Proline-Containing Dipeptides from a Marine Sponge of a *Callyspongia* Species

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A detailed analysis of the chemical constituents of a marine sponge of *Callyspongia* sp. was performed. A new proline-containing dipeptide, callyspongidipeptide A (cyclo-((*S*)-Pro-8-hydroxy-(*R*)-Ile); **1**) along with cyclo-((*S*)-Pro-(*R*)-Leu) (**3**) and seco-((*S*)-Pro-(*R*)-Val) (**2**) were isolated. The structures were determined on the basis of NMR and MS analysis, and the absolute configuration was determined by comparison of the optical rotation with known compounds.

Introduction. – Marine sponges produce numerous bioactive compounds with promising pharmaceutical properties. The genus *Callyspongia* (order Haplosclerida, family Callyspongiidae) is widely distributed and contains various bioactive constituents such as polyacetylenes [1–11], peptides [12][13], terpenoids [14–17], alkaloids [18–20], fatty acids [21–23], polyketides [24], sterols [25], peroxides [26], and butenolides [27]. Many of these secondary metabolites have diverse bioactivities such as antifouling [28][29], cytotoxic [30][31], anticancer [24], and antimicrobial [28] properties. In the course of our search for cytotoxic metabolites from marine sponges, we have discovered a new proline-containing dipeptide named callyspongidipeptide A (**1**) from *Callyspongia* sp., together with the new natural product seco-((*S*)-Pro-(*R*)-Val) (**2**) and the known compound cyclo-((*S*)-Pro-(*R*)-Leu) (**3**). The gross structures of these compounds were elucidated by the aid of COSY, HMQC, and HMBC experiments, while the absolute configuration was determined from the optical rotation. Here, we present the isolation and structure elucidation of these metabolites.

Results and Discussion. – The $CHCl_3$ soluble portion of *Callyspongia* sp. was subjected to a combination of reversed-phase flash column chromatography and RP-HPLC separation to give three compounds. The identification of **1**–**3** as dipeptides was straightforward from the MS analysis and the presence of characteristic ^{13}C-NMR chemical shifts for the amide CO groups (δ(C) 167.6–172.9) and the typical ^{1}H-NMR signals of amino acids. The presence of a proline moiety as one of the components of

1–**3** was deduced by the presence of CH_2 *multiplet*s in the spectra (δ(H) 1.91–3.55, 6 H) and by H,C correlations observed in the HMBC experiments, in particular from CH_2(3) to C(1) and C(6)[1]). The NMR spectra clearly indicated that leucine, valine, and isoleucine were the other amino acid residues in compounds **1**–**3**, respectively.

Compound **3** was obtained as colorless crystals. The ESI-MS (negative-ion mode) data showed a *pseudo*-molecular-ion peak at *m*/*z* 209 $[M-H]^-$. ^{1}H- and ^{13}C-NMR spectra of **3** were in agreement with previously reported data, which allowed to propose the diketopiperazine structure cyclo-(Pro-Leu) for compound **3** [32]. The absolute configuration was determined by the optical rotation, $[\alpha]_D^{25}$, value of **3** (−78.3), well agreeing with reported data ($[\alpha]_D = -91$) [32]. Compound **3** was thus assigned the structure cyclo-((*S*)-Pro-(*R*)-Leu)[1]) (*Fig. 1*). Compound **3** had been previously isolated from the marine sponge *Calyx cf. podatypa* [32].

