Study on Surfactin, a Cyclic Depsipeptide. II.[1] Synthesis of Surfactin B_2 Produced by *Bacillus natto* KMD 2311[2]

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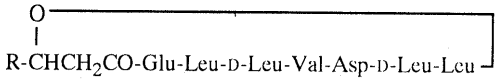
The total synthesis of surfactin B_2, a cyclic depsipeptide isolated from *Bacillus natto* KMD 2311, was achieved to elucidate the absolute configuration of its fatty acid moiety. This is the first chemical confirmation of the absolute configuration of a surfactin homolog. Two possible diastereoisomers of surfactin B_2, cyclo[D- and L-3-(Glu–Leu–D-Leu–Val–Asp–D-Leu–Leu–O)-*n*-tetradecanoyl] (1a and b), were synthesized by a solution method using mainly active ester and azide fragment condensation methods. Cyclization reaction of the partially protected linear depsipeptide containing the C-terminal *N*-succinimidyl active ester in pyridine by the high dilution method at room temperature for 3 d gave the desired cyclic depsipeptide in a high yield of about 70%. The synthetic product 1a, containing the D-isomer of 3-hydroxytetradecanoic acid as a fatty acid moiety, was identical with natural surfactin B_2.

Key words total synthesis; surfactin; surfactin homolog; D-3-hydroxytetradecanoic acid; cyclization reaction; cyclic depsipeptide

Arima *et al.*[3a] isolated surfactin from *Bacillus subtilis* IAM 1213 and showed that it has a strong surface tension-lowering activity and a potent clotting-inhibitory activity. The structure of surfactin (Fig. 1, surfactin C_1) as a cyclic depsipeptide having iso-3-hydroxypentadecanoic acid as a fatty acid was proposed by Kakinuma *et al.*[3b] Our study[1] revealed that *Bacillus natto* KMD 2311 contains at least eight homologous depsipeptides, which were deduced to be cyclic compounds with a hydroxyfatty acid, *n*-, iso- or anteiso-3-hydroxyfatty acid of carbon number 13—16, as part of the ring system. The peptide portion of the eight homologs was acyl–Glu–Leu–Leu–Val–Asp–Leu–Leu in each case. Four compounds among them were found to be identical with known surfactin homologs, A_1, B_1, B_2 and C_1. The others were novel homologs, surfactin A_2, A_3, C_2 and D, the acyl groups of which were anteiso-3-hydroxytridecanoic acid, *n*-3-hydroxytridecanoic acid, anteiso-3-hydroxypentadecanoic acid and iso-3-hydroxyhexadecanoic acid, respectively. So far, the absolute configuration of the hydroxyfatty acids of surfactin homologs has not been elucidated. In 1976, Morrison *et al.*[4] reported the synthesis of an artificial surfactin homolog "norsurfactin", which corresponds to one of the diastereoisomers of surfactin B_2. The IR spectrum of their synthetic product, [D-3-hydroxytetradecanoic acid[1]]-surfactin, was indistinguishable from that of natural surfactin, which was thought to be a mixture of surfactin homologs at that time. Our natural surfactin B_2 has a similar melting point to the synthetic norsurfactin (Table 1), but the optical rotations of the solutions in chloroform were not in agreement (Table 1). The reason for the discrepancy was not clear. It is possible to ascribe the lower rotation of their synthetic material to the presence of impurities. Since no surfactin homolog had been synthesized, we initiated a study aimed at the total synthesis of surfactin B_2, which would allow elucidation of the absolute configuration of its fatty acid moiety.



Homolog	R	
A_1	CH_3, CH_3 $CH(CH_2)_7$-	(*iso*)
A_2	CH_3CH_2, CH_3 $CH(CH_2)_6$-	(*anteiso*)
A_3	$CH_3(CH_2)_9$-	(*n*-)
B_1	CH_3, CH_3 $CH(CH_2)_8$-	(*iso*)
B_2	$CH_3(CH_2)_{10}$-	(*n*-)
C_1	CH_3, CH_3 $CH(CH_2)_9$-	(*iso*)
C_2	CH_3CH_2, CH_3 $CH(CH_2)_8$-	(*anteiso*)
D	CH_3, CH_3 $CH(CH_2)_{10}$-	(*iso*)

Fig. 1. Structures of Surfactin Homologs

Table 1. Physical Properties of Surfactin B_2, the Synthetic Products (**1a**, **b**) and Norsurfactin

	mp (°C)	$[\alpha]_D^t$ ($c=1$) $CHCl_3$ t (°C)	$[\alpha]_D^t$ ($c=1$) MeOH t (°C)	FAB-MS (m/z)	TLC Rf[a]
Surfactin B_2	137—138	+37.2 (9.0)	−36.5[b] (22.0)	1022 $(M+H)^+$	0.36
1a	136—138	+37.6 (7.5)	−37.0 (9.5)	1022 $(M+H)^+$	0.36
1b	136—138	+20.4 (12.5)	−19.6 (12.5)	1022 $(M+H)^+$	0.36
Norsurfactin[4]	138—142	+27.1 (25)	−35.2 (25)		

a) Rf^1 ($CHCl_3$: MeOH : AcOH = 95 : 5 : 3). *b*) $c=0.8$. **1a**: [D-3-Hydroxytetradecanoic acid[1]]-surfactin. **1b**: [L-3-Hydroxytetradecanoic acid[1]]-surfactin.

