Mycalamide D, a New Cytotoxic Amide from the New Zealand Marine Sponge *Mycale* Species

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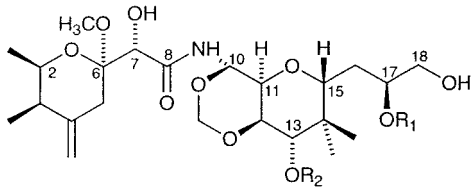
A new mycalamide, mycalamide D (**3**), has been isolated from the New Zealand marine sponge *Mycale* sp. This new metabolite, in which the C13-*O*-methyl group of mycalamide A (**1**) is replaced by a hydrogen atom, was found to be cytotoxic to a range of mammalian cell lines, with a potency approximately 20-fold less than that of **1**.

The mycalamides are potent antiviral and antitumor compounds isolated from marine sponges. Mycalamides A (**1**) and B (**2**) were originally isolated from the New Zealand marine sponge *Mycale* sp. collected in Otago Harbour on the southeast coast of the South Island.[1,2] Subsequent studies have revealed that these toxins cause reversion of *ras*-transformed cancerous cells back to normal morphology[3] and block the activation of $CD4^+$ T lymphocytes.[4] We recently examined a collection of the same *Mycale* species collected on the northeast coast of the North Island of New Zealand and found evidence of a novel mycalamide. The use of ^{1}H NMR as a guide led to the isolation of 9 mg of **1** and 4 mg of mycalamide D (**3**).



R_1 = H, R_2 = CH_3 mycalamide A **1**
R_1 = CH_3, R_2 = CH_3 mycalamide B **2**
R_1 = H, R_2 = H mycalamide D **3**

An $[M + Na]^+$ ion was observed in the electrospray HRMS of mycalamide D (**3**) consistent with a molecular formula of $C_{23}H_{39}NO_{10}$, corresponding to the loss of one carbon and two hydrogen atoms from the molecular formula of mycalamide A (**1**). Resonances attributable to all 23 carbons and 35 of the 39 hydrogens were observed in the ^{1}H and ^{13}C NMR spectra. A close inspection of the NMR spectra of mycalamides A (**1**) and D (**3**) revealed the very similar nature of the two compounds and allowed most carbons and hydrogens of **3** to be assigned to the mycalamide skeleton by direct comparison of chemical shifts. These assignments were confirmed by HSQC, HMBC, and COSY correlations similar to those recorded on **1** and are summarized in Table 1. Proton and carbon resonances attributed to the oxymethyl group at position 13 of **1** are absent from the NMR spectra of mycalamide D (**3**). Also missing are the ^{1}H resonance at δ 3.48 and carbon resonance at δ 79.11, which are replaced by a methine pair at ^{1}H δ 4.02 and ^{13}C δ 69.21. The upfield shift of 9.9 ppm for the oxymethine carbon is consistent with the replacement of a hydroxyl in **3** for the oxymethyl of **1**. A COSY correlation from the downfield H13 proton resonance at δ 4.02 to H12, and HMBC correlations to C11, C12, C14, C15, 14-CH_3ax, and 14-CH_3eq confirmed the assignment of the new hydroxyl to position 13.

Table 1. ^{13}C (75 MHz) and ^{1}H (300 MHz) NMR Data ($CDCl_3$) for Mycalamide A (**1**) and Mycalamide D (**3**)

