Pitiprolamide, a Proline-Rich Dolastatin 16 Analogue from the Marine Cyanobacterium *Lyngbya majuscula* from Guam

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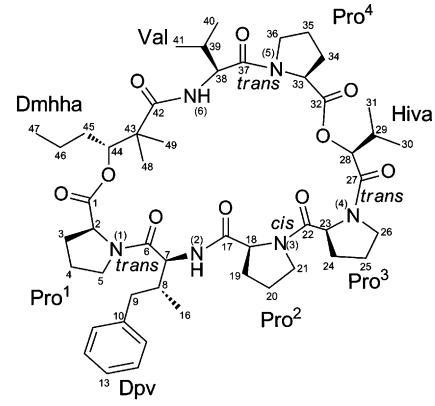
An unusual cyclic depsipeptide, pitiprolamide (**1**), was isolated from the marine cyanobacterium *Lyngbya majuscula* collected at Piti Bomb Holes, Guam. The structure was deduced using NMR, MS, X-ray crystallography, and enantioselective HPLC-MS techniques. Remarkably, proline represents half of the residues forming pitiprolamide (**1**). Other distinctive features include a 4-phenylvaline (dolaphenvaline, Dpv) moiety initially found in dolastatin 16 and the rare 2,2-dimethyl-3-hydroxyhexanoic acid (Dmhha) unit condensed in a unique sequence in one single molecule. Pitiprolamide (**1**) showed weak cytotoxic activity against HCT116 colon and MCF7 breast cancer cell lines, as well as weak antibacterial activities against *Mycobacterium tuberculosis* and *Bacillus cereus*.

The marine cyanobacterium *Lyngbya majuscula* is a prolific source of structurally diverse secondary metabolites. One of its multifarious structural themes is the establishment of cyclic peptides by utilizing a diversity of standard as well as modified amino acids.[1] Among these, proline-rich cyclic peptides represent an intriguing class. The interest in this class arises from the distinctively low activation barrier for the prolyl amide bond to switch between *cis* and *trans* conformations readily compared to other amide linkages, which would impact the three-dimensional structure of the peptide.[2] The rate of this interconversion is solvent dependent, being higher in nonpolar solvents, where the charge separation and resonance stabilization of the ground state of both isomers are lost, lowering the *cis*-to-*trans* torsion barrier.[3−5] Recently, further attention has been directed toward this prolyl isomerization, as it has been suggested to be involved in the rate-limiting steps for protein folding[6] and to act as a molecular timer in several biological and pathological processes.[7]

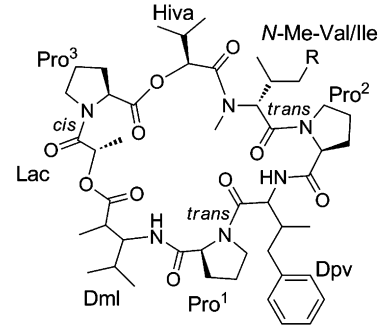
In our quest for novel drug leads from marine cyanobacteria, we isolated a novel proline-rich cyclodepsipeptide, pitiprolamide (**1**), from a collection of the cyanobacterium *L. majuscula* from Piti Bomb Holes, Guam, the same population that previously yielded pitipeptolides A and B.[8] Compound **1** is structurally related to dolastatin 16[9] (**2**) and other marine secondary metabolites isolated from marine invertebrates and cyanobacteria. Here, we describe the isolation, structure determination, and initial biological evaluation of compound **1**.

The EtOAc/MeOH extract of the cyanobacterial sample was subjected to solvent partitioning followed by silica chromatography. Using NMR-guided fractionation targeting modified peptides and depsipeptides, we identified a pitipeptolide-containing fraction, which was further subjected to purification by reversed-phase HPLC to furnish compound **1** as a colorless, amorphous solid.

