Dolastatin 3 and Two Novel Cyclic Peptides from a Palauan Collection of *Lyngbya majuscula*

Scott S. Mitchell,[†] D. John Faulkner,*[,†] Kathleen Rubins,[‡] and Frederic D. Bushman[‡]

Scripps Institution of Oceanography, University of California at San Diego, La Jolla, California 92093-0212, and Infectious Disease Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037

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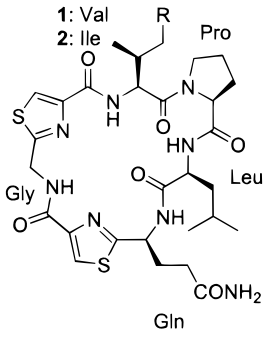
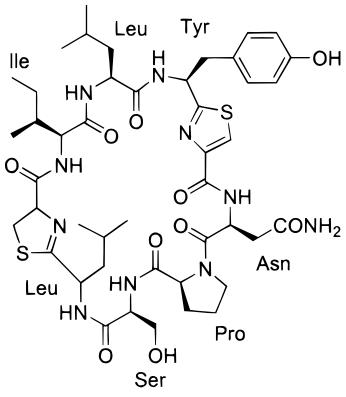
A collection of *Lyngbya majuscula* from Palau contained the peptides dolastatin 3 (**1**), homodolastatin 3 (**2**), and kororamide (**3**), together with aplysiatoxin (**4**), debromoaplysiatoxin (**5**), and oscillatoxin A (**6**). The structures of the new peptides homodolastatin 3 (**2**) and kororamide (**3**) were determined by interpretation of spectroscopic data and chemical degradation.

As part of a collaborative effort to discover new inhibitors of HIV-1 integrase from marine organisms,[1] we screened a collection of crude extracts of marine organisms from Palau. Among the few extracts that showed activity, most of which were from marine sponges, was an extract of the macroscopic cyanophyte *Lyngbya majuscula* that had been collected because it was fouling corals. *L. majuscula* has previously yielded a wide variety of natural products[2] that include the antiproliferative agents, curacin A[3] and debromoaplysiatoxin,[4] and both acyclic[5] and cyclic[6] peptides. In this paper we report the HIV-1 integrase inhibition assay-guided isolation of dolastatin 3 (**1**), which had previously been reported from the sea hare *Dolabella auricularia*,[7] and the structure elucidation of two new cyclic peptides, homodolastatin 3 (**2**) and kororamide (**3**).



1 R = H
2 R = Me



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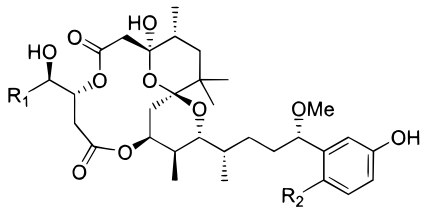
The specimen of *L. majuscula* (90−95% homogeneous) was collected in shallow water in the lagoon near Big Goby marine lake, Palau, and was immediately frozen. The crude methanolic extract of the cyanophyte was found to inhibit HIV-1 integrase in the terminal-cleavage and strand-transfer assays,[8] but did not inhibit molluscum contagiosum virus (MCV) topoisomerase.[9] The ethyl acetate-soluble material from the methanolic extract was subjected to repeated chromatography on reversed-phase supports to obtain dolastatin 3 (**1**, 4×10^{-5}% wet wt), homodolastatin 3 (**2**, 4.8×10^{-5}% wet wt), and kororamide (**3**, 4×10^{-5}% wet wt), in addition to the known metabolites aplysiatoxin (**4**), debromoaplysiatoxin (**5**), and oscillotoxin (**6**).[4,10] Each fraction obtained during the separation procedure was screened for activity against HIV-1 integrase, and it was shown that the only active material present was dolastatin 3 (**1**), which was identified by comparison of its spectral data with literature values.[7]