	mycalamide A (**1**)		mycalamide D (**3**)	
position	δ_C	δ_H (*J* Hz)	δ_C	δ_H (*J* Hz)
2	69.64	3.98 dq, 2.7,6.6	69.8	4.00 dq, 2.7,6.5
3	41.33	2.24 dq, 2.7,7.1	41.39	2.26 dq, 2.7,6.6
4	145.73		145.66	
5	33.58	2.37 s	33.85	2.38 br s
6	99.80		99.82	
7	72.52	4.33 s		4.32 s
8	171.98		171.70	
10	73.47	5.89 t, 9.9	73.82	5.89 t, 10
11	71.14	3.89 dd, 6.7,9.9	71.6	3.91 dd, 6.7,10
12	74.35	4.23 dd, 6.7,10.3	73.82	4.15 dd, 6.7,10.6
13	79.11	3.48 d, 10.3	69.21	4.02 d, 10.6
14	41.64		40.84	
15	78.94	3.62 t, 6.2	78.77	3.64 dd, 3.4,8.8
16	31.67	1.52 m, 2H	32.26	1.58 m
17	71.79	3.75 dq, 3.1,6.3	71.4	3.76 m
18	66.39	3.56 dd, 3.1,11.5	66.5	3.59 dd, 3.2,11.2
		3.38 dd, 6.3,11.5		3.39 dd, 6.1,11.2
2-CH_3	17.89	1.20 d, 6.6	17.87	1.21 d, 6.5
3-CH_3	11.94	1.00 d, 7.1	12.03	1.01 d, 6.6
4-CH_2	110.39	4.85 d, 1.6	110.57	4.85 br s
		4.74 d, 1.6		4.75 br s
14-CH_3ax	13.41	0.87 s	12.49	0.93 s
14-CH_3eq	23.01	0.98 s	22.87	1.02 s
6-OCH_3	48.77	3.30 s	49.02	3.31 s
10-OCH_2	86.82	5.15 d, 7.1	86.61	5.10 d, 6.8
		4.88 d, 7.1		4.84 d, 6.8
13-OCH_3	61.85	3.56 s		
NH9		7.55 d, 9.9		7.51 d, 10

A coupling constant of 10.6 Hz between H13 and H12 of **3** is almost identical to that of mycalamide A (**1**), establishing the same stereochemical relation of the two centers. The chemical shifts and coupling constants of all other observable ^{1}H resonances of mycalamide D (**3**) match those of mycalamide A (**1**) within experimental limits, suggesting that the relative stereochemistry of the two compounds is the same. A series of NOE correlations (see Figure 1) observed in a ROESY spectrum of **3**, similar to NOE enhancements observed previously in **1**,[1] confirm that the

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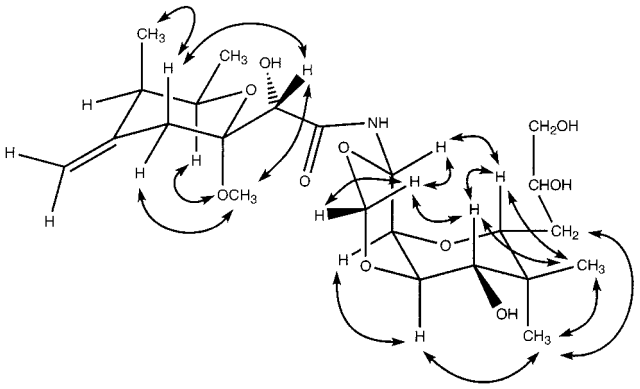

Figure 1. Relative stereochemistry of mycalamide D (**3**). NOES observed in a ROESY spectrum are illustrated with arrows.

relative stereochemistry of **3** is the same as **1**, which has been established unambiguously by synthesis.[5]

A range of different cell types was examined for their metabolic sensitivity to mycalamides A (**1**) and D (**3**), including an established, nontumorigenic pig kidney cell line (LLC−PK_1), a human lung carcinoma (H441), and a human neuroblastoma (SH−SY5Y). Both **1** and **3** were cytotoxic at nanomolar concentrations to each of these cell lines, despite the inherent differences in each cell line's growth pattern and metabolic activity (Figure 2). In all three cell lines mycalamide D (**3**) was significantly less cytotoxic than mycalamide A (**1**) (Table 2).

Although mycalamide B (**2**) was unavailable for testing in our laboratory, it has been reported to be 2−6 times more potent a toxin than mycalamide A (**1**) in the four cell lines that were examined.[6] The reported IC_{50} value in mouse lymphoma P-388 cells was 5.2 nM for **1** and 1.3 nM for **2**. In a preliminary experiment involving a single preparation, we obtained an IC_{50} value for **1** in the P-388 cell line of 4.0 ± 0.7 nM, comparing favorably with that reported previously. Mycalamide D (**3**) in our study gave an IC_{50} value for P-388 cells of 65.5 ± 5.5 nM, 20-fold less toxic than **1**. Interestingly, the cytotoxic potency of the mycalamides inversely correlates with the number of oxymethyl groups present, with mycalamide B (**2**) having three oxymethyl groups, mycalamide A (**1**) having two, and mycalamide D (**3**) having one. The polarity of the three compounds is also inversely related to methylation as evidenced by an R_f of 0.22 measured for **3** and the reported R_f values of 0.50 (**2**) and 0.35(**1**)[2] on Si gel TLC (1:19 EtOH EtOAc). We speculate that the differences in cytotoxicity of the three metabolites may reflect their ability to cross the cell membrane.

