Mycalamides C and D, Cytotoxic Compounds from the Marine Sponge *Stylinos* n. Species

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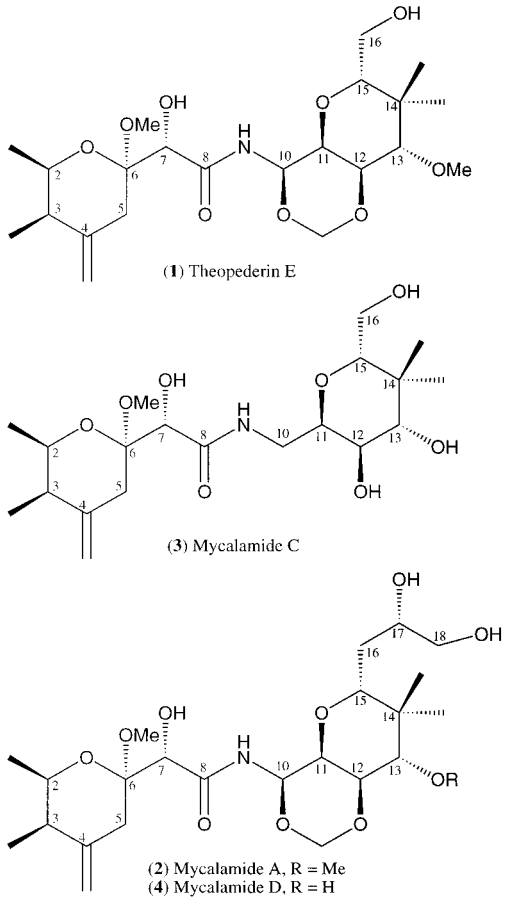
The new cytotoxic compounds, mycalamides C (**3**) and D (**4**), have been isolated from the marine sponge *Stylinos* n. sp., along with the known theopederin E (**1**) and mycalamide A (**2**).

Since the isolation of mycalamide A, a potent antiviral and cytotoxic compound from a sponge of the genus *Mycale*, a number of related metabolites have been isolated.[1] Mycalamide B was described a short time later from the same New Zealand sponge.[2] The isolation of the water-soluble onnamide A from a Japanese specimen of a *Theonella* sp. occurred at the same time as the isolation of mycalamide A[3] and was followed by the isolation of several other examples of onnamides, also from the genus *Theonella*.[4,5] More recently Fusetani et al. reported the isolation of a related series, named the theopederins, from a Japanese specimen of the genus *Theonella*.[6] These compounds all share common features with the terrestrial natural product pederin, the principal component of the blister beetle.[7]

Extracts of a soft-textured, yellow-orange sponge from Heron Island, later identified as *Stylinos* n. sp. (family Mycalidae, order Poecilosclerida, class Demospongiae), were highly active in P-388 assays (IC_{50} = 16.5 ng/mL). Fractionation of a small quantity of extract on Si gel, followed by bioassay of the resulting fractions, indicated the active components were polar. Extraction of a larger recollection of the sponge (1.12 kg) resulted in a (DCM) extract that had potent bioactivity [P-388; (IC_{50} = 22 ng/mL)]. Reversed-phase flash chromatography[8] resulted in a series of fractions that were combined on the basis of TLC, NMR, and bioassay results. The major bioactive fraction was partitioned further on a DIOL flash column, giving the known theopederin E (**1**) and mycalamide A (**2**), both having spectral data identical with those reported in the literature.[1,2,6] Reversed-phase HPLC of a more polar fraction eluting with 30% acetonitrile−water resulted in the isolation of mycalamide C (**3**) and mycalamide D (**4**).

The ^{1}H NMR spectrum of mycalamide C (**3**) was similar to that of theopederin E (**1**), although it had one less methoxy signal and no dioxolane methylene signals. LR-FABMS mass spectral measurement of **3** gave a molecular ion at 418 mass units [MH^+] for which high-resolution analysis gave the molecular formula $C_{20}H_{36}NO_8$, in agreement with the missing NMR signals. Analysis of the DQF−COSY spectrum showed correlations from the NH signal at δ 7.07 to the methylene signal at δ 3.70 (H10), and then to δ 4.15, showing that C10 was not oxygenated. Detailed analysis of the DQF-COSY and HMBC spectra showed the remainder of the carbon skeleton was identical to that of theopederin E. The methoxy signal at δ 3.29 showed an HMBC correlation to C6, establishing its position.



