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Wewakazole, a Novel Cyclic Dodecapeptide from a Papua New Guinea *Lyngbya majuscula*

Lisa M. Nogle, Brian L. Marquez, and William H. Gerwick*

College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

bill.gerwick@oregonstate.edu

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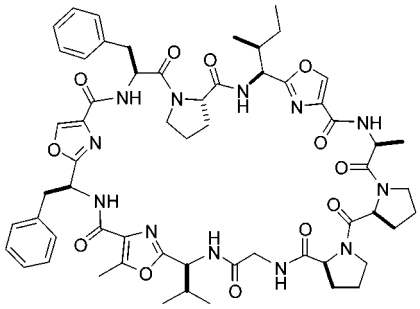
ABSTRACT



Phytochemical examination of a Papua New Guinea collection of *Lyngbya majuscula* resulted in the discovery of wewakazole (1), a novel cyclic dodecapeptide containing an unprecedented six five-membered heterocycles. Multiple NMR experiments and MS/MS data were required to assemble its planar structure because of its extensively signal-overlapped NMR spectra. In particular, a 1D HMBC was utilized to orient a three amino acid fragment that could not be placed by standard spectroscopic methods.

Lyngbya majuscula is best recognized for its production of lipopeptides, products of both polyketide synthase (PKS) and nonribosomal polypeptide synthetase (NRPS) biosynthetic origins.[1] In fact, natural products of purely amino acid derivation are comparatively rare from this genus. A phytochemical study of a Papua New Guinea collection of this cyanobacterium resulted in the discovery of wewakazole (**1**), a cyclic peptide integrating structural features uncharacteristic of *L. majuscula* secondary metabolites. In this paper, we present the isolation and structure determination of this novel marine cyanobacterial metabolite.

The *L. majuscula* strain was collected from a shoreline growth of coral (<1.5 m depth) in Wewak Bay, Papua New Guinea, in September 1998. The extract (5.9 g) was chromatographed over silica gel, and the resulting fractions were analyzed by TLC. The highly pigmented, polar fractions eluting from the VLC also possessed UV-absorbing metabolites and thus were further fractionated by LH-20 size-exclusion chromatography (elution with 100% MeOH). Additional purification by C_{18} solid-phase extraction and reversed-phase HPLC (4:1 MeOH/H_2O, Phenomenex Luna phenylhexyl 5 μm) afforded 6.9 mg of wewakazole (**1**).[2]

The ^{1}H and ^{13}C NMR spectra of wewakazole indicated that **1** was a large molecule of peptide origin. Twelve carbon resonances observed between 158 and 174 ppm, and a molecular composition of $C_{59}H_{72}N_{12}O_{12}$ determined by HRFABMS, suggested that wewakazole possessed twelve amino acid residues.

Although a high degree of spectral overlap existed in both the proton and carbon dimensions, twelve individual units were assembled from the HSQC, TOCSY, and HMBC data

(1) Gerwick, W. H.; Tan, L. T.; Sitachitta, N. In *The Alkaloids*; Cordell, G. A., Ed.; Academic Press: San Diego, 2001; Vol. 57, pp 75−184.

(2) **Wewakazole (1):** glassy oil; $[\alpha]^{21}_D$ −46.8 (*c* 0.41, MeOH); UV (MeOH) λ_{max} 213 nm (log ϵ 4.55), 221 nm (log ϵ 4.39); IR (neat) 3371, 3275, 2961, 2932, 2878, 1635, 1514, 1452 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* [M + H]$^+$ 1141.5463 (calcd for $C_{59}H_{73}N_{12}O_{12}$, 1141.5471); MS/MS fragmentation of parent ion *m/z* 1142 1114 (100), 1017 (28), 765 (96), 694 (40), 668 (77), 597 (50), 571 (43), 514 (27), 497 (39), 349 (44), 321 (54), 295 (57), 172 (69).

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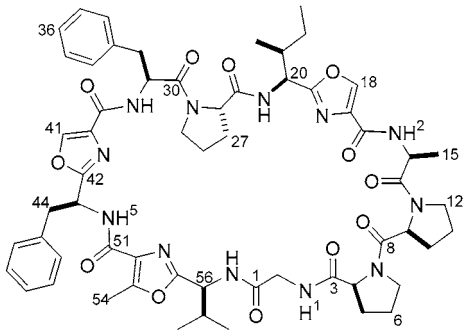
Table 1. NMR Spectral Data for Wewakazole (**1**) in $CDCl_3$

