JOURNAL OF NATURAL PRODUCTS

Article

Cite This: J. Nat. Prod. XXXX, XXX, XXX-XXX

pubs.acs.org/jnp

Genomics-Driven Discovery of Chlorinated Cyclic Hexapeptides Ulleungmycins A and B from a *Streptomyces* Species

Sangkeun Son,[†,‡] Young-Soo Hong,[†,‡] Mina Jang,[†,‡] Kyung Taek Heo,[†,‡] Byeongsan Lee,[†] Jun-Pil Jang,[†] Jong-Won Kim,[†] In-Ja Ryoo,[†] Won-Gon Kim,[‡,§] Sung-Kyun Ko,[†,‡] Bo Yeon Kim,[†,‡] Jae-Hyuk Jang,*[,†,‡] and Jong Seog Ahn*[,†,‡]

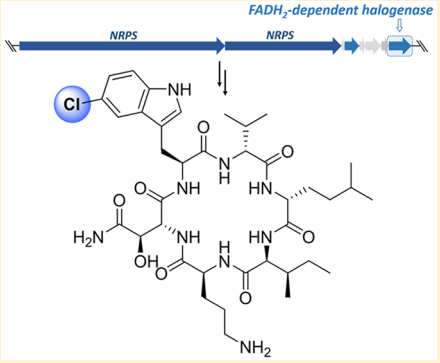
[†]Anticancer Agent Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju 28116, Korea

[‡]Department of Biomolecular Science, KRIBB School of Bioscience, Korea University of Science and Technology (UST), Daejeon 34141, Korea

[§]Superbacteria Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Korea

Supporting Information

ABSTRACT: Analysis of the genome sequence of *Streptomyces* sp. KCB13F003 showed the presence of a cryptic gene cluster encoding flavin-dependent halogenase and nonribosomal peptide synthetase. Pleiotropic approaches using multiple culture media followed by LC-MS-guided isolation and spectroscopic analysis enabled the identification of two new chlorinated cyclic hexapeptides, ulleungmycins A and B (**1** and **2**). Their structures, including absolute configurations, were determined by 1D and 2D NMR techniques, advanced Marfey's analysis, and GITC derivatization. The new peptides, featuring unusual amino acids 5-chloro-L-tryptophan and D-homoleucine, exhibited moderate antibacterial activities against Gram-positive pathogenic bacteria including methicillin-resistant and quinolone-resistant *Staphylococcus aureus*.



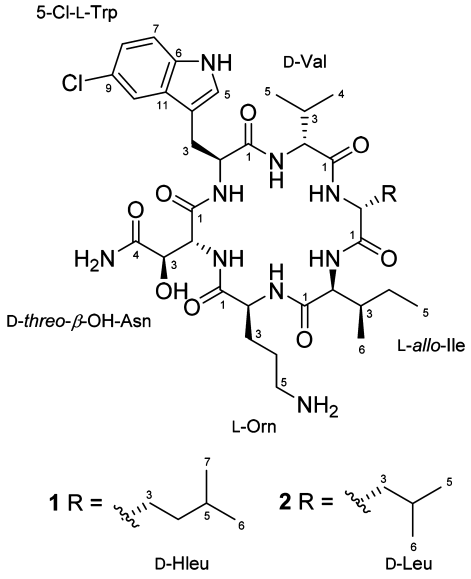
The genomics-driven discovery of novel bioactive natural products has been enabled by the reduced cost of genome sequencing and by advances in bioinformatics-based predictions of encoded compounds in biosynthetic gene clusters.[1] Nonribosomal peptides (NRPs), among the most structurally diverse and pharmaceutically important secondary metabolites, are synthesized by modular multifunctional nonribosomal peptide synthetases (NRPSs), and the core peptide structures are often subsequently modified via the action of tailoring enzymes including methyltransferases, glycosyltransferases, and halogenases, further increasing structural complexity.[2] Our earlier studies on new compounds found through LC-MS screening of bacterial culture extracts led to the discovery of new cyclic depsipeptides, ulleungamides A and B, featuring several novel residues from *Streptomyces* sp. KCB13F003.[3] To probe their biosynthetic mechanisms and further investigate the secondary metabolic potential of the strain, we turned our attention to analyzing the whole genome sequence and found that the strain harbors multiple putative biosynthetic gene clusters including polyketide synthases (natamycin), lantipeptide synthetases (planosporicin), and NRPSs (ulleungamide).[3,4] Significantly, the genome contains diverse NRPS gene clusters whose products were not detected in the culture extract, indicating that the strain could be a promising NRP producer. Among these genes, we focused on one NRPS gene cluster adjacent to the halogenase gene capable of encoding chlorinated hexapeptides, which are rarely reported natural products. The most frequently used strategies for activation of cryptic secondary metabolite gene clusters include alteration in growth conditions, heterologous expression of gene clusters, and genetic manipulation of the regulators.[5] In consideration of pleiotropic effects of growth conditions on gene expression, we screened different culture media with LC-MS analysis to detect the encoded compounds and consequently found conditions for the production of two chlorinated NRPs (**1** and **2**) whose structures are strongly correlated with the predicted domain organization of the gene cluster. Structurally, they were characterized as unusual NRPs containing 5-chloro-L-tryptophan (5-Cl-L-Trp) and D-homoleucine (D-Hleu). Here, we report the isolation, structural characterization, and biological activities as well as the biosynthetic analysis of these two new peptides.

