STRUCTURE AND SYNTHESIS OF AN IMMUNOACTIVE LIPOPEPTIDE, WS1279, OF MICROBIAL ORIGIN

Miho TANAKA,[a] Nobuharu SHIGEMATSU,[a] Yasuhiro HORI,[a] Toshio GOTO,[a] Masashi HASHIMOTO,*[,a] Yuko TSUDA,[b] and Yoshio OKADA[b]

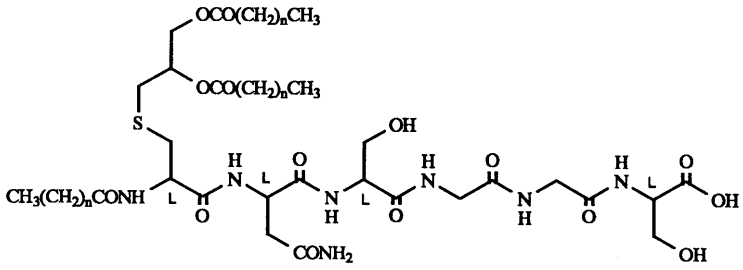
Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd.,[a] 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan and Faculty of Pharmaceutical Sciences, Kobe-Gakuin University,[b] Nishi-ku, Kobe 673, Japan

The structure of WS1279 (1) isolated from a Streptomyces as an immunoactive lipopeptide has been deduced on the basis of chemical and physical evidence and confirmed by a synthesis.

KEYWORDS Streptomyces; natural product; peptide; fatty acid; S-glycerylcysteine; immunostimulating activity

It has long been known that various bacteria and their cell wall components increase the number of colony stimulating units in the bone marrow.[1] 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 the bone marrow cells. Here we describe the structure and synthesis of this natural product.

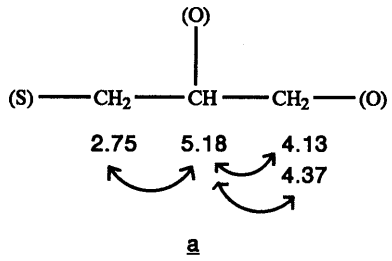
WS1279 (1) was isolated as a mixture of lipopeptides in which, although the peptide part consists of a single component, the lipid part is composed mainly of five kinds of fatty acids: FABMS of 1, m/z$(M+Na)^+$ 1306 1320, 1334(main), 1348, 1362. The presence of the three fatty acid residues in 1 was corroborated by the ^1H NMR data of 1 in $CDCl_3$-CD_3OD: δ 0.85(9H, m, 3xCH_3), 1.25(ca72H, m, $(CH_2)_n$), 1.60(6H, m, 3xβ-CH_2), 2.30(6H, m, 3xα-CH_2). Hydrolysis of 1 with 6N HCl (110°C, 18 h) followed by methylation with CH_2N_2 gave a mixture of fatty acid methyl esters. The total ion chromatogram of this mixture in GC-MS[2] showed five major peaks at 3'26", 3'31", 4'20", 4'42", and 5'17" (5:1:3:9:2) corresponding to m/z 256, 256, 270, 270, and 284, respectively. The main peak at 4'42" (m/z 270) was identified to be methyl palmitate by comparison (GC-MS)[3] with an authentic sample. Alkaline hydrolysis of 1 (1N NaOH-MeOH, r.t.) gave a product whose FABMS showed the molecular ion at m/z $858(M+Na)^+$ due to loss of two fatty acids from the molecule of 1, indicating that two of the three fatty acid residues are bonded somewhere to the peptide sequence by an ester bond and therefore the remaining one (a single fatty acid) is linked by an amide bond.



1

Amino acid analysis of the 6N HCl hydrolysate of WS1279 (1) (see above) revealed the presence of Asp, Ser, Gly, and NH_3 (1:2:2:1), plus two unknown amino acids (before Asp and between Ser and Gly).[4] Asp and Ser were determined to be L by HPLC analysis using a chiral column (Chiralpak WH).[5] L-Asp is presumed to be derived from L-Asn, because the 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 shown below. The two unidentified peaks were assumed to be due to S-glycerylcysteine itself and its degradation product (see below).

