Cyclocinamide A. An Unusual Cytotoxic Halogenated Hexapeptide from the Marine Sponge *Psammocinia*

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Characteristically, sponges from the family Thorectidae (order Dictyoceratida) contain a high proportion of terpenoids ranging from C15 and C20 to C25 types.[1] Thus, we do not normally seek members of this family during expeditions because our goal is to emphasize sponge-derived nitrogen compounds.[2] However, the thorectids are increasingly being recognized as a source of unusual nitrogen-containing compounds.[3] Surprisingly, during the routine screening of sponge extracts in a soft agar assay[4] designed to detect solid tumor selective compounds, extracts of *Psammocinia* sps. (Thorectidae) proved to be potent and partially selective in their cytotoxicity profiles. The scant previous literature on the genus *Psammocinia* describes only acyclic sesterterpenes containing both terminal tetronic acid and furan moieties.[5] Knowing that such compounds, including variabilin, were present in the extracts, but were not responsible for the activity results, prompted a bioassay-guided isolation of the active constituents. We now report the structure and cytotoxicity properties of cyclocinamide A (**1**), a minor hexapeptide of the sponge extract.

A sample of *Psammocinia* sp. 1 (coll. no. 90135) was obtained from the Milne Bay Province of Papua New Guinea (PNG).[6] A semipure fraction (WBS3) from the polar portion of the crude extract was selectively *in vitro* cytotoxic (at 35 μg/disk) to colon-38 tumor cells. This prompted several recollections of *Psammocinia* sp. 1 (coll. no. 91113, 93105) plus a collection of *Psammocinia* sp. 2 (coll. no. 93145) from Normanby and the Milne Bay regions of PNG. The polar extracts of the additional 3 collections were also each solid tumor selective to C38, and variabilin[1] was a major noncytotoxic constituent of all 4 sponges. Further workup of the coll. no. 93145 extract eventually yielded cyclocinamide A (**1**) ($[\alpha]_D$ = + 29°, *c* 0.10, MeOH) as an amorphous solid (6.4 mg, 0.005% of extract), which was partially responsible for the crude extract cytotoxicity. In order to achieve its isolation, the methanol extract was first partitioned according to our standard procedure.[7] The subsequent 50% aqueous MeOH solvent partition fraction was further purified as guided by toxicity to brine shrimp[8] and tumor cells. This consisted of chromatography on Sephadex LH20 (50:50, MeOH/CH_2Cl_2) with final purification of the bioactive fractions by HPLC (ODS, 41:7:9:43, MeOH/THF/MeCN/H_2O).

The structure elucidation commenced by establishing the molecular formula of $C_{29}H_{33}BrClN_9O_8$ from the HRFAB MS $[MH]^+$ 750.1406 (Δ0.4 mmu of calcd). The isotope cluster for the MH^+ peak exhibited a pattern characteristic of BrCl. Data contained in the ^{1}H and ^{13}C DEPT-135 NMR spectra[9] indicated 1 methyl, 5 methylene, 10 methine, and 13 quaternary carbons totaling $C_{29}H_{23}$ plus 10 heteroatom protons consisting of 9 *N*H's and 1 assumed to be an OH. The 17 unsaturations evident in the molecular formula were divided as follows. There were 7 carbonyls (7 ^{13}C NMR resonances δ 160.9−172.7 and IR 1670 cm^{-1}) and 6 trisubstituted double bonds (12 ^{13}C NMR resonances δ 108−135, 6 ^{1}H NMR resonances δ 6.87−7.67), leaving 4 rings. A polypeptide was probable from the 8 *N*H proton resonances in the region δ 6.8−8.4, and an indole moiety was intimated from the diagnostic *N*H at δ 11.10.

The task of assembling a list of substructures (**A**−**F**) was accomplished once a series of 2D NMR spectra (COSY, HMQC, and HMBC; *J* optimized for 9 Hz) were in hand. Substructure **A** (Figure 1) was identified as a 5′-bromotryptophan residue by comparison of the NMR shifts, especially at CH23 (δ 113.4/7.30 (d), J = 8.5 Hz), C25 (δ 111.1), and CH26 (δ 120.7/7.67 (d), J = 2 Hz), to those of models.[10] Additional affirmation of **A** was obtained from key ^{1}H-^{1}H COSY NMR (*N*H6 ↔ H7) and HMBC NMR correlations (C19, C20, C22, C27 → *N*H21; C7, C19 → H$_2$18/18′; C8 → H7; C19 → H20) as well as chiral TLC. The 1,2-diamino moiety **B** was established from the coupled 5-spin system identified in the ^{1}H−^{1}H COSY NMR spectrum (Table 1S, Supporting Information). The glycine residue shown as **C** was also readily assembled by the 3-spin system identified by ^{1}H−^{1}H COSY plus additional HMBC NMR correlations (from C29 and C32 to H$_2$30/30′). The uncommon isoserine residue **D** was recognized by first assigning the alcohol functionality to CH4 (δ 69.7/4.04) which was proximal to CH$_2$3−*N*H2 and C5 based on 2D NMR data. The hydroxy proton, not visible in the ^{1}H NMR spectrum (DMSO-d_6, 500 MHz), plus H$_2$3 and H$_2$10, partially obstructed by the

