Phoriospongin A and B: Two New Nematocidal Depsipeptides from the Australian Marine Sponges *Phoriospongia* sp. and *Callyspongia bilamellata*

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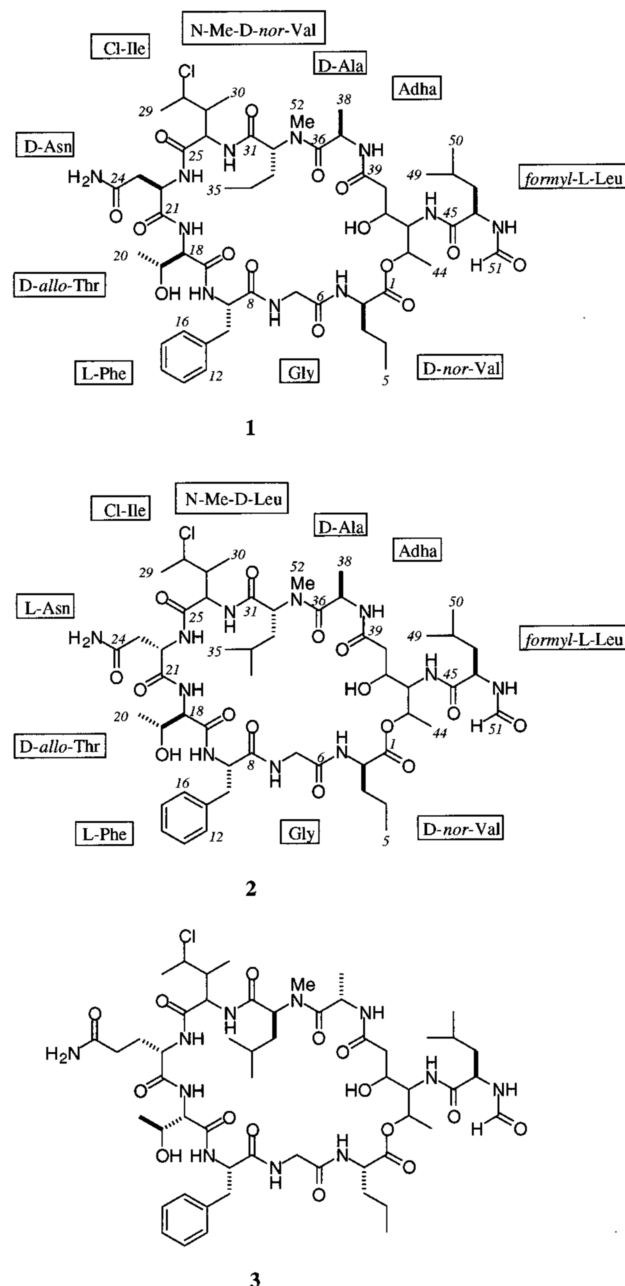
Bioassay-directed fractionation of two southern Australian sponges, *Phoriospongia* sp. and *Callyspongia bilamellata*, yielded two new nematocidal depsipeptides, identified as phoriospongins A (**1**) and B (**2**). The structures of the phoriospongins were determined by detailed spectroscopic analysis and comparison with the previously reported sponge depsipeptide cyclolithistide A (**3**), as well as ESIMS and HPLC analysis of acid hydrolysates. It is noteworthy that the unique and yet structurally related metabolites **1**−**3** are found in sponges spanning three taxonomic orders, Poescilosclerida, Haplosclerida, and Lithistida.

Marine invertebrates are well recognized for their ability to produce a wide array of novel natural products. Prominent among the more unusual metabolites reported from marine sponges in recent years are cyclic peptides, and in particular depsipeptides. While peptides are more usually recognized as biologically significant primary metabolites, sponge depsipeptides incorporate structurally diverse design elements and are generally recognized as secondary metabolites. These peptides frequently include novel amino acid residues, as well as residues of non-amino acid origin. Even the common amino acids are often present as unnatural D stereoisomers and/or *N*-methylated analogues. Not surprisingly, the structure elucidation of such compounds can be quite challenging insofar as they defy traditional amino acid sequencing strategies. During the course of our investigations into new generation agrochemical agents from marine sources we have screened a large collection of southern Australian sponges for their ability to combat the commercially significant livestock parasite *Haemonchus contortus*. To date this study has revealed many promising leads, including a selection of novel epoxylipid nematocides from the brown alga *Notheia anomola*[1−4] and the sponge alkaloids geodin A Mg salt,[5] onnamide F,[6] and the amphilactams.[7] Most recently we reported the discovery of an unprecedented class of nematocidal dithiocyanate lipids from a southern Australian *Oceanapia* sp.[8] In this report we extend the variety of structurally diverse marine nematocidal agents to include depsipeptides. Analysis of duplicate specimens of a shallow water *Phoriospongia* sp. collected by hand (snorkel) in Port Phillip Bay, Victoria, and a deeper water specimen of *Callyspongia bilamellata* collected during scientific trawling operations in the Great Australian Bight revealed the presence of two nematocidal depsipeptides. This report describes the isolation, structure elucidation, and nematocidal properties of these compounds, which have been attributed the trivial names phoriospongin A (**1**) and B (**2**).