The ^{1}H NMR spectrum of mycalamide D (**4**) showed marked similarities to that of mycalamide A (**2**), but also contained one less methoxy signal. LRFABMS measurement gave a molecular ion at 512 mass units [MNa^+], 14 mass units less than mycalamide A (**2**). High-resolution analysis corresponded to the molecular formula $C_{23}H_{39}NO_{10}Na$. Analysis of DQF−COSY and HMBC spectra showed that the carbon skeleton was identical to that of mycalamide A (**2**). The methoxy signal at δ 3.29 showed an HMBC correlation to C6, establishing its position and confirming that the methyl group was missing from the C13 oxygen. ^{1}H and ^{13}C NMR data for **1**−**4** are compared in Tables 1 and 2.

The optical rotations measured for theopederin E (**1**) (+110°) and mycalamide A (**2**) (+84°) agreed with literature

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Table 1. ^{1}H NMR Data for Compounds **1**−**4**

no.	theopederin E (**1**)[a]	mycalamide A (**2**)[a]	mycalamide C (**3**)[b]	mycalamide D (**4**)[b]
2	4.06 (dq 6.6, 2.9)	3.98 (dq 6.6, 2.7)	4.00 (dq 6.6, 2.8)	3.99 (dq 6.7, 2.7)
2−Me	1.21 (d 6.6)	1.19 (d 6.6)	1.17 (d 6.6)	1.18 (d 6.3)
3	2.27 (dq 7.1, 2.9)	2.24 (dq 7.0, 2.7)	2.23 (dq 7.2, 2.8)	2.24 (dq 6.9, 2.7)
3−Me	1.03 (d 7.1)	0.99 (d 7.0)	0.97 (d 7.2)	0.99 (d 6.9)
4═CH_2	4.86 (dd 2.0, 2.0)	4.84 (m)	4.83 (m)	4.80 (m)
	4.76 (dd 2.0, 2.0)	4.73 (m)	4.73 (m)	4.80 (m)
5	2.37 (d 14.1)	2.36 (m)	2.32 (d 14.1)	2.37 (s)
	2.21 (ddd 14.1. 2.0, 2.0)		2.18 (d 14.1)	
6−OMe	3.30 (s)	3.29 (s)	3.29 (s)	3.29 (s)
7	4.28 (s)	4.30 (s)	4.19 (s)	4.31 (s)
9	7.44 (d 9.1)	7.49 (d 9.8)	7.07 (m)	7.50 (d 10.2)
10	5.82 (dd 9.1, 9.1)	5.87 (t 9.8)	3.70 (m)	5.87 (dd 10.2, 9.9)
10−OCH_2	5.13 (d 6.9)	5.13 (d 6.9)		5.08 (d 6.8)
	4.91 (d 6.9)	4.87 (d 6.9)		4.82 (d 7.0)
11	3.85 (dd 9.1, 6.5)	3.8 (dd 9.8, 6.7)	4.15 (m)	3.89 (dd 9.6, 6.9)
12	4.21 (dd 9.8, 6.5)	4.22 (dd 10.3, 6.7)	3.89 (dd 9.6, 6.6)	4.14 (dd 10.8, 6.9)
13	3.42 (d 9.8)	3.46 (d 10.3)	3.37 (d 9.6)	4.00 (d 10.2)
13−OMe	3.57 (s)	3.55 (s)		
14−Me_{eq}	1.09 (s)	0.98 (s)	1.03 (s)	1.01 (s)
14−Me_{ax}	0.87 (s)	0.87 (s)	0.89 (s)	0.92 (s)
15	3.59 (dd 7.7, 2.4)	3.60 (dd 5.5, 4.0)	3.57 (m)	3.63 (m)
16	3.64 (ddd 11.6, 7.4, 2.4)	1.54 (m)	3.67 (m)	1.57 (m)
	3.51 (ddd 11.6, 7.7, 4.5)		3.57 (m)	
17		3.74 (m)		3.75 (m)
18		3.55 (m)		3.58 (m)
		3.38 (dd 11.2, 6.2)		3.39 (m)

[a] ^{1}H NMR data recorded at 300 MHz in $CDCl_3$ referenced at δ 7.25. [b] ^{1}H NMR data recorded at 500 MHz in $CDCl_3$ referenced at δ 7.25.

