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Nairaiamides A and B. Two Novel Di-Proline Heptapeptides Isolated from a Fijian *Lissoclinum bistratum* Ascidian.

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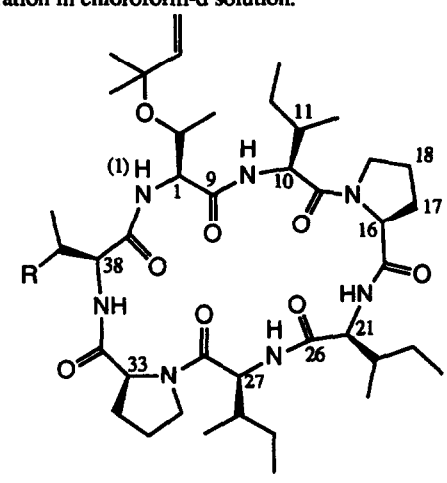
Abstract: Two new cyclic heptapeptides (**1**, **2**) have been isolated from the ascidian *Lissoclinum bistratum*, and their structures determined by a combination of spectroscopic techniques, including a natural abundance two-dimensional $^{1}H\{^{13}C,^{1}H\}$-HMQC-TOCSY experiment which was pivotal to assigning and identifying the amino acid residues. $^{1}H\{^{13}C\}$-HMBC and $[^{1}H\text{-}^{1}H]$-ROESY experiments provided the correlations necessary to sequence the peptides. In both peptides, the Ile-Pro amide bonds adopt *cis* configurations.

Introduction

Our investigation of an extract of *L. bistratum* collected at Nairai Island, Fiji, yielded two new cyclic heptapeptides, nairaiamides A and B (**1**, **2**). The peptides both possess two proline residues, a dimethylallylthreonine (daT), and several isoleucines; **1** possesses a Val residue in place of an Ile in **2**. Due to the redundancy of amino acids, the ^{1}H NMR spectra exhibited severe signal overlap. In order to assign the resonances, and thereby identify the amino acids, we performed ^{13}C-edited 2D HMQC-TOCSY NMR experiments.[1,2] Carbon-13 chemical shift data and $^{1}H\text{-}^{1}H$ NOEs (from 2D ROESY experiments) indicated that both Ile-Pro amide bonds in each peptide assume the cis configuration in chloroform-d solution.[3-5]

The nairaiamides are similar to a cyclic heptapeptide reported independently from *Axinella* sp. collected in Palau under the name axinastatin,[6] and as pseudoaxinellin from the Papua New Guinea sponge *Pseudoaxinella massa*.[7] Like the nairaiamides, this peptide also possesses two proline residues arranged in the order cyclo-Pro-X-X-Pro-X-X-X. Based on $^{1}H\text{-}^{1}H$ NOE and ^{13}C chemical shift data,[7] the Val-Pro amide bond in that peptide appears to assume the cis configuration, while the Asn-Pro amide bond assumes the trans orientation. The reports of axinastatin and pseudoaxinellin were followed several months later by the report of the cyclic heptapeptide malaysiatin, isolated from the Malaysian sponge *Pseudaxinyssa* sp., collected in Borneo.[8] This peptide possesses the exact same amino acid composition, but has been assigned a different sequence. Comparison of the ^{1}H and ^{13}C NMR data[7,8] reported to that of axinastatin/pseudoaxinellin[7] indicates they are likely the same.



1: R=CH_3, **2**: R=CH_2CH_3

Results and Discussion

Nairaiamide A. A molecular formula of $C_{42}H_{71}N_7O_8$ for **1** was established in part from HR FAB$^+$ MS, indicating 11 degrees of unsaturation. A 2D $^1H\{^{13}C,^1H\}$-HMQC-TOCSY[1,2] spectrum, in conjunction with the data from DQF-COSY, TOCSY and $^1H\{^{13}C\}$-HMQC experiments, allowed the identification of two Pro residues, three Ile, a Val and a daT residue. (For example, a correlation from the αH at 4.37 ppm to ^1H-bearing carbons at 54.14, 37.04, 24.82, 15.11 and 10.10, placed those 5 carbons in the same spin system, identifying it as an Ile residue.) This composition accounted for 10 degrees of unsaturation, the 11th resulting from the cyclic nature of the peptide. The sequence of the cyclic peptide was established based on data from two experiments: 2D $^1H\{^{13}C\}$-HMBC[9] and 2D ROESY.[10]