Results and Discussion

The EtOH extracts of duplicate *Phoriospongia* sp. from Port Phillip Bay and a specimen of *Callyspongia bilamellata* from the Great Australian Bight, Australia, were found

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to exhibit significant nematocidal activity (LD_{99} 100, 194, and 106 μg/mL, respectively) against the commercially significant livestock parasite *Haemonchus contortus*. Analytical HPLC, ^1H NMR, and ESI(+)MS analysis of the crude extracts suggested that these sponges contained a common nematocidal agent(s). The EtOH extract of one of the duplicate *Phoriospongia* sp. was decanted, concentrated in vacuo, and triturated with CH_2Cl_2, and the insoluble portion was partitioned between n-BuOH and water. The nematocidal active n-BuOH solubles were subjected to C_{18} solid-phase extraction (SPE) and HPLC to yield two new depsipeptides as the sole active agents: phoriospongins A (**1**) (0.08%, LD_{99} 8.3 μg/mL) and B (**2**) (0.1%, LD_{99} 8.3 μg/mL). Similar processing of the EtOH extracts from the remaining *Phoriospongia* sp. and *Callyspongia bilamellata* confirmed comparable concentrations of the same nematocidal agents.

High-resolution ESI(+)MS analysis of **1** revealed a pseudomolecular ion [$(M + Na)^+$, *m/z* 1158.5562] consistent with a molecular formula ($C_{52}H_{82}N_{11}O_{15}Cl$, Δ mmu = −1.6) requiring 17 double-bond equivalents (DBE). Examination of the NMR data (see Table 1) strongly suggested a modified peptide. Particularly noteworthy were the numerous deshielded amide NH resonances, a cluster of α amino acid methines, and a rich selection of aliphatic primary and secondary methyls indicative of lipophilic amino acid residues (Ala, Val, Thr, Leu, Ile, ...). The amino acid composition of **1** was partially solved by HPLC analysis of an acid hydrolysate that had been subjected to derivatization with Marfey's reagent. This approach successfully identified the common amino acids Gly and L-Phe; the less common stereoisomers D-Ala, D-Leu, D-*allo*-Thr, and D-Asp (note that acid hydrolysis does not permit differentiation between D-Asp and D-Asn in the natural product); and the rarer amino acid D-*nor*-Val. Authentic standards were prepared for the Marfey's derivatives of both enantiomers of all these amino acids and compared with the natural hydrolysate by coinjection on HPLC. Since NMR analysis indicated at least one *N*-methylated amino acid, a number of likely synthetic standards were prepared and subjected to derivatization with Marfey's reagent. HPLC analysis confirmed the presence of the Marfey's derivative of *N*-Me-D-*nor*-Val.

ESI(+)MS analysis of the underivatized acid hydrolysate supported the assignment of many of the amino acids listed above, while also revealing a chlorinated species at *m/z* 166/168 [Cl-Ile, $(M + H)^+$] and an unidentified structural unit characterized by ions at *m/z* 146 [Adha, $(M + H - H_2O)^+$] and 164 [Adha, $(M + H)^+$]. Further examination of the NMR data and comparison to the known sponge depsipeptide cyclolithistide A (**3**)[9] revealed these latter two subunits as Cl-Ile and the modified carbohydrate residue Adha. Stereochemical assignments are not possible for either of these residues at this time. ESI(−)MS analysis of the underivatized acid hydrolysate clearly identified Asp [$(M - H)^-$, *m/z* 132], although as noted earlier, this observation does not permit assignment of Asn versus Asp to **1**.

