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A Further Study of the Cytotoxic Constituents of a Milnamide-Producing Sponge

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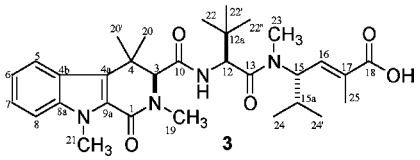
ABSTRACT



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A reinvestigation of *Auletta* sp. yielded the novel compound milnamide C (3) plus the known compounds milnamide A (1), milnamide B (hemiasterlin) (2), jasplakinolide (5), and geodiamolides A (6), D (7), E (8), and G (9). The isolation work was guided by cytoskeletal bioactivity data. Compounds 2 and 3 were shown to cause microtubule depolymerization, and 6–9 were shown to cause microfilament disruption. This biological activity and the structural elucidation of 3, including X-ray analysis, are reported here.

The alkaloids of marine sponges continue to be a fertile source of inspirational bioactive compounds.[1] Among the many that can be cited, there are three compounds that are of interest to our laboratory. First are the milnamides,[2,3] of nonribosomal peptide biosynthetic origins, isolated from various sponge sources including *Auletta* sp.,[2a,b,4] *Siphonochalina* spp.,[2b] *Hemiasterella minor*,[2c] and *Cymbastela* sp.[2d−f] Second is jasplakinolide[5a] (jaspamide)[5b] (**5**), originally isolated from *Jaspis splendens* in 1986 and subsequently found in other marine sponge genera including *Auletta* sp.,[2a] *H.*

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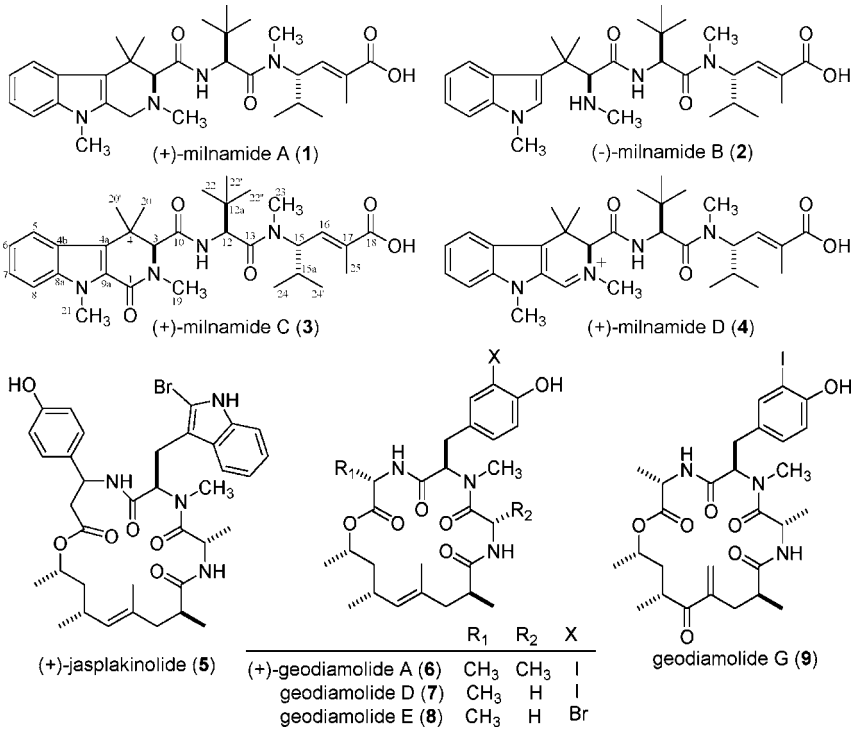

Figure 1. The different array of compounds isolated from *Auletta* sp.

minor,[2c] and *Cymbastela* sp.[2e] Third are the geodiamolides,[2c,d,f,6] which are unique because they have been isolated from both Caribbean[6a,b] and Indo-Pacific sponges.[2c,d,f,6c] This report outlines the isolation and structure elucidation of the novel compound milnamide C (**3**), along with the known compounds milnamides A (**1**) and B (hemiasterlin) (**2**), jasplakinolide (**5**), and geodiamolides A (**6**), D (**7**), E (**8**), and G (**9**) from two collections of *Auletta* sp. from Papua New Guinea (Figure 1). It was prompted by the recent communication reporting milnamide D (**4**).[2e]

