Tetrahedron Letters 59 (2018) 3806–3809

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Croissamide, a proline-rich cyclic peptide with an *N*-prenylated tryptophan from a marine cyanobacterium *Symploca* sp.

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ARTICLE INFO

Article history:
Received 4 August 2018
Revised 30 August 2018
Accepted 6 September 2018
Available online 7 September 2018

Keywords:
Croissamide
Marine cyanobacteria
Cyclic peptide
Proline-rich
Symploca

ABSTRACT

Croissamide, a proline-rich cyclic peptide that contains an *N*-prenylated tryptophan, was isolated from a marine cyanobacterium *Symploca* sp. Its gross structure was determined by spectroscopic analyses, and the absolute configuration was established based on chiral HPLC analyses of acid hydrolysates.

To date, many proline-rich cyclic peptides have been discovered from various marine organisms such as sponges, ascidians and so on. These compounds have attracted increasing interest because of their biological activities [1], including cytotoxicity [2], antitubercular activity [3], anti-HIV activity [4], repellent (antifouling) activity [5] and inhibitory activity toward NO production [6]. Among marine creatures, cyanobacteria are known to be prolific producers of peptidic natural products, and several proline-rich cyclic peptides, including wewakapeptin A [7], wewakazole B [8], pahayokolide A [9] and trichormamide A [10], have been isolated from marine cyanobacteria. Against this background, we investigated the secondary metabolites of a marine cyanobacterium *Symploca* sp. and isolated croissamide (**1**), a cyclic peptide containing 11 α-amino acids, including five prolines and one *N*-prenylated tryptophan. Here we report the isolation and structure determination of croissamide (**1**).

Marine cyanobacterial samples (1600 g, wet weight) were collected at Minna Island (called "croissant island" due to its crescent shape), Okinawa. Based on morphological observations, the cyanobacterium was identified as *Symploca* sp. (see Supplementary Data for details). This sample was extracted with methanol, and the extract was filtered, concentrated, and partitioned between EtOAc and H_2O. The EtOAc-soluble material was further partitioned between 90% aqueous MeOH and hexane. The material obtained from the aqueous MeOH portion was subjected to fractionation by reversed-phase column chromatography (ODS silica gel, MeOH–H_2O) and repeated reversed-phase HPLC to give croissamide (**1**, 10.4 mg) [11,12].

The molecular formula of **1** was found to be $C_{67}H_{92}N_{12}O_{11}$ by HRESIMS (*m*/*z* 1241.7104, calcd for $C_{67}H_{93}N_{12}O_{11}$ $[M+H]^+$ 1241.7087). The NMR data for **1** are summarized in Table 1. The ^{1}H NMR spectrum revealed the presence of a double doublet signal (δ 6.15, *J* = 17.7, 10.6 Hz) and two doublet signals (δ 5.14, *J* = 17.7 Hz, δ 5.15, *J* = 10.6 Hz) corresponding to a mono-substituted alkene. In the ^{13}C NMR spectrum, 11 carbonyl signals (δ 172.2, 172.1, 171.6, 170.9, 170.4, 170.1, 169.5, 169.1, 169.0, 168.8 and 166.6) were observed. Based on further analyses of the ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HMQC, HMBC and NOESY spectra, the presence of 11 α-amino acids: glycine (Gly), alanine (Ala), two leucines (Leu), phenylalanine (Phe), five prolines (Pro) and *N*-prenylated-tryptophan (*N*-Pre-Trp) was confirmed. The location of a prenyl group was determined as shown in Fig. 1 based on two NOESY correlations: H4 of *N*-Pre-Trp/H15 of *N*-Pre-Trp and H4 of *N*-Pre-Trp/H16 of *N*-Pre-Trp. Although significant overlap of the methylene signals derived from the five proline residues was observed on the ^{1}H NMR spectrum, we were able to distinguish among them based on analyses of the TOCSY spectrum.