Assembly of the final structure for phoriospongin A (**1**) was achieved through detailed analysis of the NMR data (see Table 1). Thus the DEPT 135, DQFCOSY, and TOCSY data supported NMR assignments, while the gHMBC revealed the following sequence of correlations: 2-NH (D-*nor*-Val) to C 6 (Gly); 7-NH (Gly) to C 8 (L-Phe); 9-NH (L-Phe) to C 17 (D-*allo*-Thr); 18-NH (D-*allo*-Thr) to C 21 (D-Asn); 22-NH (D-Asn) to C 25 (Cl-Ile); 26-NH (Cl-Ile) to C 31 (*N*-Me-D-*nor*-Val); C 52 (*N*-Me-D-*nor*-Val) to C 36 (D-Ala); 37-NH (D-Ala) to C 39 (Adha); H 43 (Adha) to C 1 (D-*nor*-Val); 42-NH (Adha) to C 45 (D-Leu); 46-NH (D-Leu) to C 51 (*formyl*). In the course of this analysis it became apparent, from consideration of the molecular formula, that phoriospongin A (**1**) incorporated a D-Asn as opposed to D-Asp residue. It is worth noting that phoriospongin A (**1**) is structurally similar to cyclolithistide A (**3**), varying in the incorporation of D-Asn versus L-Gln and *N*-Me-D-*nor*-Val versus *N*-Me-L-Leu. Curiously, not only does phoriospongin A (**1**) incorporate the less common D enantiomers of both these replacement amino acids, compared to the L enantiomers observed in cyclolithistide A (**3**), but whereas **3** is reported to contain L-*nor*-Val, L-*allo*-Thr, and L-Ala, **1** incorporates the corresponding D enantiomers. In this respect phoriospongin A (**1**) is especially rich in the less common D amino acids.

Another curious distinction between these two structurally similar marine metabolites is their response to MS/MS analysis. Whereas this technique proved invaluable in the structure elucidation of cyclolithistide A (**3**),[9] our repeated efforts on both triple quadrupole and ion trap instruments failed to achieve comparable returns for phoriospongin A (**1**). Whether this is a function of the instrument and/or operator or reflects remarkably different fragmentation characteristics remains unknown.

Phoriospongin B (**2**) proved to be a homologue of phoriospongin A (**1**), differing only in the replacement of *N*-Me-D-*nor*-Val by *N*-Me-D-Leu, and D-Asn by L-Asn. Analysis of the acid hydrolysate, with and without Marfey's derivatization, with comparison to suitable authentic and synthetic standards, as well as detailed spectroscopic analysis (see Table 2), supported the assignment for phoriospongin B (**2**) as indicated.

Sponges of the order Lithistida are well recognized for their capacity to yield novel peptides, characterized by a high proportion of D and/or *N*-methylated amino acids. The similarity between Lithistid peptides and those from microorganisms leads to speculation that Lithistid peptides might arise from symbiotic microbes.[10−13] Indeed, a bicyclic peptide from the Lithistid sponge *Theonella swinholide* has been demonstrated to be associated with natural populations of a filamentous bacteria separated from the host sponge.[14,15] The prospect that novel bioactive Lithistid peptides might be accessed by fermentation has exciting implications for the possible commercial exploitation of these complex bioactive metabolites. Whereas cyclolithistide A (**3**) is reported from a Lithistid sponge, this is not so for the phoriospongins which, as this report reveals, are found in sponges of the orders Poescilosclerida and Haplosclerida. As with speculation on Lithistid chemistry in general, the occurrence of novel cyclic depsipeptides exemplified by **1**−**3** across three taxonomic orders of sponge raises questions about the true biosynthetic origins of these compounds.

Experimental Section

General Experimental Procedures. Solid-phase extraction was carried out by using either Alltech Maxi-clean silica 900 mg cartridges or Alltech Maxi-clean C_{18} 900 mg cartridges attached to luer-lock syringes. High-performance liquid chromatography (HPLC) was performed using either a Waters 600 solvent delivery system equipped with a Waters 2700 sample manager and Waters 996 photodiode array detector, or a Waters 2790 separations module equipped with a Waters 996 photodiode array detector, Alltech 500 evaporative light scattering detector with low-temperature adapter, and Waters Fraction Collector II. Both systems operated under PC control running Waters Millennium[32] software. Chiroptical measurements ($[\alpha]_D$) were obtained on a Jasco Dip-1000 digital polarimeter in a 100 by 2 mm cell. Ultraviolet (UV) absorption

Table 1. NMR (MeOH-d_4, 400 MHz) Data for Phoriospongin A (**1**)[a]

