Structure and Synthesis of an Immunoactive Lipopeptide, WS1279, of Microbial Origin[1)]

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The structure of WS1279, isolated from *Streptomyces* sp. as an immunoactive lipopeptide, has been deduced on the basis of chemical and physical evidence as *S*-[2,3-bis(palmitoyloxy)propyl]-N^{α}-palmitoyl-Cys-Asn-Ser-Gly-Gly-Ser-OH. This was confirmed by synthesis.

Keywords *Streptomyces*; natural product; lipopeptide; *S*-2,3-dihydroxypropylcysteine; chemical synthesis; immunostimulating activity

Bacteria and cell wall components exhibit immunostimulating activity through proliferation of colony stimulating units in the bone marrow.[2,3)] In the course of our screening program for such immunoactive substances, we isolated from *Streptomyces willmorei* No. 1279 a new lipopeptide, WS1279, which stimulates the proliferation of bone marrow cells.[4)] Here, we report the structural elucidation and synthesis of this natural product.

WS1279 (I) was isolated as a mixture of lipopeptides in which the lipid part is composed of several kinds of fatty acids (Fig. 1). The fast-atom bombardment mass spectrum (FAB-MS) of I showed five main molecular ion peaks at *m/z* $(M+Na)^+$ 1306, 1320, 1334 (main), 1348 and 1362. The presence of the three fatty acid residues in I was corroborated by the ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of I in $CDCl_3$–CD_3OD: δ 0.85 (9H, m, $3\times CH_3$), 1.25 (*ca.* 72H, m, $(CH_2)_n$), 1.60 (6H, m, $3\times\beta$-CH_2), 2.30 (6H, m, $3\times\alpha$-CH_2). Hydrolysis of I with 6 N HCl followed by methylation with diazomethane gave a mixture of fatty acid methyl esters. The total ion chromatogram of this mixture in gas chromatography–mass spectrum (GC-MS) showed five major peaks at 3 min 26 s, 3 min 31 s, 4 min 20 s, 4 min 42 s and 5 min 17 s (5:1:3:9:2), corresponding to *m/z* 256, 256, 270, 270 and 284, respectively, which were identified as methyl isopentadecanoate, methyl anteisopentadecanoate, methyl isopalmitate, methyl palmitate and methyl isoheptadecanoate, respectively, by comparison GC-MS with authentic samples. Alkaline hydrolysis of I (1 N NaOH) gave a product, whose FAB-MS showed the molecular ion at *m/z* 858 $(M+Na)^+$ due to loss of two fatty acid residues from the molecule of I, indicating that two of the three fatty acid residues are bonded somewhere to the peptide sequence by ester bonds and therefore the remaining one is linked by an amide bond. The latter amide-bonded lipophilic part was found to consist of a single fatty acid, because the molecular ion of the hydrolysis product appeared as a single peak.

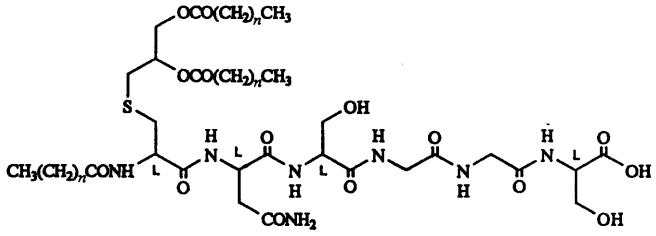

Fig. 1. Structure of WS1279

Amino acid analysis of the 6 N HCl hydrolysate of WS1279 (I) revealed the presence of Asp, Ser, Gly and NH_3 (1:2:2:1), plus two unknown compounds at 9.62 min (before Asp) and at 15.40 min (between Ser and Gly) in addition to the peak due to cystine (22.72 min). Asp and Ser were determined to be L by high performance liquid chromatography (HPLC) analysis using a chiral column (Chiralpak WH). L-Asp is presumed to have been derived from L-Asn, because an equimolar ratio of Asp and NH_3 was obtained in the amino acid analysis as described above and the C-terminal was shown to be a carboxylic acid but not a carboxamide as inferred from the result of hydrolysis with carboxypeptidase A (see below). The two unidentified peaks in the amino acid analysis were presumed to be *S*-glycerylcysteine and its degradation product.

The presence of *S*-glycerylcysteine in the molecule of WS1279 (I) was supposed on the following grounds. The ^{1}H-NMR spectrum of I showed signals at δ 2.75 (2H, m), 4.13 (1H, m), 4.37 (1H, m) and 5.18 (1H, m), and the ^{1}H–^{1}H correlation spectrum (^{1}H–^{1}H COSY) revealed that these signals are coupled to each other, indicating the partial structure shown in Fig. 2. The presence of one sulfur atom was identified by EMAX analysis [found, 2.2%; calcd. based on I ($n=14$), 2.4%]. The FAB-MS of WS1279 itself and the alkaline hydrolysis product both showed a fragment ion at *m/z* 751, which corresponds to the fragments formed by elimination of the $SCH_2CH(OR)CH_2OR$ units from the molecules. The final confirmation of the *S*-glycerylcysteine unit was obtained by comparison with a synthetic sample of *S*-[(2*RS*)-2,3-dihydroxypropyl]cysteine in amino acid analysis. The synthetic sample showed a peak corresponding to that at 9.62 min in the amino acid analysis of I, while, after hydrolysis with 6 N HCl, the synthetic sample showed an additional peak corresponding to that at 15.40 min. This material was deduced to be *S*-[(2*RS*)-2-chloro-3-hydroxypropyl]cysteine from the result of the GC-MS measurement (see below).

