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Wainunuamide, a histidine-containing proline-rich cyclic heptapeptide isolated from the Fijian marine sponge *Stylotella aurantium*

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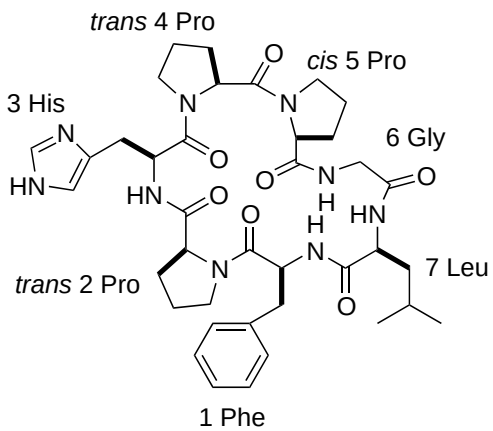
Abstract—The isolation and structure determination of an unusual cyclic heptapeptide, wainunuamide, from a Fijian marine sponge, *Stylotella aurantium*, is reported. The peptide contains three proline residues and a histidine residue, which is rare in cyclic peptides and has only previously been reported in a cyclic peptide isolated from the cyanobacterium *Oscillatoria agardhii*, suggesting a possible source of the peptide. Wainunuamide is found to have weak cytotoxic activity.

Cyclic peptides have become increasingly important because of their wide range of pharmacological activities[1] and interesting chemical structures.[2,3] They show therapeutic potential due to greater resistance to in vivo enzymatic degradation as well as greater bioavailability than non-cyclic analogues.[2] Cyclisation helps reduce conformational flexibility of the peptide backbone which enables systematic manipulation of the 3D structure to understand receptor binding in biological targets or for improvement of biological activity. An interesting class of marine cyclic peptides is represented by the proline-rich compounds usually containing seven or eight amino acid residues. The role of proline in these molecules has been linked to the control of conformation of the molecule in solution because of the restricted ϕ of proline;[4,5] a group of examples are the hymenamides,[6–9] stylopeptide,[10] axinellins,[11,12] axinastatins,[13–15] and the phakellistatins.[16–22]

When Fijian marine sponges were screened for anticancer activity, considerable activity was detected in the crude extract of *Stylotella aurantium* (Order Halichondrida; Family Halichondriidae). The sponge was collected in December 1997 at a depth of about 5 m by snorkelling from *Cakaulevu* reef, in the district of *Wainunu*, in the island of *Vanua Levu*, Fiji Islands (17° 2.609′; 178° 54.694′ E). A freeze dried sample (1.6 kg dry weight) was extracted and partitioned with the procedure previously described.[23] Anticancer activity was detected in the dichloromethane partition fraction; ID_{50}s were 0.47 and 0.45 μg/mL for A2780 ovarian tumour and K562 leukaemia cancer cells respectively. This fraction was subjected to Sephadex size exclusion chromatography (LH-20) using a mixture of methanol and dichloromethane (50/50) as eluent. Similar fractions were pooled together on the basis of TLC analysis. Analysis by ^{1}H and ^{13}C NMR indicated that one



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pooled fraction contained small peptides. Therefore this fraction was given priority for isolation work. Purification was achieved by reverse phase C18 HPLC with a mixture of acetonitrile, water and TFA (50/50/0.1) as eluent; this yielded the new compound wainunuamide (**1**, 11.2 mg), as well as the known compounds pseudoaxinellin (9.2 mg),[12] phakellistatin 2 (6.6 mg)[16] and a new conformer of phakellistatin 2 (9.5 mg). Also isolated was a new octapeptide which will be reported separately.