no.	^{13}C δ	^{1}H δ [m, J (Hz)]	DQFCOSY	TOCSY	gHMBC
1	173.4				
2	54.74	4.3 (ddd, 11.6, 8.2, 3.7)	H-3b, 2-NH	H-3a, H-3b, H-4a, H-4b, H-5, 2-NH	
3a	34.09	1.83 (m)	H-3b, H4a	H-2, H-3b, H-4a, H-4b, H-5, 2-NH	
3b		1.64 (m)	H-2, H-3a, H-4b	H-2, H-3a, H-4a, H-5, 2-NH	
4a	20.52	1.48 (m)	H-3a, H-4b, H-5	H-2, H-3a, H-3b, H-4b, H-5	
4b		1.31 (m)	H-3b, H-4a, H-5	H-2, H-3a, H-4a, H-5	
5	13.51	0.93 (t, 7.3)	H-4a, H-4b	H-2, H-3a, H-3b, H-4a, H-4b	
2-NH		7.94 (d, 8.2)	H-2	H-2, H-3a, H-3b	C-6
6	172.36				
7a	45.19	3.87 (dd, 16.5, 4.0)	H-7b	H-7b	
7b		3.67 (dd, 16.5, 4.0)	H7a	H-7a	C-6, C-8
7-NH		8.86 (t, 4.0)			C-8
8	175.67				
9	51.99	5.12 (ddd, 13.1, 10.1, 7.0)	H-10a, H-10b, 9-NH	H-10a, H-10b, 9-NH	
10a	39.23	3.07 (m)	H-9, H-10b	H-9, H-10b, 9-NH	C-8, C-9, C-11, C-12/C-16
10b		3.03 (dd, 13.1, 10.1)	H-9, H-10a	H-9, H-10a, 9-NH	C-8, C-9, C-11, C-12/C-16
11	136.94				
12,16	130.49	7.27 (m)			C-10, C-11, C-14
13,15	129.61	7.31 (m)			C-11
14	128.15	7.26 (m)			C-12/C-16
9-NH		7.98 (d, 7.0)	H-9	H-9, H-10a, H-10b	C-17
17	172.36				
18	58.75	4.93 (dd, 9.5, 6.7)	H-19, 18-NH	H-20	C-17, C-19, C-20
19	69.33	3.99 (dd, 6.7, 6.4)	H-18, H-20	H-20	C-17, C-20
20	19.56	1.18 (d, 6.4)	H-19	H-18, H-19, 19-OH	C-18, C-19
19-OH		*b*		H-20	
18-NH		7.2 (d, 9.5)	H-18		C-21
21	174.24				
22	50.71	4.95 (m)	H-23a, H-23b, 22-NH	22-NH	C-21
23a	31.68	2.74 (dd, 14.3, 9.2)	H-22, H-23b	H-23b, 24-NH$_2$	C-22, C-25
23b		2.65 (dd, 14.3, 4.9)	H-22, H-23a	H-23a, 24-NH$_2$	C-22, C-25
24	*d*				
22-NH		8.58 (d, 10.1)	H-22	H-22	C-25
24-NH$_2$		*c*		H-23a, H-23b	
25	173.49				
26	56.66	4.45 (dd, 11, 8.5)	H-27, 26-NH	H-27, H-30, 26-NH	C-25, C-27
27	42.11	2.58 (m)	H-26, H-30	H-26, H-30, 26-NH	C-25, C-26, C-30
28	59.04	4.53 (dq, 6.7, 1.2)	H-29	H-29	C-26, C-27, C-29, C-30
29	23.19	1.51 (d, 6.7)	H-28	H-28, H-30	C-27, C-28
30	10.02	0.98 (d, 6.4)	H-27	H-26, H-27, H-29, 26-NH	C-26, C-27, C-28, C-29
26-NH		7.85 (d, 8.5)	H-26	H-26, H-27, H-30	C-31
31	174.6				
32	66.83	3.54 (dd, 9.5, 4.0)	H-33a, H-33b	H-33a, H-33b, H-34a, H-34b, H-35	
33a	31.68	1.92 (m)	H-32, H-33b, H-34b, H-35	H-32, H-33b, H-34a, H-35	
33b		1.58 (m)	H-32, H-33a, H-34a	H-32, H-33a, H-34b, H-35	C-31
34a	20.69	1.04 (m)	H-33b, H-34b, H-35	H-32, H-33a, H-33b, H-34b, H-35	C-31, C-35
34b		0.77 (m)	H-33a, H-34a	H-32, H-33a, H-33b, H-34a, H-35	C-35
35	14.3	0.82 (t, 6.4)	H-33a, H-34a	H-32, H-33a, H-33b, H-34a, H-34b	C-33, C-34
52	39.88	3.06 (s)			C-32, C-36
36	174.24				
37	51.32	4.07 (dd, 7.6, 6.1)	H-38, 37-NH	H-38, 37-NH	C-36, C-38
38	17.66	1.11 (d, 7.6)	H-37	37-NH	C-37
37-NH		7.36 (d, 6.1)	H-37	H-37, H-38	C-37, C-38, C-39
39	172.36				
40a	40.89	2.56 (m)	H-40b, H-41	H-40b, H-41	C-39, C-41
40b		2.21 (m)	H-40a	H-40a, H-41	C-39
41	72.27	4.47 (m)	H-40a	H-40a, H-40b	C-45
42	56.54	4.01 (d, 9.8)	42-NH	42-NH	C-44, C-45
43	75.49	5.32 (dq, 6.4, 3.7)	H-44	H-44	C-1, C-41, C-44
44	19.17	1.28 (d, 3.7)	H 43	H-43	
42-NH		8.54 (d, 9.8)	H-42	H-42	C-45
45	175.23				
46	52.63	4.615 (m)	H-47b	H-47a, H-47b, H-49, H-50	C-45, C-47
47a	42.75	1.75 (m)	H-48	H-46, H-47b, H-48, H-49, H-50	C-46, C-48
47b		1.65 (m)	H-46	H-46, H-47a, H-49, H-50	C-46, C-48
48	26.00	1.82 (m)	H-47a, H-49, H-50	H-46, H-47a, H-49, H-50	C-47
49	23.19	1.07 (d, 6.4)	H-48	H-46, H-47a, H-47b, H-48	
50	22.53	1.02 (d, 6.4)	H-48	H-46, H-47a, H-47b, H-48	
46-NH		8.41 (d, 6.7)			C-51
51	163.71	8.15 (s)			C-46

