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Minutissamides A-D, Antiproliferative Cyclic Decapeptides from the Cultured Cyanobacterium *Anabaena minutissima*

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Abstract

Four cyclic decapeptides, minutissamides A-D (**1** - **4**), were isolated from the cultured cyanobacterium *Anabaena minutissima* (UTEX 1613). The planar structures were determined using various spectroscopic techniques including HRESIMS, and 1D and 2D NMR experiments. The absolute configurations of the α -amino acid residues were assigned using Marfey's method after acid hydrolysis. The absolute configuration of a β -amino acid residue was assigned by a combination of the advanced Marfey's method, *J*-based configurational analysis and ROE spectroscopic analysis. The structures of minutissamides A-D (**1** - **4**) were characterized by the presence of three non-standard α -amino acid residues (two α,β -dehydro- α -aminobutyric acids and one *N*-methylated Asn) and one β -amino acid residue (2-hydroxy-3-amino-4-methyl dodecanoic acid or 2-hydroxy-3-amino-4-methyl hexadecanoic acid). Minutissamides A-D (**1** - **4**) exhibited antiproliferative activity against the HT-29 human colon cancer cell line with IC₅₀ values of 2.0, 20.0, 11.8 and 22.7 μ M, respectively.

Introduction

Cyanobacteria (blue-green algae) have been shown to be prolific producers of bioactive secondary metabolites.¹⁻³ A major class of secondary metabolites from cyanobacteria is polyketide-conjugated nonribosomal peptides, commonly known as lipopeptides.^{4,5} Numerous cyclic lipopeptides have been isolated from both freshwater and marine cyanobacteria with ring sizes of up to 12 peptide residues. Cyclic deca- and undecapeptides, containing 10 and 11 residues, have been reported to exhibit a wide spectrum of biological activities, including antifungal,^{6,7} cardiotonic,^{8,9} and cytotoxic.^{10,11} A common structural feature of cyclic deca- and undecapeptides is the occurrence of one lipophilic β -amino acid residue with a structurally unique side chain.¹² Amination of fatty acids at C-3 forming β -amino acids provides the branching point via an amide bond with other α -amino acids, whereas C-2 is usually methylene, methylated or hydroxylated. Further fatty acid modifications include methylation, oxidation, chlorination and/or hydroxylation of the side chain, resulting in the high diversity of modified β -amino fatty acids. Most of the cyclic lipopeptides in this class also possess non-standard amino acids, such as *N*-methylated α -amino acids, α -hydroxy or β -hydroxy amino acids, and Dhb (α , β -dehydro- α -aminobutyric acid).

In our continuing search for secondary metabolites from laboratory-cultured cyanobacteria, we evaluated the cell extract of cultured *Anabaena minutissima* (UTEX 1613). Herein we

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Supporting Information Available: Complete NMR data tables of **2** - **4**; HPLC chromatograms of Marfey's and the advanced Marfey's derivatives of **1**; ¹H, COSY, TOCSY, HSQC and HMBC spectra of **1-4**; DEPTQ spectra of **1**, **3** and **4**; ROESY spectrum of **1**; Q-TOF Tandem MS spectrum of **1**. This information is available free of charge via the Internet at <http://pub.acs.org>.

report the isolation, structure elucidation and biological activity of four new cyclic decapeptides, named minutissamides A-D, each possessing two Dhbs, one *N*-methylated Asn and one β -amino acid, Hamd (2-hydroxy-3-amino-4-methyl dodecanoic acid) or modified Hamh (2-hydroxy-3-amino-4-methyl hexadecanoic acid). The planar structures were determined using a combination of spectroscopic techniques including HRESIMS, tandem MS, and 1D and 2D NMR (COSY, TOCSY, HSQC, HMBC and ROESY) experiments. The absolute configurations of the α -amino acid residues were determined by Marfey's method, whereas the stereoconfiguration of the Hamd residue in **1** was solved by a combination of the advanced Marfey's method, *J*-based configurational analysis and ROE correlations.

Results and Discussion

Anabaena minutissima (UTEX 1613) was grown in Z media.¹³ The freeze-dried cells were extracted with a mixture of CH₂Cl₂ and MeOH (1:1), and dried in vacuo. The cell extract was subsequently fractionated by column chromatography using Diaion HP-20 resin and an increasing amount of isopropyl alcohol (IPA) in H₂O. LC-MS analysis of the third fraction (40% IPA) indicated the presence of peptides with molecular weights of 1118, 1152, 1188 and 1190 Da. Subsequent Sephadex LH-20 column chromatography followed by reverse-phased HPLC yielded minutissamides A-D (**1** - **4**).

