약학석사학위논문

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

Clathria gombawuiensis 한국해면에서 분리한 신규 Peptide

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Abstract

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

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A new peptide, gombamide A (**1**), was isolated from the marine sponge *Clathria gombawuiensis*, collected from Korean waters. Based on 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 pyroglutamicacid (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.

Key Word : *Clathria gombawuiensis*, a Cyclic C-terminally-modified Thiohexapeptide, the Unusual Amino Acid Residues

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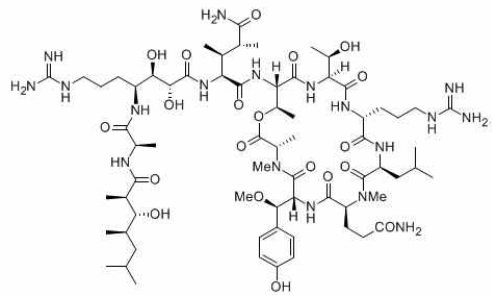
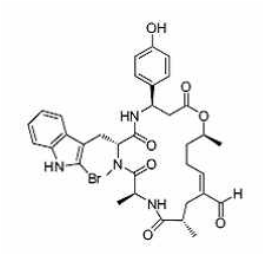
Introduction

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 *Theonellamirabilis.*[6] Morerecently, 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} 225ppm). 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.



Callipeltin A



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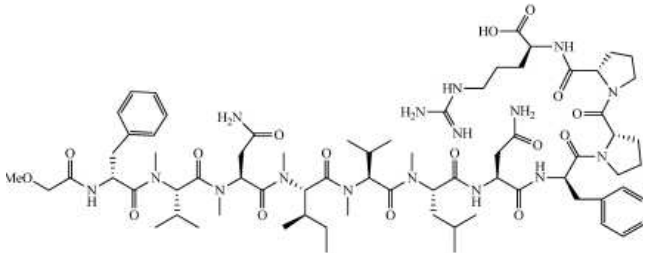
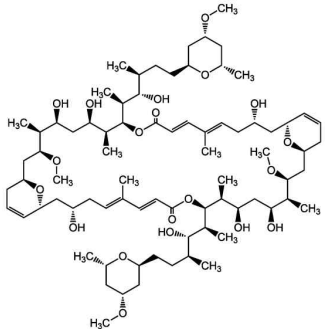
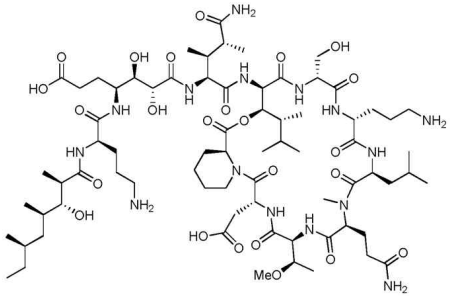


Koshikamide B



Cyclothionamide A



Homophymine A

Figure 1. Bioactive Peptides

Experimental Section

1. 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*-nitrobenzylalcohol (NBA) 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 glass prior to use.

2. 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

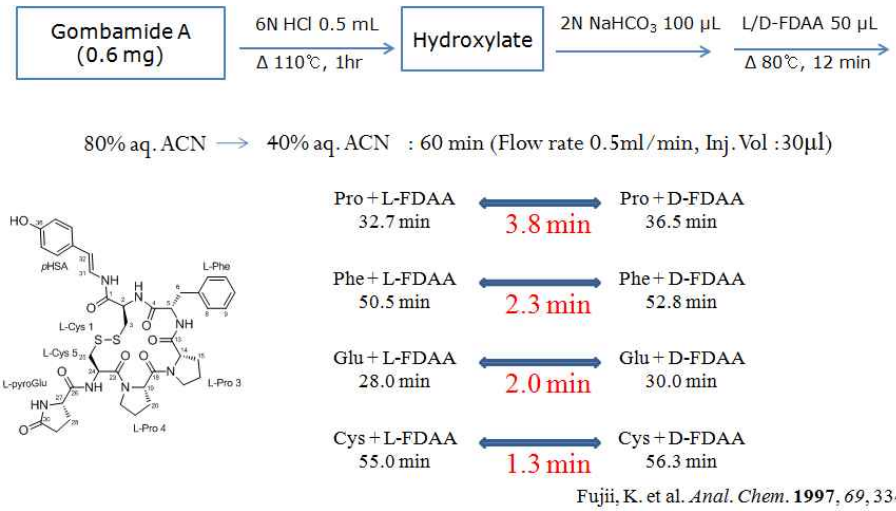
repartitioned between H_2O–MeOH (15:85) (9.53 g) and *n*-hexane (19.04 g). The former layer (9.53 g) was separated by C_{18} reversed-phase flash chromatography using a sequential mixture of H_2O and MeOH (six fractions in gradient, H_2O–MeOH, from 50:50 to 0:100), acetone, and finally EtOAc as the eluents.

Based on the results of ^1H 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 semi-preparative 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.

