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Lipodiscamides A–C, New Cytotoxic Lipopeptides from *Discodermia kiiensis*

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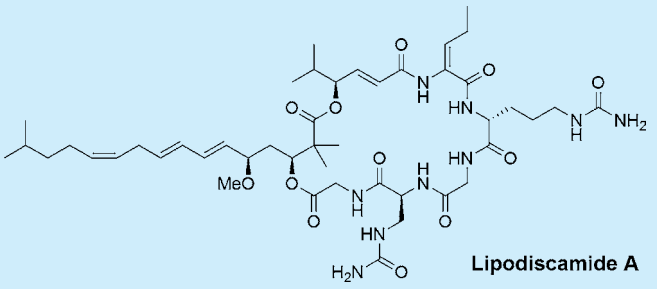
Supporting Information

ABSTRACT: Lipodiscamides A–C, three new lipodepsipeptides, were characterized from the marine sponge *Discodermia kiiensis*. These structurally rare cyclic lipodepsipeptides were found to possess an unprecedented dilactone macrocycle and, thus, represent a new family of lipopeptides. They are the only lipopeptides bearing 4*S*-hydroxy-*trans*-2-enoate, and noncanonical amino acids, L-3-ureidoalanine (Uda), *E*-dehydronorvaline (Denor), and D-citrulline (Cit). MTT assays against P388 and HeLa cells revealed the moderate cytotoxicity of all three compounds.



Lipopeptides are a class of secondary metabolite hybrids consisting of a hydrophobic tail attached to a short linear or cyclic peptide. Several bioactive lipopeptides exhibiting cytotoxic, antimicrobial, immunosuppressant, and surfactant properties are reported to be produced by various bacteria, such as *Streptomyces*, *Pseudomonas*, and *Bacillus*,[1−4] as well as fungi, including *Fusarium*, *Aspergillus*, and *Pochonia*.[5−7] In contrast, lipopeptides seem to be a rather rare family among the natural products derived from the marine sponges, even though sponges are prolific sources of biologically active metabolites, such as polyketides, peptides, and terpenoids. In the course of our search for bioactive secondary metabolites from sponges of the genus *Discodermia*, we have reported the isolation of cytotoxic cyclopeptides, calyxamides from *D. calyx*[8], and cyclolithistide from *D. japonica*.[9] Furthermore, peptidic metabolites, such as discodermins and discokiolides, have been isolated from another species, *D. kiiensis*.[10−12] Herein, we report a new family of cytotoxic and structurally unique cyclic lipodepsipeptides characterized from *D. kiiensis*.

The frozen sponge (1 kg), collected off Shikine-jima Island, was extracted with EtOH. The EtOH extract was then partitioned repeatedly between Et_2O and H_2O, and the organic extract was repartitioned between *n*-hexane and 90% aqueous MeOH. Then, the aqueous MeOH extract was subjected to flash ODS chromatography, to obtain the 80% aqueous MeOH fraction. This fraction was purified by reversed-phase HPLC on a π-NAP column, using an isocratic mode with 50 mM NH_4OAc in 85% MeOH, to afford 4.8, 1.2, and 0.4 mg of **1**−**3**, respectively (Figure 1).

The major compound, lipodiscamide A, **1**, showed an *m*/*z* at 966.5289 $[M + Na]^+$ in HR-FABMS, and its molecular formula was established as $C_{46}H_{73}N_9O_{12}$. A TLC analysis of the compound revealed that it responds positively to 10% H_2SO_4 spray, generating a blue spot that turned purple upon continued heating. When the NMR solvent was changed from CD_3OD to DMSO-d_6, five exchangeable protons between 7.5 and 10.5 ppm appeared (Figures S1, S3), which were eventually assigned to α-amino acids, establishing the peptidic nature of **1**. The olefinic protons between 5.3 and 6.6 ppm hinted at the presence of a polyketide and/or an unsaturated fatty acid moiety. In brief, the two pairs of diastereotopic methylene

