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Stylissamide X, a new proline-rich cyclic octapeptide as an inhibitor of cell migration, from an Indonesian marine sponge of *Stylissa* sp.

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ABSTRACT

A new proline-rich cyclic octapeptide named stylissamide X (**1**) was isolated from an Indonesian marine sponge of *Stylissa* sp. as an inhibitor of cell migration from the guidance of wound-healing assay. The chemical structure of stylissamide X (**1**) was determined on the basis of spectroscopic analysis, and stereostructure of the amino acids were deduced by Marfey's method. Compound **1** showed inhibitory activity against migration of HeLa cells in the ranges of 0.1–10 μM concentration through both wound-healing assay and chemotaxicell chamber assay, while the cell viability was maintained more than 75% up to 10 μM concentration of **1**.

Cancer metastasis is responsible for over 90% of cancer deaths[1] and consists of the multi-step process involving epithelial–mesenchymal transition (EMT), degradation of matrix, cell migration, and cell adhesion.[2] Then, cell migration is attracting much attention as one of the alternative strategies for development of anti-cancer chemotherapies. So far, several natural products such as withaferin A,[3] prodigiosin,[4] and migrastatin[5] have been reported to inhibit cell migration.

In the course of our study of pharmaceutical valuable substances from marine organisms, we started a search for anti-cell migration substances and isolated a new proline-rich cyclic octapeptide named stylissamide X[6] (**1**) from an Indonesian marine sponge of *Stylissa* sp. on the basis of bioassay-guided separation. In this paper, the structure elucidation of stylissamide X (**1**) and its anti-cell migration activity are presented.

The dried marine sponge of *Stylissa* sp. 05A05 (500 g), which was collected in 2005 at Biak, Indonesia, was extracted with MeOH to obtain a MeOH extract. The MeOH extract (125 g), which showed anti-cell migration activity through wound-healing assay,[7] was partitioned into a water–EtOAc mixture (1:1). The EtOAc soluble portion was further partitioned with hexane–90% aq MeOH mixture (1:1) to furnish a 90% MeOH extract (62 g) and hexane extract (36 g). On the guidance of bioassay, the 90% MeOH extract [62 g, minimum inhibitory concentration (MIC) of anti-cell migration activity = 10 μg/mL] was further fractionated by SiO_2 gel column chromatography [$CHCl_3$/MeOH/H_2O = 65:3:1 (lower phase)] to give 10 fractions (Fr. M1–Fr. M10). The active Fr. M5 (3.6 g, MIC = 1.0 μg/mL) was then separated by ODS column chromatography (MeOH–H_2O) to obtain five fractions (Fr. M51–Fr. M55). The active Fr. M53 (180 mg, MIC = 0.1 μg/mL) was further

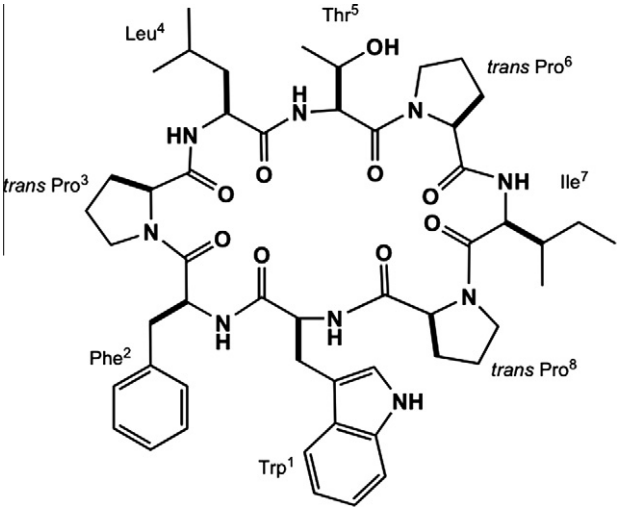

Figure 1. Chemical structure of stylissamide X (**1**).