3. Advanced Marfey's Analysis

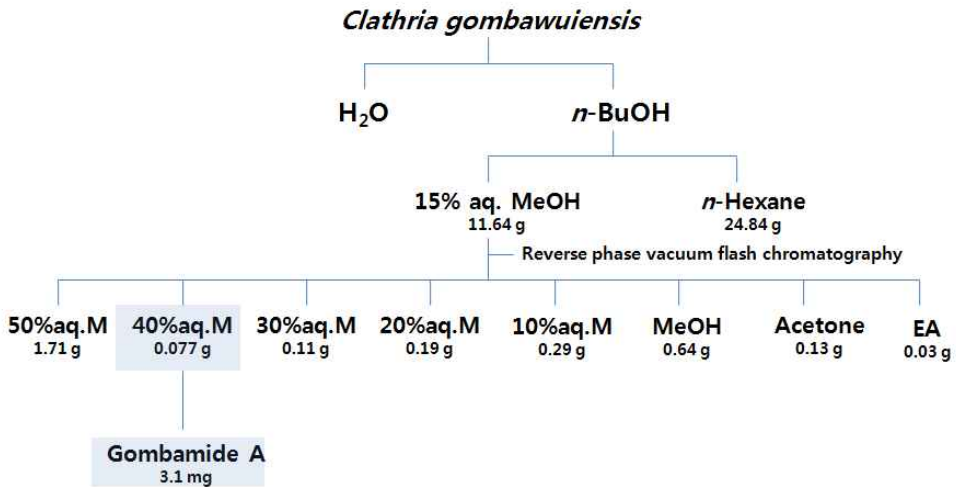
Gombamide A (0.6 mg) was dissolved in 0.5 mL of 6 N HCl and heated at 110 °C for 1 h. This solution was evaporated, and traces of HCl were removed by repeated drying under vacuum with distilled water. To the divided hydrolysate (0.3 mg), 100 μL of 1 N $NaHCO_3$ and 50μL of 1% L- or D-FDAA in acetone were added. The mixture was stirred at 80 °C for 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 min and 30.0 min for L-Glu, 32.7 min and 36.5 min for L-Pro, and 50.5 min and 52.8 min for L-Phe, 55.0 and 56.3 min for L-Cys, respectively.

These results demonstrate that all amino acids in gombamide A are in L form.



Fujii, K. et al. *Anal. Chem.* **1997**, *69*, 3346–3352.

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 Table1 ; HRFABMS *m/z* 791.2852 $[M+H]^+$ (calcd for $C_{38}H_{46}N_7O_8S_2$, 791.2849).



Scheme 1. Isolation of Gombamide A

Results

1. Compound 1

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 (< δ_H 7.6) of the ^1H NMR spectrum.

The planar structure of compound **1** was determined through a combination of COSY, TOCSY, *g*HSQC, and *g*HMBC 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 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 *g*HMBC 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 based on 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 *g*HMBC data (Figure 2). Further extension of this moiety by the *g*HMBC 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=14.8Hz) 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=10.0Hz). 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 *g*HMBC and NOESY experiments (Figure2). 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 *g*HMBC 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 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 *g*HMBC 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 *g*HMBC correlations with H-24 and H-25. Although unsupported by the *g*HMBC 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'sanalysis.[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 (*p*HSA) and pyroglutamicacid (pyroGlu) monomers. A literature study showed that the former residue or related variants have been found in a few sponge-derived peptides. The anchinopeptolides from *Anchinoetenacior*[12] and cyclotheonamide C from *Theonella swinhoei*[13] have the same *p*HSA residue, while the celenamides from *Clionacelata* have an additional *meta*-hydroxy group at the *p*HSA.[14] Pyroglutamate has been found in didemnin D from the tunicate *Tridemnumsolidum*,[15] the stephanotic acid from the plant *Stephanotis floribunda*,[16] and pyroglutamyl dipeptides and the asteropsin A from *Asteropus* spp. sponges;[17,18]

N-methylpyroglutamicacid (pyroMeGlu) was found in the kendarimide A from the sponge *Haliclona*sp.[19] Inaddition, cyclicpeptides having disulfide linkages are rarely found from sponges with the microcionamides from *Clathria abietina*,[20] the neopetrosiamides from *Neopetrosia*sp.,[21] andasteropsin A[18] being the only examples in literature, pointing to its 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 mM, respectively (the LC_{50} values of doxorubicin are 0.7 and 0.5 mM, respectively). This compound also moderately inhibited the action of Na^+/K^+-ATPase with an LC_{50} value of 17.8 mM (the LC_{50} value of ouabain is 9.4 mM).

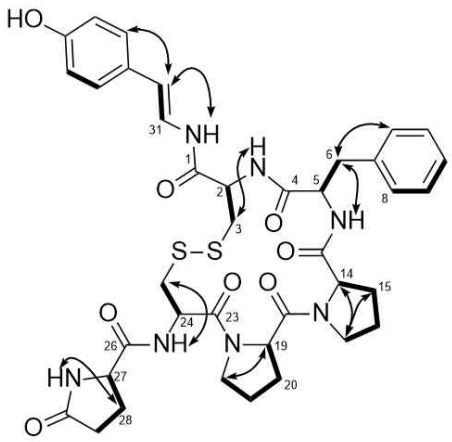


Figure 2. COSY and TOCSY correlations of Gombamide A in DMSO-d_6

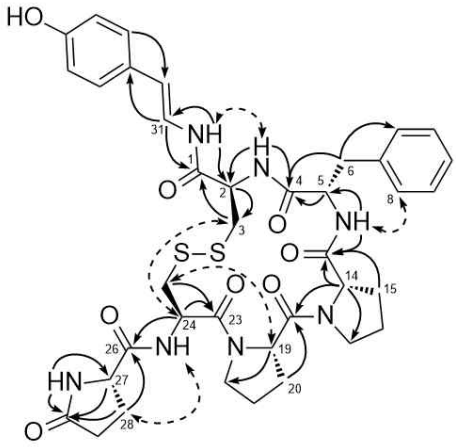
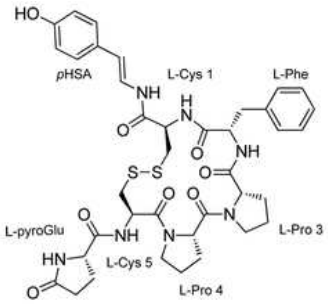


