New Cyclic Peptides with Cytotoxic Activity from the Ascidian *Lissoclinum patella*

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The isolation and structures of a new patellamide (patellamide D) and two new lissoclinamides (lissoclinamides 4 and 5) from the aplousobranch ascidian *Lissoclinum patella* are described. Structures were determined largely by using two-dimensional NMR techniques and mass spectrometry. These peptides and other members of the patellamide and lissoclinamide families that have been reported previously are found within the obligate algal symbiont of the genus *Prochloron*. The cytotoxicities of the compounds toward fibroblast and tumor cell lines are reported. One of these compounds, lissoclinamide 4, is markedly more toxic than other members of the family. Structure–activity relationships are discussed.

The Ascidiacea, marine benthic invertebrate organisms, have proven to be a rich source of cytotoxic and antiviral compounds that range from a simple urea derivative[1] and geranyl hydroquinone[2] to nitrogen heterocycles,[3] sphingosine-like compounds,[4] and complex peptides[5] and depsipeptides.[6] The depsipeptide compounds, the didemmins, isolated from *Tridemnum solidum*, have been shown to reduce the survival of mammalian cells in culture and to prolong the life span of tumor-bearing animals.[7,8] The cyclic peptides that have been isolated from *Lissoclinum patella*[5] belong to two general families of compounds, the octapeptide patellamide family, **1**, and the heptapeptide lissoclinamide family, **2**.[5] This paper reports the isolation, structure determination, and cytotoxicity of three new peptides from *L. patella*, one patellamide and two lissoclinamides. Another patellamide, ascidiacyclamide, has been reported from an unspecified ascidian.[9] This compound was also isolated in the present study along with another known patellamide, ulithiacyclamide.[5]

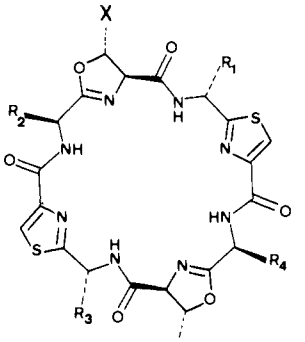
Of the known patellamides and lissoclinamides, ulithiacyclamide is the most potent cytotoxic compound. It has IC_{50} values (concentrations required to cause 50% inhibition) of 0.35 and 0.01 μg/mL for murine leukemia L1210 and human CEM cell lines, respectively.[10] Patellamides A, B, and C are approximately 10 times less cytotoxic having IC_{50} values of 2–4 μg/mL for L1210 cells.[10]

Most ascidians from which biologically active compounds have been isolated are in symbiosis with unicellular algae. For example, *Tridemnum solidum* has a Cyanophyte symbiont, *Synechocystis trididemni*,[11] whereas *L. patella* has prokaryotic algal cells of the genus *Prochloron*.[12] The *Prochloron* has been separated from its host and the presence of the cyclic peptides investigated.

Results and Discussion

Isolation and Structure of Peptides. A crude oil was initially isolated from *L. patella* after homogenization with methanol/toluene (3:1), extraction with 1 M sodium nitrate solution, chloroform extraction of the aqueous layer, and finally evaporation of the chloroform solution. The elution profile obtained when the crude oil was chromatographed on Partisil ODS-3 is shown in Figure 1. The compounds, indicated by arrows (fractions 1–5) have marked cytotoxic activity and are, in order of elution, patellamide D, ascidiacyclamide, ulithiacyclamide, lissoclinamide 4, and lissoclinamide 5.

Ascidiacyclamide, which has alternating D-valine and L-isoleucine side chains, has had its structure determined by X-ray analysis[13] and by synthesis.[14] Fracton 2 was identified as ascidiacyclamide by its ^1H and ^{13}C NMR spectra,[9] its FAB-MS, and its amino acid analysis. Similarly fraction 3 was identified as ulithiacyclamide, which was first described by Ireland and Scheuer,[15] and syn-



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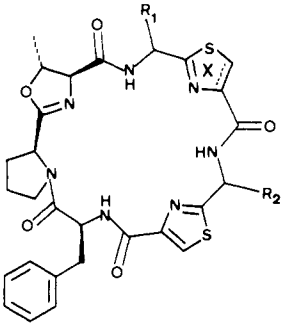
name	X	R_1	R_2	R_3	R_4
patellamide A	H	D-Val	L-Ile	D-Val	L-Ile
patellamide B	Me	D-Ala	L-Leu	D-Phe	L-Ile
patellamide C	Me	D-Ala	L-Val	D-Phe	L-Ile
patellamide D	Me	D-Ala	L-Ile	D-Phe	L-Ile
ascidiacyclamide	Me	D-Val	L-Ile	D-Val	L-Ile
ulithiacyclamide	Me	D-Leu	L-$^1/_2$Cys	D-Leu	L-$^1/_2$Cys



