Milnamide A, an Unusual Cytotoxic Tripeptide from the Marine Sponge *Auletta* cf. *constricta*[1]

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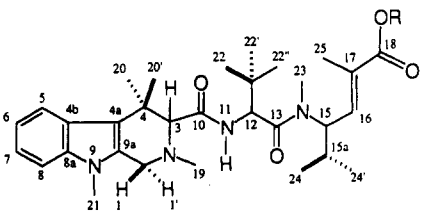
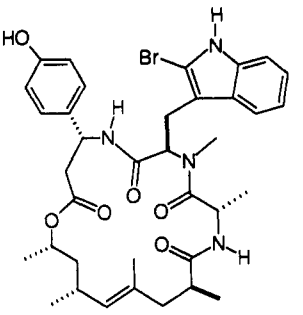
Summary: A highly methylated cytotoxic tripeptide, milnamide A (1), along with jasplakinolide (3) was isolated from Papua New Guinean collections of the Axinellida sponge *Auletta* cf. *constricta*.

Physiologically active nitrogen-containing compounds such as amino acid derivatives, alkaloids, or ketide amino acids are occasionally isolated from marine sponges. Collecting taxa which might possess these structurally interesting substances as chemotaxonomic markers represents one way to encounter them. In this context, the Choristida (*a.k.a.* Astrophorida) order of sponges has been a repeated source of ketide amino acids,[2a-c,4b] alkaloids,[2d,e,3,4c] and peptides.[4a] Isolation work on sponge extracts guided by cytotoxicity assays or mechanism-based screens have also occasionally uncovered these structural types with recent examples being reported by our group[5-8] as well as by other laboratories.[9-13] We now outline results to extend this structural motif. The cytotoxic constituents of an Axinellida sponge unexpectedly included a new tripeptide, milnamide A (1), accompanied by a known Choristida sponge ketide amino acid, jasplakinolide (3).



1 R = H
2 R = CH_3



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Chemical study of a beautiful orange sponge, *Auletta* cf. *constricta* (Axinellidae), obtained from the Milne Bay province of Papua New Guinea,[14] began when its crude extract exhibited substantial *in vitro* cytotoxicity to HT-29 cells. This sponge has been depicted underwater in an oversized book on the Great Barrier Reef[15] but was misidentified as *Clathria* sp. Solvent partitioning of the crude extract concentrated the HT-29 activity into the CH_2Cl_2 fraction whose NMR spectrum displayed resonances characteristic of jasplakinolide (3). Additional purification of the HT-29 active CH_2Cl_2 fraction afforded 3 as a major constituent (yield = 0.21%, dry weight) accompanied by 1 (yield = 0.016%, dry weight) which was clearly unrelated to 3 as its ^1H NMR (Figure 1) dramatically showed a *tert*-butyl group but not the presence of a tyrosine moiety.

A most unusual feature of the ^1H NMR spectrum of milnamide A (1)[16] was that eight methyl groups could be seen in addition to the *tert*-butyl group. The ^{13}C NMR plus the HMBC spectra revealed a total of 44 protons attached to carbons ($9CH_1$, $1CH_2$, and $11CH_3$) plus 10 carbons as CH_0 types. Intense peaks observable by HREIMS (40 eV), M^+ = 538.3511, or LRFABMS, $[M + H]^+$ = 539, corresponded to a molecular formula of $C_{31}H_{46}N_4O_4$ (Δ0.8 mmu of calcd) whose 11 unsaturations were ascribed to three carbonyls (^{13}C δ 171.9, 171.5, 170.3), five double bonds (CH_1 types: 140.3, 120.6, 119.5, 118.7, 108.9; CH_0 types: 137.4, 131.9, 131.7, 125.1, 113.3), and

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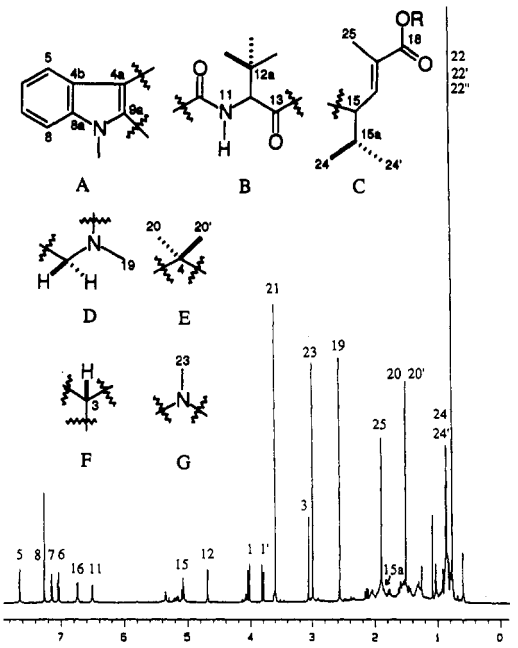


