JOURNAL OF NATURAL PRODUCTS

Note

pubs.acs.org/jnp

Wewakazole B, a Cytotoxic Cyanobactin from the Cyanobacterium *Moorea producens* Collected in the Red Sea

Julius Adam V. Lopez,[†] Sultan S. Al-Lihaibi,[‡] Walied M. Alarif,[‡] Ahmed Abdel-Lateff,[§,⊥] Yasuyuki Nogata,[∥] Kenji Washio,[†,#] Masaaki Morikawa,[†,#] and Tatsufumi Okino*[,†,#]

[†]Graduate School of Environmental Science and [#]Faculty of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

[‡]Department of Marine Chemistry, Faculty of Marine Sciences, King Abdulaziz University, P.O. Box 80207, Jeddah 21589, Saudi Arabia

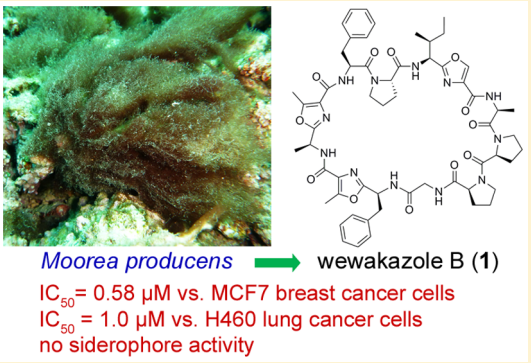
[§]Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University, P.O. Box 80260, Jeddah 21589, Saudi Arabia

[⊥]Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia 61519, Egypt

[∥]Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, Abiko 270-1194, Japan

Supporting Information

ABSTRACT: A mass spectrometry (MS)-guided isolation has led to the purification of a new cyanobactin, wewakazole B (**1**), along with the known compound curacin D from a Red Sea *Moorea producens*. The planar structure of **1** was elucidated using a combination of NMR and MS techniques. After ozonolysis and acid hydrolysis, the absolute configurations of the amino acid components of **1** were determined by chiral-phase LC-MS and HPLC analyses. Notably, compound **1** exhibited cytotoxic activity toward human MCF7 breast cancer cells (IC_{50} = 0.58 μM) and human H460 lung cancer cells (IC_{50} = 1.0 μM) and was also found to be inactive in a siderophore assay.



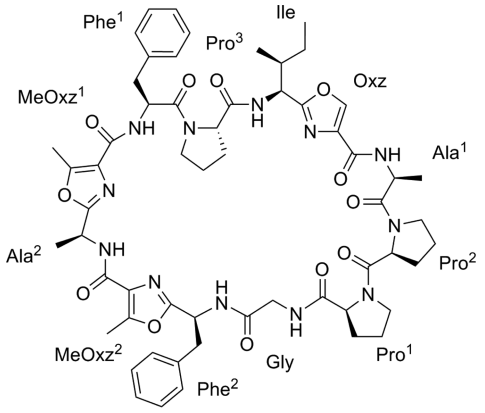
Cyanobacteria are a rich source of novel bioactive compounds.[1] In particular, *Moorea producens* (formerly *Lyngbya majuscula*)[2] strains have been reported to produce a wide variety of significant secondary metabolites, such as antillatoxins, apratoxins, curacins, jamaicamides, kalkitoxin, and lyngbyabellins.[3] In addition, a few cyanobactins have been reported from *M. producens* such as dolastatin 3, homodolastatin 3, kororamide, and wewakazole.[4,5] Cyanobactins are ribosomally synthesized and post-translationally modified peptides (RiPPs), which are produced by the post-ribosomal peptide synthesis (PRPS) pathway.[6] These peptides are solely derived from proteinogenic amino acids and are commonly formed as N−C macrocyclic systems, although highly modified short linear cyanobactins have also been reported.[7] Peptides belonging to this class are usually 6−20 amino acids in length, with many containing heterocyclized Cys, Ser, and Thr residues, whereas some contain prenylated Ser, Thr, or Tyr residues.[6,7]

The ability of *M. producens* to use multiple biosynthetic pathways to produce distinct natural products depending on its environment has enabled this cyanobacterium to maintain its status as a potential source of novel compounds.[8] With this in mind, and as part of our ongoing interest in the isolation of new cytotoxic and protease-inhibiting compounds from natural sources, we recently collected and screened samples of *M. producens* from the Red Sea. Herein, we report the isolation of a new cytotoxic cyanobactin, wewakazole B (**1**), as well as the known cytotoxic compound curacin D[9] from a sample of *M. producens* collected from the Red Sea.

