An Antifungal Cyclodepsipeptide, Cyclolithistide A, from the Sponge *Theonella swinhoei*

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A novel antifungal cyclic depsipeptide, cyclolithistide A (**1**), was isolated from a marine sponge, *Theonella swinhoei*. The structure of cyclolithistide A, which contains the unique amino acids 4-amino-3, 5-dihydroxyhexanoic acid, formyl-leucine, and chloroisoleucine, was elucidated through a combination of spectroscopic techniques. Thirteen additional specimens of *T. swinhoei* and three of *Theonella conica* were examined in this study. Many contained the two cyclic peptides, motuporin (**2**) and theonellapeptolide 1d (**3**), as major components, but none of these were a source of **1**. The rationale behind this study and some additional aspects of these unusual results are presented.

Macrocycles having both intricate structures and rich physiological properties are often isolated from marine sponges in the Lithistida order. Primarily, the natural products from Lithistida sponges examined to date fall into two classes: polyketide esters[1,2] as monomers and dimers, and cyclic polypeptides[3,4,5] ranging from cyclic depsipeptides to large-ring bicyclic peptides. There are wide ranging perspectives underlying the continuing investigation of these compounds which are unique to lithistid sponges. The presence of many stereocenters and multiple rings presents a considerable challenge to the elucidation of new structures or dereplication of known compounds.[5] Not unexpectedly, the quest for concise total syntheses of these natural products has been taken up by several groups.[5a,6] In addition, these lithistid metabolites have also been broadly used as tools to study cell biological processes.[5a,7]

Several years ago we undertook a biogeographical comparison of the lithistid sponge *Theonella swinhoei* because this species is especially rich in cyclic peptides with uncommon amino acids. The diverse array of *T. swinhoei* derived cyclic polypeptides headed by cyclotheonamides,[5h] keramamides,[5f] motuporin,[5i] perthamide B,[5j] theonellamides,[5k] theonegramide,[5b] theonellapeptolides,[5c] and theopalauamide[5l] made it an ideal candidate for such a project. A further curious circumstance is that there are close similarities between metabolites reported from *Theonella* sponges and cultured cyanobacteria. The most striking is that (−)-motuporin (**2**),[5i] from *T. swinhoei*, has a structure which differs from that of (−)-nodularin,[8] from cultured *Nodularia spumigena*, by only the *S*-valine being replaced with *S*-arginine.[7i] This raises a question about some type of parallel origin for these compounds.[3,9]

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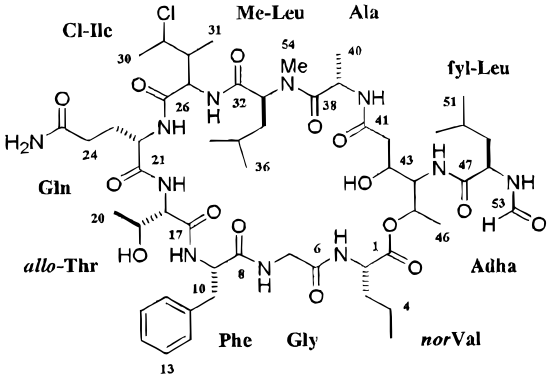
We have repeatedly encountered (−)-motuporin (**2**)[5i] and (−)-theonellapeptolide 1d (**3**)[5c] as the two major metabolites from Papua New Guinea (PNG) collections of *T. swinhoei* and *T. conica*, both heavily infested with cyanobacteria. In this paper we report on the study of 17 specimens of these two organisms, from both PNG and Indonesia, including those devoid of cyanobacteria. Interestingly, one of these specimens contained the new antifungal cyclic decapeptide, cyclolithistide A (**1**), whose structure and properties are described herein.



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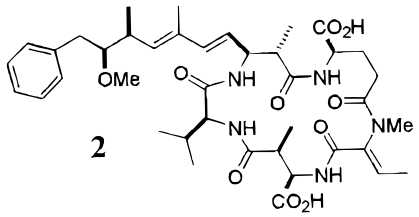

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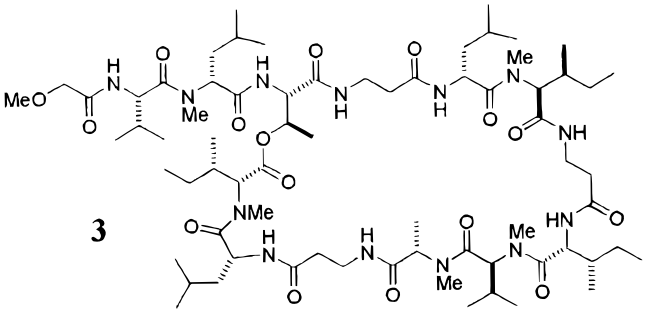

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Results and Discussions

Two methods were used to reliably identify the major constituents **2** and **3** in the various crude extracts shown in Table 1 of both *T. swinhoei* and *T. conica*. Characteristic lowfield ^{1}H NMR spectral peaks of motuporin (**2**) included the four vinyl (δ 5.3−6.9) and five aromatic (δ 7.1−7.3) protons which were repeatedly observed in the crude extracts. In a similar fashion theonellapeptolide 1d (**3**) was dereplicated by observing the intense singlet resonances (δ 2.7−3.5) for one OMe and five NMe groups. In a somewhat different approach some of the crude extracts were examined by mass spectrometry. The strategy was to use diagnostic clusters of ESI mass spectral peaks for **2** consisting of $[MH]^+$ at *m/z* 768.3 or $[MNa]^+$ *m/z* = 790.4 and for **3** $[MH]^+$ *m/z* 1405.1 (Figure S11 in Supporting Information).

The isolation of (−) cyclolithistide A (**1**) was carried out on a single specimen (coll. no. 94590, 0.1 kg, wet weight) found in a cave near Sangihe Island, Indonesia.

