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ABSTRACT

Two new cyclic heptapeptides, carteritins A and B (**1** and **2**), were isolated from the marine sponge *Stylissa carteri* along with three known cyclic heptapeptides, phakellistatin 13 (**3**) and hymenamides C and D (**4** and **5**). Their structures were elucidated based on data obtained using 2D NMR, HRESIMS, and ESIMS/MS, in addition to Marfey's analysis. Carteritin A (**1**) showed cytotoxicities against HeLa, HCT116, and RAW264 cells with IC_{50} values of 0.70–1.5 μM.

Marine organisms are rich sources of biologically active metabolites. Sponges belonging to the orders Axinellida and Halichondrida have been identified as sources of bioactive proline-rich cyclic peptides such as phakellistatins from the sponge genus *Phakellia*,[1–3] axinellins from the genus *Axinella*,[4] stylisins and stylissamides from the genus *Stylissa*,[5,6] and hymenamides from the genus *Hymeniacidon*.[7] In our search for drug leads, we have screened the extracts of marine invertebrates, including sponges and tunicates, collected in Indonesia. The EtOAc-soluble fraction of the marine sponge *Stylissa carteri* showed cytotoxicity in HeLa cells (0% survival at 10 μg/mL), and we purified the cytotoxic metabolites from this sponge. We herein describe the isolation and structural elucidation of two new proline-rich cyclic heptapeptides, carteritins A and B (**1** and **2**) (Fig. 1), along with three known cyclic heptapeptides, phakellistatin 13 (**3**) and hymenamides C and D (**4** and **5**), from the marine sponge *S. carteri* along with the determination of their cytotoxic activities.

The marine sponge (230 g, wet weight)[8] was collected by scuba at a depth of 10 m in Bangka Island, North Sulawesi, Indonesia, in September 2007, and immediately soaked in EtOH. The EtOH extract was suspended in H_2O and extracted with EtOAc. The EtOAc-soluble fraction was partitioned between hexane and 90% MeOH–H_2O. The 90% MeOH–H_2O soluble fraction (0.60 g) was subjected to SiO_2 column chromatography with a stepwise gradient elution using CH_2Cl_2/MeOH to afford three fractions: Fr. A (CH_2Cl_2/MeOH (10:1)), Fr. B (CH_2Cl_2/MeOH (85:15)), and Fr. C (CH_2Cl_2/MeOH (70:30)). Fr. A (34.7 mg) was further subjected to ODS column chromatography and eluted with MeOH/H_2O (70:30) to afford **3** (10.9 mg). Fr. B (40.0 mg) was purified by gel filtration HPLC (CH_2Cl_2/MeOH/H_2O (6:4:1)) followed by ODS HPLC (MeOH/H_2O (55:45)) to afford **2**[9] (1.1 mg). Fr. C (210 mg) was subjected to diol column chromatography (CH_2Cl_2/MeOH (9:1)) followed by gel filtration HPLC (CH_2Cl_2/MeOH/H_2O (6:4:1)) to yield **1**[9] (11 mg) and **5** (1.9 mg). Fr. C (40 mg) was subjected to gel filtration HPLC (CH_2Cl_2/MeOH/H_2O (6:4:1)) to yield **4** (6.5 mg).

The molecular formula of **1** was defined as $C_{44}H_{57}N_7O_{10}$, based on an analysis of HRFABMS. The ^1H and ^{13}C NMR spectra in DMSO-d_6 revealed the peptidic nature of **1** (Table 1). ^{13}C NMR data showed eight carbonyl and seven α-methine carbons, and the ^1H NMR spectrum displayed four doublet amide protons (δ 6.91, 7.50, 7.85, and 8.03), a hydroxyl proton (δ 12.05 (s)), and mono- and *p*-substituted phenyl ring systems. These data suggested that **1** was a heptapeptide containing a phenylalanine and tyrosine residues in addition to an amino acid residue containing a carbonyl carbon in its side chain. A detailed analysis of the COSY, TOCSY, HSQC, and HMBC spectra resulted in the identification of seven amino acid residues as Pro (3×), Phe, Ile, Glu, and Tyr. Two