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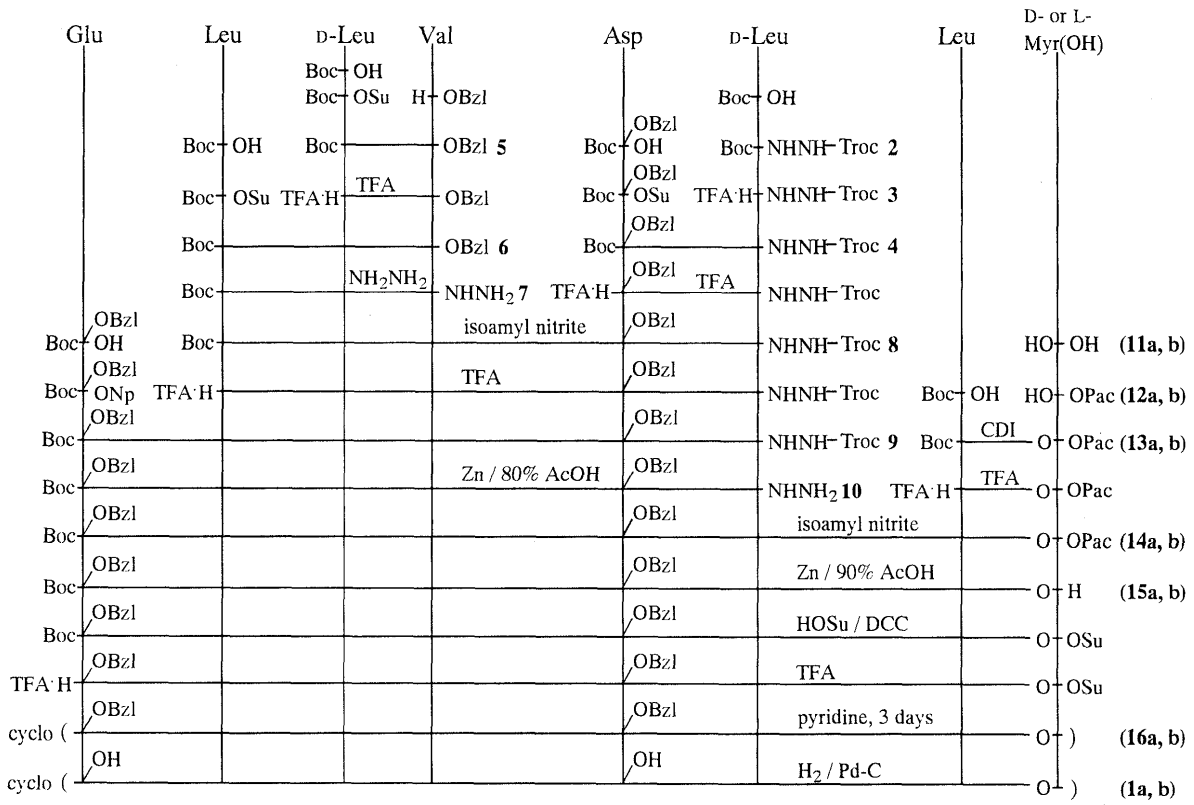

Fig. 2. Synthetic Scheme for Surfactin B_2

This paper presents the synthesis of two diastereoisomers, cyclo[D- and L-3-(Glu–Leu–D-Leu–Val–Asp–D-Leu–Leu–O)-tetradecanoyl] (**1a** and **b**) and the establishment of the identity of **1a** with natural surfactin B_2. The synthetic strategy for **1a** and **b** is shown in Fig. 2. The peptide portion was synthesized by a conventional method. The *tert*-Boc group was used for protection of the α-amino function and was later removed with anhydrous TFA. The carboxylic acid in the side chain of Glu and Asp was protected with benzyl ester, which was removed by hydrogenolysis after the formation of the lactone ring. Each of the protected peptide fragments was prepared in stepwise manner using active ester, mainly *N*-succinimidyl active ester. The C-terminal of the fragments was protected with benzyl ester or Troc-hydrazide, which was converted to hydrazide prior to fragment condensation by the azide method. The peptide chain was elongated exclusively by the azide fragment condensation method. To avoid racemization, the cyclization reaction between the amino group of Glu and the carboxyl group of the hydroxyfatty acid was conducted by the *N*-succinimidyl active ester method.

Boc–D-Leu–OH and Troc–$NHNH_2$ were coupled in THF by the mixed anhydride method using isobutyl chlorocarbonate to give Boc–D-Leu–NHNH–Troc (**2**). A solution of H–D-Leu–NHNH–Troc in AcOEt, obtained by deblocking **2** with TFA, was treated with Boc–Asp(OBzl)–OSu at room temperature overnight to give Boc–Asp(OBzl)–D-Leu–NHNH–Troc (**4**). A mixture of Boc–D-Leu–OSu and H–Val–OBzl in AcOEt was allowed to stand at room temperature to afford Boc–D-Leu–Val–OBzl (**5**). Boc–Leu–OSu was added to a AcOEt solution of the deblocked product of **5** with TFA in the usual manner and the coupling product Boc–Leu–D-Leu–Val–OBzl (**6**) was isolated and purified in the usual manner. Peptide **6** was converted to the corresponding hydrazide, Boc–Leu–D-Leu–Val–$NHNH_2$ (**7**), by treatment with hydrazine hydrate. The peptide **7** was coupled with H–Asp(OBzl)–D-Leu–NHNH–Troc derived from **4** to give Boc–Leu–D-Leu–Val–Asp(OBzl)–D-Leu–NHNH–Troc (**8**). The peptide **8** was deblocked with TFA and then allowed to react with Boc–Glu(OBzl)–ONp to give Boc–Glu(OBzl)–Leu–D-Leu–Val–Asp(OBzl)–D-Leu–NHNH–Troc (**9**), which was hydrogenated with Zn/80% AcOH to produce Boc–Glu(OBzl)–Leu–D-Leu–Val–Asp(OBzl)–D-Leu–$NHNH_2$ (**10**). The peptide **9** gave a satisfactory amino acid analysis (after acid hydrolysis), TLC and elemental analysis. The resolution of DL-3-tetradecanoic acid was effected with *d*- or *l*-ephedrine.[5] Optically active D- or L-3-hydroxytetradecanoic acid (**11a** or **b**) was converted to the corresponding phenacyl (Pac) ester (**12a** or **b**) using phenacyl bromide.[6] Phenacyl D- or L-3-(Boc–Leu–O)-tetradecanoate (**13a** or **b**) was obtained by the coupling reaction between Boc–Leu–OH and **12a** or **b** in THF with *N*,*N*′-carbonyldiimidazole. The N^{α}-deprotected product of **13a** or **b** was coupled with the azide derived from **10** to give phenacyl D- or L-[Boc–Glu(OBzl)–Leu–D-Leu–Val–Asp(OBzl)–D-Leu–Leu–O]-tetradecanoate (**14a** or **b**). Then the Pac group of **14a** or **b** was deblocked with Zn/90% AcOH[7] to afford D- or L-3-[Boc–Glu(OBzl)–Leu–D-Leu–Val–Asp(OBzl)–D-Leu–Leu–O]-tetradecanoic acid (**15a** or **b**). The acid **15a** or **b** was converted to the corresponding *N*-succinimidyl ester by the use of HOSu and DCC, and the crude product, without purification, was treated with TFA in the usual manner to remove the Boc group. The resulting TFA salt

