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A New Cytotoxic and Tubulin-Interactive Milnamide Derivative from a Marine Sponge *Cymbastela* sp.

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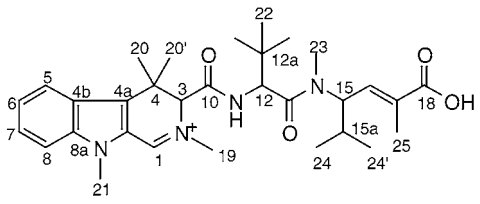
ABSTRACT



4 Milnamide D

The crude methanol extract of a marine sponge *Cymbastela* sp. collected in Papua New Guinea was selected for chemical investigation due to its significant cytotoxicity. Fractionation of the extract led to the isolation of jaspamide (1), hemiasterlin (2), milnamide A (3), and a new metabolite, milnamide D (4). The structure was solved by interpretation of NMR and mass spectra data. The cytotoxic and antitubulin activities of milnamide D (4) were evaluated.

Two families of closely related cytotoxic peptides, milnamides and hemiasterlins, have been isolated from several marine sponges. Milnamide A[1] was obtained from *Auletta* cf. *constricta* (Papua New Guinea). Hemiasterlins were independently isolated from *Hemiasterella minor*[2] (South Africa) and *Auletta* sp.,[3] *Cymbastela* sp.,[4] and *Siphonochalina* spp.[3] (Papua New Guinea). The hemiasterlins are potent antimitotic agents that inhibit the polymerization of tubulin[3,5] by binding to the Vinca-peptide domain.[6]

The sponge *Cymbastela* (order Halichondrida, family Axinellidae), collected in Milne Bay, Papua New Guinea, was investigated due to the marked activity of its crude methanolic extract in in vitro cytotoxicity assays against Caco-2 and A-431 cell lines. In this paper, we report the isolation of jaspamide (**1**),[7] hemiasterlin (**2**),[2] milnamide A (**3**),[1] and a new milnamide, milnamide D (**4**).[8] Milnamide D (**4**) binds to purified tubulin and inhibits its assembly in vitro.

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(8) Names milnamide B and C have been assigned to unpublished structures. Crews, P. Personal communication.
(9) Culture conditions and cell proliferation assay (see Experimental Section in Supporting Information): (a) HCT 116 strains were donated by Dr. Bert Vogelstein (Johns Hopkins University). (b) Carmichael, J.; DeGraff, W. G.; Gasdar, A. F. *Cancer Res.* **1987**, *47*, 936−942. (c) *GraphPad Prism 3.0*; GraphPad Software: San Diego, 2002.

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The specimen of *Cymbastela* collected from Papua New Guinea was extracted with MeOH. The crude extract was subjected to a solvent partition scheme to yield hexane, $CHCl_3$, and aqueous MeOH extracts. The $CHCl_3$ extract was subjected to reversed-phase flash column chromatography using a MeOH/H_2O gradient. The fraction eluting with (75:25) MeOH/H_2O was concentrated to provide 66.9 mg of material that was further separated by HPLC using a reversed-phase semipreparative column to provide 20.7 mg of jaspamide (**1**),[7] 2.7 mg of hemiasterlin (**2**),[2] 1.7 mg of milnamide A (**3**),[1] and 1.3 mg of milnamide D (**4**) (Figure 1).

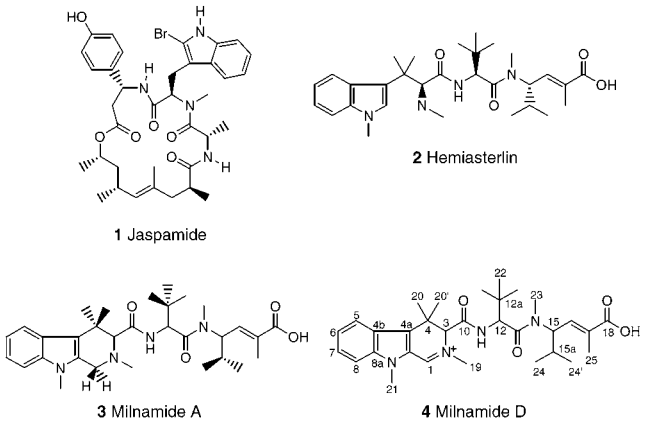

Figure 1. Jaspamide (**1**), hemiasterlin (**2**), milnamide A (**3**), and milnamide D (**4**).