	position	$^{13}C^a$	^1H (J in Hz)^a	TOCSY	HMBC^b
Gly	1	169.4			
	2	43.3	4.31, dd (17.4, 8.2)	NH-1	1, 3, 4
			3.52, dd (17.4, 4.3)		
	NH-1		6.92, m	2	3
Pro 1	3	172.9			
	4	61.4	3.74, dd (8.0, 5.1)	5, 6	3, 5, 6, 8
	5	28.6	1.57, 1.02	4, 6, 7	3, 6, 7
	6	24.8	1.82, 0.96	4, 5, 7	5, 7
	7	47.5	3.40	5, 6	6
Pro 2	8	171.2			
	9	59.1	4.62, m	10, 11, 12	8, 10
	10	27.6	2.39, 1.86	9, 11, 12	12
	11	26.2	2.09	9, 10, 12	10, 12
	12	47.7	3.85, 3.40	9, 10, 11	11, 13
Ala	13	171.0			
	14	46.5	4.79, m	15, NH-2	13, 15, 16, 17
	15	18.5	1.28, d (6.9)	14, NH-2	13, 14
	NH-2		7.89, brs	14, 15	13, 14, 16, 17
Oxz 1	16	159.8			
	17	136.2			
	18	140.8	8.04, s		17, 19
Ile	19	165.0			
	20	53.1	5.05, dd (9.2, 6.1)	21, 22, 24, NH-3	19, 21, 22, 24, 25
	21	37.7	2.19	20, 22, 23, 24	20
	22	25.8	1.43	20, 21, 23, 24	21, 223, 24
	23	11.9	0.87	21, 22	21, 22
	24	16.5	0.93	20, 21, 22	21, 22
	NH-3		9.34, d (9.4)	20	20, 25
Pro 3	25	172.1			
	26	61.5	3.34	27, 28	25, 27, 28, 29, 30
	27	31.5	2.08, 0.92	26, 28, 29	25, 26, 28, 29
	28	22.6	1.62, 1.51	26, 27, 29	26
	29	47.0	3.45	27, 28	
Phe 1	30	170.8			
	31	55.9	4.68, m	32, NH-4	30, 39
	32	37.5	3.79, dd (11.5, 3.7)	31	30, 31, 33, 34/38
			3.02, dd (11.5, 11.2)		
	33	136.9			
	34/38	130.5	7.52, d (6.6)	35/37	32, 35/37, 36
	35/37	129.2	7.30	34/38	33, 34/38
	36	128.0	7.29		35/37
	NH-4		7.86, d (7.8)	31	30, 31, 32, 39
Oxz 2	39	160.7			
	40	135.8			
	41	142.7	7.78, s		40, 42
Phe 2	42	163.6			
	43	48.8	5.62, brq (6.9)	44, NH-5	42, 44, 45
	44	40.8	3.17, dd (13.5, 6.9), 3.29	43, NH-5	42, 43, 45, 46/50
	45	135.7			
	46/50	129.7	6.90, d (7.2)	47/49, 48	44, 47/49, 48
	47/49	129.0	7.14, t (7.2)	46/50, 48	45, 46/50
	48	127.7	7.23, d (7.2)	47/49	47/49
	NH-5		7.35	43, 44	42, 43, 51
MeOxz	51	161.3			
	52	129.0			
	53	154.0			
	54	12.2	2.61, s		51, 52, 53
Val	55	162.1			
	56	53.3	4.83, m	57, 58, 59, NH-6	1, 55, 57
	57	30.8	2.33	56, 58, 59, NH-6	58
	58	19.7	0.87	56, 57, NH-6	56, 57, 59
	59	20.9	0.93	56, 57, NH-6	56, 57, 58
	NH-6		7.38	56, 57, 58, 59	1, 56, 57

[a] Calibrated to residual solvent at δ 77.23, 7.23. [b] Proton showing correlation to indicated carbon. Optimized for 4 and 8 Hz coupling.

(Table 1). Among the deduced partial structures were the standard amino acids glycine (Gly), alanine, (Ala), valine (Val), isoleucine (Ile), two phenylalanines (Phe), and three prolines (Pro). Interestingly, wewakazole also displayed the two methine singlets CH-18 (δ_C 140.8, δ_H 8.04) and CH-41 (δ_C 142.7, δ_H 7.78) indicative of two oxazole (Oxz) moieties, and the methyl singlet CH_3-54 (δ_C 12.2, δ_H 2.61), characteristic of a methyl oxazole residue (MeOxz).[3]

The heterocyclization of cysteine residues to form thiazole and thiazoline rings occurs frequently in *L. majuscula* peptides. In fact, free reduced cysteine has never been observed in its secondary metabolites.[1] Conversely, the serine and threonine counterparts, oxazoles and methyloxazoles, are rare in marine cyanobacteria, more often being detected in metabolites from marine invertebrates. HMBC correlations and chemical shift comparisons with literature values confirmed the presence of both a methyloxazole and two oxazole units in the structure of wewakazole and thus accounted for all remaining atoms in its molecular formula.



wewakazole (**1**)

An 8 Hz optimized HMBC experiment (Bruker AM400 NMR spectrometer) was used to initiate amino acid sequencing. Despite several proton and carbon resonances in **1** having close or overlapping chemical shifts, four fragments accounting for all 12 amino acid units could be constructed from these data (Figure 1). However, neither the methyloxazole (fragment D) nor the oxazole moieties (fragments C and D) showed the connectivities necessary for subsequent amino acid sequencing, and thus, additional 2D NMR experiments were conducted.