Experimental Section

General Experimental Procedures. MS were recorded on a Mariner electrospray TOF mass spectrometer. UV spectra were recorded on a HP 8452-A spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. NMR spectra were measured with a Varian Inova 300 MHz spectrometer.

Animal Material. The sponge was collected subtidally using scuba at Cape Karikari, North Island, New Zealand. A voucher specimen is stored at the National Institute of Water and Atmospheric Research (97CK/MYC/1−6). This species belongs to the genus *Mycale* (family Mycalidae, order Poecilosclerida) and appears to be similar to a specimen described previously from Otago Harbour.[1] Individuals of this species may be encrusting or massive, with a chocolate-brown ectosome, often with a purple tinge. The sponge surface often has large oscules (2−4 mm diameter) and may appear stippled due to the presence of polychaete worm tubes. The choanosome is

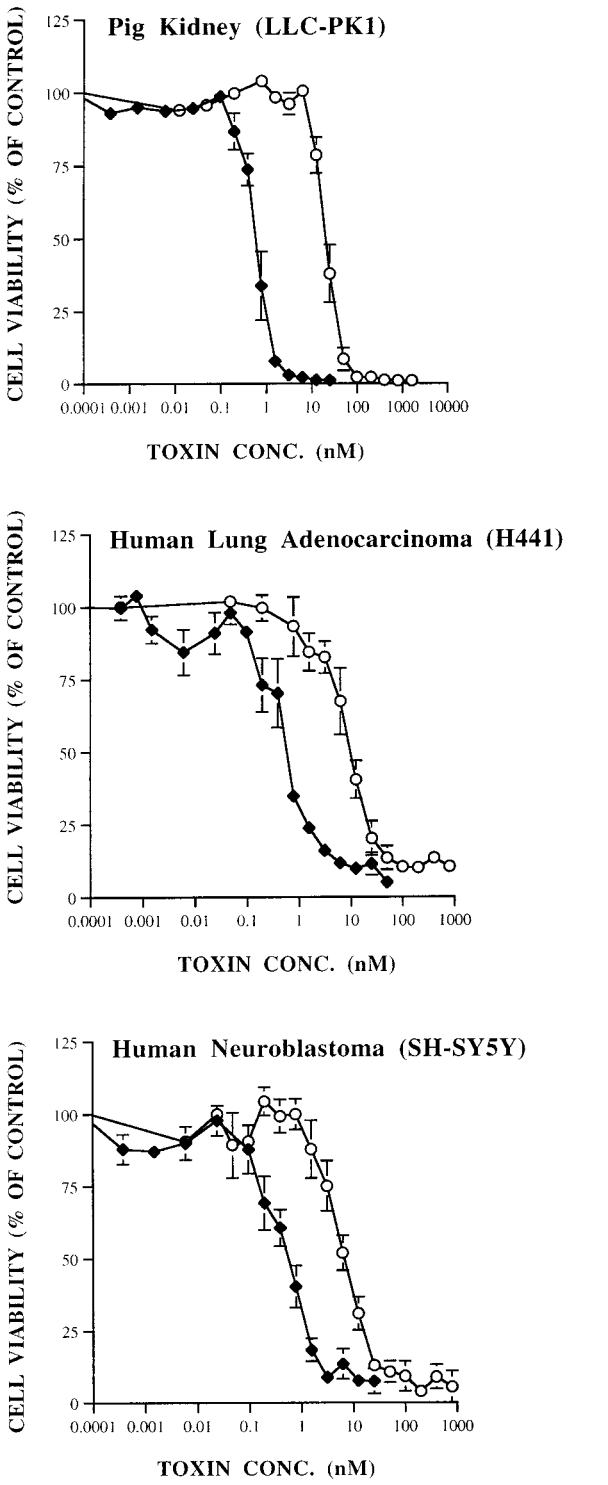

Figure 2. The cytotoxic effects of mycalamides A (**1**) and D (**3**) on cultured cells. Graphs represent cell viability as a percentage of the control (no toxin added) over a range of toxin concentrations. Values are presented as the mean ± SD. Mycalamide A (**1**) = —♦—; mycalamide D (**3**) = —○—.

light brown with a reticulate skeleton composed of tracts of subtylostyles (220−270 μm long) interspersed with microscleres: anisochelae of two size classes, 18−20 and 26−30 μm; sigmas, 20−26 μm; and raphides. The skeleton at ectosome consists of spicules identical to the choanosome, but tangentially arranged and supported by erect spicule brushes.

