# *Notes*

## Cyclonellin, a New Cyclic Octapeptide from the Marine Sponge *Axinella carteri*

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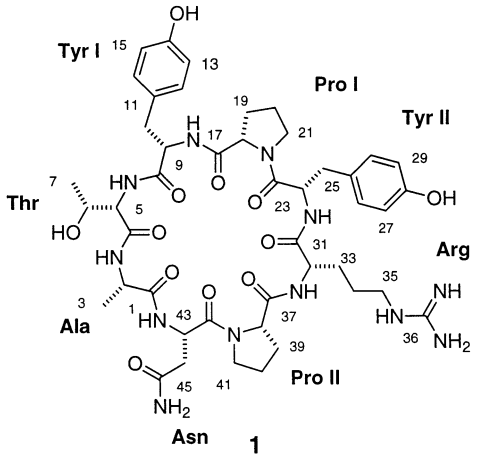
Cyclonellin (**1**), a new cyclic octapeptide, was isolated from an aqueous extract of the marine sponge *Axinella carteri*. Its structure was elucidated by interpretation of NMR spectral data of the intact compound and N-terminal Edman sequencing of linear peptide fragments obtained by partial hydrolysis of **1**. The absolute configurations of the constituent amino acids were determined by acid hydrolysis, derivitization with FDAA, and LC-MS analyses.

Marine sponges have proven to be a rich source of cyclic peptides that are diverse in both structure and bioactivity.[1,2] To date, a number of proline-rich cyclic hepta- and octapeptides have been described from the genus *Axinella*, and many of these compounds have been reported to exhibit potent antiproliferative and cytotoxic activities.[3−8] The aqueous extract of a sample of *Axinella carteri* Dendy (Axinellidae) collected from the Caroline Islands was selected for detailed chemical investigation on the basis of a pattern of differential cytotoxicity it exhibited in the U.S. National Cancer Institue (NCI)'s 60-cell-line antitumor screen.[9,10] Our investigation of this extract led to the isolation of a new cyclic octapeptide, cyclonellin (**1**), along with several previously described bromopyrrole derivatives.




**1**

A 6.0 g portion of the aqueous extract from *A. carteri* was dissolved in $H_2O$, applied to a wide-pore $C_4$ vacuum liquid chromatography column, and eluted using a step gradient with increasing concentrations of MeOH in $H_2O$. The principal cytotoxic fraction, eluted with $H_2O$−MeOH (2:1), contained a complex mixture of brominated pyrrole alkaloids. NMR and LC-MS analysis of this material revealed a series of known compounds including spongiacidins A and B[11] and axinohydantoin.[12] The fraction that eluted from the $C_4$ column with 100% $H_2O$ also exhibited cytotoxic activity, so a 4.1 g aliquot was partitioned between $H_2O$ and BuOH. The resulting BuOH-soluble material (548 mg) was chromatographed on LH-20 using MeOH−$H_2O$ (7:3), followed by gradient elution with $C_{18}$ HPLC (0−60% $CH_3CN$ in $H_2O$) to provide 15.3 mg of cyclonellin (**1**).

The positive-ion FABMS of **1** exhibited pseudomolecular ions at *m/z* 963 for $[M + H]^+$ and *m/z* 985 for $[M + Na]^+$, which defined the nominal molecular weight as 962. The molecular formula of **1** was established as $C_{45}H_{62}N_{12}O_{12}$ by high-resolution FABMS measurements of a CsI-doped sample ($[M + Cs]^+$ *m/z* 1095.3683, calcd for $C_{45}H_{62}N_{12}O_{12}Cs$, 1095.3701) in combination with detailed analyses of the $^1H$ and $^{13}C$ NMR data. The presence of nine carbonyl resonances ($\delta$ 171−175) in the $^{13}C$ NMR spectrum and six secondary amide NH resonances ($\delta$ 7.36−8.46) in the $^1H$ NMR spectrum (Table 1) suggested that **1** was a peptide metabolite. Extensive 1D and 2D NMR analyses established the presence of alanine, arginine, asparagine, threonine, two proline, and two tyrosine residues. The molecular formula of **1** required 20 degrees of unsaturation, 19 of which were accounted for by the aforementioned residues; thus **1** was a cyclic peptide.