Table 1. Summary of *Theonella* Sponges

sample no.	coll. no.[a]	location[b]	cyano-bacteria	motu-porin (**2**)	theonella-peptolide Id (**3**)
T. swinhoei					
1	89176	PNG -E	yes	yes	yes
2	90107	PNG -E	yes	yes	yes
3	90217	PNG -E	yes	yes	yes
4	93107	PNG -E	yes	yes	yes
5	93168 ecto	PNG -E	yes	yes	yes
	93169 endo	PNG -E	no	yes	yes
6	95001	PNG -E	yes	yes	yes
7	95068	PNG -E	yes	yes	yes
8	95559 ecto	PNG -E	yes	yes	yes
	95560 endo	PNG -E	no	yes	yes
9	91130	PNG -C	no	no	no
10	94509	IND -E	yes	no	no
11	94510	IND -E	yes	yes	yes
12	94533	IND -E	yes	yes	yes
13	94590	IND -C	no	no	no
14	95574	IND -C	no	no	yes
T. conica					
15	93162	PNG -E	yes	yes	yes
16	93170 ecto	PNG -E	yes	yes	yes
	93171 endo	PNG -E	no	yes	yes
17	96227	PNG -E	yes	yes	no

[a] ecto, ectosome; endo, endosome. [b] PNG, Papua New Guinea; IND, Indonesia; E, exposed; C, cave.

Visual plus microscopic examination revealed that little or no cyanobacteria were observed on the ectosome of this sponge (see Plate 1 in Supporting Information). Cyclolithistide A (**1**) was eventually obtained as an amorphous white solid (45.3 mg, 0.065% based on 69 g dry weight of sponge) from the CH_2Cl_2 solvent partition fraction of the crude extract. The extensive purification by reversed-phase gradient HPLC proceeded uneventfully and yielded material which gave an intense $[MH]^+$ peak by ESIMS at *m/z* 1164.7 which was similar to that observed on the CH_2Cl_2 solvent partition fraction (see Figure S11 in Supporting Information). Most important, the ESI spectrum of this fraction intimated that **2** (MW = 767.3) and **3** (MW = 1404.1) were absent.

The structure elucidation on cyclolithistide A (**1**) proceeded once the molecular formula was established as $C_{54}H_{86}ClN_{11}O_{15}$ (unsaturation equivalence = 17) by using a combination of MS (Figure 3) and NMR data (Tables 2 and 3). Proper interpretation of the HRFABMS $[MH]^+$ *m/z* = 1164.6080 (Δ 0.8 mmu of calcd) was possible after a Cl was added to the atom list. The APT/DEPT ^{13}C NMR spectra revealed carbon residues including 10 CH_3, 1 NMe, 9 CH_2, 16 sp^3 CH, 6 sp^2 CH (as a monosubstituted benzene and a formamide), and 12 sp^2 C for a total of $C_{54}H_{73}$. The NMe, δ_H 2.82 (s), and δ_C 33.2 (q) of this preceding list was assumed on biosynthetic grounds to be an *N*-methyl amide. Adding to the count of 73 protons from the APT and ^{1}H NMR (CD_3OD in Figures S1 and S2, Supporting Information) were an additional 11 protons observed in the DMSO-d_6 ^{1}H NMR spectrum (Table 2 and Figure S3, Supporting Information) which consisted of 9 amide NH signals between δ 7.24 and δ 8.29 and a $-C{=}O(NH_2)$ signal at δ 6.70. These data also accounted for all 11 nitrogen atoms and 11 of the 15 oxygens. The last two heteroatom-substituted hydrogens not observed by ^{1}H NMR spectroscopy were assumed to be attached to the undefined 4 oxygen atoms. To focus further on this issue, the four ^{13}C NMR CH resonances between δ 65 and δ 72 (Figures S2 and S4, Supporting Information) were closely inspected as potential carbon sites to attach the oxygens. The HMQC spectra pin-