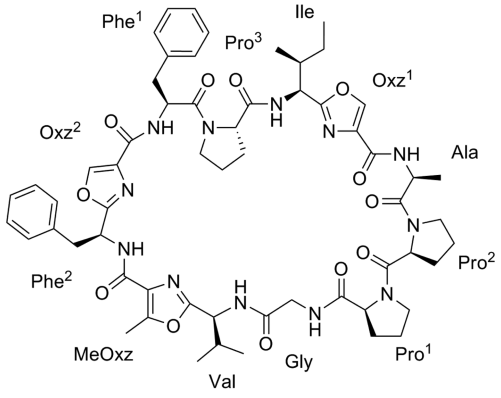
A brownish-red filamentous cyanobacterium (Figure S1) was obtained from the Red Sea near Jeddah, Saudi Arabia. This sample, which was labeled as S1301, was subsequently identified as *M. producens* based on its tuft-forming morphology[2] and a phylogenetic analysis using 16S rRNA gene sequencing (GenBank KT380828). A phylogenetic tree (Figure S2) was constructed and revealed that *M. producens* S1301 belonged to the *Moorea* spp. clade. The highest sequence homology for this material was found with the Papua New Guinean *M. producens* PNG6-9[2] (99.9%), as opposed to the apratoxin-producing Red Sea *M. producens* RS05[10] (99.3%). It is noteworthy that no masses corresponding to apratoxins or halogenated compounds were detected by LC-MS analysis. This chemical variation in *M. producens* could be attributed to several influencing factors, such as environmental conditions,

Received: January 20, 2016
Published: March 16, 2016

DOI: 10.1021/acs.jnatprod.6b00051
J. Nat. Prod. 2016, 79, 1213−1218



wewakazole B (**1**)



wewakazole (**2**)

high rates of evolution in the biosynthetic genes of *M. producens*, and horizontal gene transfer.[8]

The sample was extracted with MeOH and partitioned between EtOAc and H_2O. Initial screening showed that the EtOAc extract exhibited cytotoxic activity toward MCF7 cancer cells, while being inactive against trypsin. The EtOAc extract was therefore subjected to LC-MS profiling and silica gel column chromatography to allow for the separation of its many constituents. The fraction eluting with a 90:10 (v/v) mixture of hexane and EtOAc exhibited cytotoxicity at 10 μg/mL. Furthermore, HRMS analysis of this fraction revealed a mass ion with an m/z value of 360.2354 for $[M + H]^+$ as the major ion. This mass datum was used in conjunction with the taxonomy data described above to conduct a search of the MarinLit database,[11] which returned curacin D as the best candidate compound. Indeed, curacin D was subsequently isolated from this fraction by HPLC purification and verified by HRMS and ^{1}H NMR analysis. The fraction eluting with a 75:25 (v/v) mixture of EtOAc and MeOH also exhibited cytotoxicity, and HRMS analysis of this fraction revealed a mass ion with an m/z value of 1127.5310 for $[M + H]^+$ as a potential new compound based on a MarinLit search. The purification of this fraction by HPLC focused on this mass ion and resulted in the isolation of a new compound, wewakazole B (**1**). It is noteworthy that we were able to obtain only a small amount (0.4 mg) of **1** in this way, and it was therefore necessary to perform a reisolation procedure involving the combination of several samples with identical chemical profiles. This reisolation effort afforded an additional 0.2 mg of **1**.

The planar structure of **1** was elucidated using a combination of NMR and MS analyses. The molecular formula of **1** was determined to be $C_{58}H_{70}N_{12}O_{12}$ by ESI-TOF-MS, suggesting 30 degrees of unsaturation. The ^{1}H NMR spectrum (Table 1) revealed characteristic amide and phenyl signals (δ_H > 7.0), as well as several α-H signals (δ_H 3–6) and three sharp singlets (δ_H 2.41, 2.53, 8.02). These mass and NMR data were used to conduct a compound search of the MarinLit database. This search revealed several similarities to wewakazole (**2**),[5] and the sharp singlet peaks were subsequently identified as the methyl and methine protons of two methyloxazoles (MeOxz) and a single oxazole (Oxz), respectively. Multiplicity-edited HSQC data revealed the presence of eight α-methines, two sets of phenyl methines, six methyl groups, 13 methylenes, and a single methine. A comparison of the HSQC data for **1** with that of **2** revealed that the methine correlation (δ_C 140.6, δ_H 8.02) in **1** was missing. However, subsequent HMBC analysis revealed a correlation ($^1J_{C,H}$ = 210 Hz), as shown in Table 1. This value is characteristic of oxazole (theoretical $^1J_{C,H}$ = 209.1 Hz).[12] Taken together, these COSY, TOCSY, and HMBC data (Table 1) revealed an assembly consisting of nine common and three modified amino acid residues, including one glycine (Gly), one isoleucine (Ile), two alanines (Ala), two phenylalanines (Phe), three prolines (Pro), one oxazole (Oxz), and two methyloxazoles (MeOxz). A comparison of the amino acid compositions of **1** and **2** revealed that the latter differed from **1** by having an Oxz in place of a MeOxz and a Val rather than a second Ala.