4 R_1 = Me; R_2 = Br
5 R_1 = Me; R_2 = H
6 R_1 = R_2 = H

Homodolastatin 3 (**2**) was obtained as a white solid. The molecular formula, $C_{30}H_{42}N_8O_6S_2$, obtained from a high-resolution mass measurement of the $[M + Na]^+$ ion, suggested that **2** differed from dolastatin 3 (**1**) by the addition of a methylene group. Analysis of the ^1H and ^{13}C NMR spectra revealed signals that were characteristic of an isoleucine residue in place of the valine residue of dolastatin 3. The sequence of the amino acid residues was confirmed by analysis of the HMBC experiment. The absolute configuration of the L-isoleucine, L-proline, and L-leucine residues was determined by hydrolysis followed by GC−MS analysis of the derivatized residues on a chiral column and that of the remaining center was determined by treatment with ozone followed by hydrolysis and derivatization to obtain a peak corresponding to L-glutamate, in addition to those observed previously. Thus, homodolastatin 3 (**2**) differs from dolastatin 3 (**1**) only by the substitution of L-isoleucine for L-valine.

Kororamide (**3**) was obtained as a white solid. The molecular formula, $C_{45}H_{64}N_{10}O_{10}S_2$, which was obtained from HRMS data, clearly indicated that kororamide (**3**) was a peptide and that it was unrelated to dolastatin 3 (**1**). The ^1H NMR spectrum (Table 1) contained a signal at δ 8.29 that was assigned to a thiazole ring by interpretation of the HMBC correlations. The second sulfur atom was assigned to a thiazoline ring that gave rise to ^1H NMR signals at δ 5.11 (ddd, 1 H, J = 10, 4, 1.5 Hz), 3.72 (m, 1 H), and 3.60 (m, 1 H) and ^{13}C NMR signals at δ 75.5, 36.0, and 173.0. HMBC correlations were used to place a tyrosine

* To whom correspondence should be addressed. Tel.: (619) 534-4259. Fax: (619) 534-2997. E-mail: jfaulkner@ucsd.edu.
[†] Scripps Institution of Oceanography.
[‡] The Salk Institute.

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Table 1. NMR Data for Homodolastatin 3 (**2**) and Dolastatin 3 (**1**) in $CDCl_3$

amino acid	C#	homodolastatin 3 (**2**)			dolastatin 3 (**1**)	
		δ_C	δ_H	mult, *J* (Hz)	δ_C	δ_H
Gly-Thz	1	167.5			165.8	
	2	160.0			160.3	
	3	123.7	8.10	1 H, s	123.7	8.08
	4	148.1			148.3	
	5	40.9	5.26	1 H, d, 18,7	41.0	5.25
			4.69	1 H, d, 18,2		4.66
	NH		8.80	1 H, dd, 7,2		8.76
Gln-Thz	1	174.4			174.3	
	2	160.9			160.8	
	3	124.2	8.09	1 H, s	124.3	8.08
	4	149.0			149.0	
	5	54.7	5.56	1 H, ddd, 9,6,5	55.0	5.54
	6	28.1	2.59	1 H, m	29.6	2.54
	7	31.8	2.31	2 H, m	33.3	2.30
	8	171.1			171.1	
	NH_2		6.33	1 H, br s		6.30
			5.46	1 H, br s		5.42
	NH		8.02	1 H, d, 9		7.86
Leu	1	172.0			172.0	
	2	48.3	3.86	1 H, m	48.6	
	3	37.6	2.17	1 H, m	37.7	2.14
			1.99	1 H, m		1.95
	4	25.3	1.68	2 H, m	25.4	1.53
	5	21.1	0.96	3 H, d, 7	18.6	0.96
	6	23.3	0.94	3 H, d, 7	19.5	0.90
	NH	6.12	1 H, d, 8		5.99	
Pro	1	169.4			169.4	
	2	62.7	4.00	1 H, t, 7.5	62.6	3.98
	3	29.6	1.90	2 H, m	28.4	1.92
	4	25.4	1.80	2 H, m	25.5	1.80
	5	48.3	3.92	1 H, m	48.3	3.85
			3.73	1 H, m		3.69
Val/Ile	1	170.9			170.9	
	2	55.0	4.81	1 H, dd, 9, 4.5	55.6	4.76
	3	39.8	1.79	1 H, m	31.9	2.06
	4	25.3	1.68	2 H, m	21.2	1.05
	5	11.0	0.96	3 H, t, 7	23.3	1.06
	6	15.3	1.19	3 H, d, 7		
	NH		8.33	1 H, d, 9		8.31