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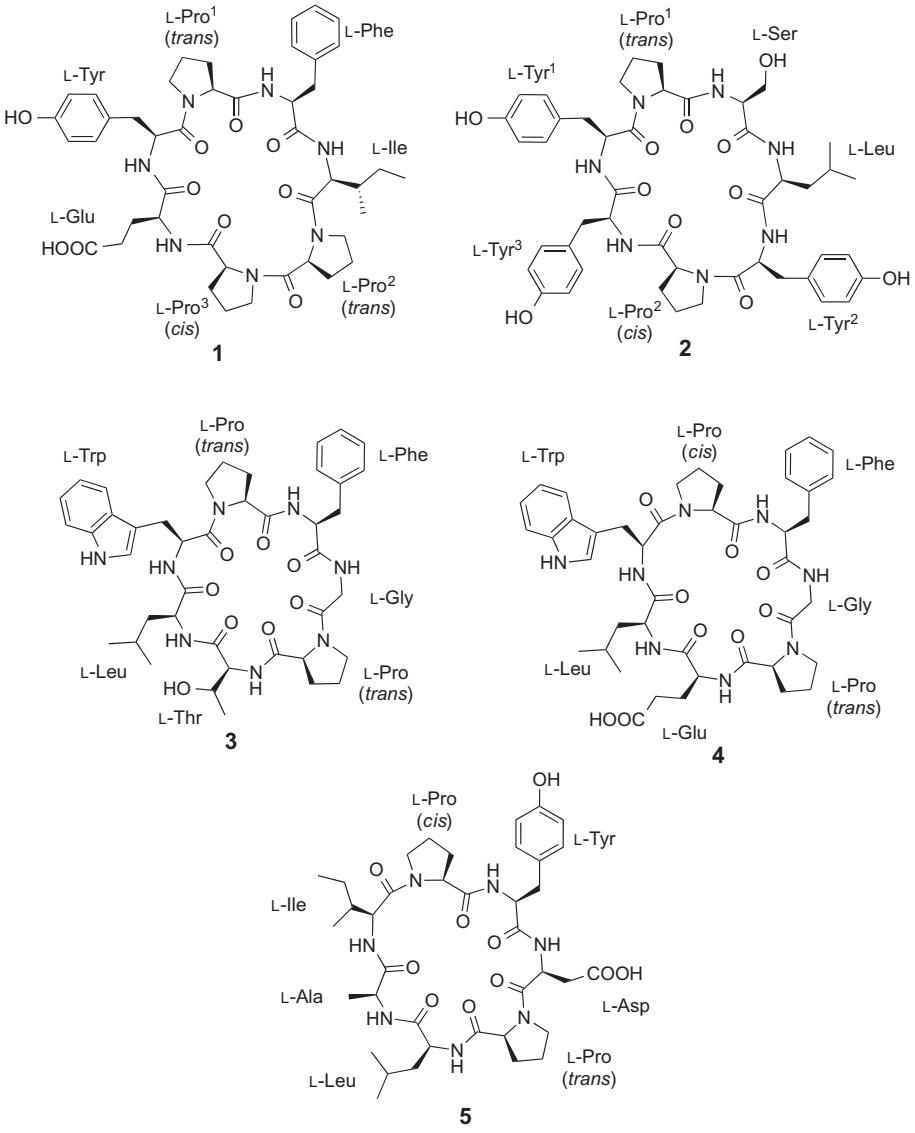

Figure 1. Structures of **1**–**5**.

fragments, Pro[1]-Phe-Ile and Pro[2]-Pro[3]-Glu-Tyr, were revealed by the HMBC correlations of Phe-NH/Pro[1]-CO, Ile-NH/Phe-CO, Pro[3]-Hα/Pro[2]-CO, Glu-NH/Pro[3]-CO, and Tyr-NH/Glu-CO (Fig. 2). The NOESY correlations between Ile-Hα/Pro[2]-Hδ and Tyr-Hα/Pro[1]-Hδ showed the sequence of **1** as *cyclo*-(Pro[1]-Phe-Ile-Pro[2]-Pro[3]-Glu-Tyr) (Fig. 2). The $\Delta\delta_{C\beta-C\gamma}$ values of three Pro residues (3.5, 3.9, and 10.0 ppm for Pro[1], Pro[2], and Pro[3], respectively) suggested the geometries of the proline amide bonds as *trans*, *trans*, and *cis*, respectively,[10] which was supported by the NOE correlations, Tyr-Hα/Pro[1]-Hδ and Ile-Hα/Pro[2]-Hδ.