The peptide sequence (Figure 1) of **1** was determined using HMBC (optimized at 4 and 8 Hz) and ROESY correlations, as well as the MS/MS fragmentation pattern (Figure 2). H-18 showed ^2J and ^3J correlations to C-17 and C-19, respectively, forming the Oxz ring. The H-20α proton of the Ile residue was correlated to C-19, whereas NH^{Ile} was correlated to C-25 of the Pro^3 residue to form the -Pro^3-Ile-Oxz- partial structure. On the basis of the ROESY correlations of NH^{Ile} and H-26 to H-31 of the Phe^1 residue, fragment A was established as -Phe^1-Pro^3-Ile-Oxz-. The structure of the initial -Phe^2-Gly-Pro^1- portion of fragment B was determined based on the following HMBC data: NH^{Phe2} to C-1, H-2a to C-1, H-2b to C-3, and H-5 to C-3. Although HMBC analysis revealed that there were no correlations to C-8, a ROESY correlation was observed from H-7 to the H-9α proton, indicating that the Pro^1 residue was connected to the Pro^2 residue. Furthermore, ROESY correlations from H-10 and H-12 to H-15 indicated that the Pro^2 residue was connected to the Ala^1 residue, with a further ROESY correlation from H-4 to H-14 completing the -Phe^2-Gly-Pro^1-Pro^2-Ala^1- fragment. Connections to the Oxz (fragment A) and MeOxz (fragments C and E) moieties were difficult to establish because of the lack of HMBC data pertaining to their correlations with neighboring amino acids, as previously reported.[5] Despite these difficulties, the cyclic structure depicted in Figure 1 was determined to be the most plausible of all of the possible combinations of fragments A–E based on the NMR and MS/MS data (Figure 2). It is noteworthy, however, that the chemical shifts of fragments C and E may be exchanged without affecting the sequence.

The proposed cyclic structure of **1** was in agreement with the MS/MS data (Figure S3) and the 30 degrees of unsaturation. The most abundant ion observed using CID had an m/z value of 1099, which was consistent with the loss of CO (28 Da). This result was also in agreement with the fragmentation pathway reported for cyclic peptides by Paizs and Suhai.[13] In the absence of a free amino terminus, protonated cyclic peptides can fragment via the loss of CO ($b_x \rightarrow a_x$), resulting in the production of sequence ions ($b_x \rightarrow b_{x-1}$ or via a $b_x - y_z$