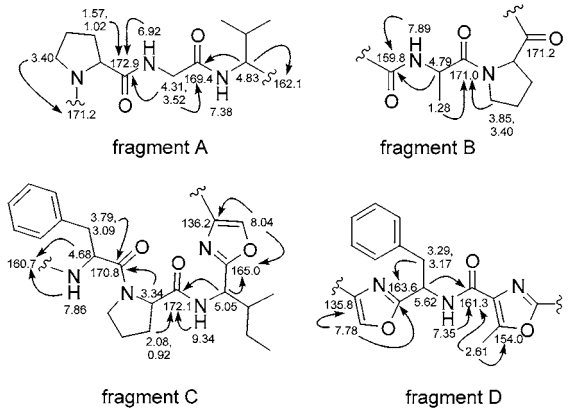


Figure 1. Partial amino acid sequencing by an 8 Hz optimized HMBC experiment.

A 4 Hz optimized HMBC (Bruker DRX600 NMR spectrometer) showed both the ^3J correlation from NH-2 to C17 and the ^4J correlation from H14 to C17, linking the alanine residue of fragment B to the oxazole of fragment C. In addition, these data more clearly distinguished between the Ala carbonyl resonance (δ_C 171.0) and that of the Pro-2 carbonyl (δ_C 171.2), allowing for connection of Pro-1 and Pro-2 and thus fragments A and B. Support for the sequencing of this portion of wewakazole was also obtained from MS/MS analysis.[2]

Although no additional HMBC connectivities or dipolar couplings were observed for either the oxazole or methyloxazole of fragment D, only two partial structures remained (A−B−C and D), and thus, there appeared only one obvious direction for connecting this cyclic peptide. However, upon further consideration, it was realized that the orientation of the three amino acid unit (fragment D) could be reversed if the proposed ^3J correlation from the Phe methylene protons at δ 3.29/3.17 (H_2-44) to C42 (δ 163.6) was actually a ^4J correlation. In this opposite orientation, all HMBC connectivities were still plausible, and therefore, alternative methods were explored to resolve this sequencing issue.

Structural analyses of peptides containing oxazole and methyloxazole residues have often noted a lack of HMBC correlations to oxazole moieties. In fact, connections through oxazoles are often assumed from molecular formula and DBE requirements.[4] However, a decoupled HMBC pulse sequence was recently reported to show such couplings, as demonstrated in the antibiotic promothiocin B.[5] This experiment was applied to **1**; unfortunately it was not successful. Ultimately, a modification of the sequence published by Meisser et al. was effectively employed in the structure elucidation of wewakazole (Figure 2).[6]

Precise irradiation of C39 (δ 160.7) was difficult to achieve, however, and thus, the carbon resonance at δ 161.3 (C51) was also irradiated. Nevertheless, only one molecular arrangement is consistent with the observed correlations. Specifically, couplings were seen to the protons at δ 7.86 (NH-4), δ 7.78 (H41), δ 7.35 (NH-5), and δ 2.61 (H_3-54). In the originally proposed orientation A, these signals represent two-, three-, or four-bond correlations (Figure 3A). However, in the alternate orientation (Figure 3B), enhancement of the proton at δ 7.78 would require an unlikely six-bond coupling from the carbon resonance at δ 161.3. This final piece of data unequivocally established the amino acid sequence of wewakazole and completed its planar structure.

The absolute stereochemistry of **1** was determined through a combination of HPLC analysis using Marfey methodology

(3) The terms "oxazole" and "methyloxazole" denote biosynthetically formed heterocycles deriving from serine and threonine, respectively.

(4) Sone, H.; Kigoshi, H.; Yamada, K. Tetrahedron **1997**, *53*, 8149−8154.

(5) Furihata, K.; Seto, H. *Tetrahedron Lett.* **1995**, *36*, 2817−2820.

(6) Meissner, A.; Sorensen, O. W. *Magn. Reson. Chem.* **2001**, *39*, 49−52.