Table 1. ^{1}H (400 MHz, δ/ppm, proton count, multiplicity, J (Hz)) and ^{13}C (100 MHz, δ/ppm, multiplicity), and 2D NMR data in CD_3OD for wainunuamide (**1**)

No.	Residue	Atom	^{13}C	^{1}H	COSY ^{1}H–^{1}H	HMBC C→H
1	Phe	α	53.3 (d)	4.85 (1H, m)	H1β_A, H1β_B, 4NH	H1β_A, H1β_B
		β	38.2 (t)	A 3.11 (1H, m)	H1α, H1β_B	H1α
				B 2.72 (1H, m)	H1α, H1β_A	
		1	138.5 (s)			H1(3/5), H1α, H1β_A, H1β_B
		2/6	130.0 (d)	7.16 (2H, d, 3.9)	H1(3/5), H1(4)	H1β_A, H1β_B
		3/5	128.9 (d)	7.15 (2H, d, 3.9)	H1(2/6)	
		4	127.1 (d)	7.10 (1H, d, 4.6)	H1(2/6)	
		CO	169.9 (s)			H1α, H1β_A, H1β_B, H2α
		NH		7.38 (1H, d, 8.1)	H1α	
2	Pro	α	60.7 (d)	4.45 (1H, m)	H2β_A	
		β	28.9 (t)	A 2.28 (1H, m)	H2α, H2β_B	H2δ_A
				B 1.04 (1H, m)	H2β_A, H2δ_A	
		γ	25.2 (t)	A 1.96 (1H, m)	H2γ_B	H2δ_A
				B 1.70 (1H, m)	H2γ_A	
		δ	48.4 (t)	A 3.58 (1H, m)	H2γ, H2δ_B	
				B 3.45 (1H, m)	H2δ_B	
		CO	172.7 (s)			H2α
3	His	α	51.0 (d)	5.05 (1H, m)	3NH, H3β_A, H3β_B	
		β	29.3(t)	A 3.58 (1H, m)	H3α, H3β_B	
				B 2.84 (1H, m)	H3α, H3β_A	
		2	130.0 (s)			H3(4)
		4	118.8 (d)	7.40 (1H, s)	H3(5)	H3β_A, H3β_B, H3(5)
		5	135.1 (d)	8.78 (1H, s)	H3(4)	
		NH		7.57 (1H, bs)	H3α	
		CO	171.5 (s)			H3α
4	Pro	α	64.3 (d)	4.15 (1H, m)	H4β_A, H4β	
		β	30.4 (t)	2.31 (1H, m)	H4α	
				1.89 (1H, m)	H4α	
		γ	25.8 (t)	A 2.16 (1H, m)	H4γ_B, H4δ	H4δ_A
				B 1.97 (1H, m)	H4γ_A	
		δ	48.0 (t)	3.84 (2H, m)	Hγ_A	
		CO	174.8 (s)			H4α
5	Pro	α	61.5 (d)	4.40 (1H, m)	H5β_A	
		β	32.8 (t)	A 2.25 (1H, m)	H5α, H5β_B	H5α
				B 2.08 (1H, m)	H5β_A	
		γ	22.7 (t)	A 1.88 (1H, m)	H5γ_B	H5α
				B 1.78 (1H, m)	H5γ_A	
		δ	48.0 (t)	A 3.66 (1H, m)	H5δ_B	H5α
				B 3.48 (1H, m)	H5δ_A	
		CO	174.7 (s)			H5α, H6α_A, H6α_B
6	Gly	α	44.7 (t)	A 3.76 (1H, m)	3NH, H6α_B	
				B 3.61 (1H, m)	H6α_A	
		NH		8.17 (1H, d, 7.8)	H3α	
		CO	170.7 (s)			H6α_A, H6α_B, H7α
7	Leu	α	53.6 (d)	4.24 (1H, m)	7NH	
		β	39.6 (t)	A 1.74 (1H, m)	H7β_B	H7α, 7δ_1CH_3, 7δ_2CH_3
				B 1.52 (1H, m)	7δ_1CH_3, 7δ_2CH_3	
		γ	26.1(d)	1.50 (1H, m)	H7β_A, H7β_B	H7α, 7δ_1CH_3, 7δ_2CH_3
		δ_1CH_3	20.8 (q)	0.82 (3H, d, 6.1)	7β_B	
		δ_2CH_3	23.1 (q)	0.87 (3H, d, 6.1)	7β_B	
		NH		8.24 (1H, d, 8.4)	H7α	
		CO	173.6 (s)			H1α, H7α