DOI: 10.1021/acs.jnatprod.6b00051
J. Nat. Prod. 2016, 79, 1213–1218

Table 1. NMR Spectroscopic Data for 1 in $CDCl_3$

unit	position	δ_C,[a] type	δ_H (J in Hz)[d]	COSY[d]/ TOCSY[d]	HMBC[e]
Gly	1	168.6,[b] C			
	2a	43.0, CH_2	4.36, dd (18.0, 8.4)	2b, NH[1]	1
	2b		3.46, m	2a, NH[1]	1, 3
	NH[1]		6.86, m		
Pro[1]	3	172.6, C			
	4	61.0, CH	3.71, m	5a, 5b	
	5a	28.0, CH_2	1.60[f]	5b, 4, 6	3
	5b		0.79, m	5a, 4, 6	3
	6a	24.5, CH_2	1.94, m	6b, 5, 7	
	6b		1.19, m	6a, 5, 7	
	7	47.1,[b] CH_2	3.46, m	6a, 6b	
Pro[2]	8	171.2, C			
	9	59.0, CH	4.59, t (7.8)	10a, 10b	
	10a	27.5, CH_2	2.31, m	9, 10b	
	10b		1.75[f]	9, 10a, 11	
	11	26.1, CH_2	1.85, m	10b, 12a, 12b	
	12a	47.3, CH_2	3.72, m	11	
	12b		3.38, m	11	
Ala[1]	13	170.3, C			
	14	46.2, CH	4.91, m	15, NH[2]	13
	15	18.8, CH_3	1.74[f]	14	13, 14
	NH[2]		7.98, d (8.4)	14	13, 16
Oxz	16	159.7, C			
	17	135.7, C			
	18	140.6, CH	8.02, s		$^1J_{CH}$ = 210 Hz, 17, 19
Ile	19	164.5, C			
	20	52.8, CH	5.04, dd (9.6, 6.6)	21, NH[3]	19
	21	37.4, CH	2.29, m	20, 22b, 24	
	22a	25.5, CH_2	1.47, m	22b, 23	
	22b		1.35, m	21, 22a, 23	
	23	11.1, CH_3	0.89, t (7.2)	22a, 22b	
	24	15.7, CH_3	0.93, d (6.6)	21	
	NH[3]		9.42, d (9.6)	20	25
Pro[3]	25	171.6, C			
	26	60.8,[b] CH	3.33, d (7.8)	27b	25
	27a	31.0, CH_2	2.11, m	27b, 28b	
	27b		0.89[f]	27a, 28a, 28b	25
	28a	22.2, CH_2	1.67[f]	27a, 27b, 28b, 29	
	28b		1.49, m	28a	
	29	46.7, CH_2	3.47, m	28a, 28b	
Phe[1]	30	171.0, C			
	31	55.1, CH	4.71, m	32, NH[4]	
	32a	37.2, CH_2	3.78, dd (12.0, 4.2)	31, 32b	33, 34/38
	32b		3.05, t (12.0)	31, 32a, NH[4]	30
	33	136.8,[b] C			
	34/38	130.1, CH	7.55, d (7.2)	35/37	35/37
	35/37	127.9, CH	7.34, t (7.2)	34/38, 36	33, 36
	36	129.2, CH	7.30, d (7.2)	35/37	35/37
	NH[4]		7.69, bs	31, 32b	
MeOxz[1g]	39	161.5,[c] C			
	40	128.9, C			
	41	154.0, C			
	42	11.7, CH_3	2.53, s		39, 40, 41
Ala[2]	43	161.3, C			
	44	42.9, CH	5.38, m	45, NH[5]	43
	45	21.1, CH_3	1.59[f]	44	43, 44
	NH[5]		7.44, d (8.4)	44	43
MeOxz[2g]	46	161.5,[c] C			
	47	128.1,[b] C			
	48	154.5, C			
	49	11.6, CH_3	2.41, s		46, 47, 48
Phe[2]	50	161.6, C			
	51	47.9, CH	5.33, m	52, NH[6]	50
	52a	38.7, CH_2	3.41, m	51, 52b, NH[6]	53, 54/58
	52b		3.11, dd (13.5, 6.0)	51, 52a, NH[6]	50, 53, 54/58
	53	136.6, C			
	54/58	129.0,[b] CH	7.08, d (6.6)	55/57	55/57, 56
	55/57	128.7, CH	7.24, d (6.6)	54/58, 56	53
	56	127.1, CH	7.21, d (6.6)	55/57	54/58
	NH[6]		7.74, d (8.4)	51, 52	1

[a]125 MHz. [b]600 MHz Jeol NMR spectrometer; optimized for a coupling of 8 Hz. [c]600 MHz Jeol NMR spectrometer; optimized for a coupling of 4 Hz. [d]600 MHz Jeol NMR spectrometer. [e]600 MHz Jeol NMR spectrometer; combined data from the optimized coupling data at 4 and 8 Hz; signals from the TOCSY experiments (underlined). [f]Signal overlap. [g]Interchangeable signals.

pathway).[13] Three sets of b/a fragment ions were observed corresponding to the three initial fragmentation sites at the N-terminus, including the Ala[1], Pro[2], and Pro[3] residues (Figures 2, S3, and S4). The Pro[2] and Pro[3] series observed in the current study were anticipated based on the proline effect.[13] The Ala[1] and Pro[2] series supported the sequence of fragment B, whereas the Pro[3] series supported the sequence from fragment A through B. Finally, the absolute configuration of **1** was determined by chiral-phase LC-MS and HPLC analyses. A comparison of the masses and retention times with those of several authentic amino acids by chiral-phase LC-MS revealed that the Ala, Phe, and Pro residues were in the L-configuration. However, this technique failed to distinguish between L-Ile and L-*allo*-Ile; therefore, a different chiral-phase column was required to separate them in conjunction with a coelution experiment (Figure S5). This technique clearly identified that **1** contained L-Ile.