Figure 3. *g*HMBC and NOESY correlations of Gombamide A in DMSO-d_6



Gombamide A

Table 1. ^{1}H and ^{13}C NMR Assignment for compound 1 in MeOD

Residue	Position	^{13}C	^{1}H (J in Hz)
L-Cys 1	1	167.4	
	2	51.5	4.62, ddd (12.0, 8.7, 3.2)
	3	41.8	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	
	5	53.2	4.88, ddd (12.7, 8.8, 4.3)
	6	33.8	3.07, dd (14.2, 12.7)
			2.96, (14.2, 4.3)
	7	138.1	
	8/12	129.5	7.39, br d (7.4)
	9/11	128.0	7.22, dd (7.7, 7.4)
	10	126.0	7.16, t (7.7)
	5-NH		9.14, d (8.8)

Residue	Position	^{13}C	^{1}H (J in Hz)
L-Pro 3	13	171.4	
	14	60.3	4.35, br d (7.4)
	15	31.1	1.83, m
			1.70, m
	16	19.7	1.31, m
			0.23, m
	17	45.8	3.22, ddd (11.0, 8.6, 3.2)
			3.02, br dd (11.0, 9.0)
L-Pro 4	18	169.4	
	19	59.3	4.86, dd (8.9, 3.3)
	20	30.3	2.23, m
			1.87, m
	21	21.8	1.77, m
			1.71, m
	22	46.4	3.50, ddd (11.7, 8.2, 4.1)
			3.35, m
L-Cys 5	23	168.0	
	24	48.5	4.59, ddd (11.6, 10.0, 5.5)
	25	37.6	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	
	27	54.9	4.12, br dd (9.1, 1.5)
	28	25.5	2.24, m
			2.09, m
	29	28.8	2.36, m
			2.06, m
	30	177.9	
	27-NH		7.93, br s
*p*HSA	31	120.4	7.15, dd (14.8, 10.0)
	32	113.6	6.26, d (14.8)
	33	126.9	
	34/38	115.6	6.71, d (8.7)
	35/37	126.5	7.18, d (8.7)
	36	156.4	
	31-NH		10.21, d (10.0)
	36-OH		9.40, s

Discussion

Sponges are widely well-known for prolific source of diverse secondary metabolites. Especially peptides, having several forms such as linear, cyclic peptides and depsipeptides, account for a relatively minor portion compared with terpenes and steroids.

However, peptides have been reported for metabolites having diverse bioactivities such as antimicrobial, antitumor, anticancer, and antituvulin.

Clathria gombawuiensis were dried at lower temperature and extracted with methanol and dichloromethane at three times respectively. The crude extract was then partitionated two different solvents, water and butanol. n-hexane and 15% aqueous methanol were also used for partition to separate compounds through their polarity. The 15% aqueous methanol layer was then fractionated on ODS flash chromatography. By using C_{18} reversed-phase HPLC twice, with Gombamide A was purified.

Following the results of NMR data, combined HSQC, COSY, TOCSY, *g*HMBC and NOESY, the metabolite was contained seven amino acid units. Each amino acids was revealed by *para*-hydroxystyrylamide, 2 cysteines, phenylalnine, 2 prolines, and pyroglutamic acid. All amino acids containing chiral center was determined L-form which were existed commonly in nature by advanced Margey's reaction.

In this study, the isolated compounds were structurally identified to be cyclic thioheptapeptide. Among peptides, cyclization with disulfide linkage is also rare in natural products. Furthermore, *para*-hydroxystyrylamide and pyroglutamic acid are also unusual amino acids. Even though Gombamide A is exhibited weak cytotoxicity

against A549 and K562, it has an outstanding metabolite as an aspect of structure.

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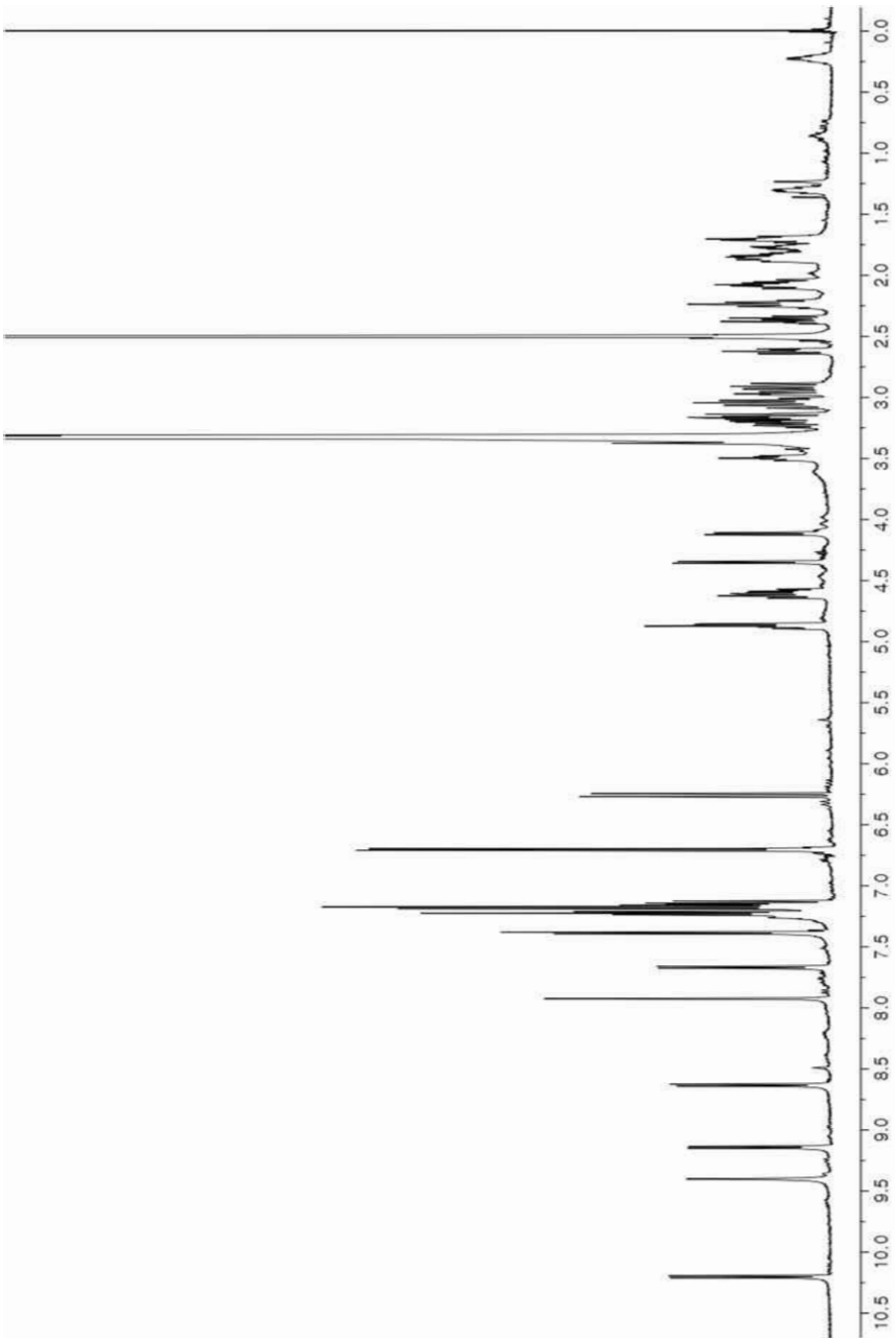