[a] ^{13}C NMR assignments are supported by a DEPT 135. [b] Overlapping with 22-NH. [c] Overlapping with 18-NH. [d] Not observed.

Table 2. NMR (MeOH-d_4, 400 MHz) Data for Phoriospongin B (**2**)[a]

no.	^{13}C δ	^{1}H δ [m, J (Hz)]	DQFCOSY	TOCSY	gHMBC
1	173.62				
2	54.78	4.29 (ddd, 11.6, 7.9, 3.6)	H-3a, H-3b, 2-NH	H-3a, H-3b, H-4a, H-4b, H-5, 2-NH	
3a	34.22	1.84 (m)	H-2, H-3b	H-2, H-3b, H-4a, H-4b, H-5, 2-NH	
3b		1.7 (m)	H 2, H-3a, H-4b	H-2, H-3a, H-4a, H-5, 2-NH	
4a	20.71	1.46 (m)	H-4b, H-5	H-2, H-3a, H-3b, H-4b, H-5	
4b		1.32 (m)	H-3b, H-4a, H-5	H-2, H-3a, H-4a, H-5	
5	13.69	0.93 (t, 7.3)	H-4a, H-4b	H-2, H-3a, H-3b, H-4a, H-4b	
2-NH		7.92 (d, 7.9)	H-2	H-2, H-3a, H-3b	C-6
6	172.65				
7a	45.21	3.86 (dd, 16.8, 3.7)	H-7b	H-7b	C-6, C-8
7b		3.66 (dd, 16.8, 3.7)	H-7a	H-7a	C-6, C-8
7-NH		8.82 (t, 3.7)			C-8
8	175.7				
9	52.69	5.12 (ddd, 12.8, 10.1, 6.7)	H-10a, H-10b, 9-NH	H-10a, H-10b, 9-NH	
10a	39.31	3.08 (m)	H-9, H-10b	H-9, H-10b, 9-NH	C-8, C-9, C-11, C-12/C-16
10b		3.02 (m)	H-9, H-10a	H-9, H-10a, 9-NH	C-8, C-9, C-11, C-12/C-16
11	137.16				
12,16	130.63	7.24 (m)			C-10, C-11, C-14
13,15	129.8	7.27 (m)			C-11
14	128.29	7.25 (m)			C-12/C-16
9-NH		8 (d, 6.7)	H-9	H-9, H-10a, H-10b	C-17
17	172.65				
18	58.74	4.93 (m)	H-19, 18-NH	H-19, H-20, 18-NH	C-17, C-19, C-20
19	69.33	4.01 (dd, 6.7, 6.4)	H-18, H-20	H-18, H-20	C-17, C-18, C-20
20	19.65	1.19 (d, 6.4)	H-19	H-18, H-19	C-18, C-19
19-OH		*b*			
18-NH		7.2 (d, 9.2)	H-18	H-18	C-21
21	174.59				
22	50.8	4.99 (m)	H-23a, H-23b, 22-NH	H-23a, H-23b, 22-NH	C-21
23a	30.46	2.75 (dd, 14.3, 9.2)	H-22, H-23b	H-22, H-23b, 24 NH_2	C-22, C-25
23b		2.65 (dd, 14.3, 5.2)	H-22, H-23a	H-22, H-23a, 24 NH_2	C-22, C-25
24	*d*				
22-NH		8.55 (d, 10.1)	H-22	H-22	C-25
24-NH_2		*c*		H-23a, H-23b	
25	173.43				
26	56.73	4.45 (dd, 11.0, 8.9)	H-27, 26-NH	H-27, H-30, 26-NH	C-25, C-27
27	42.22	2.56 (m)	H-26, H-30	H-26, H-30, 26-NH	C-25, C-26, C-30
28	59.18	4.52 (dq, 6.7, 1.2)	H-29	H-29	C-26, C-27, C-29, C-30
29	23.39	1.51 (d, 6.7)	H-28	H-28, H-30	C-27, C-28, C-30
30	10.19	0.98 (d, 6.4)	H-27	H-26, H-27, H-29, 26-NH	C-26, C-27, C-28, C-29
26-NH		7.81 (d, 8.9)	H-26	H-26, H-27, H-30	C-31
31	174.66				
32	64.84	3.63 (dd, 10.4, 7.9)	H-33a, H-33b	H-33a, H-33b, H-34, H-35, 34-Me	
33a	39.01	1.82 (m)	H-32, H-33b, H-34	H-32, H-33b, H-34, H-35, 34-Me	
33b		1.53 (m)	H-32, H-33a	H-32, H-33a, H-34, H-35, 34-Me	C-31
34	25.74	1.09 (m)	H-33a, H-35, 34-Me	H-32, H-33a, H-33b, H-35, 34-Me	C-35, 34-Me