Wewakazole B (**1**) and wewakazole (**2**)[5] are the only compounds reported to date from *M. producens* to contain both Oxz and MeOxz. In fact, the occurrence of either Oxz or

DOI: 10.1021/acs.jnatprod.6b00051
J. Nat. Prod. 2016, 79, 1213−1218

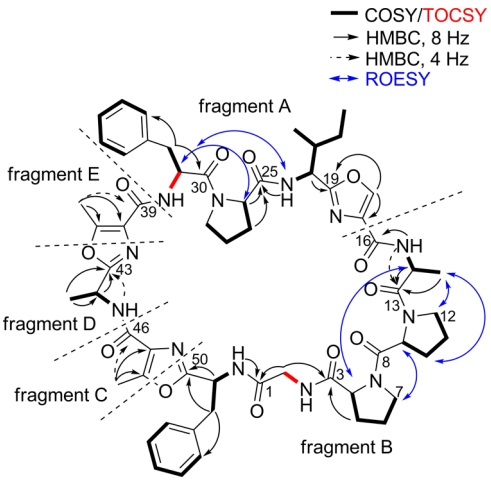


Figure 1. Key 2D NMR correlations for the partial structures of compound **1** (fragments A–E).

MeOxz in *M. producens* is very rare, whereas thiazole-containing compounds are much more common.[14] On the basis of their structures, wewakazoles most likely belong to the cyanobactin class of compounds, which have been reported to show a variety of bioactivities. The most distinct activity associated with cyanobactins is their cytotoxicity.[15] Furthermore, wewakazole B (**1**) was found to be more cytotoxic (IC_{50} = 1.0 μM) against human H460 lung cancer cells than wewakazole (**2**) (IC_{50} = 10 μM).[16] It also showed moderate cytotoxicity (IC_{50} = 0.58 μM) against human MCF7 breast cancer cells.

Cyanobactins have also been reported to exhibit transition-metal-binding activities.[6,15] Furthermore, the results of a computational analysis predicted that wewakazole (**2**) would have iron-binding activity.[17] These results and the possibility that **1** and **2** may have the same conformation because of the close similarity of their structures and NMR chemical shifts prompted us to test **1** in a CAS assay. However, the results of this analysis revealed that **1** exhibited no metal-binding activity at 89 μM, which indicated that there could be a relationship between the iron-binding activity of these compounds and their structure. Alternatively, this result may suggest that wewakazole (**2**) is not really a siderophore, but this could only be confirmed by subjecting it to the CAS assay.

Considering the many advantages associated with cyclic peptides, including their diverse functionality, high membrane permeability, ability to resist enzymatic degradation, and high bioavailability, wewakazoles represent good candidates for the design of anticancer drugs.[15,18] However, in a similar manner to other cyanobactins, the isolation of sufficient quantities of these compounds to allow for their efficient biological evaluation remains challenging. It is therefore envisioned that total synthesis or synthetic biology could be used to provide efficient access to these compounds.[15] In terms of the latter of these two options, the gene cluster responsible for the biosynthesis of cyanobactins belonging to the genus *Moorea* has not yet been fully elucidated.[19] Despite the lack of information pertaining to the biosynthesis of these compounds, the discovery of wewakazole B (**1**) represents a significant addition to our overall knowledge of bioactive cyanobactins and further highlights the versatility of *M. producens* in terms of its ability to produce exciting natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. UV and ECD spectrophotometric data were acquired on a JASCO J-720 spectropolarimeter. ^{1}H and 2D NMR spectra were recorded on a JEOL ECA600 spectrometer at 600.17 MHz, while ^{13}C NMR data were acquired using a Bruker AMX-500 spectrometer at 125.77 MHz with a 2.5 mm probe. $CDCl_3$ (D, 99.96%) from Cambridge Isotope Laboratories, Inc. was used as the solvent and internal standard (δ_H 7.24 and δ_C 77.23) for all of the NMR experiments. LC-MS analyses were conducted on an Agilent 1100 Series HPLC system coupled with a Bruker Daltonics micrOTOF-HS mass spectrometer (ESI). The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 μm, 25 °C, 0.2 mL/min) under the following conditions: 0–15 min, gradient elution of 50–80% MeCN with 0.1% (v/v) formic acid in H_2O; 15–30 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in H_2O. MS/MS fragmentation data were acquired using Thermo Scientific LTQ Orbitrap XL (ESI) and AB Sciex TOF/TOF 5800 (MALDI) instruments. The compounds were isolated on a JASCO HPLC system equipped with a PU-980 pump and a UV-970 UV/vis detector.

Biological Material. The Red Sea cyanobacterial sample S1301 was collected using scuba at a depth of 5–10 m near Jeddah, Saudi Arabia (21°41′22.01″ N; 39°00′50.90″ E), in December 2013. Foreign particles were removed by hand, and seawater was squeezed out from the samples before storing in MeOH for transportation. A small portion of this material was also preserved in 10 mL of RNAlater (Ambion) solution for genetic analysis. The sample had the characteristic tuft-forming morphology of *Moorea producens* and shared 99.9% sequence homology with *M. producens* PNG6-9[2] from Papua New Guinea. The 16S rRNA gene sequence (GenBank KT380828) of this material is currently available online. A voucher specimen (S1301) preserved in RNAlater has been deposited at Hokkaido University, Japan.