Minutissamide A (**1**) was obtained as a colorless amorphous powder. The molecular formula of **1** was determined as C₅₁H₈₃N₁₃O₁₅ by HRESIMS analysis (*m/z* 1140.6049 [M+Na]⁺). The ¹H NMR spectrum in DMSO-*d*₆ showed a signal distribution typical for lipopeptides, including several exchangeable amide NH signals (6.5 – 10.5 ppm), signals in the α -proton region (4 – 6 ppm), methylene signals (1.2 – 1.3 ppm), and numerous aliphatic doublet and triplet methyl signals (0.5 – 2.0 ppm). The structures of the amino acid residues were elucidated by interpretation of the COSY and TOCSY spectra (Figure 1 and Table 1), and indicated the presence of 10 residues, including nine α -amino acid residues and one β -amino acid residue. Six of the α -amino acid residues were identified as the standard amino acids: proline (Pro), threonine (Thr), alanine (Ala), valine (Val) and two asparagines (Asn). In addition, the structures of three non-standard amino acid residues were determined by analysis of COSY, HSQC and HMBC data (Figure 1). An HMBC correlation from the *N*-Methyl singlet (δ_{H} 2.96) to the C-2_{NMeAsn} (δ_{C} 49.4) in combination with a COSY correlation between *N*MeAsn H-2 (δ_{H} 5.53) and *N*MeAsn H₂-3 (δ_{H} 2.00 and 3.02) identified the structure of *N*-methyl asparagine (*N*MeAsn). The presence of two Dhb residues was deduced by COSY correlations observed between the methyl protons (H₃-4_{Dhb1}, δ_{H} 1.90 and H₃-4_{Dhb2}, δ_{H} 1.66) and the corresponding olefinic proton (H-3_{Dhb1}, δ_{H} 5.71 and H-3_{Dhb2}, δ_{H} 5.30), and HMBC correlations from the methyl protons (H₃-4_{Dhb1} and H₃-4_{Dhb2}) to the corresponding olefinic carbon (C-2_{Dhb1}, δ_{C} 130.1 and C-2_{Dhb2}, δ_{C} 133.0). The 2-hydroxy-3-amino-4-methyl portion in Hamd was identified by sequential COSY correlations between H-2 (δ_{H} 4.18)/H-3 (δ_{H} 3.93)/H-4 (δ_{H} 1.65), as well as COSY correlations between H-3 (δ_{H} 3.93) and 3-NH (δ_{H} 6.85) and between H-4 (δ_{H} 1.65) and 4-Me (δ_{H} 0.58). Further COSY correlations from H-4 (δ_{H} 1.65) to H₂-5 (δ_{H} 1.17 and 1.61), H₂-5 to H₂-6 (δ_{H} 1.18 and 1.24), and H₂-6 to the region of highly overlapped methylene signals (H₂-7-11, δ_{H} 1.26-1.27), corresponding to five carbons in the HSQC spectrum (δ_{C} 30.1, 29.2, 29.7, 31.8 and 22.6) and finally to the methyl triplet protons H₃-12 (δ_{H} 0.87), completed the structure of Hamd.

The sequence of the 10 residues was established by analysis of the ROESY and selective HMBC spectra (Figure 1 and Table 1). A selective HMBC spectrum for the carbonyl region was used to resolve the overlapped amide carbonyl signals.¹⁴ HMBC correlations from Dhb₂-NH (δ_{H} 9.17) to Val C-1 (δ_{C} 169.2), from Val H-2 (δ_{H} 4.29) to Hamd C-1 (δ_{C} 170.1)

and from Hamd H-3 (δ_{H} 3.93) to Pro C-1 (δ_{C} 171.9) suggested a partial sequence of Dhb₂-Val-Hamd-Pro. ROE correlations between Dhb₂-NH (δ_{H} 9.17) and Val H-2 (δ_{H} 4.29), between Val-NH (δ_{H} 6.85) and Hamd H-2 (δ_{H} 4.18), and between Hamd-NH (δ_{H} 6.85) and Pro H-2 (δ_{H} 4.19) confirmed this partial sequence. HMBC correlations from Pro H-2 (δ_{H} 4.19) to NMeAsn C-1 (δ_{C} 168.3) and from NMeAsn N-Me (δ_{H} 2.96) to Thr C-1 (δ_{C} 170.4), together with ROE correlations between Pro H₂-5 (δ_{H} 4.10) and NMeAsn H-2 (δ_{H} 5.53), and between NMeAsn N-Me (δ_{H} 2.96) and Thr H-2 (δ_{H} 4.63), further expanded the sequence to Dhb₂-Val-Hamd-Pro-NMeAsn-Thr. HMBC correlations from Thr NH (δ_{H} 7.30) to Ala C-1 (δ_{C} 172.5) and from Ala NH (δ_{H} 8.27) to Asn₁ C-1 (δ_{C} 171.2), together with ROE correlations between Thr-NH (δ_{H} 7.30) and Ala H-2 (δ_{H} 4.29), and between Ala-NH (δ_{H} 8.27) and Asn₁ H-2 (δ_{H} 4.40), established the sequence of Dhb₂-Val-Hamd-Pro-NMeAsn-Thr-Ala-Asn₁, leaving two residues (Dhb₁ and Asn₂) unassigned. The complete sequence of Dhb₂-Val-Hamd-Pro-NMeAsn-Thr-Ala-Asn₁-Dhb₁-Asn₂ was deduced by an HMBC correlation from Asn₁-NH (δ_{H} 8.45) to Dhb₁ C-1 (δ_{C} 163.4) and a ROE correlation between Dhb₁-NH (δ_{H} 10.18) and Asn₂ H-2 (δ_{H} 4.92). Lastly, HMBC correlations from Asn₂ H-2 (δ_{H} 4.92) and NH (δ_{H} 8.73) to Dhb₂ C-1 (δ_{C} 163.6) closed the ring, completing the planar structure of **1**.

Mass fragmentation analysis was performed to further verify the structure of **1**.¹⁵ The ESI-MS/MS analysis, using a quadrupole-time of flight (Q-TOF) tandem mass spectrometer, supported the proposed planar structure of **1**. The parent peak was observed at m/z [M+H]⁺ 1118.6. The ring opening occurred between Pro and NMeAsn, forming an acylium ion. The continuous fragmentation yielded the fragment ions at m/z 990.6 [M + H - NMeAsn]⁺, 889.5 [M + H - NMeAsn - Thr]⁺, 704.4 [M + H - NMeAsn - Thr - Ala - Asn₁]⁺, 621.4 [M + H - NMeAsn - Thr - Ala - Asn₁ - Dhb₁]⁺, 507.4 [M + H - NMeAsn - Thr - Ala - Asn₁ - Dhb₁ - Asn₂]⁺ and 396.3 [M + H - NMeAsn - Thr - Ala₁ - Asn₂ - Dhb₂]⁺ (Figure 2). This fragmentation pattern was in complete agreement with the structure determined by 2D NMR analysis.

