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THE STRUCTURE OF ULITHIACYCLAMIDE B. ANTITUMOR EVALUATION OF CYCLIC PEPTIDES AND MACROLIDES FROM *LISSOCLINUM PATELLA*

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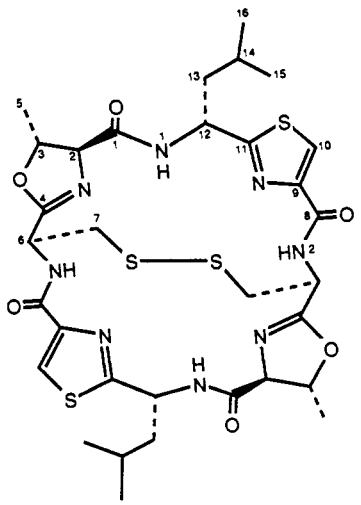
ABSTRACT.—The major cytotoxic metabolites of *Lissoclinum patella* from Pohnpei have been isolated and identified. Most of the cytotoxicity is attributed to a new cyclic peptide, ulithiacyclamide B [**4**], which is closely related to ulithiacyclamide [**1**]. The proposed structure of **4** is based on spectroscopic analysis and chemical degradation. Compound **4** exhibits an IC_{50} of 17 ng/ml against the KB cell line but does not show selective cytotoxicity against solid tumor cells in the Corbett assay.

Extracts of the ascidian *Lissoclinum patella* Gottschaldt are generally strongly cytotoxic (IC_{50}'s against KB, a human nasopharyngeal carcinoma cell line, 1.0–0.001 μg/ml). The thiazole-containing cyclic peptide ulithiacyclamide [**1**] (1,2) is responsible for most of the cytotoxicity (3) of *L. patella* from Palau. Several other thiazole-containing cyclic peptides which are much less cytotoxic, e.g., patellamides A, B [**2**], and C [**3**] (2–4), are also present in the Palauan tunicate. Ulithiacyclamide also accounts for most of the cytotoxicity of *L. patella* from Tudu Island in the Torres Straits, north of Queensland, Australia. (The Tudu Island tunicate also contained patellamides A–C.) Recently thiazole-containing macrolides, the patellazoles, have been shown to be responsible for the potent cytotoxicity of *L. patella* from Fiji (5) and Guam (6).

We report here the isolation and structure determination of the potent cytotoxin associated with *L. patella* from Pohnpei. Fractionation of the crude extract (KB $IC_{50} = 1$ μg/ml) by flash Si gel chromatography yielded several fractions which were further purified by hplc to give patellamides A, B [**2**], and C [**3**] (2–4) and a new cyclic peptide ulithiacyclamide B [**4**]. Ulithiacyclamide [**1**] was not present in this variety of *L. patella*.



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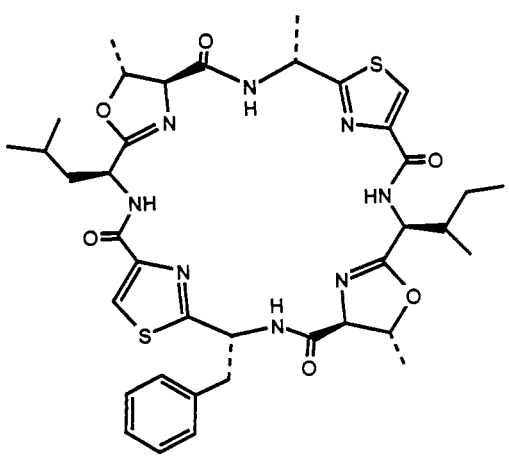

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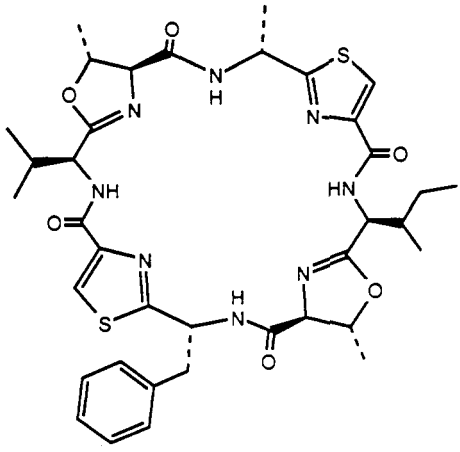

