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Termination of the structural confusion between plipastatin A1 and fengycin IX

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ABSTRACT

Plipastatin A1 and fengycin IX were experimentally proven to be identical compounds, while these had been considered as diastereomers due to the permutation of the enantiomeric pair of Tyr in most papers. The ^{1}H NMR spectrum changed to become quite similar to that of plipastatin A1, when the sample which provided resembled spectrum of fengycin IX was treated with KOAc followed by LH-20 gel filtration. Our structural investigations disclosed that the structures of these molecules should be settled into that of plipastatin A1 by Umezawa (L-Tyr4 and D-Tyr10).

1. Introduction

Plipastatin A1 is a macrocyclic depsilipopeptide which was first isolated by Umezawa et al. in 1986 from *Bacillus cereus* BMG3O2-fF67 as a phospholipase A_2 inhibitor.[1–4] In the same issue of the journal, Jung et al. independently reported fengycin from *Bacillus subtilis* strain F-29-3 as an antibiotic lipopeptide.[5] Although the final structure was not disclosed in that report, they obtained the identical amino acids and the fatty acid to those from plipastatin A1 by the acid hydrolysis. The structure of fengycin was revealed in 1999 by Budzikiewicz, that it is a diastereomer of plipastatin A1 (named fengycin IX) possessing D-Tyr4 and L-Tyr10 residues,[6] while plipastatin A1 has the reversed permutation, L-Tyr4 and D-Tyr10.[8] Due to the promising antibiotic activity of this substance and empirical safeness of the producer *Bacillus subtilis*, fengycin is expected to be a novel biocontrol[7] to produce more than 220 scientific papers and counting. Some of those papers dealt with the biogenesis of fengycins[8–17] to disclose NRP (non-ribosormal-peptide) synthase clusters. Those involved sequences suggesting racemases. However, positions of the expected racemases accorded with plipastatin A1 in spite of fengycin producers.[18,19] The enzyme cluster which rationally explains Budzikiewicz's fengycin IX has not so far been reported. These facts have brought structural confusion between these molecules. Although many papers followed Budzikiewicz's structure as fengycin IX, considerable number of recent reports mentioned that these are identical compounds[15,20–22] supposedly in order to avoid the contradiction between the structure and the biogenesis. However, there was no experimental evidence. The confusion has become more serious, because at least five other structures exist as fengycin IX (some structures did not match up to their own discussions).[8–14,16,23–25] The present studies experimentally proved that plipastatin A1 is the K^+ salt whereas fengycin IX is the free form or the TFA salts. Although these molecules gave considerably different ^{1}H NMR spectra, the quite similar ^{1}H NMR spectrum to that of fengycin IX was changed to provide nicely accorded spectrum to that of plipastatin A1 when the sample was converted into the K^+ salt. Our structural studies led a conclusion that the structures of these compounds should be settled into that of plipastatin A1 by Umezawa.

2. Results and discussions

Bacillus subtilis H336B was found to produce an antifungal cyclic peptide **1** which showed the same molecular weight 1462 as both fengycin IX and plipastatin A1 by ESIMS [1463.8019, (calcd for 1463.8038, $[M+H]^+$ $C_{72}H_{112}N_{12}O_{20}$)]. Isolation was performed by a series of conventional chromatographies (XAD-7, ODS MPLC, and ODS HPLC). Analyses involving Maefey's configurational determination[26] after acidic hydrolyses disclosed L-Glx (×3), L-Orn, L-Tyr, D-Tyr, D-*allo*-Thr, D-Ala, L-Pro, L-Ile, and 3-hydroxyhexadecanoic acid (see Figure 1). Although our amino acid analyses were not

