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Ulleungamides A and B, Modified α,β-Dehydropipecolic Acid Containing Cyclic Depsipeptides from *Streptomyces* sp. KCB13F003

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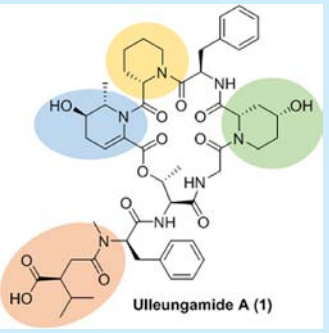
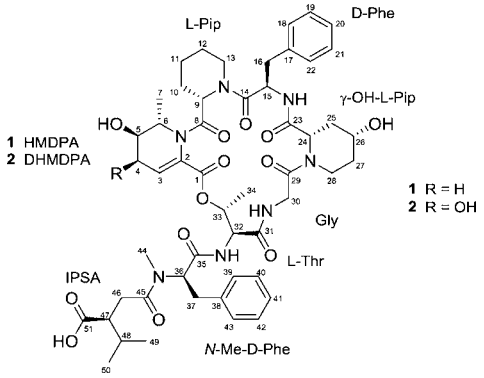
Supporting Information

ABSTRACT: Two novel cyclic depsipeptides, ulleungamides A (**1**) and B (**2**), were isolated from cultures of terrestrial *Streptomyces* sp. Their structures were determined by analyses of spectroscopic data and various chemical transformations, including modified Mosher's method, advanced Marfey's method, PGME, GITC derivatizations, and Snatzke's method. Ulleungamides were determined to be a new class of peptides bearing unprecedented units, such as 5-hydroxy-6-methyl-2,3-dehydropipecolic acid, 4,5-dihydroxy-6-methyl-2,3-dehydropipecolic acid, and amino-linked 2-isopropylsuccinic acid. Ulleungamide A displayed growth inhibitory activity against *Staphylococcus aureus* and *Salmonella typhimurium* without cytotoxicity.



Structurally unique and diverse secondary metabolites from actinomycetes have been an invaluable source of drug leads and molecular probes over the decades.[1] However, discovering novel structural scaffolds is getting more difficult due to the high rediscovery rate of known compounds.[2] To overcome this difficulty, we have focused our attention on exploration of actinomycetes from chemically less studied sites, which are potentially prolific sources of novel chemistry.[3] Although Ulleung Island, a small volcanic island located 150 km off the coast of the Korean Peninsula, has been reported as a biodiversity hot spot which harbors a variety of microorganisms together with many endemic and endangered plant species,[4] secondary metabolites of microorganisms derived from this island have not been investigated. Attracted by its probability of harboring promising strains, we isolated 208 actinomycetes from soil samples collected at Ulleung Island and screened their secondary metabolites. The preliminary chemical screening of the EtOAc extracts by LC−MS data suggested that *Streptomyces* sp. KCB13F003 produced unusual metabolites, of which UV and MS data are rare in public and in-house databases. These compounds were identified by scale-up isolation and structure elucidation as two new branched cyclic depsipeptides, ulleungamides A (**1**) and B (**2**). These novel peptides possess unprecedented residues, modified α,β-dehydropipecolic acid and amino-linked 2-isopropylsuccinic acid. The isolation, structure elucidation, and biological activity of these two new peptides are described below.



Streptomyces sp. KCB13F003 was cultured in GLY medium (30 × 250 mL) for 6 days at 28 °C, and then the metabolites were collected by the acetone extract of cells and Amberlite XAD-7 resin extract of the fermentation broth. The resulting crude extract was purified by sequential solvent partitioning, ODS vacuum flash chromatography, and reversed-phase HPLC to afford ulleungamides A (**1**) and B (**2**).

Ulleungamide A (**1**) was isolated as a white powder. The molecular formula $C_{51}H_{67}N_7O_{13}$ was determined by HRESIMS analysis (*m*/*z* 986.4861 $[M + H]^+$, calcd for $C_{51}H_{68}N_7O_{13}$,

