 31-NH/H-3. In addition, the carbonyl carbon at δ_C 167.4 was placed at C-1 due to its long-range correlations with H-2, H-3, and H-31. Similarly, the placement of the carbonyl carbon δ_C 171.0 at C-4 and the linkage between Cys-1 and Phe residues were accomplished by the correlations of 2-NH/C-2, 2-NH/C-4, H-5/C-4, and H-6/C-4. The gHMBC correlations with the H-14 and H-15 in Pro-3 placed the carbonyl carbon at δ_C 171.4 in this amino acid. An additional correlation with 5-NH assigned the Pro-3 carbonyl at C-13 and

Table 1. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR Assignments for Gombamide A (1) in DMSO-d_6

	position	δ_C	δ_H (J in Hz)
L-Cys 1	1	167.4, C	
	2	51.5, CH	4.62, ddd (12.0, 8.7, 3.2)
	3	41.8, CH_2	3.15, dd (14.1, 3.2)
			2.91, dd (14.1, 12.0)
	2-NH		7.67, d (8.7)
L-Phe	4	171.0, C	
	5	53.2, CH	4.88, ddd (12.7, 8.8, 4.3)
	6	33.8, CH_2	3.07, dd (14.2, 12.7)
			2.96, dd (14.2, 4.3)
	7	138.1, C	
	8/12	129.5, CH	7.39, br d (7.4)
	9/11	128.0, CH	7.22, dd (7.7, 7.4)
	10	126.0, CH	7.16, t (7.7)
	5-NH		9.14, d (8,8)
L-Pro 3	13	171.4, C	
	14	60.3, CH	4.35, br d (8.0)
	15	31.1, CH_2	1.83, m
			1.70, m
	16	19.7, CH_2	1.31, m
			0.23, m
	17	45.8, CH_2	3.22, ddd (11.0, 8.6, 3.2)
			3.02, br dd (11.0, 9.0)
L-Pro 4	18	169.4, C	
	19	59.3, CH	4.86, dd (8.9, 3.3)
	20	30.3, CH_2	2.23, m
			1.87, m
	21	21.8, CH_2	1.77, m
			1.71, m
	22	46.4, CH_2	3.50, ddd (11.7, 8.2, 4.1)
			3.35, m
L-Cys 5	23	168.0, C	
	24	48.5, CH	4.59, ddd (11.6, 10.0, 5.5)
	25	37.6, CH_2	3.19, dd (12.0, 5.5)
			2.62, dd (12.0, 11.6)
	24-NH		8.64, d (10.0)
L-pyroGlu	26	172.0, C	
	27	54.9, CH	4.12, br dd (9.1, 1.5)
	28	25.5, CH_2	2.24, m
			2.09, m
	29	28.8, CH_2	2.36, m
			2.06, ddd (12.5, 9.7, 2.7)
	30	177.9, C	
	27-NH		7.93, br s
*p*HSA	31	120.4, CH	7.15, dd (14.8, 10.0)
	32	113.6, CH	6.26, d (14.8)
	33	126.9, C	
	34/38	115.6, CH	6.71, d (8.7)
	35/37	126.5, CH	7.18, d (8.7)
	36	156.4, C	
	31-NH		10.21, d (10.0)
	36-OH		9.40, s

dx.doi.org/10.1021/np4003367 | *J. Nat. Prod.* 2013, 76, 1380−1383

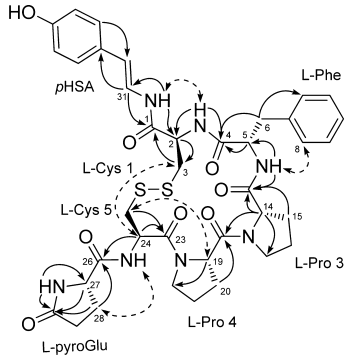

Figure 2. Key correlations of gHMBC (solid arrow) and NOESY (dashed arrow) experiments for compound **1**.

linked this residue to the Phe residue. Long-range correlations with H-14 and H-20 placed the carbonyl carbon δ_C 169.4 at C-18 and identified a peptide linkage between the Pro-3 and Pro-4 residues. However, the lack of additional gHMBC correlations hindered further identification of a peptide linkage from these data, leaving two open ends at Cys-5 and Pro-4.