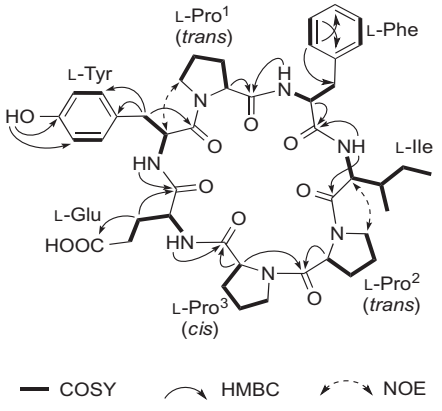

Figure 2. COSY, key HMBC, and key NOE correlations for **1**.

The molecular formula of **2** was determined as $C_{46}H_{57}N_7O_{11}$ on the basis of HRESIMS. ^{1}H and ^{13}C NMR spectra (Table 2) showed the presence of five amide protons and seven carbonyl and seven α-methine carbons, which indicated that **2** was also a heptapeptide. Seven amino acid residues were identified as Tyr (3×), Pro (2×), Ser, and Leu by a comprehensive inspection of 2D NMR spectra including COSY, HSQC, and HMBC. The HMBC correlations of Tyr[3]-NH/Pro[2]-CO, Tyr[1]-NH/Tyr[3]-CO, Ser-NH/Pro[1]-CO, Leu-NH/Ser-CO, and Tyr[2]-NH/Leu-CO revealed the presence of two fragments, Pro[1]-Ser-Leu-Tyr[2] and Pro[2]-Tyr[3]-Tyr[1] (Fig. 3). The NOESY correlations of Tyr[2]-Hα/Pro[2]-Hα and Tyr[1]-Hα/Pro[1]-Hδ established the sequence of **2** as *cyclo*-(Tyr[1]-Pro[1]-Ser-Leu-Tyr[2]-Pro[2]-Tyr[3]) (Fig. 3). The $\Delta\delta_{C\beta-C\gamma}$ values of the Pro residues (3.6 and 9.9 ppm for Pro[1] and Pro[2], respectively) and NOESY correlations of Tyr[1]-Hα/Pro[1]-Hδ and Tyr[2]-Hα/Pro[2]-Hα suggested the geometries of the proline amide bonds as *trans* and *cis*, respectively.

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for **1** in DMSO-d_6

Amino acid	Carbon	δ_C, mult	δ_H (J in Hz)	Amino acid	Carbon	δ_C, mult	δ_H (J in Hz)
Proline-1	CO	171.6, C		Proline-3	CO	171.8, C	
	α	62.7, CH	3.94, t (8.0)		α	60.3, CH	4.30, d (8.6)
	β	28.4, CH_2	1.96, m		β	31.8, CH_2	2.15, m
			1.47, m				1.99, m
	γ	24.9, CH_2	2.03, m		γ	21.8, CH_2	1.78, m
			1.89, m				1.62, m
	δ	46.8, CH_2	3.78, m		δ	46.7, CH_2	3.68, m
Phenylalanine	CO	171.0, C					3.34, m
	α	53.9, CH	4.49, td (10.0, 3.0)	Glutamic acid	CO	170.1, C	
	β	36.1, CH_2	2.94, t (13.2)		α	55.4, CH	3.86, m
			2.05, m		β	26.9, CH_2	1.81, m
	γ	138.5, C					1.61, m
	δ/δ′	128.6, CH	7.23, d (7.2)		γ	30.4, CH_2	2.10, m
	ε/ε′	128.1, CH	7.28, t (7.3)				2.00, m
	θ	126.2, CH	7.19, t (7.0)		δ	173.8, C	
	NH		7.85, d (8.3)		NH		8.03, d (6.7)
Isoleucine	CO	167.8, C		Tyrosine	CO	171.4, C	
	α	55.7, CH	4.26, t (9.2)		α	51.5, CH	4.86, td (10.5, 2.0)
	β	36.1, CH	2.04, m		β	37.0, CH_2	3.27, m
	γ	23.7, CH_2	1.48, m				2.47, t (12.5)
			1.02, m		γ	127.1, C	
	δ	11.8, CH_3	0.77, t (7.4)		δ/δ′	130.1, CH	7.09, d (8.3)
	β-Me	16.8, CH_3	0.90, d (6.3)		ε/ε′	114.9, CH	6.67, d (8.3)
	NH		6.91, d (8.0)		θ	155.9, C	
Proline-2	CO	170.3, C			OH		12.05, s
	α	58.9, CH	4.37, q (4.4)		NH		7.50, d (9.3)
	β	28.1, CH_2	2.13, m				
			1.74, m				
	γ	24.2, CH_2	1.89, m				
			1.83, m				
	δ	46.9, CH_2	3.48, m				
			3.26, m				