RESULTS AND DISCUSSION

The whole genome of *Streptomyces* sp. KCB13F003 was sequenced and assembled into two contigs, with a total size of 9.27 Mb and an overall GC content of 71.4%. Analysis of the contigs with antiSMASH software enabled us to identify the putative halogenase gene (*ulm24*) alongside an NRPS cluster (GenBank accession number MF541667) designated *ulm* in this study (Figure 1).[6] The targeted halogenase was speculated

Received: July 31, 2017

DOI: 10.1021/acs.jnatprod.7b00660
J. Nat. Prod. XXXX, XXX, XXX−XXX



to be an $FADH_2$-dependent halogenase based on the presence of the absolutely conserved motifs (GxGxxG and GWxWxIP) (Figure S1). Since a flavin reductase gene (*ulm26*) was found very close to the halogenase gene, Ulm26 was suggested to be involved in the halogenation reaction.[7] A subsequent functional prediction with the NCBI protein−protein BLAST search of the targeted halogenase showed homology to many putative or functionally established L-tryptophan halogenases, including PrnA and PyrH.[8] The gene cluster was also found to harbor two genes involved in the NRPS assembly line consisting of a total of six modules, implying incorporation of six amino acids for an encoded compound. The peptide sequence was then predicted based on the ATP-dependent adenylation (A) domain specificities of every NRPS module using the NRPSpredictor software (Table S1).[9] In the case of the A domain motif (DVAMAGAV) in module 1, it did not show a clear conserved sequence for substrate prediction. However, the similar A domain motif (DVAMAGMV) was recently reported to recognize L-Trp during the biosynthesis of hexapeptide desotamides,[10] thus suggesting the activation of the Trp or Trp-associated residues by the A domain in module 1.

At this point, due to limited prediction of exact core peptides and tailoring reactions, we prioritized the structure determination of the encoded peptides by activating the gene cluster and performing spectroscopic analyses. As none of halogen- or tryptophan-containing compounds were detected in a previous culture using glycerol-based media, we then focused our efforts to induce the expression of the gene cluster by manipulating the culture conditions and LC-MS screening. As a result of these tests, it was found that when one of the media used for production of ulleungamides was diluted (2-fold dilution), the strain produced previously unrecognized compounds exhibiting the typical indole UV spectrum and the characteristic isotopic pattern for the presence of one chlorine atom (Figure S2). Two compounds, **1** and **2** (9.0 and 4.5 mg, respectively), were then purified from the extract of the scale-up culture and subjected to detailed structural analysis.

The molecular formula of ulleungmycin A (**1**) was established as $C_{38}H_{58}N_9O_8Cl$ by HRESIMS and NMR analyses. The peptidic nature of **1** was evident from the presence of the α-protons (δ_H 4.61−3.74), amide NH signals (δ_H 8.77−7.18), and amide carbonyl signals (δ_C 173.2−169.4) observed in the 1D NMR spectra. A combination of HSQC, DQF-COSY, and HMBC experiments was used to assign the amino acid residues in the peptide. The two signals at $\delta_{C/H}$ 60.6/3.74 and 56.7/4.19 appeared to be the α-methine groups for the two amino acids Val and Ile, which were assigned by the DQF-COSY and HMBC correlations (Figure 2). The presence of an unusual