Table 2. ^{1}H and ^{13}C NMR[a] Data for Cyclolithistide A (1) in DMSO-d_6

	no.	^{13}C NMR δ (mult)	^{1}H NMR δ (mult, J (Hz), int)	^{1}H−^{1}H COSY	HMBC	ROESY[b]
n-Val	1	171.65 (s)				
	2	52.49 (d)	4.24 (bd, 8.0, 1H)	H3/3′, NH2	C3, C4, C6	H3, H4, NH2
	3	33.74 (t)	1.63, 1.52 (m, 2H)	H2, H3/3′, H4/4′		H2
	4	18.62 (t)	1.33, 1.26 (m, 2H)	H3/3′, H5		H2
	5	13.58 (q)	0.85 (t, 7.0, 3H)	H4/4′	C3, C4	
	NH2		7.68 (d, 9.5, 1H)	H2		H2, H7/7′
Gly	6	169.28 (s)				
	7	42.57 (t)	3.74,3.58 (dd, 5.5, 16.5, 2H)	H7/7′, NH7	C6, C8	H7/7′, NH2, NH7
	NH7		8.07 (bs, 1H)	H7/7′		H7
Phe	8	171.99 (s)				
	9	51.00 (d)	4.83 (bd, 7.0, 1H)	H10, NH9	C8, C11, C17	H10, H12/16, H43, H44, H45, H46, NH9
	10	37.00 (t)	2.93 (m, 2H)	H9	C8, C9	H9, H12/16
	11	137.30 (s)				
	12, 16	129.34 (d)	7.20 (m, 2H)			H9, H10
	13, 15	128.18 (d)	7.22 (m, 2H)			
	14	126.35 (d)	7.17 (m, 1H)			
	NH9		8.00 (d, 6.0, 1H)	H9	C17	H9, H18, H19
Thr	17	169.95 (s)				
	18	58.25 (d)	4.38 (d, 6.0, 1H)	H19, NH18	C17, C19, C20	H19, H20, H39, NH9
	19	66.81 (d)	3.83 (bq, 6.0, 1H)	H18, H20		H18, H20, NH9, NH18
	20	18.90 (q)	0.92 (d, 6.0, 3H)	H19	C18, C19	H18, H19
	NH18		7.95 (d, 6.0, 1H)	H18	C21	H19, H22
Gln	21	171.80 (s)				
	22	52.06 (d)	4.32 (bd, 5.5, 1H)	H23/23′	C21	H23/23′, H24, H29, H30, NH18
	23	27.82 (t)	1.91, 1.77 (m, 2H)	H22, H23/23′, H24/24′		H22, H24
	24	31.50 (t)	2.08 (m, 2H)	H23/23′	C23, C25	H22, H23/23′
	25	173.98 (s)				
	NH22		7.24 (d, 7.5, 1H)		C26	
	NH_2 25		6.70 (bs, 2H)		C24, C25	
Cl−Ile[c]	26	173.89 (s)				
	27	55.26 (d)	4.27 (d, 8.0, 1H)	H28	C28, C29, C31, C32	H28, H31
	28	40.64 (d)	2.12 (m, 1H)	H27, H29, H31	C26	H27, H29, H30, H31, NH27
	29	59.28 (d)	4.41 (bq, 6.0, 1H)	H28, H30	C27, C28, C30, C31	H22, H28, H30, H31, NH27
	30	22.94 (q)	1.45 (d, 6.0, 3H)	H29	C28, C29	H22, H28, H29, H31
	31	10.06 (q)	0.90 (d, 6.5, 3H)	H28		H27, H28, H29, H30
	NH27		8.17 (bs, 1H)			H28, H29
MeLeu	32	171.09 (s)				
	33	56.80 (d)	4.59 (bs, 1H)	H34		
	34	37.01 (t)	1.56 (m, 2H)	H33		H35, H54
	35	24.25 (d)	1.30 (m, 1H)	H36, H37		H34, H36, H37, H54
	36	23.34 (q)	0.82 (d, 6.5, 3H)	H35	C34, C35, C37	H35, H54
	37	21.51 (q)	0.76 (d, 6.5, 3H)	H35	C34, C35, C36	H35, H54
	54	33.18 (q)	2.82 (s, 3H)		C33, C38	H34, H35, H36, H37, NH39
Ala	38	172.21 (s)				
	39	48.39 (d)	4.23 (q, 7.0, 1H)	H40, NH39	C40	H18, H40, NH39
	40	18.21 (q)	1.17 (d, 7.0, 3H)	H39	C38, C39	H39, NH39
	NH39		7.60 (d, 7.0, 1H)	H39	C41	H39, H40, H54
Adha[d]	41	170.03 (s)				
	42	39.90 (t)	2.22 (m, 2H)	H43	C43	H43, H44, H45, NH44
	43	68.61 (d)	4.07 (bs, 1H)	H42, H44		H9, H42, H44, H46, NH44
	44	54.95 (d)	3.90 (m, 1H)	H43, H45, NH44		H9, H42, H43, H45, H46, NH44
	45	71.53 (d)	5.05 (bq, 6.5, 1H)	H44, H46		H9, H42, H44, H46, NH44
	46	17.40 (q)	1.10 (d, 6.5, 3H)	H45	C44, C45	H9, H43, H44, H45, NH44
	NH44		7.70 (bs, 1H)	H44	C47	H42, H43, H44, H45, H46, H48, H49, H51
fyl-Leu[e]	47	172.33 (s)				
	48	49.88 (d)	4.56 (dd, 8.0, 14.0, 1H)	H49, NH48	C47, C49, C50	H49, H50, H52, H53, NH44, NH48
	49	41.39 (t)	1.50 (m, 2H)	H48	C48, C50, C51, C52	H48, H51, H53, NH44, NH48
	50	24.34 (d)	1.60 (m, 1H)	H51, H52		H48, H53, NH48
	51	23.06 (q)	0. 90 (d, 6.5, 3H)	H50	C52	H49, NH44
	52	21.51 (q)	0.88 (d, 7.0, 3H)	H50	C50, C51	H48
	NH48		8.29 (d, 8.0, 1H)	H53	C53	H48, H49, H50
	53	160.91 (d)	8.04 (s, 1H)	NH48	C48	H48, H49, H50

[a] Recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C. [b] ROESY mixing time t_m 300 ms. [c] Chloroisoleucine. [d] 4-Amino-3,5-dihydroxyhexanoic acid. [e] Formyl-leucine.

pointed the ^{1}H resonances associated with those carbons as occurring between δ 3.83 and δ 5.05. A correlation in the HMBC NMR spectrum between δ 56.8 (C33) and δ 2.82 (H54) as well as between δ 4.59 (H33) and δ 33.2 (NMe54), plus other characteristic ^{13}C NMR shifts and ^{1}H−^{1}H COSY correlations, proved that the CH33 moiety was the α-carbon of an *N*-MeLeu subunit. Therefore, an ester and two alcohol substituents were envisioned to be