The structure of **1** was elucidated by interpreting the ^{1}H NMR, ^{13}C NMR, COSY, TOCSY, HSQC, HMBC, and ROESY spectra recorded in benzene-d_6. HRESIMS for **1** suggested a molecular formula of $C_{49}H_{72}N_6O_{10}$ (*m*/*z* 927.5209 for $[M + Na]^+$ and *m*/*z* 905.5390 for $[M + H]^+$). The low-field signals at δ_H 7.73 and 9.17 ppm suggested that these arise from amide protons. The presence of amide bonds was further supported by the assignment of α-protons ($\delta_H \approx 4-5$ ppm) and detecting their HMBC correlations



Pitiprolamide (**1**)



Dolastatin 16 (**2**) R=H
Homodolastatin 16 (**3**) R=CH_3

with neighboring carbonyls ($\delta_C \approx 165-175$ ppm). However, the chemical shifts of two carbons (C28, δ_C 77.7 and C44, δ_C 79.3 ppm) suggested their oxygenation and the presence of hydroxy acids. This further indicated that **1** could be a depsipeptide. Aromatic carbons (δ_C 126.3, 2 × 128.7, 2 × 129.8, 141.1) present were characteristic of a monosubstituted benzene.

Further analysis of the 2D NMR spectra led to the assignment of eight moieties: five proteinogenic amino acids (four proline units (Pro[1], C1−C5; Pro[2], C17−C21; Pro[3], C22−C26; Pro[4], C32−C36) and a valine unit (Val, C37−C41)), the modified amino acid dolaphenvaline (Dpv, C6−C16), and two hydroxy acids, namely, 2-hydroxy isovaleric acid (Hiva, C27−C31) and 2,2-dimethyl-3-hydroxyhexanoic acid (Dmhha, C42−C49) (Table 1). To satisfy

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Pitiprolamide (**1**) in Benzene-d_6 (δ in ppm, J in Hz) at 600 MHz (^{1}H) and 125 MHz (^{13}C) at 20 °C

unit	C/H #	δ_C	δ_H (J)	HMBC[a]
Pro[1]	1	173.9		2, 3, 44
	2	59.4	5.62, dd (8.3, 3.4)	3, 5a, 5b
	3	29.4	1.64, m	2, 5a, 5b
	4a	24.6	1.38, m	2, 3, 5b
	4b		1.86, m	
	5a	47.6	3.82, ddd (13.9, 9.2, 4.2)	2, 3, 4b
	5b		4.10, ddd (9.2, 7.7, 7.5)	
Dpv	6	170.5		7
	7	55.7	5.28, dd (11.5, 9.8)	8, 9a, 9b, 16, NH (2)
	8	39.2	2.74, m	7, 9a, 9b, 16
	9a	39.9	2.25, m	7, 16, 11/15
	9b		3.30, dd (12.9, 1.5)	
	10	141.1		16, 11/15, 12/14
	11/15	129.8	7.34, d (7.5)	16, 12/14, 13
	12/14	128.7	7.23, m	11/15, 13
	13	126.3	7.11, m	11/15, 12/14
	16	15.5	0.90, d (6.7)	7, 9a, 9b
	NH (2)		9.17, d (9.7)	
Pro[2]	17	171.6		7, 18, 19a
	18	61.9	4.33[b]	19a, 20a
	19a	32.3	1.46, m	18, 20b, 21a
	19b		2.42, m	
	20a	22.0	1.27, m	18, 21a, 21b
	20b		1.60, m	
	21a	46.5	3.51, m	18, 19b
	21b		3.64, m	
Pro[3]	22	170.8		23, 24a, 24b
	23	59.4	4.33[b]	24a, 24b, 25a, 25b, 26a, 26b, 28
	24a	28.1	1.11, m	23
	24b		1.28, m	
	25a	24.8	1.05, m	23
	25b		1.40, m	
	26a	47.2	3.17, m	24a, 24b, 25a, 25b
	26b		3.57, m	
Hiva	27	167.3		23, 28, 29
	28	77.7	4.58, d (8.6)	29, 30, 31
	29	30.7	2.27, m	28, 30, 31
	30	18.3	1.12, d (6.7)	28, 29, 31
	31	18.8	0.85, d (6.5)	28, 29, 30
Pro[4]	32	172.6		28, 33, 34
	33	59.4	4.46, dd (8.6, 6.2)	28, 34, 35a, 35b, 36a, 36b
	34	29.6	1.50, m	33, 35a, 35b, 36a, 36b
	35a	25.2	1.21, m	33, 34, 36a, 36b
	35b		0.92, m	
	36a	46.8	2.89, m	33, 34, 35a, 35b
	36b		3.00, m	
Val	37	170.1		38, 39, NH (6)
	38	55.3	5.21, dd (8.8, 1.9)	40, 41
	39	32.5	2.25, m	38, 40, 41
	40	17.4	1.54, d (6.8)	38, 39, 41
	41	21.5	1.31, d (6.7)	38, 39, 40
	NH (6)		7.73, d (8.8)	
Dmhha	42	173.9		44, 48, 49, NH (6)
	43	47.2		44, 48, 49
	44	79.3	5.01, dd (11.4, 1.6)	48, 49
	45a	32.2	1.61, m	44, 46a, 46b, 47
	45b		1.77, m	
	46a	19.5	1.05, m	44, 45a, 45b, 47
	46b		1.20, m	
	47	13.5	0.61, t (7.4)	45b, 46a
	48	23.3	1.28, s	44, 49
	49	26.1	1.73, s	44, 48

