

CONAGENIN, A LOW MOLECULAR
WEIGHT IMMUNOMODULATOR
PRODUCED BY
Streptomyces roseosporus

Sir:

In the course of screening for substances that modulate immune responses in conjunction with T cell functions, we found conagenin, a novel, low MW immunomodulator, in fermentation broths of *Streptomyces roseosporus* M1696-AF3.

The structure of conagenin was determined to be (2*S*)-*N*-[(2*R*,3*S*,4*R*)2,4-dihydroxy-3-methylpentanoyl]-2-methylserine on the basis of spectroscopic evidence (Fig. 1). In this paper, we report production and purification of conagenin, and its effect on T lymphocytes.

Fermentations for the production of conagenin were done as follows. *S. roseosporus* was cultured in 500-ml Erlenmeyer flasks containing 110 ml of the autoclaved seed medium consisting of galactose 2.0%, dextrin 2.0%, soy peptone 1.0%, corn steep liquor 0.5%, (NH₄)₂SO₄ 0.2%, CaCO₃ 0.2% and one drop of silicone. The seed medium was adjusted to pH 7.4 with 1*N* HCl and was autoclaved for 20 minutes. Fermentations were carried out on a rotary shaker (180 rpm) at 27°C for 48 hours. Three ml of the mature seed fermentation were inoculated into 500-ml flasks containing 110 ml of the autoclaved production medium consisting of maltose 2.0%, meat extract 0.5%, peptone 0.5%, yeast extract 0.3%, NaCl 0.3%, and MgSO₄·7H₂O 0.1%, supplemented with 0.1 ml of a mixture of inorganic salts consisting of CuSO₄·5H₂O 0.6%, FeSO₄·7H₂O 0.1%, MnCl₂·4H₂O 0.6% and ZnSO₄·7H₂O 0.2%. After formulation, the pH of the production medium was not adjusted, and it was autoclaved for 20 minutes. Production fermentations were carried out at 27°C for 4 days on a rotary shaker (180 rpm).

The activity of conagenin was determined by measuring the incorporation of [³H]thymidine into nylon wool-passed spleen cells (T cell-rich preparation) treated with concanavalin A (Con A). Nylon wool-passed spleen cells (NWPC) taken from F344 rats (14 weeks old) were prepared by a common

method¹⁾. The cells (1 × 10⁷ cells/ml of RPMI-1640 medium containing 5% fetal calf serum) were incubated with 5 μg of Con A in the presence of test samples of conagenin at 37°C for 4 hours, after thorough washing with α-methyl mannoside (20 mg/ml in medium). Two tenths ml of a suspension of 1 × 10⁶ cells/ml were put in each well of a microplate (Corning microplate, Iwaki Co., Ltd., Japan), and were cultured at 37°C in 5% CO₂ for 3 days. Eighteen hours before termination of culture, [³H]thymidine ([³H]TdR) was added, and its incorporation into cultured cells was measured by liquid scintillation counting. Samples inducing enhancement (>20%) of incorporation were determined to be active. Triplicate cultures were made for each determination.

Conagenin in 10 liters of a culture filtrate was adsorbed on activated carbon and eluted with 4 liters of 50% acetone. The eluate was concentrated to a syrup *in vacuo*, and it was dissolved in 2 liters of water adjusted to pH 3.0 with 1*N* HCl, then, active substance was extracted with 2 liters of *n*-butanol. After neutralization with 1*N* NaOH, the extracts were concentrated *in vacuo*. The concentrated residue (7.5 g) was dissolved in a mixture of BuOAc-BuOH-AcOH-water (6:4:1:1) (in volume), and applied to a column (50 × 150 mm) of Wakogel C-200 (Wako Pure Chemical Industries, Ltd., Japan). The column was eluted with the same solvent. After concentration, the active eluate was obtained as a yellowish powder (1.2 g). The powder dissolved, in 10% MeOH, was applied to a reverse phase HPLC column (Sensyu pak, Nucleosil 5C₁₈ 20 × 300 mm, flow rate 5 ml/minute), and was eluted with a linear gradient of 10% to 100% MeOH. The active fractions were concentrated *in vacuo* yielding a pale yellow powder which was dissolved in MeOH and crystallized.