The amino acid sequence and cyclic structure of **1** was deduced largely from HMBC and ROESY correlations between adjacent residues (Figure 1). Heteronuclear correlations between the H-α protons or amide NH signals of one residue and the carbonyl carbons of adjacent residues indicated the sequence was Asn-Ala-Thr-Tyr-Pro-Tyr-Arg-Pro. ROESY data proved particularly helpful for confirming

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**Table 1.** NMR Spectral Data for Cyclonellin (**1**) in $CD_3OH$

| | $^{13}C$[a] | $^{1}H$[b] | mult *J* (Hz) | COSY[c] | HMBC | ROESY |
|---|---|---|---|---|---|---|
| Ala | | | | | | |
| 1 | 174.68 (s) | | | | | |
| 2 | 51.27 (d) | 4.25 | dq (6.5, 6.5) | H-3, Ala NH | C-1, C-3, C-4 | H-3, Ala NH, Asn NH |
| 3 | 16.85 (q) | 1.43, 3H | d (7.5) | H-2 | C-1, C-2 | H-2, Ala NH |
| NH | | 8.46 | d (5.5) | H-2 | C-1, C-2, C-3 | H-2, H-3, H-5, H-6 |
| Thr | | | | | | |
| 4 | 172.12 (s) | | | | | |
| 5 | 58.96[d] (d) | 4.88 | m | H-6, Thr NH | C-4, C-6 | H-6, Thr NH, Ala NH |
| 6 | 71.34 (d) | 4.45 | dq (4.0, 6.0) | H-5, H-7 | C-7 | H-7, Ala NH |
| 7 | 19.18 (q) | 1.21, 3H | d (6.5) | H-6 | C-5, C-6 | H-6, H-12, H-16, Thr NH |
| NH | | 7.77 | d (9.5) | H-5 | C-5, C-8 | H-5, H-7 |
| Tyr-I | | | | | | |
| 8 | 172.78 (s) | | | | | |
| 9 | 58.98[d] (s) | 3.95 | m | H-10a, H-10b, Tyr-I NH | C-8, C-10, C-11 | H-10a, H-12, H-16, |
| 10 | 34.01 (t) | 3.28 | dd (4.0, 13.5) | H-9 | C-9, C-12, C-16 | H-9, H-12, H-16 |
| | | 3.42 | dd (12.0, 12.0) | H-9 | C-8, C-9, C-12, C-16 | H-12, H-16 |
| 11 | 130.48 (s) | | | | | |
| 12, 16 | 131.07 (d) | 6.98, 2H | d (8.0) | H-13, H-15 | C-10, C-12, C-16, C-13, C-14, C-15 | H-10a, H-10b, H-13, H-15, H-19a |
| 13, 15 | 116.18 (d) | 6.70, 2H | d (8.5) | H-12, H-16 | C-11, C-13, C-15, C-14 | H-12, H-16 |
| 14 | 157.15 (s) | | | | | |
| NH | | 8.42 | d (6.5) | H-9 | C-9, C-17 | H-18 |
| Pro-I | | | | | | |
| 17 | 174.10 (s) | | | | | |
| 18 | 62.44 (s) | 4.06 | dd (7.5, 7.5) | H-19a, H-19b | C-17, C-19, C-20, C-22 | H-19a, H-19b, Tyr-I NH |
| 19 | 30.75 (t) | 1.72 | m | H-18, H-20a | C-17, C-18, C-20 | H-12, H-16, H-18 |
| | | 1.97 | m | H-18, H-20a | C-17, C-18, C-20, C-21 | |
| 20 | 25.72 (t) | 1.93 | m | H-19a, H-21b | C-18, C-19, C-21 | |
| | | 1.99 | m | | | |
| 21 | 49.31[e] (t) | 3.72 | m | H-20a | C-18, C-19, C-20 | H-23 |
| | | 3.77 | m | H-20a | C-18, C-19, C-20 | H-23 |
| Tyr-II | | | | | | |
| 22 | 171.40 (s) | | | | | |