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986.4875) in combination with NMR data, indicating 22 degrees of unsaturation. Despite many proton and carbon signals having overlapping chemical shifts due to the presence of the minor conformer (about 50% of main conformer), each amino acid unit was identified and assembled by use of high-field NMR spectrometers (900 MHz) and nonuniform sampling (NUS) NMR processing.[5] Amide proton signals at δ_H 7.7−8.9 and α-amino methines at δ_H 4.3−5.6 in the HSQC-DEPT spectrum, together with amide and ester carbonyl carbons at δ_C 162.3−170.9 in the ^{13}C NMR spectrum, suggested a peptide nature of **1**. A comprehensive analysis of 2D NMR data (i.e., HSQC-DEPT, COSY, TOCSY, and HMBC) clearly established the presence of glycine (Gly), threonine (Thr), phenylalanine (Phe), *N*-methylphenylalanine (*N*-Me-Phe), and pipecolic acid (Pip). Of note, Phe and *N*-Me-Phe residues were especially established by HSQC-TOCSY data due to severely overlapped cross-peaks of numerous aromatic protons in the region of 7.15−7.35 ppm in COSY and TOCSY spectra (Figure 1). The proton spin system

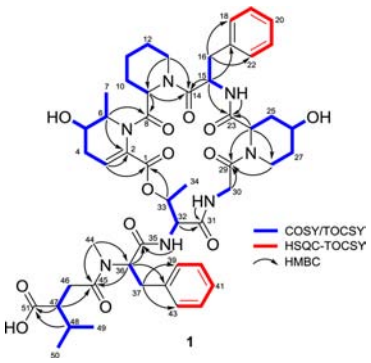

Figure 1. Key 2D NMR correlations of **1**.

from the methine proton H-24 (δ_H 4.29) to the methylene protons H_2-28 (H-24/H_2-25/H-26/26-OH/H_2-27/H_2-28) observed in the COSY spectrum and HMBC correlations from H-24 to C-23 (δ_C 170.8) and C-28 (δ_C 34.1), together with the downfield shift of C-26 (δ_C 61.9), revealed the presence of an unusual amino acid 4-hydroxypipecolic acid (γ-OH-Pip) (Figure 1). Moreover, the cross-peaks among H-3/H_2-4/H-5/5-OH/H-6/H_3-7 were observed in COSY and TOCSY spectra, which allowed the establishment of another spin system from the olefinic carbon C-3 (δ_C 120.2) to the methyl carbon C-7 (δ_C 14.5). Key HMBC correlations from the olefinic proton H-3 (δ_H 5.98) to the sp^2 quaternary carbon C-2 (δ_C 129.4) and the carbonyl carbon C-1 (δ_C 162.3) and from the downfield methine proton H-6 (δ_H 3.93) to C-2 determined the presence of unreported amino acid 5-hydroxy-6-methyl-2,3-dehydropipecolic acid (HMDPA). Furthermore, COSY correlations revealed the spin system from the methylene protons H_2-46 (δ_H 2.52 and 2.10) to the geminal dimethyl groups H_3-49 (δ_H 0.78) and H_3-50 (δ_H 0.76). This spin system was extended to the carbonyl carbons C-45 (172.0) and C-51 (δ_C 175.6) by HMBC correlations from H-47 (δ_H 2.48) to C-45 and C-51 and from H-48 (δ_H 1.73) to C-51. The presence of carboxylic acid functionality (C-51, δ_c 175.6) was suggested by the observation of a very broad singlet at δ_H 11.87 in the ^1H NMR spectrum (Figure S3, Supporting Information). In light of these data, the presence of 2-isopropylsuccinic acid (IPSA) was established.

The HMBC correlations from α-protons to amide carbonyls of adjacent amino acids led us to the establishment of the amino acid sequence of Pip-Phe-γ-OH-Pip-Gly-Thr-*N*-Me-Phe (Figure 1). Moreover, the connectivity between HMDPA and Pip was confirmed by the HMBC correlation from H-6 (δ_H 3.93) of HMDPA to the carbonyl C-8 (δ_C 170.6) of Pip. The ester linkage between the β-carbon C-33 of Thr and the carbonyl carbon C-1 of HMDPA was established by the HMBC correlation from the β-proton H-33 (δ_H 5.48) of Thr to C-1 (δ_C 162.3). In addition, HMBC correlations of H_3-44 (δ_H 2.94) and H-36 (δ_H 5.57) of *N*-Me-Phe to the amide carbonyl C-45 (δ_C 172.0) of IPSA linked the IPSA to *N*-Me-Phe. Upon these spectroscopic data, the gross structure of **1** was characterized as a 19-membered cyclic depsipeptide incorporating six amino acids including unusual residues and a branched chain terminated by the amino-linked IPSA.