Figure 4. ^{1}H spectrum of Gombamide A in DMSO-d_6

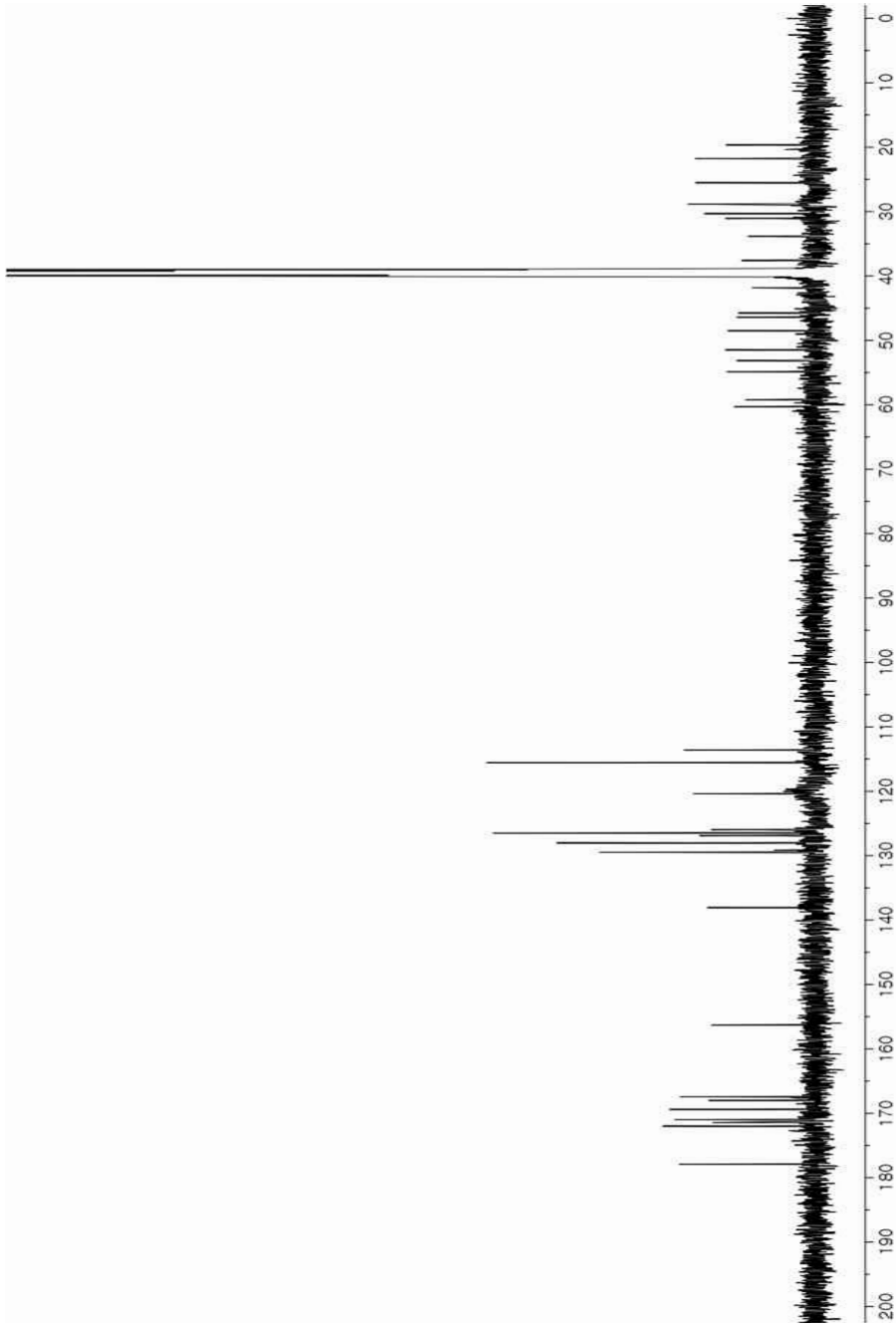

Figure 5. ^{13}C spectrum of Gombamide A in DMSO-d_6

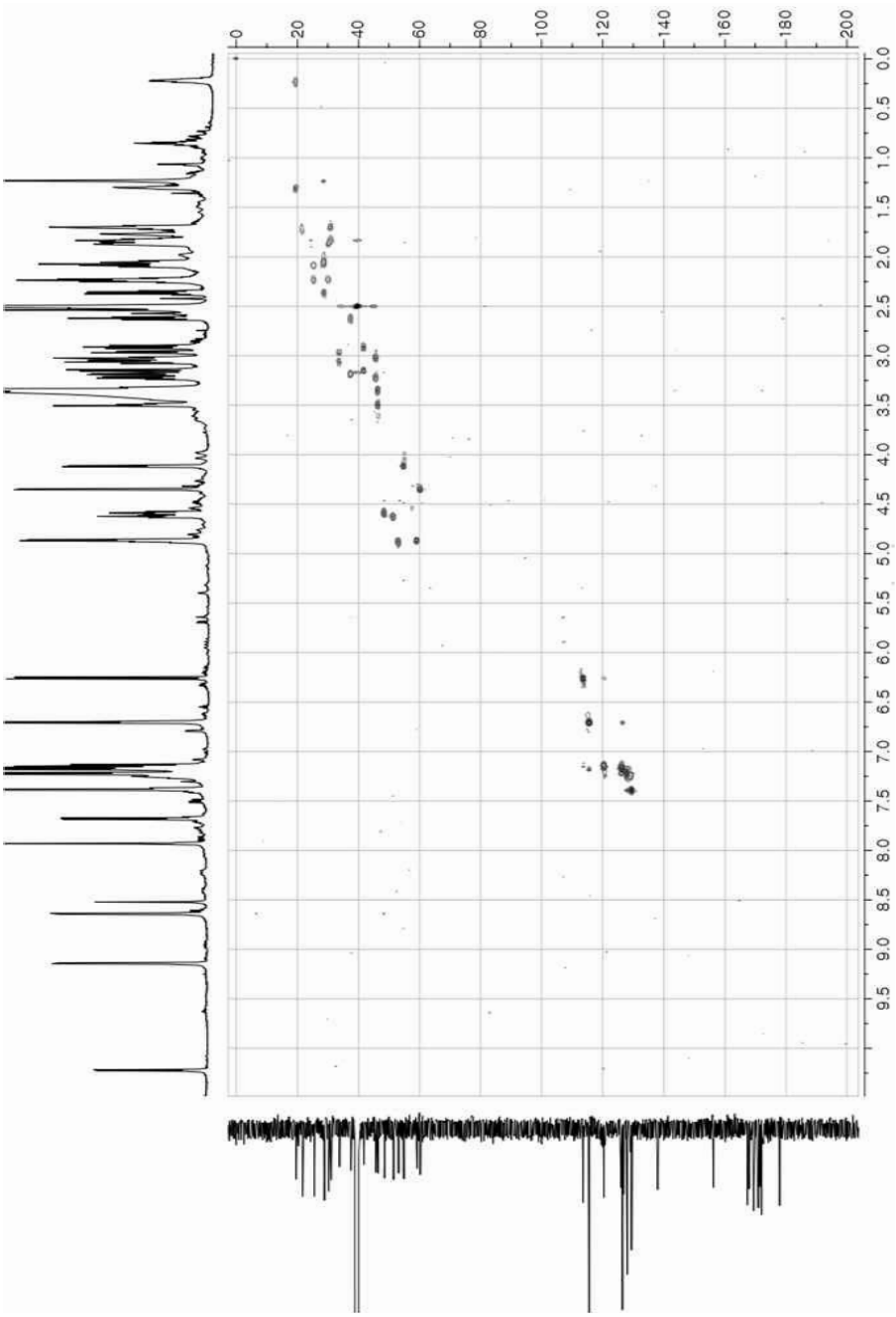

Figure 6. HSQC spectrum of Gombamide A in DMSO-d_6

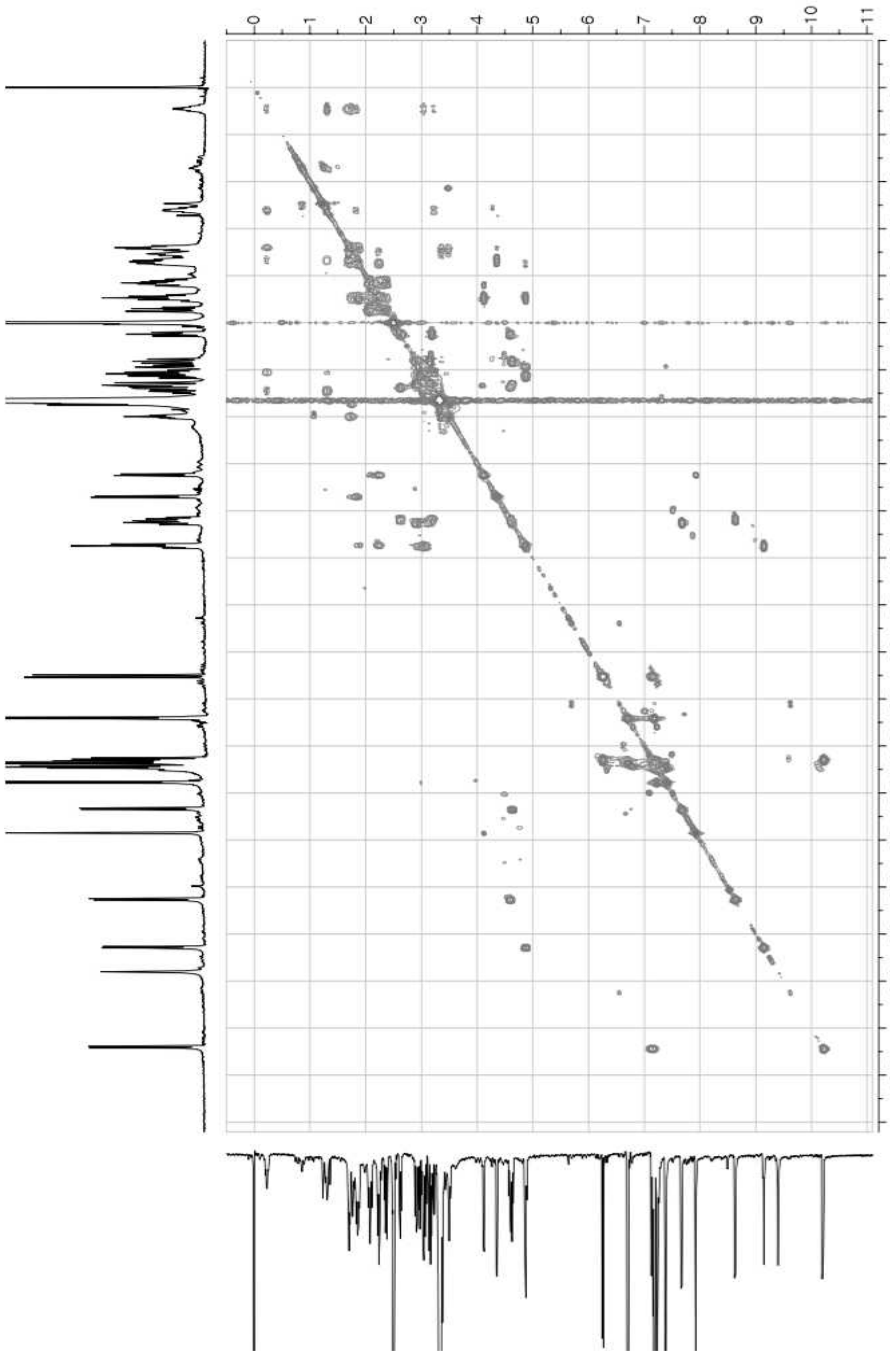

Figure 7. COSY spectrum of Gombamide A in DMSO-d_6

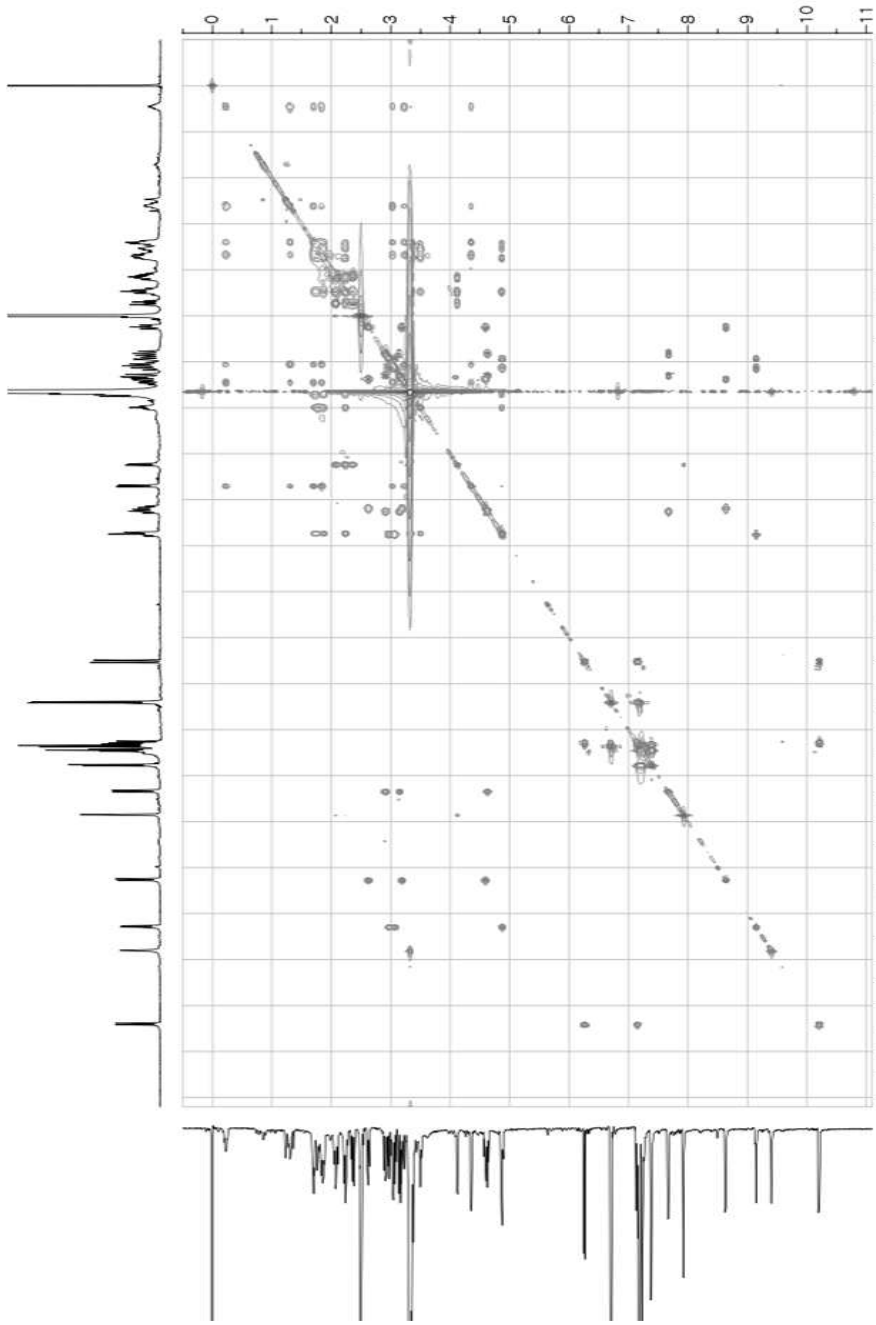

Figure 8. TOCSY spectrum of Gombamide A in DMSO-d_6

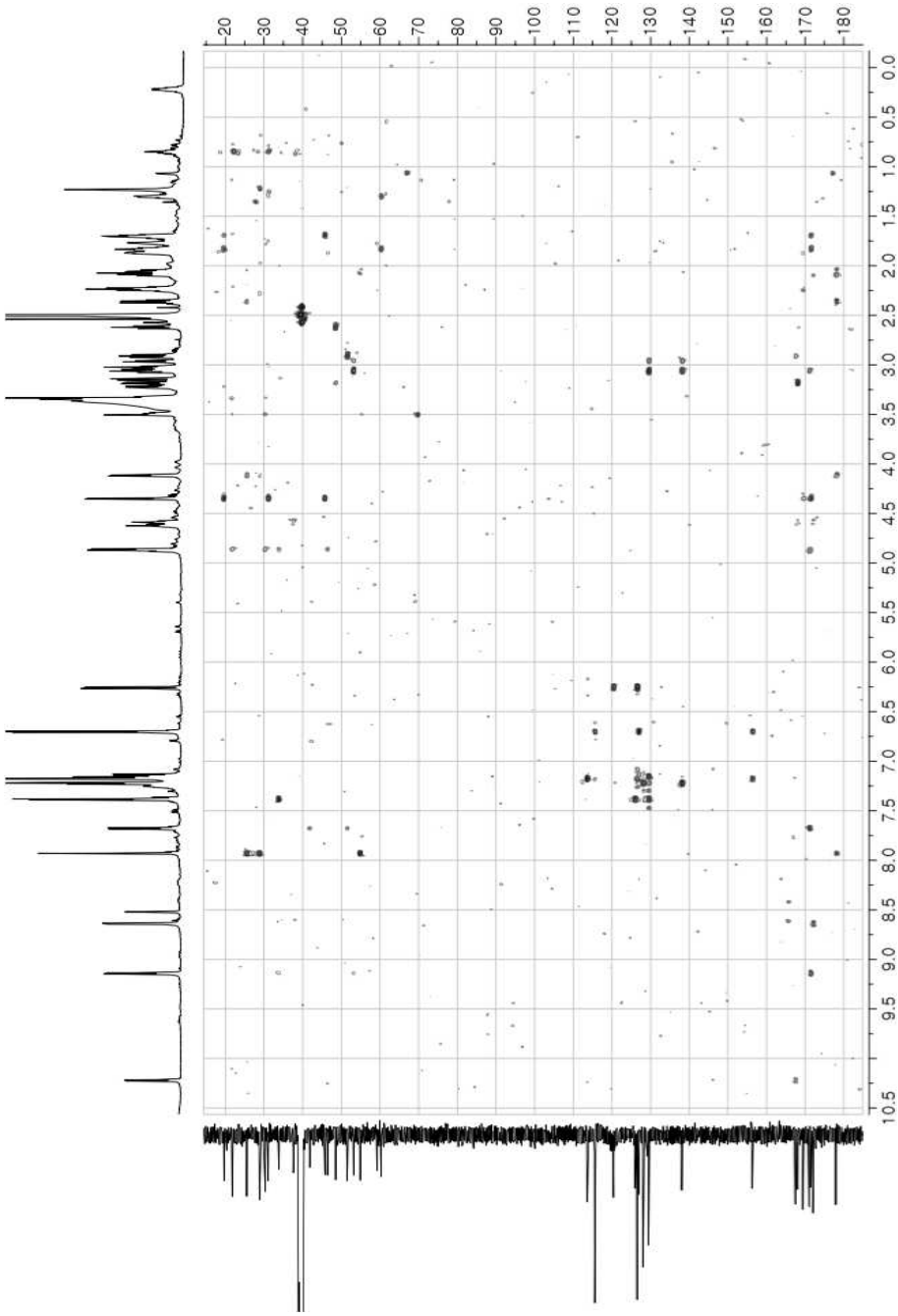