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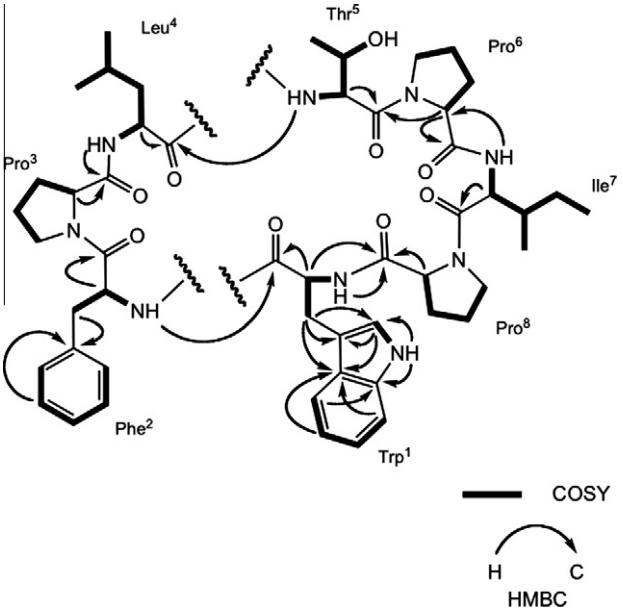

Figure 2. COSY and HMBC correlations for stylissamide X (**1**).

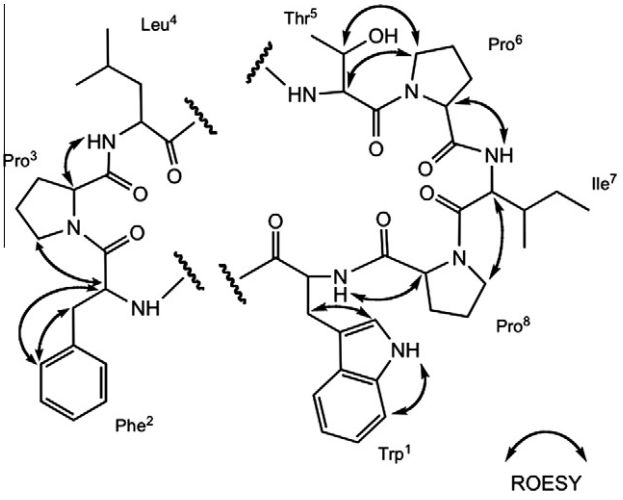

Figure 3. ROESY correlations for stylissamide X (**1**).

purified by ODS HPLC (Cosmosil MS-II, MeOH–H_2O = 8:2) to afford stylissamide X (**1**) (16 mg, 0.013% yield from the MeOH extract) (Fig. 1).

