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Cytotoxic and Tubulin-Interactive Hemiasterlins from *Auletta* sp. and *Siphonochalina* spp. Sponges†

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Abstract—Chemical and biological investigations of extracts from the sponge genus *Auletta* and two collections of *Siphonochalina* sp. have shown these organisms to be producers of the potent hemiasterlin class of antitumor agents. In addition to the previously known hemiasterlin (**1**) and hemiasterlin A (**2**), a new analogue, hemiasterlin C (**3**), was isolated and identified. The structures of **1** and **2** were assigned based on comparison to literature values, and **3** was identified on the basis of ^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC experiments. The cytotoxic and antitubulin activities of **1–3** were evaluated. In a comparative assay for inhibition of tubulin polymerization, the hemiasterlins were more potent than dolastatin 15 and equipotent with cryptophycin 1, but were somewhat less potent than dolastatin 10. © 1999 Published by Elsevier Science Ltd. Printed in Great Britain.

Introduction

In our continuing investigation of marine organisms as a source of potential antitumor compounds, our attention was drawn to three species of sponge which had been collected off the coast of Papua New Guinea and evaluated in the NCI 60 human tumor cell line primary assay.[1] One of these organisms was identified as an *Auletta* sp., while the other two were identified as *Siphonochalina* spp. COMPARE correlations[1] as high as 0.7 or greater were observed among extracts of these three organisms (Table 1). Further, COMPARE correlation analyses of the extract screening data referenced to the NCI standard agents database[1] suggested that the activity of these extracts might be due to tubulin-interactive constituents (Table 2). A literature search revealed that the only metabolites of *Siphonochalina* reported to date were a variety of acetylenic compounds and a number of novel triterpenes.[2–6] In contrast, there was only one previous report of *Auletta* in the chemical literature, documenting the isolation of milnamide (**4**), a highly modified tripeptide.[7]

Results

Bioassay-guided fractionation of the *n*BuOH partition of the *Auletta* sp. aqueous extract led to the isolation and identification of hemiasterlin (**1**) and hemiasterlin A (**2**). These assignments were supported by comparisons with the published data for these compounds obtained from *Hemiasterella minor*[8] and *Cymbastela* sp.[9] These compounds are closely related to milnamide,[7] but had not previously been reported from *Auletta*. The *n*BuOH partition fraction from the aqueous extract of *Siphonochalina* sp. was subjected to gel permeation on Sephadex LH-20, followed by HPLC (C_{18}), to afford three active compounds. The compounds with $t_R = 14.4$ min and 17.3 min were rapidly identified as hemiasterlin A and hemiasterlin, respectively, based on comparison of MS and ^{1}H NMR data for these compounds to those obtained for the *Auletta* sp. metabolites and reported literature values.[8,9] An additional compound was obtained by further HPLC of a minor peak with an intermediate retention time ($t_R = 16.8$ min) and found to be a new member of the hemiasterlin class, hemiasterlin C (**3**).

Key words: Antitumor compounds; antimitotic agents; marine metabolites; natural products; peptides.

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† Dedicated to the memory of Anna P. Talarico, who lost her battle with a brain tumor on 2 October, 1997.

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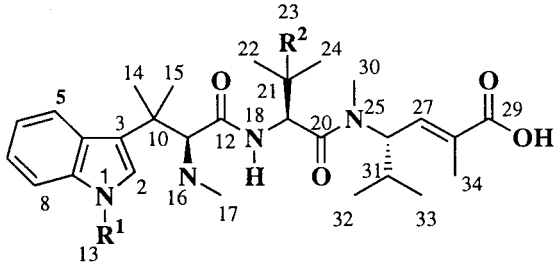
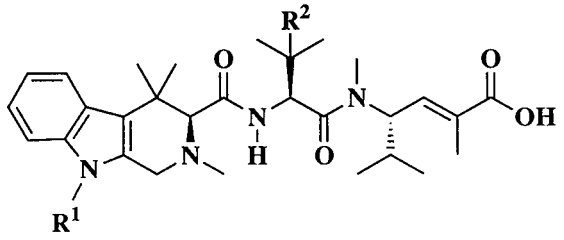
This compound gave a pseudomolecular ion (FABMS) at m/z 513 (MH^+), indicating that it was isomeric with hemiasterlin A. The ^1H NMR spectrum of this compound differed from that of hemiasterlin A in two respects. First, there was a signal at 3.8 (s, 3H), as in hemiasterlin, suggesting that N-1 was methylated. In addition, the *t*-butyl group of hemiasterlin and hemiasterlin A was replaced by a methine signal at 2.2 and nonequivalent methyl doublets (1.06 and 0.98), indicating the presence of a valine residue at amino acid position 2.