The stereochemistry of Cys in the *S*-glycerylcysteine

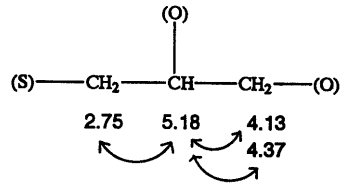

Fig. 2. Coupling in the ^{1}H–^{1}H COSY of WS1279

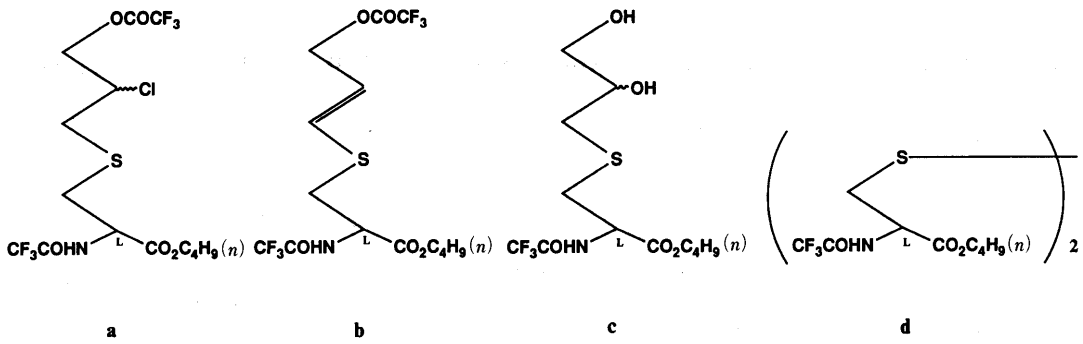

Fig. 3. Structure of Compounds (**a**, **b**, **c** and **d**) Derived from Hydrolysates of WS1279

moiety was deduced to be L as follows. The HCl hydrolysate of I was examined by HPLC on a chiral column [Crownpak CR(+)]. The hydrolysate showed peaks at 8.88, 9.49, 25.88 and 30.15 min. The former two peaks were identified as those of the diastereomeric *S*-[(2*RS*)-glyceryl]-L-cysteines by comparison with the synthetic sample. The corresponding D-cysteine derivatives, *S*-[(2*RS*)-glyceryl]-D-cysteines, were not separated and showed a peak at 4.94 min on Crownpak CR(+) under the same conditions. The degradation products of the synthetic *S*-[(2*RS*)-glyceryl]-L-cysteines also showed peaks at 25.88 and 30.15 min corresponding to the latter two peaks of the natural product under the same conditions. The structures of these products were deduced to be *S*-[(2*RS*)-2-chloro-3-hydroxypropyl]-L-cysteines as follows. The 6N HCl hydrolysate of *S*-[(2*RS*)-glyceryl]-L-cysteines was treated with 3N HCl in *n*-BuOH and then with TFAA in CH_2Cl_2 and subjected to GC-MS, which showed four main peaks in GC (scan numbers: 261, 284, 296 and 429). These peaks were estimated to show molecular weights of 461, 425, 347 and 544 from the MS data (see Experimental) and thus were assigned to have structures **a**, **b**, **c** and **d**, respectively. The peak at 15.40 min in the amino acid analysis and the peaks at 25.88 and 30.15 min in the chiral HPLC were assigned to the diastereoisomeric products **a**. Compound **b** was speculated to have been derived from during the treatments with HCl–*n*-BuOH and TFAA. Compound **c** was presumed to be a fragment ion of the tris(trifluoroacetyl) *n*-butyl ester of *S*-[(2*RS*)-glyceryl]-L-cysteine and compound **d** to be the *n*-butyl N^{α}-trifluoroacetylcystinate (cystine was observed at 22.72 min in the amino acid analysis of a hydrolysate of I). These results are summarized in Fig. 3.

In order to clarify the configuration at position 2 of the glyceryl moiety of *S*-glycerylcysteine, *S*-[(2*R*)-2,3-*O*-isopropylidene-2,3-dihydroxypropyl]-N^{α}-palmitoyl-L-Cys-OBu^t and its (2*S*) counterpart were synthesized. Although they showed single peaks on HPLC (Chiralcel OK) at 53.758 and 51.612 min, respectively, both showed, after treatment with 6N HCl, peaks identical with those at 9.62 and 15.40 min in the amino acid analysis and peaks corresponding to those at 8.88, 9.49, 25.88 and 30.15 min in the chiral HPLC [Crownpack CR(+)]. These results indicate that during the 6N HCl hydrolysis, the glyceryl moiety epimerized at position 2.

For determination of the amino acid sequence in WS1279 (I), an enzymatic degradation method was applied to I in combination with FAB-MS. After treatment of I with carboxypeptidase A, followed by alkaline hydrolysis for removal of the two palmitoyl ester groups, the resulting mixture was analyzed by FAB-MS. Cluster ions were observed at *m/z* 880, 793, 736, 679, 592 and 478 for $(M+2Na-H)^+$ and at *m/z* 858, 771, 714, 657, 570 and 456 for $(M+Na)^+$ as shown in Fig. 4. These data are consistent with the sequence Asn–Ser–Gly–Gly–Ser–OH. The hydrazine degradation of I further corroborated that Ser is the C-terminal in the sequence. The clusters at *m/z* 478 $(M+2Na-H)^+$ and 456 $(M+Na)^+$ correspond to N^{α}-palmitoyl-*S*-glycerylcysteine, which was thus found to be the N-terminal. Taking account of all the above chemical and physical data, we proposed the structure of WS1279 to be I, in which the fatty acid residues are composed mainly of palmitic acid ($n=14$).