Compound **4** was obtained as a yellow glass. The HRFABMS gave an $[M]^+$ ion at 537.3451 (calcd 537.3441) corresponding to the formula $C_{31}H_{45}N_4O_4^+$. NMR spectra of compounds **3** and **4** were very similar, but ESMS results indicated that **4** contained one less proton. Inspection of the ^1H NMR spectrum (see Table 1) indicated a change in the tetramethyltryptophan unit. In particular, the signals in **3** for the C-1 methylene group were replaced by a one-proton singlet at δ 9.50, which showed an HMQC correlation to a carbon at δ 154.0. Because of this supplementary double bond and charge in compound **4**, methyl C-19 [δ_H 3.57; δ_C 45.7] and methine C-3 [δ_H 4.81; δ_C 69.3] are shifted downfield compared to methyl C-19 and methine C-3 in milnamide A (**3**).[1] The complete structure of **4** was assigned on the basis of $^1H-^1H$, COSY, HMQC, and HMBC data. In particular, COSY data allowed us to define the same spin systems as in milnamide A (**3**): H-5/H-8; NH-11/H-12 and H-16/H-24/H-24′. Key HMBC correlations were observed between H-1 to C-9a; H-19 to C-1 and C-3; and H-3 to C-1, C-4, C-9a, C-10, and C-20. These HMBC cross-peaks placed the quaternary methylammonium group between C-1 and C-3 and the new methine between C-9a and N-2.

Compounds **2**−**4** were tested against two colorectal cancer cell lines (HCT-116, wild type, and p53-deficient mutant) (see Table 2). Milnamide D (**4**) (IC_{50} 66.8 nM) was significantly more potent than milnamide A (**3**) (IC_{50} 1652.6 nM) but less active than hemiasterlin (**2**) (IC_{50} 6.8 nM). These significant differences in the level of activity between the three compounds suggest that cyclization of the tetramethyltryptophan unit decreases cytotoxicity. However, addition of a positive charge on nitrogen partially ameliorates this effect.

In an assay for inhibition of tubulin polymerization, all three compounds showed inhibition; however, milnamide A (**3**) (IC_{50} 6.02 μM) and D (**4**) (IC_{50} 16.90 μM) were less potent than hemiasterlin (**2**) (IC_{50} 1.16 μM) (see Table 3). The reversal in the order of potency from the cytotoxicity assays suggests that milnamide D (**4**) may act by multiple mechanisms.

Table 1. ^1H and ^{13}C NMR Data for Milnamide D (**4**) (DMSO-d_6)

	Milnamide D (**4**)		
position	δ_C mult	δ_H mult (J in Hz)	HMBC
1	154.0 d	9.50 s	C-9a
3	69.3 d	4.81 s	C-1, C-4, C-9a, C-10, C-20
4	36.8 s		
4a	124.3 s		
4b	120.9 s		
5	121.1 d	7.82 d (8.3)	C-8a, C-9a
6	120.6 d	7.17 dd (7.8; 7.3)	C-4b
7	127.1 d	7.50 dd (8.8; 7.3)	C-8a
8	110.9 d	7.63 d (8.8)	C-4b
8a	140.2 s		
9a	127.1 s		
10	161.8 s		
11		8.67 d (9.8)	C-10
12	54.7 d	4.63 d (9.3)	C-12a, C-22
12a	35.0 s		
13	167.7 s		
15	55.9 d	4.84 t (10.0)	
15a	28.8 d	1.83 m	C-16, C-17, C-18
16	136.5 d	6.58 d (8.3)	C-18, C-25
17	130.2 s		
18	166.4 s		
19	45.7 q	3.57 s	C-1, C-3
20	30.0 q	1.38 s	C-3, C-4, C-9a, C-20′
20′	22.4 q	1.59 s	C-3, C-4, C-9a, C-20
21	30.2 q	3.91 s	C-4a, C-8a
22	26.5 q	0.93 s	C-12, C-12a
23	31.1 q	2.80 s	C-13
24	19.6 q	0.73 d (6.4)	C-15, C-23, C-24′
24′	19.0 q	0.51 d (6.4)	C-15, C-23, C-24
25	13.9 q	1.76 s	

Table 2. HCT-116 Cytotoxicity Assay Results[9]

	IC_{50} (nM)		
cell line	Hemiasterlin (**2**)	Milnamide A (**3**)	Milnamide D (**4**)
HCT116 p53+/+	6.8	1652.6	66.8
HCT116 p53−/−	3.0	1366.2	82.8

(10) Tubulin polymerization assay: (a) Hamel, E.; Lin, C. M. *Biochemistry* **1984**, *23*, 4173−4184. (b) Hamel, E. *Cell Biochem. Biophys.* **2003**, *38* (1), 1−22.

Table 3. Tubulin Polymerization Assay Results[10]

test compound	IC_{50} (μM)
Hemiasterlin (**2**)	1.16 ± 0.23
Milnamide A (**3**)	6.02 ± 0.29
Milnamide D (**4**)	16.90 ± 1.11

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Supporting Information Available: General experimental procedures, extraction and isolation procedures, NMR data for milnamide D, and biological assay descriptions. This material is available free of charge via the Internet at http://pubs.acs.org.

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