Stylissamide X (**1**) was obtained as a colorless solid. The ESI-TOF MS of **1** showed a pseudomolecular ion peak at *m*/*z* 974 $[M+Na]^+$. The molecular formula was determined as $C_{51}H_{69}N_9O_9$ by high-resolution (HR-) ESI-TOF MS. The IR absorptions at 3490, 3320, and 1670 cm^{-1} suggested the presence of hydroxyl group, amino group, and amide carbonyl group, respectively. The observation of the signals at 168–170 ppm in the ^{13}C NMR spectrum and the downfield signals (7.5–10.8 ppm) of the ^1H NMR spectrum indicated peptide structure of **1**. To confirm the amino acid composition of **1**, compound **1** was treated by dabsyl chloride following to acid hydrolysis.[8] HPLC analysis of the dabsylated amino acids revealed that compound **1** contained three mol of Pro, one mol each of Leu, Ile, Thr, Trp, and Phe in the structure. The existing of eight amino acids was also confirmed by NMR analysis including TOCSY, HMQC, and HMBC experiments (Fig. 2). The connectivity of these amino acids was figured out by HMBC and ROESY analysis. As shown in Figure 3, the ROESY correlations between Trp^1-NH (δ_H 8.24) and Pro^8-Hα (δ_H 3.90); Phe^2-Hα (δ_H 5.00) and Pro^3-Hδ_1 (δ_H 3.90), Pro^3-Hδ_2 (δ_H 3.60); Pro^3-Hα (δ_H 4.00) and Leu^4-NH (δ_H 8.70); Thr^5-Hα (δ_H 4.90) and Pro^6-Hδ_1 (δ_H 3.70); Thr^5-Hβ (δ_H 4.10) and Pro^6-Hδ_2 (δ_H 3.57); Pro^6-Hα (δ_H 4.40) and Ile^7-NH (δ_H 8.30); Ile^7-Hα (δ_H 4.20) and Pro^8-Hδ_1 (δ_H 3.96), Pro^8-Hδ_2 (δ_H 3.53) revealed the presence of the sequences of Phe^2-Pro^3-Leu^4 and Thr^5-Pro^6-Ile^7-Pro^8-Trp^1. In addition, the HMBC correlations (Fig. 2) between Phe^2-NH (δ_H 8.10) and Trp^1-CO (δ_C 170.1); Trp^1-Hα (δ_H 3.68) and Trp^1-CO; Thr^5-NH (δ_H 7.50) and Leu^4-CO (δ_C 170.1); Leu^4-Hα (δ_H 3.56) and Leu^4-CO proved that stylissamide X (**1**) has *cyclo*-(Trp-Phe-Pro-Leu-Thr-Pro-Ile-Pro) structure. Stereostructure of amino acids was confirmed by HPLC analysis of the derivatized amino acids by 5-fluoro-2,4-dinitrophenyl-L-alanine-amide (Marfey's reagent).[9] The result of HPLC analysis indicated that all the constituting amino acids in **1** were found to be L-amino acids. In addition, geometry of the peptidic linkages at the proline residues was figured out on the basis of the ^{13}C chemical shift difference between the proline β and γ carbons ($\Delta\delta_{\beta-\gamma}$). The small differences (Pro^3 $\Delta\delta_{\beta-\gamma}$ = 5.0, Pro^6 $\Delta\delta_{\beta-\gamma}$ = 4.4, Pro^8 $\Delta\delta_{\beta-\gamma}$ = 3.6) indicated that all the proline peptide bonds were *trans* geometry.[10,11,12c] The ROESY correlations between Phe^2-Hα and Pro^3-Hδ_1, Pro^3-Hδ_2; Ile^7-Hα and Pro^8-Hδ_1, Pro^8-Hδ_2; Thr^5-Hα and Pro^6-Hδ_1, Pro^6-Hδ_2 also supported the *trans* geometry of these proline peptidic linkages. Taken together, the gross structure of stylissamide X (**1**) was elucidated as shown in Figure 1. All the proton- and carbon-signals were assigned as shown

Table 1
^1H and ^{13}C NMR data for stylissamide X (**1**)

Residue	δ_c[a]	δ_H[b]	Residue	δ_c[a]	δ_H[b]
Trp^1			Leu^4		
α	57.0	3.68 (m)	α	52.9	3.56 (m)
β	23.4	3.64 (m)	β	36.1	1.67 (m), 2.15 (m)
		3.16 (dd, 12.5, 3.0)	γ	24.7	1.47 (m)
2	123.5	6.96 (s)	δ_1 CH_3	23.3	0.88 (d, 7.0)
3	111.2		δ_2 CH_3	18.5	0.85 (d, 6.5)
4	136.0		CO	170.1	
5	111.3	7.30 (d, 8.0)	NH		8.70 (d, 6.0)
6	120.8	7.00 (t, 7.5)			
7	118.1	6.92 (dd, 8.0, 7.0)	Thr^5		
8	117.9	7.40 (d, 7.5)	α	56.3	4.90 (dd, 9.0, 3.0)
9	127.0		β	67.6	4.10 (m)
CO	170.1		β OH		5.50 (d, 12.5)
NH (indole)		10.8 (d, 1.5)	γ CH_3	21.0	0.94 (d, 6.0)
NH		8.24 (d, 6.5)	CO	168.9	
			NH		7.50 (d, 9.5)

(*continued on next page*)

