Isolation of Calyculins, Calyculinamides, and Swinholide H from the New Zealand Deep-Water Marine Sponge *Lamellomorpha strongylata*

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As part of a New Zealand-based search for biologically active metabolites from marine organisms, we instigated an investigation into the chemistry of deeper-water (>−40 m) invertebrates. The initial collection in 1983 led ultimately to the isolation of a series of new and known halichondrins,[1] potent antimitotic agents. The second collection, also by benthic dredging, was along the Chatham Rise off the East Coast of South Island of New Zealand in 1995. Cytotoxicity screening of extracts from this collection against the P388 cell line yielded an incidence of activity ∼2×-higher (37%) than the average of the previous 13 years of collecting (>5000 shallow-water samples), confirming the potential of both deeper-water invertebrates and the Chatham Rise as a rich source for biologically active organisms. The most potent extracts belonged to a sponge identified as *Lamellomorpha strongylata*.[2,3] We report here the isolation of three novel cytotoxic compounds from this organism as well as a surprising result from an examination of the active components from a further organism from the Chatham Rise collection.

Results and Discussion

L. strongylata is a relatively abundant, massive, thick and sometimes folded, or incurved Epipolasid sponge supported by a stout stalk. It was collected by benthic dredging at −80 to −100 m along the top of the Mernoo Bank, within the Chatham Rise convergence zone. Repeated extraction of frozen specimens with methanol:dichloromethane yielded a dark brown oil. Bioassay-guided purification of the most active components in the extract by a sequence of ODS, DIOL (Merck), and Sephadex LH-20 chromatographies yielded a mixture of the previously reported calyculins A, B, E, and F[4,5] (**1**−**4**; 6.9×10^{-3} % wet weight), a mixture of calyculinamides A and B (**5** and **6**, 3.7×10^{-3} % wet weight), swinholide H (**7**, 8.5×10^{-3} % wet weight), and a mixture of theonellapeptolides (**9**, 3.8×10^{-2} % wet weight). Final purification of the various isomers of the calyculins (**1**−**4**) was carried out using ODS-HPLC (methanol−water mixtures), and the structures of the known metabolites were confirmed by comparison to literature values.[4,5]The new calyculin derivatives **5** and **6** were first evidenced by a more polar band of activity in the DIOL chromatographic step, as well as an additional peak in the ODS-HPLC profile of the crude extract that had identical UV-absorbance characteristics with that of calyculin A (photodiode array (PDA) detection). HRFABMS on the cesium adduct of each compound indicated identical molecular formulas of $C_{50}H_{83}O_{16}N_4P$, corresponding to hydration of the calyculin A skeleton. The ^{1}H NMR spectrum of **5** was virtually identical to that of **1** except for H2 and H4, both of which were considerably shifted downfield, while the ^{1}H NMR spectrum of **6** was nearly identical to that of **2**, showing downfield shifts for H2 and Me50 and an upfield shift for H4 (see Table 1). The ^{13}C NMR spectra indicated the presence of carbonyl resonances at δ 168.4 and 168.9 for **5** and **6**, respectively, while the resonances of C2 (as determined by the HSMQC[6] spectra) at δ 117.9 (**5**) and 119.5 (**6**) were shifted downfield 23 and 25 ppm, respectively, from that reported for **1**. These spectral features can be accounted for by hydration of the nitrile functionality of **1** and **2** to an amide in **5** and **6**. Complete assignment of the ^{1}H and ^{13}C NMR spectra were accomplished using standard 2D NMR techniques (COSY, TOCSY, HSMQC, and HMBC; see Table 1). The relative stereochemistry for **5** and **6** has been assigned as shown based on the similarity of J_{HH} values with those reported for **1** and **2**, while the absolute stereochemistry depicted is assumed, based on the sign of rotation (−) obtained. Preparation of suitable crystals in order to confirm the stereochemistry by X-ray diffraction analysis is underway.