The difficulty in distinguishing correlations to N2H and N7H (7.96 and 7.97 ppm, respectively) was resolved with several pieces of evidence: The HMBC trace at 7.96 ppm (F1 trace) contained carbon signals at 170.89 and 169.74 ppm, while the trace at 7.97 ppm possessed only the 170.89 ppm carbon signal. This placed N2H adjacent to C9 and N7H next to C37. This assignment is supported by an HMBC correlation from C9 to H10 (4.37 ppm), the α proton of that residue. (The proton at 4.37 ppm was unambiguously assigned as the α proton adjacent to N2H by the DQF-COSY, TOCSY and HMQC-TOCSY data.)

Because the proline residues lack amide protons, the ^1H-^{13}C HMBC approach could not be used to establish connectivities across these residues. In those cases strong correlations in a 2D ROESY spectrum provided the necessary information. Cross peaks from H10 (δ 4.37) to H16 (δ 4.57) and from H27 (δ 4.12) to H33 (δ 4.45) established the connectivity across the C15-N3 and C32-N6 bonds, completing the sequence assignment. Complete ^1H and ^{13}C NMR resonance assignments are summarized on Table 1. These strong αH to αH NOEs are also indicative of cis orientations about both Ile-Pro amide bonds. This conclusion is supported by ^{13}C chemical shift data: the difference in chemical shifts of the β and γ carbons ($\Delta\delta_{\beta\gamma}$) of the Pro residues are 9.69 and 9.47 ppm, respectively.[3-5]

Nairaiamide B. High resolution FAB$^+$ MS permitted a molecular formula of $C_{43}H_{73}N_7O_8$ for **2**, indicating that it possessed one more methylene unit than **1**. In this case there was no signal overlap in the amide region of the ^1H spectrum; unfortunately, two carbonyl carbons (C32, C37) shared identical chemical shifts in the amide region of the ^{13}C spectrum. Once again, due to severe overlap in the aliphatic region of the spectrum a 2D $^1H\{^{13}C\}$-HMQC-TOCSY experiment was necessary to assign the resonances and identify the amino acids. Analysis of the data permitted the identification of two Pro residues, four Ile and one daT.

As was the case for nairaiamide A, HMBC and ROESY data were used to sequence the cyclic peptide. The signal overlap of C32 and C37 (171.72 ppm) was resolved based on several other available correlations. Strong ROESY correlations between the α protons of the Pro and their preceding residues established the connectivities across the C15-N3 and C32-N6 bonds. The complete ^1H and ^{13}C assignments are summarized in Table 1. The $\Delta\delta_{\beta\gamma}$ for the Pro residues are 9.7 and 9.3 ppm, respectively, once again supporting *cis*-X-Pro conformations.

Experimental Section

General. NMR spectra were acquired on a Varian Unity 500 spectrometer and referenced to residual undeuterated solvent (^1H), or to solvent signals (^{13}C). High and low resolution FAB$^+$ mass measurements were done on a Finnigan MAT 95 High Resolution Gas Chromatograph/Mass Spectrometer. UV and IR spectra were obtained on Hewlett Packard HP8452A Diode Array and Perkin Elmer 16000 FTIR spectrophotometers, respectively. Optical rotations were measured on a Jasco DIP-370 polarimeter.

Table 1. ^{1}H and ^{13}C NMR Resonance Assignments for Nairaiamides A and B.