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https://doi.org/10.1016/j.tetlet.2018.09.016
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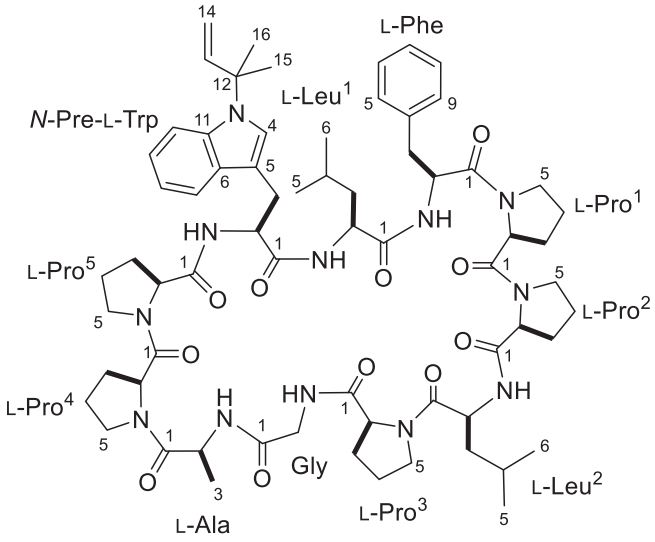

croissamide (1)

The sequences of these partial structures were determined based on HMBC and NOESY data (Table 1 and Fig. 1). Five HMBC correlations, NH of Leu^2/C-1 of Pro^2, NH of Gly/C-1 of Pro^3, NH of Ala/C-1 of Gly, NH of Leu^1/C-1 of *N*-Pre-Trp, and NH of Phe/C-1 of Leu^1, were observed. Moreover, 11 NOESY correlations, H2 of Pro^1/H5a of Pro^2, H2 of Pro^1/H5b of Pro^2, H2 of Leu^2/H5a of Pro^3, H2 of Leu^2/H5b of Pro^3, H2 of Pro^3/NH of Gly, H2 of Pro^4/H5a of Pro^5, H2 of Pro^4/H5b of Pro^5, H2 of Pro^5/NH of *N*-Pre-Trp, H3a of Pro^5/NH of *N*-Pre-Trp, H3b of Pro^5/NH of *N*-Pre-Trp and NH of Leu^1/H2 of *N*-Pre-Trp, were observed. Based on these observations, the presence of two partial sequences, Pro^1 – Pro^2 – Leu^2 – Pro^3 – Gly – Ala and Pro^4 – Pro^5 – *N*-Pre-Trp – Leu^1 – Phe, was clarified. In addition, based on the molecular formula and the degree of unsaturation, **1** was considered to be a cyclic peptide: Pro^1 and Pro^4 must be connected to Phe and Ala, respectively. Thus, the gross structure of **1** was determined as shown in Fig. 1.

The absolute configuration of **1** was determined as follows. The stereochemistry of all the α-amino acid residues was assigned to be L-form based on chiral HPLC analyses of the hydrolysate of **1**. With regard to *N*-Pre-Trp, Trp was obtained due to elimination of the prenyl group during acid hydrolysis, which was used to determine the stereochemistry.

In several solvents such as $CDCl_3$ and CD_3OD, croissamide (**1**) existed as a complex mixture of several conformers, probably due to restricted rotation of the amide bonds in the five proline residues. However, in DMSO-d_6, a single conformer of **1** was observed. Thus, we examined the conformation of each amide bond in the five proline residues in DMSO-d_6. According to previous reports, it is possible to determine the geometries of amide

Table 1
NMR data for croissamide (**1**) in DMSO-d_6.[a]