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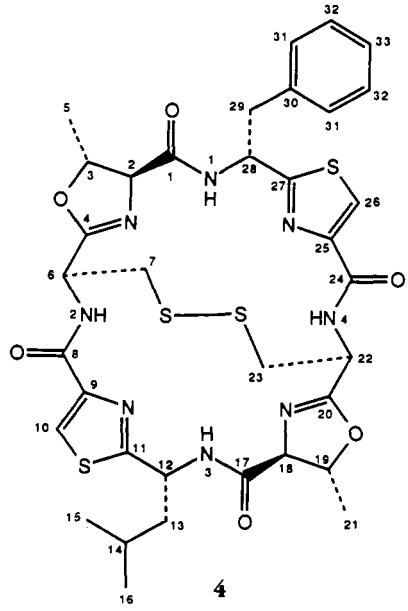

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The eims and positive ion fabms of ulithiacyclamide B [**4**] indicated a mol wt of 796 daltons. The ir spectrum showed intense absorptions at 3359, 1673, and 1653 cm^{-1}, indicative of a peptide. The ^{1}H- and ^{13}C-nmr spectral data for ulithiacyclamide B [**4**] closely resembled the spectral data reported for ulithiacyclamide [**1**] (2) (Tables 1 and 2) but revealed that ulithiacyclamide B lacked the symmetry associated with structure **1** and possessed a phenyl group [^{1}H nmr 7.10 (m, 2H), 7.25 (m, 3H) ppm; ^{13}C nmr 127.2 (d), 128.5 (d, 2C), 129.6 (d, 2C), 135.8 (s) ppm]. Comparison of the nmr data of **4** with appropriate patellamide models in addition to ulithiacyclamide [**1**] (1) and decoupling experiments suggested that **4** consisted of (a) a thiazole ring inserted between C-1 and C-2 of an *N*-substituted leucyl unit, (b) a second thiazole ring inserted between C-1 and C-2 of a phenylalanine unit, and (c) a methyl-substituted oxazoline ring inserted between each C-1–C-2 bond of a cystine unit. The molecular composition of ulithiacyclamide B was therefore $C_{35}H_{40}N_8O_6S_4$, and this was supported by an ei high resolution mass measurement. A COSY spectrum and selective proton-proton decoupling experiments supported the proposed partial structures a–c and established that the oxazoline protons on C-2 and C-18 were homoallylically coupled to the cystine protons on C-6 and C-22 ($J = 1.5$ Hz). A CSCM (7) experiment coupled with a HMBC (8) experiment (results shown in Table 3) confirmed the presence of the three structural units a–c and allowed their connectivities into a total gross structure. The HMBC experiment, however, indicated that the ^{13}C chemical shift assignments for C-8, C-9 and C-11 in the literature (1,2) for ulithiacyclamide [**1**] were incorrect. [The chemical shifts are comparable to ones reported for the amide carbonyl (163.7 ppm), C-4 (149.1 ppm), and C-2′ (172.3 ppm) of the bithiazole fragment in bleomycin A_2 (9).]

The gross structure of ulithiacyclamide B [**4**] was confirmed by chemical degradation. Hydrolysis with 5.5 M HCl yielded cystine, threonine, and the thiazole amino acids **5** and **6**. The absolute configuration of **4** was established using a modification of the method developed by Biskupiak and Ireland (10) for cyclic peptides containing 2-(1-aminoalkyl)thiazole-4-carboxylic acid residues. Reaction of **4** with $^{1}O_2$ generated a dithioozanide adduct which decomposed on acid hydrolysis to L-threonine, L-cystine, D-leucine, and D-phenylalanine. The absolute configurations of the amino acids were determined by treating the amino acid hydrolyzate with Marfey's reagent (11) and

TABLE 1. Comparison of ^{1}H-nmr Chemical Shifts and Coupling Constants for Ulithiacyclamide B [**4**] and Ulithiacyclamide [**1**].

