Supporting material

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Manuscript title : A new cytotoxic and tubulin-interactive milnamide derivative from a marine sponge *Cymbastela* sp.

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Enclosed material :

NMR data for Milnamide D (**4**) in DMSO- d_6

S1 ^1H NMR (500 MHz)

S2 COSY (500 MHz)

S3 ^{13}C NMR (100 MHz)

S4 HSQC (125 MHz)

S5 HMBC (125 MHz)

S6-S7 Experimental section

S1 ^1H NMR Milnamide D (500 MHz, $\text{DMSO}-d_6$)

S2 COSY NMR Milnamide D (500 MHz, DMSO- d_6)

S3 ^{13}C NMR Milnamide D (100 MHz, $\text{DMSO-}d_6$)

S4 HSQC NMR Milnamide D (125 MHz, DMSO-*d*₆)

S5 HMBC NMR Milnamide D (125 MHz, DMSO-*d*₆)

Experimental section :

General Experimental Procedures. UV spectra were determined in MeOH on a Hewlett-Packard 8452A diode array spectrophotometer. IR spectra were recorded on a JASCO FTIR-420 spectrophotometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. NMR spectra were acquired in DMSO- d_6 . A Varian instrument (500 MHz for ^1H and 100 and 125 MHz for ^{13}C NMR) with a Nalorac MDBG 3 mm probe and spinner was used. Mass spectra were performed on Finnigan MAT 95 (FABMS) and ThermoFinnigan LCQ-Classic (ESIMS) spectrometers.

Animal material. The specimen of *Cymbastela* sp. was collected in Milne Bay (S 10° 21.55' E 150° 44.70'), Papua New Guinea, in 2001. The sponge was identified by Mary Kay Harper, and a voucher specimen (PNG01-5-54) is held at the University of Utah.

Extraction and isolation. Frozen sponge material (800 g) was extracted with MeOH three times. The MeOH extracts were combined, filtered, and evaporated to dryness *in vacuo* to give a dark brown residue (8.4 g). This residue was dissolved in 10% water in MeOH (200 mL) and partitioned against hexane (3 × 200 mL). The water content of the MeOH phase was adjusted to 35% by adding 80 mL of water before partitioning against CHCl_3 (3 × 200 mL). This solvent partition scheme yield hexane (400.6 mg), CHCl_3 (748.3 mg) and 30% aq MeOH (7.2 g) extracts. The CHCl_3 extract was fractionated by C_{18} flash column chromatography using MeOH gradients in H_2O . The fraction eluted with (75:25) MeOH/ H_2O (66.9 mg) was further purified by C_{18} HPLC using a $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ gradient with 0.05% TFA (30:70 to 85:15 over 30 min) to afford jaspamide (**1**, 20.7 mg), hemiasterlin (**2**, 2.7 mg), milnamide A (**3**, 1.7 mg) and milnamide D (**4**, 1.3 mg).