Table 1 (*continued*)

Residue	δ_C[a]	δ_H[b]	Residue	δ_C[a]	δ_H[b]
Phe[2]					
α	51.0	5.00 (m)	Pro[6]		
β	36.4	2.96 (dd, 14.5, 4.0)	α	59.2	4.40 (dd, 8.5, 4.0)
		2.86 (dd, 14.5, 9.0)	β	29.1	1.95 (m), 2.06 (m)
1	138.1		γ	24.7	1.74 (m)[c], 1.84 (m)[c]
2/6	129.2	7.25 (m)	δ_1	47.0	3.70 (m)
3/5	127.8	7.21 (m)	δ_2		3.57 (m)
4	125.9	7.10 (t, 7.5)	CO	170.6	
CO	169.8				
NH		8.10 (d, 9.5)	Ile[7]		
			α	53.9	4.20 (t, 9.5)
Pro[3]			β	35.0	1.98 (m)
α	60.6	4.00 (t, 7.5)	β CH_3	24.6	0.86 (d, 7.0)
β	29.7	1.74 (m)[c], 2.13 (m)	γ	14.4	1.03 (m), 1.30 (m)[c]
γ	24.7	1.84 (m)[c], 2.05 (m)	δ CH_3	9.7	0.65 (t, 7.5)
δ_1	47.8	3.90 (m)[c]	CO	170.4	
δ_2		3.60 (m)	NH		8.30 (d, 8.0)
CO	170.7				
			Pro[8]		
			α	60.9	3.90 (m)[c]
			β	28.4	1.94 (m), 2.00 (m)
			γ	24.8	1.30 (m)[c], 1.77 (m)
			δ_1	47.5	3.96 (m)
			δ_2		3.53 (m)
			CO	170.9	

[a] ^{13}C NMR: δ_C (ppm), (150 MHz, DMSO-d6).
[b] ^{1}H NMR: δ_H (ppm, *J* in Hz), (600 MHz, DMSO-d6).
[c] Overlapping resonances.

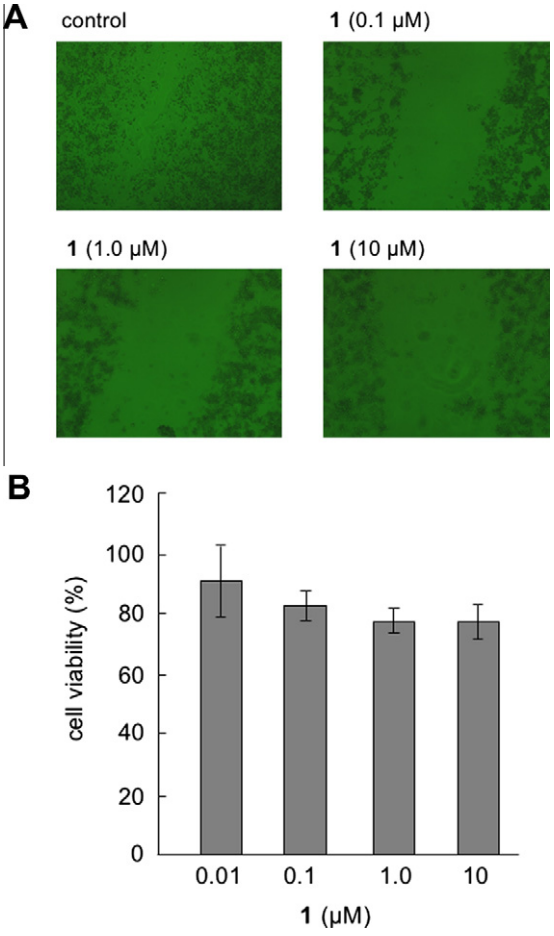

Figure 4. Anti-migration activity and anti-proliferative activity of stylissamide X (**1**). (A) Anti-migration activity of **1** by wound-healing assay, (B) anti-proliferative activity of **1** by MTT method.