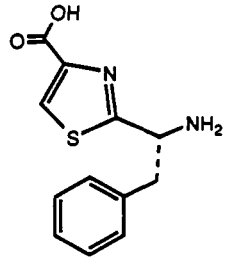
Proton	Compound	
	4	**1**[a]
H-2	4.227 (dd, J = 7.3, 1.5, 1H)	4.05 (dd, J = 8, 2, 2H)
H-3	4.72 (dq, J = 7.3, 6.2, 1H)	4.71 (dq, J = 8, 7, 2H)
H-5	1.467 (d, J = 6.2, 3H)	1.10 (d, J = 7, 6H)
H-6	5.23 (dddd, J = 8.7, 5.6, 4.3, 1.5, 1H)	5.24 (dddd, J = 9, 6, 4, 2, 2H)
H-7	3.13 (dd, J = − 14.5, 4.3, 1H)	3.02 (dd, J = 14, 4, 2H)
	3.38 (brdd, J = − 14.5, 5.6, 1H)[b]	3.22 (dd, J = 14, 6, 2H)
H-10	8.10 (s, 1H)	7.72 (s, 2H)
H-12	5.46 (td, J = 9.4, 8.4, 6.4, 1H)	5.36 (m, 2H)
H-13	1.925 (ddd, J = − 13.8, 8.4, 6.6, 1H)	1.66 (m, 4H)
	1.758 (dt, J = − 13.8, 6.6, 6.4, 1H)	
H-14	1.69 (nonet, J = 6.6, 1H)	1.35 (m, 2H)
H-15	1.012 (d, J = 6.6, 3H)	0.90 (d, J = 7, 6H)
H-16	1.003 (d, J = 6.6, 3H)	0.78 (d, J = 7, 6H)
H-18	4.244 (dd, J = 6.7, 1.5, 1H)	
H-19	4.74 (dq, J = 6.7, 6.2, 1H)	
H-21	1.461 (d, J = 6.2, 3H)	
H-22	5.18 (dddd, J = 9.1, 7, 4, 1.5, 1H)	
H-23	3.19 (m, J = − 13, 2H)	
H-26	8.02 (s, 1H)	
H-28	5.58 (td, J = 8.6, 5.6, 1H)	
H-29	3.13 (dd, J = − 13.2, 8.6, 1H)	
	3.39 (dd, J = − 13.2, 5.6, 1H)	
H-31	7.10 (m, 2H)	
H-32	7.25 (m, 2H)	
H-33	7.25 (m, 1H)	
N-1	7.83 (d, J = 8.6, 1H)	7.70 (d, J = 9, 2H)
N-2	8.29 (d, J = 8.7, 1H)	8.50 (d, J = 9, 2H)
N-3	7.57 (d, J = 9.4, 1H)	
N-4	8.25 (d, J = 9.1, 1H)	

[a]Values from Sesin *et al.* (2); spectrum determined at 270 and 200 MHz in $CDCl_3$.

[b]Broadness not due to long-range coupling, but probably to slowly interconverting rotational conformers involving the phenyl group.

analyzing the mixture of derivatives by hplc analysis (retention time comparisons and coinjections with standards). Because the leucine and phenylalanine were both D, both thiazole amino acids possessed *R* configurations. The absolute stereochemistry of ulithiacyclamide B was therefore as depicted in structure [**4**].