Swinholide H (**7**) was present as an additional cytotoxic metabolite in the extract of *L. strongylata*. The molecular formula was established as $C_{80}H_{136}O_{20}$ by HRFABMS (cesium adduct). That, in combination with a UV maximum at 270 nm, appropriate searching of the MarinLit database,[7] and examination of the ^{1}H NMR spectrum, indicated the compound was closely related to the previously reported symmetric, dimeric dilactone swinholide A (**8**).[8−10] The presence of an additional methoxyl resonance at δ 3.43 in the ^{1}H NMR spectrum, coupled with an additional, deshielded methyl resonance at δ 58.4 in the ^{13}C NMR spectrum, and a molecular formula corresponding to two CH_2 units more than that of **8** indicated that **7** was a symmetrically *O*-methylated version of swinholide A. Assignment of the ^{1}H and ^{13}C spectra of **7** by 2D NMR methods (see Table 1) revealed that the resonance of H7 (δ 3.72) had moved upfield by δ 0.42 with respect to **8** while the corresponding carbon resonance (77.3 ppm) had shifted downfield by 10.7 ppm. HMBC correlations between the methoxyl protons (δ 3.43) and the two C6 methylene protons (δ 2.18 and 2.60), to the C7 carbon resonance at δ 77.3 confirmed the site of *O*-methylation. Once again the relative and absolute stereochemistries have been drawn as shown based on the similarity of J_{HH} values between **7** and **8** and the sign of rotation (−). Again, it is anticipated that preparation of a suitable crystalline derivative will confirm these stereochemical assignments.

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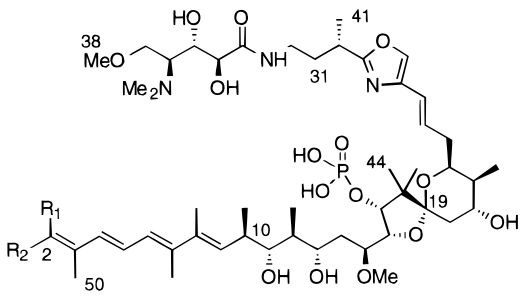
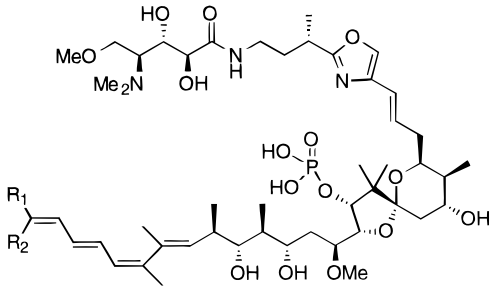
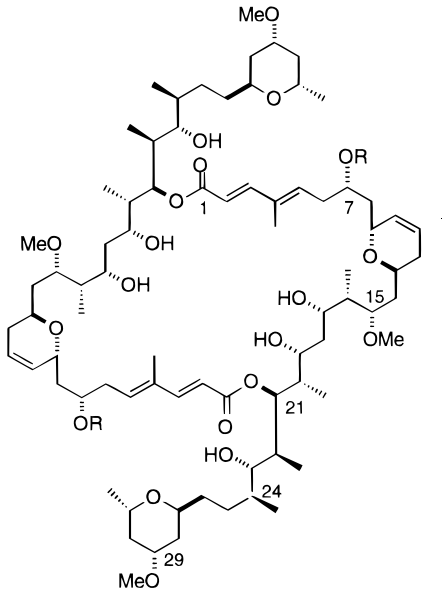
Chart 1



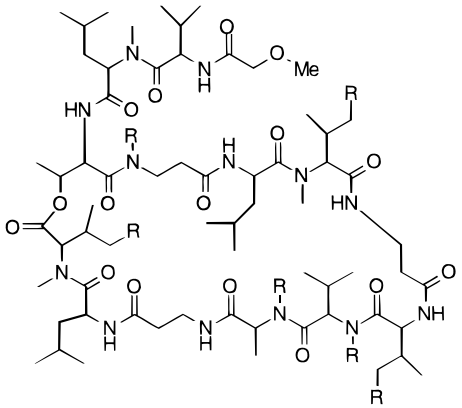
1 R_1=CN, R_2=H
2 R_1=H, R_2=CN
5 R_1=$CONH_2$, R_2=H
6 R_1=H, R_2=$CONH_2$



3 R_1=CN, R_2=H
4 R_1=H, R_2=CN



7 R=Me
8 R=H



9 R=H, Me

By examination of the ^{1}H NMR spectra, from LR and HRFABMS data, and searching of MarinLit, a third cytotoxic fraction from the *L. strongylata* extract was identified as a mixture (ODS-HPLC) of cyclic peptide lactones very closely related to the reported theonellapeptolides (**9**).[11,12] Full structural elucidation of these compounds is continuing and will be reported at a later date.