The stereochemistries of 10 stereogenic centers of **1** were established by applying various chemical and spectroscopic methods based on the chemical properties of chiral functional groups. The absolute configurations of two secondary carbinol centers, C-5 in HMDPA and C-26 in γ-OH-Pip, were determined by the modified Mosher's method.[6] First, carboxylic acid was methylated by exhaustive treatment of **1** with TMS-CHN_2 to give a methyl ester (**1b**), which was subsequently converted to *S*- and *R*-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters (**1c** and **1d**) by treatment with *R*- and *S*-MTPA-Cl. The distribution of the signs of the $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) of bis-*S*- and bis-*R*-MTPA esters (**1c** and **1d**) allowed us to assign the 5*R* and 26*R* configurations (Figure 2). The absolute configuration of

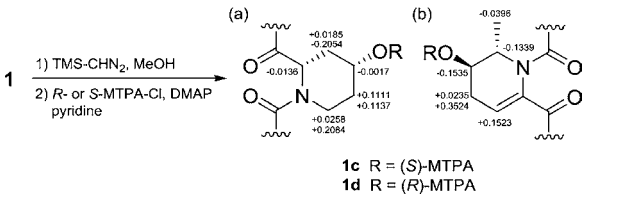

Figure 2. $\Delta\delta_{S-R}$ values obtained for bis-*S*- and bis-*R*-MTPA esters (**1c** and **1d**): (a) 4-hydroxypipecolic acid; (b) 5-hydroxy-6-methyl-2,3-dehydropipecolic acid.

C-24 could be defined by $^3J_{HH}$ coupling constants and ROESY experiment of **1c**. In the ^1H NMR spectrum of **1c**, the oxymethine proton H-26 (δ_H 5.25) appeared as a quintet with a coupling of 4.5 Hz, indicating an equatorial position of H-26. The methine proton H-24 (δ_H 4.33) was then suggested to be present at the equatorial position on the basis of its triplet signal with a coupling of 4.5 Hz, which was consistent with the presence of ROESY cross-peaks of H-24 with the adjacent methylene H_2-25 and the lack of correlations with H_2-28. These established the *S* configuration at C-24, thus indicating the presence of (2*S*, 4*R*)-4-hydroxypipecolic acid.

The absolute configuration of C-6 was also determined by a combination of extensive ROESY analysis and $^3J_{HH}$ couplings. Even though broad and overlapping ^1H signals in various solvents (DMSO-d_6, $CDCl_3$, pyridine-d_5, and CD_3OD) prevented the determination of coupling constants of HMDPA, they could be obtained from resolved cross-peak multiplets in DQF-COSY.[7] An equatorial position for H-5 was established on the basis of ROESY correlations of H-5 with H_2-4, H-6, and H_3-7 (Figure 3). In addition, the small vicinal coupling constant between H-5 and H-6 (3.2 Hz) indicated an pseudoequatorial position of H-6, and the ROESY correlation between H_3-7 and H-4a revealed the dipseudoaxial relationship of H_3-7 and H-4a, confirming a half-chair conformation of HMDPA. The absolute configuration of C-6 was therefore assigned as *S*. The stereochemistry of the α-

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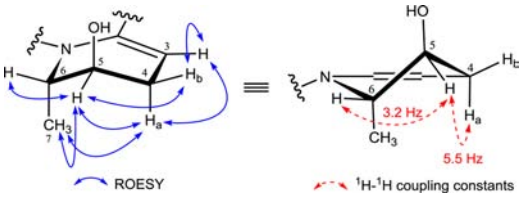

Figure 3. ROESY correlations and $^3J_{HH}$ coupling constants of 5-hydroxy-6-methyl-2,3-dehydropipecolic acid in **1**.

carbons of Pip, Phe, Thr, and *N*-Me-Phe was assigned by advanced Marfey's analysis.[8] HPLC comparison of L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) derivatives of the acid hydrolysate of **1** indicated the presence of L-Pip, D-Phe, and *N*-Me-D-Phe and established the absolute configuration of the α-carbon in Thr as *S*. Moreover, the same retention time of FDLA derivatives of commercial (2*S*, 4*R*)-4-hydroxypipecolic acid with those of γ-OH-Pip in **1** further confirmed the absolute configuration of C-24 as *S*.