Unit	Position	δ_C[b]	δ_H[c] (*J* in Hz)	COSY	Selected HMBC (H → C)	Selected NOESY
Leu^1	1	170.9				
	2	52.2	4.16, m	3a, 3b, NH	1	
	3a	41.0	1.11, m	2, 3b, 4		
	3b		1.24, m	2, 3a, 4		
	4	24.5	1.27, m	3a, 3b, 5, 6		
	5	21.5	0.71, d (6.3)	4		
	6	22.3	0.80, d (6.3)	4		
	NH		8.23, d (10.1)	2	1 (*N*-Pre-Trp)	2 (*N*-Pre-Trp)
Phe	1	169.1[d]				
	2	50.6	4.70, m	3a, 3b, NH	1	
	3a	38.9	2.76, dd (13.1, 7.1)	2, 3b	4, 5, 9	
	3b		2.85, dd (13.1, 7.1)	2, 3a	4, 5, 9	
	4	137.2				
	5/9	129.4	7.16, m	6, 8		
	6/8	128.0	7.23, m	5, 9, 7		
	7	126.2	7.17, m	6, 8		
	NH		7.32, m	2	1 (Leu^1)	
Pro^1	1	169.5				
	2	58.3	4.61, brd (8.4)	3a, 3b	1	5a (Pro^2), 5b (Pro^2)
	3a	30.1	1.76, m	2, 3b, 4		
	3b		2.16, m	2, 3a, 4		
	4	21.5	1.78–1.84, m	3a, 3b, 5a, 5b		
	5a	46.3	3.29, m	4, 5b		
	5b		3.69, m	4, 5a		
Pro^2	1	171.6				
	2	59.1	4.40, m	3a, 3b	1	
	3a	27.6	1.21, m	2, 3b, 4a, 4b		
	3b		1.87, m	2, 3a, 4a, 4b		
	4a	25.4	1.94, m	3a, 3b, 4b, 5a, 5b		
	4b		2.10, m	3a, 3b, 4a, 5a, 5b		
	5a	47.5	3.42, m	4a, 4b, 5b		2 (Pro^1)
	5b		3.53, m	4a, 4b, 5a		2 (Pro^1)
Leu^2	1	170.1				
	2	48.0	4.68, m	3a, 3b, NH	1	5a (Pro^3), 5b(Pro^3)
	3a	43.1	1.20, m	2, 3b, 4		
	3b		1.27, m	2, 3a, 4		
	4	23.5	1.50, m	3a, 3b, 5, 6		
	5	23.3	0.56, d (6.2)	4		
	6	21.8	0.65, d (6.2)	4		
	NH		7.81, d (9.1)	2	1 (Pro^2)	

(*continued on next page*)

Table 1 (*continued*)