Conagenin was obtained as a colorless crystal (34.8 mg) with a mp range of 159~161°C, and no characteristic UV absorption. The IR spectrum is shown in Fig. 2. [α]_D²⁷ is +55.4°. The molecular formula, C₁₀H₁₉NO₆ was determined by elemental analysis (Found: C 47.96, H 7.67, N 5.64, O 38.19), and HR-MS (*m/z* 250.1291 (M+H)⁺). Conagenin is soluble in water and MeOH, and insoluble in CHCl₃, EtOAc and hexane.

The stereo-structure, including absolute configuration, was elucidated by X-ray diffraction methods. The crystals of conagenin were grown in a mixed solution of water and methanol as monoclinic, space group *P*2₁, crystals, with the

Fig. 1. Structure of conagenin.

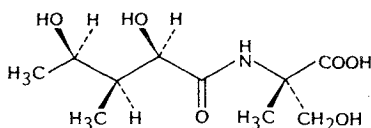


Fig. 2. IR spectrum of conagenin.

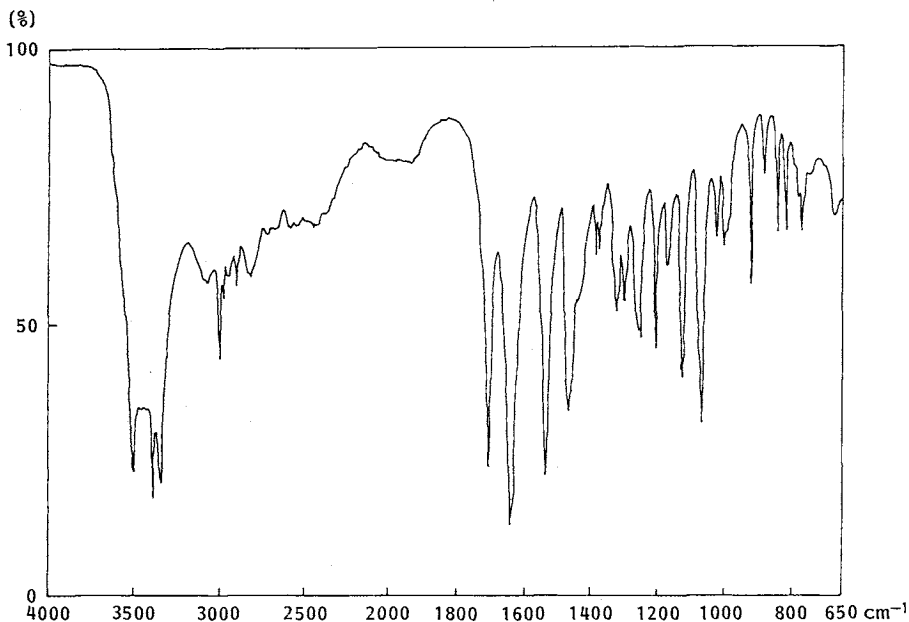
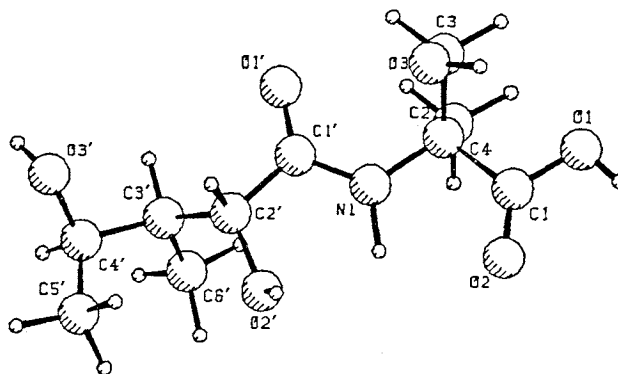


Fig. 3. Molecular structure of conagenin including absolute configuration drawn using PLUTO program.