Gene Sequencing. The details of these procedures are reported in the Supporting Information.

Extraction and Isolation. The sample was homogenized and extracted three times with MeOH. The MeOH extract (1 g) was dried

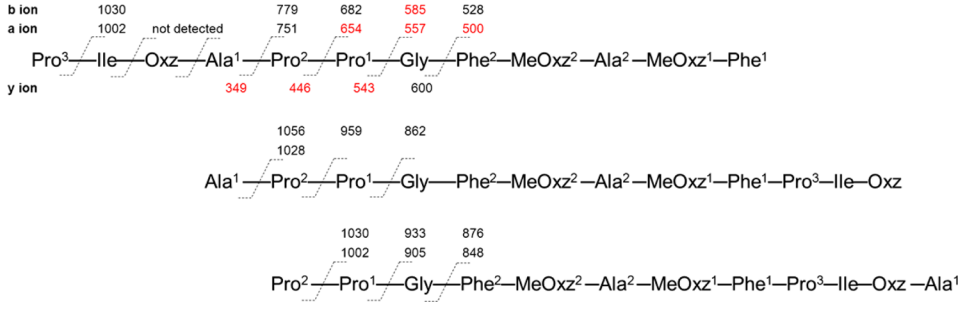


Figure 2. Key MS/MS data for compound **1**, which were obtained using LTQ Orbitrap XL and MALDI TOF/TOF systems. The mass data present in red were observed only on the MALDI TOF/TOF system.

DOI: 10.1021/acs.jnatprod.6b00051
J. Nat. Prod. 2016, 79, 1213–1218

in vacuo and was partitioned between EtOAc and H_2O. The EtOAc and H_2O extracts were then concentrated *in vacuo* and were subjected to trypsin inhibition and cytotoxicity assays, as well as being profiled by LC-MS analysis. The EtOAc fraction (346 mg) was found to be cytotoxic and was subsequently fractionated by column chromatography over silica gel eluting with a stepwise gradient composed of hexane and EtOAc (95:5, 90:10, 75:25, 50:50, 25:75 v/v), followed by EtOAc and MeOH (100:0, 75:25, 50:50, 25:75, 100 v/v). The 90:10 hexane/EtOAc fraction (25 mg) contained the cytotoxic compound curacin D by LC-MS analysis, but apparent degradation prevented its isolation. The fraction (105 mg) that eluted with a 75:25 (v/v) mixture of EtOAc and MeOH was also cytotoxic. Subsequent LC-MS analysis of this fraction revealed a distinct peak with m/z values of 1127 and 564, which were attributed to $[M + H]^+$ and $[M + 2H]^{2+}$ ions. Focusing on these ions, an MS-guided isolation was performed using semipreparative RP HPLC (Cosmosil C_{18}-AR, 10 × 250 mm, 5 μm, 3 mL/min, UV detection at 210 nm, gradient: 0−20 min 30−80% MeCN, 20−40 min 80% MeCN). The fraction that eluted at 18 min was further purified by RP HPLC (Cosmosil C_{18}-MS, 4.6 × 250 mm, 5 μm, 1 mL/min, UV detection at 210 nm, gradient: 0−20 min 50−70% MeCN) to yield pure wewakazole B (**1**, 0.4 mg, t_R 9.4 min). We subsequently combined four other samples that had similar chemical profiles and purified the combined material using the MS-guided isolation method mentioned above. This method provided access to an additional 0.2 mg of **1**. This process also resulted in the successful isolation of curacin D (0.4 mg, t_R 25.0 min) by subjecting the 90:10 hexane/EtOAc fraction (13 mg) from the combined samples to RP HPLC (Cosmosil C_{18}-MS, 4.6 × 250 mm, 5 μm, 1 mL/min, UV detection at 224 nm, gradient: 0−20 min 50−100% MeCN, 20−30 min 100% MeCN).

*Wewakazole B (**1**):* light yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 276 (4.25), 312 (4.26) nm; ECD (c 1.18 mM, MeOH), λ_{max} ($\Delta\varepsilon$) 241 (−0.0717), 205 (−0.564), 202 (−0.561) nm (Figure S15); ^1H and ^{13}C NMR data, see Table 1; ESITOFMS m/z 1127.5310 $[M + H]^+$ (calcd for $C_{58}H_{71}N_{12}O_{12}$, 1127.5314).