Table 3. ^{1}H and ^{13}C NMR[a] Data for Cyclolithistide A (1) in CD_3OD

	no.	^{13}C NMR δ (mult)	^{1}H NMR δ (mult, J (Hz), int)	^{1}H−^{1}H COSY	HMBC	ROESY[b]
n-Val	1	173.92 (s)				
	2	55.18 (d)	4.23 (dd, 3.5, 11.5, 1H)	H3/3′	C3	H3, H4
	3	34.13 (t)	1.84, 1.68 (m, 2H)	H2, H3/3′		H2
	4	20.80 (t)	1.50, 1.34 (m, 2H)	H4/4′, H5		H2
	5	13.64 (q)	0.95 (t, 7.0, 3H)	H4/4′	C3, C4	
	NH2					
Gly	6	172.62 (s)				
	7	45.38 (t)	3.87, 3.67 (dd, 5.5, 16.5, 2H)	H7/7′		
	NH7					
Phe	8	174.56 (s)				
	9	52.00 (d)	5.10 (dd, 5.0, 10.5, 1H)	H10/10′		H10, H12/16
	10	39.31 (t)	3.09, 3.02 (dd, 5.0, 10.5, 2H)	H9	C9	H9, H12/16
	11	137.05 (s)				
	12, 16	130.57 (d)	7.27 (m, 2H)		C10, C14	H9, H10
	13, 15	129.75 (d)	7.31 (m, 2H)		C11	
	14	126.35 (d)	7.26 (m, 1H)			
	NH9					
Thr	17	172.62 (s)				
	18	58.42 (d)	4.93 (d, 7.0, 1H)	H19	C19, C20	H19, H20, H45
	19	69.44 (d)	4.00 (m, 1H)	H18, H20		H18
	20	20.05 (q)	1.22 (d, 6.5, 3H)	H19	C18, C19	H18
	NH18					
Gln	21	174.08 (s)				
	22	52.61 (d)	4.58 (t, 3.5, 1H)	H23/23′		H23, H24
	23	30.86 (t)	2.12, 2.07 (m, 2H)	H22		H22
	24	32.29 (t)	2.31, 2.21 (m, 2H)	H24/24′	C22, C23, C25	H22
	25	178.09 (s)				
	NH22					
	NH_2 25					
Cl−Ile[c]	26	175.52 (s)				
	27	56.56 (d)	4.45 (d, 11.0, 1H)	H28	C28	H31, H35, H36, H37
	28	42.10 (d)	2.59 (m, 1H)	H27, H31		H29, H30, H31
	29	59.17 (d)	4.53 (dq, 1.5, 7.0, 1H)	H30	C31	H28, H30
	30	23.32 (q)	1.51 (d, 7.0, 3H)	H29	C28, C29	H28, H29
	31	10.12 (q)	0.98 (d, 6.5, 3H)	H28	C27, C28, C29	H27, H28
	NH27					
MeLeu	32	173.31 (s)				
	33	65.07 (d)	3.56 (dd, 4.5, 8.5, 1H)	H34/34′		H34, H36, H37, H54
	34	38.96 (t)	1.87, 1.54 (m, 2H)	H33		H33, H34/34′, H36, H37
	35	25.66 (d)	1.08 (bs, 1H)	H36, H37		H27, H54
	36	24.01 (q)	0.80 (d, 6.5, 3H)	H35	C34, C35	H27, H33, H34, H54
	37	21.86 (q)	0.80 (d, 6.5, 3H)	H35	C34, C35	H27, H33, H34, H54
	54	39.78 (q)	3.05 (s, 3H)		C33, C38	H33, H35, H36, H37
Ala	38	174.56 (s)				
	39	51.41 (d)	4.06 (q, 7.0, 1H)	H40	C40	H40, H43
	40	17.61 (q)	1.09 (d, 7.0, 3H)	H39	C38, C39	H39
	NH39					
Adha[d]	41	173.21 (s)				
	42	40.74 (t)	2.56, 2.17 (dd, 2.5, 11.0, 2H)	H42/42′, H43		H42/42′, H43, H44
	43	72.36 (d)	4.47 (ddd, 2.5, 3.0, 11.0, 1H)	H42/42′, H44	C42	H39, H42′, H44, H45
	44	56.65 (d)	3.98 (bd, 3.0, 1H)	H43	C43, C46, C47	H42′, H43, H45
	45	75.58 (d)	5.30 (dq, 3.0, 6.5, 1H)	H46		H18, H43, H44, H46
	46	19.32 (q)	1.26 (d, 6.5, 3H)	H45	C44, C45	H45
	NH44					
fyl-Leu[e]	47	175.14 (s)				
	48	52.61 (d)	4.61 (dd, 6.5, 13.5, 1H)	H49	C49	H49, H52
	49	42.90 (t)	1.72 (m, 2H)	H48		H48, H51, H52
	50	26.11 (d)	1.80 (m, 1H)	H51, H52		H51, H52
	51	23.32 (q)	1.07 (d, 6.5, 3H)	H50	C49, C50, C52	H49, H50
	52	22.69 (q)	1.02 (d, 6.5, 3H)	H50	C49, C50, C51	H48, H49, H50
	NH48					
	53	163.65 (d)	8.16 (s, 1H)		C48	

[a] Recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C. [b] ROESY mixing time t_m 300 ms. [c] Chloroisoleucine. [d] 4-Amino-3,5-dihydroxyhexanoic acid. [e] Formyl-leucine.

attached at the remaining three carbons (δ 66.8, 68.6, 71.5). Relative ^{1}H NMR chemical shifts suggested that the ester was attached to the CH at δ 5.05 (H45) and an OH group was linked to each CH at δ 4.07 (H43) and δ 3.83 (H19).

Cyclolithistide A was seen to contain one macrocyclic ring once the unsaturation from the benzene ring and 12 carbonyl groups had been reconciled. A combination of ^{1}H−^{1}H COSY, HMQC, and HMBC data revealed nine separate spin systems for the RCH portion of subunits RCH(N)(C=O)Z. As outlined below, another spin system consisted of a −(O=C)O−CH(CH_3)−CH(NHCO)−CH(OH)−CH_2−C(=O)− array. The next challenge was to more fully define each of these 10 residues and then link them together in an unequivocal way. Analysis of the COSY, HMQC, and HMBC data (Tables 2 and 3) pro-