Minutissamide A (**1**) contained three different types of stereogenic elements, the geometric configuration of Dhbs, the configurations of the α -amino acid residues and the configurations of the three consecutive asymmetric centers in the Hamd residue. A number of techniques were used for the assignment of stereoconfiguration of **1**, including Marfey's method and the advanced Marfey's method, *J*-based configurational analysis and ROE correlations. A strong ROE correlation between the respective NH (δ_{H} 10.18 or δ_{H} 9.17) and the corresponding olefinic proton (H-3, δ_{H} 5.71 or δ_{H} 5.30) was observed for both Dhb₁ and Dhb₂, assigning the geometric configuration of both double bonds as *E* (Figure 1). The absolute configurations of the common amino acids and NMeAsn were assigned as L-Pro, L-Thr, L-Asn₁, L-Asn₂, L-Val, D-Ala and L-NMeAsn by chromatographic comparison of Marfey's derivatives of the acid hydrolysate of **1** and appropriate amino acid standards.¹⁶ The relative configurations of the three asymmetric centers (C-2, C-3 and C-4) in the Hamd residue were established by combination of *J*-based configurational analysis and ROE correlations (Figure 3). Homonuclear (³*J*_{H-H}) coupling constants were acquired from the DQF-COSY spectrum, and the phase-sensitive HMBC spectrum was used to obtain the heteronuclear (²*J*_{CH} and ³*J*_{CH}) coupling constants.¹⁷ The small ³*J*_{HH} (5.6 Hz) between H-2 and H-3, the large ³*J*_{CH} (5.4 Hz) between H-3 and C-1, and the large ²*J*_{CH} (-5.1 Hz) between H-3 and C-2 allowed for two possible conformations (A2 and A6) out of the six conformations shown in Figure 3a. The conformation A6 was found to be a correct conformation based on a ROE correlation observed between H-2 and H-4, assigning the relative configuration between C-2 and C-3 as "*erythro*." The same method was applied for the assignment of the relative configuration between C-3 and C-4. On the basis of the large ³*J*_{HH} (11.0 Hz) measured between H-3 and H-4, only two conformations (B3 and B4) were possible as shown in Figure 3b. A ROE correlation observed between H-2 and H₂-5

confirmed B3 as the correct confirmation assigning the relative configuration between C-3 and C-4 as “*threo*.” The nearly identical carbon chemical shifts of C-1, C-2, C-3 and C-4 of Hamd (δ_C 170.1, 70.0, 56.6 and 32.6) to those of Hamh (δ_C 169.8, 69.9, 56.2 and 32.4) in puwainaphycin E provided further evidence for this relative configuration.¹⁰ Because the relative configurations of C2-C3 and C3-C4 were *erythro* and *threo*, respectively, the absolute configurations of the three stereogenic centers (C-2, C-3 and C-4) in the Hamd residue could be either “*RRS*” or “*SSR*”. The advanced Marfey’s method was applied to assign the absolute configuration of the Hamd residue.^{18, 19} The acid hydrolysate of **1** was derivatized with L- and DL-FDLA, respectively. Subsequent LC-MS analysis of DL-FDLA derivatives identified two peaks of the FDLA derivatives of Hamd (m/z 538 [M-H][−]) at 43.3 and 48.8 min, while the L-FDLA derivative of Hamd resulted in one peak at 48.8 min. Previous application of this method for the determination of the absolute configuration of Ahda (amino-2-hydroxy decanoic acid) identified that the R-L and S-D derivatives are more hydrophobic than the R-D and S-L derivatives.²⁰ In our experiment, the L-FDLA derivative of **1** corresponded to the later peak of DL-FDLA derivatives, suggesting the presence of R-L. As a result, the absolute configuration of C-3 in Hamd was assigned as *R*, which allowed for the assignment of the absolute configurations at C-2, C-3 and C-4 in Hamd as “*RRS*.”

Minutissamide B (**2**) was obtained as a colorless amorphous powder. The molecular formula of **2** was deduced as C₅₁H₈₂ClN₁₃O₁₅ on the basis of HRESIMS analysis (m/z 1174.5680 [M+Na]⁺). A M+2 isotope peak verified the presence of chlorine in **2**. The ¹H and ¹³C NMR spectra of **2** were almost identical to those of **1** except for the Hamd residue, where the terminal methyl proton signal had been replaced by a new triplet methylene resonance at 3.62 ppm. This indicated that chlorine was positioned on the terminal carbon of Hamd, thus re-naming it Hamcd (2-hydroxy-3-amino-4-methyl-12-chloro dodecanoic acid). The sequential COSY correlation network of H₂-12/H₂-11/H₂-10 and the slightly down-fielded chemical shifts of H₂-11 (δ_H 1.70) and H₂-10 (δ_H 1.37) further supported the structure of **2**. The stereoconfiguration of **2** was determined by comparison of proton and carbon chemical shifts as well as specific rotation to those of **1**. The carbon chemical shifts of all of the stereogenic centers in **2** were similar to those observed for **1** (deviation, < 0.2 ppm), suggesting the same relative configurations. The specific rotations of both **1** and **2** displayed positive values (**1**, [α]_D +4; **2**, [α]_D +5). On the basis of these data and a shared biosynthesis, we propose that the absolute configurations of all of the stereogenic centers in **2** are the same as those found in **1**.