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quantitative enough, for example due to partial *O*-derivatizations of Tyr residue with DNS [5-(dimethylamino)naphthalene-1-sulfonyl] chromophore[27] and DAA [2,4-dinitrophenyl-5-L-alanine amide] chiral auxiliary group,[26] the three Glx substructures could be disclosed by ^{1}H and ^{13}C NMR spectra (see Supplementary data). Suggested molecular formula led us to assign those to be one glutamine and two glutamic acids. These results consisted well with both fengycin IX and plipastatin A1. The NMR spectral data of **1** accorded well with those of Budzikiewicz's fengycin IX both in CD_3OH and DMSO-d_6,[28] when acidic conditions (H_2O–CH_3CN, in the presence of TFA) were employed in the final ODS-HPLC purification. These conditions give samples as free acid forms or TFA salts. However, these spectra obviously disaccorded with those of plipastatin A1.[4,29] Since Umezawa isolated plipastatins as the K^+ salts, **1** was converted into the K^+ salt by treating with aqueous KOAc followed by LH-20 gel filtration. Interestingly, these treatments dramatically changed the ^{1}H NMR profile in DMSO-d_6 as shown in Figure 2, for example, the α-proton signal in Ala7 appeared at 5.02 ppm in the K^+ salt, while it resonated at 4.58 ppm before the treatments. These signals could be assigned distinctly by observing COSY correlations with doublet methyl signals (1.11 and 1.25 ppm, respectively). The spectrum after the treatments became to accord well to that of plipastatin A1 in the Umezawa's report (see Supplementary data). No structural change occurred during these operations, which was confirmed by the LCMS retention time and the MS spectral profile. These results clearly indicated that plipastatin A1 and fengycin IX possess identical framework but they are different forms, the free acid form or the TFA salt for fengycin IX and the K^+ salt form for plipastatinn A1.[30] These findings grossly contradicted the precedent knowledge that plipastatin A1 and fengycin IX were diastereomers due to the pair of enantiomeric Tyr residues (Tyr4 and Tyr10). In other words, the above results suggested that either (in some case, both) structure of these molecules is wrong. In order to resolve the above confliction, we determined the full structure of **1** independently.

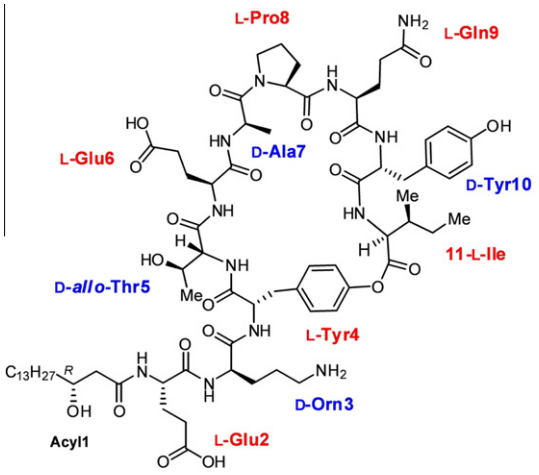


Figure 1. Structure of **1**.

A mono-cyclic structure was suggested based on the degree of unsaturation as well as the amino acid residues involved. Basic methanolysis/hydrolysis took place smoothly under mild conditions to give the methanol adduct **2** (*m/z* 1495.8350) and hydrolysate **3** (*m/z* 1481.8117), respectively, disclosing that **1** is depsipeptide. Peptide bonding should not be cleaved under these conditions. Acyclic molecule results only fragmentation by solvolysis. A doublet signal due to the *ortho*-position of the phenol ring on one Tyr residue appeared at considerably higher frequency (7.21 ppm) than that of corresponding protons on the other Tyr (6.75 ppm) in **1**. However, the hydrolysis neutered it to display both of them at around 6.73 ppm. These observations revealed that the phenolic hydroxy group in one Tyr is assignable to the lactone ring. The carboxy part of the lactone was determined to be Ile11 by

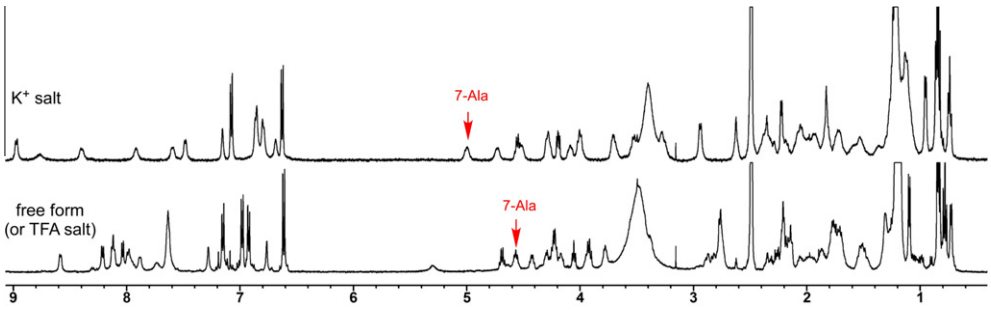


Figure 2. ^{1}H NMR spectra of **1** in DMSO-d_6 (500 MHz, upper: K^+ salt, lower: free acid form).