was cyclized without further purification. Cyclization was achieved by a high dilution method[8] in pyridine at room temperature for 3 d. The protected cyclization product, cyclo{D- or L-3-[Glu(OBzl)–Leu–D-Leu–Val–Asp(OBzl)–D-Leu–Leu–O]-tetradecanoyl} (**16a** or **b**), was obtained in 72.9% or 71.2% yield after purification by column chromatography (Dowex 50W × 8 with MeOH : H_2O = 5 : 1 and Sephadex LH-20 with MeOH). Finally, the Bzl groups of **16a** or **b** were stripped off by hydrogenation with H_2/Pd–C in the usual manner in 90% AcOH. The desired products, **1a** or **b**, were obtained after purification by RP-HPLC using Cosmosil $5C_{18}$-P (250 × 20 mm, i.d.) (Fig. 3). The homogeneity of each synthetic compound was ascertained by analytical RP-HPLC and TLC. Their structures were characterized by FAB-MS (Fig. 4), amino acid analysis of acid hydrolysates, and elemental analyses. On the basis of the physical properties given in Table 1, the two synthetic products are identical except for the specific rotations.

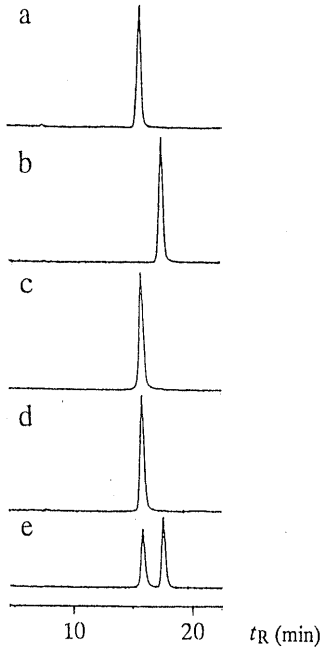

Fig. 3. HPLC Profiles of Natural Surfactin B_2, and Synthetic Products **1a** and **b**

RP-HPLC conditions: column, Cosmosil $5C_{18}$-P (250 mm × 4.6 mm i.d.); eluate, MeCN : H_2O : AcOH (80 : 20 : 1); flow rate, 1 ml/min; detection, UV 230 nm. a) **1a**, b) **1b**, c) natural surfactin B_2, d) natural surfactin B_2 + **1a** (1 : 1), e) natural surfactin B_2 + **1b** (1 : 1).

A direct comparison of the properties of our synthetic peptidelipid with those of natural surfactin B_2 was made by HPLC. The retention time of natural surfactin B_2 (Fig. 3c) on HPLC was identical with that of **1a** (Fig. 3a). Moreover, an equimolar mixture of natural surfactin B_2 and **1a** ran as a single symmetrical peak (Fig. 3d), whereas an equimolar mixture of natural surfactin B_2 and **1b** gave two peaks (Fig. 3e). Further evidence for the identity of **1a** with natural surfactin B_2 came from a comparison of their specific rotations, melting points and mass spectra (Fig. 4), all of which were closely similar. Those results indicate clearly that natural surfactin B_2 is identical with **1a**, which has D-3-hydroxytetradecanoic acid as the fatty acid moiety.

Compounds **1a** and **b** were compared with natural surfactin B_2 and sodium dodecyl sulfate (SDS) for cytolytic activity[9] against Ehrlich ascites carcinoma cells and hemolytic activity against erythrocytes. The results (Table 2) also support the conclusion that sufactin B_2 is identical with **1a**.

Experimental

General All melting points were determined on a Yanagimoto MP-J3 micromelting point apparatus without correction. Optical rotation was measured with a Nippon Bunko DIP-370 polarimeter. Infrared (IR) spectra were measured with a Nippon Bunko IRA-2 spectrometer, FAB-MS with a JEOL JMS-DX-300 mass spectrometer, and ^{1}H-NMR spectra on a JEOL JNM-M-100 spectrometer with tetramethylsilane as the internal standard. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. The solvents systems used for TLC (Kiesel gel GF_{254}, Merck) were $CHCl_3$: MeOH : AcOH (95 : 5 : 3, Rf^1), $CHCl_3$: MeOH (95 : 5, Rf^2), AcOEt (Rf^3), AcOEt : *n*-hexane (2 : 1, Rf^4), *n*-hexane : $CHCl_3$: dioxane (7 : 2 : 1, Rf^5). Column chromatography was carried out on Kiesel gel 60 (70—230 mesh, Merck). Acid hydrolysis of samples was conducted with twice-distilled 6 N HCl at 110 °C for 24 h in evacuated sealed tubes,

Table 2. Cytolytic Activity against Ehrlich Ascites Carcinoma Cells and Hemolytic Activity against Erythrocytes Given as Minimum Effective Concentration (μg/ml) of Surfactin B_2, Synthetic Products (**1a**, **b**) and SDS

	Cytolytic activity	Hemolytic activity
Surfactin B_2	100	10
1a	100	10
1b	150	25
SDS[a]	—[b]	25

Data are based on two experimental runs. *a*) SDS: sodium dodecyl sulfate. *b*) No activity at 200 μg/ml.