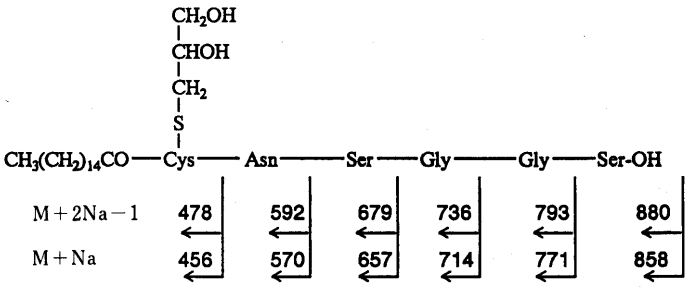

Fig. 4. Cluster Ions in the FAB-MS of the Mixture Obtained by Digestion of WS1279 with Carboxypeptidase A

A final confirmation of the structure I was obtained by a synthesis of I ($n=14$) as a diastereomeric mixture at position 2 of the glyceryl moiety as follows. The peptide part was prepared as shown in Fig. 5. Starting with H–Ser(Bu^t)–OBu^t,[5] Z–Gly–ONp[6] was coupled to give Z–Gly–Ser(Bu^t)–OBu^t. The Z group was removed by catalytic hydrogenation and the resulting amine was coupled with Z–Gly–ONp to give Z–Gly–Gly–Ser(Bu^t)–OBu^t. After removal of the Z group, Z–Ser(Bu^t)–OH[5] was coupled by the DCC–HOBt method[7] to afford Z–Ser(Bu^t)–Gly–Gly–Ser(Bu^t)–OBu^t. After removal of the Z group, the resulting peptide was coupled with Z–Asn–ONp[8] to give Z–Asn–Ser(Bu^t)–Gly–Gly–Ser(Bu^t)–OBu^t. Removal of the Z group afforded H–Asn–Ser(Bu^t)–Gly–Gly–Ser(Bu^t)–OBu^t in an analytically pure form. This peptide was coupled with *S*-[(2*RS*)-2,3-bis(palmitoyloxy)propyl]-N^{α}-palmitoyl–Cys–OH by the DCC–HOBt method described by Wiesmueller *et al.*,[9] to give *S*-[(2*RS*)-2,3-bis(palmitoyloxy)propyl]-N^{α}-palmitoyl–Cys–Asn–Ser(Bu^t)–Gly–Gly–Ser(Bu^t)–OBu^t. The final deprotection was carried out by treatment with

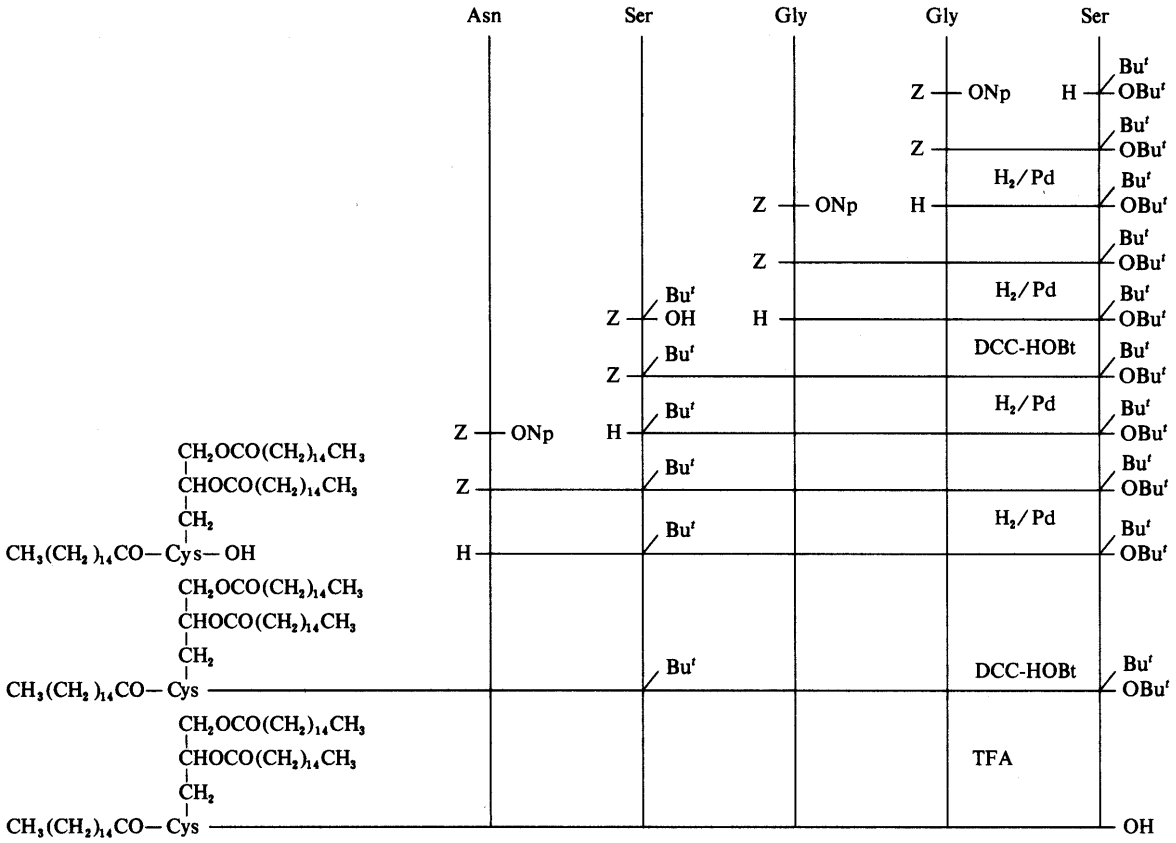

Fig. 5. Synthetic Route to WS1279

trifluoroacetic acid (TFA) to give *S*-[(2*RS*)-2,3-bis(palmitoyloxy)propyl]-N^{α}-palmitoyl–Cys–Asn–Ser–Gly–Gly–Ser–OH.