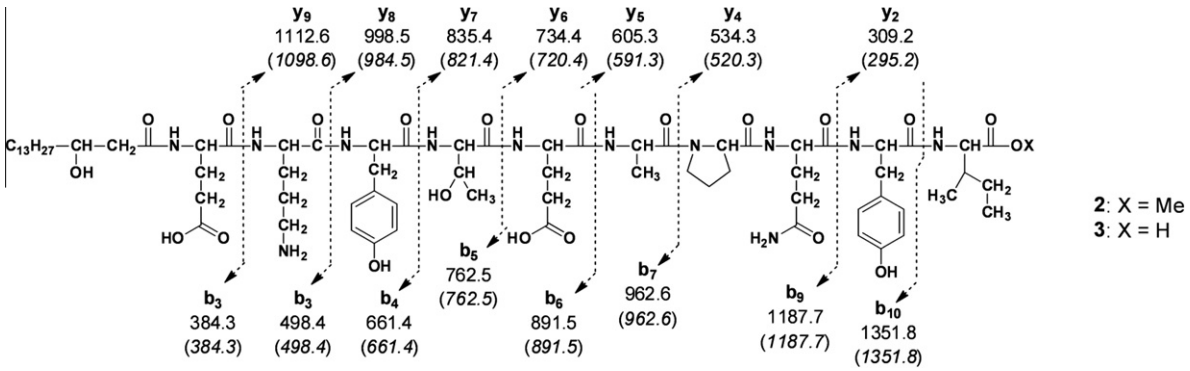
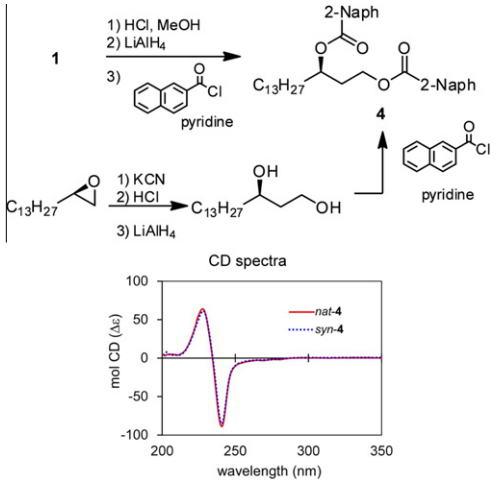
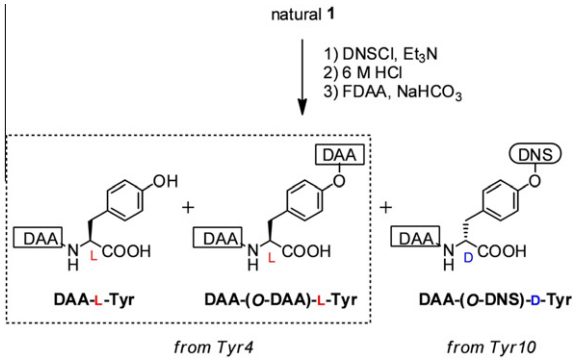


Figure 3. Structures and observed CID-MS/MS fragment ions of the methanol adduct **2** and those of hydrolysate **3** (in brackets).



Scheme 1. Determination of the configuration for the Acyl1 moiety.



Scheme 2. Determination of the configurations for Tyr4 and tyr10 residues.

identifying Ile-OMe substructure in methanolysis product **2** in the ESI-CID-MS/MS spectrum as shown in Figure 3. The CID of **2** (target ion: *m/z* 1495.8) provided a fragment ion at 309.2 which was assigned as y_2 ion ($[Tyr\text{-}Ile\text{-}OMe+H]^+$). The corresponding ion was observed at 295.2 when hydrolysate **3** was employed (target ion: *m/z* 1481.8). On the other hand **2** and **3** gave the common b_9 and b_{10} ions at *m/z* 1187.1 and 1351.8, respectively. These also indicate Tyr-Ile-OMe substructure in **2**.

Amino acid sequence was determined also with ESI-CID-MS/MS analyses. In the similar manner as above, comparison of the MS/MS spectra between **2** and **3** undoubtedly distinguished the *C*-terminus and *N*-terminus ions. Although b_8 and y_3 ions were missing in these spectra, these were assigned to be a dipeptide unit Pro and Gln (total residue mass = 225.1 u) based on the mass differences between y_9 and y_7 as well as between y_4 and y_2 (both $\Delta m/z$ = 225.1 u). The order of these two amino acid residues was determined to be Pro8-Gln9 based on the oxazolidine intermediate theory in the ESI MS/MS which suggests that CID cleavages hardly occur at the *C*-side of proline residues.[31] These analyses revealed the sequence Acyl1-Glu2-Orn3-Tyr4-*allo*-Thr5-Glu6-Ala7-Pro8-Gln9-Tyr10-Ile11. As described above, one of the two phenolic groups should form the lactone ring. We determined that Tyr4 should be the responsible residue. Lactone ring with Tyr10 might logically be possible, but it bends the tyrosine aromatic ring to bring extraordinary strain.