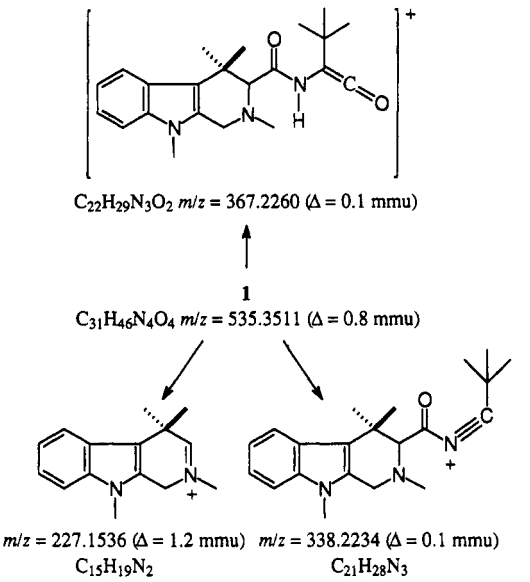
Figure 1. ^{1}H NMR of milnamide A (1) at 500 MHz.

three rings. The two heteroatom protons (HREIMS tally of H_{46}, APT count of H_{44}) were eventually assigned to secondary amino and carboxylic acid resonances. An exchangeable NH was observed to be vicinally coupled to H12. The carboxyl at δ 170.3 displayed two intense HMBC correlations to the vinylic H16 and Me25 groups, and chemical evidence for the carboxylic acid moiety was provided by conversion of 1 to its corresponding methyl ester **2**. An observation that was at first perplexing concerned the CH group with NMR chemical shifts of δ 75.6(d)/3.04(s) in the range consistent with an oxygen attachment. This group was eventually concluded to be attached to nitrogen and part of a β-carboline residue based on long-range HMBC correlations from it (H3) to C1/C4/C4a/C10/Me20 as shown in Figures S2a and S2b (supplementary material).

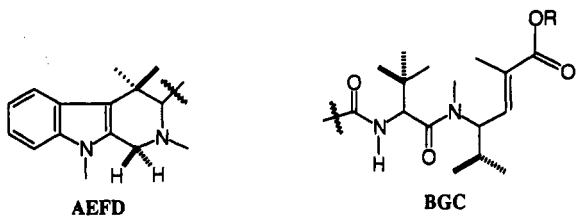
Three major substructures **A–C** were assembled based on first ^{1}H–^{1}H COSY[16] and TOCSY[16] NMR spectra, which showed three separate sets of mutually coupled proton spin systems. The HMBC data of Figure S2 (supplementary material) were next used to add the *N*Me to the indole of **A** and the *tert*-butyl and carbonyl groups to **B** and confirmed the placement of the vinyl Me in **C**. An additional fragment, **D**, was identified from HMBC correlations, and the fragments **E–G** accounted for the remaining atoms.