1 $R^1 = R^2 = CH_3$

2 $R^1 = H$, $R^2 = CH_3$

3 $R^1 = CH_3$, $R^2 = H$



4 $R^1 = R^2 = CH_3$

Table 1. GI_{50} COMPARE coefficients[1] for selected extracts

NSC	C12082[a]	C12326[b]	C12344[b]
C12082	1.00	0.71	0.70
C12326	0.71	1.00	0.77
C12344	0.70	0.77	1.00

[a] *Auletta* sp.
[b] *Siphonochalina* sp.

Table 2. COMPARE correlations of *n*-BuOH soluble extract of *Auletta* sp. (C12082) to antimitotic standard agents

Standard agent NSC	Common name	Correlation coefficient[a] GI_{50}	TGI
153858	Maytansine	0.79	0.69
49842	Vinblastine sulfate	0.76	0.72
332598	Rhizoxin	0.72	0.63
67574	Vincristine sulfate	0.65	0.64
125973	Paclitaxel	0.60	0.56

[a] Pearson correlation coefficients.

A series of NMR experiments, including HSQC, COSY, and HMBC, was performed and the results are summarized in Table 3. The presence of a methyl group attached to N-1 was supported by HMBC correlations from C-2 and C-9 to H_3-13. COSY correlations from H_3-22 and H_3-24 to H-21 and between H-21 and H-19, in addition to HMBC correlations of C-19, C-24, and C-19 to H_3-22 and from C-22, C-24, C-19, and C-20 to H-21, supported assignment of the structure as shown.

As mentioned previously, the two *Siphonochalina* spp. gave very strong COMPARE correlations to one another. Therefore, the second extract was subjected to the same separation steps as the first and very similar chromatographic profiles were obtained. This extract was found to contain the same three hemiasterlins (**1–3**). In addition to the hemiasterlins, each of these three extracts (*Auletta* and two *Siphonochalina*) was also shown to contain the geodiamolide series of cyclic depsipeptides,[10] which had also been reported, along with hemiasterlin, from *Cymbastela* sp.[11] No evidence of milnamide[7] or hemiasterlin B[9] was found in any of the extracts we studied.

Further testing of the hemiasterlins against a selected subset of human tumor cell lines confirmed potent cytotoxicity (Table 4). In an in vitro time course assay, using the SF539 (CNS) and OVCAR-3 (ovarian) tumor cell lines, none of the hemiasterlins exhibited an immediate cytotoxic effect (assay after 1 h of exposure of cells to drugs). Evidence of significant drug binding

Table 3. ^1H and ^{13}C NMR data for hemiasterlin C (**3**) in CD_3OD at 500 MHZ

Carbon no.	^1H NMR[a]	^{13}C NMR[b]	COSY	HMBC
2	7.12 (s)	129.0		3, 13
3		117.5		
4		126.0		
5	8.04 (d, 10)	120.6	6	4
6	7.14 (t, 10)	120.1	5, 7	4, 5, 7
7	7.25 (t, 10)	122.8	6, 8	
8	7.42 (d, 10)	111.2	7	
9		139.4		
10		38.7		
11	4.37 (s)	69.6		
12		165.8		
13	3.80 (s)	32.4		2, 9
14	1.40 (s)	22.0		3, 10, 11, 15
15	1.60 (s)	27.0		3, 10, 11, 14
17	2.41 (s)	34.0		11
19	4.74 (d, 8)	56.9	21	20, 21, 22, 23
20		173.0		
21	2.14 (m)	29.8	19, 22, 23	19, 22, 23
22	1.06 (d, 7.5)	26.5	21	19, 21, 23
24	0.98 (d, 7.5)	18.7	21	19, 21, 22
26	5.02 (dd, 9,8)	58.5	27, 31	27, 31
27	6.75 (d, 9)	138.0	26	26, 31, 34
28		133.7		
29		170.7		
30	3.07 (s)	31.3		20, 26
31	2.04 (m)	30.5	26, 32, 33	26, 27, 32, 33
32	0.91 (d, 7.5)	19.3	31	26, 31, 33
33	0.90 (d, 7.5)	14.3	31	26, 31, 32
34	1.88 (s)	13.6		27, 28, 29

[a] δ (multiplicity, *J* in HZ).
[b] δ.