The presence of S-glycerylcysteine in the molecule of WS1279 (1) was proposed on the following grounds. The ^{1}H NMR spectrum of 1 showed signals at δ 2.75(2H, m), 4.13(1H, m), 4.37(1H, m), and 5.18(1H, m), which were coupled to each other (^{1}H,^{1}H-COSY), indicating the partial structure a (Fig. 1). The presence of one sulfur atom was identified by EMAX analysis (found, 2.0%; calcd. based on 1 (n=14), 2.4%). The FAB mass spectra of WS1279 itself and the alkaline hydrolysis product both showed fragment ions at m/z 751, which correspond 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-((2RS)-2,3-dihydroxypropyl)cysteine[6] in amino acid analysis. The synthetic sample thus showed a peak corresponding to that of 9.62 min in the amino acid analysis of 1, while, after acid hydrolysis (6N HCl, 110°C, 18h), the sample showed an additional peak corresponding to that of 15.40 min, although its structure was not identified.[7] The stereochemistry of Cys in the S-glycerylcysteine moiety was deduced to be L as follows. The HCl hydrolysate of 1 was examined by HPLC on a chiral column (Crownpak CR).[8] The hydrolysate showed peaks at 8.88 and 9.49 min,[9] which were shown to be due to diastereomeric S-((2RS)-glyceryl)-L-cysteines by comparison with the synthetic sample.[10,11]



a

Fig. 1. Couplings in the ^{1}H,^{1}H-COSY Spectrum of WS1279

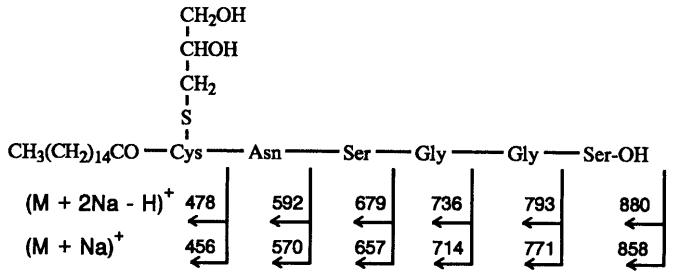


Fig. 2. Cluster Ions in the FABMS of the Mixture Obtained by Digestion of WS1279 with CarboxypeptidaseA Followed by Alkaline Hydrolysis

To determine the amino acid sequence in WS1279 (1), an enzyme degradation method was applied to 1 in combination with FABMS. Thus, after treatment of 1 with carboxypeptidase A (pH 7.0, 37°C), followed by alkaline hydrolysis for removal of the two palmitoyl ester groups, the resulting mixture was analyzed by FABMS. 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. 2. These data indicated the sequence of -Asn-Ser-Gly-Gly-Ser-OH. The hydrazine degradation of 1 further corroborated that Ser is the C-terminal in the sequence.[12] The clusters at m/z 478$(M+2Na-H)^+$ and 456$(M+Na)^+$ correspond to N-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 as being 1 in which the fatty acid residues are composed mainly of palmitic acid (n=14).

A final confirmation for structure 1 was obtained by a synthesis of 1 (n=14) as a diastereomeric mixture at the 2 position of the glyceryl moiety as follows. The pentapeptide 3 was prepared in a stepwise manner, starting from H-Ser(Bu^t)-OBu^t and using Z for the temporary amino protecting group, by the p-nitrophenyl

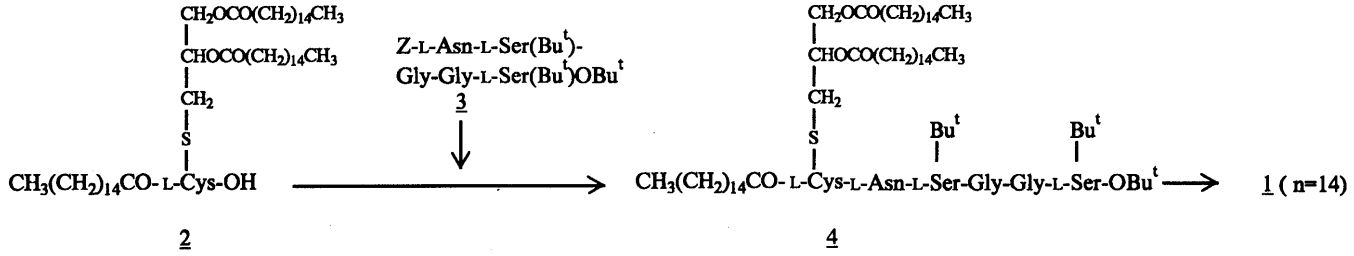


Chart 1

active ester method except for coupling of Z-Ser(Bu^t)-OH which was condensed by the DCC-HOBt procedure. In each coupling reaction, the Z group was removed by hydrogenolysis on Pd-black. The total yield of 3 was 30%.[13] S-((2RS)-Bis(palmitoyloxy)propyl)-N-palmitoyl-L-Cys-OH (2) was prepared according to the method described by Wiesmüller et al.[14] and condensed with 3 (after removal of the Z group) by the DCC-HOBt procedure in CH_2Cl_2-DMF to give the protected lipopeptide 4 corresponding to the full sequence of 1 (80% yield).[15] The final treatment of 4 with TFA for deprotection of the tert-Bu ether and ester groups afforded 1 (n=14) as a diastereomeric mixture (ca 1 : 1) in 62% yield.[16]