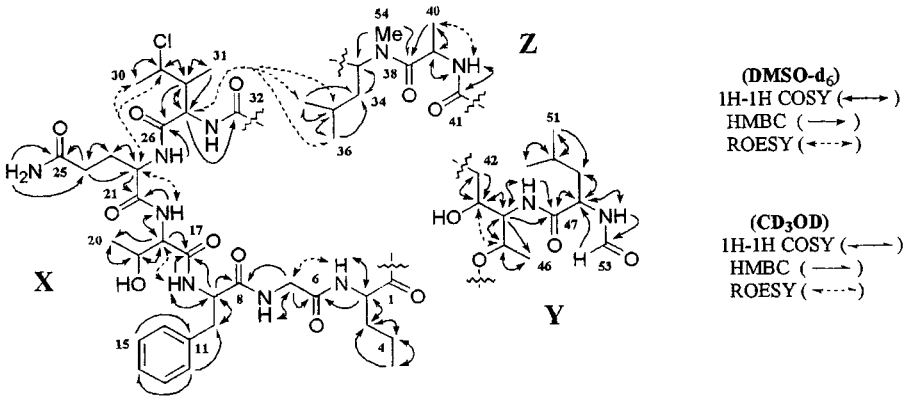

Figure 1. Partial structures **X**−**Z**, with selected 2D NMR correlations.

vided a concise way to proceed. Seven known amino acids were quickly identified and included Ala, *N*-MeLeu, Gln, Thr, Phe, Gly, and *nor*Val.

Three new amino acid residues were identified as follows. A 4-amino-3,5-dihydroxyhexanoic acid (Adha) framework was proposed using Me46 (δ 17.4/δ 1.10) as the anchor point to trace ^{1}H−^{1}H COSY and HMBC connectivities from it through to C41. The first part of a side chain was pieced together by noting that the amide NH (δ 7.70) was coupled to H44 (δ 3.90) of the Adha moiety and also to carbonyl C47 (δ 172.3) of the formyl-Leu piece. The hydroxyl of Adha was positioned at C43, and the lactone was attached at C45 based on respective ^{1}H NMR shifts of protons at δ 4.07 (H43) and δ 5.05 (H45). With the proximity of the Adha to the formyl-Leu carbonyl established, it was relatively straightforward to define the remainder of this latter subunit using ^{1}H−^{1}H COSY data. For example, diagnostic correlations were seen from the formamide NH (δ 8.29) to the formate H53 (δ 8.04) and to H48 (δ 4.56). This latter proton in turn defined the remainder of the spin system through the isopropyl group. A similar conclusion was derived by observing HMBC correlations from the δ 4.56 (H48) to C47 (δ 172.3), to C49 (δ 41.4), and to C50 (δ 24.3) while the formyl proton H53 (δ 8.04) exhibited an HMBC correlation peak to C48 (δ 49.9). At this point the chlorine was unaccounted for in the substructures but it seemed to be attached to the CH at δ 59.3/δ 4.41. Additional connectivities to this CH by HMBC and ^{1}H−^{1}H COSY NMR correlations nicely paved the way to propose a chloroisoleucine. A definitive argument for this assignment was based on the good agreement observed (2 ppm or less except at Me31) between the experimental and calculated (ACD/CNMR) ^{13}C shifts.

The 10 amino acid subunits could be united into the substructures **X**, **Y**, and **Z** (Figure 1). This was accomplished using the above 2D NMR data along with correlations observed in ROESY spectra (Figures S5 and S6, Supporting Information). The hexapeptide substructure **X** was assembled on the basis of 2D NMR correlations, shown in Figure 1, which included ^{1}H−^{1}H COSY, HMBC, and ROESY data. Similarly, the dipeptide substructure **Y** was justified on the basis of the ^{1}H−^{1}H COSY and HMBC data which included correlations involving the two NH groups and the formate H resonance. The *N*-MeLeu and Ala amino acids (as shown in **1**) were linked to give the dipeptide substructure **Z**, justified by numerous 2D NMR correlations observed to each of the four methyl groups.

The subunits **X**, **Y**, and **Z** could in principle be combined in eight possible ways which could be further distinguished after comparing experimental ^{13}C NMR data to those of appropriate models. Two distinctive functional groups, an α-keto amide and a carbamate, were present in seven of these probable structures, but both groups were absent in the eighth possibility. The α-keto amide, contained in four of the candidates, was plausible because it occurs in *Theonella* metabolites including orbiculamide A,[10] keramamides B−J,[5f] and the cyclotheonamides.[5h] Fortunately, α-keto amides are easily recognized by distinctive carbonyl ^{13}C NMR resonances at δ 196 and δ 161.[5f,h,11] Such signals were not observed in the spectra of cyclolithistide A. The carbamate group, a part of the other three structures, has not been previously observed in *Theonella* derived compounds, but it has been represented in other lithistid natural products.[2b,e] The ^{13}C NMR chemical shifts for carbamates occur in the range of δ 154 to δ 158,[2b,e,12] but analogous to the situation above, no such resonances were observed in the data of **1**. The paucity of long-range NOE's complicated the linking of all three partial structures. There were however, NOE correlations from the α-proton (H27) of the Cl−Ile to protons (H35, Me36, and Me37) of the *N*-MeLeu, which justified connecting structure **X**−**Z** by a bond from C32 to C33. Consequently, the gross structure of **1** represented the only reasonable possibility.

At this point we turned to mass spectrometry data to further confirm the sequences proposed in substructures **X**−**Z**. Identical ESI MS/MS fragmentation patterns were present in the data obtained on a triple quadrupole spectrometer (University of Illinois) or on a magnetic sector instrument (Mayo Clinic). In each MS/MS determination the $[MH]^+$ ion *m*/*z* 1164.8 was selected for further collision, but attempts to establish the amino acid sequence from the observed fragments using either standard software packages or by manual analysis were not fruitful. Alternatively, the isotope cluster characteristic of Cl provided a reliable way to trace and accurately analyze a subset of the fragment ions. The $[MH]^+$ *m*/*z* cluster at 1164.8 shown in Figure 2a il-