Milnamide D (4) was obtained as a yellow glass ; $[\alpha]_D^{25} +156.0^\circ$ (c 0.390 ; MeOH) ; UV (MeOH) λ_{max} (ε) 190 (4580) 208 (31028) 246 (9834) 286 (1931) 382 (11925) 452 (1455) nm ; IR (film) ν_{max} 3377, 2960, 2933, 2873, 1674, 1637, 1539, 1471, 1419, 1365, 1317, 1279, 1238, 1201, 1136, 1088 cm^{-1} ; ^1H NMR (500 MHz, DMSO- d_6) δ : 9.50 (1H, s, H-1) ; 4.81 (1H, s, H-3) ; 7.82 (1H, d, J = 8.3 Hz, H-5) ; 7.17 (1H, dd, J = 7.8, 7.3 Hz, H-6) ; 7.50 (1H, dd, J = 8.8, 7.3 Hz, H-7) ; 7.63 (1H, d, J = 8.8 Hz, H-8) ; 8.67 (1H, d, J = 9.8 Hz, H-11) ; 4.63 (1H, d, J = 9.3 Hz, H-12) ; 4.84 (1H, t, J = 10 Hz, H-15) ; 1.83 (1H, m, H-15a) ; 6.58 (1H, d, J = 8.3 Hz, H-16) ; 3.57 (3H, s, H-19) ; 1.38 (3H, s, H-20) ; 1.59 (3H, s, H-20') ; 3.91 (3H, s, H-21) ; 0.93 (9H, s, H-22) ; 2.80 (3H, s, H-23) ; 0.73 (3H, d, J = 6.4 Hz, H-24) ; 0.51 (3H, d, J = 6.4 Hz, H-24') ; 1.76 (3H, s, H-25) ; ^{13}C NMR (100 MHz, DMSO- d_6) δ : 154.0 (C-1) ; 69.3 (C-3) ; 36.8 (C-4) ; 124.3 (C-4a) ; 120.9 (C-4b) ; 121.1 (C-5) ; 120.6 (C-6) ; 127.1 (C-7) ; 110.9 (C-8) ; 140.2 (C-8a) ; 127.1 (C-9a) ; 161.8 (C-10) ; 54.7 (C-12) ; 35.0 (C-12a) ; 167.7 (C-13) ; 55.9 (C-15) ; 28.8 (C-15a) ; 136.5 (C-16) ; 130.2 (C-17) ; 166.4 (C-18) ; 45.7 (C-19) ; 30.0 (C-20) ; 22.4 (C-20') ; 30.2 (C-21) ; 26.5 (C-22) ; 31.1 (C-23) ; 19.6 (C-24) ; 19.0 (C-24') ; 13.9 (C-25) ; (+)-LRESIMS m/z $[\text{M}]^+$ 537.4; (+)-LRFABMS m/z $[\text{M}]^+$ 537.3 ; HRFABMS m/z 537.3451 (calcd for $\text{C}_{31}\text{H}_{45}\text{N}_4\text{O}_4$, 537.3441, Δ -1.0 mmu).

Cells and Culture Conditions. All cell cultures were grown at 37 °C, 5% CO₂ and maximal humidity (by equilibration) and cultured according to standard protocols.[ATCC, 2002] All media reagents were purchased from Invitrogen Corporation (Carlsbad, CA). The HCT 116 strains were donated by Dr. Bert Vogelstein (Johns Hopkins University) and grown in McCoy's 5A media supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 1% v/v MEM (Minimal Essential Media) sodium pyruvate, 1% v/v penicillin-streptomycin and 1% v/v L-glutamine. The MDA-MB cell lines were purchased from American Type Culture Collection (Manassas, VA) and grown in MEM supplemented with 10% v/v heat-inactivated FBS, 1% v/v MEM sodium pyruvate, 1% v/v penicillin-streptomycin and 1% v/v L-glutamine. Cells were counted using a Beckman Coulter cell counter (Fullerton, CA).

Cell Proliferation Assay. The ability of milnamide D to inhibit cellular growth was determined using an MTT assay. For the HCT 116 cell lines, 4000 cells per well were plated in 96-well plates in 200 µL of the appropriate media (see above) and allowed to grow overnight. Serial dilutions of drug were then added to wells in quadruplicate. Control wells were treated with vehicle (DMSO). After 48 h of incubation at 37 °C and 5% CO₂, the media was removed and 90 µL fresh media was added along with 10 µL of 2.5 mg/mL aqueous 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT). The cells were then incubated for 4 h under the same conditions as above. The media was then removed and 100 µL DMSO added to each well. The amount of formazan resulting from MTT metabolism was measured by observing absorbance at 540 nm using a Labsystems Multiskan Plus plate reader. Using the absorbance in the vehicle treated lanes as 100% survival (1.00 fractional survival) the fractional survival of each treatment was calculated. The amount of compound needed to cause a 50% inhibition in growth (IC₅₀) was determined using the graphing program Prism.

Tubulin Polymerization Assay. Electrophoretically homogeneous tubulin was purified from bovine brain as described previously. Tubulin polymerization was measured using the conditions of the standard assay described previously, except no preincubation step was used.