| 23 | 52.91 (d) | 5.09 | ddd (4.0, 10.0, 10.0) | H-24a, H-24b, Tyr-II NH | C-22, C-24, C-25, C-31 | H-21a, H-21b, H-24a, H-24b, H-26, H-30, Tyr-II NH |
| 24 | 38.76 (t) | 2.88 | dd (10.0, 14.0) | H-23 | C-22, C-23, C-25, C-26, C-30 | H-23, H-26, H-30 |
| | | 2.92 | dd (4.0, 14.0) | H-23 | | H-23, H-26, H-30 |
| 25 | 129.14 (s) | | | | | |
| 26, 30 | 131.61 (d) | 6.96, 2H | d (7.0) | H-27, H-29 | C-24, C-26, C-27, C-28, C-29, C-30 | H-23, H-24a, H-24b, H-27, H-29 |
| 27,29 | 116.11 (d) | 6.64, 2H | d (8.0) | H-26, H-30 | C-25, C-27, C-28, C-29 | H-26, H-30 |
| 28 | 157.05 (s) | | | | | |
| NH | | 8.08 | d (9.5) | H-23 | C-23, C-31 | H-23 |
| Arg | | | | | | |
| 31 | 173.68 (s) | | | | | |
| 32 | 55.32 (d) | 3.96 | m | H-33a, H-33b, Arg NH | C-31, C-33, C-34 | H-33a, H-33b, Arg NH |
| 33 | 28.98 (t) | 1.35 | m | H-32, H-34b | C-34 | H-32 |
| | | 1.42 | m | H-32 | C-34 | H-32 |
| 34 | 26.49 (t) | 1.35 | m | H-35a, H-35b | C-33 | H-35a, H-35b |
| | | 1.43 | m | H-33a, H-35a, H-35b | C-33 | H-35a, H-35b |
| 35 | 41.68 (t) | 3.03 | m | H-34a, H-34b, $\delta$ NH | C-33, C-34 | H-34a, H-34b, $\delta$ NH |
| | | 3.07 | m | H-34a, H-34b, $\delta$ NH | C-33, C-34 | H-34a, H-34b, $\delta$ NH |
| 36 | 158.71 (s) | | | | | |
| NH | | 8.11 | d (7.5) | H-32 | C-32, C-33, C-37 | H-32 |
| $\delta$ NH | | 7.36 | t (5.5) | H-35a, H-35b | C-34, C-35, C-36 | H-35a, H-35b |
| Pro-II | | | | | | |
| 37 | 174.60 (s) | | | | | |
| 38 | 62.96 (d) | 4.20 | dd (6.0, 9.0) | H-39a, H-39b | C-37, C-39, C-40 | H-39b |
| 39 | 30.91 (t) | 1.87 | m | H-38, $H_2$-40 | C-37, C-38, C-40, C-41 | |
| | | 2.26 | m | H-38, $H_2$-40 | C-37, C-38, C-40, C-41 | H-38 |
| 40 | 25.67 (t) | 1.93, 2H | m | H-39a, H-39b, H-41a, H-41b | C-38, C-39, C-41 | H-41a, H-41b |
| 41 | 49.09 (t) | 3.72 | m | $H_2$-40 | C-38, C-39, C-40 | $H_2$-40, H-43 |
| | | 3.98 | m | $H_2$-40 | C-38, C-39, C-40 | $H_2$-40, H-43 |
| Asn | | | | | | |
| 42 | 173.08 (s) | | | | | |
| 43 | 48.56 (d) | 4.95 | ddd (3.5, 9.0, 12.5) | H-44a, H-44b, Asn NH | C-42, C-44 | H-41a, H-41b, H-44a |
| 44 | 39.24 (t) | 2.65 | dd (3.0, 14.5) | H-43 | C-42, C-43, C-45 | H-43, $\gamma$ NHb |
| | | 3.21 | dd (12.5, 15.0) | H-43 | C-42, C-43, C-45 | |
| 45 | 175.09 (s) | | | | | |
| NH | | 8.73 | d (9.0) | H-43 | C-1 | H-2, H-44b, H-43 |
| $\gamma$ NHa | | 7.15 | bs | $\gamma$ NHb | C-44, C-45 | $\gamma$ NHb |
| $\gamma$NHb | | 7.98 | bs | $\gamma$NHa | C-45 | H-44a, $\gamma$NHa |