Wainunuamide (**1**) showed a molecular ion peak at 746.3979 $[M+H]^+$, 0.1 mmu from the value calculated for $C_{38}H_{51}N_9O_7$.[24] The ^{13}C and DEPT-135 spectra of wainunuamide exhibited seven amide carbonyls and seven α-methine carbons characteristic of a peptide. The amino acid composition was confirmed by HPLC analyses of the acid hydrolysate, which showed it to contain three Pro residues and one each of Leu, His, Gly and Phe. The stereochemistry of all residues was found to be L by chiral TLC on the acid hydrolysate.[25] The spin systems of each amino acid residue were assigned from ^1H, ^{13}C, HSQC and HSQC-TOCSY experiments (Table 1).

Since only 17 of the calculated 18 degrees of unsaturation could be accounted for by the functionalities present in the seven individual amino acids, it was obvious that **1** was a cyclic peptide. The sequence of amino acids was assigned on the basis of 2D HMBC and MS-MS fragmentation data.

Carbonyl carbons within each residue were assigned from HMBC correlations between the C=O and their respective α-protons (Table 1). Quaternary carbons in the Phe[1] and His[3] sidechains were assigned by HMBC correlations (Table 1) and by reference to known values for these residues. Cross peaks were observed in the HMBC spectrum between Phe[1]-CO (δ_C 169.9)/Pro[2]-Hα (δ_H 4.45), Leu[7]-CO (δ_C 173.6)/Phe[1]-Hα (δ_H 4.85), Pro[5]-CO (δ_C 174.7)/Gly[6]-$H\alpha_A$ (δ_H 3.76) and Gly[6]-$H\alpha_B$ (δ_C 3.61) establishing the partial sequences of Leu[7]-Phe[1]-Pro[2] and Pro[5]-Gly[6]. An HMBC correlation from Gly[6]-CO (δ_C 170.7) to Leu[7]-Hα (δ_H 4.24) combined these partial sequences to give Pro[5]-Gly[6]-Leu[7]-Phe[1]-Pro[2]. Despite the overlap between Pro[4]-CO (δ_C 174.8) and Pro[5]-CO (δ_C 174.7), the HMBC spectrum suggested that Pro[4] was connected to Pro[5]. This was confirmed by MS^n fragmentation where masses corresponding to loss of one histidine, two prolines and one each of leucine and glycine was observed in the spectrum. The spectrum of wainunuamide contained $[M+H]^+$ at m/z 746, the fragmentation of which was followed by MS^n. The ESI-MS^n of wainunuamide showed preferential fragmentation at any one of the three proline amide bonds which gave a complex series of fragment ions. A few major fragmentation pathways emerged (Fig. 1). One started with the opening of the macrocycle after loss of CO followed by loss of 268 amu due to Leu[7]-Gly[6]-Pro[5] plus H, leaving m/z 450 (Pro[4]-His[3]-Pro[2]-Phe[1]), which then lost 147 amu (Phe[1]). Another pathway left a major fragment m/z 502 corresponding to His[3]-Pro[4]-Pro[5]-Gly[6]-Leu[7] plus H, whereas yet another pathway left m/z 484 corresponding to Phe[1]-Leu[7]-Gly[6]-Pro[5]-Pro[4] plus H.