Table 2. ^{13}C NMR Data for Compounds **1**−**4**

no.	theopederin E (**1**)[a]	mycalamide A (**2**)[a]	mycalamide C (**3**)[b]	mycalamide D (**4**)[b]
2	69.3 (1)[c]	69.7 (1)[c]	69.9 (CH)[d]	70.0 (CH)[d]
2−Me	18.3 (3)	17.9 (3)	17.8 (CH_3)	17.5 (CH_3)
3	41.2 (1)	41.3 (1)	41.3 (CH)	41.0 (CH)
3−Me	12.3 (3)	12.0 (3)	11.9 (CH_3)	11.5 (CH_3)
4	144.8 (0)	145.4 (0)	145.6 (C)	144.0 (C)
4═CH_2	110.9 (2)	110.4 (2)	110.4 (CH_2)	110.5 (CH_2)
5	33.0 (2)	33.7 (2)	32.9 (CH_2)	33.0 (CH_2)
6	99.7 (0)	99.6 (0)	99.9 (C)	99.5 (C)
6−OMe	48.8 (3)	48.9 (3)	48.4 (CH_3)	49.0 (CH_3)
7	71.0 (1)	72.8 (1)	71.0 (CH)	73 (CH)
8	171.7 (0)	171.5 (0)	171.8 (C)	171.5 (C)
10	74.6 (1)	73.6 (1)	36.0 (CH_2)	73.5 (CH)
10−OCH_2	86.7 (2)	86.7 (2)		86.5 (CH_2)
11	71.0 (1)	71.2 (1)	75.7 (CH)	72.0 (CH)
12	74.2 (1	74.3 (1)	69.0 (CH)	74.0 (CH)
13	79.4 (1)	79.0 (1)	76.8 (CH)	69.0 (CH)
13−OMe	61.3 (3)	61.8 (3)		
14	39.6 (0)	41.6 (0)	38.8 (C)	40.1 (C)
14−Me_{eq}	23.4 (3)	23.1 (3)	23.2 (CH_3)	22.5 (CH_3)
14−Me_{ax}	14.6 (3)	13.5 (3)	13.9 (CH_3)	12.2 (CH_3)
15	80.2 (1)	78.9 (1)	78.4 (CH)	78.5 (CH)
16	61.5 (2)	32.0 (2)	61.6 (CH_2)	31.0 (CH_2)
17		71.5 (1)		71.8 (CH)
18		66.4 (2)		66.0 (CH_2)

[a] ^{13}C NMR data recorded at 75 MHz in $CDCl_3$ referenced at 77.0 ppm. [b] ^{13}C NMR data recorded at 125 MHz in $CDCl_3$ referenced at 77.0 ppm. [c] Number of attached protons as determined by APT. [d] Carbon type determined by DEPT.

values (+136° and +110°, respectively).[1,6] The absolute stereochemistry of mycalamide A (**2**) has been determined by total synthesis.[9] The small amounts of **3** and **4** isolated, together with their instability, prevented accurate measurement of $[\alpha]_D$ values. Assuming common stereochemistry for a series of metabolites from the same sponge species, the series **1**−**4** has been provisionally assigned as shown. The attempts made to measure $[\alpha]_D$ values for **3** and **4** gave positive rotations, supporting this assignment.

Theopederin E (**1**) and the isolated mycalamides A (**2**), C (**3**), and D (**4**) were assayed against the P-388 murine leukemia cell line, giving IC_{50} values of 0.9, 1.1, 95.0, and 35.0 ng/mL, respectively. The effect of structure on the biological activity of mycalamides and related molecules has been studied in some detail; the presence of the C7 to C10 portion, including the amide linkage, is vital for activity.[10] The activity of **3** is noteworthy in light of the poor activity of onnamide E, which is also not oxygenated at C10.[4] The biological activities of the des-*O*-methyl analogues **3** and **4** show that the absence of the C13 *O*-methyl group decreases the activity of mycalamide-like metabolites by an order of magnitude.

The isolation of such closely related metabolites as the mycalamides, theopederins, and onnamides from three different genera of marine sponges raises the interesting question of the origin of these compounds. Cell separation studies are in progress in our laboratory to investigate the

cellular origin of these biologically potent molecules in the sponge *Stylinos* n. sp.

Experimental Section

General Experimental Procedures. These have been given previously.[8,11,12] FABMS measurements were performed at the National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda.