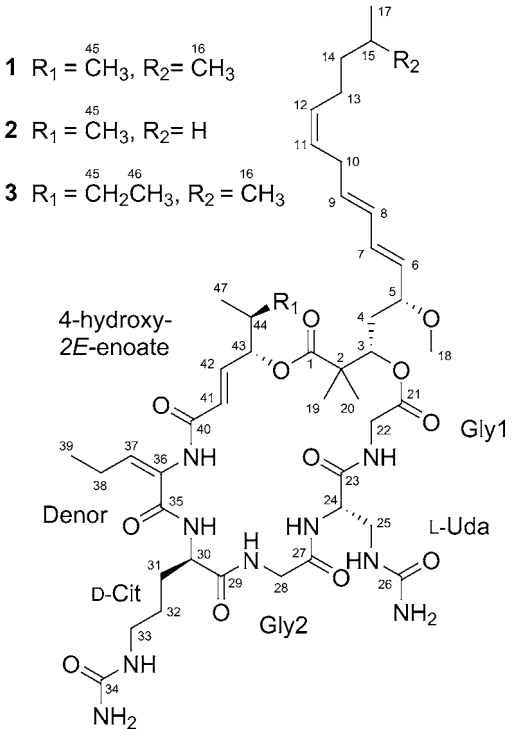


Figure 1. Structures of lipodiscamides A–C (**1**−**3**).

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protons at δ_H 4.12 and 4.29 (Figure S1) indicated the presence of two glycine subunits. The two proton signals at δ_H 4.25 and 4.37 appeared to be the α-protons of two other amino acids, which were subsequently inferred to be citrulline (Cit) and 3-ureidoalanine (Uda) by COSY and HMBC correlations (Table S1). Due to the heavily overlapped signals between 1.4 and 1.7 ppm observed with DMSO-d_6, other NMR solvents and numerous solvent mixtures were tested to achieve better resolution within this region and to ascertain the number of methylenes. Eventually, a 2:1 mixture of DMSO-d_6/CD_3CN was found to be effective for this purpose (Figures S1−S4) and also resolved all exchangeable protons (δ_H 5.31, 2H; δ_H 5.56, 2H; δ_H 5.92, 2H) of the two ureido groups. In addition to the NMR spectral data, the presence of the ureido functionality was confirmed by its color reaction with 0.06 M dimethylaminobenzaldehyde under strongly acidic conditions, which generated a yellow-colored Schiff base.[13] The presence of the dehydronorvaline (Denor) residue was then established by the COSY correlations from H-37 to H-39 and the appearance of HMBC correlations of its amide proton with the carbonyl at C-35 and with the two olefinic carbons C-36 and C-37 using the 2:1 DMSO-d_6/CD_3CN mixture (Figure S11). Its *trans* geometry was deduced by the presence of a NOESY correlation between the olefinic proton H-37 and its amide proton (Figure S12). On the other hand, the COSY correlations from H-41 to H-45 and the HMBC correlations of H-41 and H-42 with the carbonyl at C-40 confirmed the presence of the 4-hydroxy-5-methylhexa-2-enoyl moiety. The large $^3J_{HH}$ coupling constant (15.3 Hz) between H-41 and H-42 led to the assignment of a *trans* stereochemistry (Table S1). As for the C_{19} unsaturated fatty acyl chain, the C-1 to C-11 and C-12 to C-17 fragments were established by COSY correlations, and the connection between these two fragments was confirmed by the HMBC correlations of H-10, H-13, and H-14 with C-12 (Figure 2).

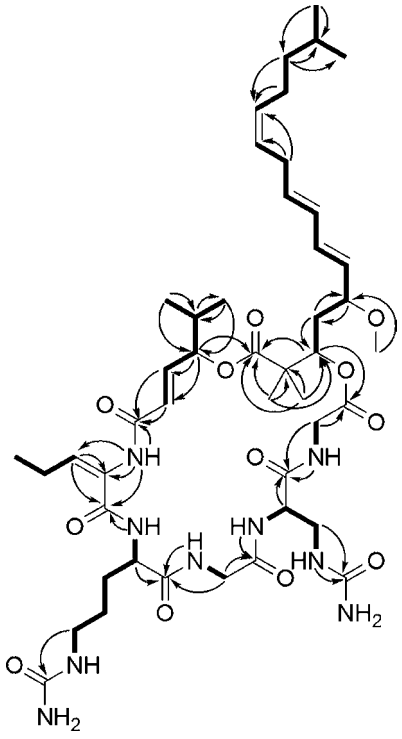

Figure 2. Key COSY and HMBC correlations of **1**.