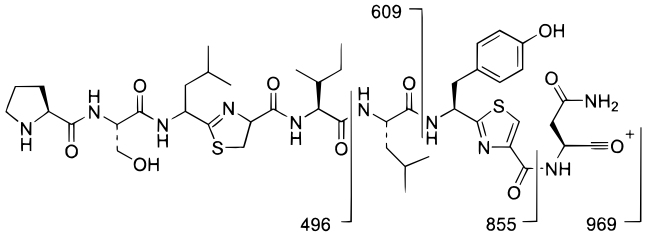

Figure 1. Fragmentation pattern observed by MALDI-TOF MS/MS.

residue adjacent to the thiazole ring and a leucine residue adjacent to the thiazoline ring. The TOCSY and HMQC data revealed the presence of leucine, isoleucine, serine, proline, and glutamine residues, and their sequence was established predominantly from the HMBC data (Table 1). Although there was no HMBC correlation linking the proline and asparagine residues, a series of strong ROESY correlations between the H-2 signal of asparagine and the H-5 protons of proline were observed as expected for a *trans*-proline amide bond. Additional evidence for several amino acid connectivities was observed by MALDI-TOF tandem mass spectrometry (Figure 1).

The absolute configurations of the L-leucine, L-isoleucine, L-serine, L-proline, and L-asparagine residues in kororamide (**3**) were determined by hydrolysis, derivatization, and analysis by GC−MS on a chiral column. The absolute configuration of the L-tyrosine residue adjacent to thiazole ring was established by subjecting kororamide (**3**) to ozonolysis followed by hydrolysis, derivatization, and GC−MS analysis to obtain two equivalents of L-aspartic acid. The absolute stereochemistry of the leucine−thiazoline unit was not determined due to the shortage of material and the lack of reliable methods.

When first isolated, dolastatin 3 (**1**) inhibited HIV-1 integrase at IC_{50} values of 5 mM for the terminal-cleavage and 4.1 mM for the strand-transfer reactions.[8] Quite remarkably, homodolatatin 3 (**2**), which only differs from dolastatin 3 (**1**) by replacement of a valine residue by isoleucine, showed no activity at 100 mg/mL in either integrase assay. However, after several months the samples were repurified and the assays repeated to obtain quite different results that indicated an appreciable loss of activity for dolastatin 3 (**1**) and a low level of activity for homodolastatin 3 (**2**). To determine whether the loss of activity was due to small experimental differences or errors in weighing, the samples were again repurified and the assays repeated under identical conditions. This resulted in a further drop in the activity of dolastatin 3 (**1**). Because the lowered activity seemed in some way related to the concentration of the original stock solutions, we subjected a solution of dolastatin 3 (**1**) in DMSO (4.4 mg/mL) to the transfer procedures used in the bioassay but without further diluting the solution. Measurement of the intensities of the UV maxima in the original and final solutions revealed a 31% loss of material during transfer. We, therefore, believe that the peptide sticks to the plastic wells and pipet tips. Given these problems in assaying the material and the fact that dolastatin 3 (**1**) is known to be cytotoxic,[7] it has not been investigated further as an integrase inhibitor.

The isolation of dolastatin 3 (**1**) from *L. majuscula* further supports the proposal of Harrigan et al.,[11] who isolated dolastatin 12 from an assemblage of *L. majuscula* and *Schizothrix calcicola* from Guam, that the dolastatin peptides previously isolated from the sea hare *D. auricularia* were of dietary origin and were probably obtained from cyanobacteria such as *L. majuscula*.