Minutissamide C (**3**) was obtained as a colorless amorphous powder. The HRESIMS analysis (m/z 1210.6440 [M+Na]⁺) suggested the molecular formula of **3** to be C₅₅H₈₉N₁₃O₁₆. Detailed comparison of the NMR spectra indicated that compounds **1** and **3** share the same cyclic decapeptide core structure. The difference was the replacement of the dodecanoic acid residue found in **1** with an oxidized hexadecanoic acid residue in **3** (Table 2). The ¹H NMR spectrum of **3** displayed three additional methylene protons, H₂-12 (δ_H 1.44), H₂-13 (δ_H 2.38) and H₂-15 (δ_H 2.40), as compared to **1**. In addition to three methylene carbon signals (δ_C 23.6, δ_C 41.9 and δ_C 35.4), a signal characteristic of a ketone (δ_C 211.5) was also apparent in the ¹³C NMR spectrum. The molecular weight difference of 70 between **3** and **1** corresponded to three methylenes and one ketone, indicating the presence of a Hamoh (2-hydroxy-3-amino-4-methyl-14-oxo hexadecanoic acid) moiety in **3**. The location of the ketone group at C-14 in Hamoh was determined by the proton chemical shifts of H₂-13 (δ_H 2.38) and H₂-15 (δ_H 2.40), and a TOCSY correlation observed between H₂-15 (δ_H 2.40) and H₃-16 (δ_H 0.90). An HMBC correlation from the terminal methyl protons (H₃-16, δ_H 0.90) to the ketone carbon (C-14, δ_C 211.5) further confirmed this location. The stereoconfiguration of **3** was assigned by comparison of spectroscopic data to those of **1**. No significant difference (deviation, < 0.1 ppm) was found for the ¹³C chemical shifts of all of the stereogenic centers in **3** when compared to **1**. Also, compound **3** displayed

a similarly small positive specific rotation ($[\alpha]_D +3$) to **1**. Together, these data suggested the absolute configurations of the stereogenic centers in **3** to be identical to those of **1**.

Minutissamide D (**4**) was also obtained as a colorless amorphous powder. The HRESIMS spectrum of **4** displayed a major ion peak at 1212.6632 $[M+Na]^+$, suggesting a molecular formula of $C_{55}H_{91}N_{13}O_{16}$. The 1H and ^{13}C NMR spectra of **4** were almost identical to those of **3**, except that the ketone in the Hamoh moiety had been replaced by resonances for a carbinol moiety (δ_H 3.28 and δ_C 71.5). This, together with the molecular weight difference of two between **4** and **3**, suggested that the ketone in Hamoh of **3** had been reduced to a hydroxy group in **4**, forming a Hamhh residue (2-hydroxy-3-amino-4-methyl-14-hydroxy hexadecanoic acid). The structure of Hamhh was further supported by analysis of the COSY and HMBC spectra. Sequential COSY correlations between H_3-16 (δ_H 0.83)/ H_2-15 (δ_H 1.16 and 1.35)/ $H-14$ (δ_H 3.28) and an HMBC correlation from H_3-16 (δ_H 0.83) to C-14 (δ_C 71.5) placed the hydroxy group at C-14. The absolute configurations of the amino acid residues and the three asymmetric centers in the Hamhh residue appeared to be the same as those found for **1** by comparison of ^{13}C chemical shifts and specific rotation ($[\alpha]_D +2$). Compound **4** possessed one more stereogenic hydroxy-bearing carbinol carbon (C-14) in the Hamhh residue. Mosher's ester analysis was attempted in an effort to assign the absolute configuration of this carbinol center. However, the presence of three hydroxy groups (one in Thr and two in Hamhh) resulted in a mixture of products and a very complex 1H NMR spectrum of the Mosher's ester products of **4**. Hydrolysis of **4** followed by isolation of the Hamhh residue would be necessary for Mosher's ester analysis, but was not attempted due to the limited amount of sample available. Thus, the absolute configuration of the C-13 carbinol carbon in Hamhh could not be assigned conclusively.

The structures of minutissamides A-D (**1-4**) were characterized by the presence of a β -amino acid residue, a 2-hydroxy-3-amino-4-methyl dodecanoic or hexadecanoic acid, and three nonstandard amino acid residues (*N*MeAsn and two Dhbs). The β -amino acid residues were further modified by chlorination in **2**, by oxidation to ketone in **3** or by hydroxylation in **4**. Interestingly, the structures of minutissamides A-D (**1-4**) revealed some similarities to the cyclic decapeptides puwainaphycins A-E, isolated from a Hawaiian terrestrial *Anabaena* sp.⁹ The sequence of the five residues in the puwainaphycins, including Dhb, Val, β -amino acid unit, Pro and *N*MeAsn, was conserved in the minutissamides. The other five amino acid residues, OMT (O-methyl-Thr), Gly, Gln, Thr-2 (or Val-2) and Thr-1 found in the puwainaphycins, were replaced by Thr, Ala, Asn1, Dhb1 and Asn2 in the minutissamides. The core structure of the lipophilic β -amino acid residues, characterized by 2-hydroxy-3-amino-4-methyl substitution, as well as the chlorination and oxidation patterns were also similar to those observed in the puwainaphycins.