Understanding the NMR and MS properties of **1** provided the basis for the characterization of the other analogues. The ^{1}H NMR spectra containing eight distinct methyl groups in addition to the *tert*-butyl group are extremely diagnostic of the structural backbone seen in **1**. In addition, the HREIMS $m/z = 538.3511$ $[M]^{+\bullet}$ (Δ 0.8 mmu of calcd for $C_{31}H_{46}N_4O_4$) plus the fragmentation cascade to $m/z = 367.2260$ ($C_{27}H_{29}N_3O_2$) and 227.1536 ($C_{15}H_{19}N_2O$) provides a signature for the presence of the β-carboline substructure seen in **1**.[2a]

Our structural analysis of milnamide B (**2**) began with the HRFABMS $m/z = 527.3601$ $[M + H]^+$ (Δ −0.4 mmu of calcd for $C_{30}H_{46}N_4O_4$). The DEPT spectra indicated that **2** did not contain the CH_2 group present in **1**, which is consistent with a proposal of a missing β-carboline ring. The remaining properties were in concurrence with that of hemiasterlin, reported from *H. minor*.[2c] Furthermore, this compound was cited to have an $[\alpha]_D = -95°$ (*c* 0.06, MeOH),[2c] whereas the same compound from *Cymbastela* sp. was found to have an $[\alpha]_D = -76°$ (*c* 0.07, MeOH)[2d] and we found our sample of **2** to have an $[\alpha]_D = -90°$ (*c* 0.08, CH_2Cl_2). Because the specific rotations for all three samples have the same sign and their NMR data are indistinguishable, it is clear that all three compounds are the same. Although the stereocenter at C-3 was not assigned in the original publications, the subsequent total synthesis resolved this point.[3a]

Milnamide C (**3**) was obtained as a yellow solid, and the molecular formula of **3** was established by HRESITOFMS to be $C_{31}H_{44}N_4O_5$ (m/z 553.3335 $[M + H]^+$, Δ −5.0 mmu of calcd). Compared with the molecular formula of **1**, there was the loss of two protons and the gain of one oxygen, as well as one additional degree of unsaturation. The NMR data of **3** (Table 1) was very similar to that of **1** and **2**. The most striking difference in the NMR data of **3** compared to that of **1** was the lack of an AB pattern, indicative of the C-1 methylene in **1**, which was replaced by a new low field signal for a carbonyl at δ 163.9 in **3**. On the basis of these data a carbonyl-containing tetrahydro-β-carbolinone moiety was proposed for **3**. Key gHMBC correlations observed from H-3 (δ 4.11) to C-1 (δ 163.9) and H-19 (δ 34.3) to C-1 (δ 163.9) facilitated the assignment of the carbonyl between N-2 and C-9a. The assignment of the carbonyl at C-1 was supported

(4) Taxonomic identification of this new species was performed by Dr. R. van Soest. He identified this sponge as an *Auletta* sp. 1. Taxonomic details will be provided at a later date in a full paper (personal communication).

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Table 1. NMR Data of Milnamide C (**3**) in MeOH-d_4[a]

no.	δ_C	δ_H (J in Hz)	^{1}H−^{1}H COSY	gHMBC[b]
1	163.9			
3	75.5	4.11 s		1, 4a, 4, 10, 20
4	37.4			
4a	125.3			
4b	124.5			
5	122.4	7.70 ddd (0.9, 0.9, 8.3)	6	8a, 4a
6	121.2	7.06 ddd (0.9, 7.1, 8.3)	5, 7	4b
7	125.5	7.27 ddd (1.2, 7.1, 8.1)	6, 8	8a, 5
8	111.6	7.42 ddd (0.9, 0.9, 8.6)	7	4b, 6
8a	141.0			
9a	126.0			
10	171.5			
11		8.03 d (8.9)[c]		
12	56.8	4.77 d (9.1)[c]		12a, 13, 22
12a	36.3			
13	172.7			
15	58.4	4.96 dd (10.1)	15a, 16	
15a	31.0	1.84 m	15, 24, 24′	
16	138.4	6.60 dd (1.4, 9.5)	15, 25	
17	134.9			
18	172.3			
19	34.3	3.03 s		1, 3
20	30.8[d]	1.49 s		3, 4, 4a, 20′
20′	24.9[d]	1.64 s		3, 4, 4a, 20
21	31.9	4.09 s		8a, 9a
22	27.0	0.95 s		12, 12a, 22
23	31.7	2.91 s		13, 15
24	19.9[e]	0.81 d (6.6)	15a	15, 15a, 24′
24′	19.6[e]	0.60 d (6.6)	15a	15, 15a, 24
25	14.6	1.86 d (1.4)	16	16, 17, 18

[a] Recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C. [b] Proton showing correlation to indicated carbon. [c] Deuterium exchange resulted in the loss of the signal for H-11 and the loss of the multiplicity of H-12. [d,e] Assignments are interchangeable.