To determine the absolute configuration of the β-carbon at C-33 in Thr, we performed the derivatization of acid hydrolysate of **1** with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC).[9] By comparison of chromatograms with GITC derivatives of authentic standards L-Thr and L-*allo*-Thr, the *R* configuration of the β-carbon in Thr was established, demonstrating that **1** contained L-Thr. Finally, the absolute configuration of C-47 was determined by applying the phenylglycine methyl ester (PGME) method.[10] The $\Delta\delta_H$ values ($\Delta\delta_H = \delta_S - \delta_R$) obtained from the *S*- and *R*-PGME amides (**1e** and **1f**) assigned the *S* configuration of C-47 (Figure 4).

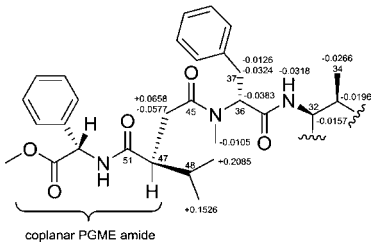

Figure 4. $\Delta\delta_{S\text{-}R}$ values around C-47 of 2-isopropylsuccinic acid obtained for *S*- and *R*-PGME amides (**1e** and **1f**).

Ulleungamide B (**2**) was isolated as a white powder, and the HRESIMS analysis showed an ion peak at *m*/*z* 1024.4642 [M + Na]$^+$ ($C_{51}H_{67}N_7O_{14}$, calcd for $C_{51}H_{67}N_7O_{14}Na$, 1024.4644). The ^{1}H and ^{13}C NMR spectra similar to those of **1**, together with an additional 16 amu, suggested that **2** is likely the hydroxy derivative of **1**. The 2D NMR analysis clearly revealed that the methylene group C-4 was replaced by the oxygenated methine group (δ_C 62.9, δ_H 4.16), indicating the presence of 4,5-dihydroxy-6-methyl-2,3-dehydropipecolic acid (DHMDPA).

The absolute configurations in L-Pip, D-Phe, *N*-Me-D-Phe, and L-Thr in **2** were determined by using Marfey's analysis and GITC derivatization with the same manner as **1**. The absolute configurations of C-24 and C-26 in γ-OH-Pip of **2** were suggested to be *S* and *R* configurations, respectively, on the basis of the chemical shifts, ROESY correlations, and the retention time of the FDLA derivatives being virtually identical to those of γ-OH-Pip of **1**. In addition, a PGME method assigned the *S* configuration of C-47 (Figure S34, Supporting Information). To determine the stereochemistry of DHMDPA, we conducted ROESY analysis and Snatzke's method. In the ROESY spectrum, observed correlations (H-5/H-4, H-6, H_3-7 and H-4/H_3-7) suggested an equatorial orientation of H-5 and a dipseudoaxial relationship of H_3-7 and H-4. This observation was consistent with the couplings of ^3J(H-4, H-5) = 5.3 Hz and ^3J(H-5, H-6) = 3.2 Hz, indicating the relative configuration of the stereogenic centers in DHMDPA as 4*R**,5*S**,6*S**. Then, the absolute configuration of DHMDPA was assigned by applying the Snatzke's method using the in situ complex of dimolybdenum tetraacetate [$Mo_2(OAc)_4$].[11] The Mo_2 complex with the 1,2-diol moiety shows the diagnostic bands in the induced CD (ICD) spectrum, which represent the chirality of the 1,2-diol moiety expressed by the sign of the O−C−C−O dihedral angle. The positive Cotton effect at 323 nm ($\Delta\varepsilon$, + 5.0) and negative Cotton effect at 282 nm ($\Delta\varepsilon$, − 8.6) of cottonogenic derivative of **2** indicated the positive O−C−C−O dihedral angle (Figure 5).

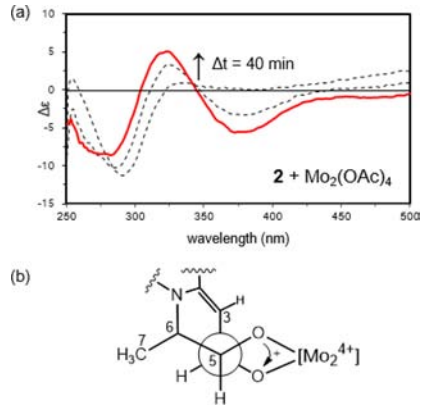

Figure 5. Determination of absolute configuration of 4,5-diol in **2** by Snatzke's method. (a) Time evolution of ICD spectra of **2** in solution of dimolybdenum tetraacetate in DMSO. (b) The sign of the O−C−C-O dihedral angle in the cottonogenic derivative.