Minutissamides A-D (**1-4**) were evaluated for their antiproliferative activity against the HT-29 human colon cancer cell line. Minutissamide A (**1**) displayed antiproliferative activity with an IC_{50} value of 2.0 M, whereas lower activity was observed for minutissamides B-D (**2-4**) with IC_{50} values of 20.0, 11.8 and 22.7 M, respectively. All four minutissamides have identical cyclic peptide cores and only differ in the lipophilic β -amino acid residue. For example, the only structural difference between minutissamides A (**1**) and B (**2**) was the presence of a chlorine atom at C-12 of this residue, but minutissamide A (**1**) was found to be 10-fold more active in the HT-29 assay. This suggests that the β -amino acid residue plays an important role for antiproliferative activity of these compounds.

Experimental Section

General Experimental Procedures

Optical rotations were measured using a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Shimadzu UV spectrometer UV2401 and scanned from 190 to 360 nm. 1D and 2D NMR spectra were obtained on a Bruker Avance DRX 600 MHz NMR spectrometer with a 5 mm CPTXI Z-gradient probe and a Bruker Avance II 900MHz NMR spectrometer with a 5 mm ATM CPTCI Z-gradient probe. ^1H and ^{13}C NMR chemical shifts were referenced to the DMSO- d_6 solvent signals (δ_{H} 2.50 and δ_{C} 39.51, respectively). A mixing time of 60 ms was set for the TOCSY experiments and 200 ms for the T-ROESY experiment. The HMBC spectra were recorded with the $^3J_{\text{C-H}}$ set to 8 Hz, and the HSQC spectra were collected with the $^1J_{\text{C-H}}$ set to 140 Hz. High-resolution ESI mass spectra were obtained using a Shimadzu IT-TOF LC mass spectrometer. Tandem mass analysis was performed using a Micromass Q-TOF LC mass spectrometer.

Biological Material

Anabaena minutissima was acquired from the Culture Collection of Algae at the University of Texas at Austin (UTEX 1613). The cyanobacterium was grown in 20 L flasks containing 18 L inorganic media (Z media).¹³ Cultures were illuminated with fluorescent lamps at 1.03 klx. The temperature of the culture room was maintained at 22 °C. After 6-8 weeks, the biomass of cyanobacteria was harvested by centrifugation and then freeze-dried.

Extraction and Isolation

The freeze-dried biomass (5.2 g) from the total 54 L (3×18 L) culture was harvested and extracted with CH_2Cl_2 -MeOH (1:1) and concentrated in vacuo to yield 1.1 g of the extract. The extract was fractionated using Diaion HP-20 with increasing amounts of IPA in H_2O to generate eight sub-fractions (0, 20, 40, 60, 70, 80, 90, 100 % aqueous IPA). Fraction 3 (40 % IPA) indicated the presence of four peptides by LC-MS. This fraction was further purified by Sephadex LH-20 column chromatography in MeOH. Fractions 7-9 containing the peptides were subjected to reverse-phased HPLC (Varian C_8 semi-preparative column, 10 mm × 250 mm, 3 mL/min) eluting with a linear gradient from 60 to 80% aqueous MeOH for 45 min. Minutissamides A-D (**1** - **4**) were eluted at 37.9 min (**1**, 1.4 mg), 31.6 min (**2**, 0.4 mg), 30.0 min (**3**, 1.0 mg) and 33.5 min (**4**, 0.7 mg), respectively.

Minutissamide A (**1**)

colorless amorphous powder; $[\alpha]_{\text{D}}^{25} + 4$ (c 0.06, MeOH); UV (MeOH) λ_{max} (log ϵ) 243 (3.45) nm; IR (neat) 3317 (br), 2925, 2861, 1681, 1635 (br), 1561, 1541 cm^{-1} ; ^1H and ^{13}C NMR (see Table 1); HRESIMS m/z 1140.6049 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{51}\text{H}_{83}\text{N}_{13}\text{O}_{15}\text{Na}$, 1140.6029)

Minutissamide B (**2**)

colorless amorphous powder; $[\alpha]_{\text{D}}^{25} + 5$ (c 0.02, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (4.24) nm; IR (neat) 3315 (br), 2926, 2852, 1670, 1647 (br), 1545, 1516, 1459, 1402 cm^{-1} ; ^1H and ^{13}C NMR (see Table 1); HRESIMS m/z 1174.5680 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{51}\text{H}_{83}\text{ClN}_{13}\text{O}_{15}\text{Na}$, 1174.5640)

Minutissamide C (**3**)

colorless amorphous powder; $[\alpha]_{\text{D}}^{25} + 3$ (c 0.07, MeOH); UV (MeOH) λ_{max} (log ϵ) 242 (2.62) nm; IR (neat) 3315 (br), 2926, 2857, 1664, 1630 (br), 1533, 1447, 1402 cm^{-1} ; ^1H and ^{13}C NMR (see Table 2); HRESIMS m/z 1210.6440 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{55}\text{H}_{89}\text{N}_{13}\text{O}_{16}\text{Na}$, 1210.6448)

Minutissamide D (4)

colorless amorphous powder; $[\alpha]_D^{25} + 2$ (*c* 0.05, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 240 (3.94) nm; IR (neat) 3320 (br), 2926, 2852, 1664, 1636 (br), 1539, 1453, 1379 cm^{-1} ; ^1H and ^{13}C NMR (see Table 2); HRESIMS m/z 1212.6632 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{55}\text{H}_{91}\text{N}_{13}\text{O}_{16}\text{Na}$, 1212.6604)