Table 4. Results of in vitro cytotoxicity assay[a]

Cell line	IC_{50} (μg/mL)		
	Hemiasterlin (**1**)	Hemiasterlin A (**2**)	Hemiasterlin C (**3**)
A498	0.0224	0.3158	0.5321
OVCAR-3	1×10^{-6}	0.0024	0.0066
SF539	0.0013	0.0061	0.0775
COLO-205	0.0001	0.0009	0.0087
NCI-H460	1×10^{-6}	0.0001	0.0015
A549	–[b]	–[b]	–[b]
LOX	1.5984	–[b]	1.1135
MDA-MB-435	0.0154	–[b]	0.4002

[a] High dose 10 μg/mL; log dilutions.
[b] Activity reached a plateau above the 50% level.

and/or cellular uptake was observed when cells were washed free of unbound drugs after 1 h of exposure and allowed to incubate for the full 2 day period of the assay. However, the cytotoxic effect was not as great as that observed in cells exposed continuously to drugs for 2 days (data not shown).

Anderson et al.[12] reported that hemiasterlin and hemiasterlin A caused treated cells to accumulate in mitotic arrest, and that hemiasterlin A treatment resulted in the disappearance of intracellular microtubules. Ireland[13] subsequently disclosed inhibition of microtubule assembly by hemiasterlin and noncompetitive inhibition of radiolabeled vinblastine binding to tubulin. These findings are consistent with the COMPARE analysis reported above, and are essentially identical to those described for the antimitotic peptides dolastatin 10[14] and cryptophycin 1.[15,16] Therefore, we decided to compare tubulin interactions of the hemiasterlins and these previously studied peptides. We also included dolastatin 15, which inhibits polymerization but not the binding of vinca alkaloids to tubulin in this comparative study.[17]

Before initiating these studies, however, we noted that the reported molar extinction coefficients of hemiasterlin,[9] hemiasterlin A,[9] and milnamide A[7] were about 1/3 to 1/2 that expected for a peptide containing a tryptophanyl residue.[18] We therefore performed a detailed spectroscopic analysis, comparing the absorbance spectrum of hemiasterlin A with those of tryptophan (Calbiochem) and *N*-methyltryptophan (abrine, Sigma) (Fig. 1a) and the absorbance spectra of hemiasterlin and hemiasterlin C with that of 1-methyltryptophan (Sigma) (Fig. 1b). The spectra of the peptides were reasonably concordant with those of the appropriate amino acids, although the peak wavelength of the hemiasterlin C preparation was at about 285 nm as compared with 289 nm for 1-methyltryptophan and the hemiasterlin preparation. We, therefore, used extinction coefficients derived from the amino acids to establish the concentrations of the stock solutions of the peptides (in DMSO) used in studies with tubulin (see Fig. 1 legend for details).

Table 5 presents the initial comparative study of the three hemiasterlins with the previously characterized antimitotic peptides dolastatins 10 and 15 and cryptophycin 1. We examined the effects of the peptides on tubulin assembly in 0.8 M glutamate.[19] We determined the concentration of each drug required to inhibit assembly by 50% (IC_{50} value) following a 20 min incubation at 30°C. As in previous studies, dolastatin 10 was modestly more potent than cryptophycin 1[16] and much more so than dolastatin 15.[17] Quantitatively, the three hemiasterlins had activities similar to each other and to cryptophycin 1. Although hemiasterlin was nominally