Figure 9. *g*HMBC spectrum of Gombamide A in DMSO-d_6

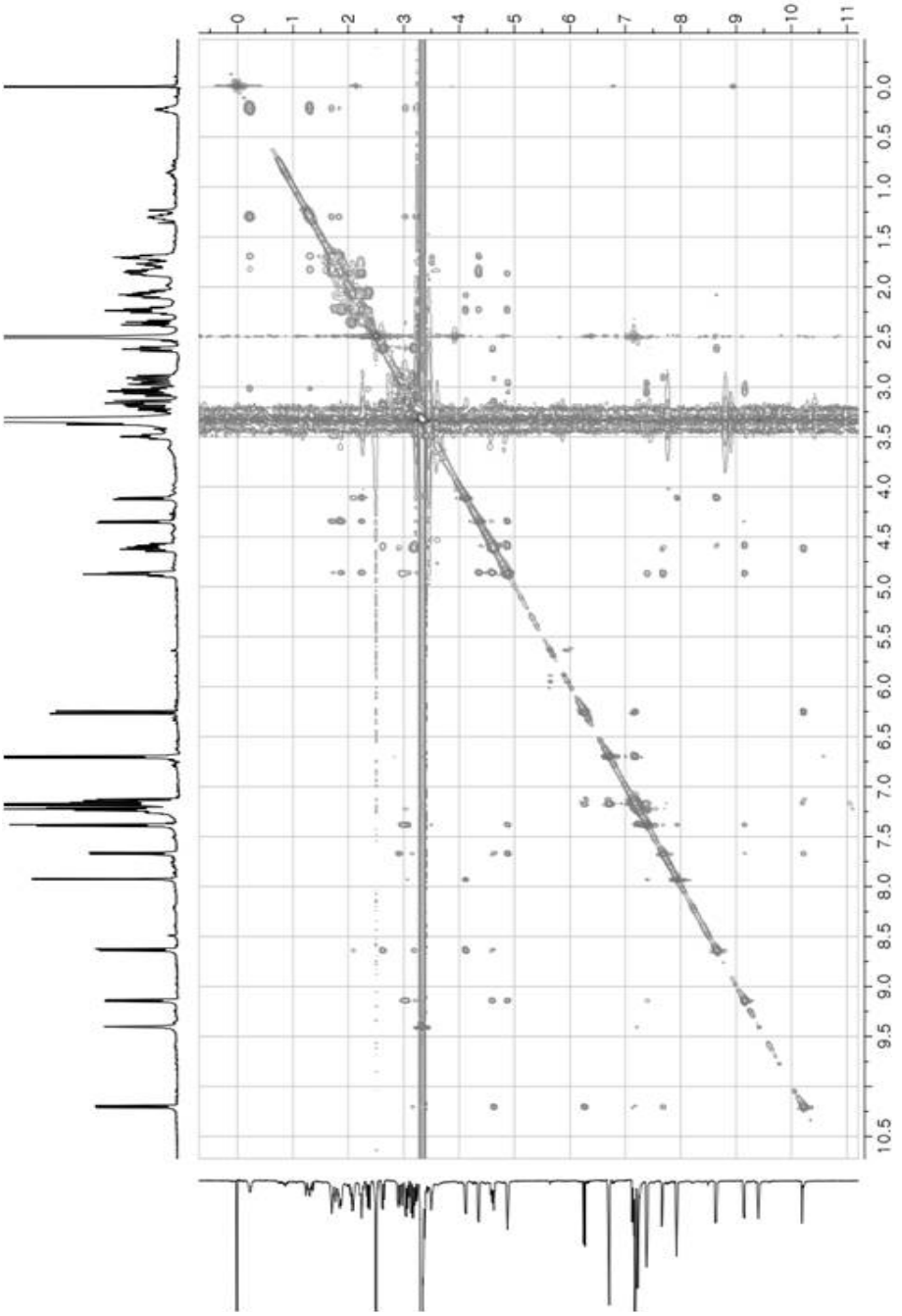

Figure 10. NOESY spectrum of Gombamide A in DMSO-d_6

국문초록

Clathria gombawuiensis 한국해면에서 분리한 신규 Peptide

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2006년도 가거도에서 채집한 해면 *Clathria gombawuiensis*의 추출물로부터 유기물질의 극성에 따라 1종의 물질을 분리하였다. 이는 여러 분광학적 자료를 토대로 peptide 계열의 신규 물질로 규명되었다.

NMR 분석을 통하여 2개의 Cysteine, 2개의 Proline, 1개의 Phenylalanine, 1개의 pyroGlutamate, 1개의 para-hydroxystyrylamide의 총 7개의 아미노산으로 이루어져 있음을 밝혀냈다. 