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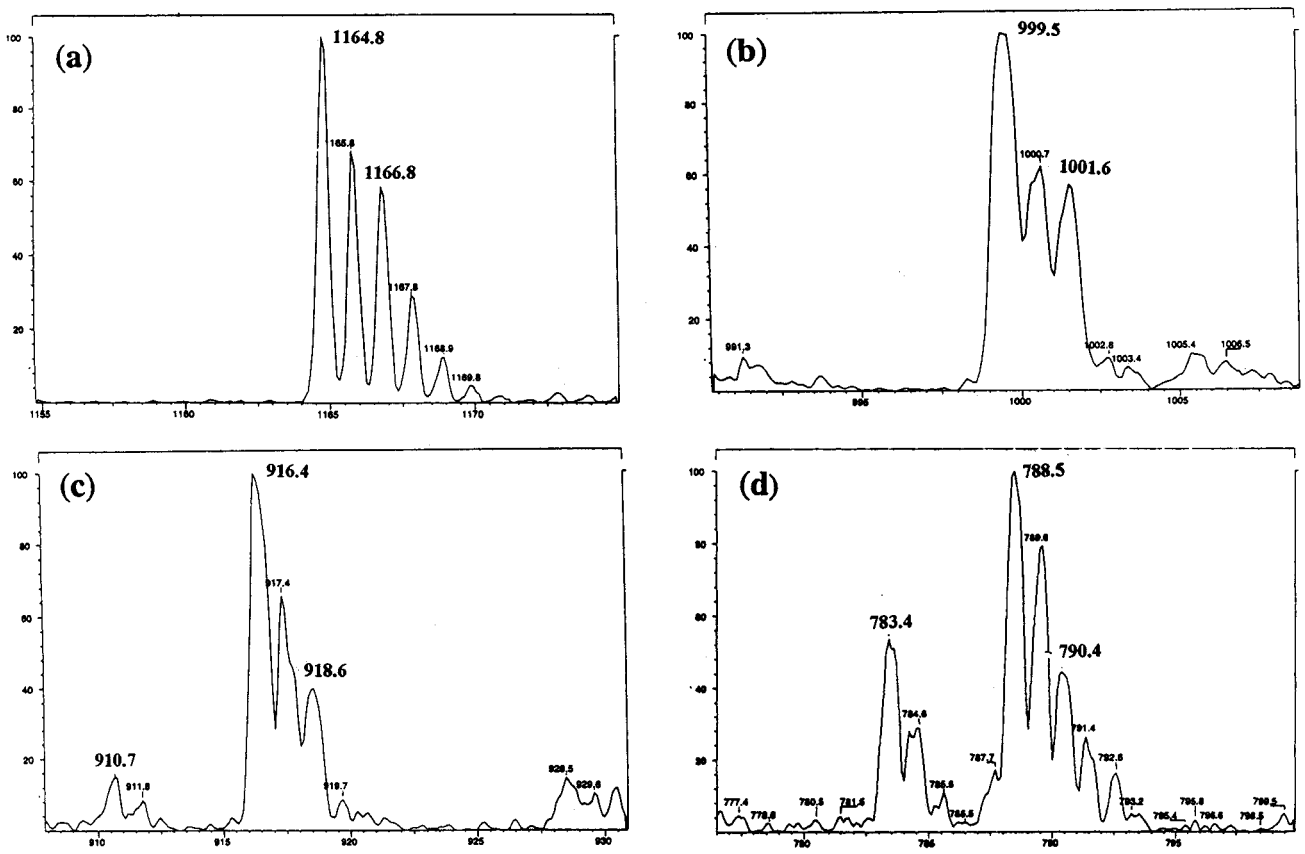

Figure 2. MS/MS expansions of the molecular ion and selected Cl-containing fragments.

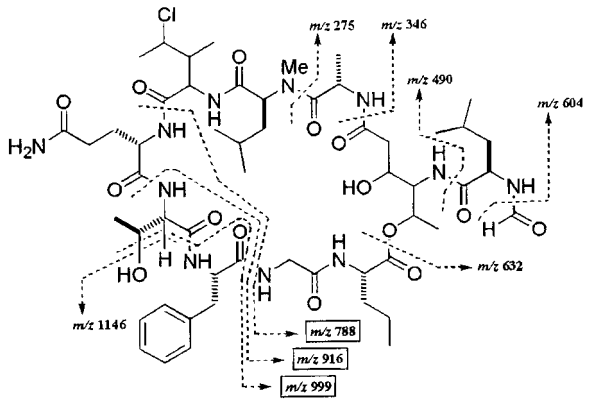

Figure 3. Summary of fragmentations from MS/MS on the *m*/*z* 1164.8 $[MH]^+$ of cyclolithistide A (**1**).

lustrates the diagnostic pattern containing the enhanced intensity $[MH + 2]^+$ peak. Further shown in Figure 2 are enhanced $[MH + 2]^+$ isotope profiles for Cl-containing fragment ion clusters at *m*/*z* 999.5, 916.4, and 788.5. By contrast, the isotope clusters at *m*/*z* 910.7 and 783.4 reveal that a Cl is not present. The *m*/*z* 999.5 cluster arises from the molecular ion undergoing fragmentation by a dehydration plus loss of the Phe as shown in Figure 3. Similarly, the *m*/*z* 916.4 is explained by the extrusion of Phe-Thr while the *m*/*z* 788.5 represents the loss of Phe-Thr-Gln. These data are consistent with the sequence of partial structure **X**. Further useful ESI MS/MS fragmentation information was obtained from the other fragment ions outlined in Figure 3. The *m*/*z* 632 arises from the loss of Gly-*nor*Val from the *m*/*z* 788 fragment, which is followed by the expulsion of CO to give *m*/*z* 604. Further fragmentation from this ion results in the loss of Leu, Adha, and Ala producing the *m*/*z* of 490, 346, and 275, respectively. Collectively, these data are consistent with the sequence of partial structures **Y**, **Z** and the overall structure of cyclolithistide A (**1**).