The geometry about the diene from C-6 to C-9 was deduced to be both *trans* according to the splitting patterns and the $^3J_{HH}$ coupling constants, which could be observed with CD_3OD (Table S2). On the other hand, the double bond between H-11 and H-12 was inferred to have a *cis* configuration on the basis of the small $^3J_{HH}$ coupling constant (10.8 Hz) observed in DMSO-d_6 (Table S3). Finally, the connections between all subunits were corroborated by the 2D NMR spectral data (Figure 2). Hence, the planar structure of lipodiscamide A was determined to be **1**.

In contrast, the molecular formula of lipodiscamide B, **2**, was established to be $C_{45}H_{71}N_9O_{12}$ by HR-FABMS, giving an m/z at 952.5121 $[M + Na]^+$. In terms of its mass, **2** appeared to have one less CH_2 unit than **1**. This assumption was clearly supported by its ^1H NMR spectrum in CD_3OD (Figure S14), which was identical to that of **1**, except for the integration of its highest field methyl group CH_3-16 (δ_H 0.90). These protons were assigned to the terminal methyl group of the fatty acyl chain and corresponded to three protons in **2**. In contrast, the corresponding region in **1** had an integral intensity of six protons (CH_3-16 and17). Furthermore, its 2D NMR spectral data were superimposable with those of **1**, except for the appearance of HMQC correlations between the methylene protons H_2-14 (δ_H 1.31) and C-14 (δ_C 30.1), and H_2-15 (δ_H 1.32) and C-15 (δ_C 32.1) (Table S2), providing additional evidence that **2** differed from **1** only at the terminal portion of the long chain acyl group. As such, the planar structure of lipodiscamide B was established as **2**.

Finally, the molecular formula of lipodiscamide C, **3**, was determined to be $C_{47}H_{75}N_9O_{12}$ by HR-FABMS, yielding an m/z at 980.5436 $[M + Na]^+$. Based on this, **3** was expected to be one CH_2 unit larger than **1**. In CD_3OD, the downfield shifts of H-43 (δ_H 4.94 in **1** to δ_H 5.12 in **3**) and H-44 (δ_H 6.53 in **1** to δ_H 6.57 in **3**) (Figure S21) suggested that the only difference between the two compounds exists in the 4-hydroxy-2-enoyl subunit. As evidenced by the COSY and HMBC correlations in DMSO-d_6 (Table S3), where the least overlapping of the signals from this moiety was found, the 4-hydroxy-5-methylhex-2-enoyl subunit in **1** is replaced by a 4-hydroxy-5-methylhept-2-enoate in **3**. Aside from this particular difference between **1** and **3**, their spectral data were unequivocally indistinguishable from one another. Therefore, lipodiscamide C was designated as the planar structure **3**.

To determine the absolute stereochemistry at the α-carbons in Cit and 3-Uda, acid hydrolysis was performed by heating each lipopeptide in 6 M HCl at 110 °C for 24 h. This process converted the ureido amino acids to ornithine (Orn) and 2,3-diaminopropionic acid (Dpr),[14] respectively, which were then derivatized to the corresponding *N*-trifluoroacetyl/methyl esters and analyzed by chiral-phase GC-MS. Standard amino acids were derivatized and analyzed in the same manner. Consequently, the results showed that Orn, and therefore Cit, was in the D-form in all three compounds. However, 3-Uda underwent racemization through a 5-dihydrouracil intermediate,[13] as expected under the acidic reaction conditions, yielding both the L- and D-products in a 3:2 ratio. To resolve this problem, mild acid hydrolysis was performed with a shorter reaction time of 6 h. This condition circumvented the acid-induced racemization, and L-Dpr was solely detected (Figure S27). As a result, all three compounds were determined to possess L-3-Uda.