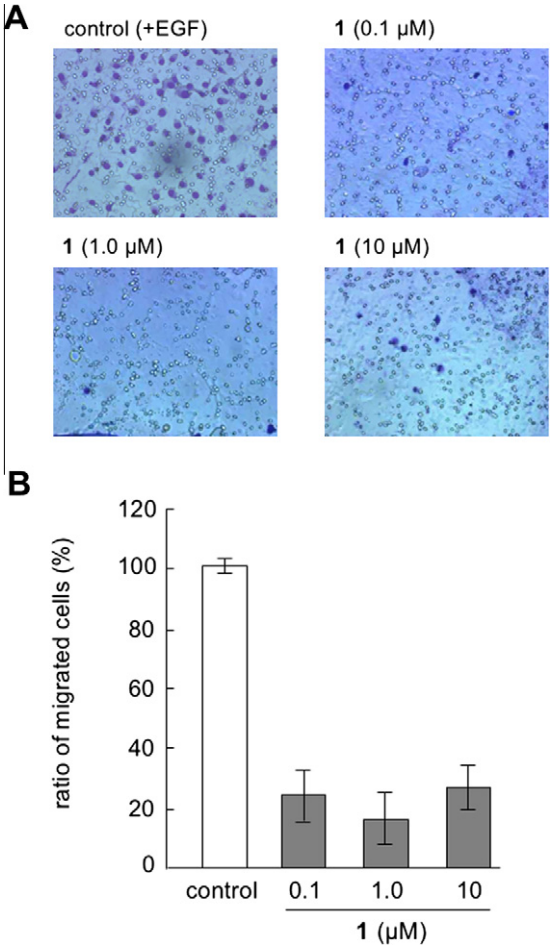

Figure 5. Effect of stylissamide X (**1**) on the migration of HeLa cells induced by EGF. HeLa cells (3×10^5 cells) were treated with the indicated concentrations of **1** and stimulated with EGF (50 ng/mL). After 24 h, the migrated cells to the reverse side of the membrane filter were counted in the six different microscopic fields. Representative figure of the migrated HeLa cells are shown (A). Data were shown by percentage based on the migrated cell number of the EGF-stimulated HeLa cells (B).

in Table 1. Stylissamide X (**1**) is structurally classified to a group of the proline-rich cyclic peptides, which are characterized to consist of seven or eight amino acids containing two or three proline residues and have been isolated from various genera of marine sponges such as *Axinella*,[12] *Stylotella*,[13] *Phakellia*,[14] *Hymeniacidon*,[15] and *Stylissa*.[16] So far, these cyclic peptides have been found to exhibit moderate cytotoxic activity and weak antimicrobial activity.

Stylissamide X (**1**) inhibited migration of HeLa cells in the concentration ranging from 0.1 μM to 10 μM through wound-healing assay, whereas cell viability of HeLa cells were maintained more than 75% up to 10 μM of **1** as shown in Figure 4. In addition, stylissamide X (**1**) also inhibited EGF-induced migration of HeLa cells[17] from 0.1 μM to 10 μM concentration of **1** through chemotaxicell chamber assay (Fig. 5). Further detailed evaluation of the anti-migration activity of tumor cells is under way.