Unit	Position	δ_C[b]	δ_H[c] (*J* in Hz)	COSY	Selected HMBC (H → C)	Selected NOESY
Pro^3	1	170.4				
	2	58.9	4.45, m	3a, 3b	1	NH (Gly)
	3a	30.3	1.70, m	2, 3b, 4		
	3b		2.12, m	2, 3a, 4		
	4	23.9	1.80–1.87, m	3a, 3b, 5a, 5b		
	5a	46.6	3.44, m	4, 5b		2 (Leu^2)
	5b		3.62, m	4, 5a		2 (Leu^2)
Gly	1	166.6				
	2a	42.1	3.30, m	2b, NH	1	
	2b		3.86, dd (18.2, 6.1)	2a, NH	1	
	NH		7.56, d (6.1)	2a, 2b	1 (Pro^3)	2 (Pro^3)
Ala	1	169.0[d]				
	2	44.4	4.81, m	3, NH	1	
	3	18.1	1.05, d (6.4)	2		
	NH		8.27, d (9.6)	2	1 (Gly)	
Pro^4	1	168.8				
	2	58.0	4.88, brd (8.7)	3a, 3b	1	5a (Pro^5), 5b (Pro^5)
	3a	30.3	2.20, m	2, 3b, 4		
	3b		2.00, m	2, 3a, 4		
	4	21.5	1.78–1.83, m	3a, 3b, 5a, 5b		
	5a	46.6	3.33, m	4, 5b		
	5b		3.47, m	4, 5a		
Pro^5	1	172.1				
	2	58.7	4.19, brd (7.7)	3a, 3b		NH (*N*-Pre-Trp)
	3a	29.7	2.37, m	2, 3b, 4a, 4b		NH (*N*-Pre-Trp)
	3b		1.74, m	2, 3a, 4a, 4b		NH (*N*-Pre-Trp)
	4a	23.9	1.65, m	3a, 3b, 4b, 5a, 5b		
	4b		1.90, m	3a, 3b, 4a, 5a, 5b		
	5a	46.6	3.41, m	4a, 4b, 5b		2 (Pro^4)
	5b		3.65, m	4a, 4b, 5a		2 (Pro^4)
N-Pre-Trp	1	172.2				
	2	51.2	4.81, m	3a, 3b, NH	1	NH (Leu^1)
	3a	29.0	3.09, m	2, 3b	4, 5, 6	
	3b		3.22, m	2, 3a	4, 5, 6	
	4	121.9	7.23, s		5, 6	15, 16
	5	111.0				
	6	129.4				
	7	119.0	7.55, d (8.1)	8	5, 11	
	8	117.6	6.87, dd (8.1, 7.6)	7, 9		
	9	120.4	6.98, dd (8.4, 7.6)	8, 10		
	10	112.9	7.36, d (8.4)	9	6	
	11	135.1				
	12	58.4				
	13	144.8	6.15, dd (17.7, 10.6)	14a, 14b	12	
	14a	112.9	5.14, d (17.7)	13		
	14b		5.15, d (10.6)	13		
	15	27.6	1.61, s		12, 13, 16	4
	16	27.4	1.65, s		12, 13, 15	4
	NH		8.17, d (10.1)	2		2 (Pro^5), 3a (Pro^5), 3b (Pro^5)

[a] ^{1}H–^{13}C connectivities were determined by the HMQC method.
[b] Measured at 100 MHz.
[c] Measured at 400 MHz.
[d] These carbon signals are interchangeable.

bonds at proline residues on the basis of the ^{13}C chemical shift difference between the proline β and γ positions ($\Delta\delta_{\beta-\gamma}$) [13,14]. The large differences observed at Pro^1 ($\Delta\delta_{\beta-\gamma}$ = 8.6 ppm) and Pro^4 ($\Delta\delta_{\beta-\gamma}$ = 8.8 ppm) indicated that their peptide bonds were in a *cis* geometry as shown in Fig. 1. On the other hand, the geometries of peptide bonds of Pro^2, Pro^3, and Pro^5 could not be determined clearly based on their $\Delta\delta_{\beta-\gamma}$ values (2.2, 6.4, and 5.8 ppm for Pro^2, Pro^3, and Pro^5, respectively). However, the geometries of their peptide bonds were determined to be *trans*, based on the following NOESY correlations: H2 of Pro^1 and H5a/H5b of Pro^2; H2 of Leu^2 and H5a/H5b of Pro^3; H2 of Pro^4 and H5a/H5b of Pro^5.

Croissamide (**1**) did not inhibit the growth of human cancer cells, HeLa and HL60, at 10 μM. Meanwhile, **1** showed weak inhibitory activity against NO production in LPS-stimulated RAW 264.3 cells [15]. Although we tested for additional biological activities of **1**, such as anti-malarial activity, protease-inhibitory activity and anti-bacterial activity, **1** did not show any significant activities.

In conclusion, croissamide (**1**), a new cyclic peptide, was isolated from a marine cyanobacterium, *Symploca* sp. The structure of **1** was established by spectroscopic analyses and chiral HPLC analyses of acid hydrolysates. The structure of croissamide (**1**) contains five prolines and one *N*-prenylated tryptophan. So far, a number of prenylated peptides have been reported, such as hexamollamide [16], trunkamide [17] and prenylagaramides [18]. Prenylated positions of these compounds are mainly oxygen atoms in serine, threonin and tyrosine residues. Regarding tryptophan, *C*-prenylation is dominating as found in kawaguchipeptin A [19], and there are few reports on natural compounds possessing *N*-prenylated tryptophans [20]. To the best of our knowledge, **1** is the first cyanobacterial compound that possesses an *N*-prenylated