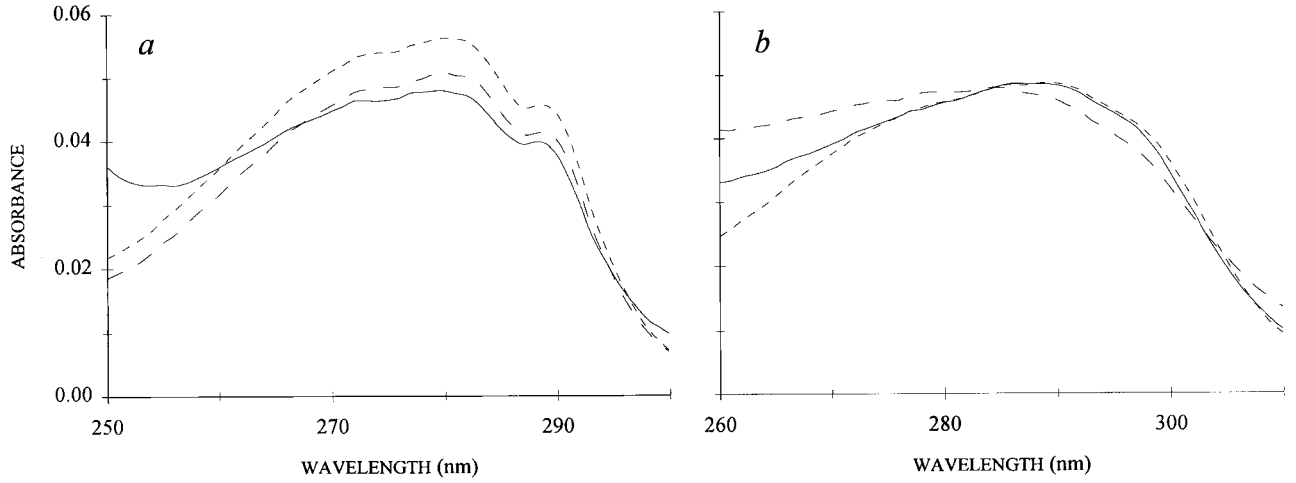


Figure 1. Absorbance spectra comparisons. Stock solutions (in DMSO) were diluted into 500 μL of 0.1 M 4-morpholine ethanesulfonate, pH 6.9, 0.5 mM $MgCl_2$, and additional DMSO to obtain 520 μL final sample volume (3.85% DMSO). (a) The absorbance spectrum of hemiasterlin A (solid curve) was compared with that of 10 μM L-tryptophan (*small dashes*) and 10 μM *N*-methyl-L-tryptophan (L-abrine; *large dashes*). Hemiasterlin A lacks the N-1 methyl group, as do tryptophan and abrine, but, as in the hemiasterlins, the alpha carbon of abrine is methylated. The peak absorbance for tryptophan, abrine, and hemiasterlin A is at 280 nm, but the absorbance for abrine is 10% lower than that for tryptophan, based on reagent mass. The extinction coefficient obtained for abrine was 5070, and this value was used to determine concentrations of the hemiasterlin A stock solution. (b) The absorbance spectra of hemiasterlin (*solid curve*) and hemiasterlin C (*large dashes*) were compared to that of 10 μM 1-methyl-DL-tryptophan (*small dashes*), since the 1-methyl group is present on all three compounds. The extinction coefficient obtained for 1-methyl-DL-tryptophan was 4860 at 289 nm, and this value was used to determine concentrations of the hemiasterlin and hemiasterlin C stock solutions.

Table 5. Tubulin polymerization assay results[a]

Test compound	IC_{50} (μM)
Hemiasterlin (**1**)	0.98 ± 0.02
Hemiasterlin (**2**)	1.1 ± 0.01
Hemiasterlin (**3**)	1.3 ± 0.01
Dolastatin 10	0.59 ± 0.05
Dolastatin 15	5.4 ± 1.0
Cryptophycin 1	1.1 ± 0.07

[a] Reaction mixtures (0.24 mL) contained 10 μM (1.0 mg/mL) purified bovine brain tubulin 0.8 M monosodium glutamate (pH of 2 M stock solution adjusted to pH 6.6 with HCl), varying concentrations of drug, and 4% (v/v) dimethyl sulfoxide (as drug solvent). Reaction mixtures were preincubated for 15 min at 30°C and then chilled on ice. GTP was added in 10 μL to 0.4 mM, and the reaction mixtures were tranferred to cuvettes held at 0°C by electronic temperature controllers in Gilford 250 spectrophotometers. Baselines were established, and the temperature raised to 30°C (over~1 min). Assembly was followed for 20 min at 30°C, and the drug concentration required to inhibit extent of the reaction by 50% was determined from the data.[19]

most potent and hemiasterlin C least potent among these four peptides, our experience with this assay is that quantitative differences between drugs are not significant unless there is at least a 20% difference in IC_{50} values. We are presently comparing the biochemical properties of the hemiasterlins in greater detail to each other and to other antimitotic peptides.

Discussion

Dolastatin 10, an analogue of dolastatin 15 (Cemadotin), and an analogue of cryptophycin 1 (cryptophycin 52) are all currently in early clinical trials. The structures of these larger peptides are considerably more complex than those of the hemiasterlins. The structural motif of the hemiasterlins would appear to be readily amenable to the rapid generation of analogues by combinatorial chemistry as a potential route to lead-optimization for further preclinical development.