The synthetic sample was identical with the natural product on thin layer chromatography (TLC) and HPLC. It showed activity to stimulate the colony stimulating factor (CSF)-induced proliferation of bone marrow cells *in vitro* (concentration giving 50% increase: synthetic, 0.38 μg/ml; natural, 0.33 μg/ml). This discrepancy in activity between the synthetic sample and the natural product was probably due to the following reasons: 1) the synthetic sample was a diastereomeric mixture; 2) the synthetic sample was of higher purity and therefore was less soluble in H_2O.[10] Furthermore, it was revealed that the peptides Z–Asn–Ser–Gly–Gly–Ser–OH, H–Asn–Ser(Bu^t)–Gly–Gly–Ser(Bu^t)–OBu^t and H–Asn–Ser–Gly–Gly–Ser–OH did not exhibit any stimulating activity at the concentration of 50 μg/ml, although *S*-[(2*RS*)-2,3-bis(palmitoyloxy)propyl]-N^{α}-palmitoyl–Cys–OH exhibited stimulating activity at the concentration of 5 μg/ml. These results indicate that the lipid peptide structure with a certain chain length is required for manifestation of full stimulating activity. Full details of their activity will be reported elsewhere.

Experimental

The melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co., Ltd.). Amino acid compositions of acid hydrolysates (6 N HCl, 110 °C, 18 h) were determined with an amino acid analyzer (K-101 AS, Kyowa Seimitsu). HPLC was conducted with a Shimadzu LC-4A instrument. ^{1}H-NMR spectra were measured with a Bruker AM 400 wb spectrometer, MS with a VG Analytical ZAB-SE and GC–MS with a ZAB-SE instrument equipped with a Hewlett Packard 5890 computer. For column chromatography, a Toyo SF-160 fraction collector was used, if necessary. In TLC (Kieselgel G, Merck), Rf^1, Rf^2, Rf^3, Rf^4 and Rf^5 values refer to the systems of $CHCl_3$, MeOH and H_2O (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (89 : 10 : 1), $CHCl_3$, MeOH, H_2O (8 : 3 : 1, lower phase), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase) and *n*-BuOH, AcOH, pyridine and H_2O, (1 : 1 : 1 : 1), respectively.

WS1279 (I) WS1279 was isolated as a powder from a fermentation broth of *Streptomyces willmorei* No. 1279.[4] TLC: Rf^3 0.10. HPLC: column, Ultrasphere (4.6 mm i.d. × 250 mm); eluant, MeOH–aq. $HClO_4$ (pH 2) (93 : 7); flow rate, 1 ml/min; temperature, 50 °C; detection, UV 210 nm; retention time, 15.4 min. Amino acid analysis of an acid hydrolysate: Asp (11.26 min), Ser (12.89 min), Gly (19.14 min), NH_3 (39.38 min) (1 : 2 : 2 : 1), cystine (22.72 min), plus two unknown peaks (9.62 and 15.40 min). FAB-MS *m/z*: $(M+Na)^+$ 1306, 1320, 1334 (main), 1348, 1362; fragment ion, *m/z* 751. ^{1}H-NMR ($CDCl_3$–CD_3OD) δ: 0.85 (9H, m, 3 × CH_3), 1.25 (*ca.* 72H, m, $(CH_2)_n$), 1.60 (6H, m, 3 × β-CH_2), 2.30 (6H, m, 3 × α-CH_2). EMAX analysis: Calcd 2.4% (n = 14 in I), Found: 2.2%.

Hydrolysis of WS1279 with 6 N HCl WS1279 (0.1 mg) was hydrolyzed with 6 N HCl (0.8 ml) at 110 °C in a sealed tube for 18 h. The reaction mixture was evaporated to dryness, and the residue was dissolved in 0.1 N HCl and the solution was extracted with AcOEt. The extract was evaporated and the residue was dissolved in MeOH and treated with CH_2N_2 in ether. This mixture was subjected to GC–MS. GC conditions: column, Quadrex (methyl silicone, 0.25 mm i.d. × 25 m × 0.25 μm film); oven temperature, 150—260 °C (10 °C/min); injection temperature, 270 °C; inlet pressure, 10 psi; carrier gas, He at 40 ml/min; retention times, 3 min 26 s, 3 min 31 s, 4 min 20 s, 4 min 42 s and 5 min 17 s (5 : 1 : 3 : 9 : 2), which were identified as those of methyl isopentadecanoate (*m/z* 256), methyl anteisopentadecanoate (*m/z* 256), methyl isopalmitate (*m/z* 270), methyl palmitate (*m/z* 270), and methyl isoheptadecanoate (*m/z* 284), respectively, by comparison with authentic samples. The 0.1 N HCl layer was evaporated and the residue was again dissolved in 0.1 N HCl (20 μl). A 10 μl portion of this solution was subjected to HPLC using Chiralpack WH (4 mm i.d. × 250 mm); eluant, 0.25 mM $CuSO_4$; flow rate, 1 ml/min; column temperature, 50 °C; detection, UV 254 nm; retention time, 43.33, 25.75 min (L-Asp, 43.33; D-Asp, 35.45; L-Ser, 25.75; D-Ser, 17.82 min). The remaining 10 μl portion of the 0.1 N HCl solution was subjected to HPLC using Crownpack CR(+) (4 mm i.d. × 150 mm); eluant, aq. $HClO_4$ (pH 1.0); flow rate, 0.4 ml/min; column temperature, 0 °C; detection, UV 200 nm; retention times, 8.88, 9.49, 25.88 and 30.15 min. The former two peaks

were identified as diastereomeric *S*-[(2*RS*)-glyceryl]-L-cysteines by comparison with a synthetic sample (see below). For identification of the latter two peaks, see the data on hydrolysis of *S*-[(2*RS*)-glyceryl]-L-cysteines with 6 N HCl.

Hydrolysis of WS1279 with NaOH WS1279 (1 mg) was hydrolysed with 1 N NaOH–MeOH (1 : 1, 0.5 ml) at room temperature for 2 h. A portion of the hydrolysate was subjected to FAB-MS *m/z*: 858 $(M+Na)^+$.