These results determined the absolute configuration of DHMDPA to be 4*R*,5*S*,6*S*. When we performed the same experiment using **1**, it did not exhibit any diagnostic bands, demonstrating that Cotton effects of **2** in a solution of $Mo_2(OAc)_4$ in DMSO are clearly due to the Mo_2 complex with 4,5-diol (Figure S35, Supporting Information). Consequently, it is concluded that the same stereochemistry is present in the common stereogenic centers of **1** and **2**. This result was further supported by comparable optical rotation values and CD spectra of **1** and **2** (Figures S39 and S40, Supporting Information).

Structurally, the presence of two sets of signals observed in 1D NMR data of **1** and **2** could be explained by the cis/trans amide bond conformation or by the inversion of the piperidine ring of pipecolic acid units.[12] The major differences of chemical shifts between conformers were observed dominantly in branched units, *N*-Me-D-Phe and IPSA, and groups that are close to them in space (C-3 and C-33). Extensive ROESY analysis revealed that the major and minor conformers adopted trans and cis conformations of the amide bond between *N*-Me-Phe and IPSA, respectively, suggesting that cis/trans isomerization would be responsible for the conformational equilibrium of **1** and **2** (Figure S36, Supporting Information).

Compounds **1** and **2** were tested for their growth inhibitory activity against cancer (HeLa, MCF-7, and PC-3) and normal (NRK, MRC-5, and 267B1) cell lines, but none of them exhibited pronounced activities up to 50 μM. Ulleungamides were also evaluated for antibacterial and antifungal activities against various pathogenic microbes by a disk diffusion assay. Compound **1**

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showed inhibition zones only against *Staphylococcus aureus* (**1**; 14 mm at 25 μg/disk, kanamycin; 15 mm at 25 μg/disk) and *Salmonella typhimurium* (**1**; 9 mm at 50 μg/disk, chloramphenicol; 17 mm at 50 μg/disk), while **2** was inactive against all tested organisms up to a concentration of 100 μg/disk, suggesting that ulleungamide A (**1**) has a selective antibacterial activity and the presence of a hydroxy group at position C-4 severely reduces the activity.

Ulleungamides A (**1**) and B (**2**) are a new class of cyclic depsipeptides featuring several unique structural features. They contain multiple pipecolic acid-derived residues, including Pip, γ-OH-Pip, HMDPA, and DHMDPA. Pipecolic acid, cyclic nonproteogenic amino acid, is a key constituent of numerous pharmaceutically important secondary metabolites, such as rapamycin and FK506.[13] Sandramycin, quinaldopeptin, and petriellin A also have been reported to possess multiple pipecolic acids,[14] but ulleungamides are the first example of having three residues of Pip and Pip derivatives. The peptide metabolites that contain the γ-OH-Pip residue are quite rare with only two examples, which were found in virginiamycin S_5 and MBJ-0110.[15] Moreover, the presence of HMDPA and DHMDPA was not reported. On the basis of chemical structures, they are likely to be formed via modification of a pipecolic acid by *C*-methylation, hydroxylation, and desaturation during or after nonribosomal peptide chain elongation. HMDPA and DHMDPA represent new examples of natural α,β-dehydroamino acids, which have been shown to contribute to conformational properties and undergo chemical reactions that can influence the bioactivity of peptide molecules.[16] The significant differences in antimicrobial activity between **1** and **2** imply that chemical properties of these modified α,β-dehydroamino acids contribute significantly to the activity of ulleungamides. Owing to their structural and functional characteristics, pipecolic acid derivatives have been used for scaffolds to design bioactive compounds, e.g., a HIV inhibitor Palinavir and a thrombin inhibitor argatroban.[17] Further studies on identification of the gene cluster of HMDPA and DHMDPA might provide a route to the combinatorial biosynthesis of highly modified pipecolic acids to generate compounds with interesting bioactivity. In addition, the presence of an IPSA unit is also unprecedented. It could be originated from 2-isopropyl malate, which is an intermediate of leucine biosynthesis,[18] but its origin should be further investigated.

The discovery of ulleungamides is another example supporting the potential of microorganisms from previously uninvestigated sites and especially those which represent unique biodiversity to produce novel secondary metabolites. Further investigation on the other actinomyces derived from Ulleung Island based on bioactivity and structural novelty is currently underway.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01969.

Experimental procedures and spectral data of **1** and **2** (NMR, HRESIMS, and CD spectroscopic data) (PDF)

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Notes

The authors declare no competing financial interest.

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