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(9) ^{13}C NMR (125 MHz, DMSO-d_6) δ: 172.7 (s, C8), 171.9 (s, C16), 170.9 (s, C5), 170.1 (s, C1), 169.2 (s, C29), 168.8 (s, C12), 160.9 (s, C32), 134.8 (s, C22), 129.1 (s, C27), 125.3 (d, C20), 124.9 (s, C33), 124.9 (d, C36), 123.4 (d, C24), 120.7 (d, C26), 113.4 (d, C23), 111.8 (d, C34), 111.1 (s, C25), 109.6 (s, C19), 108.6 (s, C35), 69.7 (d, C4), 54.3 (d, C11), 53.3 (d, C7), 49.5 (d, C14), 42.8 (t, C3), 42.3 (t, C30), 40.3 (t, C10), 36.5 (t, C15), 36.4 (q, C38), 27.8 (d, C18). ^{1}H NMR data in CD_3OD or DMSO-d_6 plus ^{1}H−^{1}H COSY, HMBC (J = 9 or J = 4 Hz) are in Table 1S (Supporting Information).

(10) Table 3S (Supporting Information).

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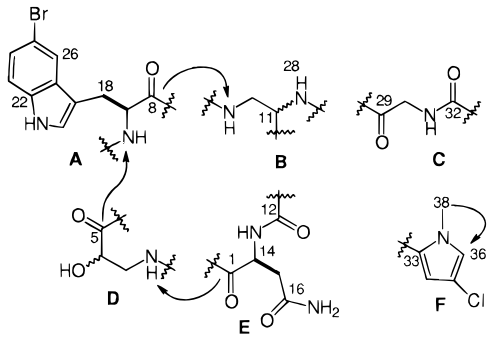
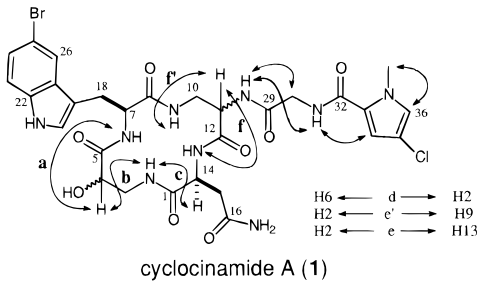


Figure 1. Partial structures **A**−**F** of cyclocinamide A (**1**). Arrows indicate diagnostic HMBC correlations with J_{CH} optimized for 4 Hz.



cyclocinamide A (**1**)

Figure 2. Important NOE correlations for **1**.

water signal, were all clearly observed by water suppression (45 °C, DMSO-d_6, 500 MHz; see the Supporting Information). Substructure **E**, an extended asparagine residue, was assembled by the ^{1}H−^{1}H COSY data which identified the spin system of *N*H13−H14−H$_2$15, by the 2D HMBC correlations from C12 to *N*H13 and by the HMBC correlations from C1/C16 to H$_2$15. Additionally, the nonequivalent *N*H$_2$17 protons exhibited the characteristic chemical shifts of a primary amide functionality (δ 6.80 and 7.28). The remaining atoms, C$_5$H$_5$NCl, were joined together in array **F**. HMBC correlations from H34 and H36 to the 4 vinyl carbons established the conjugated diene containing 2 Hs (J = 2 Hz), which were envisioned to be part of a pyrrole ring. While the *N*Me had to be attached to CH36, as supported by HMBC data, a clear decision could not be made about the placement of Cl at C34 or C35.

The connections shown in **1** between substructures **A**−**F** were achieved by analysis of additional HMBC and NOESY NMR data. Especially important was an HMBC experiment where J was optimized for 4 Hz[11] (Figure 1 and Supporting Information) and as shown these data allowed the substructures to be sequenced as a **E**−**D**−**A**−**B** fusion. The final connection between C11 and C12 to complete the 14-membered ring of cyclocinamide A (**1**) was made because the shift of CH11 (δ 54.3/4.33) indicated this was an α-position of an amino acid and that C12 (δ 168.8) was a normal amino acid type carbonyl. Similarly, the shift of C29 (δ 169.2) suggested it was also a normal amide carbonyl presumably attached to *N*28. Especially important were the strong NOEs from *N*H28 to H$_2$30 and *N*H31 (Figure 2). The fusion of **C** and **F** to complete the side chain at C11 was addressed next. The shift of the amide carbonyl C32 (δ 160.9) indicated it was adjacent to an unsaturated moiety which could be accommodated by attaching it to C33 of **F**, and also confirmed that C32 was not connected to C11 of **B**. The **C**−**F** substructure is analogous to that found in mauritamide A (with a C=O at δ 159.7 and *N*Me at δ 35.4).[12] Finally, the NOE from *N*H31 to H34 meant that the Cl had to be attached to C35 and further supported the union of **C** and **F**.