It is of particular interest to note that calyculins, swinholides, and theonellapeptolides have been reported previously as metabolites from sponges in the order Lithistida,[4,5,8−13] while the genus *Lamellomorpha* has been classified as an Epipolasid sponge, since it lacks the characteristic, interlocked desmas present in Lithistid sponges. Furthermore, while swinholides and theonellapeptolides have both been reported from *Theonella swinhoei*, this is the first time that all three classes of compounds have been isolated from a single species.

Swinholide H (**7**) has also been detected by ODS-HPLC in the bioactive fractions from a second sponge from the collection, a Dictyoceratid species, *Tedania diversiraphidiphora*, collected at a separate site on the Mernoo Bank. Recently, Faulkner and Bewley have reported that in the Lithistid sponge *Theonella swinhoei*, swinholide A (**8**) is localized to a population of mixed heterotrophic eubacteria.[13] We therefore suggest that the presence of **7** in two unrelated species from this collection offers evidence for the possibility of nonspecific symbiosis in marine invertebrates.

The swinholides had previously been established to be actin-interactive agents[14] and the calyculins to be protein phosphatase 2A inhibitors,[15] but neither class of compound had previously been evaluated by the NCI. Testing of swinholide H (**7**) and calyculinamide A and B (**5** and **6**) (as a mixture of the geometric isomers)[16] in the NCI's *in vitro* 60 cell line screening system established that each compound was a potent cell-growth inhibitor with a high degree of differential activity against a range of cell lines (see Experimental Section). In contrast calyculin A and B (**1** and **2**) (also tested as a mixture of

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(16) The tetraene moiety of the calyculins photoisomerizes very readily.[4]

Table 1. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR Chemical Shift Data for Compounds 5−7[a]