Extraction and Isolation. The sponge (850 g wet wt) was macerated and extracted twice with MeOH (2 L) for 24 h. The second and first methanolic extracts were passed through a column of HP20 (20 × 2.5 cm) equilibrated with MeOH. The

Table 2. Mean IC_{50} Values[a] for Mycalamide A (**1**) and Mycalamide D (**3**) on Cultured Cells[b]

cell type	mycalamide A (**1**) $IC_{50} \pm SD$	mycalamide D (**3**) $IC_{50} \pm SD$
pig kidney LLC−PK_1	0.65 ± 0.27 nM ($n = 6$)	19.43 ± 10.76 nM ($n = 7$)
human lung adenocarcinoma H441	0.46 ± 0.14 nM ($n = 6$)	9.30 ± 3.96 nM ($n = 6$)
human neuroblastoma SH−SY5Y	0.52 ± 0.22 nM ($n = 6$)	6.42 ± 1.65 nM ($n = 6$)

[a] For each of the cell lines examined, **1** was a more potent cytotoxin than **3** by at least a factor of 10. **1** vs **3** Students' unpaired *t*-test (df 10−11) $p < 0.0001$ for LLC−PK_1; $p < 0.0003$ for H441; $p < 0.0001$ for SH−SY5Y. [b] IC_{50} values for each toxin are shown (nM) for each cell line ± the SD. The number (*n*) of experiments from which IC_{50} values were calculated is shown in parentheses.

eluents were combined and passed back through the same column. The resulting eluent was diluted with H_2O (200 mL) and passed back through the column. Finally, the eluent was diluted with H_2O (9.5 L) and passed back through the column. The column was then washed with H_2O (200 mL) and eluted with 150 mL fractions of (a) 20% acetone−H_2O, (b) 55% acetone−H_2O, and (c) 100% acetone. Fraction b was concentrated to dryness to yield a viscous brown oil (190 mg). The oil was dissolved in MeOH (20 mL) and passed through an Amberchrom column (500 mg) equilibrated with MeOH. The eluent was then diluted with H_2O (60 mL) and passed back through the column. The Amberchrom was then washed with H_2O (10 mL) and transferred to the top of a 20 cm × 2.5 cm Amberchrom column equilibrated with 10% acetone−H_2O. The column was then eluted with increasing concentrations of acetone in H_2O. The 30−32% fractions were concentrated to yield 4 mg of mycalamide D (**3**) as a colorless oil. The 38−40% fractions were concentrated to yield 9 mg of mycalamide A (**1**).

Mycalamide D (3): oil; $[\alpha]^{20}_D$ +41° (*c* 0.3 $CHCl_3$); IR (KBr) ν_{max} 3421, 2966, 1734, 1684, 1542, 1388, 1076, 1027; ^1H and ^{13}C NMR (Table 1); ESHRMS *m*/*z* 512.2483 (calcd for $C_{23}H_{39}NO_{10}Na$ 512.2472).

Cytotoxicity Assays. The cytotoxicity of mycalamides A and D was initially tested in three cell lines: LLC−PK_1 (pig kidney), H441 (human lung adenocarcinoma), and SH−SY5Y (human neuroblastoma). Cell lines were maintained in Dulbecco's modified Eagle's medium−F12 medium (50:50) (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 μg/mL penicillin G, and 50 μg/mL streptomycin sulfate. Briefly, cultures were established in 96-well plates at 3000, 5000, and 10 000 cells/well for LLC−PK_1, H441, and SH−SY5Y cell lines, respectively. The different seeding densities were used to compensate for different growth rates of the cells in culture. After 96-h exposure to the toxins, cell viability was determined by the methylthiazolyltetrazolium bromide (MTT) colorimetric assay.[6] MTT standard curves were determined for each cell line, and the MTT absorbance over a range of cell densities was found to be linear for each. Data were analyzed with the SYSTAT statistical program using a nonlinear model fit, and IC_{50} values were calculated using a Logit−Log plot.[7]

Supporting Information Available: NMR spectra of **1**, including ^1H, ^{13}C, COSY, and HSQC. NMR spectra of **3**, including ^1H, ^{13}C, DEPT, COSY, ROESY, HSQC, and HMBC. This material is available free of charge via the Internet at http://pubs.acs.org.

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