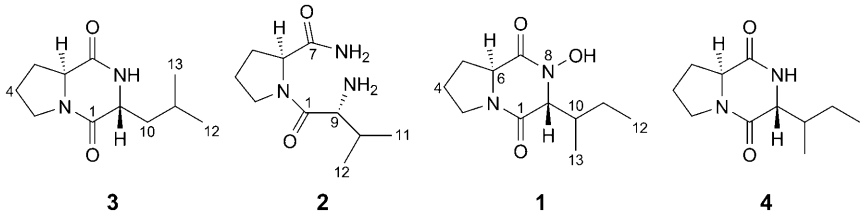

Fig. 1. *Structures of* **1**–**4**

Compound **2** was isolated as colorless crystals. The molecular formula $C_{10}H_{19}N_3O_2$ was established by ESI-MS. Initial analysis of NMR data indicated the presence of two CO groups (δ(C) 167.6, C(7) and 172.6, C(1)[1])), an N-bearing CH_2 group (δ(H) 3.50–3.54 and 3.55–3.59, CH_2(3); δ(C) 46.2), two N-bearing CH groups (δ(H) 4.05, H–C(6), and 4.23, H–C(9); δ(C) 61.6 and 60.1), and two Me groups (δ(H) 0.95, *d*, *J* = 6.9, Me(12), and 1.11, *d*, *J* = 7.3, Me(11)) (*Table*). Interpretation of COSY data allowed to establish the connectivity of C(9)–C(12). Both Me(11) and Me(12) displayed HMBC correlations to the quaternary CO C-atom resonating at δ(C) 172.6 (C(1)). Moreover, the signals at δ(H) 2.50 (*dq*, H–C(10)) and 4.23 (*t*, H–C(9)) showed the presence of a Val moiety in this compound. The absolute configuration was determined from the $[\alpha]_D^{25}$ value of **2** (−91.7), well agreeing with the reported data, $[\alpha]_D = -74$ [32]. Thus, compound **2** was assigned the structure seco-((*S*)-Pro-(*R*)-Val)[1]). Biogenetic considerations also support the absolute configuration of **2** to be identical to that of **3**. Compound **2** has already been obtained as an intermediate in the synthesis of peptides [33][34].

Compound **1** was obtained as colorless crystals. The molecular formula of **1** was established as $C_{11}H_{18}N_2O_3$ on the basis of HR-ESI-MS, which was sixteen mass units higher than cyclo-(Pro-Ile) [32][35][36]. This difference suggested the presence of one OH group. The OH group was also supported by the absorption band at 3207 cm^{-1} in the IR spectrum. The ^{1}H-NMR spectrum showed a secondary Me *doublet* at δ(H) 0.95 (J = 6.9, Me(13)), and a terminal Me *triplet* at δ(H) 1.07 (J = 6.9, Me(12)), indicating the presence of an anteiso branched chain. In addition, the signals at δ(H) 4.22 (*t*, *J* =

[1]) Arbitrary atom numbering. For systematic names, see *Exper. Part.*

Table. *^{1}H- and ^{13}C-NMR Data of Compounds* **1**–**3**[1]). At 500/125 MHz, resp., in CD_3OD; δ in ppm, J in Hz.

	3		**2**		**1**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
C(1)		172.9		172.6		172.6
CH_2(3)	3.54–3.51 (*m*)	46.5	3.59–3.55 (*m*), 3.54–3.50 (*m*)	46.2	3.60–3.53 (*m*), 3.52–3.49 (*m*)	46.2
CH_2(4)	1.94–1.87 (*m*), 2.06–1.99 (*m*)	23.6	2.05–2.01 (*m*), 1.98–1.94 (*m*)	23.3	2.06–2.02 (*m*), 1.98–1.91 (*m*)	23.3
CH_2(5)	2.35–2.31 (*m*), 1.96–1.87 (*m*)	29.1	2.35–2.33 (*m*), 1.98–1.94 (*m*)	29.6	2.35–2.32 (*m*), 2.00–1.92 (*m*)	29.6
H–C(6)	4.15 (br. *s*)	60.3	4.05 (br. *s*)	61.6	4.10 (br. *s*)	61.3
C(7)		168.9		167.6		167.6
H–C(9)	4.29 (*t*, *J* = 7.1)	54.7	4.23 (*t*, *J* = 7.0)	60.1	4.22 (*t*, *J* = 7.0)	60.0
CH_2(10) or H–C(10)	1.99–1.94 (*m*), 1.57–1.53 (*m*)	39.4	2.50 (*dq*, *J* = 7.0, 2.5)	30.0	2.20–2.16 (*m*)	25.5
H–C(11), Me(11), or CH_2(11)	2.06–2.01 (*m*)	25.7	1.11 (*d*, *J* = 7.3)	18.9	1.49–1.43 (*m*), 1.37–1.31 (*m*)	37.1
Me(12)	1.01 (*d*, *J* = 6.5)	23.4	0.95 (*d*, *J* = 6.9)	16.7	1.07 (*t*, *J* = 6.9)	15.6
Me(13)	0.97 (*d*, *J* = 6.5)	22.2			0.95 (*d*, *J* = 6.9)	12.6

7.0, H–C(9)), 2.20–2.16 (*m*, H–C(10)), and 1.49–1.43 (*m*, 1 H of CH_2(11)), 1.37–1.31 (*m*, 1 H of CH_2(11)) allowed to propose an Ile moiety for this compound. This conclusion was further supported by 2D-NMR data. H–C(10) (δ(H) 2.20–2.16) showed long-range coupling to C(1) (δ(C) 172.6) and C(9) (δ(C) 60.0). Further key HMBC correlations are shown in *Fig. 2*. The assignments of the C- and H-atoms were supported by COSY, HMQC, and HMBC experiments.