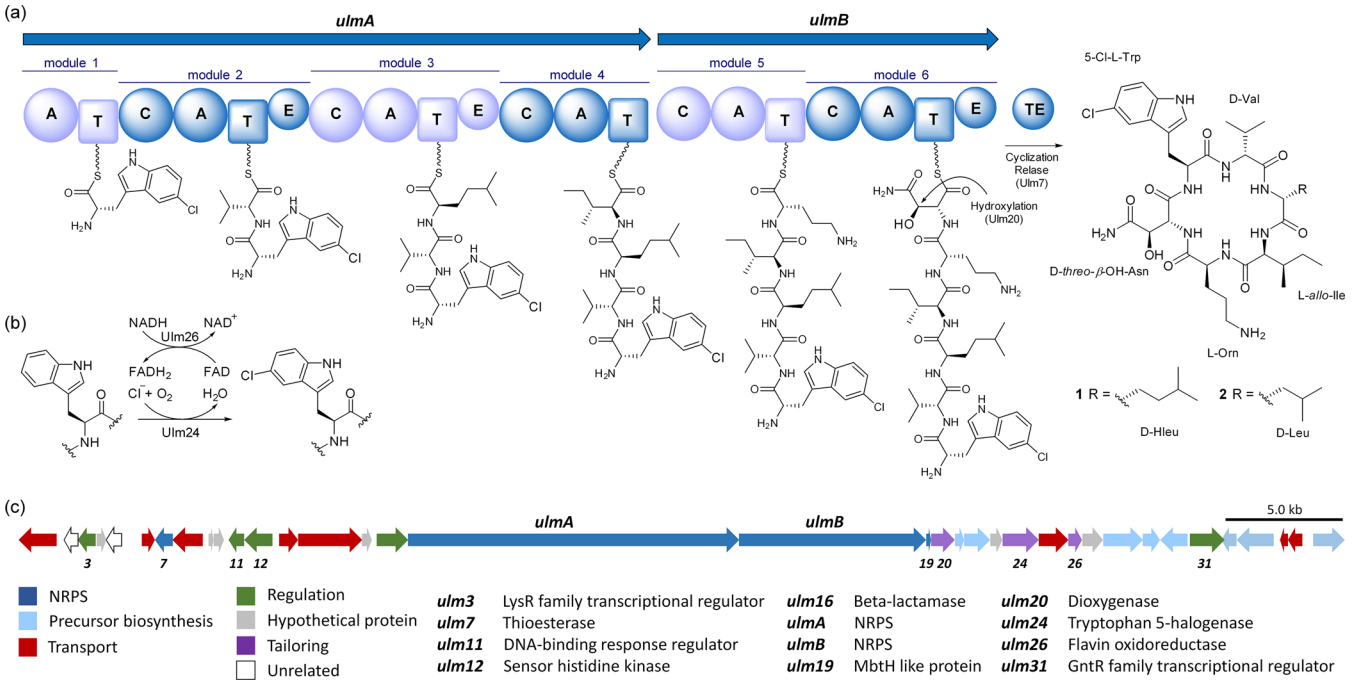


Figure 1. Analysis of the *ulm* gene cluster. (a) Proposed biosynthetic pathway for the ulleungmycins. (b) Chlorination of tryptophan by $FADH_2$-dependent halogenase (Ulm24) and flavin reductase (Ulm26). (c) Genetic organization of the *ulm* gene cluster. Genes are color-coded according to their proposed functions.

DOI: 10.1021/acs.jnatprod.7b00660
J. Nat. Prod. XXXX, XXX, XXX−XXX

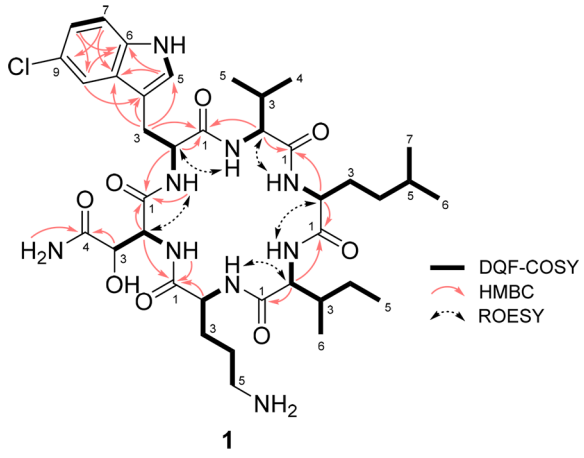


Figure 2. Key 2D NMR correlations used for determining the structure of **1**.

amino acid, 5-Cl-Trp, was revealed as follows. Three spin systems could be constructed by the DQF-COSY spectra analysis: 2-$NH_{Cl\text{-}Trp}$ to H_2-$3_{Cl\text{-}Trp}$; H-$5_{Cl\text{-}Trp}$ to 5-$NH_{Cl\text{-}Trp}$; and H-$7_{Cl\text{-}Trp}$ to H-$8_{Cl\text{-}Trp}$ (Figure 2). Key HMBC correlations from H_2-$3_{Cl\text{-}Trp}$ to the carbonyl C-$1_{Cl\text{-}Trp}$, sp^2 nonprotonated carbons C-$4_{Cl\text{-}Trp}$ and C-$11_{Cl\text{-}Trp}$, and sp^2 methine C-$5_{Cl\text{-}Trp}$, together with a characteristic chemical shift of 5-$NH_{Cl\text{-}Trp}$ (δ_H 11.1), suggested the presence of an indole ring. Strong $^3J_{C,H}$ HMBC correlations of the aromatic protons (H-5/C-6; H-7/C-9, C-11; H-8/C-6, C-10; H-10/C-4, C-6, C-8) clearly identified the indole moiety. The nonprotonated aromatic carbon C-$9_{Cl\text{-}Trp}$ (δ_C 122.9), which was confirmed by no correlation in the HSQC spectrum and *meta*-coupled H-$10_{Cl\text{-}Trp}$ (J = 1.7 Hz), was assigned as having a chlorine atom, establishing this residue as 5-Cl-Trp. On the other hand, the DQF-COSY correlations from the amide 2-NH_{Hleu} to the terminal *gem*-dimethyls H_3-6_{Hleu} and H_3-7_{Hleu} and the HMBC correlation of H-2_{Hleu} to the amide carbonyl C-1_{Hleu} confirmed the presence of the rare amino acid homoleucine (Hleu).