[a] Protons showing long-range correlation to indicated carbon. [b] Multiplicity could not be unambiguously deduced because the peaks for those two protons as well as peaks from a minor conformer are overlapping.

the molecular requirements from the MS analysis, compound **1** had to be a cyclic depsipeptide.

The sequence of the residues forming **1** was determined by HMBC correlations (Supporting Information Figure S1) along with MS-MS fragmentation data (Supporting Information Figure S2). Furthermore, as the compound crystallized out of an aqueous MeOH solution, the implied sequence was confirmed by X-ray crystallography.

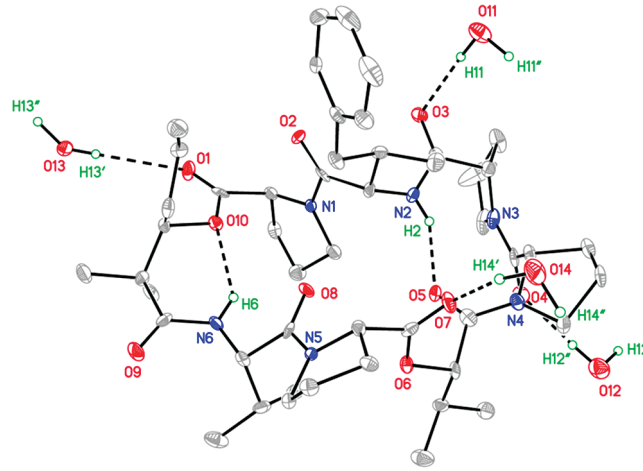

Figure 1. ORTEP diagram for pitiprolamide (**1**) showing the molecule and water solvent molecules. Hydrogen bonding is shown as dashed lines.

The availability of an X-ray crystal structure for **1** simplified its stereochemical assignment by establishing the relative configuration. Enantioselective amino acid analysis of the acid hydrolysate by HPLC-MS established the L-configuration for all Pro units and for Val. Consequently, the Dmhha unit had a 3*R* and Hiva had an *S* configuration. The Dpv moiety had a 2*S*,3*R* configuration. This is the first crystal structure of a Dpv-containing compound.

Because **1** crystallized from an aqueous solvent, hydration was detected in the crystal structure. Four water molecules formed H-bonds with the four carbonyls in Dmhha, Hiva, Pro^2, and Pro^3 units. Those H-bonds provide further conformational stability observed in polar solvents as mentioned before. Moreover, two intramolecular hydrogen bonds were observed. The first was between the amide proton in Val and the hydroxy-derived oxygen in the Dmhha unit. The second one occurred between the other amide proton in the Dpv unit and the carbonyl in the Hiva unit. This latter 4→1 type H-bond granted the cyclic peptide a β-turn, enclosing the *cis*-amide linked Pro^2-Pro^3 residues as shown in Figure 1. This matches what is known about the contribution of proline to various turn types, as well as the induction of a turn by *cis* Pro-Pro sequences.[10]