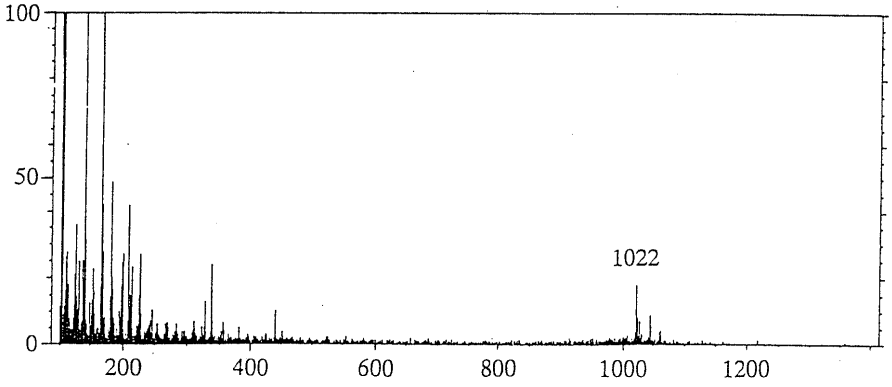

Fig. 4. Mass Spectrum of the Synthetic Products (**1a, b**)

and amino acid analysis was performed on a Hitachi KLA-5 amino acid analyzer system. HPLC was run on a Hitachi 638-30 liquid chromatograph with a Hitachi 635 M UV monitor.

Reagents Unless otherwise stated, all reagents and solvents were obtained commercially as reagent grade products and used without further purification. Boc-protected amino acids, HOSu, DCC and TFA were purchased from Peptide Institute Inc., Japan.

Peptide Synthesis The α-amino function of amino acids was protected by the Boc group. The γ- and β-carboxyl groups of Glu and Asp were protected by the Bzl group. Troc was used for the protection of hydrazide function. The protecting group for fatty acid was Pac for the carboxyl group.

Boc-D-Leu-NHNH-Troc (2) Isobutyl chlorocarbonate (6.83 ml, 50 mmol) was added to a solution of Boc-D-Leu-OH·H_2O (13.7 g, 55 mmol) and TEA (7 ml, 50 mmol) in dry THF (250 ml) under ice-cooling. The mixture was stirred for 30 min under ice-cooling and then Troc-$NHNH_2$[10] (10.4 g, 50 mmol) in THF (150 ml) was added thereto. The reaction mixture was stirred for 2 h under ice-cooling and evaporated *in vacuo*. The residue obtained was dissolved in AcOEt (600 ml) and this solution was washed successively with ice-cold 10% citric acid (300 ml), H_2O (200 ml), 1 N $NaHCO_3$ (300 ml) and saturated NaCl solution (200 ml), then dried over Na_2SO_4 and evaporated. The crystalline residue was reprecipitated from Et_2O–petroleum ether to give **2** (20.0 g, 94.8%). Rf^1 0.65, mp 91.0—93.0 °C, $[\alpha]_D^{8.5}$ +37.4° ($c=1$, MeOH). MS *m/z*: 363 $(M-C_4H_8)^+$, 307 $(M-2\times C_4H_8)^+$. *Anal.* Calcd for $C_{14}H_{24}Cl_3N_3O_5$: C, 39.97; H, 5.75; N, 9.99. Found: C, 40.11; H, 5.82; N, 9.66.

H-D-Leu-NHNH-Troc·TFA (3) A mixture of **2** (14.7 g, 35 mmol) and anisole (7.0 ml) was cooled to −20°C and TFA (35 ml) was added thereto. The mixture was stirred at 0 °C for 1 h, concentrated to a small volume *in vacuo* and then solidified by the addition of petroleum ether. The solid was collected by filtration and dried over KOH to give **3** (14.8 g, 97.3%), mp 159.5—161.5 °C.

Boc-Asp(OBzl)-D-Leu-NHNH-Troc (4) TEA (0.64 ml, 4.62 mmol) was added to a solution of **3** (2.01 g, 4.62 mmol) in DMF (2.1 ml) at −20°C, followed by AcOEt (42 ml) and Boc-Asp(OBzl)-OSu (1.76 g, 4.2 mmol) in AcOEt (3.8 ml). The reaction mixture was stirred at room temperature overnight and washed successively with H_2O (5 ml), ice-cold 0.5 N HCl (10 ml), ice-cold H_2O (5 ml), ice-cold 1 N $NaHCO_3$ (10 ml) and H_2O (5 ml × 2) and then dried over Na_2SO_4. The organic layer was evaporated *in vacuo* to give a solid, which was reprecipitated with AcOEt–petroleum ether to give **4** (2.25 g, 75.6%). Rf^1 0.65, mp 78—82 °C, $[\alpha]_D^{17}$ +17.5° ($c=1$, MeOH). MS *m/z*: 624 (M^+). *Anal.* Calcd for $C_{25}H_{35}Cl_3N_4O_8$·AcOEt: C, 48.78; H, 6.07; N, 7.85. Found: C, 48.90; H, 6.15; N, 7.86. Amino acid ratios in an acid hydrolysate: Asp 1.05, Leu 1.00.

Boc-D-Leu-Val-OBzl (5) In the same manner as described for **4**, H-Val-OBzl·TosH (20.8 g, 55 mmol) with TEA (7.7 ml, 55 mmol) in DMF (25 ml) and AcOEt (500 ml) was coupled with Boc-D-Leu-OSu (16.4 g, 50 mmol) derived from Boc-D-Leu-OH using DCC and HOSu. The resulting reprecipitated solid was purified by column chromatography on silica gel with $CHCl_3$: MeOH (95 : 5), followed by recrystallization from petroleum ether, to give **5** (15.7 g, 74.8%). Rf^1 0.94, mp 79.0—80.0 °C, $[\alpha]_D^{26}$ +7.8° ($c=2$, MeOH). MS *m/z*: 420 (M^+). *Anal.* Calcd for $C_{22}H_{36}N_2O_5$: C, 65.69; H, 8.63; N, 6.66. Found: C, 65.96; H, 8.79; N, 6.69. Amino acid ratios in an acid hydrolysate: Val 1.00, Leu 1.00.

