
Notes

**WS1279, A NOVEL LIPOPEPTIDE ISOLATED
FROM *Streptomyces willmorei*
FERMENTATION, ISOLATION AND
PHYSICO-CHEMICAL PROPERTIES**

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The current approach to the treatment of a majority of advanced neoplastic disease involves the use of surgery, radiation and systematic chemotherapy. However, bone marrow toxicity is a factor limiting the success of radiation and chemotherapeutic drugs because patients often become highly vulnerable to bacterial or fungal infections after treatment^{1,2}. As a result, rescue from myelotoxicity is a major concern in the treatment of these patients and a biological response modifier capable of facilitating the recovery of hematopoietic competency will be clinically important³.

In the course of our screening program for myelopoietic substances from microbial products, *Streptomyces willmorei* No. 1279 was found to produce a novel lipopeptide, WS1279. In this paper we report fermentation, isolation and physico-chemical properties of WS1279.

The producing organism, strain No. 1279, was isolated from a soil sample collected in Kagawa-prefecture, Japan, and strain No. 1279 was identified as a strain of *Streptomyces willmorei* and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces willmorei* No. 1279 and the accession No. FERM P-11405.

For the production of WS1279 the *Streptomyces willmorei* No. 1279 was inoculated into a 500-ml flask (first seed culture) containing 160 ml of seed medium composed of corn starch 1.0%, glycerol

1.0%, glucose 0.5%, Pharmamedia (Traders Protein) 0.5%, dried yeast 0.5%, corn steep liquor 0.5%, and CaCO₃ 0.2% (pH 6.5). The flasks were shaken on a rotary shaker (220 rpm, 5.1-cm throw) at 30°C for 3 days. One ml of the first seed culture was transferred to 500-ml flasks (second seed culture) containing 160 ml of the seed medium with the same composition described above. After cultivating for 1 day, 2 liters of the second seed culture was transferred to a 200-liter jar fermentor containing 140 liters of production medium composed of soluble starch 3.0%, peanut powder 0.5%, soybean meal 0.5%, dried yeast 0.5%, K₂HPO₄ 0.5%, KH₂PO₄ 0.05%, and Ca(OH)₂ 0.3%. The fermentation was carried out at 30°C for 4 days under aeration of 140 liters per minute and agitation of 250 rpm. The production of WS1279 was monitored to determine the mitogenic activity of bone marrow cells of BDF₁ mice.

The procedure for purification of WS1279 is as follows. The broth filtrate (1,200 liters) was adjusted to pH 2 with 6N HCl and extracted with EtOAc (120 liters, twice). The EtOAc layer was concentrated (100 liters) and extracted with 5% NaHCO₃ (50 liters). The aqueous extract was adjusted to pH 2, followed by EtOAc extraction (50 liters, twice). The extract was concentrated and chromatographed on 3.75 liters of Silicar CC-4 (Mallinckrodt). The column was eluted with hexane (4 liters), hexane-EtOAc (3:1, 8 liters), EtOAc (8 liters) and finally EtOAc-acetone (1:1, 16 liters). WS1279 eluted with EtOAc-acetone (1:1). Active fraction was concentrated to dryness to yield an oily material (60 g). The oily material was dissolved in 300 ml of methanol, and applied to a LiChroprep RP-8 column (Lobar column, size B, E. Merck) and eluted with MeOH. The active fraction was concentrated, applied to a LiChroprep RP-8 column and eluted with MeOH again. The active eluate was concentrated to dryness to give a brown powder (250 mg). The powder was finally purified by Toyopearl HW-40 SF (Tosoh) column chromatography (solvent; MeOH), and WS1279 was obtained as a white powder (20 mg).

The physico-chemical properties of WS1279 are summarized in Table 1.

The FAB-MS spectrum of WS1279 showed five

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Table 1. Physico-chemical data for WS1279.

| | |
|---|--|
| Appearance | White powder |
| Solubility | |
| Slightly soluble: | CHCl ₃ , MeOH, DMSO |
| Insoluble: | H ₂ O |
| TLC ^a R _f | 0.1 |
| ¹ H NMR δ (ppm) ^b | 0.85 (9H, m), 1.25 (72H, m), 1.60 (6H, m), 2.30 (6H, m), 2.8 (5H, m), 3.06 (1H, dd, <i>J</i> = 5.5, 14), 3.75~4.0 (8H, m), 4.13 (1H, dd, <i>J</i> = 6.5, 12), 4.31 (1H, m), 4.37 (1H, m), 4.45 (1H, m), 4.51 (1H, m), 4.65 (1H, m), 5.18 (1H, m) |
| FAB-MS | 1,306, 1,320, 1,334 (main), (<i>m/z</i> (M + Na) ⁺) 1,348, 1,362 |
| EMAX analysis | S (2.2%) |
| Amino acid analysis ^c | Asp, Ser, Gly, NH ₃ (1:2:2:1), two unknown amino acids |
| Fatty acid analysis | Isopentadecanoate, anteisopentadecanoate, isopalmitate, palmitate, isoheptadecanoate (5:1:3:9:2) |

^a Kieselgel G (E. Merck), CHCl₃ - MeOH - H₂O (8:3:1, lower phase).