	calyculinamide A (**5**)		calyculinamide B (**6**)		swinholide H (**7**)	
carbon no.	δ ^{1}H (mult, *J* Hz)	δ ^{13}C	δ ^{1}H (mult, *J* Hz)	δ ^{13}C	δ ^{1}H (mult, *J* Hz)	δ ^{13}C
1[b]		168.4		168.9		169.9
2	5.58 (s)	117.9	5.72 (s)	119.5	5.79 (d, 15.5)	113.9
3		148.1[b]		150.2[b]	7.58 (d, 15.6)	152.6
4	7.76 (d, 15.5)	128.9	6.20 (d, 15.2)	131.1		134.0[b]
4-Me	–	–	–	–	1.82 (s)	12.3
5	6.90 (dd, 11.0,15.5)	132.2	6.94 (dd, 11.3, 15.2)	131.2	6.15 (br t, 7.5)	140.6
6	6.38 (d, 11.0)	125.0	6.28 (d, 11.3)	123.9	2.60 (m), 2.18 (m)	35.4
7		135.0[b]		134.7[b]	3.72	77.3
7-OMe	–	–	–	–	3.43 (s)	58.4
8		142.0[b]		142.1[b]	1.43, 1.79	39.9
9	6.13 (m)	130.9	6.20 (m)	131.1	4.47 (br d, 10.7)	69.7
10	2.75	35.8	2.74	35.8	5.65 (br d, 10.2)	130.2
11	3.48	80.8	3.57	80.6	5.78 (br d, 10.2)	123.9
12	1.45 (m)	42.1	1.45 (m)	42.1	1.90, 1.95	31.7
13	3.44	75.0	3.44	75.0	3.48	64.3
14	1.56, 1.80	38.4	1.56, 1.80	38.4	1.59, 1.80	37.0
15	3.75	77.2	3.75	77.4	3.92	77.2
15-OMe	–	–	–	–	3.36 (s)	57.1
16	4.00	84.8	3.98	84.9	1.47	42.9
16-Me	–	–	–	–	0.80 (d, 7.0)	9.3
17	4.07	81.8	4.07	81.9	3.78	73.9
18		50.1[b]		50.1[b]	1.60	38.5
19		108.7[b]		108.8[b]	3.92	71.2
20	1.59, 1.81	29.6	1.59, 1.81	29.6	1.73	40.7
20-OMe	–	–	–	–	0.93 (d, 7.0)	9.4
21	3.85	71.4	3.85	71.5	5.40 (br d, 10.4)	74.6
22	1.70	37.7	1.70	37.7	1.92	37.4
22-Me	–	–	–	–	0.85 (d, 7.0)	9.2
23	4.32 (br d, 12.0)	67.1	4.33 (br d, 12.0)	67.1	3.06 (br d, 9.6)	76.2
24	1.95, 2.48 (br t)	36.0	1.95, 2.48 (br t)	36.0	1.65	33.3
24-Me	–	–	–	–	0.96 (d, 6.7)	17.7
25	6.94 (m)	132.6	6.94 (m)	132.6	1.23, 1.76	24.1
26	6.10 (d, 17.5)	116.1	6.10 (d, 17.5)	116.1	1.23, 1.86	29.3
27		137.2[b]		137.3[b]	4.00	71.5
28	7.32 (s)	133.8	7.32 (s)	133.8	1.60, 1.81	35.0
29		169.9[b]		169.8[b]	3.50	73.4
29-OMe	–	–	–	–	3.33 (s)	55.3
30	3.21 (m)	28.4	3.22 (m)	28.5	1.14, 1.97	38.8
31	1.77, 1.91	33.6	1.78, 1.91	33.6	3.67	64.7
31-Me	–	–	–	–	1.19 (d, 6.1)	21.9
32	3.05 (br d), 4.04	34.4	3.05 (br d), 4.04	34.4		
33		176.3[b]		176.3[b]		
34	4.49 (d, 10.1)	73.5	4.49 (d, 10.0)	73.5		
35	3.60	68.7	3.60	68.8		
36	4.04	63.6	4.04	63.8		
37	3.76, 3.95	65.6	3.68, 3.95	65.6		
38	3.41 (s)	59.0	3.42 (s)	59.0		
39	2.89 (br s)	37.5	2.89 (br s)	37.5		
40	2.81 (br s)	44.4	2.80 (br, s)	44.4		
41	1.32 (d, 6.6)	17.6	1.31 (d, 6.8)	17.6		
42	0.87 (d, 7.3)	10.9	0.87 (d, 7.1)	10.9		
43	1.24 (s)	17.6	1.24 (s)	17.6		
44	0.91 (s)	22.4	0.91 (s)	22.4		
45	3.50 (s)	61.0	3.51 (s)	60.9		
46	0.59 (d, 6.6)	12.8	0.58 (d, 6.8)	12.7		
47	1.04 (d, 6.9)	18.0	1.05 (d, 6.8)	17.8		
48	1.84 (s)	13.8	1.85 (s)	13.0		
49	2.00 (s)	13.8	2.04 (s)	14.0		
50	2.01 (s)	20.9	2.33 (s)	13.0		
NH	8.30 (br d, 9.0)		8.30 (br d, 9.0)			
OH	5.42, 6.05, 6.55, 7.66		5.34, 6.06, 6.28, 6.60, 7.70			

[a] Values in ppm relative to C*H*Cl$_3$ (δ7.25) and *C*DCl$_3$ (δ 77.0), J_{HH} in hertz. [b] Assignment by HMBC correlations.

geometric isomers), while also potent cytotoxins, showed markedly less differential. Both swinholide H (**7**) and the calyculinamides (**5** and **6**) have been selected by the US National Cancer Institute (NCI) for further preclinical evaluation.

Experimental Section

Collection. Specimens were collected in February 1995 by benthic dredging (−80 to −100 m) from the Mernoo Bank, Chatham Rise, New Zealand. The animals were frozen immediately, transferred to our laboratory, and kept frozen at −20 °C until extraction. Duplicate voucher specimens were prepared for each sample collected and are stored in the museum at NIWA, Wellington, New Zealand. Taxonomy on the collection was by Dr. C. N. Battershill, NIWA, and (in part) by Professor Dame P. Bergquist, University of Auckland.