[a] Multiplicities determined using the DEPT pulse sequence. [b] With geminal protons, the smaller $\delta$-value is given the 'a' designation, the larger $\delta$-value is given the 'b' designation [c] Geminal couplings not shown. [d] Assignments may be reversed. [e] Signal obscured by solvent; assigned from HSQC data.

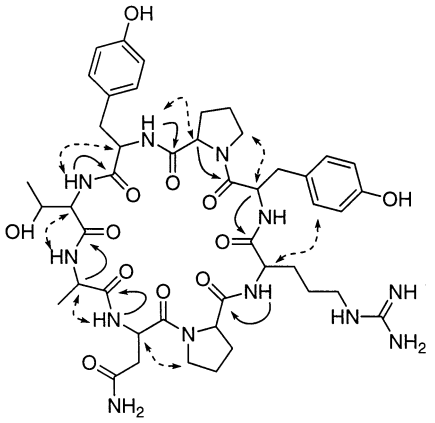

**Figure 1.** Key HMBC (→) and ROESY (<- - ->) correlations for cyclonellin (**1**).

the location of the proline residues. ROESY interactions were observed between the tyrosine-II H-α (δ 5.09) and the $H_2$-γ protons (δ 3.72, 3.77) assigned to proline-I and between the asparagine H-α (δ 4.95) and the proline-II $H_2$-γ protons (δ 3.72 and 3.98). These latter correlations helped establish the final Pro-Asn peptide bond, which allowed assignment of the macrocyclic structure of **1**. Mild acid hydrolysis (1.2 N HCl, 105 °C, 1 h) of the cyclic peptide followed by $C_{18}$ HPLC led to the recovery of a full length linear peptide, which resulted from cleavage of the Asn-Pro-II peptide bond, and two smaller fragments (Pro-Arg-Tyr and Pro-Tyr-Thr-Ala-Asn) formed by a subsequent cut at the Pro-I-Tyr-II linkage. Under these hydrolysis conditions asparagine was converted to aspartic acid, as evidenced by MS analysis and subsequent sequencing efforts. The amino acid sequence of the linear octapeptide and the two smaller peptide fragments was determined by automated N-terminal Edman degradation. These results were fully consistent with the assigned amino acid sequence of cyclonellin (**1**).

The absolute stereochemistry of cyclonellin was determined by complete acid hydrolysis of **1** and Marfey's analysis[13] of the resulting amino acids. The acid hydrolysate was derivatized with *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA), and then LC-MS comparison of the derivatives from **1** with the FDAA derivatives of appropriate D- and L-amino acid standards established that all of the residues in **1** had an L-configuration. Comparison of the threonine derivative obtained from **1** with FDAA derivatives of the four diastereomers of threonine confirmed the presence of L-threonine (2*S*, 3*R*) in cyclonellin.

Cyclonellin (**1**) was evaluated in an in vitro cytotoxicity assay[14] that utilized COLO-205 (colon) and OVCAR-3 (ovarian) human tumor cell lines, and it was inactive at a high test concentration of 50 μg/mL. This result was somewhat surprising, since cyclonellin was purified from a cytotoxic chromatography fraction that by $^1$H NMR did not appear to have other significant constituents present. However, there have been several previous reports of naturally occurring, proline-rich cyclic peptides that were initially described as having cytotoxic activity, but subsequent synthetic samples were not cytotoxic.[15−18] This apparent discrepancy has been attributed to potent trace contaminants that complexed with and co-purified with the natural peptides. In the case of cyclonellin, which is more polar than most other proline-rich cyclic peptides due to the presence of arginine, asparagine, threonine, and two tyrosine residues, the peptide was apparently separated from the other cyctotoxic constituent(s) during the final HPLC purification step. The identity of the cytotoxic agent that co-chromatographed with cyclonellin throughout most of the purification process is still unknown.

## Experimental Section

**General Experimental Procedures.** Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Beckman DU 640 spectrophotometer. IR spectra were obtained on KBr disks in a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. NMR spectra were acquired in $CD_3OH$ on a Varian INOVA spectrometer operating at 500 MHz for $^1$H and 125 MHz for $^{13}$C, and referenced to the residual nondeuterated solvent. FABMS were recorded on a JEOL SX102 mass spectrometer using glycerol or magic bullet as matrix. Electrospray mass spectra were obtained on a Hewlett-Packard HP1100 integrated LC-MS system. HPLC was performed on a Waters 600E system with a Waters 990 DAD, monitoring at 220 nm. Amino acid sequences were determined by sequential N-terminal Edman degradation using an Applied Biosystems model 494 sequencer according to the protocols of the manufacturer.

**Sponge Material.** Samples of the marine sponge *Axinella carteri* were collected by P. L. Colin (Coral Reef Research Foundation) off Chuuk, Micronesia. The sponge material was frozen shortly after collection and maintained frozen prior to extraction. A voucher specimen for this collection (0CDN1635) is maintained at the Smithsonian Institution, Washington, D.C.

**Extraction and Isolation.** The frozen samples were ground into a coarse powder (248 g) and extracted with $H_2O$ to yield 41.7 g of crude extract after lyophilization. A 6.0 g portion of the crude aqueous extract was dissolved in $H_2O$, applied to a wide-pore $C_4$ column (5 × 8 cm), and sequentially eluted with $H_2O$ (100%), $H_2O$−MeOH (2:1), $H_2O$−MeOH (1:2), and MeOH (100%) under vacuum. A 4.1 g aliquot of the 100% $H_2O$ fraction was taken up in 300 mL of $H_2O$ and partitioned with 3 × 300 mL of BuOH, and the resulting BuOH layers were combined and dried (548 mg). The BuOH fraction was purified by gel permeation chromatography on Sephadex LH-20 (3 × 100 cm) eluting with $H_2O$−MeOH (7:3), followed by $C_{18}$ HPLC (Dynamax, 8 μm particle size, 1 × 25 cm) employing a linear gradient from 0 to 60% MeCN in $H_2O$ (0.1% TFA) over 30 min (3 mL/min) to yield compound **1** (15.3 mg, $t_R$ 20.3 min).