The process of establishing the absolute stereochemistry of cyclolithistide A (**1**) began by a chiral TLC analysis which enabled eight centers to be designated as 2*S*, 9*S*, 18*S*, 19*S*, 22*S*, 33*S*, 39*S*, and 48*R*. This was accomplished by acid hydrolysis of cyclolithistide A (**1**), followed by HPLC separation of the amino acids, and then comparing chiral reverse-phase TLC R_f values to those of optically pure standards. The amino acids *nor*Val, Phe, Gln, *N*-MeLeu, and Ala could all be assigned the *S* configuration. Similarly, the configuration of the Thr was found to be *S-allo* while the formyl-Leu side chain was determined to be *R*.

The ROESY NMR data also offered information about the stereochemistry. Complementary sets of cross-peaks were visible in spectra obtained in DMSO-d_6 and CD_3-OD (Tables 2 and 3). Collectively, these data suggested the close spatial proximity for several amino acid α-protons by observing dipolar couplings between H9 (δ 4.83) and H43 (δ 4.07), H9 and H44 (δ 3.90), H9 and H45 (δ 5.05), H22 (δ 4.32) and H29 (δ 4.41), and H22 and Me30 (δ 1.45) in DMSO-d_6 as well as H18 (δ 4.93) and H45 (δ 5.30), H39 (δ 4.06) and H43 (δ 4.47) in CD_3OD. While mutual ROESY correlations suggested an appropriate assignment of stereochemistry to the Cl−Ile and Adha residues, molecular modeling of cyclolithistide A proved inconclusive. They are therefore left unassigned at this time.

Continuing the theme of bioactivity observed for lithistid peptides, it is important to note that cyclolithistide A exhibited significant antifungal activity against *Candida albicans* (ATCC 24433) in the agar disk diffusion assay. At a dose of 20 μg/disk, cyclolithistide A produced a zone of inhibition which was equal to 90% of the 100 μg/disk standard nystatin. Compound **1** showed no antibacterial activity against *Escherichia coli* or *Bacillus subtilis*, nor was there any activity in the NCI 60 cell line assays.

In total, we examined 17 specimens of *Theonella* collected by SCUBA from Papua New Guinea and Indonesia. Both *T. swinhoei* and *T. conica* were gathered from diverse shallow-water reef environments, and they were examined in parallel because of their very similar morphologies. Of additional note was that a majority of these sponges were obtained from exposed sites (coded as E, Table 1) and as expected these were correspondingly rich in cyanobacteria. All of the E *T. swinhoei* sponges from Papua New Guinea, which includes sample nos. 1−8 of Table 1, were a source of both motuporin (**2**) and theonellapeptolide 1d (**3**) plus other theonellapeptolides. Similarly both compounds were present in two of the three E specimens of *T. conica*. However, the presence of these compounds was sporadic from the Indonesia E *T. swinhoei*. Only one specimen, sample no. 13, afforded cyclolithistide A (**1**), and it was unaccompanied by either **2** or **3**.

The possibility that the mixtures of **2** and **3** might be tied to cyanobacterial content was further investigated by employing different workup and/or collection strategies. In several cases we separated the sponge ectosome (rich in cyanobacteria) from the endosome (depleted in cyanobacteria) soon after the sponges were brought to the surface. Members of both species were treated in this manner and included three specimens (entries 5, 8, and 16 of Table 1). Both **2** and **3** were major components in each of the resultant six extracts. Crude extracts were also prepared for three collections of *T. swinhoei* obtained from the roofs of caves (entries 9, 13, and 14 of Table 1). As expected, these possessed little to no cyanobacteria, and the samples included taxa from both Papua New Guinea (coll. no. 91130) and Indonesia (coll. nos. 94590 and 95574). Our expectation was that each of these sponges would provide a profile of the metabolites produced by *T. swinhoei* when the chlorophyll-containing symbionts were absent or present in very small amounts. As predicted, motuporin (**2**) was absent from all three of these crude extracts. Interestingly, in one case (coll. no. 95574) mixtures of theonellapeptolides were observed but these compounds were absent from the other two specimens. Another interesting aspect of our study is that only one organism, coll. no. 89176, was observed to contain macrolides in small concentrations consisting of preswinholide A.[13] The swinholide family of compounds have been repeatedly observed from *T. swinhoei*.[9]

Conclusions

We, as well as others, continue to find the sponge genus *Theonella* to be fascinating from several viewpoints. The discovery of cyclolithistide A (**1**) extends the structural motif of halogenated cyclic peptides observed from *Theonella* sponges. To date, all of the past halogenated compounds, except keramamide A and the recently isolated microsclerodermin C (having an indole ring Cl atom),[5f,14] reported from this genus carry a Br on the indole ring, and recent examples include theonegramide,[5b] the theonellamides,[5k] orbiculamide A,[11] and the keramides B−H.[3] There are three unusual amino acid residues present in **1**. One of these, the formyl-Leu, is somewhat analogous to the formyl-isoserine present in keramamides F−J.[5f] There is a biosynthetic similarity between the Adha residue and isostatine which is a subunit in didemnin B obtained from a tunicate.[15] The other unusual amino acid, Cl−Ile, does not appear to have precedents.[16]

Some of our results are in contrast to many prior investigations on the species *T. swinhoei*. First, we were unable to observe, in the specimen which afforded cyclolithistide A, the more common nonhalogenated cyclic peptides including **2** and **3**. Also, no members of the swinholide family were seen in our crude extracts. Conveniently, cyclolithistide A (**1**) was easily visible in the semipure solvent partition fractions. While these data do not answer the question as to the true producer of certain similar metabolites, they do appear to remove photosynthetic symbionts of *Theonella swinhoei* as architects of cyclolithistide A.