Configuration of Acyl1 moiety was next determined. Budzikiewicz determined *R*-configuration for this part by analogy with other bacterial lipopeptides, there was however no experimental evidence. Umezawa had concluded the same configuration by measuring the specific rotation value of methyl 3-hydroxyhexadecanoate ($[\alpha]_D$ −35) obtained by the acidic methanolysis of plipastatin A1. However much smaller value ($[\alpha]_D$ −6.0) was also reported for this molecule in the recent synthetic paper.[32] This suggested low accuracy for our particular case because only small quantity of the sample was available from the natural product. We investigated that with circular dichroism (CD) by taking advantage of its high sensitivity. After acidic methanolysis, the resulting methyl 3-hydroxyhexadecanoate was reduced with $LiAlH_4$ to give hexadecane-1,3-diol as shown in Scheme 1. With expecting large $\Delta\varepsilon$ values,[33,34] we introduced 2-naphthoyl group as the chromophore to give bis-1,3-*O*-(2-naphthoyl) derivative *nat*-**4**. In spite of small amount (870 μg based on UV absorption), this sample showed a typical negative split Cotton effect at 230 nm with reliable quality. The authentic sample (*R*)-**4** was prepared by conventional transformations from (*R*)-pentadecen-1-2-oxide which was readily provided by asymmetric dihydroxylation of 1-pentadecene with AD-mixβ followed by a selective 1-sulfonylation and an epoxide formation under basic conditions. The ^1H NMR of (*R*)-**4** was identical to that of *nat*-**4**. Comparison of the CD profiles led us to undoubtedly establish the (*R*)-configuration for Acyl1 moiety.

These results corroborated both Umezawa's plipastatin A1 and Budzikiewicz's fengycin IX. The permutation of D-Tyr and L-Tyr was only the argument which remained to be determined. In other words, either structure of these molecules is identical to **1,** and the other is incorrect in the order of D- and L-Tyr residues. Umezawa concluded D-Tyr10 by observing *O*-DNP-D-Tyr (DNP = 2,4-dinitrophenyl group) by the acidic hydrolysis after introduction of DNP group into plipastatin A1. However, they also observed Gln from the hydrolysate **3** by carboxypeptidase Y digestion. Since this enzyme is well established to cleave off L-amino acids selectively, D-Tyr10 should terminate the digestion before releasing L-Gln9. Budzikiewicz reached the other conclusion by detecting only D-Tyr by the acidic hydrolysis after CrO_3 oxidation of fengycin IX. Since these experiments involved ambiguity as described, we investigated that with a certain methodology.

As shown in Scheme 2, 10-Tyr was labeled with DNS group. This condition also furnished DNS on the δ-amine group of Orn3, affording the bis-DNS derivative giving single LCMS signal (*m/z* 1929.9). Subsequent acidic hydrolysis librated L-Tyr and D-(*O*-DNS)-Tyr. Their configurations were determined by LCMS after Marfey's DAA derivatization. Since the phenolic OH in Tyr was also reacted under the conditions, a mixture of DAA-(*O*-DAA)-L-Tyr, DAA-L-Tyr, and DAA-(*O*-DNS)-D-Tyr was obtained by these transformations. Authentic DAA-L-Tyr, DAA-(*O*-DNS)-L-Tyr, and DAA-(*O*-DAA)-L-Tyr as well as the corresponding D-Tyr derivatives were prepared from *N*-Boc-L-Tyr and *N*-Boc-D-Tyr, respectively. The LCMS ion chromatograms choosing *m/z* 434.1, 686.2, and 667.2 detected DAA-L-Tyr, DAA-(*O*-DAA)-L-Tyr, and DAA-(*O*-DNS)-D-Tyr, respectively, as shown in Table 1. Standing the reaction mixture without neutralization allowed a gradual isomerization of the amino acid moieties[26] to provide the corresponding diastereomers. These were also helpful in LCMS peak assignments. These analyses distinctly disclosed L- and D-configurations for Tyr4 and Tyr10, respectively. As described, the full structure of **1** was thus established as shown in Figure 1 which consists with Umezawa's plipastatin A1 but not with Budzikiewicz's fengycin IX.