The ^{1}H NMR spectrum of **1** in benzene-d_6 revealed the presence of two isomers showing a slow interconversion over time (Supporting Information Figure S3) due to prolyl amide isomerization, which complicated the structure elucidation process. The difference in chemical shifts of the β and γ carbon signals in Pro^2 ($\Delta\delta_{\beta\gamma}$ = 10.2 ppm) suggested a *cis* orientation for the Pro^2-Pro^3 linkage for the major conformer in the NMR solvent benzene-d_6. The bonds preceding the three other Pro units appeared to be *trans*, concluded from the lower $\Delta\delta_{\beta\gamma}$ values (less than 6 ppm).[2] This matches the X-ray crystal structure and the ROESY correlations, pointing out the similarity between the solution and the solid-state structures.

Pitiprolamide (**1**) is a dolastatin 16 analogue that is comprised of unusual units. Pitiprolamide (**1**) is structurally related to dolastatin 16 (**2**), which was reported more than 10 years ago from the sea hare *Dolabella auricularia*.[9] To the best of our knowledge, compound **1** is the second dolastatin 16 analogue from a marine cyanobacterium. Homodolastatin 16 (**3**) has been isolated from a different collection of *L. majuscula* from Kenya.[11] The isolation of **1** from *L. majuscula* adds to the growing body of evidence regarding the cyanobacterial biosynthetic origin of dolastatin 16 and other dolastatins as well. Kulokekahilide-1 is another dolastatin 16 analogue from the marine mollusk *Philinopsis speciosa* assumed to be of dietary cyanobacterial origin.[12]

The building blocks of dolastatin 16 and its three analogues share several features (Figure 2). First, they are all octa-bidepsipeptides.

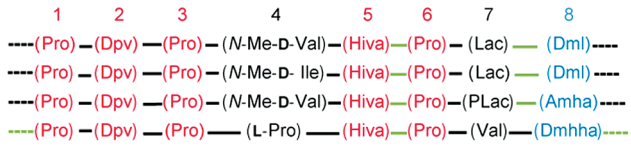

Figure 2. Residue sequences for dolastatin 16, homodolastatin 16, kulokekahilide-1, and compound **1** (from top to bottom). Conserved units are shown in red. Substituted hexanoic and isohexanoic acid residues are shown in blue. Green lines indicate ester linkages. Dotted lines indicate connected residues. The configuration of the fourth unit in all the analogues is noted in bold, indicating configurational change at that position.

Second, five conserved residues could be noted in the four structural analogues: Pro-Dpv-Pro and Hiva-Pro. Third, the four compounds share derivatives of hexanoic and isohexanoic acids at the same position. Nonetheless, there are some distinctive differences characterizing **1**. Compound **1** has four Pro residues, which corresponds to 50% of all the residues. Also, it has a β-hydroxy hexanoic acid derivative versus β-amino hexanoic and isohexanoic acid derivatives in the other three analogues. This difference shifts the position of the second ester linkage in **1**, as shown in Figure 2. Moreover, a configurational change is prominent, where Pro[3] in **1**, which replaces the *N*-Me-D-Val/Ile in the other analogues, has an L-configuration.

Compound **1** also shares some structural features with other marine cyanobacterial secondary metabolites. For example, the rare Dmhha residue in **1** was first reported in guineamides E and F[13] and was recently illustrated in palmyramide A.[14] Also, the proline-rich aspect in **1** has been characterized in some other secondary metabolites reported from different marine sponges; examples include phakellistatins,[15] stylisins,[16] and stylissamides.[17] However, **1** contains all of these rare structural characteristics in one molecule.