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name	X	R_1	R_2
lissoclinamide 1	thiazole	L-Val	D-Ile
ulicyclamide	thiazole	L-Ile	D-Ala
lissoclinamide 2	thiazoline	D-Ile	D-Ala
lissoclinamide 3	thiazoline	D-Ile	L-Ala
lissoclinamide 4	thiazoline	Val	Phe
lissoclinamide 5	thiazole	Val	phe

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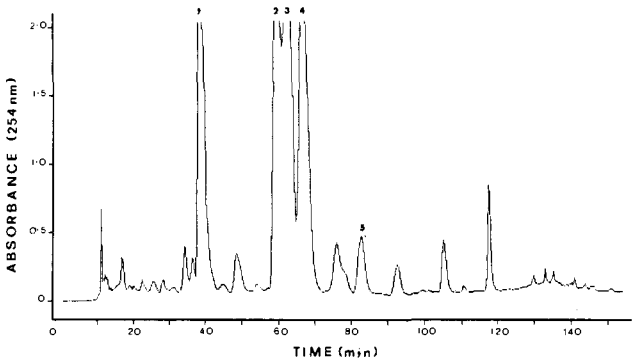

Figure 1. HPLC of *L. patella* extract. The extract was applied to a Whatman Partisil 10-ODS-3 column (C-18) in methanol/water (77:23) and eluted as described in the Experimental Section. The cytotoxic compounds, numbered 1–5, are patellamide D, ascidiacyclamide, ulithiacyclamide, lissoclinamide 4, and lissoclinamide 5, respectively.

thesized by Shioiri and co-workers.[16] The other three compounds have new structures that have been named in accordance with the nomenclature adopted by Ireland's group.[5]

Patellamide D, **3** ($C_{38}H_{48}N_8O_6S_2$), is very similar in structure to patellamide B except that a leucine is replaced by an isoleucine at position 6. HR-EI mass spectroscopy gave a molecular weight of 776.3138 (calcd 776.3128). Positive ion FAB-MS gave $(M + H)^+$ 777. Acid hydrolysis of patellamide D followed by derivatization to the *N*-pentafluoropropionyl isopropyl esters and chiral gas chromatography yielded L-threonine and L-isoleucine in equimolar amounts. Attempts to determine the stereochemistry of the thiazole amino acids were unsuccessful.[17]

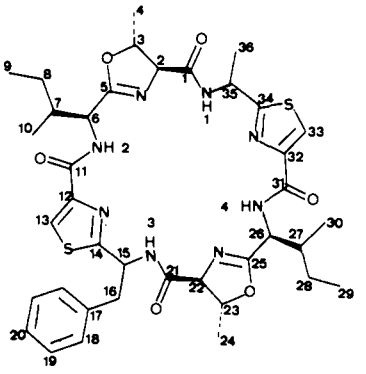

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Table I. NMR Assignments for Patellamide D (**3**)

carbon no.	$\delta(^{13}C)$	$\delta(^1H)$	multiplicity (^1H)	coupling constant (^1H–^1H): *J*, Hz
1	173.08[a]			
2	73.65	4.34	d	2.8
3	82.47	4.97	dq	6.5, 2.8
4	21.20	1.47	d (3 H)	6.5
5	168.30[b]			
6	53.20	4.72	dd	10.9, 7.6
7	33.08	2.21	m	
8	25.01	1.61, 1.37	m, m	
9	8.87	0.89	t (3 H)	7.4
10	15.12	1.03	d (3 H)	6.8
11	161.85[c]			
12	147.81[d]			
13	123.84	7.44	s	
14	173.03[a]			
15	52.29	5.49	dt	14.0, 9.8
16	40.95	3.30, 3.43	dd, dd	13.9, 5.8; 13.9, 10.0
17	136.32			
18	129.31 (2C)	7.26–7.40	m	
19	128.76 (2C)	7.26–7.40	m	
20	127.17	7.26–7.40	m	
21	172.67[a]			
22	73.65	4.24	d	2.5
23	82.40	5.00	dq	6.5, 2.8
24	21.16	1.43	d (3 H)	6.5
25	168.32[b]			
26	53.20	4.76	dd	11.1, 7.5
27	33.08	2.21	m	
28	24.98	1.40, 1.61	m	
29	8.80	0.87	t (3 H)	7.4
30	15.10	1.03	d (3 H)	6.8
31	161.77[c]			
32	147.49[d]			
33	123.69	7.51	s	
34	170.82[a]			
35	46.66	5.35	dq	9.8, 7.0
36	20.83	1.73	d (3 H)	7.0
NH(1)	7.58		d	9.8
NH(2)	7.55		d	10.7
NH(3)	7.65		d	9.8
NH(4)	7.57		d	11.1

[a–d] Interchangeable assignments.