Enzyme Degradation of WS1279 with Carboxypeptidase A WS1279 (1 mg) was treated with carboxypeptidase A (0.2 mg) in 5% $NaHCO_3$ (1 ml, pH 7.0) at 20 °C for 10 d. A 1 N NaOH solution (0.2 ml) was added to the mixture and, after 2 h at room temperature, a portion of the mixture was subjected to FAB-MS *m/z*: 880, 793, 736, 679, 592 and 478 for $(M+2Na-H)^+$ and 858, 771, 714, 657, 570 and 456 for $(M+Na)^+$.

Degradation of WS1279 with Hydrazine WS1279 (0.1 mg) was hydrazinolyzed with dry hydrazine (0.8 ml) in a sealed tube at 100 °C for 6 h. The reaction mixture was lyophilized and the residue was dissolved in 0.1 N HCl and applied to an amino acid analyzer: the detected amino acid was Ser.

Hydrolysis of *S*-[(2*RS*)-Glyceryl]-L-cysteines with 6 N HCl *S*-[(2*RS*)-Glyceryl]-L-cysteines (0.1 mg) were hydrolyzed with 6 N HCl (0.8 ml) in the same manner as described above and then the mixture was evaporated to dryness. Amino acid analysis showed two peaks corresponding to those (9.62 and 15.40 min) seen in the amino acid analysis of WS1279. HPLC on Crownpack CR(+) under conditions similar to those for the 6 N HCl hydrolysate of I showed four peaks corresponding to those at 8.88, 9.49, 25.88 and 30.15 min. For identification of the former two peaks, see the data on hydrolysis of WS1279 with 6 N HCl. For identification of the latter two peaks, the following analysis was carried out. The hydrolysate was treated with 3 N HCl in *n*-BuOH (500 μl) at 100 °C for 15 min. The mixture was again evaporated to dryness and the residue was treated with TFAA (200 μl) in CH_2Cl_2 (600 μl) at 150 °C for 5 min. This reaction mixture was subjected to GC–MS: GC column, Ultra 1 (0.32 mm i.d. × 25 m × 0.1 μm film); oven temperature, 100—300 °C (10 °C/min); injection temperature, 270 °C; inlet pressure, 0.7 kgf/cm^2; reagent gas, NH_3(Cl): scan numbers, 261, 284, 296 and 429, which were assigned to be due to **a** [*m/z* 479 and 481 $(M+18)^+$], **b** [*m/z* 426 $(M+1)^+$, 443 $(M+18)^+$ and 461 $(M+36)^+$], **c** [*m/z* 347 M^+, 365 $(M+18)^+$ and 383 $(M+36)^+$] and **d** [*m/z* 544 M^+, 562 $(M+18)^+$]. The peaks at 25.88 and 30.15 min in the chiral HPLC were assigned to **a** (see the text, Fig. 3).

***S*-[(2*RS*)-2,3-Bis(palmitoyloxy)propyl]-*N*$^\alpha$-palmitoyl-Cys-OH** The title compound was prepared according to the method described by Wiesmueller *et al.*[9]

Z-Gly-Ser(Bu^t)-OBu^t (1) Z-Gly-ONp (7.4 g, 0.022 ml) and H-Ser(Bu^t)-OBu^t [prepared from Z-Ser(Bu^t)-OBu^t (13.7 g, 0.040 mol) by catalytic hydrogenation over a Pd catalyst] were dissolved in DMF (50 ml) and the reaction mixture was stirred at room temperature for 48 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give an oily material. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (5 × 26 cm), equilibrated and eluted with $CHCl_3$. The eluate (2000—3000 ml) evaporated to dryness. Petroleum ether was added to the residue to provide the purified oily material, yield 8.2 g (89%), Rf^1 0.73, Rf^2 0.63. *Anal.* Calcd for $C_{21}H_{32}N_2O_6$: C, 61.7; H, 7.90; N, 6.86. Found: C, 61.7; H, 7.93; N, 6.79. Amino acid ratios in an acid hydrolysate: $Ser_{1.00}Gly_{1.00}$ (average recovery 85%).

Z-Gly-Gly-Ser(Bu^t)-OBu^t (2) The title compound was prepared from Z-Gly-ONp (6.3 g, 0.019 mol) and H-Gly-Ser(Bu^t)-OBu^t [prepared from **1** (8.2 g, 0.020 mol)] in the same manner as described for the synthesis of **1**. The crude material was recrystallized from AcOEt and ether, yield 5.7 g (64%), mp 92—98 °C, $[\alpha]_D^{22}$ +5.8° (*c* = 1.0, MeOH), Rf^1 0.50, Rf^2 0.44. *Anal.* Calcd for $C_{23}H_{35}N_3O_7$: C, 59.3; H, 7.58; N, 9.03. Found: C, 59.4; H, 7.66; N, 9.05.

Z-Ser(Bu^t)-Gly-Gly-Ser(Bu^t)-OBu^t (3) Z-Ser(Bu^t)-OH [prepared from Z-Ser(Bu^t)-OH·DCHA (5.7 g, 0.012 mol)], H-Gly-Gly-Ser(Bu^t)-OBu^t [prepared from **2** (5.6 g, 0.012 mol)] and HOBt (1.6 g, 0.012 mol) were dissolved in DMF (80 ml) and cooled with ice-salt. DCC (2.7 g, 0.013 mol) was added to the above cold solution. The reaction mixture was stirred at 4 °C overnight. After removal of dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 10% citric acid and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to give a solid mass, which was collected by filtration. The crude material in $CHCl_3$ (5 ml) was applied to a silica gel column (4 × 34 cm), equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (1500 ml), 0.5% MeOH in $CHCl_3$ (1200 ml) and then 1% MeOH in $CHCl_3$ (600 ml). The eluates with 0.5% MeOH in $CHCl_3$ (900—1200 ml) and 1% MeOH in $CHCl_3$ (600 ml) were evaporated to dryness. Petroleum ether was added to the residue to give a white powder, yield 4.7 g (64%), mp 148—151 °C, $[\alpha]_D^{22}$ +5.8° (*c* = 0.9, MeOH), Rf^1 0.57, Rf^2 0.46. *Anal.* Calcd for $C_{30}H_{48}N_4O_9$: C, 59.2; H, 7.95; N, 9.20. Found: C, 59.0; H, 8.05; N, 9.29.