**Cyclonellin (1):** amorphous white solid; $[\alpha]_D$ −92.8° (*c* 0.25, MeOH); UV (EtOH) $\lambda_{max}$ (log ε) 218 (4.44), 276 (3.88) nm; IR (film, KBr) $\nu_{max}$ 3278, 1668, 1516, 1450, 1203, 1133 cm$^{-1}$; $^1$H and $^{13}$C NMR data, see Table 1; FABMS, positive ion, *m/z* 963.5 $[M + H]^+$, *m/z* 985.5 $[M + Na]^+$; HRFABMS, CsI-doped sample, *m/z* 1095.3683 $[M + Cs]^+$, calcd for $C_{45}H_{62}N_{12}O_{12}Cs$, 1095.3701 (Δ −1.8 mmu).

**Acid Hydrolysis and LC-MS Analysis of FDAA Derivitives.**[13] The peptide (100 μg) was dissolved in 6 N HCl (constant boiling, 0.5 mL), degassed, and heated at 105−108 °C for 16 h under vacuum. The solvent was removed in vacuo, and the residue washed with $H_2O$ and dried in vacuo to give the hydrolysis product. The hydrolysis product (50 μg) was dissolved in 15 μL of 6% TEA (in MeCN−$H_2O$, 1:1) and treated with 7.5 μL of 1% *N*-(3-fluoro-4,6-dinitrophenyl)-L-alaninamide (FDAA) in acetone at 40 °C for 1 h. The reaction mixture was diluted with 45 μL of $H_2O$ and an aliquot applied to a $C_8$ column (Zorbax 300SB, 2.1 × 150 mm, 5 μm) eluting with a linear gradient (0−50%) of MeCN in 5% $MeCO_2H$ over 30 min (0.25 mL/min, 50 °C). FDAA-derivitized amino acids were detected by absorption at 340 nm and by MSD (positive ion, mass range 100−1000 Da) and compared with similarly derivatized commercially available amino acid standards. Amino acid standards with retention times (min) shown in parentheses: L-Ala (18.03), D-Ala (21.10), L-Arg (13.48), D-Arg (15.63), L-Asp (14.29), d-Asp (16.97), L-Pro (18.91), D-Pro (20.22), L-Thr (13.80), L-*allo*-Thr (14.86), D-Thr (18.82), D-*allo*-Thr (16.84), L-Tyr (21.83), D-Tyr (23.81). The amino acids observed and retention times (min) for the FDAA derivitives of the hydrolysate of **1**: L-Ala (18.00), L-Arg (13.17), L-Asp

(14.24, Asn is converted to Asp during acid hydrolysis), L-Pro (18.90), L-Thr (13.54), L-Tyr (21.83).

**Partial Hydrolysis of 1, LC-MS, and Edman Sequencing.** The peptide (0.1 mg) was dissolved in 200 $\mu$L of 1.2 N HCl and heated at 105 °C for 1 h. The solution was then diluted with 600 $\mu$L of $H_2O$ and stored frozen (−20 °C) prior to analysis. The hydrolysate (50 $\mu$L) was chromatographed in dupicate by linear gradient $C_{18}$ HPLC (Dynamax, 4.6 × 250 mm, 8 $\mu$m) employing 0−50% MeCN in $H_2O$ (0.1% TFA) over 30 min (1.5 mL/min). Individual peaks were collected from the first run and chromatographed by LC-MS to identify the linear peptide fragments: $[M + H]^+$ *m*/*z* 566.2, appropriate for Pro-Tyr-Thr-Ala-Asp(Asn), $t_R = 13.1$ min; $[M + H]^+$ *m*/*z* 435.2, Pro-Arg-Tyr, $t_R$=15.8 min; $[M + H]^+$ *m*/*z* 982, Pro-Arg-Tyr-Pro-Tyr-Thr-Ala-Asp(Asn), $t_R = 16.2$ min. The HPLC peaks corresponding to the full linear peptide and the two peptide fragments were collected in the second HPLC run and subsequently analyzed by N-terminal Edman degradation on a gas phase automated amino acid sequencer.

**Cytotoxicity Evaluations.** Chromatography fractions and purified compounds were dissolved in DMSO−$H_2O$ (1:1) and assayed in an in vitro cytotoxicity assay against colon (COLO-205) and ovarian (OVCAR-3) human tumor cell lines. Experimental details of the assay have been published elsewhere.[14]

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