35	24.03	0.81 (d, 6.4)	H-34	H-32, H-33a, H-33b, H-34	C-31, C-34
34-Me	21.94	0.81 (d, 6.4)	H-34	H-32, H-33a, H-33b, H-34	C-31, C-34
52	39.56	3.05 (s)			C-32, C-37
36	174.59				
37	51.43	4.06 (dd, 7.0, 6.1)	H-38, 37-NH	H-38, 37-NH	C-36, C-38
38	17.74	1.12 (d, 7.0)	H-37	H-37, 37-NH	C-36, C-37
37-NH		7.36 (d, 6.1)	H-37	H-37, H-38	C-37, C-38, C-39
39	172.46				
40a	40.94	2.57 (m)	H-40b, H-41	H-40b, H-41	C-39, C-41
40b		2.22 (m)	H-40a	H-40a, H-41	C-39, C-41
41	72.31	4.46 (m)	H-40a	H-40a, H-40b	C-45
42	56.73	3.99 (bs)	42-NH	42-NH	C-43, C-45
43	75.6	5.32 (dq, 6.4, 3.0)	H 44	H-44	C-1, C-42, C-44
44	19.33	1.26 (d, 6.4)	H-43	H-43	C-43
42-NH		8.53 (d, 9.8)	H-42	H-42	C-45
45	175.28				
46	52.69	4.63 (dd, 8.5, 5.8)	H-47a, H-47b	H-47a, H-47b, H-49, H-50	C-45, C-47
47a	42.9	1.73 (m)	H-46, H-47b	H-46, H-47b, H-49, H-50	C-46, C-48
47b		1.68 (m)	H-46, H-47a	H-46, H-47a, H-49, H-50	C-46, C-48
48	26.16	1.78 (m)	H-49, H-50	H-49, H-50	C-47
49	23.35	1.07 (d, 6.4)	H-48	H-46, H-47a, H-47b, H-48	C-47, C-48, C-50
50	22.7	1.02 (d, 6.4)	H-48	H-46, H-47a, H-47b, H-48	C-47, C-48, C-49
46-NH		8.41 (d, 6.7)			C-51
51	163.71	8.15 (s)			C-46

[a] ^{13}C NMR assignments are supported by a DEPT 135. [b] Overlapping with 22-NH. [c] Overlapping with 18-NH. [d] Not observed.

spectra were obtained using a Hitachi Model 150-20 double beam spectrophotometer, while infrared (IR) spectra were acquired using a Bio-rad FTS 165 FT-IR spectrometer under PC control running Bio-rad Win-IR software. ^{1}H and ^{13}C NMR

spectra were performed on either a Varian Inova 400, Varian Unity 300, or Varian Unity 400 plus spectrometer, in the solvents indicated and referenced to residual ^{1}H signals in the deuterated solvents. ESIMS were acquired using a Waters 2790 separations module equipped with a Micromass ZMD mass detector. High-resolution ESIMS measurements were obtained on a Bruker BioApex 47E FT mass spectrometer at a cone voltage of 100 kV. Nematocidal screening was performed using published procedures.[21]

Animal Material. Duplicate specimens of a *Phoriospongia* sp. (Museum of Victoria Registry Numbers MVF79997 and MVF79998) (extracted dry weight 70.8 and 59.1 g) were obtained in February 1998 by hand (snorkel) at Indented Head Reef, Port Phillip Bay, Australia. A specimen of *Callyspongia bilamellata* (MVF83556) (extracted dry weight 10.6 g) was obtained in July 1995 at a depth of 42 m by epibenthic sled during a scientific cruise aboard the RV *Franklin* in the Great Australian Bight, Australia.