Experimental Section

General Experimental Procedures. The NMR spectra were recorded at 250 or 500 MHz for ^{1}H and 62.9 or 125.7 MHz for ^{13}C. Multiplicities of ^{13}C NMR peaks were determined from APT and DEPT experiments. Standard pulse sequences were employed for the APT, DEPT, HMQC, and HMBC experiments. Phase sensitive ROESY spectra were measured with a mixing time t_m of 300 ms. Low- and high-resolution FAB, electrospray, and MS/MS mass spectra were obtained from the Mayo Clinic, University of Illinois, or Finnigan mass spectrometry facilities. High-performance liquid chromatography (HPLC) was performed on ODS columns. Advanced Chemistry Development (ACD Labs Software) calculated ^{13}C NMR shifts: C26 (δ 170), C27 (δ 53), C28 (δ 40), C29 (δ 58), C30 (δ 26), C31 (δ 16).

Collection and Identification. The 17 sponge specimens listed in Table 1 were collected by SCUBA from depths which varied from 15 to 30 m. Two of the samples identified by Dr. M. C. Diaz (UCSC IMS) as *Theonella swinhoei* Sollas 1888 (order Lithistida, family Theonellidae) were carefully examined for this taxonomic determination. These included coll. nos. 90107 and 91130 which were characterized by comparing their skeletal properties to those described in the literature.[17,18] The gross morphologies of these specimens were used to identify the remaining sponges concluded to be *T. swinhoei*. A complete examination was also conducted on two of the samples identified as *Theonella conica* (Kieschnick 1896) (order Lithistida, family Theonellidae) which included coll. nos. 93170 and 93171. These specimens reasonably matched the properties described in the literature for *T. conica*,[17] which has a more irregular and convoluted surface than in *T. swinhoei*. However, there are unpublished debates on the possibility that

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swinhoei and *conica* might actually be conspecific. The *T. swinhoei* (coll. no. 94590) which yielded **1** was collected in November from the roof of a cave at a depth of 25−30 m near Sangihe Island off Northern Sulawesi, Indonesia. Voucher specimens and photographs of all organisms are archived at U.C. Santa Cruz.

Extraction and Isolation. The specimen (coll. no. 94590, 0.1 kg) was preserved by being immersed in a 50:50 alcohol/H_2O solution. After approximately 24 h this solution was decanted and discarded. The damp organism was placed in a Nalgene bottle and shipped back to the home lab at ambient temperature. Next 100% MeOH was added, and the organism was soaked for 24 h. This procedure was repeated two more times. The crude oil was then successively partitioned between equal volumes of aqueous MeOH, percent adjusted to produce a biphasic solution, and hexanes followed by CH_2Cl_2. The remaining water solubles were extracted with *sec*-BuOH. The further workup of coll. no. 94590 was as follows. The CH_2Cl_2 fraction was subjected to Sephadex LH-20 gel filtration chromatography in 50:50 CH_2Cl_2/MeOH giving seven fractions. The third fraction was then subjected to reverse-phase gradient HPLC on a C18 preparative column (MeOH/H_2O, 65:35 to 100% MeOH) to afford **1** (45.3 mg, 0.065% dry weight of sponge). Motuporin (**2**) was initially isolated from *T. swinhoei* (coll. no. 93169) as a light tan amorphous solid (65.1 mg, 0.023% dry weight of sponge) with $[\alpha] = -81.3°$. Theonellapeptolide 1d (**3**) was also isolated from *T. swinhoei* (coll. no. 93169) as a white amorphous solid (108.8 mg, 0.038% dry weight of sponge) with $[\alpha] = -65.8°$. The literature values of rotation are $[\alpha] = -83.8°$ and $[\alpha] = -68°$ for motuporin and theonellapeptolide 1d, respectively.

Determination of Absolute Stereochemistry. A 0.3 mg portion of cyclolithistide A was dissolved in 6 N HCl (1.0 mL) and heated in an evacuated vial at 110 °C for 12 h. The reaction mixture was evaporated in a stream of nitrogen prior to vacuum-drying. HPLC separation accomplished using an ODS (4.6 mm × 25 cm) analytical column, elution with 100% water (2 mL/min), and UV detection at $\lambda = 210$ nm. Chiral TLC analysis using Chiralplate Macherey-Nagel. Eluent = 7:3 EtOH/H_2O. R_f values for standards: D-norvaline 0.64, L-norvaline 0.70; D-*N*-Me-leucine 0.62, L-*N*-Me-leucine 0.68; and D-leucine 0.59, L-leucine 0.64. R_f values found: norvaline 0.70, *N*-Me-leucine 0.68, and leucine 0.59. Eluent = 1:1:4 MeOH/H_2O/CH_3CN. R_f values for standards: D-phenylalanine 0.53, L-phenylalanine 0.66; D-glutamic acid 0.58, L-glutamic acid 0.61; D-alanine 0.48, L-alanine 0.52; and D-*allo*-threonine 0.40, L-*allo*-threonine 0.44. R_f values found: phenylalanine 0.66, glutamic acid 0.61, alanine 0.52, and L-*allo*-threonine 0.44.

Cyclolithistide A (1): white amorphous solid (45.3 mg); mp 174 °C (dec); $[\alpha] = -29.3$ ($c = 0.015$, MeOH); UV (MeOH) λ_{max} 220, 266 nm; IR (film) 3412, 1720, 1644, 1541, 1016, 642 cm^{-1}; LRFABMS, positive ion, *m*/*z* (relative intensity) 1202 $[M + K + H]^+$ (15), 1186 $[M + Na + H]^+$ (50), 1164 $[M + H]^+$ (100); HRFABMS 1164.6080 $[M + H]^+ = C_{54}H_{87}ClN_{11}O_{15}$ (Δ 0.8 mmu of calcd). ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125.7 MHz, DMSO-d_6), see Table 2. ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125.7 MHz, CD_3OD), see Table 3.

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Supporting Information Available: ^1H and ^{13}C NMR spectra of cyclolithistide A, as well as selected 2D NMR spectra (15 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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