Boc-Leu-D-Leu-Val-OBzl (6) A solution of **5** (2.33 g, 5.54 mmol) in anisole (1.0 ml) was deprotected with TFA (15 ml) in the same manner as described for **3** to give H-D-Leu-Val-OBzl·TFA (2.22 g, 5.12 mmol). In the same manner as described for **4**, the resulting TFA salt with TEA (0.71 ml) in DMF (3 ml) and AcOEt (25 ml) was coupled with Boc-Leu-OSu (1.68 g, 5.12 mmol) in AcOEt (10 ml) and the reaction mixture was treated to give **6**, which was reprecipitated from $CHCl_3$–Et_2O. Yield 1.74 g (63.8%), Rf^1 0.63, mp 142.5—144.0°C, $[\alpha]_D^{28}$ −5.6° ($c=2$, MeOH). MS *m/z*: 533 (M^+). *Anal.* Calcd for $C_{29}H_{47}N_3O_6$: C, 65.26; H, 8.88; N, 7.87. Found: C, 65.24; H, 8.87; N, 7.87. Amino acid ratios in an acid hydrolysate: Val 1.00, Leu 1.85.

Boc-Leu-D-Leu-Val-NHNH$_2$ (7) A solution of **6** (5.33 g, 10 mmol) was dissolved in MeOH (40 ml), then 100% hydrazine hydrate (5 ml, 100 mmol) was added. The mixture was left overnight at room temperature, and the product was solidified by addition of H_2O (100 ml). Recrystallization from MeOH gave **7** (4.55 g, 99.6%). mp 199—199.5 °C, $[\alpha]_D^{26}$ −4.4° ($c=0.5$, DMF), MS *m/z*: 457 (M^+). *Anal.* Calcd for $C_{22}H_{43}N_5O_6$: C, 57.74; H, 9.47; N, 15.30. Found: C, 57.83; H, 9.47; N, 15.14.

Boc-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-NHNH-Troc (8) Compound **4** (4.97 g, 7 mmol) was partially deblocked with TFA (14 ml) in the presence of anisole (1.4 ml) in the same manner as described for **3** to give H-Asp(OBzl)-D-Leu-NHNH-Troc·TFA. Then **7** (3.20 g, 7 mmol) was converted in the usual manner to the azide in DMF (14 ml) using 6 N HCl in dioxane (4.67 ml, 28 mmol) and isoamyl nitrite (1.15 ml, 8.4 mmol) at −20 °C for 30 min. The solution was neutralized with TEA at −20°C, and allowed to react with H-Asp(OBzl)-D-Leu-NHNH-Troc·TFA in DMF (14 ml) and TEA (0.97 ml, 7 mmol). The mixture was stirred and adjusted to pH 7—8 with TEA at 4 °C for 4 d. After addition of ice-cold H_2O (150 ml), the resulting crude product was collected, washed with H_2O and dried (P_2O_5). Reprecipitation from MeOH–petroleum ether gave **8** (3.28 g, 48.4%). Rf^1 0.59, mp 217—218 °C, $[\alpha]_D^{15.5}$ +13° ($c=1$, MeOH), $[\alpha]_D^{8.5}$ +76.8° ($c=1$, $CHCl_3$). FAB-MS *m/z*: 850 $(M+H-C_5H_8O_2)^+$. *Anal.* Calcd for $C_{42}H_{66}Cl_3N_7O_{11}$·$H_2O$: C, 52.04; H, 7.07; N, 10.11. Found: C, 52.30; H, 6.93; N, 10.08. Amino acid ratios in an acid hydrolysate: Asp 1.00, Val 0.96, Leu 3.00.

Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-NHNH-Troc (9) Compound **8** (11.0 g, 11.3 mmol) was partially deblocked with TFA (23 ml) in the presence of anisole (2.2 ml) in the same manner as described for **3** to give H-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-NHNH-Troc·TFA. The TFA salt in DMF (5.7 ml) was neutralized with TEA (1.58 ml, 11.3 mmol) at 0 °C, diluted with AcOEt (140 ml) and then coupled with Boc-Glu(OBzl)-ONp (5.18 g, 11.3 mmol) at room temperature for 12 h. The solvents were evaporated *in vacuo* and the residue was triturated with H_2O (150 ml). The rubber-like product was collected, washed with H_2O and dried (P_2O_5). It was purified by column chromatography on silica gel with $CHCl_3$: MeOH (95 : 5). The main fractions were collected, concentrated to a small volume, and dissolved in $CHCl_3$ (300 ml). The solution was washed successively with 1 N $NaHCO_3$ (100 ml, 50 ml), saturated NaCl solution (50 ml), ice-cold 5% citric acid (50 ml) and saturated NaCl solution (50 ml), dried over Na_2SO_4 and evaporated. The residue was solidified with *n*-hexane. The crude product was purified by column chromatography on silica gel with AcOEt. The main fraction was concentrated and solidified from AcOEt–petroleum ether. Reprecipitation from AcOEt–petroleum ether gave **9** (4.93 g, 37.3%). Rf^1 0.59, Rf^2 0.54, Rf^3 0.84, mp 188—190 °C, $[\alpha]_D^{20.5}$ +134° ($c=1$, MeOH). FAB-MS *m/z*: 1069 $(M+H-C_5H_8O_2)^+$. *Anal.* Calcd for $C_{54}H_{79}Cl_3N_8O_{14}$: C, 55.41; H, 6.80; N, 9.57. Found: C, 55.16; H, 6.79; N, 9.47. Amino acid ratios in an acid hydrolysate: Asp 0.99, Glu 1.00, Val 0.92, Leu 3.15.

Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-NHNH$_2$ (10) Compound **9** (2.34 g, 2 mmol) in 80% AcOH (25 ml) was partially deblocked with Zn dust (1.3 g, 20 mmol) for 48 h at room temperature. The reaction mixture was filtered and the filtrate was added to ice-cold H_2O (250 ml). The resulting solid was collected, washed with ice-cold H_2O and dried (P_2O_5). Reprecipitation from MeOH–petroleum ether gave **10** (1.79 g, 90.0%). Rf^1 0.61, Rf^3 0.74, Rf^4 0.30, mp 177—178.5 °C, $[\alpha]_D^{11}$ −9.4° ($c=1$, DMF). FAB-MS *m/z*: 895 $(M+H-C_5H_8O_2)^+$.

D-3-Hydroxytetradecanoic Acid (11a) and L-3-Hydroxytetradecanoic Acid (11b) Compound **11a** or **11b** was prepared by ephedrine resolution of the corresponding racemic acid according to the literature.[6] Physical data are as follows: **11a**–*l*-ephedrine salt: mp 95.5—97 °C, $[\alpha]_D^{13}$ −29.8° ($c=1$, $CHCl_3$) [ref. 6: mp 96.5—97.5 °C, $[\alpha]_D^{16}$ −31.1° ($c=1$, $CHCl_3$)]. **11a**: mp 72.0—73.0 °C, $[\alpha]_D^{17.5}$ −16.8° ($c=1$, $CHCl_3$), $[\alpha]_D^{12.5}$ +4.2° ($c=1$, MeOH), $[\alpha]_D^{17}$ −10.2° ($c=1$, pyridine) [ref. 6 mp 73.4—74.5 °C, $[\alpha]_D^{25}$ −10.4° ($c=1$, pyridine); ref. 5 mp 74—75 °C, $[\alpha]_D^{25}$ −15.4° ($c=2$, $CHCl_3$)]. **11b**-*d*-ephedrine salt: mp 95.5—96.5 °C, $[\alpha]_D^{13}$ +30.6° ($c=1$, $CHCl_3$) [ref. 6 mp 96—97.5°C, $[\alpha]_D^{16}$ +30.0° ($c=1$, $CHCl_3$)]. **11b**: mp 72.0—73.0 °C, $[\alpha]_D^{16}$ +16.8° ($c=1$, $CHCl_3$), $[\alpha]_D^{12.5}$ −4.2° ($c=1$, MeOH), $[\alpha]_D^{12.5}$ +10.3° ($c=1$, pyridine) [ref. 6: mp 72—73.5 °C, $[\alpha]_D^{25}$ +10.6° ($c=1$, pyridine); ref. 5 mp 74—75 °C, $[\alpha]_D^{25}$ +15.4° ($c=2$, $CHCl_3$)].

Phenacyl D-3-Hydroxytetradecanoate (12a) Phenacyl bromide (1.79 g, 9 mmol) was added to a solution of **11a** (2.20 g, 9 mmol) in EtOH (38.7 ml) and anhydrous Na_2CO_3 (0.48 g, 4.5 mmol) in H_2O (6.39 ml), and the mixture was refluxed for 2 h. After cooling, the reaction mixture was diluted with H_2O (180 ml) and the resulting ester was collected, washed with H_2O and dried over P_2O_5. Recrystallization from EtOH–H_2O gave **12a** (3.10 g, 95.2%) as long filament-like crystals. Rf^1 0.81, mp 69.5—71.0 °C, $[\alpha]_D^{15}$ 0° ($c=1$, MeOH). MS *m/z*: 362 (M^+). *Anal.* Calcd for $C_{22}H_{34}O_4$: C, 72.89; H, 9.45. Found: C, 72.89; H, 9.60. IR ν_{max}^{Nujol} cm^{-1}: 3500 (OH), 1708 (ester C=O), 1688 (C=O). ^{1}H-NMR

($CDCl_3$) δ: 0.86 (3H, t, J=5.5 Hz, $-CH_3$), 1.03—1.90 [20H, br, $-(CH_2)_{10}-$], 2.51—2.67 [2H, m, $-CH(OH)-C\underline{H}_2-COO-$], 3.27—3.51 (1H, br, -OH), 3.95—4.22 [1H, m, $-C\underline{H}(OH)-CH_2-$], 5.24 and 5.44 (2H, AB q, J=16 Hz, $-C\underline{H}_2-CO-Ph$), 7.32—7.61 (3H, m, *meta* and *para* Ar-H), 7.79—7.95 (2H, m, *ortho* Ar-H).

Phenacyl L-3-Hydroxytetradecanoate (12b) This compound was prepared from **11b** (2.20 g, 9 mmol) in the same manner as described for **12a** as long filament-like crystals. Yield 2.99 g (91.8%), Rf^1 0.78, mp 69.0—70.5 °C, $[\alpha]_D^{15}$ 0° (c=1, MeOH). MS m/z: 362 (M^+). *Anal.* Calcd for $C_{22}H_{34}O_4$: C, 72.89; H, 9.45. Found: C, 72.89; H, 9.55.

Phenacyl D-3-(Boc-Leu-O)-tetradecanoate (13a) A solution of Boc-Leu-OH (3.74 g, 15 mmol) in dry THF (34.5 ml) was treated with CDI (3.65 g, 22.5 mmol) with stirring at 0 °C for 1 h. Then **12a** (2.53 g, 7.5 mmol) in cold dry THF (30 ml) was added and the reaction mixture was stirred at 0 °C for 6 h and then at room temperature overnight. Next, H_2O (1 ml) was added to the reaction mixture, the solvents were evaporated off and the resulting residue was dissolved in ether (50 ml). This solution was washed successively with ice-cold H_2O (5 ml), ice-cold 10% citric acid (10 ml), ice-cold H_2O (5 ml), ice-cold 1 N $NaHCO_3$ (10 ml), and ice-cold H_2O (5 ml × 2). The organic layer was dried over Na_2SO_4 and evaporated. The resulting residue was purified by column chromatography on silica gel with *n*-hexane: $CHCl_3$: dioxane (7:2:1). The main fractions were collected, concentrated to a small volume, and purified by column chromatography on silica gel with benzene: AcOEt (97:3). The main fraction was evaporated to give oily **13a** (3.20 g, 74.2%), Rf^5 0.57. MS m/z: 575 (M^+).