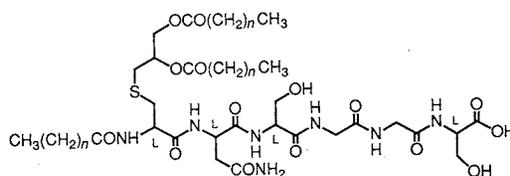
^b 400 MHz, CDCl₃ - CD₃OD, *J* = Hz.

^c Asp and Ser were determined to be L by a chiral HPLC.

main molecular ion peaks at *m/z* (M + Na)⁺ 1,306, 1,320, 1,334 (main), 1,348 and 1,362, indicating that WS1279 is a mixture of five main congeners. The ¹H NMR spectrum exhibited signals due to fatty acids δ 0.85 (9H, m, 3 × CH₃), 1.25 (*ca.* 72H, m, (CH₂)_{*n*}), 1.60 (6H, m, 3 × β-CH₂), 2.30 (6H, m, 3 × α-CH₂), indicating the presence of the three fatty acid residues in the molecules of WS1279. These fatty acids were identified by the GC-MS measurement. Hydrolysis of WS1279 with 6N HCl followed by methylation with diazomethane gave a mixture of fatty acid methyl esters. The total ion chromatogram of this mixture showed five major peaks at 206, 211, 260, 282 and 317 seconds in a ratio 5:1:3:9:2, corresponding to *m/z* 256, 256, 270, 270 and 284, respectively. These peaks were identified as methyl isopentadecanoate, methyl anteisopentadecanoate, methyl isopalmitate, methyl palmitate and methyl isoheptadecanoate, respectively.

Amino acid analysis of the 6N HCl hydrolysate of WS1279 showed the presence of Asp, Ser, Gly and NH₃ (1:2:2:1), plus two unknown amino acids. The latter two amino acids were identified as

Fig. 1. Structure of WS1279.

Table 2. Effects of WS1279, lipopolysaccharide (LPS) and muramyl dipeptide (MDP) on the proliferation of bone marrow cells of BDF₁ mice.

| Drug | ED ₅₀ (μg/ml) ^a |
|--------|---------------------------------------|
| WS1279 | 0.33 |
| LPS | ND ^b (0.01) |
| MDP | ND ^b (5) |

Bone marrow cells were prepared from BDF₁ mice. The proliferating activity of bone marrow cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method⁶. In brief, the 25 μl of bone marrow cell suspension (3 × 10⁶ cells/ml) and 50 μl of assay solution in DULBECCO's modified EAGLE's medium containing 10% heat inactivated fetal bovine serum, 100 u/ml benzylpenicillin, 100 μg/ml streptomycin, 5 × 10⁻⁵ M 2-mercaptoethanol and 2.5% L-929-conditioned medium were mixed per well of 96-well flat-bottomed microtiter plate (Sumitomo Bakelite) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 4 days. Then, 10 μl of 5 mg/ml MTT in DULBECCO's phosphate-buffered saline was added per microtiter well and incubated for 6 hours. To each well 150 μl of 0.04N HCl in isopropanol were added to solubilize the reduced MTT and the absorbance was measured at 550 nm with reference at 660 nm.

^a ED₅₀ was indicated the dose of 50% increase of bone marrow proliferation.

^b Efficacy was under 50%, ED₅₀ could not be determined.

The number in parentheses represents maximum response at the indicated dose.

S-glycylcysteine and its degradation product as described in the preceding paper^{4,5}. Asp and Ser were determined to be L by HPLC analysis using a chiral column. The presence of one sulfur atom was identified by EMAX analysis (found, 2.2%). These physical data and further degradation experiments suggested the structure of WS1279 to be as depicted in Fig. 1 (*n* = 14), which was finally confirmed by synthesis⁵.

Bone marrow growth-stimulating activity of WS1279 *in vitro* is shown in Table 2. We compared with lipopolysaccharide (LPS, *Escherichia coli* 055: B5, Sigma) and muramyl dipeptide (MDP, prepared in our laboratory) in the bone marrow growth-

stimulating activity. LPS and MDP are components of bacterial outer membrane, which modulate the immunological activities^{7,8}). WS1279 stimulated the proliferation of bone marrow cells and its ED₅₀ was 0.33 µg/ml. LPS and MDP could not be determined their ED₅₀ values.

Lipoprotein from the outer membrane of *Escherichia coli* is a mitogen towards mouse lymphocytes⁹). It was characterized and sequenced by BRAUN and coworkers¹⁰). The molecular structure responsible for the mitogenicity of the molecule resides in its *N*-terminal fatty acid-containing part; lipopeptide fragments which carried three fatty acids bound to glycercylcysteine attached to an oligopeptide chain of three to five amino acids were mitogenically active. JOHNSON *et al.*¹¹) demonstrated that the synthetic lipopeptide segment *S*-[2,3-bis(palmitoyloxy)-(2*R,S*)-propyl]-*N*-palmitoyl-(*R*)-Cys-Ser-Ser-Asn-Ala was a potent B-lymphocyte mitogen. The structure of WS1279 resembles that of synthetic peptide derivative of bacterial lipoprotein, however, the most important difference between these two compounds is as follows; WS1279 was isolated from the culture broth of *Streptomyces willmorei* as a secondary metabolite, while synthetic lipopeptide was a component of bacterial outer membrane.

JOHNSON *et al.* reported that the synthetic derivative of bacterial lipopeptide was a spleen cell mitogen¹¹) but they did not study on the bone marrow cell proliferating activity of this synthetic sample. Further biological studies on WS1279 will be reported in another paper.

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