The structure was unambiguously assigned from detailed analysis of ^1H and ^{13}C NMR spectra and 2D COSY 45 and ^1H–^{13}C shift correlation experiments. The NMR data are given in Table I. The assignments were made from the analysis itself and from a comparison of the data with that for patellamides and lissoclinamides of known structure. Five-bond homoallylic coupling is particularly important in the determination of the structure. In the COSY 45 spectrum, long-range coupling is observed between H-2 and H-6, H-2 and H-35, H-22 and H-26, H-22 and H-15, H-13, and H-33, and H-15 and H-35, thus establishing the sequence of amino acids around the ring.

Lissoclinamide 4 ($C_{38}H_{43}N_7O_5S_2$), **4**, and lissoclinamide 5 ($C_{38}H_{41}N_7O_5S_2$), **5**, have similar structures except that **4** has a thiazoline and a thiazole ring whereas **5** has two thiazole rings. Accurate mass measurements by HREIMS gave a molecular ion mass of 741.2830 for **4** (calcd 741.2767) and 739.2636 for **5** (calcd 739.2610). Positive ion FAB-MS gave $(M + H)^+$ 742 and 740, respectively. Acid hydrolysis of lissoclinamide 4 yielded L-threonine, L-proline, L-phenylalanine, L-cysteine, and DL-valine. In our hands, acid hydrolysis of amino acids adjacent to thiazoline rings leads to racemization. Hydrolysis of lissoclinamide 5 yielded L-threonine, L-proline, and L-phenylalanine. No

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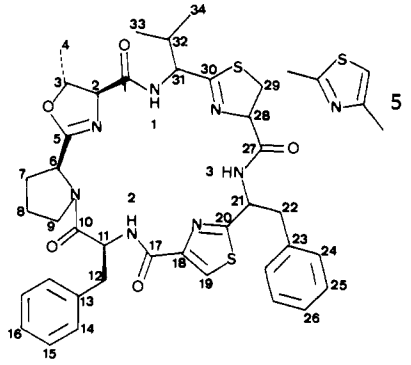
Table II. NMR Assignments for Lissoclinamide 4 (4)[a]

carbon no.	δ(^{13}C)	δ(^{1}H)	multiplicity (^{1}H)	coupling constant (^{1}H–^{1}H): *J*, Hz
1	171.38			
2	75.24	4.29	d	4.5
3	82.34	4.85	m	
4	21.85	1.46	d (3 H)	6.2
5	169.28			
6	56.76	4.65	t	10.0
7	28.53	1.85, 2.15	m, m	
8	25.30	1.73	m (2 H)	
9	46.98	2.00, 3.25	m, m	
10	170.41			
11	54.16	4.80	m	
12	40.41	2.78, 3.38	dd, dd	13.2, 10.1; 13.2, 4.3
13	136.11			
14	129.94 (2 C)	7.05–7.40	m	
15	128.64 (2 C)	7.05–7.40	m	
11	127.28	7.05–7.40	m	
17	159.65			
18	148.38			
19	123.08	7.93	s	
20	167.84			
21	54.31	5.40	m	
22	42.45	2.88, 3.65	dd, dd	13.3, 9.3; 13.3, 4.2
23	135.95			
24	129.92 (2 C)	7.05–7.40	m	
25	128.61 (2 C)	7.05–7.40	m	
26	127.20	7.05–7.40	m	
27	170.56			
28	80.10	5.28	t	9.7
29	33.96	3.75, 3.59	dd, dd	11.4, 9.4; 11.4, 10.2
30	173.38			
31	55.48	4.65	t	8.0
32	33.28	2.38	m	
33	19.41	0.87	d (3 H)	6.7
34	20.08	0.93	d (3 H)	6.4
NH (1)		7.79	d	10.1
NH(2)		8.65	d	6.5
NH(3)		8.52	d	5.9

[a] These assignments were determined by ^{1}H–^{1}H COSY 45, ^{1}H–^{13}C COSY, ^{1}H–^{13}C COLOC experiments.

cysteine or valine was found as expected from the presence of two thiazole rings.