	Nairaiamide A			Nairaiamide B		
C #	δ (ppm)	^{1}H (mult, Hz)[a]	LR ^{1}H-^{13}C[b]	δ (ppm)	^{1}H (mult, Hz)[a]	LR ^{1}H-^{13}C[b]
N1H	-	7.36 (d, 4.6)	C42	-	7.32 (d, 4.5)	C43
1	57.09	4.23 (m)	C9	57.06	4.22 (m)	C9
2	65.80	4.30 (m)	C4, C9	65.77	4.28 (m)	C9
3	16.29	0.85 (d)	-	16.17	0.84 (d)	C4
4	77.54	-	-	77.51	-	-
5	27.41	1.37 (s)	-	27.39	1.34 (s)	-
6	25.09	1.42 (s)	-	25.08	1.40 (s)	-
7	142.96	5.90 (dd, 17.5, 10.8)	-	142.95	5.89 (dd, 10.8, 17.4)	-
8	114.38	5.26 (dd, 17.5, 1.0), 5.12 (dd, 10.8, 1.0)	-	114.36	5.28 (dd, 17.4, 1.0), 5.11 (dd, 10.8, 1.0)	-
9	169.91	-	N2H, H2, H3	169.91	-	N2H, H2, H3
N2H	-	7.96 (d, 9.3)	C9	-	7.95 (d, 9.5)	C9
10	54.14	4.37 (dd, 9.7, 9.3)§	C15	54.24	4.32 (dd, 9.6, 9.5)§	C15
11	37.04	1.78 (m)	-	36.94	1.76 (m)	-
12	15.11	0.83 (d)	-	15.12	0.81 (d)	-
13	24.82	1.54 (m), 1.16 (m)	-	24.86	1.50 (m), 1.13 (m)	-
14	10.10	0.83 (m)	-	10.09	0.81 (m)	-
15	172.59	-	H10	172.56	-	H10
16	61.70	4.57 (d, 7.6)§	C20	61.72	4.55 (d, 7.4)§	C20
17	31.72	2.45 (dd, 7.3, 12.7), 2.04 (m)	-	31.70	2.42 (m), 2.02 (m)	-
18	22.03	2.01 (m), 1.72 (m)	-	21.99	2.00 (m), 1.69 (m)	-
19	46.31†	3.64 (m), 3.46 (m)	-	46.29†	3.62 (m), 3.42 (m)	-
20	172.16	-	N4H, H16	172.19	-	N4H, H16
N4H	-	6.12 (d, 7.2)	C20	-	6.15 (d, 9.1)	C20
21	56.33	4.21 (m)	C26	56.22	4.20 (m)	C26
22	32.46	1.86 (m)	-	32.59	1.84 (m)	-
23	16.47	0.90 (d)	-	16.40	0.88 (d)	-
24	24.58	1.47 (m), 0.89 (m)	-	24.54	1.45 (m), 0.86 (m)	-
25	10.50	0.81 (m)	-	10.58	0.81 (m)	-
26	171.31	-	N5H, H21	171.5	-	N5H, H21
N5H	-	7.49 (d, 4.2)	C26	-	7.50 (d, 4.2)	C26
27	57.31	4.12 (dd, 6.2, 4.4)¥	C32	57.28	4.08 (dd, 4.3, 6.5)¥	C32
28	35.86	1.74 (m)	-	35.88	1.71 (m)	-
29	16.00	0.94 (d)	-	15.93	0.91 (d)	-
30	24.40	1.63 (m), 1.29 (m)	-	24.45	1.62 (m), 1.27 (m)	-
31	11.02	0.88 (m)	-	10.94	0.86 (m)	-
32	170.75	-	H27	170.72•	-	H27
33	60.95	4.45 (d, 7.3)¥	C37	60.98	4.42 (d, 7.1)¥	C37
34	31.24	2.59 (m), 1.90 (m)	-	31.13	2.60 (m), 1.86 (m)	-
35	21.77	1.90 (m), 1.68 (m)	-	21.80	1.89 (m), 1.65 (m)	-
36	46.26†	3.64 (m), 3.46 (m)	-	46.23†	3.62 (m), 3.42 (m)	-
37	170.89	-	N7H, H33	170.72•	-	N7H, H33
N7H	-	7.97 (d, 7.9)	C37	-	8.08 (d, 7.9)	C37
38	62.70	3.93 (dd, 8.4, 7.9)	C42	61.53	3.92 (dd, 7.9, 9.5)	C43
39	29.68	2.19 (m)	-	35.63	1.96 (m)	-
40	19.38	0.94 (d)	-	15.47	0.89 (d)	-
41	19.20	0.95 (d)	-	25.78	1.45 (m), 1.18 (m)	-
42	171.42	-	N1H, H38	10.66	0.81 (m)	-
43	-	-	-	171.54	-	N1H, C38

[a] ^{1}H-^{1}H couplings measured in 1D ^{1}H spectrum. [b] $^{1}H\{^{13}C\}$-HMBC correlations important for deriving peptide sequence. §, ¥ ^{1}H signals that display strong ROESY crosspeaks. † Interchangeable assignments. • Identical chemical shifts.