Z-Asn-Ser(Bu^t)-Gly-Gly-Ser(Bu^t)-OBu^t (4) Z-Asn-ONp (1.5 g, 3.8 mmol) and H-Ser(Bu^t)-Gly-Gly-Ser(Bu^t)-OBu^t [prepared from **3** (2.3 g, 3.8 mmol)] were dissolved in DMF (20 ml) and the reaction mixture was stirred at room temperature for 48 h. After removal of the solvent, AcOEt was added to the residue to give a gelatinous precipitate, which was collected by filtration. The crude material in EtOH (5 ml) was applied to a Sephadex LH-20 column (3.5 × 127 cm), equilibrated and eluted with EtOH. Individual fractions (8 g each) were collected and the solvent of the effluent (tube Nos. 34—42) was evaporated off. Ether was added to the residue to give a white precipitate, which was collected by filtration, yield 1.9 g (69%), mp 142—145 °C, Rf^1 0.34, Rf^2 0.26, $[\alpha]_D^{22}$ −1.2° (*c* = 1.0, DMF). *Anal.* Calcd for $C_{34}H_{54}N_6O_{11}$: C, 56.5; H, 7.53; N, 11.6. Found: C, 56.3; H, 7.97; N, 11.3. Amino acid ratios in an acid hydrolysate: $Asp_{1.1}Ser_{2.0}Gly_{2.0}$ (average recovery 80%).

H-Asn-Ser(Bu^t)-Gly-Gly-Ser(Bu^t)-OBu^t (5) Z-Asn-Ser(Bu^t)-Gly-Gly-Ser(Bu^t)-OBu^t (**4**) (1.5 g, 2.5 mmol) in MeOH (50 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, ether and petroleum ether were added to the residue to give a white precipitate, which was collected by filtration, yield 1.1 g (91%), mp 62—78 °C, $[\alpha]_D^{22}$ +0.2° (*c* = 0.5, DMF), Rf^3 0.11. *Anal.* Calcd for $C_{26}H_{48}N_6O_9$: C, 53.1; H, 8.21; N, 14.3. Found: C, 52.8; H, 8.26; N, 14.0.

S-[(2*RS*)-2,3-Bis(palmitoyloxy)propyl]-*N*$^\alpha$-palmitoyl-Cys-Asn-Ser(Bu^t)-Gly-Gly-Ser(Bu^t)-OBu^t (6) *S*-[(2*RS*)-2,3-Bis(palmitoyloxy)propyl]-*N*$^\alpha$-palmitoyl-cysteine (1.0 g, 1.0 mmol), HOBt (0.14 g, 1.0 mmol) in dichloromethane (20 ml) and DMF (3 ml) were cooled with ice-salt. DCC (0.24 g, 1.2 mmol) was added to the solution. After 30 min, compound **5** (0.58 g, 1.0 mmol) in dichloromethane (20 ml) and DMF (2 ml) was added to the above cold solution. The reaction mixture was stirred at room temperature for 15 h. After removal of the solvent and dicyclohexylurea, the residue was dissolved in $CHCl_3$ (4 ml) and MeOH (10 ml) and the solution was allowed to stand at −30 °C for 4 h. The resultant precipitate was collected by filtration. This material was dissolved in $CHCl_3$ (3 ml) and MeOH (6 ml). After 3 h at −30 °C, the colorless protected hexapeptide was collected by filtration and washed with $CHCl_3$ and MeOH, yield 1.2 g (80%), mp 179—187 °C, $[\alpha]_D^{22}$ −0.49° (*c* = 0.5, DMF), Rf^1 0.51. *Anal.* Calcd for $C_{80}H_{149}N_7O_{15}S\cdot 2H_2O$: C, 63.3; H, 10.2; N, 6.46. Found: C, 63.7; H, 10.1; N, 6.62. Amino acid ratios in an acid hydrolysate: $Asp_{1.1}Ser_{1.8}Gly_{2.0}$ (average recovery 83%); *S*-[(2*RS*)-2,3-dihydroxy]-Cys-OH appeared at a position before Asp.

***S*-[(2*RS*)-2,3-Bis(palmitoyloxy)propyl]-*N*$^\alpha$-palmitoyl-Cys-Asn-Ser-Gly-Gly-Ser-OH (7)** A solution of **6** (1.0 g, 0.70 mmol) in TFA (5.4 ml) was stored at room temperature for 3 h. After removal of the TFA, water (20 ml) was added and evaporated. The residue was dissolved in warm $CHCl_3$ (8 ml). Addition of MeOH (5 ml) afforded a precipitate. After 12 h at −30 °C, the colorless material was collected by filtration and washed with MeOH, yield 0.56 g (62%), mp 200.5—203.5 °C, Rf^3 0.10. *Anal.* Calcd for $C_{68}H_{125}N_7O_{15}S\cdot 3H_2O$: C, 59.8; H, 9.66; N, 7.17. Found: C, 59.6; H, 9.40; N, 7.41. Amino acid ratios in an acid hydrolysate: $Asp_{0.94}Ser_{2.0}Gly_{2.0}$ (average recovery 94%), *S*-[(2*RS*)-2,3-dihydroxypropyl]-Cys-OH appeared at a position before Asp.