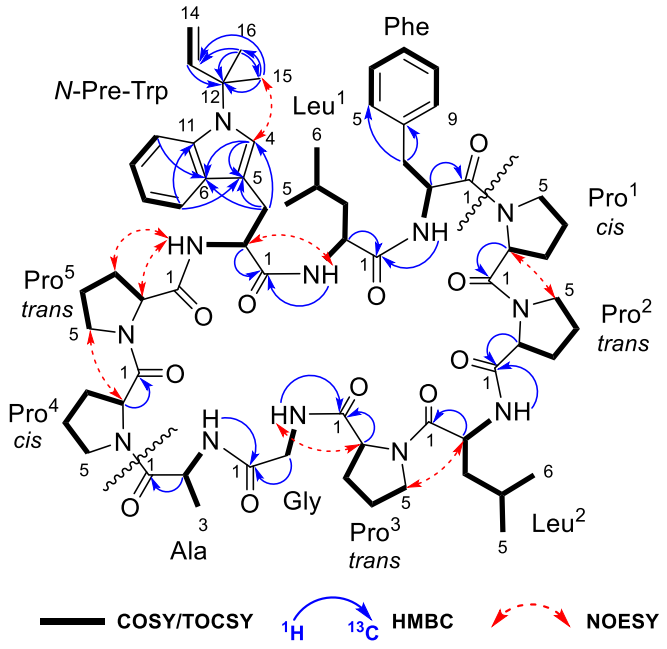

Fig. 1. Gross structure of croissamide (**1**) based on 2D NMR correlations.

tryptophan. Despite several efforts, we have not yet detected any significant biological activities of **1**. Further biological evaluations of croissamide (**1**) are ongoing in our laboratory.

Acknowledgement

This work was supported by JSPS KAKENHI Grant Number 18K14346.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tetlet.2018.09.016.

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[11] The detailed isolation procedures of croissamide (**1**) were as follows: Marine cyanobacterial samples were collected at Minna Island, Okinawa Prefecture, Japan, at a depth of 0–1 m in March 2018. The collected cyanobacterium (1600 g) was extracted with methanol (3 L) for 1 week. The extract was filtered, and the filtrate was concentrated. The residue was partitioned between ethyl acetate (3 × 0.3 L) and water (0.3 L). The material obtained from the organic layer was partitioned between 90% aqueous methanol (0.3 L) and hexane (3 × 0.3 L). The aqueous methanol fraction (355 mg) was first separated by column chromatography on ODS (4.0 g) eluted with 40% methanol, 60% methanol, 80% methanol, and methanol. The fraction (111 mg) eluted with 80% methanol was subjected to HPLC [Conditions for HPLC separation: column, Cosmosil 5C_{18}MS-II (ϕ20 × 250 mm); flow rate 5 mL/min; detection, UV 215 nm; solvent 80% MeOH] in three batches to give a fraction that contained croissamide (**1**) (46.8 mg, t_R = 32.0–47.6 min). This fraction was further separated by repeated HPLC [Cosmosil 5PE-MS (ϕ 20 × 250 mm); flow rate 5 mL/min; detection, UV 215 nm; solvent 90% MeOH] to give croissamide (**1**) (10.4 mg, t_R = 35.8 min).
[12] *Croissamide* (***1***): colorless amorphous solid; $[\alpha]_D^{28}$ −108 (*c* 0.33, CH_3OH); IR (neat) 3313, 2955, 1636, 1525, 1455, 747 cm^{-1}; ^1H NMR, ^{13}C NMR, COSY, HMQC, HMBC and NOESY data, see Table 1; HRESIMS *m*/*z* 1241.7104 $[M+H]^+$ (calcd for $C_{51}H_{75}N_6O_{12}$, 1241.7087).
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