Ulithiacyclamide and ulithiacyclamide B exhibited IC_{50}'s of 35 ng/ml and 17 ng/ml, respectively, against the KB cell line (12) (Table 4). Preulicyclamide isolated from



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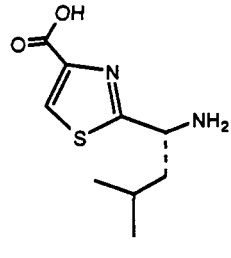

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TABLE 2. Comparison of ^{13}C-nmr Chemical Shifts for Ulithiacyclamide B [**4**] and Ulithiacyclamide [**1**].

Carbon	Compound	
	4[a]	**1**[b]
C-1	169.8 (s)	170.0[c]
C-2	74.2 (d)	74.1
C-3	81.7 (d)	81.7
C-4	167.7 (s)	167.3
C-5	21.8 (q)	22.1
C-6	48.4 (d)	48.4[d]
C-7	46.7 (t)	46.5
C-8	160.2 (s)	160.1
C-9	149.1 (s)	149.2
C-10	124.2 (d)	124.1
C-11	170.6 (s)	170.5[c]
C-12	48.4 (d)	48.5[d]
C-13	46.3 (t)	46.5
C-14	25.0 (d)	25.3
C-15	22.5 (q)	22.7[e]
C-16	22.3 (q)	22.8[e]
C-17	170.0 (s)	
C-18	73.8 (d)	
C-19	81.6 (d)	
C-20	167.9 (s)	
C-21	21.7 (q)	
C-22	47.7 (d)	
C-23	45.7 (t)	
C-24	160.0 (s)	
C-25	148.5 (s)	
C-26	124.6 (d)	
C-27	168.5 (s)	
C-28	52.0 (d)	
C-29	42.8 (t)	
C-30	135.8 (s)	
C-31	129.6 (d) (2C)	
C-32	128.5 (d) (2C)	
C-33	127.2 (d)	

[a]Coupling patterns determined from the fully coupled spectrum; chemical shifts based on CSCM and HMBC data.

[b]See Sesin *et al.* (2); spectrum determined at 90 and 66 MHz in $CDCl_3$. Chemical shifts of C-8, C-9, and C-11 based on HMBC data for **4**.

[c–e]Assignments may be interchanged.

Palauan *L. patella* and patellamides A, B, and C (2) were at least two orders of magnitude less active whereas patellazole B was about a hundred times more cytotoxic.

None of the compounds showed selective cytotoxicity against solid tumor cell lines in the Corbett disk diffusion assay (13,14) (Table 5). In the assay each compound was absorbed onto a 6-mm filter paper disk which was then placed on top of a soft agar plate that had been seeded with the leukemia, solid tumor, or low malignancy cell line. Agents showing a significantly larger zone of inhibition for the solid tumor cell lines than for the leukemia and low malignancy cell lines passed this primary screen and were selected as candidates for evaluation against solid tumors in vivo. A zone differential of >250 zone units, where 6 mm equals 200 zone units (Zu), has been shown to correlate frequently with activity in vivo (15). Ulithiacyclamide B and patellazoles A and B

TABLE 3. Proton-Detected ^{1}H-^{13}C Multiple-Bond Correlation (HMBC) Data for Ulithiacyclamide B [4].[a]

Proton	Carbon	Proton	Carbon
2	1, 4, 5	19	17, 20
3	1, 4	21	18, 19
5	2, 3	22	20, 23, 24
6	4, 7, 8	23	20, 22
7	4	26	24, 25, 27
10	8, 9, 11	28	1, 27, 29, 30
12	11, 14, 17	29	27, 28, 30, 31
13	11, 12, 14, 15, 16	31	29, 31, 32, 33
14	12, 15, 16	N1	1
15	13, 14, 16	N2	4, 8
16	13, 14, 15	N3	12, 17
18	17, 19, 20, 21	N4	24

[a]A mixing time of 95 msec was used. Cross peaks from the NH protons to the carbons two and three bonds away and from protons on methines attached to the amide NHs and to the amide carbonyl carbons were not observed when the HMBC spectrum was determined in wet $CDCl_3$.

showed essentially equal cytotoxicity against the leukemia (murine L1210), solid tumor (murine colon adenocarcinoma C38 and human colon adenocarcinoma H116), and low malignancy (murine) cell lines. This suggested that these compounds were general cytotoxins and therefore would probably be too toxic to be active against solid tumors in vivo. The patellamides were marginally but very weakly active against L1210, and preulicyclamide showed no activity in the Corbett assay at 100 μg/disk.