The additional spin system comprising 2-$NH_{OH\text{-}Asn}$, H-$2_{OH\text{-}Asn}$, and the oxygenated methine H-$3_{OH\text{-}Asn}$ (δ_H 4.41) was obtained from DQF-COSY correlations, indicating the oxygenated β-carbon. A β-OH-Asn residue was then identified through the HMBC correlations from H-$3_{OH\text{-}Asn}$ and two mutually coupled broad singlets 4-$NH_{2OH\text{-}Asn}$ to the carbonyl C-$4_{OH\text{-}Asn}$ (δ_C 173.2). The last proton sequence starting from 2-NH_{Orn} through the α-proton H-2_{Orn} and two methylenes H_2-3_{Orn} and H_2-4_{Orn} to the aminated methylene H_2-5_{Orn} (δ_H 2.71) established the ornithine residue, assigning a total of six amino acid residues. The amino acid sequence of β-OH-Asn/5-Cl-

Table 1. ^{13}C (225 MHz) and ^1H (900 MHz) NMR Spectroscopic Data for 1 and 2 in DMSO-d_6

		1		2	
	position	δ_C, type	δ_H, mult (J in Hz)	δ_C, type	δ_H, mult (J in Hz)
5-Cl-L-Trp	1	171.2, C		171.5, C	
	2	53.5, CH	4.61, dd (15.1, 7.7)	53.6, CH	4.61, dd (14.9, 8.0)
	3a	27.1, CH_2	3.11, dd (14.2, 7.9)	27.5, CH_2	3.09, dd (14.1, 8.3)
	3b		2.83, dd (14.2, 6.6)		2.83, dd (14.1, 6.3)
	4	109.9, C		109.9, C	
	5	125.5, CH	7.13, s	125.4, CH	7.13, ovl[a]
	6	134.5, C		134.5, C	
	7	112.7, CH	7.31, d (8.5)	112.7, CH	7.32, d (8.5)
	8	120.6, CH	7.03, dd (8.5, 1.9)	120.6, CH	7.03, dd (8.5, 1.9)
	9	122.9, C		122.9, C	
	10	117.5, CH	7.54, d (1.7)	117.5, CH	7.54, d (1.7)
	11	128.4, C		128.4, C	
	2-NH		7.90, ovl[a]		7.85, d (8.0)
	5-NH		11.1, s		11.08, s
D-Val	1	170.9, C		171.1, C	
	2	60.6, CH	3.74, t (7.7)	60.8, CH	3.70, t (7.4)
	3	29.1, CH	1.88, m	28.9, CH	1.89, m
	4	18.9, CH_3	0.67, d (6.8)	18.9, CH_3	0.66, d (6.8)
	5	18.5, CH_3	0.69, d (6.7)	18.6, CH_3	0.68, d (6.7)
	2-NH		8.7, br s		8.46, br s
D-Hleu	1	172.2, C		172.1, C	
	2	52.8, CH	4.25, m	51.2, CH	4.25, ovl[a]
	3a	28.7, CH_2	1.75, m	39.2, CH_2	1.59, m
	3b		1.61, m		1.54, m
	4a	34.2, CH_2	1.08, m	24.1, CH	1.49, m
	4b		1.06, m		
	5	27.0, CH	1.45, tt (13.3, 6.7)	21.3, CH_3	0.78, d (6.5)
	6	22.5, CH_3	0.80, ovl[a]	22.9, CH_3	0.84, d (6.6)
	7	22.2, CH_3	0.80, ovl[a]		
	2-NH		7.82, br s		8.10, br s
L-*allo*-Ile	1	171.7, C		171.8, C	
	2	56.7, CH	4.19, t (6.9)	56.5, CH	4.25, ovl[a]
	3	36.0, CH	1.91, m	36.2, CH	1.90, ovl[a]
	4a	25.5, CH_2	1.34, td (13.9, 7.0)	25.4, CH_2	1.34, m
	4b		1.18, m		1.12, tt (15.0, 7.3)
	5	11.3, CH_3	0.86, t (7.4)	11.1, CH_3	0.87, t (7.4)
	6	14.5, CH_3	0.84, d (6.4)	14.4, CH_3	0.83, d (6.9)
	2-NH		7.90, s		7.65, br d (6.0)
L-Orn	1	170.5, C		170.2, C	
	2	52.9, CH	3.99, br s	53.4, CH	3.82, br s
	3a	27.1, CH_2	1.89, ovl[a]	26.6, CH_2	1.90, ovl[a]
	3b		1.69, m		1.70, m
	4a	25.1, CH_2	1.51, m	25.3, CH_2	1.51, ovl[a]
	4b		1.47, m		1.47, ovl[a]
	5	38.8, CH_2	2.71, br s	39.0, CH_2	2.72, br s
	2-NH		8.77, br s		8.95, br s
	5-NH_2		8.39, br s		8.39, br s
D-*threo*-β-OH-Asn	1	169.4, C		169.5, C	
	2	55.7, CH	4.54, dd (8.3, 1.8)	55.6, CH	4.56, dd (8.4, 1.4)
	3	71.3, CH	4.41, d (2.1)	71.1, CH	4.44, d (1.8)
	4	173.2, C		173.1, C	
	2-NH		7.34, br s		7.21, d (5.7)
	4-NH_2		7.35, br s		7.34, br s
			7.18, br s		7.13, ovl[a]