A taxonomic description of MVF79997 and MVF79998 is as follows: growth form macrobenthic, massive, irregular-lobate; color in life pale orange-gray; color in EtOH gray; texture compressible, arenaceous, friable; surface opaque, dimpled, papillose, "brain-like"; oscules conspicuous, discrete, marginal on lobes, with membranous lip; spicules megascleres strongyles vestigial (130−150 × 2 μm); microscleres none; ectosome a distinct crust 100−400 μm in thickness of regular small-grain detritus; choanosome an anastamosing reticulation of tracts of detritus bound by spongin with strongyles occasionally forming whispy, plumose tracts and foreign spicular detritus scattered throughout the collagenous matrix.

A taxonomic description of MVF83556 is as follows: growth form macrobenthic with a thick basal attachment, thickly flabelliform-cup-shaped, 10−15 mm thick, lamellate; color on deck dark beige; color in EtOH brown; texture compressible, harsh, exuding mucus; oscules small and scattered; surface translucent, microscopically hispid, coarsely rugose; megascleres oxeas hastate with axial canal, occasionally stylote (80−120 × 5−8 μm); ectosome a single tangential, detachable layer of oxeas overlying subectosomal tufts of oxeas; choanosome a regular, isodictyal (polygonal) reticulation of spicules bundles with a few primary fibers, up to 100 μm in thickness, curving out toward and perpendicular to the surface, cored by multispicular tracts of oxeas. Interstitial collagen abundant and granular.

Extraction and Isolation. The frozen sponges were transported to the laboratory, where they were thawed, documented, diced, and stored in EtOH at −20 °C until required. A portion of the decanted EtOH extract (~50%) from one of the duplicate *Phoriospongia* sp. was concentrated in vacuo to yield a dark oil (2.2 g) that displayed nematocidal activity (LD_{99} 100 μg/mL). This material was triturated with CH_2Cl_2 and the insoluble portion partitioned between n-BuOH and water. The n-BuOH solubles (150 mg, 6.8%) were further fractionated by C_{18} solid-phase extraction (SPE) (10% stepwise gradient elution from H_2O to MeOH) and C_{18} HPLC (2 mL/min isocratic elution with 15% H_2O/MeOH through a 5 μm Phenomenex C_{18} 250 × 10 mm column) to yield the novel depsipeptides phoriospongin A (**1**) (1.7 mg, 0.08%, LD_{99} 8.3 μg/mL) and phoriospongin B (**2**) (2.3 mg, 0.10%, LD_{99} 8.3 μg/mL). The remaining duplicate *Phoriospongia* sp. was processed as above to yield a comparable recovery of phoriospongins A and B. The specimen of *Callyspongia bilamellata* yielded EtOH extractables (1.3 gm), which partitioned to nematocidal n-BuOH solubles (75 mg, 3.4%, LD_{99} 63 μg/mL) that were further fractionated by C_{18} SPE to yield a mixture of phoriospongins A and B (1.3 mg, 0.1%). This mixture was analyzed by analytical HPLC, ^{1}H NMR, and ESI(+)MS. Percentage yields are calculated against the total mass of crude extractables.

Phoriospongin A (1): colorless solid; $[\alpha]^{20}_D$ +18° (*c* 0.08, MeOH); IR (KBr) ν_{max} 3348 (br), 2925, 1702−1566, 1460−1384 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 204 (13750), 229 (4100), 273 (1300); ^{1}H NMR (MeOH-d_4, 400 MHz), see Table 1; ^{13}C NMR (MeOH-d_4, 100 MHz), see Table 1; ESI(+)MS (20 kV) *m*/*z* 1136 (M + H)$^+$; HRESI(+)MS 1158.5562 [(M + Na)$^+$, $C_{52}H_{82}N_{11}O_{15}ClNa$ requires 1158.5578].

Phoriospongin B (2): colorless solid; $[\alpha]^{20}_D$ −6.2° (*c* 0.13, MeOH); IR (KBr) ν_{max} 3371 (br), 2928, 1725−1546 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 207 (16350), 229 (3750), 273 (1000); ^{1}H NMR (MeOH-d_4, 400 MHz), see Table 2; ^{13}C NMR (MeOH-d_4, 100 MHz), see Table 2; ESI(+)MS (20 kV) *m*/*z* 1150 (M + H)$^+$; HRESI(+)MS 1172.5701 [(M + Na)$^+$, $C_{53}H_{84}N_{11}O_{15}ClNa$ requires 1172.5734].