With the identification of *Siphonochalina* as a previously unknown source, the hemiasterlins have now been identified from four different sponge genera, some rather distantly related. That, and the potential biomedical importance of these unique, highly modified tripeptides, may justify efforts to further elucidate the biosynthetic origin of these compounds.

Experimental

General

NMR spectra were recorded on a Varian VXR 500 spectrometer, and chemical shifts were referenced relative to the residual undeuterated solvent signal. Proton-detected heteronuclear correlations were measured using HSQC (optimized for $^1J_{HC} = 140$ Hz) and HMBC (optimized for $^nJ_{HC} = 8$ Hz) pulse sequences. Whenever possible, proton–proton connectivities were confirmed by COSY 45 NMR experiments. Infrared spectra were obtained on a Perkin–Elmer Spectrum 2000 FT-IR spectrometer. Optical rotations were measured with a Perkin–Elmer 241 polarimeter. Mass spectra were recorded with a JEOL SX102 spectrometer. Ultraviolet spectra were obtained on a Beckman DU7400 spectrophotometer. Dolastatins 10 and 15 were generous gifts of G. R. Pettit, Arizona State University, and cryptophycin 1 was kindly provided by Merck Research Laboratories.

Animal material

Samples of *Auletta* sp. (0CDN1807) and of *Siphonochalina* spp. (0CDN1949 and 0CDN1944) were collected off the coast of Papua New Guinea by the Coral Reef Foundation, under contract to the NCI. Voucher specimens from these collections are maintained at the Smithsonian Sorting Center, Suitland, MD.

General extraction procedures

Fresh samples were frozen immediately after collection and stored at −20°C until processed. Frozen samples were ground to a coarse powder with dry ice and extracted with H_2O at 4°C. After the aqueous extracts were removed by centrifugation and lyophilization, the marc was also freeze-dried and sequentially extracted with MeOH/CH_2Cl_2 (1/1) and MeOH. The organic extracts were then combined and evaporated at reduced pressure.

Fractionation of *Auletta* sp

A 1.26 g aliquot of the aqueous extract was partitioned between *n*BuOH and H_2O; the activity was concentrated in the *n*BuOH soluble portion (183 mg). This fraction was allowed to permeate through a Sephadex LH-20 column (2.5×122 cm) eluted with MeOH and monitored at 250 nm. The active fraction (44 mg) was then subjected to HPLC (Rainin Microsorb C_{18}, 220 nm, 3.5 mL/min) with linear gradient elution from 45 to 75% CH_3CN in 0.05% aqueous TFA over 20 min, to afford hemiasterlin ($t_R = 17.3$ min, 5 mg) and hemiasterlin A ($t_R = 14.4$ min, 3 mg). ^{1}H NMR and mass spectral data for these compounds were in good agreement with published values.

Fractionation of *Siphonochalina* sp

An 8 g aliquot of the aqueous extract was partitioned as described above for *Auletta* sp. The activity was likewise concentrated in the *n*BuOH soluble portion (797 mg). This fraction was permeated through a Sephadex LH-20 column (2.5×122 cm), which was eluted with MeOH and monitored at 220 nm. The active fraction (140 mg) was subjected to a second Sephadex LH-20 column (2×110 cm) eluted with MeOH/H_2O (9/1) and monitored at 220 nm. The active fraction (79 mg) was subjected to HPLC using the gradient described above on a Rainin Microsorb C_{18} column (2.5×30 cm with guard) at a flow rate of 10 mL/min to afford hemiasterlin ($t_R = 17.3$ min, 11 mg) and hemiasterlin A ($t_R = 14.4$ min, 3 mg), as well as a third active fraction ($t_R = 16.8$ min, 4 mg). This fraction was subjected to a second round of

HPLC separation (Rainin Microsorb C_{18}, 220 nm, 1.0 mL/min) using a linear gradient elution from 20 to 50% CH_3CN in 0.05% aqueous TFA over 20 min to afford hemiasterlin C ($t_R = 6.5$ min, 1.5 mg), $[\alpha]_D - 18.8°$ (*c* 0.11, MeOH); IR ($CHCl_3$) 4215, 3688, 3620, 3021, 2400, 2361, 1717, 1653, 1521, 1420 cm^{-1}; HRFABMS, *m/z* 513.3461 [MH^+], calculated for $C_{29}H_{45}N_4O_4$, 513.3441; NMR data, Table 3.

Similar treatment of 10 g of the other *Siphonochalina* sp. aqueous extract yielded **1** (18 mg), **2** (9 mg) and **3** (1 mg).

Acknowledgements

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