Table 2
^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data for **2** in DMSO-d_6

Amino acid	Carbon	δ_C, mult	δ_H (J in Hz)	Amino acid	Carbon	δ_C, mult	δ_H (J in Hz)
Tyrosine-1	CO	171.7, C		Tyrosine-2	CO	170.2, C	
	α	51.5, CH	4.91, m		α	53.7, CH	4.16, m
	β	37.2, CH_2	3.24, m		β	35.4, CH_2	2.87, dd (14.4, 7.2)
			2.57, m				2.74, dd (14.4, 7.8)
	γ	126.8, C			γ	126.3, C	
	δ/δ′	130.6, CH	7.08, d (8.4)		δ/δ′	129.8, CH	6.96, d (8.4)
	ε/ε′	115.1, CH	6.62, d (8.4)		ε/ε′	115.4, CH	6.69, d (8.4)
	θ	155.9, C			θ	156.0, C	
	OH		9.14, s		OH		9.24, s
	NH		7.79, d (8.5)		NH		9.14
Proline-1	CO	171.1, C		Proline-2	CO	170.1, C	
	α	62.2, CH	4.19, m		α	60.0, CH	3.49, d (8.4)
	β	28.7, CH_2	2.25, m		β	30.4, CH_2	1.52, m
			1.85, m				1.03, m
	γ	25.1, CH_2	2.06, m		γ	20.5, CH_2	1.26, m
			1.95, m				0.43, m
	δ	47.1, CH_2	3.82, m		δ	46.0, CH_2	3.06, m
			3.78, m				2.81, m
Serine	CO	169.0, C		Tyrosine-3	CO	169.9, C	
	α	55.1, CH	4.22, m		α	57.0, CH	3.97, m
	β	61.0, CH_2	3.78, m		β	37.2, CH_2	2.56, m
			3.67, m				2.51, m
	OH		4.92		γ	127.5, C	
	NH		7.17, d (8.1)		δ/δ′	129.3, CH	6.81, d (8.4)
Leucine	CO	170.8, C			ε/ε′	114.8, CH	6.61, d (8.4)
	α	51.5, CH	4.42, m		θ	156.1, C	
	β	40.2, CH_2	1.34, m		OH		9.21, s
			1.74, m		NH		7.57, d (7.9)
	γ	24.6, CH	1.56, m				
	δ	23.5, CH_3	0.90, d (7.4)				
	δ′	22.0, CH_3	0.94, d (6.3)				

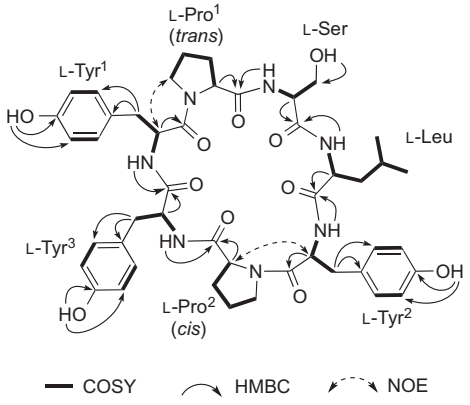

Figure 3. COSY, key HMBC, and key NOE correlations for **2**.

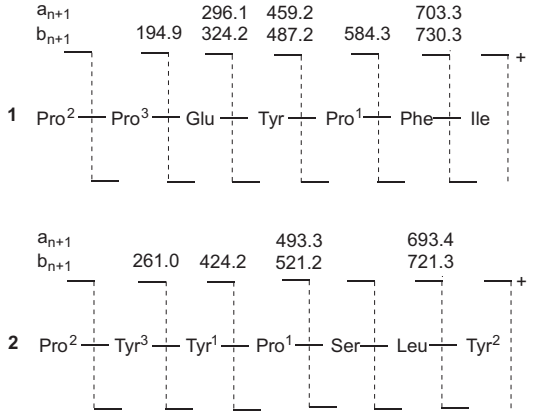

Figure 4. ESIMS/MS sequence ions (m/z) for protonated molecules $[M+H]^+$ of **1** and **2**.