Z-Asn-Ser-Gly-Gly-Ser-OH (8) A solution of **4** (350 mg, 0.48 mmol) in TFA (3.7 ml) containing anisole (0.52 ml) was stirred at 0 °C for 10 min and at room temperature for 90 min. Ether was added to the solution to give a precipitate, which was collected by filtration and washed with EtOH, yield 230 mg (89%), mp 161—164 °C, $[\alpha]_D^{22}$ −4.1° (*c* = 0.9, DMF), Rf^4 0.25. *Anal.* Calcd for $C_{22}H_{30}N_6O_{11}\cdot 1.5H_2O$: C, 45.4; H, 5.71; N, 14.4. Found: C, 45.8; H, 5.71; N, 14.1. Amino acid ratios in an acid hydrolysate: $Asp_{1.1}Ser_{2.0}Gly_{2.0}$ (average recovery 60%).

H-Asn-Ser-Gly-Gly-Ser-OH (9) Compound **8** (150 mg, 0.28 mmol) in 50% AcOH (20 ml) was hydrogenated over a Pd catalyst. After removal of the Pd, the solvent was removed by lyophilization to give an amorphous powder, yield 110 mg (78%), $[\alpha]_D^{22}$ −2.5° (*c* = 1.0, 1 N HCl), Rf^5 0.57. Amino acid ratios in an acid hydrolysate: $Asp_{1.0}Ser_{2.0}Gly_{1.9}$ (average recovery 73%).

(Boc-Cys-OBu^t)$_2$ Boc-ON (3.4 g, 14 mmol) in dioxane (20 ml) was added to a solution of $(H-Cys-OBu^t)_2$[9] (2.4 g, 6.8 mmol) in dioxane (10 ml) containing *N*-methylmorpholine (3.0 ml, 28 mmol). The reaction mixture was stirred at room temperature for 15 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10%

citric acid and water, dried over Na_2SO_4 and evaporated down. The crude material in $CHCl_3$ (3 ml) was applied to a silica gel column (2.5 × 34 cm), equilibrated and eluted with $CHCl_3$. The eluate (150—300 ml) was evaporated to dryness to give an oily material, yield 0.50 g (13%), Rf^1 0.74.

(Boc-D-Cys-OBu^t)$_2$ $(Boc)_2O$ (4.0 g, 19 mmol) in dioxane (20 ml) was added to a solution of (H-D-Cys-OBu^t)$_2$ (3.0 g, 8.2 mmol) [prepared from D-cystine (2.0 g, 8.2 mmol) according to the method previously described[9]] in dioxane (10 ml) containing *N*-methylmorpholine (2.0 ml, 19 mmol). The reaction mixture was stirred at room temperature for 15 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down. The crude material in $CHCl_3$ (3 ml) was applied to a silica gel column (2.5 × 25.5 cm), equilibrated and eluted with $CHCl_3$. The eluate (30—200 ml) was evaporated to dryness to give an oily material. Petroleum ether was added to the residue to give a white precipitate, yield 1.3 g (28%), mp 92.5—94 °C, $[\alpha]_D^{22}$ −15.4° (c=0.4, $CHCl_3$), Rf^1 0.74. *Anal.* Calcd for $C_{24}H_{44}N_2O_8S_2$: C, 52.2; H, 8.02; N, 5.06. Found: C, 51.9; H, 8.03; N, 5.10.

Boc-Cys-OBu^t (Boc-Cys-OBu^t)$_2$ (0.50 g, 0.90 mmol) in $CHCl_3$ (20 ml) was reduced with dithioerythritol (0.59 g, 3.8 mmol) in the presence of Et_3N (0.39 ml, 2.8 mmol). After being stirred at room temperature for 2 h under N_2, the solution was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down. The oily residue was dried over P_2O_5 *in vacuo*, yield 0.50 mg (100%), Rf^1 0.60.

Boc-D-Cys-OBu^t The title compound was prepared from (Boc-D-Cys-OBu^t)$_2$ (0.50 g, 0.90 mmol) in the same manner as described above. The oily material was dried over P_2O_5 *in vacuo*. Petroleum ether was added to the residue to give a white precipitate, yield 0.33 g (67%), mp 46—49 °C, $[\alpha]_D^{22}$ −17.1° (c=1.0, $CHCl_3$), Rf^1 0.60. *Anal.* Calcd for $C_{12}H_{23}NO_4S$: C, 52.0; H, 8.35; N, 5.04. Found: C, 51.8; H, 8.46; N, 4.92.

***S*-[(2*RS*)-2,3-Dihydroxypropyl]-*N*$^\alpha$-Boc-Cys-OBu^t** 3-Bromo-1,2-propanediol (1.8 ml, 18 mmol) was added to a solution of Boc-Cys-OBu^t (0.50 g, 1.8 mmol) in DMF (10 ml) containing Et_3N (2.5 ml, 18 mmol). The reaction mixture was stirred at 80 °C for 1 h and at room temperature for 1 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over Na_2SO_4 and evaporated down to give an oily material, which was dried over P_2O_5 *in vacuo*, yield 0.30 g (47%), Rf^1 0.48.

***S*-[(2*RS*)-2,3-Dihydroxypropyl]-*N*$^\alpha$-Boc-D-Cys-OBu^t** The title compound was prepared from Boc-D-Cys-OBu^t (0.32 g, 1.2 mmol) in the same manner as described above. The crude material in $CHCl_3$ (3 ml) was applied to a silica gel column (2.5 × 31.5 cm), equilibrated with $CHCl_3$ and eluted with $CHCl_3$ (600 ml) and then 1% MeOH in $CHCl_3$ (1000 ml). Individual fractions (10 g each) were collected and the solvent of the effluent (tube Nos. 90—180) was removed by evaporation. Petroleum ether was added to the residue to give an oily material, which was dried over P_2O_5 *in vacuo*, yield 0.20 g (47%), Rf^1 0.48.