Experimental Section

General Methods. The IR and UV spectra were recorded on Perkin-Elmer 1600 and Lambda 3B instruments, respectively. CD measurements were carried out on a modified Cary 61 spectropolarimeter.[12] ^{13}C NMR spectra were recorded on a Varian Gemini 400 MHz spectrometer, and ^1H NMR, HMQC, and HMBC experiments were recorded on a Varian Inova 300 MHz spectrometer. Chemical shifts are reported in parts per million, referenced to residual solvent peaks, and coupling constants (*J*) are reported in hertz. High-resolution MALDI Fourier transform mass spectra were run on an Ionspec FTMS mass spectrometer at the mass spectrometry facility at the Scripps Research Institute.

Biological Material. The sample (95−079) containing 90−95% *L. majuscula*, contaminated with minor amounts of *Phormidium* sp. or *Schizothrix* sp., was collected by snorkelling in shallow water in the lagoon near Big Goby marine lake, Palau, and was immediately frozen. The identification was made by Mary Ann Roberts, Oregon State University. A voucher specimen has been retained by the corresponding author and is available on request.

Extraction and Purification. The frozen material (3 kg wet wt) was extracted with MeOH (4 × 1 L), and the extracts were concentrated to obtain a gum (10 g), which was partitioned between EtOAc and H_2O. The extracts were screened for inhibition of HIV-1 integrase, and the EtOAc extract was found to be active. The organic extract was purified using flash chromatography on a C_{18} reversed-phase support using a stepwise gradient from H_2O to MeOH as eluent. The only active fraction was a peptide-containing fraction that eluted with 40% MeOH in H_2O. The peptides were separated by HPLC on a C_{18} reversed-phase column, using a linear gradient

Table 2. NMR Assignments for Kororamide (**3**) in DMSO-d_6

amino acid	C#	δ_C	δ_H	mult, *J* (Hz)	HMBC
Tyr-Thz	1	160.6			
	2	146.7			
	3	126.0	8.29	1 H, s	C-2, C-4
	4	147.0			
	5	52.0	5.31	1 H, dd, 12,8	C-4, C-6, C-7
	6	39.0	2.92	1 H, m	
			2.88	1 H, m	
	7	131.0			
	8	117.5	6.96	1 H, d, 8.5	
	9	129.0	6.69	1 H, d, 8.5	
	10	156.0			
	NH		7.07	1 H, d, 7, 5	C-4, C-5, C-1(Leu)
	OH		9.35	1 H, br s	
Leu	1	171.9			
	2	51.6	4.08	1 H, ddd, 9.5,7, 2.5	
	3	34.5	1.70	2 H, m	
	4	24.0	1.55	1 H, m	
	5	23.0	0.90	3 H, d, 8	
	6	20.5	0.78	3 H, d, 8	
	NH		9.03	1 H, d, 7	C-2, C-1(Ile)
Ile	1	172.4			
	2	59.9	4.34	1 H, dd, 10, 7	C-1, C-3, C-4, C-6, C-1(Thn)
	3	34.5	1.70	1 H, m	
	4	24.1	1.32	2 H, m	
	5	13.2	0.80	3 H, t, 7.5	
	6	13.5	0.93	3 H, d, 7.5	
	NH		7.56	1 H, d, 7.5	
Leu-Thn	1	170.2			
	2	75.5	5.11	1 H, ddd, 10, 4, 1.5	
	3	36.0	3.72	1 H, m	C-1, C-2, C-4
			3.60	1 H, m	
	4	173.0			
	5	48.2	4.79	1 H, m	C-4
	6	36.9	1.52	1 H, m	
			1.38	1 H, m	
	7	24.5	1.60	1 H, m	
	8	20.5	0.86	3 H, d, 8	
	9	20.5	0.86	3 H, d, 8	
	NH		7.68	1 H, d, 10	C-1(Ser)
Ser	1	168.9			
	2	57.2	4.21	1 H, td, 8.5, 3	
	3	59.8	3.85	1 H, m	
			3.70	1 H, m	
	NH		7.56	1 H, d, 8.5	C-1(Pro)
	OH		4.95	1 H, m	
Pro	1	170.6			
	2	56.1	4.38	1 H, t, 6	C-1, C-3, C-4
	3	28.0	2.00	2 H, m	
	4	27.9	1.88	2 H, m	
	5	46.5	3.68	1 H, m	
			3.45	1 H, m	
Asn	1	169.8			
	2	48.2	4.92	1 H, ddd, 6.5, 6, 2.5	
	3	39.0	2.90	1 H, m	C-1, C-4
			2.71	1 H, m	
	4	172.3			
	NH2		7.75	1 H, br s	
			7.20	1 H, br s	
	NH		8.30	1 H, d, 6.5	C-1, C-2, C-1(Thz)