[a]Signals were overlapped with other signals.

DOI: 10.1021/acs.jnatprod.7b00660
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Trp/Val/Hleu/Ile/Orn was corroborated by the $^3J_{C,H}$ HMBC correlations from the α-protons to the carbonyl carbons of the adjacent residues, and the ROESY correlations between the α-protons and the amide protons supported this result. The ROESY cross-peak between H-2$_{Ile}$ (δ_H 4.19) and 2-NH$_{Orn}$ (δ_H 8.77) established the ring closure and completed the planar structure of **1** as a chlorinated cyclic hexapeptide.

The absolute configuration of the amino acids in **1** was determined by advanced Marfey's analysis following acid hydrolysis.[11] The standards for 5-Cl-Trp and Hleu were obtained by acid hydrolysis of commercially available L-*N*-Boc-5-chlorotryptophan and Fmoc-D-homoleucine, respectively. LC-MS chromatographic comparison of the hydrolysis products of **1**, after derivatization with L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide), enabled us to assign 5-Cl-L-Trp, D-Val, D-Hleu, L-Ile, and L-Orn. Marfey's analysis revealed that the β-OH-Asn of **1** underwent hydrolysis to β-OH-Asp under the acid hydrolysis conditions, which is a frequently observed phenomenon during the preparation of the acid hydrolysates.[12] To precisely determine its absolute configuration, the L- and D-FDLA derivatives of the authentic standards L-*threo*-β-OH-Asp and L-*erythro*-β-OH-Asp were prepared. The L- and D-FDLA derivatives of β-OH-Asp from the hydrolysate of **1** were found to elute at the same time as the D- and L-FDLA derivatives of the L-*threo*-β-OH-Asp, respectively. This was taken as evidence for the absolute configuration of β-OH-Asn, thus indicating the presence of D-*threo*-β-OH-Asn in **1**. The *threo* configuration was further supported by *J*-based configuration analysis using vicinal coupling constants (from HECADE and *J*-resolved HMBC spectra) and ROESY correlations (Figure S3).[13] In addition, chromatographic comparisons of GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) derivatives of the acid hydrolysate of **1** and those of authentic L-Ile and L-*allo*-Ile demonstrated that **1** contains L-*allo*-Ile.[14]

The molecular formula of ulleungmycin B (**2**) was established as $C_{38}H_{58}N_9O_8Cl$ by HRESIMS, which is 14 amu less than the mass of **1**. The 1D and HSQC NMR data of **2** closely resembled those of **1**, except for a missing methylene group. The DQF-COSY and HMBC NMR data confirmed that **2** had Leu instead of Hleu. LC-MS analyses of the FDLA and GITC derivatives for the acid hydrolysate of **2** established the configuration of amino acids and enabled us to conclude that both compounds shared the same absolute configuration.

Ulleungmycins were active against multiple strains of Gram-positive bacteria including antibiotic-resistant strains, MRSA (methicillin-resistant *Staphylococcus aureus*), and QRSA (quinolone-resistant *Staphylococcus aureus*), as well as antibiotic-susceptible strains, with MIC values in the range of 8−32 μg/mL (Table 2).

Ulleungmycins A and B are structurally characterized by the unusual amino acids 5-Cl-L-Trp and D-Hleu. Although an indole ring chlorinated in the 5 position has been observed from pyrroindomycin B as well as bisindole alkaloids, such as cladoniamides and lynamicins,[15] the unique 5-Cl-L-Trp residue in peptide metabolites was previously described only once in the lantibiotic NAI-107.[16] The presence of Hleu is attributed to the low specificity of biosynthetic enzymes involved in the synthesis of branched chain amino acids valine, isoleucine, and leucine.[17] Although L-Hleu has been found in a limited number of metabolites such as longicatenamycins and mycoplanecins,[18] D-Hleu-containing metabolites have not been previously reported. Additionally, a fungal dodecadepsipeptide decatransin possessing three Hleu residues was reported recently but without stereochemical assignment.[19] Ulleungmycins A and B are most closely related to two antibacterial cyclic hexapeptides, desotamides and longicatenamycins.[10,18a] Of note, the gene clusters for the ulleungmycins (*ulm*) and desotamides (*dsa*) show considerable homology, but tailoring enzymes, such as halogenase and dioxygenase, are absent in the *dsa* gene cluster (Table S3). Ulleungmycins also share a similar amino acid composition with longicatenamycins which were isolated as a mixture of peptidic congeners. The absolute configurations of the amino acids in longicatenamycins are opposite those of ulleungmycins. The majority of the absolute configurations of the amino acids in longicatenamycins were solved by ORD or specific rotations of the pure or mixed residues.[20] The total synthesis of longicatenamycin A has reproduced its biological activity.[21]