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The NMR data for the new lissoclinamides are given in Tables II and III. In the 2D COSY 45 NMR spectrum of lissoclinamide 4, long-range coupling is observed between H-2 and H-6 and between H-29 and H-31. Due to the coincidence of the proton resonances at H-6 and H-31, it was not possible to obtain sequence information from long-range ^{1}H–^{1}H couplings. To deduce the sequence and unambiguously assign the carbonyl and other quaternary carbon resonances, a COLOC experiment was performed.[18] A partial contour plot of this experiment showing the quaternary carbons is seen in Figure 2. The COSY 45 spectrum of lissoclinamide 5 showed only one long-range

Table III. NMR Assignments for Lissoclinamide 5 (5)[a]

carbon no.	δ(^{13}C)	δ(^{1}H)	multiplicity (^{1}H)	coupling constant (^{1}H–^{1}H): *J*, Hz
1	171.43			
2	75.28	4.30	d	4.2
3	82.61	4.89	m	
4	21.80	1.47	d (3 H)	6.2
5	169.70			
6	56.61	4.58	t	7.7
7	28.78	2.13, 1.89	m, m	
8	25.11	1.75	m (2 H)	
9	47.15	2.12, 3.27	m, m	
10	171.06			
11	53.97	4.89	m	
12	40.79	2.91, 3.27	dd, dd	13.0, 10.1; 13.0, 4.4
13	136.10			
14	129.74 (2 C)	7.14–7.52	m	
15	128.66 (2 C)	7.14–7.52	m	
16	127.15	7.14–7.52	m	
17	159.87			
18	148.07			
19	123.08	7.90	s	
20	167.72			
21	54.53	5.43	m	
22	42.84	2.76, 3.89	dd, dd	13.0, 9.8; 13.0, 4.6
23	136.25			
24	129.18 (2 C)			
25	128.69 (2 C)			
26	127.29			
27	160.58			
28	150.50			
29	122.95	8.08	s	
30	168.73			
31	55.24	5.19	t	10.4
32	32.97	2.76	m	
33	19.98	1.09	d (3 H)	6.5
34	20.26	0.79	d (3 H)	6.5
NH(1)		7.92	d	10.2
NH(2)		8.71	d	5.6
NH(3)		9.23	d	7.6

[a] The assignments were established by ^{1}H–^{1}H COSY 45, ^{1}H–^{13}C COSY, and ^{1}H–^{13}C COLOC experiments.

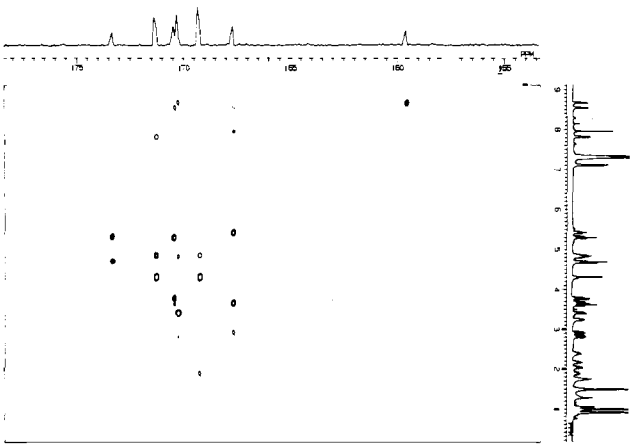

Figure 2. Partial contour plot of the ^{1}H–^{13}C COLOC spectrum of lissoclinamide 4 in $CDCl_3$ showing correlations of the quaternary carbon atoms.

coupling between H-2 and H-6, so a COLOC experiment was again necessary to determine the sequence of amino acids. The correlations yielding sequence information for lissoclinamides 4 and 5 are shown in Table IV.

Conformational Information. The vicinal coupling constant ^{3}J(NHCH) is related to the dihedral angle between NH and α-CH by a Karplus-type relationship.[19]

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Table IV. $^{1}H/^{13}C$ Correlations from the COLOC Experiments

lissoclinamide 4 (4)	lissoclinamide 5 (5)
C1-H2	C1-H2
C1-NH(1)	C1-NH(1)
C5-H2	C1-H3
C5-H3	C5-H2
C5-H7	C5-H7
C10-H11	C10-H12
C10-NH(2)	C13-H12
C13-H12	C14-H12
C14-H12	C17-NH(2)
C17-NH(2)	C18-H19
C18-H19	C20-H21
C20-H21	C20-H22
C20-H22	C20-H19
C20-H19	C27-NH(3)
C23-H22	C28-H29
C24-H22	C30-H29
C27-NH(3)	C30-H31
C27-H28	
C30-H28	
C30-H31	