Determination of Amino Acid Configurations

Approximately 0.3 mg of minutissamide A was hydrolyzed using 6 N HCl (500 μL) in a high-pressure tube for 16 h at 110 $^{\circ}\text{C}$. The hydrolysate was dried under vacuum and re-dissolved in H_2O . This procedure was repeated three times to completely remove the remaining HCl. For the derivatization with Marfey's reagent (FDAA), the hydrolysate (0.1 mg) or amino acid standards was dissolved in 50 μL of H_2O , and 20 μL of 1N NaHCO_3 and 110 μL of acetone were added. The reaction was initiated by adding 20 μL of FDAA solution (10 mg/mL w/v in acetone), proceeded for 1 h at 40 $^{\circ}\text{C}$ and was quenched by adding 20 μL of 1N HCl. The reactant was dried under vacuum and re-dissolved in CH_3CN for HPLC analysis. Chromatographic analysis of the FDAA derivatives was performed on an Alltech C_{18} reverse-phased column (5 μm , 250 $\times$ 4.6 mm) with the flow rate of 1.0 mL/min. Aqueous CH_3CN containing 0.1% formic acid (FA) was used as a mobile phase eluting with a linear gradient from 10% to 100% (A: H_2O containing 0.1% FA and B: CH_3CN containing 0.1% FA, gradient condition: 0 min-10% B, 5 min-10% B, 35 min-20% B, 65 min-30% B, 70 min-60% B, 72 min-100% B and 80 min-100% B). The absolute configurations of the amino acids were assigned by comparing the retention times with those of the corresponding amino acid standards. The retention times of the FDAA derivatives of the authentic amino acid standards were identified at 30.70 (L-Thr and N-Me-D-Asp), 32.30 (L-Asp), 36.17 (N-Me-L-Asp), 36.94 (D-Asp), 41.06 (L-Ala), 42.54 (D-Thr), 44.57 (L-Pro), 49.84 (D-Pro), 51.76 (D-Ala), 59.09 (L-Val) and 71.45 (D-Val) min. The FDAA derivatives of the amino acids from the hydrolysate of **1** showed the peaks at 32.30, 36.17, 44.57, 51.76 and 59.09 min corresponding to L-Asp, N-Me-L-Asp, L-Pro and L-Val, respectively. The peak at 30.70 min was identified as L-Thr by LC-MS analysis (m/z 372, $[\text{M} + \text{H}]^+$).

Advanced Marfey's Analysis

L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) was synthesized using the protocol published for Marfey's analysis.²¹ The acid hydrolysate (0.2 mg) was separated into two equal portion for derivatization with either L-FDLA or DL-FDLA. Each portion was dissolved in 50 μL of H_2O and then mixed with 20 μL of 1N NaHCO_3 and 110 μL of acetone. Finally, 20 μL of L-FDLA or DL-FDLA (10 mg/mL w/v in acetone) was added, and the mixtures were heated to 40 $^{\circ}\text{C}$ for 1 h. The reaction mixtures were cooled to room temp., and 20 μL of 1N HCl was added to quench the reaction. After drying, the FDLA derivatives were dissolved in CH_3CN for LC-MS analysis. The chromatograms of L-FDLA and DL-FDLA derivatives were compared for the assignment of the absolute configuration of Hamd in **1**. LC-MS analysis was performed using a Varian reverse-phased C_{18} column (250 $\times$ 2.0 mm) at the flow rate of 0.4 mL/min. The linear gradient eluting from 20% to 75% aqueous CH_3CN (0.1% FA) for 50 min was used as a mobile phase, and ESI was used as the ionization method. Two peaks corresponding to the L- and D-FDLA derivatives of Hamd were observed at 43.33 and 48.84 min (m/z 538, $[\text{M}-\text{H}]^-$), respectively. The L-FDLA derivative of Hamd gave one peak at 48.84 min.

HT-29 Antiproliferative Assay

Antiproliferative activity against the HT-29 cancer cell line was performed according to established protocols.²²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Key 2D correlations of minutissamide A (**1**) that were used for the determination of the planar structure of **1**.

Figure 2.
ESI-MS/MS fragmentation of minutissamide A (1).

Figure 3.

Newman projections for (a) C-2/C-3 and (b) C-3/C-4. All possible relative conformations are shown. The DQF-COSY and phase-sensitive HMBC spectra were used for the calculation of homo- and hetero nuclear coupling constants. Labels below projections denote the predicted size of coupling constants. The predicted values that are consistent with observed values are highlighted by a box. Observed rOe correlations are presented as double-headed arrows: (a) H-2/H-4 and (b) H-2/H₂-5.

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Chart 1.