이 중 pyroGlutamate와 para-hydroxystyrylamide의 경우 특이적 아미노산으로서 그 구조적 신규성이 뛰어나다 할 수 있다. 각각의 아미노산은 gHMBC와 NOESY, TOCSY 분석을 통하여 연결할 수 있었다. Advanced Marfey's analysis를 통하여 6개의 아미노산은 L-form을 하고 있음을 알았다. 2개의 Cysteine은 NOESY correlation과 HRFABMS로 이황화결합을 하고 있음을 확인하였다. 대체적으로 이황화 결합을 가지고 있는 천연물의 경우 생리활성에서 좋은 결과를 보이는 경우가 많았으나 이 물질의 경우 그리 뛰

어난 결과를 얻을 수 없었다. 또한 소량으로 분리정제 되었기 때문에 여러 가지 생리활성을 확인해 볼 수 없었다. 이 물질은 Journal of Natural Products에 2013년 6월 23일에 최종 publish되었다.

주요어 : *Clathria gombawuiensis*, 특이적 아미노산, 이황화결합, peptide

학번 : 2012-21601

감사의 글

2011년 10월 24일 처음 실험실에 들어와 새로운 환경에 지레 겁먹고 적응하지 못하던 일이 엊그제 같은데, 어느덧 벌써 4학기가 종료되고 석사 논문의 감사의 글을 쓰고 있자니 감회가 새롭습니다. 지금도 눈을 감고 있으면 졸업한 여러 선배들, 굵직굵직한 사건들이 주마등처럼 스쳐지나갑니다. 지난 2년은 저에게 있어 과학도가 되기 위한 발판이 되는 기회였습니다.

매주 진행되는 랩미팅 시간은 또 다른 교육의 장이었고, 아버지 같은 신종헌 교수님께 인간적으로나 사회적으로 많은 가르침을 받을 수 있어서 진심으로 감사드립니다. 혼난 적도 많았지만, 지나고 생각해보면 다 제게 도움이 되는 일임을 깨닫습니다. 마음속 깊이 항상 프로가 되라는 말씀 되새기며 살아가도록 노력하겠습니다.