Absolute Configuration. Ozonolysis (O_3, CH_2Cl_2, 5 min) was performed using approximately 0.1 mg of **1**. The product was evaporated to dryness and hydrolyzed (6 N HCl, 110 °C for 15 h). The hydrolysate was dried *in vacuo* and reconstituted in H_2O (500 μL), and the resulting aqueous solution was analyzed by LC-MS (Supelco Chirobiotic TAG 2.1 × 250 mm, 0.3 mL/min, 25 °C, 1:1 (v/v) mixture of 0.1% aqueous formic acid and 1% (w/v) NH_4OAc in MeOH). The masses and retention times of the standard amino acids (m/z, L-, D-) were as follows: Ala (m/z 90, 4.1 min, 5.8 min), Pro (m/z 116, 6.8 min, 17.1 min), and Phe (m/z 166, 5.2 min, 7.0 min). The hydrolysate gave the following results: 4.2 min (m/z 90), 6.8 min (m/z 116), and 5.2 min (m/z 166), corresponding to L-Ala, L-Pro, and L-Phe, respectively. However, this method failed to successfully separate the stereoisomers of Ile (L-*allo*-, L-, D-*allo*-, D-). This issue was therefore resolved by chiral-phase HPLC[20] (Sumichiral OA-5000 column, 4.6 × 150 mm, 1 mL/min, 40 °C, UV detection at 254 nm, isocratic elution, 5:95 (v/v) mixture of MeOH and 2.0 mM $CuSO_4$ in H_2O) with the following retention times: L-*allo*-, 18.27 min; L-, 21.75 min; D-*allo*-, 30.00 min; and D-, 37.61 min. The subsequent coelution of the hydrolysate (Figure S5) with the standards clearly showed that L-Ile (21.70 min) was the true configuration.

Cytotoxicity Assay. A standard MTT assay was performed based on the method reported by Mosmann.[21] Briefly, MCF7 breast cancer cells (Culture Collections, Public Health England) and H460 lung cancer cells (ATCC HTB-177) were maintained in RPMI-1640 medium (Wako) with 10% fetal bovine serum (FBS) (BioWest). Cancer cells were seeded into 96-well plates at a density of 1.0×10^4 cells per well. After 24 h at 37 °C with 5% CO_2, the cells were treated with sample solutions containing different concentrations of the test material and incubated for 72 h under the same conditions. The medium was removed and replaced with 100 μL of MTT in RPMI-1640 with 10% FBS (0.5 mg/mL), and the cells were then incubated for 3 h at 37 °C with 5% CO_2. The MTT solution was aspirated, and the formazan crystals were dissolved in DMSO. After 10 min of incubation, the optical density at 570 nm was measured using a Thermo Labsystems Multiskan JX plate reader. Cisplatin (Sigma-Aldrich) was used as the positive control, with IC_{50} values of 8.4 and 9.0 μM against MCF7 and H460 cancer cells, respectively.

Trypsin Inhibition Assay. Trypsin inhibition was assessed as previously described.[22]

Siderophore Assay. The CAS assay was conducted as previously reported in the literature[23] with the following modification. The CAS shuttle solution was loaded in a 96-well plate, followed by the samples and controls, which were added to give concentrations of 10 and 100 μg/mL at a final volume of 50 μL. After 45 min of equilibration, the absorbance at 630 nm was measured using a Thermo Labsystems Multiskan JX plate reader. Deferoxamine (Sigma-Aldrich) was used as the positive control (IC_{50} = 5.1 μM).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00051.

Figures S1−S15; details of 16S rRNA gene sequencing and phylogenetic analysis experiments; 1D/2D NMR and MS/MS spectra for wewakazole B (**1**) together with the chiral-phase HPLC chromatograms; ECD spectrum and cytotoxicity dose−response curve data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81 11 706 4519. Fax: +81 11 706 4867. E-mail: okino@ees.hokudai.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was conducted as part of the National Science, Technology and Innovation Plan (MAARIFAH)−the King Abdulaziz City for Science and Technology of the Kingdom of Saudi Arabia (award number 11-ENV1540-03). We thank Dr. K. Kaneko for his guidance on the DNA experiments. We also thank Dr. E. Fukushi (GC-MS and NMR Laboratory) and Dr. Y. Kumaki (High-Resolution NMR Laboratory), Hokkaido University, for the NMR analyses. We are grateful to Dr. M. Kurasaki of the Faculty of Environmental Earth Science, Hokkaido University, for providing access to their cell culture facilities. We also thank Dr. M. Kita of Tsukuba University for his advice concerning our MS/MS experiments and Dr. T. Yoshizawa of Hokkaido University for the use of the MALDI TOF/TOF instrument.