Next, to elucidate the absolute configurations at C-3 and C-5, all four diastereomeric standards of **6′** were synthetically

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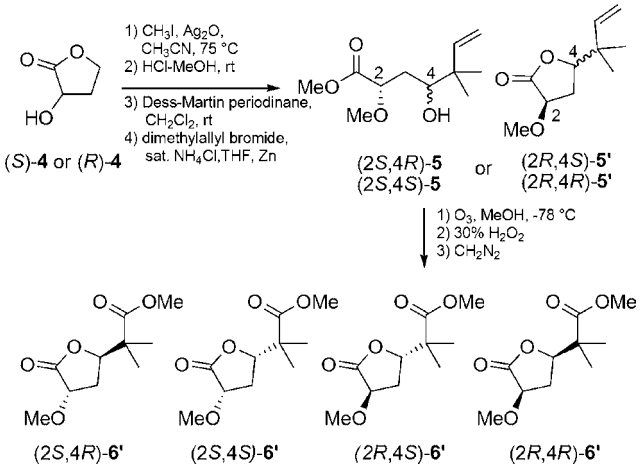
prepared from optically pure **4** (Scheme 1).[15−17] In the case of (*S*)-**4**, a mixture of (2*S*,4*R*)- and (2*S*,4*S*)-**5** was obtained. These

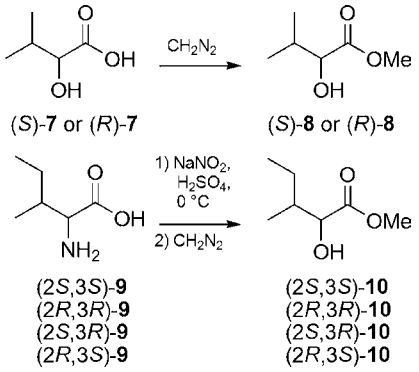
Scheme 1. Synthesis of the Stereoisomers of 6′



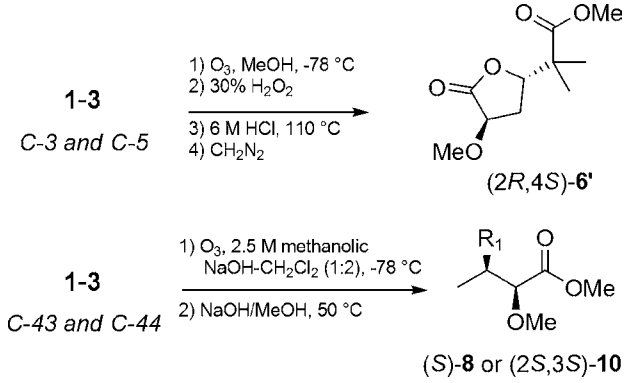
diastereomers were separated by silica gel chromatography, and their absolute configurations were determined by reaction with 2-methoxyphenylacetic acid (MPA) (Figure S28). On the other hand, (*R*)-**4** produced a mixture of the allylated lactones (2*R*,4*S*)- and (2*R*,4*R*)-**5**′, which were also separated by silica gel chromatography. Their relative configurations were then determined by NOESY analyses (Figures S29−30). Afterward, **5**/**5**′ were subjected to ozonolysis, oxidative workup, and methyl esterification. These reactions yielded all possible stereoisomers of **6**′, which were analyzed by chiral-phase GC-MS (Figure S31). In addition, because the *syn*-isomers were only partially lactonized, the acyclic dimethylester products (2*S*,4*S*)-**6** and (2*R*,4*R*)-**6** were also detected together with their respective lactones. The lipopeptides were subjected to ozonolysis at −78 °C, followed by oxidative workup, acid hydrolysis, and methyl esterification (Scheme 3). The derivatized natural products were subsequently analyzed by chiral-phase GC-MS and gave a peak that coeluted with (2*R*,4*S*)-**6**′ (Figure S32). Hence, the 3*S*,5*R*-configuration was assigned to all three compounds.