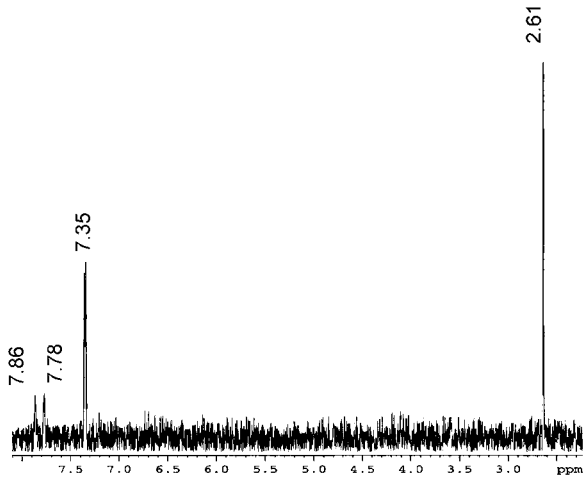


Figure 2. 1D HMBC of **1**. A total of 32K scans were acquired with selective irradiation at C39 (δ 160.7) on a 13.8 mM solution of wewakazole (6.3 mg dissolved in 0.4 mL of $CDCl_3$). The delay for the evolution of long-range heteronuclear coupling was optimized for 4 Hz (125 ms), and the spectrum was processed with 1 Hz line broadening. Irradiation of both C39 and C51 (δ 161.3) displayed couplings to NH-4 (δ 7.86), H41 (δ 7.78), NH-5 (δ 7.35), and H_3-54 (δ 2.61).

and chiral GCMS analysis using *N*-pentafluoropropyl isopropyl ester (PFPA) derivatives. By Marfey analysis, the retention times of the derivatized residues in the hydrolysate of **1** matched those of L-Ala, L-Val and L-Phe. However, the retention times for the L-Ile and L-*allo*-Ile standards were identical, as were those of the D-Ile and D-*allo*-Ile standards. Thus, we could only conclude that the isoleucine residue of **1** was either of L- or L-*allo* configuration. Additionally, though Marfey analysis of the hydrolysate indicated the presence of at least one L-Pro residue, the underivatized Marfey reagent eluted coincidently with the D-Pro standard, precluding stereochemical analysis of the three proline residues of **1** by this method. Chiral GCMS was employed to resolve these final stereochemical issues. Analysis of the PFPA derivatized wewakazole hydrolysate identified the Ile and all three proline residues to be of L-configuration and confirmed the L-stereochemistry for the Ala, Val, and Phe moieties.

Isolation and structure elucidation of wewakazole further demonstrates the enormous potential of cyanobacteria, and especially *L. majuscula*, to produce novel secondary metabolites. While *L. majuscula* is known for its synthesis of

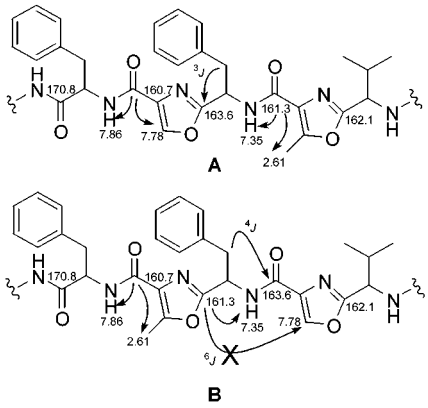


Figure 3. Possible orientations for the MeOxz-Phe-Oxz fragment in **1** based on either a (A) ^3J coupling or (B) a ^4J coupling from δ_H 3.29/3.17 to δ_C 163.6.

metabolites deriving from mixed PKS/NRPS origins, wewakazole represents an exceptional class of compound for this species, deriving solely from amino acids and comprised of residues atypical for this marine cyanobacterium. While a small number of oxazole-containing metabolites have been reported from terrestrial and freshwater cyanobacteria, the occurrence of oxazole and methyloxazole residues in **1** is exceptional for marine cyanobacterial chemistry.[7] Most significantly, the presence of six heterocyclic rings in wewakazole is without precedent in marine-derived cyclic peptides.

Acknowledgment. Financial support from the NIH (CA52955 and GM63554) is gratefully acknowledged. We thank R. T. Williamson for helpful discussions, B. Arbogast and the OSU EHSC for mass spectral data and the Government of Papua New Guinea for permission to make the cyanobacterial collection.

Supporting Information Available: Experimental data for **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) (a) Admi, V.; Afek, U.; Carmeli, S. *J. Nat. Prod.* **1996**, *59*, 396−399. (b) Ogino, J.; Moore, R. E.; Patterson, G. M.; Smith, C. D. *J. Nat. Prod.* **1996**, *59*, 581−586. (c) Nagatsu, A.; Kajitani, H.; Sakakibara, J. *Tetrahedron Lett.* **1995**, *36*, 4097−4100. (d) Ishida, K.; Nakagawa, H.; Murakami, M. *J. Nat. Prod.* **2000**, *63*, 1315−1317.