Table 1

NMR Spectroscopic Data for Minutissamides A and B (1 and 2) in DMSO- d_6

	Minutissamide A (1)					Minutissamide B (2) ^a		
	δ_C^b	δ_H^c	mult. (J in Hz)	COSY	HMBC	ROESY	δ_C^f	δ_H^e mult. (J in Hz)
1	170.1						170.0	
2	70.0	4.18	d (4.8)	3	3		70.0	4.18 overlapped
3	56.6	3.93	m	2, 4	1 ^e , 2, 1 _{Pro} ^e		56.6	3.92 M
4	32.6	1.65	m	3, 5, 4-Me			32.6	1.65 m
Hamd or Hamed								
5	33.8	1.17	m	4, 6			33.7	1.17 m
		1.61	m					1.61 m
6	25.9	1.18	m	5, 6			25.9	1.16 m
		1.24	m					1.23 m
7	30.1	1.26	m	6, 8			30.1	1.23 m
8	29.2	1.26	m	7, 9			29.7	1.23 m
9	29.7	1.26	m	8, 10			29.1	1.26 m
10	31.8	1.26	m	10, 11			26.6	1.37 m
11	22.6	1.27	m	11, 12			32.4	1.70 m
12	14.5	0.87	t (7.2)	11	10, 11		45.8	3.62 t (6.6)
2-OH		5.51	br s	2				nd ^d
3-NH		6.85	partly overlapped	3	1 _{Pro} ^e	3, 2 _{Pro}		6.88 partly overlapped
4-Me	16.3	0.58	d (6.6)	4	3, 4, 5	3	16.4	0.57 d (6.6)
Pro							171.9	
1	171.9							
2	60.5	4.19	m	3	3, 4, 1 _{NMeAsn}		60.4	4.18 m
3	30.7	1.91	m	2, 4	1		30.7	1.92 m
4	24.2	1.85	m	3, 5			24.2	1.83 m
		1.92	m					1.92 m
5	47.3	3.26	m	4	2	2 _{NMeAsn}	47.3	3.25 m
		4.10	m					4.09 m
NMeAsn							168.3	
1	168.3							

Minutissamide A (1)										Minutissamide B (2) ^a		
		δ_C^b	δ_H^c	mult. (J in Hz)	COSY	HMBC	ROESY	δ_C^f	δ_H^c	mult. (J in Hz)		
Thr	2	49.4	5.53	dd (12.0, 3.0)	3	1 ^e , 3, 4 ^e	5 _{Pro} , 3, N-Me	49.3	5.53	brd (12.0)		
	3	34.4	2.00	m	2, 4	1 ^e , 2 ^e , 4 ^e		34.4	2.00	m		
	4	172.1	3.02	dd (15.6, 12.0)					3.01	m		
	N-Me	30.7	2.96	s		2, 1 _{Thr} ^e	2 _{Thr} , 3 _{Thr}	30.7	2.95	s		
	NH ₂		5.92	s		3			6.03	m		
			7.52	s					7.52	m		
	1	170.4						170.4				
	2	55.3	4.63	dd (8.4, 3.6)	2-NH, 3	1 ^e , 3, 1 _{Ala} ^e	N-Me, 3, 4	55.3	4.62	m		
	3	66.8	3.92	m	2, 4			66.8	3.92	m		
	4	20.0	1.00	d (6.0)	3	2, 3		19.9	0.99	d (6.6)		
Ala	3-OH		5.13	br s						nd ^d		
	NH		7.30	d (7.8)	2	1 _{Ala}	2, 3		7.30	d (8.4)		
	1	172.5						172.5				
	2	48.9	4.29	q (7.2)	2-NH, 3	1 ^e , 1 _{Asn1} ^e		48.7	4.28	m		
	3	17.3	1.23	d (7.2)	2	1 ^e , 2		17.3	1.22	d (7.2)		
	NH		8.27	d (8.4)	2	2, 1 _{Asn1}	2, 2 _{Asn1} , NH _{Thr} , NH _{Asn1}					
	1	171.2						171.2				
	2	50.5	4.40	m	2-NH, 3	3	3	50.3	4.39	m		
	3	36.2	2.56	m	2	1 ^e , 2, 4 ^e		36.2	2.53	m		
	4	173.2	2.87	dd (18.6, 7.2)					2.88	dd (16.8, 7.2)		
Asn1	NH		8.45	d (7.2)	2	1 _{Dhb1}	2		8.45	d (7.2)		
	1	163.4										
	2	130.1										
	NH ₂		7.04	s		3			7.04	s		
			7.58	s					7.59	s		
	1	163.4						163.4				
	2	130.1						130.1				

		Minutissamide A (1)				Minutissamide B (2) ^a			
		δ_C^b	δ_H^c	mult. (J in Hz)	COSY	HMBC	ROESY	δ_C^f	δ_H^c
									mult. (J in Hz)
Asn2	3	125.6	5.71	q (7.2)	4	1, 2, 4	NH	125.6	5.71
	4	13.7	1.90	d (7.2)	3	2, 3		13.6	1.89
	NH		10.18	s		1, 2 _{Asn2} ^e	3, 2 _{Asn2}		10.20
									s
Dhb2	1	172.4						172.4	
	2	49.4	4.92	q (7.2)	2-NH, 3	1 ^e , 3, 1 _{Dhb2} ^e	3	49.4	4.91
	3	39.1	2.67	dd (15.0, 7.2)	2	1 ^e , 2, 4 ^e		39.2	2.67
	4	172.6	2.77	dd (15.0, 7.8)				172.7	2.77
NH									dd (15.0, 7.2)
									dd (15.0, 7.8)
NH ₂									
Val	1	163.6	8.73	d (7.8)	2	1 _{Dhb2}		163.6	8.74
	2	133.0	7.11	s		3		133.0	7.12
	3	116.5	5.30	q (7.2)	4	1, 2, 4	NH	116.4	5.29
	4	13.5	1.66	d (7.2)	3	2, 3		13.3	1.65
Val									d (7.2)
Val	1	169.2	9.17	s		1 ^e , 3, 1 _{Val} ^e	3, 2 _{Val}	169.2	9.21
	2	56.2	4.29	d (10.8)	2-NH, 3	1 ^e , 1 _{Hamd} ^e		56.2	4.28
	3	33.1	1.75	m	2, 4	1 ^e		33.1	1.74
	4	19.4	0.83	d (6.6)	3	2, 3, 4 ^e		19.3	0.82
Val	4 ^e	19.1	0.90	d (6.6)	3	2, 3, 4		19.0	0.89
									d (6.6)
Val	NH		6.85	partly overlapped	2		2 _{Hamd}	6.88	partly overlapped

^a Complete NMR table for minutissamide B (2) including 2D NMR data can be found in the Supporting Information

^b Carbon chemical shifts were assigned from DEPT-Q spectrum measured at 226 MHz

^c Measured at 600 MHz

^d nd: Not detected

ϵ Assigned from the selective HMBC spectrum

f Assigned from the HSQC and HMBC spectra.