3. Conclusion

We succeeded in disclosing that fengycin IX and plipastatin A1 are identical compounds although these had been considered as diastereomers at the two Tyr residues. Although reported their NMR spectra showed discordance, it could be explained by their

Table 1
Comparison of LCMS retention times of DAA-Tyr derivatives

DAA-amino acids	$[M+H]^+$ (*m/z*)	Retention time (min)			Judged configuration
		Authentic L-isomer	Authentic D-isomer	Signals from **1**	
DAA-Tyr	434.1	20.1	22.3	20.4	L
DAA-(*O*-DAA)-Tyr	686.2	38.9	44.1	39.7	L
DAA-(*O*-DNS)-Tyr	667.2	47.9	50.3	50.3	D

Conditions: Inertsil ODS 2.1 mm × 100 mm, 20–100% CH_3CN/H_2O for 80 min (containing 0.1% HCOOH) 0.20 mL/min flow.

forms; plipastatin is the K^+ salt, while fengycin is the free form or the TFA salts. The present studies disclosed that structures of these molecules should be settled into that of plipastatin A1 by Umezawa. This structure showed more advantageous to rationally explain fengycin biosynthesis than the precedent structure by Budzikiewicz. The confusion was probably caused by the preoccupation that these two must be different molecules because of disaccording NMR profiles. Many groups utilized MS/MS spectra in fengycin/plipastatin identifications. Of course mass spectrometry has brought incredible progress in the last decade to enable us determine molecules within a short period. However, instant structural conclusions only by LC–MS/MS magnified the confusion in this particular case. In other words, the present studies rediscover the importance of detailed structural elucidation based on chemical degradations and derivatizations. Our results experimentally terminate the structural ambiguity between these compounds, and would somehow accelerates the fengycin/plipastatin researches such as detailed lactonization mechanism in the biosynthesis,[12] application as a functional molecule,[35] and detailed mechanistic studies of the biological activity.[36]

Budzikiewicz's fengycin IX does not exist. The NMR spectrum of this virtual diastereomer would show different profile from the natural product, and it will be the final proof for this argument. Synthesis of this isomer is undergoing in our laboratories.

4. Experimental

4.1. Fermentation and isolation

Bacillus subtilis H336B was isolated as a contaminant from a fungal culture and was identified through BLAST search of Genbank based on 16S rRNA gene sequence. It was deposited at the Japan Collection of Microorganisms of Riken Bioresource Center as JCM 18293. The bacterium was cultured in a medium prepared from glycerol (100 g), meat extract (20 g), polypeptone (20 g), yeast extract (40 g), NaCl (8.0 g), $MgSO_4{\cdot}7H_2O$ (2.0 g), K_2HPO_4 (2.0 g), $CaCO_3$ (12.8 g), and H_2O (4.0 L) under shaking conditions (110 rpm). After 3 days, the culture broth was filtered in suction through a Celite pad and the filtrate was concentrated under reduced pressure until the whole volume became 1.0 L. The resulting aqueous suspension was loaded on an Amberlite XAD-7HP (70 mm I.D. × 700 mm) column and eluted with 30%, 50%, 90%, and 100% aqueous methanol solution. The activity was recovered in 50% and 90% aqueous methanol fractions to give a residue (231 mg) after lyophilization. Then, the residue was charged on Waters ODS Sep-Pak® Vac 35 cc (10 g). After washing with 50% aqueous methanol, the active substance was eluted with 90% aqueous methanol. After concentration, the residue was further subjected to medium pressured liquid chromatography with UltraPack (ODS, Size B, Yamazen Co.) with 85% aqueous methanol. The active fractions were combined and concentrated under reduced pressure to give crude **1** (27 mg). Pure **1** (12 mg) was obtained by preparative ODS HPLC (Sunfire™ Prep C18 OBD™ 19 mm I.D. × 150 mm, 45–75% CH_3CN/H_2O containing 0.1% TFA for 15 min, 10.0 mL/min flow) followed by lyophilization. t_R = 8.5 min (above conditions). ESIMS (rel. int.%) *m/z* 1485.7915 (4, calcd for 1485.7857, $[M+Na]^+$ $C_{72}H_{110}N_{12}O_{20}Na$), 1463.8019, (25, calcd for 1463.8038, $[M+H]^+$ $C_{72}H_{112}N_{12}O_{20}$), 751.3769 (9.0, calcd for 751.3837 $[M+H+K]^{2+}$, $C_{72}H_{111}N_{12}O_{20}K$), 743.3934 (11, calcd for 739.3967 $[M+H+Na]^{2+}$, $C_{72}H_{111}N_{12}O_{20}Na$), 732.4006 (100, calcd for 738.4005, $[M+2H]^{2+}$, $C_{72}H_{111}N_{12}O_{20}$). The detailed NMR spectral data are shown in Supplementary data.

4.2. Transformation into the K^+ salt

Pure **1** (4.4 mg) was diluted with aqueous KOAc solution (200 mg in 10 mL) and the resulting solution was lyophilized. After the obtained powder was dissolved in a minimum amount of H_2O (ca. 300 μL), gel filtration was performed with LH-20 (25 mm I.D. × 300 mm) to give the K^+ salt (ca 2.2 mg) after lyophilization. No reaction occurred during these operations, which was verified by LCMS.