In a leukemia versus normal cell assay (Valeriote assay), none of the compounds tested (patellamides B and C, ulithiacyclamide B, and patellazoles A and B) showed selective activity (zone differential of >250 Zu) against either a lymphocytic leukemia (L1210) or an acute myelogenous leukemia (C1498) compared with a normal myeloid-committed stem cell (CFU-GM). The results of the Valeriote assay suggested that none of these compounds, if found to be active in vivo, would reduce myelosuppression or offer a distinct advantage over any clinically used antileukemic agent such as cytosine arabinoside (ara-C).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ^{1}H- and ^{13}C-nmr spectra were recorded on General Electric Omega-500 and QE-300 instruments in $CDCl_3$. ^{1}H chemical shifts are referenced to the residual $CHCl_3$ signal (7.24 ppm) and ^{13}C chemical shifts are referenced to the solvent peak (77.0 ppm). Eims and fabms (in monothioglycerol matrix) were determined on a VG 70-SE mass spectrometer. Ir spectra were re-

TABLE 4. Cytotoxicity of Compounds from *Lissoclinum patella* against KB Cells.

Agent	IC_{50} (ng/ml)
Ulithiacyclamide B [4]	17
Ulithiacyclamide [1]	35
Patellamide A	3000
Patellamide B [2]	>4000
Patellamide C [3]	6000
Preulicyclamide	10000
Patellazole A	10
Patellazole B	0.3

TABLE 5. Evaluation of Compounds from *Lissoclinum patella* for Selective Cytotoxicity, using the Corbett Assay, and Comparison with Clinically-Used Drugs.

Agent	Dose (μg/disk)	Tumor Cell Lines—Activity in Zone Units			
		L1210	C38	H116	Low Malignancy Cell Line
Ulithiacyclamide B	10	270	290	280–300	260
Patellamide A	100	150–240	160	0	0–100
Patellamide B	100	50–220	150	0–30	0–30
Patellamide C	200	260–340	170	0–100	0–30
Preulicyclamide	100	0	0	0	0
Patellazole A	5	240–330	320–500	370–450	300–450
Patellazole B	5	300–370	460	460	150–310
5-Fluorouracil	1.5	0	800		
Cytosine arabinoside (ara-C)	0.15	550–770	[a]		

[a]Ara-C shows 0 Zu against murine pancreatic adenocarcinoma #03 (13).

corded in KBr on a Perkin-Elmer 1420 Ratio Recording Infrared Spectrometer. Uv spectra were recorded on a Perkin-Elmer Lambda 4C UV/VIS Spectrophotometer. Optical rotations were determined on a Rudolph Research Autopol II polarimeter in a 5-cm microcell.

Hplc purifications were performed using Alltech Econosphere C18, 5μ, 150 mm × 4.6 mm, and Alltech Econosil C18, 10μ, 250 mm × 10 mm columns for analytical and preparative separations, respectively, and an Eyela uv Detector (Model UV-10). The hplc solvents were either hplc grade or ACS grade distilled from appropriate drying and cleaning agents.

Hplc analysis of amino acid derivatives employed a 100 mm × 4.6 mm (5μ) Pierce RP-18 column (fitted with a 15 mm × 4.6 mm precolumn) with a Perkin-Elmer Series 4 Liquid Chromatograph solvent delivery system and Perkin-Elmer ISS-100 autosampler. Perkin-Elmer 7500 Professional and IBM Personal XT computers controlled the pumps and the autosampler and recorded the uv spectra observed with Kratos Spectroflow 757 Absorbance and Bromma LKB 2140 Rapid Spectral uv detectors. Ozone was generated with a Welsbach Ozonator.