A long-range correlation with H-28 assigned the carbonyl carbon δ_C 172.0 at C-26 of the pyroGlu residue (Figure 2). Further correlations of this carbonyl carbon with H-24 and 24-NH connected the pyroGlu with Cys-5, supported by a NOESY cross-peak at 24-NH/H-28. The only remaining carbon at δ_C 168.0 was placed at C-23 of the Cys-5 residue by its gHMBC correlations with H-24 and H-25. Although unsupported by the gHMBC data, the linkages of Cys-5 with Cys-1 and Pro-4 were indicated by the NOESY cross-peaks at H-3/H-24 and H-19/H-25. Cys-5 was therefore connected to Cys-1 by a disulfide bond, while it was also connected to Pro-4 by a peptide bond.

The confirmation of amino acid residues as well as the absolute configuration of each residue in compound **1** was achieved by advanced Marfey's analysis.[11] After acid hydrolysis of **1**, the ESI-LC/MS analysis of the hydrolysate adducts with L- and D-FDAA clearly confirmed the NMR-based amino acid residue assignments, except for pyroGlu, which was converted to Glu through acid hydrolysis of the γ-lactam ring. The comparison of LC retention times between L- and D-FDAA-derivatized hydrolysates assigned L configurations to all of the amino acid residues. Thus, the structure of gombamide A (**1**) was unambiguously determined to be a cyclic thiopeptide.

In addition to the common amino acid residues, gombamide A (**1**) possessed *para*-hydroxystyrylamide and pyroglutamic acid monomers. A literature study showed that the former residue or related variants have been found in a few sponge-derived peptides. The anchinopeptolides from *Anchinoe tenacior*[12] and cyclotheonamide C from *Theonella swinhoei*[13] have the same *p*HSA residue, while the celenamides from *Cliona celata* have an additional *meta*-hydroxy group at the *p*HSA.[14] Pyroglutamate has been found in didemnin D from the tunicate *Tridemnum solidum*,[15] the stephanotic acid from the plant *Stephanotis floribunda*,[16] and pyroglutamyl dipeptides and asteropsin A from *Asteropus* spp. sponges;[17,18] *N*-methylpyroglutamic acid (pyroMeGlu) was found in kendarimide A from the sponge *Haliclona* sp.[19] In addition, cyclic peptides having disulfide linkages are rarely found from sponges, with the microcionamides from *Clathria abietina*,[20] the neopetrosiamides from *Neopetrosia* sp.,[21] and asteropsin A[18] being the only examples in literature, pointing to their rarity.

Sponge-derived peptides exhibit diverse bioactivities. In our experiments, gombamide A exhibited weak cytotoxicity against the K562 and A549 cell lines with LC_{50} values of 6.9 and 7.1 μM, respectively (the LC_{50} values of doxorubicin are 0.7 and 0.5 μM, respectively). This compound also moderately inhibited the action of Na^+/K^+-ATPase with an LC_{50} value of 17.8 μM (the LC_{50} value of ouabain is 9.4 μM).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1020 polarimeter using a 1 cm cell. UV spectra were recorded on a Hitachi U-3010 spectrophotometer, and IR spectra were recorded on a JASCO 300E FT-IR spectrometer. NMR spectra were recorded in DMSO-d_6 containing Me_4Si as an internal standard on Bruker Avance 600 spectrometers. Proton and carbon NMRs were measured at 600 and 150 MHz, respectively. Mass spectrometric data were obtained at the Korea Basic Science Institute (Daegu, Korea) and were acquired using a JEOL JMS 700 mass spectrometer with *meta*-nitrobenzyl alcohol as a matrix for the FABMS. Low-resolution ESIMS data were recorded on an Agilent Technologies 6130 quadrupole mass spectrometer with an Agilent Technologies 1200 series HPLC. HPLC was performed on a SpectraSystem p2000 equipped with a SpectraSystem RI-150 refractive index detector. All solvents were spectroscopic grade or distilled in a glassware prior to use.