by the downfield shift of C-4a in **3** as would be expected for the β position in an α,β-unsaturated carbonyl. The other logical alternative of locating C-1 between N-2 and C-3 was ruled out on the basis of calculated ^{13}C chemical shifts for two model substructures (**3i** and **3ii**) (Figure 2).[7]

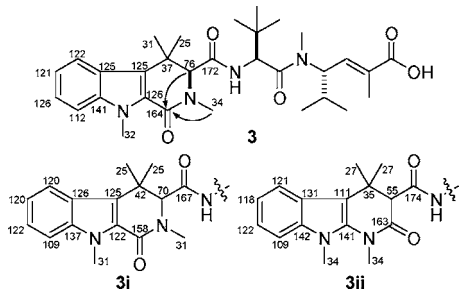

Figure 2. Selected gHMBC correlations, experimental ^{13}C NMR data, and calculated ^{13}C NMR data for model substructures of milnamide C (**3**).

After considerable effort, suitable crystals were obtained, thus allowing for X-ray crystallographic analysis that confirmed our proposed structure for **3** (CCDC No. 228754). The ORTEP diagram (Figure 3) revealed that the relative stereochemistry of **3** is consistent with that previously published for **2**.[2d,g] Further, the absolute stereochemistry that is shown for **1** and **3** in Figure 1 is based on the conclusions derived from the total synthesis of **2**.[3a] In view of these conclusions it is appealing to consider that the absolute stereochemistry of milnamide D (**4**) should be as shown here (Figure 1).

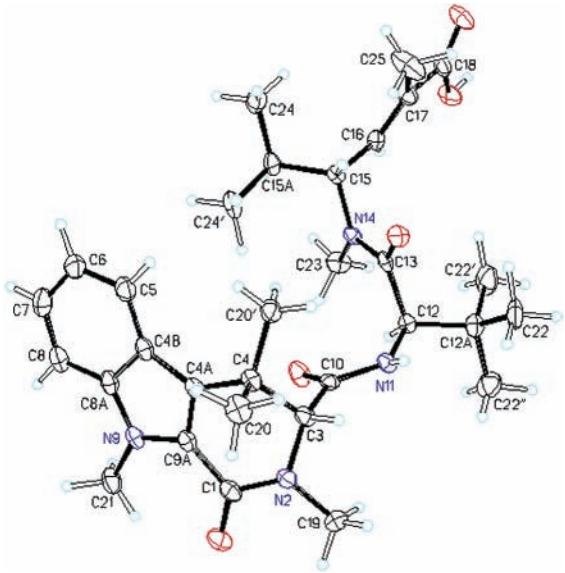

Figure 3. X-ray crystal structure of **3**. Crystallographic data have been deposited at the Cambridge Crystallographic Data Center (CCDC No. 228754). Copies of the data can be obtained free of charge at http://www.ccdc.cam.ac.uk/.

The remaining six compounds isolated consisted of the known compounds milnamide A (**1**), jasplakinolide (**5**), and geodiamolides A (**6**), D (**7**), E (**8**), and G (**9**). The isolation work of **1** was previously reported,[2a] and **5**−**9** were all identified on the basis of comparison of the NMR and HRMS data obtained to that in the literature.[2d,5a,6a,c] Although the milnamides and geodiamolides have been found co-occurring in both *H. minor*[2c] and *Cymbastela* sp. sponges,[2d,f] this is the first report of them from an *Auletta* sp. sponge. On the basis of the morphological similarities of the *Cymbastela* sp. and *Auletta* sp. sponges[4] and the chemotaxonomic data presented here, a revision in their taxonomy may be warranted.

The last aspect of our study was to evaluate **2**, **3**, and **6**−**9** for biological activity in a cytoskeletal assay, as well as to evaluate **2**, **3**, **7**, and **8** for cytotoxicity toward MDA-MB-435 cancer cells.[8,9] Table 2 presents a comparison of this activity, and Figures 4 and 5 shows the cytoskeletal disruption observed with **2**, **3**, and **8**. Our results are consistent with those of previous studies[3] showing hemiasterlin (mil-

(7) *ACD/CNMR Predictor*, version 2.51; Advanced Chemistry Development, Inc.: Toronto, Canada, 1997.

(8) Tinley, T. L.; Randall-Hlubek, D. A.; Leal, R. M.; Jackson, E. M.; Cessac, J. W.; Quada, J. C., Jr.; Hemscheidt, T. K.; Mooberry, S. L. *Cancer Res.* **2003**, *63*, 3211−3220.