from H_2O to MeCN as eluent, with additional cleanup by HPLC (**1**, 40:60 MeCN−H_2O; **2** and **3**, 1:1 MeCN−H_2O), to obtain dolastatin 3 (**1**, 1.2 mg, 4×10^{-5}% wet wt), homodolastatin 3 (**2**, 1.5 mg, 4.8×10^{-5}% wet wt), and kororamide (**3**, 1.2 mg, 4×10^{-5}% wet wt). The flash chromatography fraction eluted with 60% MeOH in H_2O was separated by HPLC on a C_{18} reversed-phase column, using a linear gradient from H_2O to MeCN as eluent, to obtain aplysiatoxin (**4**, 1.4 mg, 4.5×10^{-5}%), debromoaplysiatoxin (**5**, 2.0 mg, 6.4×10^{-5}%), and oscillotoxin (**6**, 0.8 mg, 2.6×10^{-5}%), all of which were identified by comparison of spectral data with literature values.[4,10]

Homodolastatin 3 (2): white solid; UV (MeOH) 240 nm (ϵ 7000); ^{1}H NMR (300 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (400 MHz, $CDCl_3$) see Table 1; ESIMS *m*/*z* 675; HRMS (MALDI-TOF) *m*/*z* 675.2740 (calcd for $C_{30}H_{42}N_8O_6S_2Na$, 675.2780).

Kororamide (3): white solid; CD (MeOH) min 198 nm (θ −1500 deg·cm^2·dmol^{-1}), cross 227 nm, max 233 nm (θ 900 deg·cm^2·dmol^{-1}); UV (MeOH) 238 nm (ϵ 6400) ^{1}H NMR (300 MHz, $CDCl_3$) see Table 2; ^{13}C NMR (400 MHz, $CDCl_3$) see Table 2; ESI/MS *m*/*z*; HRMS (MALDI-TOF) *m*/*z* 969.4325 (calcd for $C_{45}H_{65}N_{10}O_{10}S_2$, 969.4327).

Determination of the Absolute Configuration of Amino Acid Residues. Solutions of peptide **2** or **3** (50−100 μg) in 6 N HCl (800 μL) in tightly capped thick-walled vials were heated at 90 °C for 16 h. The solvent was removed by heating under a stream of dry N_2, and the samples were redissolved in dry 2-propanol (400 μL). After adding acetyl chloride (100

μL) to this solution, the vial was immediatly capped and heated to 110 °C for 45 min. After removing the solvents under dry N_2, the residue was redissolved in dry CH_2Cl_2 (400 μL), pentafluoropropionic acid (400 μL) was added, and the vial was quickly capped. The solution was kept at 100 °C for 15 min, the solvents were removed under N_2, and the samples were dissolved in EtOAc (300 μL) for analysis by GC−MS using an Alltech Chirasil−Val capillary column. The oven temperature was ramped from 50 to 220 °C at 4 °C/min, and the identity of each peak was confirmed by co-injection with a standard that had been derivatized using the protocol described above.

The stereochemistry of thiazole amino acids was determined by ozonolysis of the thiazole rings followed by oxidation to yield standard amino acids with retention of the original stereochemistry. Peptides **2** and **3** (100 μg) were dissolved in MeOH (3 mL), and a stream of ozone in oxygen was bubbled through the stirred solution at −78 °C for 15 min. The solvents were removed in vacuo and the residue was treated with H_2O_2−HCO_2H (200 μL of a 1:2 solution) and warmed to 70 °C for 20 min. The resulting residues were then hydrolyzed and derivatized as described above.

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