Analtech Uniplate Si gel HLF plates, Whatman KC8F octyl reversed-phase plates (200 μ), and Whatman K2 microcrystalline cellulose analytical tlc precoated plates (250 μ) were used for preparative and analytical tlc. Either Aldrich Si Gel (230–400 mesh) or Alltech extract-clean silica bond-elute columns (500 mg/2.8 ml) were used for flash chromatography, and Alltech extract-clean C-18 columns (500 mg/2.8 ml) were used for pre-hplc clean-up of samples.

ISOLATION.—*L. patella* (SG-51) (16) was collected from dead coral outside and north of Palikir Pass, Pohnpei at a depth of 20–40 ft. The freeze-dried tunicate (194 g) was extracted with CH_2Cl_2-EtOH (1:1) to give 4.11 g of a dark green oil. The extract (1.2 g) was fractionated by flash chromatography on a 20 cm × 30 mm column of Si gel using successively 5% EtOAc/hexane, 10% EtOAc/hexane, 25% EtOAc/hexane, 50% EtOAc/hexane, EtOAc, and 10% MeOH in EtOAc (250 ml of each). The fraction eluting with 100% EtOAc contained patellamide A and ulithiacyclamide B [**4**], and the fraction eluting with 10% MeOH/EtOAc contained patellamides B [**2**] and C [**3**]. Reversed-phase hplc purification of these two fractions using 70% MeOH/H_2O and 60% MeOH/H_2O, respectively, yielded patellamide A (52 mg), ulithiacyclamide B [**4**] (27 mg), and patellamides B [**2**] (31 mg) and C [**3**] (25 mg), all as amorphous solids. Patellamides A, B, and C and ulithiacyclamide B exhibited R_f values of 0.54, 0.53, 0.48, and 0.63, respectively, on a silica tlc plate using 5% MeOH/CH_2Cl_2 and could be visualized by either short wave uv light (254 nm) or exposure to iodine vapor.

Two samples of *L. patella* (SG-70 and SG-71) were collected from Tudu Island. The CH_2Cl_2-EtOH (1:1) extracts (0.219 g and 0.103 g) of the freeze-dried tunicates yielded 35 and 29 mg of ulithiacyclamide [**1**], respectively, using the same procedure. Small amounts of patellamides A, B, and C were also isolated from the Tuduan variety.

Voucher specimens of the tunicates from Pohnpei and Tudu Island are being retained at the University of Guam Marine Laboratory.

PHYSICAL AND SPECTRAL DATA FOR ULITHIACYCLAMIDE B [**4**].—Amorphous solid: $[\alpha]^{24.5}D + 117°$ ($c = 0.17$, MeOH); uv (MeOH) λ max 202 nm (ε 154897), 235 (63291); ir (KBr) 3359 br, 2931, 2901, 2845, 1673, 1653, 1540, 1489, 1456, 1377, 1174, 1039 cm^{-1}; ^1H nmr see Table 1; ^{13}C nmr see Table 2; eims *m/z* (rel. int.) 796 (14), 763 (17), 730 (80), 702 (21), 639 (29); hreims *m/z*

796.1969 ($C_{35}H_{40}N_8O_6S_4$, −1.5 mmu error), 763.2096 ($C_{35}H_{39}N_8O_6S_3$, 5.9 mmu error), 730.2253 ($C_{35}H_{38}N_8O_6S_2$, 10.2 mmu error), 639.1812 ($C_{28}H_{31}N_8O_6S_2$, −0.4 mmu error); fabms *m/z* $[MH]^+$ 797, $[MK]^+$ 835.