Animal Material. Specimens of *Clathria gombawuiensis* (voucher number 06SH5-2) were collected by hand using scuba equipment off the shore of Gageo-do, Korea, at a depth of 25 m on September 9–11, 2006. The sponge was thickly encrusted, and its color was red while still alive. The choanosomal skeleton is regularly plumo-reticulated, with well-developed spongin fibers forming regular anastomoses of differentiated primary and secondary spongin fibers. For the spicules, megascleres were composed of thick styles (320–400 × 18–20 μm), slender styles (290–33 × 8–11 μm), subtylotes (200–280 × 8–9 μm), and acanthostyles (90–170 × 8–10 μm), while microscleres were composed of large toxa (130–18 μm), small toxa (10–12 μm), and palmate isochelae (20–25 μm). These morphological features agreed well with those reported in the literature.[10] A voucher specimen (registry no. Spo. 66) is deposited at the Natural History Museum, Hannam University, Korea, under the curatorship of C.J.S.

Extraction and Isolation. The freshly collected specimens were immediately frozen and stored at −25 °C until use. The lyophilized specimens were macerated and repeatedly extracted with MeOH (2 L × 3) and CH_2Cl_2 (2 L × 3). The combined extracts (66.65 g) were successively partitioned between *n*-BuOH (30.49 g) and H_2O (35.31 g); the former fraction was repartitioned between H_2O–MeOH (15:85) (9.53 g) and *n*–hexane (19.04 g). The former layer (9.53 g) was separated by C_{18} reversed-phase flash chromatography using a sequential mixture of H_2O and MeOH (six fractions in gradient, H_2O–MeOH, from 50:50 to 0:100), acetone, and finally EtOAc as the eluents.

On the basis of the results of ^{1}H NMR and cytotoxicity analyses, the fraction that eluted with H_2O–MeOH (40:60; 0.14 g) was chosen for separation. This fraction was separated by semipreparative reversed-phase HPLC (YMC-ODS column, 10 mm × 250 mm; H_2O–MeOH, 45:55). Further purification by reversed-phase HPLC (YMC-ODS column, 4.6 mm × 250 mm; H_2O–MeOH, 50:50) yielded compound **1** as a yellow, amorphous solid. The final isolated amount was 3.1 mg.

Gombamide A (1): yellow, amorphous solid; $[\alpha]_D^{25}$ +17.3 (*c* 0.50, MeOH); UV (MeOH) λ_{max} (log ε) 285 (4.02), 309 (3.80) nm; IR (ZnSe) ν_{max} 3273, 2950, 1635, 1509, 1453 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 791.2852 $[M + H]^+$ (calcd for $C_{38}H_{46}N_7O_8S_2$, 791.2849).

Advanced Marfey's Analysis of Compound 1. Gombamide A (0.6 mg) was dissolved in 0.5 mL of 6 N HCl and heated at 110 °C for 1 h. This solution was evaporated, and traces of HCl were removed by repeated drying under vacuum with distilled water. To the divided hydrolysate (0.3 mg) were added 100 μL of 1 N $NaHCO_3$ and 50 μL of 1% L- or D-FDAA in acetone. The mixture was stirred at 80 °C for

dx.doi.org/10.1021/np4003367 | J. Nat. Prod. 2013, 76, 1380–1383

12 min. After the reaction was quenched by the addition of 50 μL of 2 N HCl, the mixture was analyzed by ESI-LC/MS to assign the chirality of the amino acids. The retention times of the L- and D-FDAA-derivatized hydrolysates were 28.0 and 30.0 min for L-Glu, 32.7 and 36.5 min for L-Pro, 50.5 and 52.8 min for L-Phe, and 55.0 and 56.3 min for L-Cys, respectively. These results demonstrate that all amino acids in gombamide A are in L form.

Biological Assays. The cytotoxicity assays were performed in accordance with literature protocols.[22,23] Na^+/K^+-ATPase inhibition assay was performed according to previously described methods.[24]

■ ASSOCIATED CONTENT

Supporting Information

^1H, ^{13}C, and 2-D NMR spectra of gombamide A are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to the Basic Science Research Institute in Daegu, Korea, for providing mass data. This study was supported by National Research Foundation of Korea (NRF) grants funded by the Korean government (MEST, Ministry of Education, Science and Technology) (M1A5A1-2010-0020429 and 2009-0083533).

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