The structure was thus confirmed as *cyclo*(Phe[1]-Pro[2]-His[3]-Pro[4]-Pro[5]-Gly[6]-Leu[7]). The geometry of the peptidic linkages was assigned on the basis of the differences in ^{13}C chemical shifts of the Cβ and Cγ of the proline residues. ^{13}C NMR data of wainunuamide

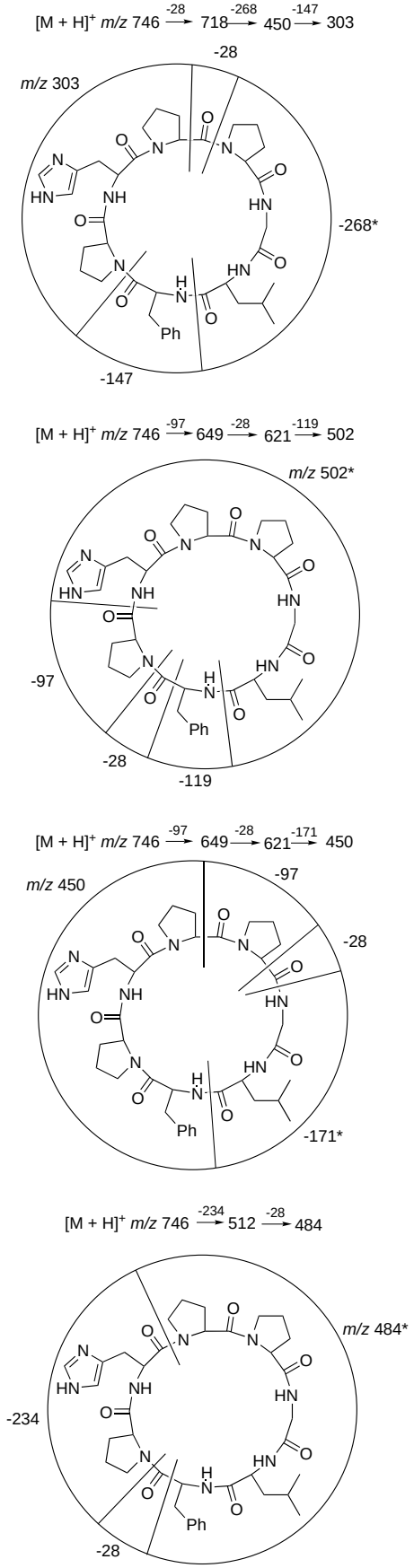

Figure 1. Major fragmentation pathways of **1** in ESI-MS^n. Starred masses are protonated, adding one mass unit to the predicted fragment mass.

indicated two proline peptide bonds were *trans* as shown by the small ^{13}C NMR chemical shifts difference of $Pro^2\Delta\delta_{C\beta\text{-}C\gamma}=3.2$, $Pro^4\Delta\delta_{C\beta\text{-}C\gamma}=4.6$ and one *cis* as indicated by the large ^{13}C chemical shift difference of $Pro^5\Delta\delta_{C\beta\text{-}C\gamma}=10.2$.[26,27] Adjacent *cis* and *trans* Pro residues were also found in phakellistatin 8 and were found to be powerful β-turn inducers.[4]

Wainunuamide (**1**) is a new histidine-containing cyclic peptide. The only other example in the literature of a cyclic peptide containing histidine and prolines is the compound agardhipeptin isolated from the cyanobacterium *Oscillatoria agardhii*.[28] This suggests that the source of **1** in *Stylotella aurantium* may be a cyanobacterial species. Compound **1** shows weak cytotoxic activity with ID_{50}s of 19.15 and 18.36 μg/mL for A2780 ovarian tumour and K562 leukaemia cancer cells, respectively.

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24. *Wainunuamide* (**1**) Colourless oil, 8.5 mg (0.00053%) yield); $[\alpha]_D^{25}$ −64.1° (*c* 0.011 MeOH); UV (100% MeOH) λ_{max} 279 (ε 3167); IR (cm^{-1}) 1673, 1435, 1200, 1137, 841. LRESIMS *m/z* 746.3 $[M+H]^+$ and HRESIMS *m/z* 746.3979 $[M+H]^+$ Δ 0.1 mmu from calculated for $C_{38}H_{52}O_7N_9$. NMR data (Table 1).
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