HYDROLYSIS WITH 5.5 M HCL.—The peptide (5 mg) in 5.5 M HCl (1 ml) (freshly distilled, constant boiling HCl) was heated at 108° with stirring for 18 h in a threaded Pyrex tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness and traces of HCl removed from the residual hydrolyzate by repeated evaporation from 10 ml volumes of H_2O. Silica and cellulose tlc *n*-BuOH–H_2O–HOAc (4:1:1) comparisons of the amino acids in the ulithiacyclamide B hydrolyzate with either authentic amino acids or amino acids generated from the hydrolyses of patellamide B [**2**] and ulithiacyclamide [**1**] suggested the presence of cystine, threonine, and thiazole amino acids **5** and **6**. These amino acids were separated on cellulose preparative tlc plates *n*-BuOH–H_2O–AcOH (4:1:1) and were identified unambiguously by ^1H nmr and eims.

GENERAL PROCEDURE FOR REACTION OF PEPTIDES WITH $^1O_2$ AND SUBSEQUENT WORKUP.—Triphenyl phosphite (1 g, 3.2 mmol) was dissolved in CH_2Cl_2 (50 ml) and cooled to −77° in a dry ice/Me_2CO bath. A stream of ozone was bubbled through the solution until a deep blue ozone color persisted. A stream of N_2 was then bubbled through the solution at −77° to remove excess ozone. A 3-ml aliquot of the ozone adduct solution was added directly to a second flask containing the peptide (2 mg) in CH_2Cl_2 (2 ml) at −77°. [After attempting several experimental modifications we concluded that deactivation of $^1O_2$ in Hawaii was too rapid to allow for direct transfer of $^1O_2$ with a connecting hose adaptor as was achieved by Biskupiak and Ireland (10).] The reaction mixture was allowed to warm to room temperature. Vigorous bubbling was observed. After gas evolution had ceased, the reaction mixture (incomplete reaction) was immediately purified on a silica Bond-Elut column. Triphenyl phosphate was eluted with 100% CH_2Cl_2, and the products of interest (and starting material) were eluted with 100% EtOAc. In the case of the EtOAc fraction, the solvent was removed under a stream of N_2, and the crude mixture was treated directly with 5.5 M HCl (1 ml) for 12 h with stirring at 108° in a Pyrex threaded tube sealed with a Teflon screw cap. The cooled reaction mixture was evaporated to dryness three times from 10-ml volumes of H_2O.

DERIVATIZATION OF AMINO ACIDS WITH MARFEY'S REAGENT AND HPLC ANALYSIS.—To a 1-ml vial containing 2 μmol of the pure amino acid standard in 40 μl of H_2O was added 2.8 μmol of 5-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (10) in 80 μl of Me_2CO followed by 20 μl of 1 N $NaHCO_3$. The mixture was heated for 1 h at 40°. After cooling to room temperature, 10 μl of 2 N HCl was added and the resulting solution was filtered through a 4.5-μ filter and stored in the dark until hplc analysis.

To prepare FDAA derivatives of the amino acids in the peptide hydrolyzates, a 40-μl aliquot containing 0.3 mg of the amino acid mixture was reacted with 4.2 μmol of FDAA in 115 μl of Me_2CO as described above. A 5-μl aliquot of the resulting mixture of FDAA derivatives was analyzed by reversed-phase hplc. A linear gradient of triethylammonium phosphate (50 mM, pH 3.0)-MeCN (9:1) and MeCN, with 0% MeCN at the start ↦ 40% MeCN over 40 min (flow rate 2 ml/min) was used to separate the FDAA derivatives which were detected by uv at 340 nm. Each peak in the chromatographic trace was identified by comparing its retention time with that of the FDAA derivative of the pure amino acid standard and by coinjection. The $^1O_2$ oxidized ulithiacyclamide B [**4**] hydrolyzate showed peaks at 12.35, 24.52, 30.22, and 31.90 min. The amino acid standards gave the following retention times in minutes: 12.32 for L-, 12.52 for L-allo-, 16.68 for D- and 14.52 for D-allo-threonine; 24.52 for L- and 29.35 for D-cystine; 25.08 for L- and 30.18 for D-phenylalanine; and 26.02 for L- and 31.85 for D-leucine. In all cases a peak at 17.35 min was observed which was attributed to excess FDAA.

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