4.3. Amino acid analysis

Pure **1** (500 μg) was treated with 6.0 M HCl (500 μL) in a sealed tube at 110 °C for 12 h. After water (3.0 mL) was added, the resulting solution was lyophilized. The residue was stirred with 5-(dimethylamino)naphthalene-1-sulfonyl chloride (DNSCl, 600 μg, 2.22 μmol) in a mixture of 0.5 M $NaHCO_3$ (100 μL) and acetone (100 μL) at room temperature for 14 h. The resulting solution was directly analyzed with LCMS [Inertsil® ODS (2.1 mm I.D. × 100 mm, 30–100% H_2O/CH_3CN for 15 min containing 0.1% HCOOH, 0.20 mL/min flow) equipped with a UV detector (350 nm). The following amino acid derivatives were found; DNS-Glu (t_R = 5.8 min, *m/z* 381.11), DNS-*allo*-Thr (t_R = 5.8 min, *m/z* 353.12), DNS-Tyr (t_R = 7.5 min, *m/z* 415.13), DNS-Ala (t_R = 9.3 min, *m/z* 323.10), DNS-Pro (t_R = 11.5 min, *m/z* 349.12), DNS-Ile (t_R = 13.4 min, *m/z* 345.15), $(DNS)_2$-Orn (t_R = 15.3 min, *m/z* 599.20), and $(DNS)_2$-Tyr (t_R = 18.6 min, *m/z* 648.19).

4.4. Determinations of the configurations for constituting amino acids

Acid degradation of **1** (500 μg) was performed in the similar manner as described above. The residue was stirred with (5-fluoro-2,4-dinitrophenyl)-L-alanine amide (L-FDAA, 300 μg) and $NaHCO_3$ (21 mg) in a mixture of H_2O (500 μL) and acetone (200 μL) at 40 °C for 90 min. After cooling, the reaction mixture was directly analyzed by LCMS with ODS column. The retention times and MS-profiles were checked by authentic amino acids. The retention times and configurational judgments were summarized in Supplementary data.

4.5. Determination of configuration of 3-hydroxyhecadecanoate acid (Acyl1) moiety

Natural **1** (14.1 mg) was heated in 10% HCl/MeOH (1.5 mL) at 60 °C for 12 h. The mixture was poured in H_2O (15 mL) and

extracted with AcOEt (15 mL). The organic solution was washed with brine (15 mL), dried over $MgSO_4$ and then concentrated under reduced pressure. Methyl 3-hydroxyhexadecanoate (1.3 mg) was isolated by preparative TLC (AcOEt/hexane = 20:80, R_f = 0.6). ^{1}H NMR (in $CDCl_3$) δ 0.88 (3H, t, 6.9 Hz), 1.25 (20H), 1.50–1.57 (2H, m), 2.41 (1H, dd, J = 9.1, 16.4 Hz), 2.52 (1H, dd, J = 3.0, 16.4 Hz), 3.71 (3H, s), 4.00 (1H, m). After the methyl ester thus obtained was dissolved in THF (1.0 mL), $LiAlH_4$ (2.0 mg) was added at room temperature and the mixture was stirred for 30 min. Methanol (2 drops) was added to destroy excess reagent and the resulting mixture was further stirred for additional 30 min. The mixture was poured in H_2O (15 mL) and extracted with AcOEt (15 mL). The organic solution was washed with brine (15 mL), dried over $MgSO_4$ and then concentrated under reduced pressure. After the residue was dissolved in pyridine without purification, 2-naphthoyl chloride (5.0 mg) was added at 60 °C and the mixture was stirred for 2 h at the same temperature. The mixture was concentrated and then diluted with AcOEt (10 mL). The resulting solution was washed with H_2O (10 mL) and brine (15 mL) successively, and then dried over $MgSO_4$. After concentration under reduced pressure, HPLC purification [Capcell Pak C8 UG120 (5 μm), 4.6 mm I.D. × 250 mm, CH_3CN/H_2O, 50:50–100:0 for 15 min, then keeping 100:0 for 15 min (containing 0.1% TFA), 1.0 mL/min flow] afforded **4** at t_R = 21.7 min. Co-injection with synthetic authentic sample confirm the HPLC peak. The yield was estimated to be ca. 870 μg based on the UV absorbance at 262 nm (ε 124500). ^{1}H NMR ($CDCl_3$) δ 0.80 (3H, t, J = 6.9 Hz), 1.15 (16H, m), 1.28, 1.39 (each 2H, m), 1.69, 1.77 (each 1H, m), 2.22 (2H, br q, J = 6.5 Hz), 4.48 (2H, m), 5.39 (1H, br quint, J = 6.5 Hz), 7.36–7.52 (4H, m), 7.66–7.83 (6H, m), 7.92, 7.97 (each br dd. J = 1.6, 8.6 Hz), 8.45, 8.50 (each br s). The ^{1}H NMR spectrum was identical to that of synthetic authentic sample. ESIMS m/z 567.36 $[M+H]^+$.