***S*-[(2*RS*)-2,3-Dihydroxypropyl]-L-Cys-OH** *S*-[(2*RS*)-2,3-Dihydroxypropyl]-*N*$^\alpha$-Boc-Cys-OBu^t (0.30 g, 0.85 mmol) was dissolved in TFA (6.5 ml) containing thioanisole (1.0 ml) and the reaction mixture was stirred at room temperature for 1 h. Ether (25 ml) and petroleum ether (25 ml) were added to the reaction mixture under ice-cooling to yield an oily material. After removal of the solvent by decantation, the residue was dissolved in EtOH (10 ml). The pH of the solution was adjusted to 7 using Et_3N to yield a precipitate, which was collected by centrifugation, yield 26 mg (17%), mp 160—166 °C, $[\alpha]_D^{22}$ −8.6° (c=0.7, 0.1 N HCl), Rf^3 0.14, Rf^4 0.60. *Anal.* Calcd for $C_6H_{13}NO_4S$: C, 36.9; H, 6.70; N, 7.17. Found: C, 36.8; H, 6.81; N, 6.98. Chiral HPLC [Crownpack RC(+)] under the same conditions as those for the 6 N HCl hydrolysate of I showed two peaks corresponding to those at 8.88 and 9.49 min in the analysis of the hydrolysate of I.

***S*-[(2*RS*)-2,3-Dihydroxypropyl]-D-Cys-OH** The title compound was prepared from *S*-[(2*RS*)-2,3-dihydroxypropyl]-D-Cys-OBu^t (0.20 g, 0.57 mmol) in the same manner as described above, yield 11 mg (9.4%), mp 171—176 °C, $[\alpha]_D^{22}$ +4.5° (c=0.5, 0.1 N HCl), Rf^3 0.14, Rf^4 0.60. *Anal.* Calcd for $C_6H_{13}NO_4S$: C, 36.9; H, 6.70; N, 7.17. Found: C, 36.5; H, 6.72; N, 6.83. Chiral HPLC [Crownpack CR(+)] under the same conditions as those for the 6 N HCl hydrolysate of I: retention time, 4.94 min.

(2*S*)-1-*O*-Tosyl-2,3-*O*-isopropylideneglycerine Tosyl chloride (7.2 g, 38 mmol) was added to a solution of (2*S*)-2,3-*O*-isopropylideneglycerine (5.0 g, 38 mmol, Aldrich) in dry pyridine (30 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down to give an oily material, which was dried over P_2O_5 *in vacuo*, yield 10.3 g (95%), Rf^1 0.70. IR: 1170, 1375 (SO_2) cm^{-1}.

(2*R*)-1-*O*-Tosyl-2,3-*O*-isopropylideneglycerine The title compound was prepared from (2*R*)-2,3-*O*-isopropylideneglycerine (5.0 g, 38 mmol, Aldrich) in the same manner as described above, yield 10.1 g (93%), Rf^1 0.70. IR: 1170, 1375 (SO_2) cm^{-1}.

***S*-[(2*S*)-2,3-*O*-Isopropylidene-2,3-dihydroxypropyl]-*N*$^\alpha$-palmitoyl-Cys-OBu^t** *N*$^\alpha$-Palmitoyl-Cys-OBu^t (1.7 g, 4.1 mmol) and K_2CO_3 (0.56 g, 4.1 mmol) were added to a refluxing solution of (2*S*)-1-*O*-tosyl-2,3-isopropylideneglycerine (2.0 g, 7.1 mmol) in EtOH (50 ml). After 10 h, the EtOH was removed by evaporation. The residue was extracted with AcOEt. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated down to give an oily product. The crude product in $CHCl_3$ (2 ml) was applied to a silica gel column (2.8 × 30 cm), equilibrated and eluted with $CHCl_3$. The eluate (800—900 ml) was evaporated to dryness to give an oily material, yield 0.10 g (4.6%), $[\alpha]_D^{22}$ +18.0° (c=1.7, $CHCl_3$), Rf^2 0.73. Exact MS Calcd for $C_{29}H_{55}NO_5S$+H: 530.3879. Found: 530.3865. Amino acid analysis of an acid hydrolysate showed peaks corresponding to those (9.63 and 15.40 min) in the amino acid analysis of the acid hydrolysate of WS1279. HPLC of the 6 N HCl hydrolysate on Crownpack CR(+) showed four peaks corresponding to those (8.88, 9.42, 25.88 and 30.15 min) in the HPLC of the 6 N HCl hydrolysate of WS1279.

***S*-[(2*R*)-2,3-*O*-Isopropylidene-2,3-dihydroxypropyl]-*N*$^\alpha$-palmitoyl-Cys-OBu^t** The title compound was prepared from *N*$^\alpha$-palmitoyl-Cys-OBu^t (1.0 g, 2.4 mmol) and (2*R*)-1-*O*-tosyl-2,3-isopropylideneglycerine (1.2 g, 4.1 mmol) in the same manner as described above, yield 50 mg (3.8%), $[\alpha]_D^{22}$ −5.3° (c=1.2, $CHCl_3$), Rf^2 0.73. Exact MS Calcd for $C_{29}H_{55}NO_5S$ +H: 530.3879. Found: 530.3886. Amino acid analysis and HPLC on Crownpack CR(+) of the 6 N HCl hydrolysate showed the same peaks as those of the 2*S* counterpart.

References and Notes

1) All amino acid residues are of L-configuration except in the case of glycine unless otherwise indicated. The abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6** 362 (1967); *ibid.*, **11**, 1726 (1972). Other abbreviations used are: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyl ester; OBu^t, *tert*-butyl ester; ONp, *p*-nitrophenyl ester; Bu^t, *tert*-butyl; DCC, *N*,*N*'-dicyclohexylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; AcOEt, ethyl acetate; DMF, dimethylformamide; TFAA, trifluoroacetic anhydride.
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