Acknowledgements

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6. *Stylissamide X (**1**)*: Colorless solid. $[\alpha]_D^{20}$ −77 (*c* = 0.2, $CHCl_3$). IR ν_{max} (KBr) cm^{-1}: 3490, 3320, 1670. UV λ_{max} (MeOH) nm (ε): 209 (7700). ESI-MS: *m*/*z* 974 $[M+Na]^+$. High resolution ESI-MS: Calcd for $C_{51}H_{69}N_9O_9Na$: *m*/*z* 974.5116. Found 974.5107. ^1H NMR (600 MHz, DMSO-d6, δ_H), ^{13}C NMR (150 MHz, DMSO-d6, δ_C) spectra: as shown in Table 1.
7. (a) Liang, C. C.; Park, A. Y.; Guan, J. L. *Nat. Protoc.* **2007**, *2*, 329; (b) Wound-healing assay was performed according to the method described by Liang, et al. [Ref. [7a]]. Briefly, the suspension of HeLa cells in DMEM supplemented with 10% FBS were plated into each well of 96-well plates (1×10^5 cells/well/100 μL), and were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C to allow the forming of confluent monolayer. After 24 h, the monolayer was scratched with a pipette tip, washed with culture medium to remove floating cells, and photographed. Then, the cells were treated with testing samples for 24 h. The cells were photographed again to evaluate whether the cells treated with testing samples were able to migrate from the cutting edge of wound or not. The anti-migration activity of testing samples was determined as a minimum concentration of testing samples to inhibit the migration of cells completely.
8. Stylissamide X (**1**) (0.5 mg) were treated with 2 mL of 1.2 N HCl aq and heated at 110 °C for 12 h. The reaction mixture was dried under reduced pressure to obtain crude amino acids. The crude amino acids from stylissamide X (**1**) and authentic amino acids were dissolved in 40 μL of 50 mM sodium bicarbonate (pH 8.1) in glass tubes respectively. Then, 80 μL of freshly prepared 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) solution (4 mM in acetonitrile) was added to each sample. The samples were sealed with caps and Parafilm. The samples were then heated at 70 °C for 10 min. After dabsylation, the samples were analyzed by reversed-phase HPLC under the following condition; Cosmosil $5C_{18}$ AR, (4.6 mm i.d. × 250 mm), a 50 min linear gradient from acetonitrile–25 mM sodium acetate buffer (pH 6.5) = 3:7 to 1:1, 30 °C, 1 mL/min, and detection at 436 nm. Authentic dabsylated Thr, Pro, Ile, Leu, Trp, and Phe were eluted with retention times of 24.8, 30.8, 37.2, 38.4, 40.4, and 42.4 min, respectively.
9. The crude amino acids from stylissamide X (**1**) and L- and D-authentic amino acids were dissolved in 100 μL of 0.5 M sodium bicarbonate in 3-mL screw-cap glass tubes, respectively. Then, 100 μL of 5-fluoro-2,4-dinitrophenyl-L-alanine amide (L-FDAA, Marfey's reagent, 10 mg/mL in acetone) was added to each sample. The samples were sealed with caps and incubated at 70 °C for 60 min. After addition of 20 μL of 1 N HCl aq, the reaction mixture was diluted with methanol to suitable volumes (10–15 fold dilution). An aliquot of the L-FDAA derivatives was analyzed by HPLC under the following condition; Cosmosil πNAP, (4.6 mm i.d. × 250 mm), a 80 min linear gradient from acetonitrile–H_2O containing 0.1% TFA = 1:9 to 1:1, 1.5 mL/min, and detection at 340 nm. Authentic derivatized L-Thr, D-Thr, L-Pro, D-Pro, L-Ile, D-Ile, L-Leu, D-Leu, L-Phe, D-Phe, L-Trp, and D-Trp were eluted with retention times of 39.6, 43.2, 47.6, 51.2, 62.8, 68.8, 63.6, 68.8, 66.4, 71.6, 64.8, and 68.4 min, respectively.
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17. A polycarbonate filter of the inner chamber (Chemotaxicell chamber, 8 μm) was soaked in fibronectin solution (1.3 μg/mL) for 1 h at 37 °C and dried in vacuo. Then, HeLa cells (3.0×10^5 cells) were suspended in FBS free-DMEM containing the indicated concentration of **1** and seeded in the inner chamber. The inner chamber was put into the outer chamber (24-well plate), which was filled with FBS free-DMEM containing Epidermal Growth Factor (EGF) (50 ng/mL). After 24 h incubation at 37 °C, the non-migrated cells on the upper surface of the filter were removed by wiping with cotton swabs, and the filter was fixed with 70% EtOH and stained with Giemsa. The cells, which migrated through the filter to the reverse side, were counted manually at six different areas under a microscope. Data were shown by percentage based on the migrated cell number of EGF-stimulated HeLa cells.