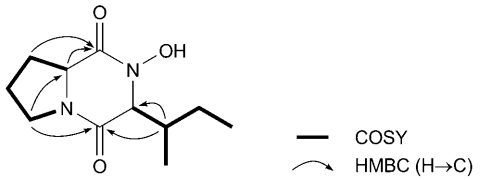

Fig. 2. *COSY* (—) *and HMBC* (H → C) *data of compound* **1**

The dry sample of **1** has been stored at $-20°$ for three months. On standing, **1** decomposed to give several degradation products. One of the major degradation products (**4**) was identified as cyclo-((*S*)-Pro-(*R*)-Ile). The absolute configuration was determined from the $[\alpha]_D^{25}$ value of **4** (−97.8), well agreeing with reported data ($[\alpha]_D$ = −109) [32]. Thus, compound **4** was assigned as cyclo-((*S*)-Pro-(*R*)-Ile), and the absolute configuration of **1** was accordingly deduced.

Compounds **1**–**3** were tested for their cytotoxic activity against SGC-7901 (human stomach cancer), HepG2 (human liver cancer), and HeLa (human ovarian cancer) cell lines using the MTT method. Compounds **1**–**3** were not active ($IC_{50} > 100$ μg/ml) against the three human tumor cell lines.

Proline-containing dipeptides have been isolated from a variety of biological sources such as microorganisms, sponges, and a variety of tissues and body fluids, as well as processed food and beverages [37]. A few marine bacteria are known to produce diketopiperazines in culture. For example, five diketopiperazines have been isolated from a cyanobacterium isolated from the sponge *Calyx cf. podatypa* [32], while other diketopiperazines were reported in the marine bacteria *Pseudomonas aeruginosa* [35], *Halobacillus litoralis Ys3106* [38], and *Streptomyces* sp. [39][40], and in the bacterial strains CF-20 and C-148 [36]. In this context, whether compounds **1**–**3** are produced by the sponge or by symbiotic bacteria remains to be established. The wide range of biological properties reported for diketopiperazines points to various therapeutic possibilities. Some of the most important biological activities of diketopiperazines are related to the inhibition of plasminogen activator inhibitor-1 (PAI-1) and alteration of cardiovascular and blood-clotting functions. They also have activities as antitumour, antiviral, antifungal, antibacterial, and antihyperglycaemic agents and affinities for calcium channels and opioid, GABAergic, serotoninergic 5-HT1A, and oxytocin receptors [41]. Cyclo-(4-hydroxy-Pro-Phe) showed cytotoxicity against tsFT210 cell line [42].

Experimental Part

General. Column chromatography (CC): *YMC Gel* (*ODS-A*, 12 nm, S-50 μm). TLC: silica gel *GF254* (*Qingdao Marine Chemical Factory*, Qingdao, P. R. China). Analytical HPLC: *Hitachi L-2400* HPLC system; *YMC ODS-H80* column (250 × 4.6 mm i.d., 4 μm) coupled to an *Alltech ELSD 800* detector; semi-prep. HPLC was performed on a *Hitachi L-2400* HPLC system, using a *YMC ODS-H80* column (250 × 10 mm i.d., 4 μm) coupled to an *Alltech ELSD 800* detector with flow-splitter valve (*Parker, NS*) set at a split ratio of 20 : 1 (collector : detector). Spots were detected on TLC under UV light or by heating after spraying with 5% H_2SO_4 in EtOH (*v*/*v*). M.p.: *X-6* micro-melting point apparatus (uncorrected). Optical rotations: *Jasco DIP-370* digital polarimeter. IR Spectra: *Jasco FT/IR-410* spectrometer. NMR Spectra: *Bruker AC 500 NMR* spectrometer, with Me_4Si as an internal standard. ESI-MS Spectra: *Agilent 1200 LC-MS* spectrometer. HR-ESI-MS Spectra: *Bruker Daltonics APEX II 47e* spectrometer.