■ REFERENCES

(1) Nunnery, J. K.; Mevers, E.; Gerwick, W. H. *Curr. Opin. Biotechnol.* **2010**, *21*, 787−793.

(2) Engene, N.; Rottacker, E. C.; Kastovsky, J.; Byrum, T.; Choi, H.; Ellisman, M. H.; Komarek, J.; Gerwick, W. H. *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 1171−1178.

(3) Ramaswamy, A. V.; Flatt, P. M.; Edwards, D. J.; Simmons, T. L.; Han, B.; Gerwick, W. H. In *Frontiers in Marine Biotechnology*; Proksch, P.; Muller, E. G., Eds.; Horizon Bioscience: Norwich, U.K., 2006; pp 175−224.

(4) Mitchell, S. S.; Faulkner, D. J.; Rubins, K.; Bushman, F. D. *J. Nat. Prod.* **2000**, *63*, 279−282.

(5) Nogle, L. M.; Marquez, B. L.; Gerwick, W. H. *Org. Lett.* **2003**, *5*, 3−6.

(6) Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.;

DOI: 10.1021/acs.jnatprod.6b00051
J. Nat. Prod. 2016, 79, 1213−1218

Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio, S.; Dorrestein, P. C.; Entian, K.-D.; Fischbach, M. A.; Garavelli, J. S.; Göransson, U.; Gruber, C. W.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, M.; Kelly, W. L.; Klinman, J. P.; Kuipers, O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Müller, R.; Nair, S. K.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J. T.; Rebuffat, S.; Ross, R. P.; Sahl, H.-G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Süssmuth, R. D.; Tagg, J. R.; Tang, G.-L.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel, S. C.; Willey, J. M.; van der Donk, W. A. *Nat. Prod. Rep.* **2013**, *30*, 108−160.

(7) Leikoski, N.; Liu, L.; Jokela, J.; Wahlsten, M.; Gugger, M.; Calteau, A.; Permi, P.; Kerfeld, C. A.; Sivonen, K.; Fewer, D. P. *Chem. Biol.* **2013**, *20*, 1033−1043.

(8) Thacker, R. W.; Paul, V. J. *Appl. Environ. Microbiol.* **2004**, *70*, 3305−3312.

(9) Márquez, B.; Verdier-Pinard, P.; Hamel, E.; Gerwick, W. H. *Phytochemistry* **1998**, *49*, 2387−2389.

(10) Thornburg, C. C.; Cowley, E. S.; Sikorska, J.; Shaala, L. A.; Ishmael, J. E.; Youssef, D. T. A.; McPhail, K. L. *J. Nat. Prod.* **2013**, *76*, 1781−1788.

(11) MarinLit. URL http://www.pubs.rsc.org/marinlit/ (accessed Aug 24, 2015).

(12) Kalinowski, H.; Berger, S.; Braun, S. *Carbon-13 NMR Spectroscopy*; John Wiley & Sons: Great Britain, 1988; p 506.

(13) Paizs, B.; Suhai, S. *Mass Spectrom. Rev.* **2005**, *24*, 508−548.

(14) Han, B.; McPhail, K. L.; Gross, H.; Goeger, D. E.; Mooberry, S. L.; Gerwick, W. H. *Tetrahedron* **2005**, *61*, 11723−11729.

(15) Martins, J.; Vasconcelos, V. *Mar. Drugs* **2015**, *13*, 6910−6946.

(16) Malloy, K. L.; Villa, F. A.; Engene, N.; Matainaho, T.; Gerwick, L.; Gerwick, W. H. *J. Nat. Prod.* **2011**, *74*, 95−98.

(17) Manning, T. J.; Thomas, J.; Osiro, S.; Smith, J.; Abadi, G.; Noble, L.; Phillips, D. *Nat. Prod. Res.* **2008**, *22*, 399−413.

(18) Driggers, E. M.; Hale, J.; Lee, J.; Terrent, N. K. *Nat. Rev. Drug Discovery* **2008**, *7*, 608−624.

(19) Donia, M. S.; Schmidt, E. W. *Chem. Biol.* **2011**, *18*, 508−519.

(20) Kisugi, T.; Okino, T. *J. Nat. Prod.* **2009**, *72*, 777−781.

(21) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.

(22) Shin, H. J.; Murakami, M.; Matsuda, H.; Yamaguchi, K. *Tetrahedron* **1996**, *52*, 8159−8168.

(23) Schwyn, B.; Neilands, J. B. *Anal. Biochem.* **1987**, *160*, 47−56.

DOI: 10.1021/acs.jnatprod.6b00051
J. Nat. Prod. 2016, 79, 1213−1218