(11) Generally, only two bond *N*H to C=O HMBC correlations are observed in medium-sized cyclic peptides: Kessler, H.; Oschkinat, H.; Loosli, H.-R. In *Two-Dimensional NMR Spectroscopy-Applications for Chemists and Biochemists*, Croasmun, W. R., Carlson, R. M. K., Eds.; VCH: New York, 1987; pp 259−297.

The issue of establishing the absolute stereochemistry of the 4 chiral centers was considered using several approaches. The most definitive data was derived from chiral TLC[13] analysis which allowed 7(*S*),14(*S*) stereochemistry to be established. Acid hydrolysis of cyclocinamide A (0.1 mg) was undertaken with 6 N HCl in the presence of phenol (3% v/v) to prevent degradation of the indole ring in tryptophan.[14] Chiral TLC R_f values of the hydrolysate identified (*S*)-bromotryptophan and (*S*)-asparagine.[15] The possibility that the 6 NOE cross-peaks (a, b, c, d, e/e′, f/f′) and 4 vicinal $^3J_{3,4}$, $^3J_{10,11}$ couplings observed (Figure 2 and Supporting Information) could be used to assign the remaining stereocenters at C4 and C11 was pursued. The plan was to compare the distance and dihedral angle constraints against values predicted by computer molecular modeling for the minimum-energy conformations of the 4 possible diastereomers: 4(*R*)/11(*R*), 4(*R*)/11(*S*), 4(*S*)/11(*R*), 4(*S*)/11(*S*). However, efforts to assign stereochemistry by this approach proved inconclusive because each diastereomer provided a fit to the experimental data (Table 2S, Supporting Information). The minute quantity of **1** remaining prevented further experiments. Thus, the absolute configurations at C4[16] and C11 are unassigned but might be suspected as being *S* on the basis of biogenetic arguments.

The isolation of **1** was greatly facilitated by the favorable cytotoxicity profiles of semipure extract fractions in the soft agar disk diffusion assay. For example, a fraction (FMS4) of coll. no. 93145 displayed zone sizes in the primary assay[4,17] as follows: [μg/disk, L1210/C38/M17-Adr/CX1] 100, 400/940/580/400 vs 5-fluorouracil (standard chemotherapeutic agent); [μg/disk, L1210/C38/CX1] 2.5, 40/900/0 (which both represent significant selective cytotoxicity). Purified **1** exhibited the following profile, [μg/disk, L1210/C38/M17-Adr/CX1] 50, 0/500/0/0, and retained the robust tumor selectivity against C38 observed in the crude extract fractions. Only 2 mg of **1** was available for follow-up via an *in vivo* experiment. Thus, a single mouse with early stage M-16 (mammary adenocarcinoma no. 16) was given a total dose of 20 mg/kg, but the response was no activity and no toxicity. Such experiments will be continued in the future using material being prepared by total synthesis in order to test for antitumor activity *in vivo* at the maximum tolerated dose.

Overall, the structure of cyclocinamide A has no parallel among known natural products. Its tetrapeptide core, consisting of a 14-membered ring, along with the dipeptide side chain terminating in the proline-derived *N*-methylchloropyrrole are both extremely distinctive.

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Supporting Information Available: 1D NMR (^{1}H, ^{13}C, and DEPT 135) and 2D NMR (COSY, HMQC, HMBC, NOESY), Table 1S (1D and 2D ^{1}H and ^{13}C NMR data), FABMS, and Table 2S−4S (18 pages). See any current masthead page for ordering and Internet access instructions.

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(15) Table 4S (Supporting Information).
(16) For L-(*S*)-isoserine, see: Williams, T. M.; Crumbie, R.; Mosher, H. S. *J. Org. Chem.* **1985**, *50*, 91−97 and references within.
(17) A zone differential of 250 (=6.5 mm) units or greater is the basis for denoting an agent as either a solid tumor or a leukemia-selective compound. A 1 log dilution produces a 330 unit zone change on average. Thus, a 250 unit differential represents approximately an 8-fold change in sensitivity.