4.6. Determination of the configurations for Tyr4 and Tyr10

A solution of **1** (1.0 mg) was stirred with dansyl chloride (1.8 mg) and triethylamine (20 μL) in acetonitrile (500 μL) at room temperature for 3 h. The reaction was monitored with LCMS. After 3 h, the starting **1** (m/z 1463) totally disappeared and a signal (m/z 1929.91, suggesting bis-DNS product) was newly observed in the LCMS. After concentration, the residue was dissolved in 6 M aqueous HCl solution (500 μL) and heated at 110 °C for 6 h. Water (3.0 mL) was added to the mixture and the resulting solution was lyophilized. The residue was stirred with L-FDAA (10 mg) and $NaHCO_3$ (21 mg) in a mixture of H_2O (500 μL) and acetone (200 μL) at 40 °C for 90 min. The reaction mixture was directly analyzed by LCMS [Inertsil ODS 2.1 mm I.D. × 100 mm, 20–100% CH_3CN/H_2O for 80 min (containing 0.1% HCOOH) 0.20 mL/min flow]. The DAA-L-Tyr-OH and DAA-D-(*O*-DNS)-Tyr-OH were detected at 20.4 (m/z 343.13) and 50.3 min (m/z 667.18), respectively. The corresponding diastereomers DAA-D-Tyr-OH and DAA-L-(*O*-DNS)-Tyr-OH appeared at 22.3 and 47.9 min, those were established employing authentic samples.