Journal of Natural Products에 논문을 투고함에 있어 많은 도움을 주신 오동찬 교수님께도 감사의 말씀을 전합니다. 수업을 통해서도 많은 지식을 얻을 수 있어서 큰 도움이 되었습니다. 교수님의 열린 사고는 우물 안의 개구리 같던 저를 한 단계 도약시키는 계기가 되었습니다. 자주 찾아뵙진 못했지만 바쁘신 와중에도 논문을 심사해 주시고 저희들의 물질 활성을 봐주시는 오기봉 교수님께도 감사의 말씀 전합니다.

그리고 2년 넘게 실험실 생활을 해오면서 가족보다 더 오랜 하루를 함께하는 실험실 선후배님들께도 진심으로 고마운 마음을 전합니다. 여러분들과 함께하며 실험적으로나 인격적으로 성숙해질 수 있었습니다.

다른 걱정 없이 실험에 매진할 수 있게 지지해 주신 아버지 어머니께도 감사의 말씀을 전합니다. 스물아홉이 된 지금의 제가 존재할 수 있었던 것은 부모님의 사랑이 없으면 불가능했을 것이라 생각합니다.

앞으로도 어제보다 나은 내일을 살기위해 노력하고 주변 사람을 둘러보고 감싸줄 수 있는 사람이 되도록 노력하며 살아가겠습니다.