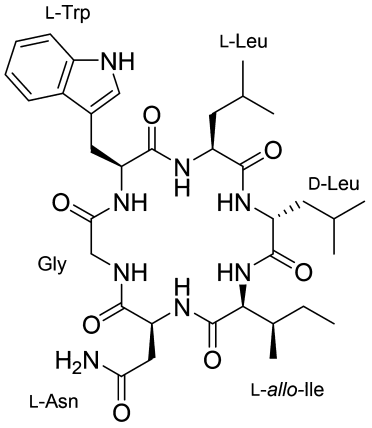
Table 2. Antibacterial Activities of 1 and 2

strain	MIC (μg/mL)		
	1	**2**	Cip[a]
Staphylococcus aureus RN 4220	16	16	0.125
MRSA CCARM 3167	16	32	4
QRSA CCARM 3505	8	16	32
Bacillus subtilis KCTC 1021	8	8	0.015
Streptococcus pneumoniae KCTC 5412	16	8	0.5
Enterococcus faecalis KCTC 5191	8	8	1
Escherichia coli CCARM 1356	128	64	64
Pseudomonas aeruginosa KCTC 2004	128	128	0.125

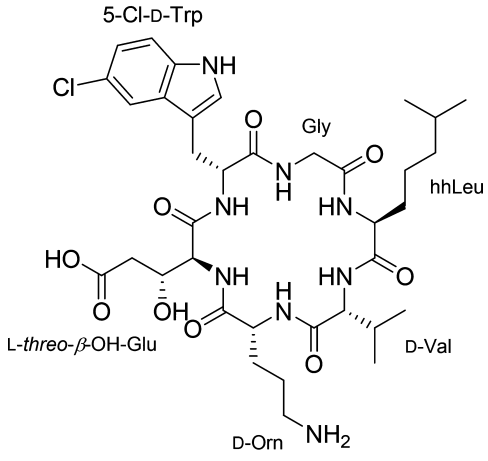
[a]Ciprofloxacin was used as a positive control.

On the basis of the information from the chemical structures and the putative biosynthetic gene cluster, we deduced a biosynthetic model for the ulleungmycins. Although the boundaries of the gene cluster were not established in this study, the genes were annotated to be involved in the biosynthesis of the ulleungmycins based on BLAST-based functional prediction (Figure 1 and Table S2). The number of NRPS modules of the gene cluster was in accordance with the number of amino acid residues, and predicted incorporation of Asn and Val based on the A domain specificities was also consistent with the sequence of β-OH-Asn and Val in the ulleungmycins. The presence of epimerase (E) domains embedded in modules 2, 3, and 6 matched the *R*-configured amino acids Val, Hleu/Leu, and *threo*-β-OH-Asn, suggesting that compounds **1** and **2** are the products of the *ulm* cluster. Furthermore, we investigated the presence of the dechlorinated analogues by analyzing the LC-MS data, and corresponding minor peaks were found in the extract (Figure S4). Thus, although the stage at which L-Trp is chlorinated is not yet clear, it is suggested that nonchlorinated L-Trp could be accepted by the A domain of module 1. It is hypothesized that thioester cleavage and release might be accomplished by the discrete thioesterase (TE) Ulm7 due to the absence of a C-terminal TE domain in the termination module 6. In addition, the gene *ulm20* codes for an enzyme with sequence similarity to TauD, the nonheme iron enzyme taurine hydroxylase.[22] Ulm20 also shows homology to SyrP from the syringomycin biosynthetic cluster, which catalyzes β-hydroxylation of Asp tethered to the PCP domain.[23] This fact, together with the significant degree of the substrate prediction score for Asn, suggested that hydroxylation might occur by Ulm20 once Asn is bound to the PCP domain as a thioester. The gene cluster further contains *ulm31*, whose predicted product is a closely related

DOI: 10.1021/acs.jnatprod.7b00660
J. Nat. Prod. XXXX, XXX, XXX−XXX



desotamide A



longicatenamycin A

homologue to the global regulator GntR. Mutations in the GntR family regulator of *Streptomyces* were shown to activate a cryptic gene cluster or enhance the production of antibiotics.[24] The regulatory role of GntR, which responds to nutritional signals, together with the two-component regulatory system (*ulm11* and *ulm12*) and the LysR family transcriptional regulator (*ulm3*), is a possible candidate to explain the different production profiles of ulleungmycins depending on the culture media.[25]

Screening tailoring enzymes, especially halogenases, which have the potential to diversify the substrate structures, is considered to be a promising genome-driven method to find new halogen-containing metabolites, although only a limited number of case studies have been reported.[26] The discovery of ulleungmycins provides evidence that the combination of genome-mining approaches focusing on tailoring enzymes and bioinformatic tools for predicting encoded compounds is an effective strategy to access new chemical entities.