Phenacyl L-3-(Boc-Leu-O)-tetradecanoate (13b) The same procedure as used for **13a** gave oily **13b** from **12b** (2.53 g, 7.5 mmol). Yield 2.86 g (66.2%), Rf^5 0.60. MS m/z: 575 (M^+).

Phenacyl D-3-[Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoate (14a) Compound **13a** (1.94 g, 3.37 mmol) was partially deblocked with TFA (10.25 ml) in the presence of anisole (0.68 ml) in the same manner as described for **3** to give phenacyl D-3-(H-Leu-O)-tetradecanoate·TFA. Then **10** (3.05 g, 3.07 mmol) was converted to the azide in DMF (6.5 ml) using 4 N HCl in dioxane (3.17 ml, 13.5 mmol) and isoamyl nitrite (0.50 ml, 3.68 mmol) in the same manner as described for **8**. The azide was allowed to react with phenacyl D-3-(H-Leu-O)-tetradecanoate·TFA (*ca.* 3.37 mmol) in DMF (6.5 ml) and TEA (0.47 ml, 3.37 mmol) at 4 °C for 48 h. The solvents were evaporated *in vacuo* and the resulting residue was dissolved in AcOEt (32.6 ml). This solution was washed successively with ice-cold 5% citric acid (10 ml), ice-cold H_2O (5 ml), ice-cold 1 N $NaHCO_3$ (10 ml), ice-cold H_2O (5 ml) and saturated NaCl (5 ml). The organic layer was dried over Na_2SO_4 and evaporated. The resulting residue was purified by column chromatography on silica gel with $CHCl_3$: MeOH : AcOH (95 : 5 : 3). The desired product was collected, concentrated to a small volume and dissolved in AcOEt. This solution was washed with H_2O, dried over Na_2SO_4 and evaporated *in vacuo*. Reprecipitation of the product from AcOEt–ether–petroleum ether gave **14a** (2.91 g, 66.2%), Rf^1 0.63, mp 131—132 °C, $[\alpha]_D^{9.5}$ +6.4° (c=1, MeOH). FAB-MS m/z: 1338 $(M+H-C_5H_8O_2)^+$.

Phenacyl L-3-[Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoate (14b) The same procedure as used for **14a** gave **14b** from **13b** (1.94 g, 3.37 mmol) and **10** (3.05 g, 3.07 mmol). Yield 1.99 g (45.2%), Rf^1 0.72, mp 116—118 °C, $[\alpha]_D^{9.5}$ +6.8° (c=1, MeOH). FAB-MS m/z: 1338 $(M+H-C_5H_8O_2)^+$.

D-3-[Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoic Acid (15a) A solution of **14a** (1.87 g, 1.3 mmol) in 90% AcOH (39 ml) containing Zn dust (1.7 g, 26 mmol) was stirred at 0 °C for 1 h and at room temperature overnight.[8] It was filtered and the filtrate was evaporated *in vacuo*. The resulting residue was dissolved in AcOEt (100 ml) and this solution was washed successively with H_2O (20 ml × 2) and saturated NaCl (40 ml). The organic layer was dried over Na_2SO_4 and evaporated. The resulting residue was purified by column chromatography on silica gel with $CHCl_3$: MeOH (95 : 5). The desired fraction was collected, and concentrated to a small volume. Reprecipitation from AcOEt–*n*-hexane gave **15a** (1.23 g, 70.8%), Rf^2 0.22, Rf^4 0.73, mp 111—113 °C, $[\alpha]_D^{8.5}$ +2.0° (c=1, MeOH). FAB-MS m/z: 1220 $(M+H-C_5H_8O_2)^+$. *Anal.* Calcd for $C_{71}H_{113}N_7O_{16}\cdot H_2O$: C, 63.70; H, 8.66; N, 7.32. Found: C, 63.46; H, 8.65; N, 7.59. Amino acid ratios in an acid hydrolysate: Asp 0.99, Glu 1.02, Val 1.00, Leu 4.02.

L-3-[Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoic Acid (15b) The same procedure as used for **15a** gave **15b** from **14b** (1.87 g, 1.3 mmol). Yield 1.18 g (67.6%), Rf^2 0.29, Rf^4 0.74, mp 121—123 °C, $[\alpha]_D^{7.5}$ +8.4° (c=1, MeOH). FAB-MS m/z: 1220 $(M+H-C_5H_8O_2)^+$. *Anal.* Calcd for $C_{71}H_{113}N_7O_{16}\cdot H_2O$: C, 63.70; H, 8.66; N, 7.32. Found: C, 63.69; H, 8.65; N, 7.72. Amino acid ratios in an acid hydrolysate: Asp 0.99, Glu 1.04, Val 1.00, Leu 3.97.

Cyclo{D-3-[Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoyl} (16a) HOSu (121 mg, 1.05 mmol) and DCC (187 mg, 0.91 mmol) were added to a solution of **15a** (0.936 g, 0.7 mmol) in DMF (3.5 ml) at 0 °C and the mixture was stirred at 4 °C for 48 h. The solvent was evaporated *in vacuo* and the resulting residue was dissolved in $CHCl_3$. The insoluble material was filtered off and the filtrate was concentrated to a small volume. The residue was triturated with H_2O, and the solid was collected and dried over P_2O_5 to give crude succinimidyl D-3-[Boc-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoate (1.01 g, *ca.* 0.7 mmol). The succinimidyl ester was partially deblocked with TFA (4 ml) in the presence of anisole (0.4 ml) in the same manner as described for **3** to give oily succinimidyl D-3-[H-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoate·TFA. A solution of succinimidyl D-3-[H-Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoate·TFA (*ca.* 0.7 mmol) in DMF (7 ml) was added dropwise to pyridine (210 ml) with stirring during 5 h at room temperature and the reaction mixture was kept at room temperature for 72 h, then evaporated *in vacuo*.[9] The resulting residue was triturated with ice-cold H_2O containing one drop of 10% AcOH and the solid was dried to give crude **16a**, which was purified by column chromatography on Dowex 50W × 8 (1.8 × 11 cm) with MeOH : H_2O (5 : 1). The desired fractions were collected, concentrated to a small volume and subjected to gel filtration on a Sephadex LH-20 (3.0 × 50 cm) with MeOH. Fractions (3 ml each) containing the desired material (fraction No. 46—55) were collected and evaporated. Reprecipitation from ether–petroleum ether gave **16a** (0.622 g, 72.9%), Rf^1 0.82, mp 101—103 °C, $[\alpha]_D^{11.5}$ −26.6° (c=1, MeOH). FAB-MS m/z: 1202 $(M+H)^+$. *Anal.* Calcd for $C_{66}H_{103}N_7O_{13}$: C, 65.92; H, 8.63; N, 8.15. Found: C, 65.60; H, 8.43; N, 8.09.