4.7. Preparation of syn-4

Pentadecene (231 mg, 1.10 μmol) was stirred with AD-mixβ (2.0 g) in a mixture of H_2O (5.0 mL) and 2-methyl-2-propanol (5.0 mL) for 3 h at 0 °C. The mixture was poured in H_2O (50 mL) and extracted with ether (30 mL × 3). The ethereal solution was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The residue was diluted with hexane (3.0 mL) and stood at room temperature for 12 h to give (*R*)-pentadecan-1,2-diol as plates (232 mg, 950 μmol, 86%). Mp 63 °C (from hexane), $[\alpha]_D^{24}$ +6.3 (c 0.72, MeOH), ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.7 Hz), 1.23–1.35 (21H), 1.45 (3H, m) 3.43 (1H, dd, J = 7.7, 11.0 Hz), 3.66 (1H, dd, J = 3.1, 11.0 Hz), 3.72 (1H, m), ^{13}C NMR ($CDCl_3$) δ 14.09, 22.67, 25.52, 29.34, 29.53, 29.57, 29.63, 29.64 (for three carbons), 29.67, 31.91, 33.20, 66.84, 72.32. The optical purity was determined by converting its bisMTPA ester to confirm that it was more than 95% ee. The diol (150 mg, 614 μmol) was stirred with mesitylenesulfonyl chloride (171 mg, 730 μmol) and pyridine (150 μL, 1.83 μmol) at room temperature for 12 h. The mixture was poured in H_2O and extracted with ether (×3). The ethereal solution was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. Silica gel column chromatography of the residue gave (*S*)-2-hydroxytetradecyl mesitylenesulfonate as an oil (135 mg, 51%), (*R*)-1-hydroxypentadecan-2-yl mesitylenesulfonate (21.0 mg, 8%), and the recovered diol (35.0 mg, 23%). ^{1}H NMR for 1-sulfonate ($CDCl_3$) δ 0.88 (3H, t, J = 7.0 Hz), 1.25 (20H) 1.28 (1H, m), 1.42 (3H, m), 2.06 (1H, br d, J = 4.2 Hz), 2.32 (3H, s), 2.04 (6H, s), 3.82 (1H, dd, J = 7.2, 9.5 Hz), 3.85 (1H, m), 3.99 (1H, dd, J = 2.2, 9.5 Hz), 6.98 (2H, s). The obtained 1-sulfonate (135 mg, 316 μmol) was stirred with K_2CO_3 (50 mg) in methanol (2.0 mL) at room temperature for 2 h. The suspension was poured in H_2O and extracted with ether (×3). The ethereal solution was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. Silica gel column chromatography of the residue gave (*S*)-2-pentadecene oxide (70 mg, 98%). ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 7.0 Hz), 1.26 (20H), 1.44 (2H, m), 1.53 (2H, m), 2.46 (1H, dd, J = 2.8, 5.0 Hz), 2.74 (1H, dd, J = 4.0, 5.0 Hz), 2.90 (1H, m). A solution of the epoxide (70 mg, 310 μmol) was stirred with KCNe (70 mg, 310 μmol) in MeOH (2.0 mL) at 40 °C for 16 h. The mixture was poured in H_2O (100 mL) and extracted with ether (80 mL × 3). The ethereal solution was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. Silica gel column chromatography of the residue gave (*S*)-3-hydroxyhexadecanenitrile as plates (65 mg, 83%). Mp ca. 30 °C (from cold hexane), ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz), 1.24–1.34 (21H), 1.44 (1H, m), 1.59 (2H, m), 1.94 (1H, br d, J = 5.0 Hz), 2.49 (1H, dd, J = 6.4, 16.7 Hz), 2.57 (1H, dd, J = 4.6, 16.7 Hz), 3.95 (1H, m). The obtained nitrile (65 mg, 237 μmol) was stirred in 12 M HCl (5.0 mL) at 75 °C for 30 min. After cooling, the mixture was diluted with H_2O (10 mL) and extracted with AcOEt (20 mL × 3). The combined organic solution was washed with brine, dried over $MgSO_4$ and the concentrated under reduced pressure to give the crude carboxylic acid (65 mg). ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.2–1.6 (24H), 2.48 (1H, dd, J = 8.7, 16.5 Hz), 2.58 (1H, dd, J = 3.0, 16.5 Hz), 4.03 (1H, m). After the crude carboxylic acid (24 mg, 88.1 μmol) was diluted with THF (2.0 mL), $LiAlH_4$ (8.0 mg) was added and the mixture was stirred at room temperature for 2 h. After the mixture was cooled in an ice bath, methanol (three drops) was added to decompose the excess reagent. The mixture was poured in 2 M aqueous HCl solution and extracted with AcOEt (10 mL × 3). The combined organic solution was washed with brine (15 mL), dried over $MgSO_4$ and then concentrated under reduced pressure. Silica gel column chromatography of the residue with AcOEt/hexane (40:60) gave (*R*)-hexadecane-1,3-diol (21 mg, 92%). ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.9 Hz), 1.23–1.35 (22H), 1.48 (2H, m), 1.70 (2H, m), 2.26, 2.31 (each 1H, br), 3.86 (3H, m). The 1,3-diol thus obtained (15 mg, 58 μmol) was stirred with 2-naphtoylchloride (25 mg, 131 μmol) in pyridine (1.0 mL) at 65 °C for 2 h. After concentration, silica gel column chromatography of the residue (hexane/AcOEt = 7:93) gave the synthetic **4** (22.0 mg, 67%). UV 230.5 nm (ε = 124,500, $c = 1.02 \times 10^{-5}$ mol/L, CH_3CN), ^{1}H NMR ($CDCl_3$) δ 0.87 (3H, t, J = 6.9 Hz), 1.20–1.50 (22H), 1.82 (2H, m), 2.28 (2H, m), 4.52 (1H, dt, J = 6.6, 11.2 Hz), 4.58 (1H, ddd, J = 4.1, 6.2, 11.2 Hz), 7.52 (4H), 7.76 (1H, br d, J = 8.7 Hz), 7.81 (4H), 7.88 (1H, br d, J = 8.7 Hz), 7.99 (1H, dd, J = 1.7, 8.7 Hz), 8.04 (1H, dd, J = 1.7, 8.7 Hz), 8.52, 8.58 (each 1H, br s). APCIMS m/z 567.36.

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Supplementary data

Supplementary data (summary for structural confusion of fengycin in papers, comparison of TIC and mass spectra between **1** and **3**, NMR spectra (^{1}H, ^{13}C, DEPT-135, COSY, HMQC, HMBC and HSQC-TOXSY) of **1**, comparison of ^{1}H and ^{13}C NMR signal assignments of **1** (free form) in CD_3OH with those reported by Budzikiewicz, comparison of the ^{1}H NMR between **1** and **3** in CD_3OD, ^{1}H NMR spectral comparison between **1** and plipastatin by Umezawa in DMSO-d_6, COSY spectrum of **1** (K^+ salt, free acid) in DMSO-d_6, CID-MS/MS spectra and assignments of **2** and **3**. ^{1}H NMR spectrum of **4**, tables for the HPLC retention times in configurational determination of the amino acids, experimental detail for the preparation of the *syn*-**4**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.04.040.

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