EXPERIMENTAL SECTION

General Experimental Procedures. The specific rotation was obtained in MeOH using a JASCO P-1020 polarimeter. UV spectra were recorded in MeOH using an Optizen 2120 UV spectrophotometer. 1D and 2D NMR spectra were measured on a Bruker Biospin Advance II 900 NMR spectrometer (900 MHz for ^{1}H and 225 MHz for ^{13}C) at the Korea Basic Science Institute (KBSI) in Ochang, Korea. Chemical shifts were referenced to the respective residual solvent peaks (δ_H 2.50 and δ_C 39.51 for DMSO-d_6). HRESIMS data were obtained on a Waters SYNAPT G2 Q-TOF mass spectrometer at the KBSI in Ochang, Korea. Semipreparative reversed-phase HPLC was carried out using a Young Lin YL9100 HPLC system equipped with a Young Lin YL9160 PDA detector. LC-MS was performed with a Thermo LTQ XL linear ion trap attached to an ESI source that was connected to a Thermo Scientific Dionex Ultimate 3000 Rapid Separation LC system using a Waters HSS T3 column (Waters, 2.1 × 150 mm, 2.5 μm). Cosmosil 75C18 was used for ODS flash chromatography.

Microorganism Culture and Compound Purification Methods. The strain *Streptomyces* sp. KCB13F003 was isolated and identified as previously described.[3] The strain maintained on an SY agar medium was used for a large-scale culture. Two agar plugs of the strain were directly inoculated into a 1 L baffled Erlenmeyer flask containing 200 mL of a modified GLY medium (7.9 mL of glycerol, 5 g of lactose, 2.5 g of malt extract, 2.5 g of yeast extract, and 0.5 g of $CaCO_3$ per 1 L of distilled water). The cultures were shaken at 28 °C and 125 rpm for 132 h. The cells were centrifuged for 25 min at 5000 rpm and then extracted with 1 L of acetone and filtered. Following the removal of acetone, the aqueous solution was extracted three times with an equal volume of ethyl acetate. The supernatant was also extracted three times with an equal volume of ethyl acetate. The crude extracts of the supernatant and cells were combined and fractionated by ODS chromatography eluting with a stepwise gradient elution of MeOH−H_2O [2:8, 4:6, 6:4, 8:2, 10:0 (v/v)] to yield 10 fractions. Fractions 8 and 9, eluted with 80% and 100% MeOH, respectively, contained the desired materials. Fraction 9 was subjected to HPLC purification (Cosmosil Cholester, 10 × 250 mm, 5 μm; flow rate 3 mL/min; 25−40% CH_3CN−H_2O containing 0.05% formic acid over 20 min) to afford compound **1** (9.0 mg, t_R 14.6 min). Fraction 8 was purified by HPLC (Cosmosil Cholester, 10 × 250 mm, 5 μm; flow rate 3 mL/min; 25−40% CH_3CN−H_2O containing 0.05% formic acid over 20 min) to afford compound **2** (4.5 mg, t_R 12.0 min).

*Ulleungmycin A (**1**):* white powder; $[\alpha]_D^{22}$ +21.7 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.4), 227 (3.5), 290 (2.7); for NMR data, see Table 1; HRESIMS *m*/*z* 804.4166 [M + H]$^+$ (calcd for $C_{38}H_{59}N_9O_8Cl$, 804.4175).

*Ulleungmycin B (**2**):* white powder; $[\alpha]_D^{22}$ +38.2 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 207 (3.4), 227 (3.4), 290 (2.7); for NMR data, see Table 1; HRESIMS *m*/*z* 790.4018 [M + H]$^+$ (calcd for $C_{37}H_{57}N_9O_8Cl$, 790.4019).