Finally, to establish the absolute stereochemistry at C-43 in **1**−**2**, standards were prepared by the methyl esterification of commercially available, optically pure 2-hydroxyisovaleric acid (hiv) **7**. In addition, in the cases of C-43 and C-44 in **3**, the isoleucine stereoisomers **9** were diazotized and hydrolyzed to afford the corresponding 2-hydroxy-3-methylpentanoic acids (hmpa),[18] which were converted to the respective methyl esters **10** (Scheme 2). Meanwhile, the three lipopeptides were individually subjected to ozonolysis in a 1:2 mixture of 2.5 M methanolic NaOH and CH_2Cl_2 at −78 °C, thus cleaving the $\Delta^{41,42}$ olefin to a methyl ester (Scheme 3).[19] We decided to resort to such conditions because conventional ozonolysis, as well as OsO_4-catalyzed cleavage with Oxone,[20] failed to break this double bond. Subsequently, methanolysis was performed by heating the reaction mixture at 50 °C for 1 h. These reactions conveniently converted the 4-hydroxy-2*E*-enoyl substituents in **1** and **2** to the hiv methyl ester **8**, and that in **3** to the hmpa methyl ester **10**. The ozonolyzed methanolysates were then analyzed by GC-MS, and the retention times were compared with the appropriate synthetic standard. Based on the chiral-phase GC-MS results, **1** and **2** possess an *S*-

Scheme 2. Synthesis of the Stereoisomers of 8 and 10



Scheme 3. Degradation Schemes of 1−3 To Determine the Absolute Configurations at C-3, C-5, C-43 (and C-44)



configuration at C-43, since the methyl 2-hydroxyisovalerate detected in their ozonolyzed methanolysates coeluted with synthetic (*S*)-**8** (Figure S33). In the case of **3**, a *syn*-stereochemistry was initially assigned to the C-43/C-44 bond using nonchiral GC-MS conditions (Figure S34). On the other hand, under the chiral conditions employed, the derivatized **3** lacked a peak corresponding to **10**. Thus, the heating at 50 °C was continued overnight, and the subsequent chiral-phase GC-MS analysis revealed the presence of (2*R*,3*S*)- and (2*S*,3*S*)-**10** (Figure S35). This led to the conclusion that C-44 should have an *S*-stereochemistry. As for the C-43 position, it also possesses an *S*-configuration on the basis of the following: (1) nonchiral GC-MS analysis established a *syn*-stereochemistry; (2) **1** and **2** both have an *S*-stereochemistry at this position, making it more biogenetically plausible to have a 43*S*-stereochemistry, as well. Therefore, the complete structures were concluded to be those depicted in Figure 1.

Lipodiscamides A−C are structurally unique cyclic lipopentapeptides with the 3-hydroxyl group of the fatty acyl chain attached to the C-terminus of the amino acid chain by an ester linkage forming a macrocylic lactone. They are the first characterized D-Cit-bearing lipopeptides.[14,21−23] In addition, there is only one example of a secondary metabolite bearing a 3-Uda residue and only one report of the occurrence of Denor, which was found in a thiopeptide antibiotic.[24−26] The α,β-dehydroamino acids are known to be components of bioactive peptides, and their geometrical configurations significantly affect their bioactivity. Although the *Z*-isomer is thermodynamically more stable,[27,28] lipodiscamides have the unusual *E*-Denor residue. Furthermore, the presence of the 4-hydroxy-2*E*-enoyl unit is unprecedented. The same subunit, but with the *cis*

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geometry, exists in antitumor substances isolated from a *Pseudomonas* sp.[29] Interestingly, in lipodiscamides, this 4-hydroxyl group is linked to the fatty acyl chain by a second ester bond within the macrocycle. As such, lipodiscamides serve as the first examples of a new family of dilactone lipopeptides.

In terms of bioactivity, lipodiscamides A–C showed moderate cytotoxicity against P388 murine leukemia cells, with IC_{50} values of 23, 20, and 31 μM, and moderate to weak cytotoxicity against HeLa cells, with IC_{50} values of 18, 26, and 46 μM, respectively. However, they did not exhibit antimicrobial activity against *Bacillus cereus*, methicillin-sensitive *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. This unexpected lack of antimicrobial activity might be due to the insufficient overall positive charge contributed by the carbamoylated functionalities of these molecules.

■ ASSOCIATED CONTENT

Supporting Information

Description of experimental procedures and spectroscopic data of **1**–**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

The abstract graphic has been updated and the R2 substituents in compounds 1 and 2 in Figure 1 have been corrected. The revised version was re-posted on June 20, 2014.