Because natural products emerged from evolutionary selection as biologically active compounds,[18] we initiated bioactivity assessment for **1**. This cyclic depsipeptide showed weak cytotoxic activity against HCT116 colorectal carcinoma and MCF7 breast adenocarcinoma cell lines (IC_{50} 33 μM for both). While dolastatin 16, homodolastatin 16, and kulokekahilide-1 showed more potent cytotoxic activities against different cancer cell lines,[9,11,12] the structural differences in the four analogues could signify the relationship between some residues and cytotoxicity. Compound **1** showed weak antibacterial activity against *Mycobacterium tuberculosis* starting at 50 μg in a disk diffusion assay (Supporting Information Table S1) and against *Bacillus cereus* starting at 1 μM in a microtiter plate-based assay with an approximate IC_{50} value of 70 μM. No antibacterial activities were detected against either *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a Perkin-Elmer 341 polarimeter. UV and optical density were measured on a SpectraMax M5 (Molecular Devices), and IR data were obtained on a Perkin-Elmer Spectrum One FT-IR spectrometer. The ^{13}C NMR spectrum was recorded on a Bruker 500 MHz spectrometer operating at 125 MHz. ^1H and 2D NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer. All spectra were obtained in benzene-d_6 using residual solvent signals (δ_H 7.16, δ_C 128.06 ppm) as internal standards. HSQC and HMBC experiments were optimized for $^1J_{CH} = 145$ and $^1J_{CH} = 7$ Hz, respectively. The ^1H NMR spectrum in acetone-d_6 was recorded on a Varian Mercury 400 MHz spectrometer using residual solvent signal (δ_H 2.05 ppm) as a reference. HRMS data were recorded on an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector in positive ion mode. LC-MS data were obtained using an API 3200 triple quadrupole MS (Applied Biosystems) equipped with a Shimadzu LC system. ESIMS fragmentation data were recorded on an API 3200 by direct injection with a syringe driver.

Extraction and Isolation. *Lyngbya majuscula* was collected from Piti Bomb Holes, Guam, in February 2000. The freeze-dried organism was extracted three times with EtOAc−MeOH (1:1) to afford an organic extract (35.5 g). The resulting extract was partitioned between hexanes and 80% aqueous MeOH, the methanolic phase was evaporated to dryness, and the residue was further partitioned between *n*-BuOH and H_2O. After concentrating the *n*-BuOH extract in vacuo, the resulting residue (7.2 g) was subjected to flash chromatography over silica gel, eluting with CH_2Cl_2 followed by increasing gradients of *i*-PrOH in CH_2Cl_2, and finally with MeOH. The fraction eluting with 4% *i*-PrOH/CH_2Cl_2 was fractionated on a semipreparative reversed-phase HPLC column (YMC-Pack ODS-AQ, 250 × 10 mm, 5 μm, 2 mL/min; UV detection at 220/254 nm) using a MeOH/H_2O linear gradient (75% to 100% over 30 min and then 100% MeOH for 10 min). The fraction eluting between t_R 19.2 and 20.2 min was then repurified using semipreparative reversed-phase HPLC (Luna C18, 250 × 10 mm, 5 μm, 2.0 mL/min; UV detection at 200/220 nm) using a MeOH/H_2O linear gradient (75% to 100% over 20 min followed by 100% MeOH for 10 min) to afford compound **1** (22 mg) at t_R 19 min.

Pitiprolamide (1): colorless, amorphous solid; $[\alpha]^{20}_D$ −65 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.41) nm; IR (film) ν_{max} 3749, 2964, 2876, 1734, 1643, 1546, 1522, 1502, 1436, 1282, 1190, 1092 cm^{-1}; ^1H NMR, ^{13}C NMR, and HMBC data, see Table 1; HRESI/APCIMS *m/z* 927.5209 $[M + Na]^+$ (calcd $C_{49}H_{72}N_6O_{10}Na$ 927.5201) and *m/z* 905.5390 $[M + H]^+$ (calcd $C_{49}H_{73}N_6O_{10}$ 905.5383).