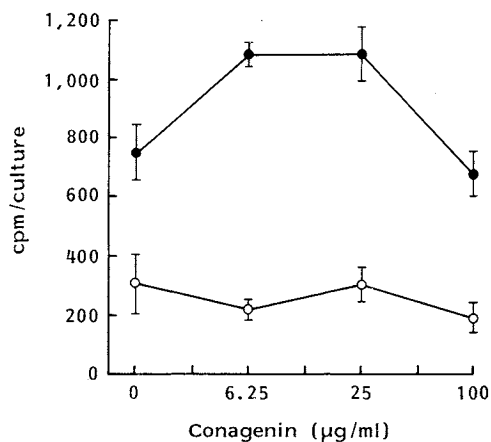
lattice constants of $a=8.646(5)$, $b=9.642(7)$, $c=7.528(7)$ Å, $\beta=93.85(5)^\circ$, $D_x=1.322$ gcm $^{-3}$, $Z=2$. Intensities of 2,958 reflections including 955 Friedel pairs and symmetry equivalent reflections were measured on a Philips PW1100 diffractometer using graphite monochromated Cu K α radiation. Of these, 2,720 reflections in the 2θ range 6° through 156° were above the $2\sigma(I)$ level, and were regarded as observed. The disagreement of the observed structure factors between 995 Friedel pairs, $(R_F)^*_{\text{Friedel}}$ was 0.055, while that between 307 symmetry equivalent pairs, $(R_F)^*_{\text{symm}}$ was 0.020. The struc-

ture was determined by the direct method, and was refined by the method of least-squares with block-diagonal matrix approximations. 19H atoms were found on the difference electron density map, and were refined along with 17 heavier atoms. Absolute configuration was determined by the anomalous dispersion method using the dispersion effect of C, N and O atoms for Cu K α radiation. Of the 12 Friedel pairs which showed the ratio of their structure factors differing more than 3% from unity (for both calculated and observed structure factors), 10 pairs clearly indicated the absolute

* $(R_F)_{\text{Friedel}} = 2\sum[F_o(hkl) - F_o(\bar{h}\bar{k}\bar{l})] / \sum[F_o(hkl) + F_o(\bar{h}\bar{k}\bar{l})]$, $(R_F)_{\text{symm}} = 2\sum[F_o(hkl) - F_o(h'k'l')] / \sum[F_o(hkl) + F_o(h'k'l')]$, where hkl and $h'k'l'$ are symmetry equivalent reflections.

Fig. 4. Effect of conagenin on proliferation of rat splenic T cells.

● Con A treated, ○ non-treated.



configuration given in Fig. 3²). The final R value for 1,330 independent structure factors was 0.076 allowing for the dispersion corrections.

The effect of conagenin on rat splenic T cell proliferation *in vitro* was shown in Fig. 4 by the method described earlier. Conagenin at concentrations of 6.25 to 25 µg/ml increased the incorporation of [³H]TdR into Con A-treated cells. No increased incorporation was observed at a concentration of 100 µg/ml. It did not show the effect on non-treated cells. The mechanisms of action are now under study.

Although some of low molecular immunomodulators are known to be inhibitors of enzymes located on cell surface such as aminopeptidases, carboxypeptidases, alkaline phosphatases and esterases^{3~7}), conagenin had no inhibitory effect on these enzymes (IC₅₀ > 100 µg/ml). It had no antimicrobial activity at the concentration of 100 µg/ml. Conagenin, administered at 500 mg/kg by the iv route, did not show any toxicity to ICR mice.

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References

- ISHIZUKA, M.; J. SATO, Y. SUGIYAMA, T. TAKEUCHI & H. UMEZAWA: Mitogenic effect of bestatin on lymphocytes. *J. Antibiotics* 33: 653~662, 1980
- MOTHERWELL, W. D. S. & W. CLEGG: *In A Program for Drawing Molecular and Crystal Structures*, PLUTO 78. Univ. of Cambridge, England, 1978
- UMEZAWA, H.; T. AOYAGI, H. SUDA, M. HAMADA & T. TAKEUCHI: Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiotics* 29: 97~99, 1976
- AOYAGI, T.; T. YAMAMOTO, K. KOJIRI, F. KOJIMA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Forphenicine, an inhibitor of alkaline phosphatase Produced by actinomycetes. *J. Antibiotics* 31: 244~246, 1978
- AOYAGI, T.; H. TOBE, F. KOJIMA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Amastatin, an inhibitor of aminopeptidase A, Produced by actinomycetes. *J. Antibiotics* 31: 636~638, 1978
- UMEZAWA, H.; T. AOYAGI, K. UOTANI, M. HAMADA, T. TAKEUCHI & S. TAKAHASHI: Ebelactone, an inhibitor of esterase, produced by actinomycetes. *J. Antibiotics* 33: 1594~1596, 1980
- UMEZAWA, H.; T. AOYAGI, K. OGAWA, H. IINUMA, H. NAGANAWA, M. HAMADA & T. TAKEUCHI: Histargin, a new inhibitor of carboxypeptidase B, produced by actinomycetes. *J. Antibiotics* 37: 1088~1090, 1984