Animal Material. The sponge was collected by hand in July 2005, off the coast of Hainan Island, P. R. China. The specimen was identified by Dr. *Kyung Jin Lee*, Invertebrate Research Division, National Institute of Biological Resources, Environmental Research Complex, Incheon, Korea. A voucher specimen (0507002) was deposited with the Key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Extraction and Isolation. The sponge (10 kg) was extracted three times with 95% EtOH (50 l) at r.t. for 3 d. The extract was concentrated under reduced pressure, and partitioned between H_2O (4 l) and $CHCl_3$ (4 l); the $CHCl_3$ layer (405 g) was further partitioned between 85% EtOH (4 l) and petroleum ether (PE; 4 l) to yield 85% EtOH (98 g) and PE (270 g) fractions. The 85% EtOH fraction was subjected to semi-prep. HPLC, eluting with a solvent system of 40 → 100% MeOH, to afford 25 fractions. *Fr. 1* (4.7 g) was further separated by reversed-phase flash CC, eluting with 10 → 50% MeOH, to afford 13 subfractions (*Frs. e1*–*e13*). *Fr. e5* was further purified with semi-prep. HPLC, eluting with MeOH/H_2O (15 : 85) at a flow rate of 2 ml min^{-1}, to afford compound **2** (t_R = 17.6 min, 8.1 mg). *Fr. 2* (2.2 g) was further separated by semi-prep. HPLC, eluting with 10 → 50% MeOH, to afford 12 subfractions (*Frs. g1*–*g12*). *Frs. g2*–*g4* were combined and further separated by semi-prep. HPLC, eluting with MeOH/H_2O (25 : 75) at a flow rate of 2 ml min^{-1}, to provide compounds **1** (t_R = 18.5 min, 62 mg) and **3** (t_R = 20.5 min, 56 mg).

*Cyclo-((*S*)-Pro-(*R*)-Leu)* (= *(3*R*,8a*S*)-Hexahydro-3-(2-methylpropyl)pyrrolo[1,2-*a*]pyrazine-1,4-dione*; **3**). Colorless crystals. $[\alpha]_D^{25} = -78.3$ (*c* = 0.03, MeOH). ^{1}H- and ^{13}C-NMR data: see *Table.* ESI-MS (neg.): 209 ($[M-H]^-$).

*Seco-((*S*)-Pro-(*R*)-Val)* (=D-*Valylprolinamide*; **2**). Colorless crystals. $[\alpha]_D^{25} = -91.7$ ($c = 0.04$, MeOH). ^{1}H- and ^{13}C-NMR data: see *Table*. ESI-MS (neg.): 212 ($[M-H]^-$).

Callyspongidipeptide A (= *(3*R*,8a*S*)-3-(Butan-2-yl)-hexahydro-2-hydroxypyrrolo[1,2-*a*]pyrazine-1,4-dione*; **1**). Colorless crystals (MeOH). M.p. 103.4–106.0°. R_f (SiO_2; $CHCl_3$/MeOH 9:1): 0.72. $[\alpha]_D^{25} = -17.8$ ($c = 0.03$, MeOH). IR (film): 3207, 2965, 1661, 1434, 829. ^{1}H- and ^{13}C-NMR data: *Table*. ESI-MS (neg.): 225 ($[M-H]^-$). HR-ESI-MS: 227.1404 ($[M+H]^+$, $C_{11}H_{19}N_2O_3^+$; calc. 227.1396).

*Cyclo-((*S*)-Pro-(*R*)-Ile)* (= *(3*R*,8a*S*)-3-(Butan-2-yl)hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione*; **4**). Colorless crystals (MeOH). $[\alpha]_D^{25} = -97.8$ ($c = 0.01$, MeOH). ^{1}H- and ^{13}C-NMR data are in agreement with literature values [32]. ESI-MS (neg.): 209 ($[M-H]^-$).

Measurement of Cytotoxicity. The cytotoxicity of compounds **1**–**3** was tested on the HeLa (human ovarian cancer), SGC-7901 (human stomach cancer), and HepG2 (human liver cancer) cell lines using the MTT method. Cell suspensions (180 µl) were seeded in 96-well plates at densities of 1.0×10^5 cells per well with test compound (20 µl) added from DMSO stock soln. After 3 d of culture, attached cells were incubated with MTT (10 µl, 4 h) and subsequently solubilized in 10% SDS/*N*,*N*-dimethylformamide (DMF) soln. (100 µl, 10 h). The absorbance was measured at 570 nm using a microplate reader.

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