Isolation. The ascidian was collected by SCUBA at depths of 35 feet in Fijian waters and identified as *Lissoclinum* sp. by Dr. Françoise Monniot, Museum National d'Histoire Naturelle Paris, France. The freeze-dried ascidian (98 grams) was homogenized, extracted with methanol (1.2 L), and the resulting extract reduced to 150 mL and subjected to a solvent partition: extract with hexanes (2 x 150 mL), add 10 mL H_2O and extract with CCl_4 (2 x 150 mL), add 30 mL H_2O and extract with $CHCl_3$ (2 x 150 mL), yielding four fractions of 182 mg, 458 mg, 514 mg and 1.9 g, respectively. Silica flash chromatography of the CCl_4 fraction (2 x 30 cm, Silica Gel 60 Å, Baxter S/P, 230-400 Mesh, stepped gradient elution 100% $CHCl_3$ to 50% MeOH), followed by reversed-phase flash chromatography (2 x 18 cm, ODS-3 EM Lichroprep C18, stepped gradient elution, 20% to 100% MeOH/H_2O) and reversed phase HPLC (1 x 25 cm, Rainin Dynamax 60A, 85% MeOH/H_2O, 3 mL/min, RI detection), yielded 39.5 mg (0.04 % yield) and 51.4 mg (0.05 % yield) of **1** and **2**, respectively.

$^1H\{^{13}C\text{-}^1H\}$-HMQC-TOCSY spectra. ^1H-detected ^1H-^{13}C correlated HMQC-TOCSY data were acquired with 1472 complex points in F2 and 1024 points in F1, with 16 transients per increment and Waltz decoupling of ^{13}C during the 192 ms acquisition. The spectral width in the ^{13}C dimension included only the protonated carbons (17644.5 Hz for **1**, and 17597.9 Hz for **2**), while the full ^1H spectral width was acquired in F2 (3842.8 and 3838.4 Hz for **1** and **2**, respectively). The HMQC portion of the pulse sequence was optimized for a $^1J_{CH}$ of 140 Hz, and the isotropic mixing time in the TOCSY portion was 50 ms.

Stereochemistry. The peptides (1 mg; 1.25 x 10^{-3} mmol **1** and 1.23 x 10^{-3} mmol **2**) were dissolved in 2 mL 6N HCl in a glass bomb sealed with a Teflon screw cap, and placed in an oven at 104 °C for 20 h. After repeated dissolution in H_2O and re-evaporation in vacuo, the residues were resuspended in 300 µL H_2O, 200 µL of which were derivatized with (1-flouro-2,4-dinitrophen-5-yl) L-alanineamide (FDAA).[11] HPLC analysis (Waters NOVAPAK C_{18} 4.6 x 100 mm column, isocratic elution 20% MeCN in 50 mM triethylammonium phosphate, pH 3.0, 1.0 mL/min, UV detection λ = 340 nm) against similarly derivatized standards established L-Thr (from daT), L-Pro, L-Val and L-Ile for **1**, and L-Thr, L-Pro and L-Ile for **2**.

Nairaiamide A. Clear glass, 39.5 mg. Low resolution FAB MS $(M-H)^-$ 800, HR FAB$^+$ MS $(M+H)^+$ 802.5426 ($C_{42}H_{72}N_7O_8$, Δ = 1.67 mmu), IR (thin film) 3330, 2965, 2358, 1660, 1504, 1416 cm^{-1}, UV (CH_2Cl_2) λ_{max} 230 nm (ε 2060), $[\alpha]_D^{25}$ -116° (c = 2.15, CH_2Cl_2).

Nairaiamide B. Clear glass, 51.4 mg. Low resolution FAB$^-$ MS $(M-H)^-$ 814 da, high resolution FAB$^+$ MS $(M+H)^+$ 816.5591 ($C_{43}H_{74}N_7O_8$, Δ 0.79 mmu, IR (thin film) 3330, 2965, 2359, 1659, 1504, 1416 cm^{-1}, UV (CH_2Cl_2) λ_{max} 230 nm (ε 2580), $[\alpha]_D^{25}$ -140° (c = 2.06, CH_2Cl_2).

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