Table 2

¹H and ¹³C NMR Data for Minutissamides C and D (**3** and **4**) in DMSO-*d*₆

	Minutissamide C (3) ^a					Minutissamide D (4) ^a				
	δ_C	δ_H	b	c	mult. (J in Hz)	δ_C	δ_H	b	c	mult. (J in Hz)
Hamoh or Hamhh	1		170.0			170.1				
	2		70.0	4.17	d (4.8)	70.0	4.18			d (4.2)
	3		56.5	3.92	m	56.6	3.92			m
	4		32.6	1.65	m	32.6	1.65			m
	5		33.8	1.17	m	33.9	1.17			m
				1.62	m		1.61			m
	6		25.9	1.16	m	25.9	1.16			m
				1.23	m		1.23			m
	7		30.1	1.23	m	30.1	1.25			m
	8		29.1	1.23	m	29.1	1.25			m
	9		29.7	1.23	m	29.7	1.25			m
Pro	10		29.4	1.23	m	29.4	1.25			m
	11		29.4	1.23	m	29.4	1.25			m
	12		23.6	1.44	q (7.2)	25.8	1.16			m
							1.35			m
	13		41.9	2.38	t (7.2)	35.4	1.26			m
							1.32			m
	14		211.5			71.5	3.28			m
	15		35.4	2.40	q (7.8)	30.1	1.16			m
							1.35			m
	16		8.2	0.90	t (7.8)	10.6	0.83			t (7.2)
	2-OH				nd ^d					nd ^d
3-NH			6.84	partly overlapped		6.87			partly overlapped	
4-CH ₃		16.4	0.57	d (6.6)	16.5	0.57			d (6.6)	
1		171.9			172.0					
2		60.6	4.18	m	60.6	4.18			m	

	Minutissamide C (3) ^a				Minutissamide D (4) ^a			
	δ_C	δ_H	δ_C	mult. (J in Hz)	δ_C	δ_H	δ_C	mult. (J in Hz)
NMeAsn	3	30.8	1.92	m	30.8	1.92	m	
	4	24.2	1.84	m	24.3	1.83	m	
	5	47.3	1.91	m	47.3	1.91	m	
			3.26	m		3.25	m	
			4.10	m		4.09	m	
Thr	1	170.4			170.4			
	2	55.3	4.62	dd (9.0, 3.6)	55.3	4.62	dd (8.4, 3.6)	
	3	66.8	3.93	m	66.8	3.91	m	
	4	20.0	0.99	d (6.0)	20.0	0.98	d (7.8)	
3-OH				nd ^d			nd ^d	
Ala	NH		7.29	d (9.0)		7.30	d (7.8)	
	1	172.6			172.6			
	2	48.9	4.28	m	49.0	4.28	m	
	3	17.3	1.22	d (7.2)	17.4	1.22	d (7.2)	
AsnI	NH		8.26	d (8.4)		8.26	d (8.4)	
	1	171.2			171.2			
	2	50.5	4.39	td (7.2, 3.0)	50.5	4.39	td (7.2, 3.0)	
	3	36.2	2.53	m	36.2	2.52	m	
NH	4	173.2	2.87	dd (16.8, 7.2)	173.2	2.87	dd (16.8, 7.2)	
			8.45	d (7.2)		8.44	d (7.2)	

		Minutissamide C (3) ^a				Minutissamide D (4) ^a			
		δ_C	δ_H	c	mult. (J in Hz)	δ_C	δ_H	c	mult. (J in Hz)
Dhb1	NH ₂		7.04	s			7.04	s	
	1	163.4				163.4			
	2	130.1				130.1			
	3	125.6	5.70	q (7.2)		125.8	5.70	q (7.8)	
	4	13.7	1.89	d (7.2)		13.7	1.89	d (7.8)	
Asn2	NH		10.18	s			10.19	s	
	1	172.4				172.4			
	2	49.4	4.90	q (7.2)		49.5	4.91	q (7.2)	
	3	39.0	2.66	dd (15.0, 7.2)		39.2	2.67	dd (15.0, 7.2)	
	4	172.6	2.76	dd (15.0, 7.8)			2.76	dd (15.0, 7.8)	
Dhb2	NH		8.73	d (7.8)			8.73	d (7.8)	
	1	163.6				163.7			
	2	133.0				133.0			
	3	116.4	5.29	q (7.2)		116.5	5.29	q (7.2)	
	4	13.5	1.65	d (7.2)		13.5	1.64	d (7.2)	
Val	NH		9.18	s			9.19	s	
	1	169.2				169.3			
	2	56.2	4.28	m		56.3	4.28	m	
	3	33.1	1.74	m		33.1	1.74	m	
	4	19.4	0.82	d (6.6)		19.4	0.82	d (6.6)	
	4'	19.1	0.89	d (6.6)		19.1	0.89	d (6.6)	
	NH		6.84	partly overlapped			6.86	partly overlapped	

^aComplete NMR tables for minutissamides C (3) and D (4) including 2D NMR data can be found in the Supporting Information

^bCarbon chemical shifts were assigned from DEPT-Q spectrum measured at 226 MHz

^cMeasured at 600 MHz