Advanced Marfey's Analysis. Compounds **1** and **2** (0.1 mg each) were separately hydrolyzed with 6 N HCl (0.5 mL) at 100 °C for 1 h. The solution was dried repeatedly *in vacuo* and dissolved in 1 N $NaHCO_3$ (100 μL). To a solution was added a Marfey's solution of 1% L-FDLA (5-fluoro-2,4-dinitrophenyl-5-L-leucine amide) in acetone (100 μL). The reaction mixture was allowed to react at 40 °C for 1 h and quenched with 20 μL of 2 N HCl. After 2-fold dilutions in CH_3CN, the solution was analyzed by LC-MS (Waters HSS T3 column, 2.1 × 150 mm; flow rate 0.3 mL/min; 5−100% CH_3CN−H_2O containing 0.05% formic acid over 15 min). D-FDLA derivatives were prepared and analyzed in the same manner as described above. An optimized separation condition (Waters HSS T3 column, 2.1 × 150 mm; flow rate 0.3 mL/min; CH_3CN−H_2O containing 0.05% formic acid; 5% at 0−5 min, 5−40% at 5−65 min, and 40−100% at 65−70 min) was used to resolve FDLA derivatives of β-OH-Asp. Retention times (t_R, min) of the L- and D-FDLA derivatives for compound **1** were as follows: 5-Cl-L-Trp 13.55, 14.23; D-Val 14.01, 12.54; D-Hleu 15.33, 13.66; L-*allo*-Ile 12.96, 14.65; L-Orn (bis derivative) 14.52, 14.23; D-*threo*-β-OH-Asp 46.41, 46.59. Retention times (t_R, min) of the L- and D-FDLA derivatives for compound **2** were as follows: 5-Cl-L-Trp 13.54, 14.24; D-Val 13.99, 12.57; D-Leu 14.7, 13.1; L-*allo*-Ile 12.96, 14.65; L-Orn (bis derivative) 14.51, 14.24; D-*threo*-β-OH-Asp 46.41, 46.59. Retention times (t_R, min) of the L- and D-FDLA derivatives of L-*threo*-β-OH-Asp and L-*erythro*-β-OH-Asp were as follows: L-*threo*-β-OH-Asp 46.5, 46.35; L-*erythro*-β-OH-Asp 52.66, 51.14.

GITC Derivatization. The acid hydrolysates of **1** and **2** (0.1 mg each) were separately dissolved in triethylamine (6% w/v in acetone, 200 μL) and a GITC solution (1% w/v in acetone, 200 μL). The reaction mixture was stirred at room temperature for 20 min. After adding 5% acetic acid (200 μL), the sample was directly analyzed on

DOI: 10.1021/acs.jnatprod.7b00660
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the LC-MS (Waters HSS T3 column, 2.1 × 150 mm; flow rate 0.3 mL/min; CH_3CN-H_2O containing 0.05% formic acid; 5% at 0−5 min, 5−40% at 5−65 min, and 40−100% at 65−70 min). The retention time for the GITC derivatives of **1** and **2** was 59.26 min. GITC derivatives of authentic L-Ile and L-*allo*-Ile were prepared and analyzed in the same manner (t_R = 59.48 and 59.24 min, respectively).

Antibacterial Activity Assay. The whole-cell antimicrobial activity was determined using broth microdilution as described previously.[27] Drug-resistant pathogens including methicillin-resistant *S. aureus* (MRSA) CCARM 3167, quinolone-resistant *S. aureus* (QRSA) CCARM 3505, and *Escherichia coli* CCARM 1356 were obtained from the Culture Collection of Antimicrobial Resistant Microbes of Korea. Most of the test strains were grown to mid log phase in Mueller−Hinton broth and diluted 1000-fold in the same medium. Cells (10^5/mL) were inoculated into Mueller−Hinton broth and dispensed at 0.2 mL/well in 96-well microtiter plates. *Streptococcus pneumonia* were grown in Todd−Hewitt medium instead of Mueller−Hinton broth. Since test compounds and ciprofloxacin (Sigma) were soluble in DMSO, they were prepared in DMSO, the final concentration of which did not exceed 0.05%. Cells were treated with either 0.05% DMSO as vehicle control or test samples. The MICs were determined in triplicate by serial 2-fold dilutions of the test compounds. The MIC was defined as the concentration of a test compound that completely inhibited cell growth during a 24 h incubation at 37 °C. Bacterial growth was determined by measuring the absorption at 650 nm using a microtiter enzyme-linked immunosorbent assay (ELISA) reader.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00660.

Supplementary tables and figures for gene cluster analyses, HRESIMS, and NMR spectra (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel (J.-H. Jang): +82-43-240-6164. Fax: +82-43-240-6169. E-mail: jangjh@kribb.re.kr.

*Tel (J.-S. Ahn): +82-43-240-6160. Fax: +82-43-240-6169. E-mail: jsahn@kribb.re.kr.

ORCID

Young-Soo Hong: 0000-0001-7768-2239

Jae-Hyuk Jang: 0000-0002-4363-4252

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Research Council of Science & Technology (NST) granted by the International Joint Research Project (ASIA-16-001) and KRIBB Research Initiative Program of Korea. This work was also supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT; Ministry of Science ICT) (NRF-2017R1C1B2002602). We thank the Korea Basic Science Institute, Ochang, Korea, for providing the NMR (900 and 800 MHz) and HRESIMS. We would also like to express our gratitude to Prof. Y. Igarashi and N. Oku (Toyama Prefectural University) for kindly providing us with L-*threo*-β-OH-Asp and L-*erythro*-β-OH-Asp standards.

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