Sponge Material. *Stylinos* n. sp. (QM species number 1856) order Poecilosclerida, family Mycalidae (voucher sample no. QM G312725). Description: Thickly bulbous encrusting on unconsolidated coral rubble. Color: orange-tan-brown alive, with small white flecks scattered over the surface; light beige in ethanol. Oscules: large, up to 8 mm diameter, collapsing out of water; surface covered by numerous small ostia, up to 1 mm in diameter. Texture: soft, mucous. Surface: opaque, membranous, optically smooth, even and unornamented apart from visible fibers below surface. No specialized ectosomal skeleton present, with tips of ascending spicule bundles barely protruding through the surface, although surface may have a thin layer of fine sand and detritus scattered. Choanosomal skeleton composed of large, meandering, light fibers fully cored with large sand grains permeating the entire choanosome. Loose, plumose, paucispicular tracts of subtylostyles extend through the choanosome, between the tracts of sand, becoming more pronounced near the surface, forming brush-like terminations under the surface membrane; collagen within the mesohyl is moderately dense, relatively homogeneous, containing some scattered detritus and many free subtylostyles. Megascleres are a single category of straight, slender subtylostyles with prominent tyles (215−228 × 4−7 μm in dimension). Distribution: This species has so far only been recorded from the southern (Capricorn-Bunker Group) and mid-sectors of the Great Barrier Reef (Myrmidon Reef), but possibly has a more widespread distribution. Notes: This species is probably new to science. However, it is inappropriate to create a new taxon for it until a thorough revision of the Indo-Pacific species has been undertaken. *Stylinos* has traditionally been included with the Halichondrida, whereas it clearly belongs with the mycalids in skeletal structure and megasclere geometry ('mycalostyles'), though it lacks the characteristic anisochelae microscleres of other mycalids. This assessment is clearly supported by its possession of mycalamides.

Extraction and Isolation. *Stylinos* n. sp. (1.12 kg) was sliced finely and extracted with DCM−MeOH (1:1, 5 × 700 mL), and the combined extracts were evaporated to an aqueous residue, which was extracted with hexane (3 × 300 mL), then DCM (3 × 300 mL). The combined hexane layers were dried over $MgSO_4$ and evaporated to give 9.84 g (0.88%) of an orange residue. The DCM layers were combined and dried over $MgSO_4$ and evaporated to give 3.05 g (0.27%) of an orange residue. The samples were assayed for anti-P-388 activity, and the DCM fraction was significantly more active. Fractionation of the DCM extract on a reversed-phase flash column eluting with 30% MeOH−H_2O stepwise through to DCM gave a series of fractions. These fractions were combined on the basis of TLC and NMR analysis and bioassay results. The active fraction was applied to a DIOL flash column eluting with DCM to EtOAc, which gave pure theopederin E (**1**) (87 mg, 7.8 × 10^{-3}%), mycalamide A (**1**) (12.7 mg, 1.13 × 10^{-3}%), and another fraction containing two components. These compounds were separated on reversed-phase HPLC eluting with 30% acetonitrile−water to give mycalamide C (**3**) (2.0 mg, 1.8 × 10^{-4}%) and mycalamide D (**4**) (1.4 mg, 1.25 × 10^{-4}%). Mycalamides and related compounds are acid sensitive, so to prevent decomposition NMR spectra were run in $CDCl_3$ with 0.1% pyridine-d_5. CAUTION: Mycalamide-rich samples cause adverse skin reactions.

Mycalamide C (3). *N*-(3,4-Dihydroxy-6-hydroxymethyl-5,5-dimethyl-tetrahydro-pyran-2-ylmethyl)-2-hydroxy-2-(2-methoxy-5,6-dimethyl-4-methylene-tetrahydro-pyran-2-yl)-acetamide (**3**): ^{1}H NMR data ($CDCl_3$), see Table 1; ^{13}C NMR data ($CDCl_3$), see Table 2; HRFABMS (positive ion), found *m*/*z* 418.2431, calcd for $C_{20}H_{36}NO_8^+$ 418.2441; LRFABMS (positive ion), 418.3 [MH^+], 386.2 [$M - CH_3O$]$^+$.

Mycalamide D (4). *N*-[6-(2,3-Dihydroxypropyl)-8-hydroxy-7,7-dimethyl-hexahydro-pyrano[3,2-*d*][1,3]dioxin-4-yl]-2-hydroxy-2-(2-methoxy-5,6-dimethyl-4-methylene-tetrahydro-pyran-2-yl)-acetamide (**4**): ^{1}H NMR data ($CDCl_3$), see Table 1; ^{13}C NMR data ($CDCl_3$), see Table 2; HRFABMS (positive ion), found *m*/*z* 512.2484, calcd for $C_{23}H_{39}NO_{10}Na^+$ 512.2472; LRFABMS (positive ion), 512.3 [MNa^+], 458.3 ($M - CH_3O$)$^+$.

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