Table 3
Cytotoxic activities (IC_{50}, μM) of **1**–**5**

Compd	HeLa	HCT116	RAW264
1	0.70	1.3	1.5
2	>50	>50	>50
3	15	>50	>50
4	25	5.0	>50
5	>50	>50	>50

The sequences of **1** and **2** were confirmed by an ESIMS/MS fragmentation analysis (Fig. 4). The absolute configurations of all amino acid residues in **1** and **2** were determined as L by Marfey's method[11] after acidic hydrolysis.[12]

The isolated heptapeptides **1**–**5** were evaluated for cytotoxicities against a human cervical cancer cell line (HeLa), human colon cancer cell line (HCT116), and murine macrophage line (RAW264) (Table 3).[13] Carteritin A (**1**) showed strong cytotoxicities against the three cell lines with IC_{50} values of 0.70, 1.3, and 1.5 μM, respectively, whereas carteritin B (**2**) and hymenamide D (**5**) were not cytotoxic against these cell lines, even at 50 μM. Phakellistatin 13 (**3**) exhibited moderate cytotoxicity against HeLa cells with an IC_{50} value of 15 μM, while hymenamide C (**4**) was cytotoxic against HeLa and HCT 116 cells with IC_{50} values of 25 and 5.0 μM, respectively. Although **1**–**5** were similar proline-rich heptapeptides, marked differences were observed in their cytotoxicities.

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Supplementary data

Supplementary data (experimental details and NMR spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.02.031.

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8. The marine sponge, *Stylissa carteri*, was collected by scuba at a depth of 10 m in Bangka Island, North Sulawesi, Indonesia, in September 2007 and immediately soaked in EtOH. A voucher specimen (RMNH POR 10164) of the sponge has been deposited in the Naturalis Biodiversity Center, the Netherlands.
9. **1**: A yellow amorphous solid; $[\alpha]_D^{21}$ −125 (*c* 1.10, MeOH); UV (MeOH) λ_{max} (log ε) 278 (3.3), 230 (sh.) (3.99) nm; IR (film) ν_{max} 3315, 2965, 2930, 2878, 1720, 1625, 1517, and 1443 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRFABMS m/z 844.4326 $[M+H]^+$ (calcd for $C_{44}H_{58}N_7O_{10}$, 844.4245). **2**: A colorless amorphous solid; $[\alpha]_D^{21}$ −82 (*c* 0.60, MeOH); UV (MeOH) λ_{max} (log ε) 278 (3.55), 226 (4.27) nm; IR (film) ν_{max} 3299, 2961, 2926, 1630, 1516, 1449, 1346, 1238, and 1102 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS m/z 906.3968 $[M+Na]^+$ (calcd for $C_{46}H_{57}N_7O_{11}Na$, 906.4012).
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12. Each compound (0.5 mg of **1** and 0.1 mg of **2**) was hydrolyzed by 6 M HCl at 108 °C for 24 h. Each residue was dissolved in 100 μL of 6% TEA in H_2O and reacted with 200 μL of 1% L-FDAA in acetone at 50 °C for 1 h. The reaction mixture was then neutralized by the addition of 30 μL of 2 M HCl and dried under a vacuum. The residue was dissolved in 500 μL DMSO and subjected to an analysis by reverse phase HPLC: column, Cosmosil C_{18} MS (4.6 × 250 mm); solvent system, gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (50:50:0.1) in 50 min for analyses of Pro, Ile, Leu, Phe, Tyr, and Glu and from H_2O/TFA (100:0.1) to MeOH/H_2O/TFA (50:50:0.1) in 60 min for an analysis of Ser; flow rate, 1 mL/min; detector, UV (340 nm). The retention times of the standard amino acids were as follows: D-Glu, 40.5 min; L-Glu, 40.0 min; D-Pro, 43.2 min; L-Pro, 42.2 min; D-Tyr, 45.6 min; L-Tyr, 44.8 min; D-Ile, 55.0 min; L-Ile, 51.2 min; D-Phe, 54.2 min; L-Phe, 51.8 min; D-Leu, 55.3 min; L-Leu, 51.9 min; D-Ser, 57.6 min; L-Ser, 56.0 min.
13. The cytotoxicity test was performed using HeLa, HCT116, and RAW264 cell lines. Cells were grown in MEM (SIGMA), DMEM/F12 (1:1) (GIBCO), and MEMα with L-glutamine and phenol red (Wako) medium, respectively, supplemented with 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) under a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were seeded on 96-well microplates (3×10^3 cells/well) and pre-cultured for one day. Medium was replaced with that containing test compounds at various concentrations, and the cells were further cultured at 37 °C for three days. Medium was then replaced with 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (200 μg/mL in medium) and cells were incubated under the same conditions for 3 h. After the addition of 200 μL of DMSO, optical density at 570 nm was measured with a microplate reader.