Isolation. ***Lamellomorpha strongylata.*** The frozen sponge (1 kg) was homogenized and extracted with methanol:dichloromethane (3:1; 3 × 2 L). The combined extracts were concentrated to an aqueous suspension (~400 mL) and partitioned with dichloromethane (3 × 250 mL). Due to the labile geometric

isomerization of the calyculins, all subsequent steps were performed in a darkened room with all columns and flasks wrapped in aluminum foil.[16] After removal of solvent, the dichloromethane solubles (6.31 g) were coated onto freshly prepared ODS silica (10 g) and packed on to a low pressure column of freshly prepared ODS silica (100 g, 8 × 20 cm) and eluted using a steep, step-gradient pattern with the first eluent being the aqueous partition. The column was subsequently eluted with water−methanol mixtures ranging from 100% water to 100% methanol, followed by stripping the column with methanol:dichloromethane (1:1) and dichloromethane. All biological activity eluted in the methanol:water (4:1) to methanol: dichloromethane (1:1) fractions. The combined active fractions (2.2 g) were coated onto DIOL (Merck, 3.2 g) and subjected to a second low pressure column chromatography (Merck DIOL, 50 g, 5 × 20 cm) with a step gradient elution pattern using hexane-dichloromethane mixtures. Fractions eluting at 30−50% dichloromethane contained calyculins A, B, E, and F (**1**−**4**) and swinholide H (**7**), while fractions eluting at 70−100% dichloromethane were found to contain calyculinamides A and B (**5** and **6**) and a mixture of theonellapeptolides (**9**). Repeated Sephadex LH-20 chromatography of these fractions resulted in the isolation of swinholide H (85 mg), a mixture of calyculins (69 mg), a mixture of calyculinamides (37 mg), and a mixture of theonellapeptolides (380 mg). Final purification by RP HPLC (Alltech ODS silica, 5 μm pore size, 20 cm length) using methanol−water mixtures afforded pure stereoisomers **5** and **6** for characterization.

Tedania diversiraphidiphora. The frozen sponge (250 g) was extracted and partitioned as described above. The dichloromethane solubles (0.8 g) were chromatographed, as described above, on a freshly prepared ODS silica column (10 g). The fraction eluting with methanol was analyzed by RP HPLC (ODS). Identical retention times and UV spectra (PDA detection) identified the presence of swinholide H (**7**).

Calyculinamide A (5): colorless solid; $[\alpha]^{20}_D$ −41° (c 0.53, EtOH); UV λ_{max} (EtOH) 201 (ϵ 32000), 226 (ϵ 30000), 337 nm (ϵ 39000); IR ν_{max} (film) 3735, 3648, 3324, 2935, 1651, 1591, 1064 cm^{-1}; HRFABMS (M + Cs)$^+$ obsd m/z 1159.4580, Δ −1.6 mmu; ^{1}H and ^{13}C NMR data, see Table 1.

Calyculinamide B (6): colorless solid; $[\alpha]^{20}_D$ −27° (c 0.145, EtOH); UV λ_{max} (EtOH) 227 (ϵ 26000), 335 nm (ϵ 45600); IR ν_{max} (film) 3734, 3316, 2935, 1652, 1588, 1065 cm^{-1}; HRFABMS (M + Cs)$^+$ obsd m/z 1159.4580, Δ −1.6 mmu; ^{1}H and ^{13}C NMR data, see Table 1. The mean graph cytotoxicity data for **5** and **6** was as follows: $\log_{10}$ GI$_{50}$ −10.14 (0.46, 1.49); $\log_{10}$ TGI$_{50}$ −9.60 (1.00, 3.04); log LC$_{50}$ −9.09 (1.51, 4.00) M (Δ, range).[17] For comparison purposes the equivalent data for the calyculin A plus B mixture (**1** and **2**) was: $\log_{10}$ GI$_{50}$ −9.56 (0.38, 2.94); $\log_{10}$ TGI$_{50}$ −9.22 (0.40, 2.62); log LC$_{50}$ −8.87 (0.43, 2.30) M (Δ, range).

Swinholide H (7): colorless solid; mp 90−91 °C; $[\alpha]^{20}_D$ −82° (*c* 0.165, EtOH); UV λ_{max} (EtOH) 270 nm (ϵ 51000); IR (film) ν_{max} 3736, 3444, 3032, 2938, 2826, 1682, 1615 cm^{-1}; HRFABMS (M + Cs)$^+$ obsd m/z 1549.8663, Δ −1.6 mmu; ^{1}H and ^{13}C NMR data, see Table 1. The mean graph cytotoxicity data was as follows: $\log_{10}$ GI$_{50}$ −8.00 (0.60, 1.49); $\log_{10}$ TGI$_{50}$ −6.71 (1.90, 4.00); log LC$_{50}$ −5.60 (1.81, 2.80) M (Δ, range).

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Supporting Information Available: Copies of ^{1}H and ^{13}C NMR spectra of **5**−**7** (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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