For patellamide D the coupling constants are NH(1)–CH-(35) 9.8, NH(2)–CH(6) 10.7, NH(3)–CH(15) 9.8, NH(4)–CH(26) 11.1 Hz. These values are similar to the corresponding constants for patellamide B: 9, not given, 10, and 11 Hz.[5] The similarity is not surprising considering that the peptides differ only in one residue: L-leucine in patellamide B is replaced by L-isoleucine in patellamide D. As the thiazole amino acids are D in all the patellamides studied to date, it is assumed that this applies also to patellamide D. The similarity in the coupling constants supports that assumption. The "thiazole" coupling constants (J = 9.8 Hz) correspond to dihedral angles $180 > \theta \geq 155°$ and the "oxazoline" constants (J = 10.7, 11.1 Hz) correspond to dihedral angles $180 > \theta \geq 160°$. Ascidiacyclamide has the relative sizes of the coupling constants reversed, with values 10.55, 7.9, 10.55, and 7.9 Hz.[19] The corresponding dihedral angles are $180 > \phi \geq 160°$ (thiazole) and $160 > \theta \geq 145°$ (oxazoline). X-ray analysis gave 170° and 156° for these two angles.[20] These three patellamides have structures in solution approximating the solid-state structure found for ascidiacyclamide[19] in which the peptide N–H protons and the heterocyclic nitrogens are pointing toward the center of the molecule, orientations ideal for chelation to metal ions.

For lissoclinamides 1–5 and ulicyclamide the coupling constant that corresponds to J(NH(1)–CH(31)) in 4 is of the order of 10 Hz, i.e. a dihedral angle of $180 > \theta \geq 155°$, irrespective of the absolute configuration at α-CH.[5] The other two coupling constants are significantly less. For lissoclinamide 4 and 5 they are J(NH(2)–CH(11)) = 6.5 and 5.6 Hz and J(NH(3)–CH(21)) = 5.9 and 7.6 Hz, respectively. These correspond to dihedral angles of about 130° (5–6 Hz) to 140° (7.6 Hz) or 40° (5–6 Hz) to 20° (7.6 Hz).

The α-CH–β-CH_2 vicinal coupling constants for the phenylalanine residues can be analyzed by the method of Feeney to yield populations of the rotamers about α-C–β-C.[21] For patellamide D the two vicinal coupling constants of 10.0 and 5.8 Hz yield one acceptable solution with the rotamer in which the phenyl group is between CO and α-CH (rotamer I) possessing a mole fraction of 0.8 and the rotamer in which it lies between α-CH and NH (rotamer II) a mole fraction of 0.2. For lissoclinamide 4, the coupling constants for the phenylalanine residues at 11 and 21 give two sets of solutions for each residue. These are presented as follows: site, rotamer I mole fraction, rotamer II mole fraction, rotamer III mole fraction. Lissoclinamide 4:11, 0.2, 0.8, 0; 11, 0.8, 0, 0.2; 21, 0.2, 0.7, 0.1; 21, 0.6, 0.1, 0.3. Lissoclinamide 5: 11, 0.2, 0.8, 0; 11, 0.7, 0.1, 0.2; 21, 0.2, 0.8, 0; 21, 0.8, 0, 0.2.

Variation in Major Fractions Isolated. The proportions and structures of the toxic peptides isolated from *L. patella* seem to be dependent on the geographical location of the collected animals. In the first paper on the subject, ulithiacyclamide and ulicyclamide were reported to be the major constituents from specimens collected on reef flats near Korror Island, Palau Islands.[15] Subsequently, Ireland and co-workers reported that the patellamides A, B, C and ulithiacyclamide were the major components from *L. patella* collected at Eil Malk Island, Palau Islands.[10] Further work on this species yielded lissoclinamides 1, 2, 3 as additional components.[22] Hamamoto and his co-workers reported ulithiacyclamide and ascidiacyclamide from an unspecified ascidian from Rodda Reef, Queensland, Australia.[9] Over a 4-year period we have reproducibly found as the major constituents patellamide D, ascidiacyclamide, ulithiacyclamide, and lissoclinamides 4 and 5 from specimens collected at different locations and depths on the Heron Island reef. The yields of the compounds were 2.6%, 1.9%, 2.1%, 1.3%, and 0.8% of the dried extract respectively (100 g of frozen animal yielded 190 mg of dried extract). It has been found essential to process the organisms, or at least to freeze them, as quickly as possible. If they are left in an aerated salt water aquarium for over 12 h, these toxic compounds cannot be isolated. This could be associated with the viability of the organism itself or of the symbiont *Prochloron*. Ireland has recently found that *L. patella* collected from Fijian reefs yielded no cyclic peptides even if frozen quickly after collection.[23] He suggests that in this case the symbiont is not *Prochloron* but a blue-green alga.