Acid Hydrolysis. Phoriospongins A (**1**) (1.4 mg) and B (**2**) (1.8 mg) were treated separately with 6 N HCl (2.8 and 3.6 mL, respectively) and the resulting solutions stirred at 110 °C for 24 h, after which the reaction mixtures were dried in vacuo and redissolved in water (700 and 900 μL, respectively). These acid hydrolysates were then subjected to ESIMS analysis, followed by derivatization and HPLC analysis.

ESIMS Analysis of the Acid Hydrolysates. Acid hydrolysates obtained from phoriospongins A (**1**) and B (**2**) were submitted to ESIMS analysis using direct infusion into a Micromass ZMD. In the case of the hydrolysate from **1** significant ions were observed in the ESI(+)MS at *m*/*z* 118 (Val), 120 (Thr),132 (Leu and *N*-Me-*nor*-Val), 146+164 (Adha), 166 (Phe), and 166/168 (Cl-Ile). The hydrolysate from **2** revealed ions in the ESI(+)MS at *m*/*z* 118 (Val), 120 (Thr), 132 (Leu), 146+164 (Adha and *N*-Me-Leu), 166 (Phe), and 166/168 (Cl-Ile). Instrumental limitations prevented observation of ions for Gly and Ala in either hydrolysate. ESI(−)MS analysis of both hydrolysates revealed a significant ion at *m*/*z* 132 (Asp: note that the presence of ions for Asp in the hydrolysate does not differentiate between Asn and Asp in the phoriospongins).

Marfey's Analysis. To 50 μL of each acid hydrolysate (or authentic amino acid standard at comparable concentration) was added 20 μL of 1 M sodium bicarbonate and 100 μL of 1% l-FDAA in acetone. The solutions were stirred at 37 °C for 60 min, after which the mixtures were neutralized with 20 μL of 1 N HCl and the derivatized samples diluted with 810 μL of acetonitrile. HPLC analysis involved elution of 5 μL of the derivatized solutions through a Phenomenex Luna C_{18}(2) 4.6 × 150 mm column, with a 1 mL/min 45 min linear gradient from 15% B in A to 45% B in A (solvent A: 0.1 M aqueous ammonium acetate solution to pH 3 with the addition of TFA; solvent B: MeCN) with UV detection at 340 nm.

Authentic Standards. Retention times (t_R, min) of authentic amino acid Marfey's derivatives were L-Asp (8.77), L-*allo*-Thr (9.52), D-Asp (10.42), D-*allo*-Thr (11.58), Gly (12.19), L-Ala (13.45), D-Ala (18.33), L-*nor*-Val (23.41), *N*-Me-L-*nor*-Val (27.55), L-Phe (28.02), L-Leu (29.28), *N*-Me-D-*nor*-Val (29.32), D-*nor*-Val (30.42), D-Phe (33.81), *N*-Me-L-Leu (34.07), D-Leu (36.10), and *N*-Me-D-Leu (36.68).

Phoriospongin A Hydrolysate. Retention times of the Marfey's derivatives in the acid hydrolysate of **1** were 11.01 (D-Asp), 11.54 (D-*allo*-Thr), 12.21 (Gly), 18.26 (D-Ala), 27.95 (L-Phe), 29.46 (*N*-Me-D-*nor*-Val), 30.16 (D-*nor*-Val), and 35.98 (D-Leu) min.

Phoriospongin B Hydrolysate. Retention times of the Marfey's derivatives in the acid hydrolysate of **2** were 9.12 (L-Asp), 11.62 (D-*allo*-Thr), 12.17 (Gly), 18.28 (D-Ala), 28.09 (L-Phe), 30.25 (D-*nor*-Val), 34.12 (*N*-Me-L-Leu), and 36.03 (D-Leu) min.

Synthesis of *N*-Me-Leu. Synthetic samples of *N*-Me-L-Leu (336 mg, 15.2%) and *N*-Me-D-Leu (234 mg, 42.3%) were prepared by the method of Grieco et al.[16] These amino acid analogues proved to be identical by ^{1}H NMR, ESI(+)MS, and $[\alpha]_D$ with literature values.[17,18]

Synthesis of *N*-Me-*nor*-Val. Synthetic samples of *N*-Me-D-nor-Val (35 mg, 8.03%) and *N*-Me-L-nor-Val (42 mg, 7.7%) were prepared by the method of Golebiowski et al.[19] These amino acid analogues proved to be identical by ^{1}H NMR, ESI-(+)MS, and $[\alpha]_D$ with literature values.[20]

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