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Table 2. Cytoxicity and Cytoskeletal Bioactivity Data of **2**, **3**, and **6**−**9**[8,9]

compound	IC_{50} in MDA-MB-435 μg/mL, $n = 3$	mechanism of action
milnamide B (**2**)	$1.48 (\pm 0.4) \times 10^{-4}$	microtubule depolymerization
milnamide C (**3**)	0.32 ± 0.02	microtubule depolymerization
geodiamolide A (**6**)	nd[a]	microfilament disruption
geodiamolide D (**7**)	0.08 ± 0.03	microfilament disruption
geodiamolide E (**8**)	0.25 ± 0.05	microfilament disruption
geodiamolide G (**9**)	nd[a]	microfilament disruption

[a] Not determined.

namide B) (**2**) to be a potent antimitotic agent that induces microtubule depolymerization similar to that caused by vinblastine. As expected, we observed **2** to induce classic microtubule depolymerization, as evidenced by total loss of cellular microtubules yet retention of cellular shape (Figure 4C) when compared to control (Figure 4A). Micronucleation

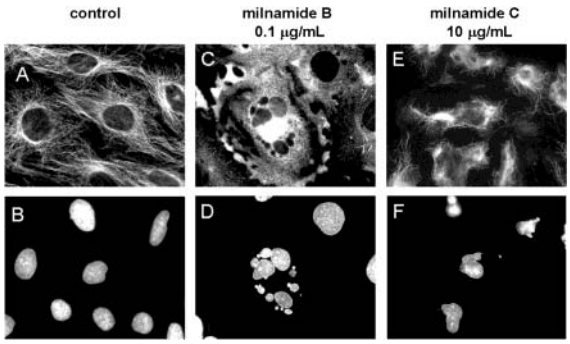

Figure 4. Effects of milnamide B (**2**) and C (**3**) on microtubules (C and E) and on nuclear structure (D and F). Compound-induced changes in microtubules were evaluated by immunofluorescence techniques.[8]

was also observed in cells treated with **2** (Figure 4D). Micronucleation is the breakdown of the nucleus into smaller membrane-bound fragments and is a hallmark of microtubule disruptors. Also as expected, milnamide C (**3**) displayed microtubule cytoskeletal activity as evidenced by a loss of cellular microtubules at the cell periphery (Figure 4E). This

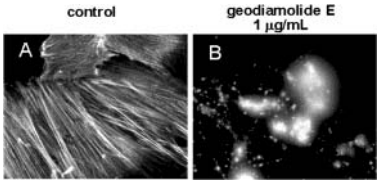

Figure 5. Microfilament disruption induced by geodiamolide E (**8**). Changes in microfilaments were evaluated using a phalloidin assay as described previously.[9]

is consistent with the effects of low concentrations of microtubule depolymerizers, yet this compound also caused some cell shape changes and cellular shrinkage concomitant with nuclear shrinkage (Figure 4E and F). Compound **3** is significantly less potent than **2** against MDA-MB-435 cancer cells with IC_{50} values of 3.2×10^{-1} and 1.48×10^{-4} μg/mL, respectively. The four geodiamolides (**6**−**9**) caused disruption of the cellular microfilament network. Compounds **7** and **8** induced microfilament disruptions (Figure 5 and data not shown) and were effective inhibitors of cellular proliferation in MDA-MB-435 cancer cells with IC_{50} values of 8.0×10^{-2} and 2.5×10^{-1} μg/mL, respectively. Compounds **6** and **9** were also tested and caused microfilament disruption (Table 2); however, insufficient material was available to allow for IC_{50} determination.

One interesting aspect of our research on *Auletta* sp. sponges is that **1**−**3** have been found in collections obtained from Milne Bay, Papua New Guinea, but not in collections from the Solomon Islands. However, **5** was seen in all of these collections.[10] Another interesting note is that the 2,3,4,9-tetrahydro-β-carbolin-1-one moiety seen in milnamide C (**3**), although very rare in sponge-derived alkaloids, has been observed in compounds from *Hyrtios*[1a] and an undescribed *Petrosiidae* sponge.[11] Additionally, the saturated β-carboline moiety seen in milnamide A (**1**) is not common in sponge-derived compounds and has been found in a small number of sponge metabolites including keramamine C.[12] A regular β-carboline substructure is more common and is present in a number of compounds including the manzamines[1b,13] and the reticulatines.[1c] There is one additional important difference among these metabolites. The extensive methylation in the milnamides suggests a biosynthetic pathway that should be of interest for further genetic manipulation especially with regards to the many methyl-transferases at work.[14]

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Supporting Information Available: Experimental procedures, ^{1}H and ^{13}C NMR spectra of **2** and **3**, and X-ray data of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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