Isolated *Prochloron* cells were extracted by using the same method as the whole organism and gave the same set of cytotoxic compounds as the whole organism. The amounts of the peptides in the isolated *Prochloron* were compared with amounts obtained from the remaining host animal. On a weight to wet weight basis the amounts in *Prochloron* were equal to or greater than the amounts in the host animal alone, the variation being caused by unknown factors perhaps seasonal or geographical. The exact site of synthesis of these compounds, whether in the algal cell or the host ascidian, is an intriguing question which awaits further experimentation. The suite of compounds is not characteristic of the algal cell because a completely different set of compounds was isolated from *Lissoclinium bistratum* which contains the same *Prochloron* symbiont.[24,25]

Cytotoxicity. The cytotoxicity of these compounds was determined by incorporation of [*methyl*-^{3}H]thymidine into human cell lines 5 days after a 1-h exposure (Figure 3). Two cell lines were used: MRC5CV1, a control fibroblast cell line, and T24, a bladder carcinoma line. In order to compare our results with those of others, the cytotoxicity of ulithiacyclamide (kindly provided by Dr. Chris Ireland)

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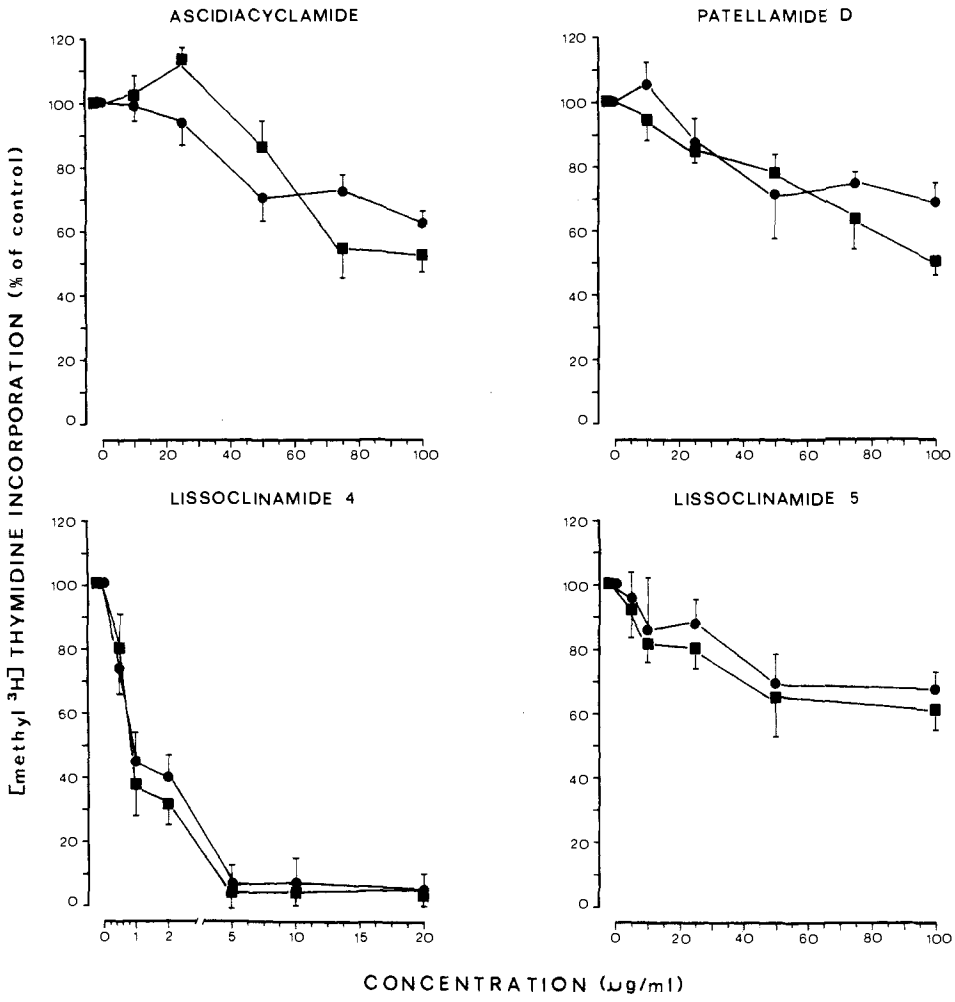