(16) Milnamide A (1): $[\alpha]^{27}_D$ + 28.8 (*c* 0.50, CH_2Cl_2); UV λ_{max} (MeOH) 223 (ϵ 14 700), 285 (ϵ 2810), 294 (ϵ 3070), 304 (ϵ 3030), 380 (ϵ 1260) nm; IR (film) ν_{max} 3319, 2964, 2864, 1680, 1634, 1470, 1368, 1244, 1085 cm^{-1}; HREIMS M^+ 538.3511 = $C_{31}H_{46}N_4O_4$ (Δ 0.8 mmu of calcd); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (1H, d, *J* = 7.9 Hz, H5), 7.26 (1H, d, *J* = 8.1 Hz, H8), 7.15 (1H, dd, *J* = 6.8 and 8.1 Hz, H7), 7.03 (1H, dd, *J* = 6.8 and 7.9 Hz, H6), 6.72 (1H, d, *J* = 10 Hz, H16), 6.52 (1H, d, *J* = 8.8 Hz, H11), 5.12 (1H, t, *J* = 10 Hz, H15), 4.75 (1H, d, *J* = 8.8 Hz, H12), 4.02 (1H, d, *J* = 14.9 Hz, H1), 3.83 (1H, d, *J* = 14.9 Hz, H1′), 3.59 (3H, s, H21), 3.04 (1H, s, H3), 2.98 (3H, s, H23), 2.60 (3H, s, H19), 1.89 (3H, s, H25), 1.89 (1H, m, H15a), 1.48 (6H, s, H20), 0.86 (3H, d, *J* = 6.9 Hz, H24), 0.82 (3H, d, *J* = 6.9 Hz, H24′), 0.81 (9H, s, H22); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.9 s (C13), 171.5 s (C10), 170.3 s (C18), 140.3 d (C16), 137.4 s (C8a), 131.9 s (C17), 131.7 s (C9a), 125.1 s (C4b), 120.6 d (C7), 119.5 d (C5), 118.7 d (C6), 113.3 s (C4a), 108.9 d (C8), 75.6 d (C3), 56.4 d (C15), 54.7 d (C12), 48.3 t (C1), 43.8 q (C19), 35.4 s (C4), 34.7 s (C12a), 31.2 q (C23), 30.1 q (C20), 29.7 d (C15a), 29.2 q (C21), 26.4 q (C22), 24.9 q (C20′), 19.3 q (C24), 19.1 q (C24′), 13.7 q (C25). For additional NMR data see Figures S1 (^{13}C NMR), S2 (HMBC), S3 (^{1}H–^{1}H COSY), and S4 (^{1}H–^{1}H TOCSY) (supplementary material); for additional MS data see Scheme 1.

Scheme 1. Mass Spectral Behavior of Milnamide A (1)



Several redundant HMBC NMR correlations were used to join the substructures introduced above. Two larger moieties, **BGC** and **AEFD**, were assigned from HMBC



correlations from $H_3$23 to C13 and C15, from $H_3$19 to C3, from $H_3$20 to C3 and C4a, and from H1/1′ to C9a. The clear HMBC correlation observed from H3 to C10 justified the merging of this final pair of substructures as shown in the complete structure of 1. Additional support for this overall structure was provided by the HREIMS spectral fragmentation pattern summarized in Scheme 1. Very limited stereochemical elements could be defined at this point. The ROESY correlations to H3 suggested that this proton was axial.

Milnamide A (1) has a skeleton without prior precedent as it incorporates a β-carboline joined to a dipeptide fragment. There is a distant structural analogy between 1 and the nephilatoxins from spiders which are potent neurotoxins.[17] The β-carboline moiety of 1 is highly methylated and is unlike any carboline described in the literature.[18] This substructure is present in only two other families of sponge-derived alkaloids which includes the manzamines[19] and the reticulatines.[20] To be investigated in the future is the absolute chirality of the additional biosynthetic subunits. These include the *tert*-leucine (=

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pseudoleucine), which has been observed in the discodermins,[21] and the new vinylogous amino acid[22] we call homo vinylogous valine (homo V-val), comparable to V-tyr found in the cyclotheonamides[23] and synthetic carbamates.[24]

There are additional aspects about our work on *Auletta* that require further comment. The cytotoxicity bioassay guided us to the isolation of two structurally diverse cytotoxins. Jasplakinolide (3) has quite interesting antitumor properties and is selectively potent against three types of cell lines: CNS, renal, and prostate. Recently, preliminary *in vivo* results have shown that jasplakinolide can provide a 28% T/C reduction of PC3 prostate tumors in mice. Alternatively, milnamide A appears to be broadly cytotoxic as it exhibited *in vitro* IC_{50}'s μg/mL of 4.1 (A549), 2.8 (HT-29), 3.3 (B16/F10), and 0.74 (P388). Finally, it was unexpected to find that both a Halichondrida and an Astrophorida sponge are a source of jasplakinolide. This observation does, however, parallel an intriguing circumstance that the geodiamolides are observed from both Astrophorida and Halichondrida sponges,[25] and overall these observations are in contrast to the patterns of sponge chemotaxonomy[26] where certain biosynthetic classes are often restricted to a single taxonomic order.[27]

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Supplementary Material Available: Figures S1–S4 (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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