Cyclo{L-3-[Glu(OBzl)-Leu-D-Leu-Val-Asp(OBzl)-D-Leu-Leu-O]-tetradecanoyl} (16b) The same procedure as used for **16a** gave **16b** from **15b** (0.936 g, 0.7 mmol). Yield 0.608 g (71.2%), Rf^1 0.71, mp 85—87 °C, $[\alpha]_D^{11.5}$ −13.4° (c=1, MeOH). FAB-MS m/z: 1202 $(M+H)^+$. *Anal.* Calcd for $C_{66}H_{103}N_7O_{13}$: C, 65.92; H, 8.63; N, 8.15. Found: C, 65.97; H, 8.80; N, 8.03.

Cyclo[D-3-(Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu-O)-tetradecanoyl] (1a) Compound **16a** (0.366 g, 0.3 mmol) was hydrogenated in 90% AcOH (6 ml) in the presence of Pd-black for 4 h. The catalyst was removed by filtration and the filtrate was evaporated. The resulting residue was reprecipitated from ether–petroleum ether to give crude **1a** (0.276 g, 89.0%). The crude product was purified by preparative HPLC (Cosmosil $5C_{18}$-P, 250 × 20 mm, i.d.) with MeCN : H_2O : AcOH (80 : 20 : 1) and the main peak fractions were collected. The organic solvents were evaporated *in vacuo* and the remaining aquous emulsion was lyophilized and dried (KOH). The result of analytical HPLC is shown in Fig. 3a. Rf^1 0.36, mp 136—138 °C, $[\alpha]_D^{9.5}$ −37.0° (c=1, MeOH), $[\alpha]_D^{7.5}$ +37.6° (c=1, $CHCl_3$). FAB-MS m/z: 1022 $(M+H)^+$. *Anal.* Calcd for $C_{52}H_{91}N_7O_{13}$: C, 61.09; H, 8.97; N, 9.59. Found: C, 60.83; H, 8.60; N, 9.94. Amino acid ratios in an acid hydrolysate: Asp 0.99, Glu 0.95, Val 1.00, Leu 3.98.

Cyclo[L-3-(Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu-O)-tetradecanoyl] (1b) The same procedure as used for **1a** gave **1b** from **16b** (0.366 g, 0.3 mmol). The result of analytical HPLC is shown in Fig. 3b. Yield 0.283 g (92.4%), Rf^1 0.36, mp 136—138 °C, $[\alpha]_D^{12.5}$ −19.6° (c=1, MeOH), $[\alpha]_D^{12.5}$ +20.4° (c=1, $CHCl_3$). FAB-MS m/z: 1022 $(M+H)^+$. *Anal.* Calcd for $C_{52}H_{91}N_7O_{13}$: C, 61.09; H, 8.97; N, 9.59. Found: C, 60.94; H, 8.71; N, 9.90. Amino acid ratios in an acid hydrolysate: Asp 0.98, Glu 1.06, Val 1.00, Leu 4.16.

Cytolytic Activity against Ehrlich Ascites Carcinoma Cells The bioassay method used for this study was essentially the same as that previously described.[9] Ehrlich ascites carcinoma cell suspension was prepared in phosphate-buffered saline (PBS) (pH 7.4) instead of phosphate buffer (pH 7.2). Mixtures of tumor cell suspension (4×10^7 cells/ml PBS, 40 μl), trypsin[11] solution (1.25 mg/ml PBS, 20 μl), EDTA solution (2.5 mg/ml PBS, 20 μl), and sample solution (1, 0.75, 0.5, 0.25, 0.1, and 0.05 mg/ml PBS, 20 μl), were incubated at 37 °C for 2 h, then centrifuged at 3000 rpm for 10 min, and the presence (negative activity) or absence (positive activity) of precipitated cells was noted. Minimum cytolytic concentrations of surfactin B_2, **1a**, **1b** and SDS are listed in Table 2.

Hemolytic Activity against Erythrocytes Human blood (1 ml) was

centrifuged at 2500 rpm for 5 min. Precipitated erythrocytes were collected, washed 3 times with 2 ml each of PBS by centrifugation and suspended in PBS (10 ml). Mixtures of erythrocyte suspension (5×10^5 erythrocytes/ml PBS, 50 μl) and sample solution (0.2, 0.1, 0.05, 0.02, 0.01, and 0.005 mg/ml PBS, 50 μl) were incubated at 37°C for 2 h, then centrifuged at 2500 rpm for 5 min and the presence (negative activity) or absence (positive activity) of precipitated erythrocytes was noted. Minimum hemolytic concentrations of surfactin B_2, **1a**, **b** and SDS are listed in Table 2.

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References and Notes

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2) Amino acids and their derivatives mentioned in this paper are of L-configuration unless otherwise indicated. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9—37 (1984). Other abbreviations used are: Boc, *tert*-butoxycarbonyl; HOSu, *N*-hydroxysuccinimide; OSu, *N*-succinimidyl ester, ONp, 4-nitrophenyl ester; OBzl, benzyl ester; Troc, 2,2,2-trichloroethyloxycarbonyl; Tos, 4-toluenesulfonyl; DCC, *N*,*N'*-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; TEA, triethylamine; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography.
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