Figure 3. Effect of cytotoxic compounds on cell viability. Control cells (MRC5CV1, ●) and tumor cells (T24, ■) were exposed for 1 h to the indicated concentrations of the four compounds examined. Five days later the incorporation of [*methyl*-^{3}H]thymidine was assessed and expressed as a percentage of the incorporation of untreated cells (100%). Each experiment was performed three times with duplicate assays at each point. The error bars show the standard error of the mean.

was determined in our system by using both a 1-h and continuous exposure. The IC_{50} values obtained for ulithiacyclamide are 0.22 and 0.15 μg/mL (1-h exposure) for MRC5CV1 and T24 cell lines, respectively, and 0.04 and 0.10 μg/mL (continuous exposure). These values compare favorably with those previously reported for this compound under continuous exposure.[10]

The most cytotoxic of the new compounds presented here is lissoclinamide 4, which reduced survival in both T24 and MRC5CV1 cells to approximately 5% of the untreated values at 5 μg/mL (IC_{50} ≈ 0.8 μg/mL). There was no statistical difference in the sensitivity of the control and tumor cell lines to any of the compounds tested. Interestingly lissoclinamide 5, which differs from lissoclinamide 4 only by the presence of a thiazole instead of a thiazoline ring, is much less cytotoxic (by 2 orders of magnitude). Also Ireland reported that lissoclinamides 1–3 displayed only borderline cytotoxicity with IC_{50} values greater than 10 μg/mL, using L1210 cells.[22] Lissoclinamides 2 and 3 have the same basic structure as lissoclinamide 4 but display reduced cytotoxicity. The differences lie in the substituents at positions 21 and 31 in 4: lissoclinamides 2 and 3 have a D-isoleucine at 31 and D- or L-alanine at 21, whereas lissoclinamide 4 has valine at 31 and phenylalanine at 21. Lissoclinamides 4 and 5 have similar orientations of the aromatic side chains and the cyclic peptide rings have similar conformations. Studies of the exact modes of action of the lissoclinamides might provide a clue to the difference in activity.

Experimental Section

Collection of Animals. Specimens of *L. patella* were collected from Heron Island Reef on the Great Barrier Reef, Queensland, Australia. The species was verified by Dr. P. Kott, Queensland Museum. Animals were cleared of debris, cut into pieces about 2 × 2 cm, and frozen at −20 °C, within 4 h of collection.

A prokaryotic algal symbiont was found associated with *L. patella* and identified as *Prochloron* by Dr. G. Cox, University of Sydney, on the basis of ultrastructural features together with biochemical data.[25]

Extraction of Compounds. A typical sample size for extraction, 250 g (wet weight) of frozen ascidian, was homogenized in a Waring Blendor with 1 L of a 3:1 methanol/toluene solution. The homogenate was filtered and the filtrate was extracted with 1 M sodium nitrate solution (300 mL).[26] The aqueous layer was extracted with chloroform (3 × 100 mL) and the chloroform dried over anhydrous sodium sulfate. The chloroform was evaporated to dryness, yielding a dark greenish brown oil (yield 300 mg). A total of 7 kg of *L. patella* was processed in this way, yielding approximately 10 g of crude extract. For the extraction of *Prochloron*, the algal cells were isolated by slicing the colony horizontally and gently washing the cells with filtered sea water into a collection dish. After centrifugation the *Prochloron* cells were

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frozen at -20 °C. The extraction procedure was the same as described above.

Chromatography. The crude extract was dissolved in methanol/water (77:23) and applied to a Whatman Partisil ODS-3 9 mm × 500 mm preparative HPLC column. Elution was effected by means of a concave gradient from 77 to 100% methanol over 120 min and the absorbance of the eluate was monitored with a Waters 990 Photodiode Array detector. Fractions corresponding to the various peaks were pooled, evaporated to dryness, and subjected to further purification, either by rechromatography on the same column under isocratic conditions or by chromatography on Sephadex LH-20 using the same solvent. Complete separation of peaks 2 and 3 (Figure 1) required chromatography on silica gel 60 using dichloromethane/ethyl acetate (3:2).