The synthetic sample was identified with the natural product on TLC and HPLC.[17] It showed an activity in stimulation of the CSF-induced proliferation of the bone marrow cells in vitro (50% increase: synthetic, 0.38 μg/ml: natural, 0.32 μg/ml).[18]

REFERENCES AND NOTES

1) a) R. S. Foster, Cancer Res., 38, 2666 (1978); b) R. S. Foster, B. R. McPherson, and D. A. Browdie, Cancer Res., 37, 1349 (1977).
2) Quadrex, methyl silicone; 25m x 0.25mm x 0.25μm film; 150 - 260°C (10°C/min).
3) The peaks at 3'26", 3'31", 4'20", and 5'17" were identified to be methyl isopentadecanoate, methyl anteisopentadecanoate, methyl isopalmitate, and methyl isoheptadecanoate, respectively.
4) Retention times of the two unidentified peaks: 9.62 and 15.40 min (Asp, 11.26; Ser, 12.89; Gly, 19.19).
5) Chiralpak WH(4.0 x 250mm); eluent, 0.25mM $CuSO_4$; flow rate, 1 ml/min; temp, 50°C; retention time, L-Asp, 43.33; D-Asp, 35.45; L-Ser, 25.75; D-Ser, 17.82 min.
6) Prepared from (Boc-Cys-OBu^t)$_2$ via 1) disulfide reduction, 2) reaction with 3-bromo-1,2-propanediol, and 3) removal of the protecting groups by treatment with TFA: mp 160°C; TLC (silica gel), Rf 0.60 (n-BuOH-AcOH-pyridine-H_2O (4:1:1:2)).
7) Details will be reported in a forthcoming full paper.
8) Crownpak CR (4.0 x 150mm); eluent, aqueous $HClO_4$ (pH 1.0); flow rate, 0.4 ml/min; temp, 0°C; detection, UV 200 nm.
9) Two unknown peaks were also observed at 25.88 and 30.15 min. These were found to be due to degradation products of S-glycerylcysteine. Details will be reported in a forthcoming full paper.
10) S-((2RS)-Glyceryl)-D-cysteines were also prepared (mp 171-176°C; TLC (silica gel), Rf 0.60 (n-BuOH-AcOH-pyridine-H_2O (4:1:1:2))), which were not separated and showed a peak at 4.94 min on Crownpak CR under the same conditions.
11) The natural product seemed to be epimerized at the 2 position of the glyceryl moiety during the acid hydrolysis.
12) When the hydrazine degradation method was applied to 1, Ser was detected as the C-terminal amino acid.
13) The protected pentapeptide 3: mp 142-145.5°C; $[\alpha]_D^{22}$ -1.2° (c 0.1, DMF); TLC (silica gel), Rf 0.34 ($CHCl_3$-MeOH-H_2O (89:10:1)): amino acid analysis, Asp 1.1, Ser 2.0, Gly 2.0.
14) H.-H. Wiesmüller, W. Bessler, and G. Jung, Hoppe-Seylers Z. Physiol. Chem., 364, 593 (1983).
15) The protected WS1279 (4): mp 179-187°C; TLC (silica gel), Rf 0.51 ($CHCl_3$-MeOH-H_2 (89:10:1)); amino acid analysis, Asp 1.1, Ser 1.8, Gly 2.0.
16) The synthetic WS1279 (1,n=14): mp 200.5-203.5°C; TLC (silica gel), Rf 0.10 (n-BuOH-AcOH-H_2O (4:1:5, upper phase)); amino acid analysis, Asp 0.94, Ser 2.0, Gly 2.0. Anal. Calcd for $C_{68}H_{125}N_7O_{15}S$ $3H_2O$: C, 59.8; H, 9.66; N, 7.17. Found: C, 59.6; H, 9.40; N, 7.41.
17) For TLC, see ref. 16. HPLC: Ultrasphere (4.6 x 250 mm); eluent, MeOH-aqueous $HClO_4$ (pH 2) (97:3); flow rate, 1.0 ml/min; temp, 50°C; detection, UV 210 nm; retention time, 15.4 min.
18) This difference in activity between the synthetic sample and the natural product was probably due to the following points: 1) the synthetic sample was a diastereomeric mixture; 2) the synthetic sample was of higher purity and therefore was less soluble in H_2O.

(Received April 18, 1990)