Acid Hydrolysis and Enantioselective Amino Acid Analysis by HPLC/MS. A sample of **1** (0.1 mg) was treated with 6 N HCl (0.5 mL) at 110 °C for 24 h. The hydrolysate was concentrated to dryness, resuspended in 100 μL of H_2O, and then analyzed by enantioselective HPLC [column: Chirobiotic TAG (250 × 4.6 mm), Supelco; solvent: MeOH/10 mM NH_4OAc (40:60, pH 5.6); flow rate 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan)]. The retention times (t_R min; MRM ion pair, parent→product) of the authentic amino acids were as follows: L-Pro (14.8; 116→70), D-Pro (41.4), L-Val (8.5; 118→72), D-Val (16.0). The hydrolysate of **1** showed peaks corresponding to L-Pro and L-Val at t_R 14.8 and 8.5, respectively. The MS parameters used were as follows: DP 32, EP 4, CE 21.8, CXP 2.8, CUR 50, CAD medium, IS 4500, TEM 750, GS1 65, and GS2 65.

Cell Viability Assays. Cell culture medium was purchased from Invitrogen, and fetal bovine serum (FBS) from Hyclone. Cells were propagated and maintained in DMEM supplemented with 10% FBS in 37 °C humidified air and 5% CO_2. Cells were seeded in 96-well plates (MCF7 10 500 cells/well; HCT116 10 000 cells/well). After 24 h, cells were treated with various concentrations of compound **1** or solvent control (1% EtOH). After 48 h of incubation, cell viability was measured using MTT according to the manufacturer's instructions (Promega).

Antibacterial Assays. Pitiprolamide (**1**) was tested against *Mycobacterium tuberculosis* [ATCC # 25177] in a disk diffusion assay. The compound was dissolved in EtOH, and three different amounts were loaded on sterile filter paper discs. The dry discs where applied to an inoculated agar plate and incubated for 12 days at 37 °C, after which the diameter of the zone of inhibition was measured. The microtiter plate-based assay method[19] was used to test the antibacterial activity of **1** against three human pathogenic bacteria: *Bacillus cereus* [ATCC # 10987], *Staphylococcus aureus* [ATCC # 25923], and *Pseudomonas aeruginosa* [ATCC # BAA-47]. The former organism was grown in LB broth medium at 30 °C, while the two latter strains were grown in the same medium at 37 °C. For each organism, a standard curve was constructed and used to interpret the assay data.

X-ray Crystallography.[20] $C_{49}H_{79}N_6O_{13.5}$, M_r = 968.18, orthorhombic, $P2_12_12_1$, a = 10.534(2) Å, b = 14.952(3) Å, c = 33.511(7) Å, V = 5278.0(19) Å^3, Z = 0, D_{calc} = 1.218 g cm^{-3}, Mo Kα (λ = 0.71073 Å), T = 100 K. Data were collected at 100 K on a Bruker DUO system equipped with an APEX II area detector and a graphite monochromator utilizing Mo Kα radiation (λ = 0.71073 Å). Cell parameters were refined using 3973 reflections. A hemisphere of data was collected using the ω-scan method (0.5° frame width). Absorption corrections by integration were applied based on measured indexed crystal faces. The structure was solved by the direct methods in SHELXTL6 and refined using full-matrix least-squares. The non-H atoms were treated anisotropically, whereas the hydrogen atoms were calculated in ideal positions and were riding on their respective carbon atoms. In addition to the molecule, there are three and a half water

molecules in the asymmetric unit (one water refined with 50% occupancy). The water protons were obtained from a difference Fourier map and were treated riding on their parent oxygen atoms. The amino protons were also obtained from a difference Fourier map and were refined freely. All of those protons are involved in a network of hydrogen bonds, as can be seen in the Supporting Information, Table S4. A total of 622 parameters were refined in the final cycle of refinement using 3819 reflections with $I > 2\sigma(I)$ to yield R_1 and wR_2 of 4.99% and 6.26%, respectively.

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Supporting Information Available: Additional experimental details on antibacterial assays including Table S1, NMR spectra, Figures S1, S2, and S3, stereo ORTEP drawing (Figure S4) and packing drawing (Figure S5), and X-ray diffraction data (Tables S2−S4) for compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC accession no. 801619). Copies of the data can be obtained, free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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