NMR Spectroscopy. Spectra were obtained with a C-5 dual ^1H, ^{13}C probe in a JEOL GX400 spectrometer. Compounds were dissolved in deuterated chloroform (Merck) and chemical shifts were derived relative to tetramethylsilane (TMS). For homonuclear correlated experiments (COSY 45), the following parameters were used: Patellamide D: spectral width, 3189 Hz, data matrix, 512 × 2048, scans 32, recycle time 4 s, zero filling in ν_1 domain. Lissoclinamide 4: spectral width 3360 Hz, data matrix 512 × 1024, scans 48, recycle time 3.15 s, zero filling in both domains. Lissoclinamide 5: spectral width 3931 Hz, data matrix 256 × 1024, scans 128, recycle time 4 s, zero filling in both dimensions. The overall time of accumulation was 16–24 h. Sine-bell apodization functions were used. For the heteronuclear correlated experiments the following parameters were used. Patellamide D: data matrix 128 × 4096, scans 192, recycle time 4 s. Lissoclinamide 4: data matrix 128 × 4096, scans 680, recycle time 1.8 s. Lissoclinamide 5: data matrix 64 × 4096, scans 2030, recycle time 1.7 s. Fixed delays of $\Delta1(^1/_2J)$ and $\Delta2(^1/_4J)$ were set for J = 138 Hz. Both dimensions were zero filled and multiplied by a sine-bell function before transformation. The COLOC[18] experiments on lissoclinamides 4 and 5 were performed with delay times $\Delta1$ = 25 ms and $\Delta2$ = 30 ms, data matrix 128 × 4096, recycle time 1.722 s, and zero filling in the ν_1 domain. A sine-bell apodization function was applied before transformation.

Mass Spectroscopy. A Kratos MS25RFA instrument was used. For the FAB-MS an Iontech saddle-field FAB source was used with argon gas. Samples were dissolved in methanol at a concentration of 1 mg/mL and diluted 5 times with glycerol for FAB analysis. High-resolution electron-impact mass spectra (HREIMS) were recorded at 70 keV and a resolving power of 3000.

Acid Hydrolysis and Chiral Gas Chromatography. Peptides (0.3 μmol) were hydrolyzed in 6 M HCl (1 mL) and mercaptoethane (100 μL) in vacuo for 3–24 h at 110 °C. Mercaptoethane was added to prevent oxidation of cysteine during hydrolysis. After evaporation to dryness the amino acids were converted to their *N*-pentafluoropropionyl isopropyl esters as described by Frank and co-workers[27] and applied to a Chirasil-Val GC column for separation of D and L isomers.[28]

Cytotoxicity Testing. Two cell lines were used: MRC5CV1 (SV40-transformed human fibroblasts) as control and T24 (transitional cell carcinoma of the bladder) as a tumor line. Cells were maintained in RPMI-1640 medium (Commonwealth Serum Laboratories) containing 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were plated at a density of 5 × 10^3 cells/16-mm well, 24 h prior to cytotoxicity testing. Initial screening for cytotoxicity was performed by microscopic assessment of cultures 5–7 days after exposure to various concentrations of the compounds for 1 h. Compounds that showed cytotoxicity at this level were studied in more detail by examining their effects on the incorporation of [*methyl*-^3H]thymidine into DNA. This method is more rapid and considerably less tedious than the clonogenic assay and has been shown to reflect reliably cell survival when compared to data obtained from the clonogenic assay.[29] Cells were exposed to various concentrations of the compounds for 1 h and after 5 days were labeled with [*methyl*-^3H]thymidine (10 μCi/mL, 40 Ci/mmol, Amersham) for 4 h, after which they were extracted with cold 10% trichloroacetic acid (TCA). The TCA-insoluble material was collected onto glass fiber filters and washed with cold 5% TCA followed by 100% ethanol prior to liquid scintillation counting. Each experiment was performed three times with duplicate assays at each point. The statistical significance of differences between mean values was determined by using the student's *t* test.

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Novel Cytotoxic Compounds from the Ascidian *Lissoclinum bistratum*

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The isolation and structures of two new cyclic hexapeptides and two new macrocyclic ethers from the aplousobranch ascidian *Lissoclinum bistratum* are described. Their structures were determined by two-dimensional NMR techniques. The hexapeptides, named bistratamide A and bistratamide B, differ only by the presence or absence of one double bond. They were tested for cytotoxicity toward human fibroblast and tumor cell lines and displayed similar toxicities to the octapeptides called patellamides from *Lissoclinum patella*. The peptides are found within the obligate algal symbiont *Prochloron* but clearly differ from peptides isolated from the same *Prochloron* of *L. patella*. The macrocyclic ethers isolated from *L. bistratum* are exceedingly potent in cytotoxicity. They have been named bistratenes A and B, and structures for these compounds are proposed.

Recent research has confirmed that marine organisms are a valuable source of new organic compounds with potential use as antineoplastic agents.[1] The didemnid ascidians in particular have been a rich source of cytotoxic amino acid–derived metabolites.[2,3] In a related paper[4] we

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