NOTES

Cytovaricin B, a New Inhibitor of JAK-STAT Signal Transduction Produced by *Streptomyces torulosus*

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Cytokines govern the growth, differentiation and functional activity of cells in the haematopoietic and immune systems. Of these, interleukin-3 (IL-3) has a broad range of function, with actions on cells at various stages of haematopoietic development[1].

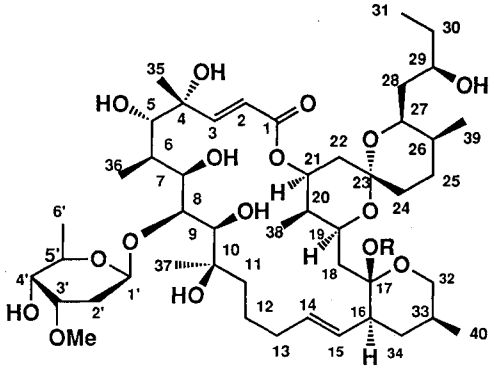
Recently, attention has been focused on the role of the Janus kinase (JAK) family of kinases in cytokine action. Many cytokine receptors have now been shown to associate physically with and activate specific JAK kinases[2]. JAK kinases regulate latent, cytoplasmic transcription factors termed STATs (Signal transducers and activators of transcription). IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) activate JAK2 and STAT5[3,4]. Because many cytokines induce tyrosine phosphorylation in lymphoid progenitor cells, and constitutive tyrosine phosphorylation is commonly observed in B-lineage leukaemias[5,6], attempts have been made to develop protein tyrosine kinase (PTK) blockers of leukaemia cell growth[7,8]. Specific tyrosine kinase blockers of JAK2 were reported to selectively inhibit leukaemic cell growth *in vitro* and *in vivo* by inducing programmed cell death, with no deleterious effect on normal haematopoiesis[9].

In order to study the role of STAT5 in IL-3 signaling, and to find new growth inhibitors of leukaemic cells, we have screened inhibitors of STAT5 from microbial metabolites using a luciferase reporter system in IL-3 dependent Ba/F3 cells. The cells were transfected with reporter plasmid consisting of luciferase gene under the control of the β-casein promoter having STAT5 binding sites. In this system, the expression of the luciferase activity is expected to be suppressed by inhibitors of JAK-STAT signal transduction.

In the course of our screening program using this system, we have found a new compound, cytovaricin B (**1**) from the culture broth of *Streptomyces torulosus* 3197-GM1. In this paper, we report the fermentation, isolation, physico-chemical properties, structure elucidation and inhibitory activity of **1**.

Streptomyces torulosus 3197-GM1, which was isolated from a soil sample collected in Iwaki, Fukushima Prefecture, Japan, was inoculated in 500-ml Erlenmeyer flasks containing 100 ml of a medium comprising of starch 2.5%, soybean meal 1.5%, dry yeast 0.2%, and $CaCO_3$ 0.4%, the pH being adjusted to 6.2 before sterilization. The fermentation was carried out at 27°C for 5 days on a rotary shaker. The supernatant of cultured broth (1.6 liter) was extracted twice with ethyl acetate (1 liter × 2) at pH 7. The organic layer was concentrated to dryness and the residue was applied to a silica gel column (2 × 30 cm, Wakogel C-200). After washing the column with hexane (200 ml), the active material was eluted with hexane-ethyl acetate (3:1, 300 ml) and *n*-hexane-ethyl acetate (2:1, 300 ml). The combined active eluate was concentrated to give a crude material, which was subjected to Toyopearl HW-40 column (2 × 70 cm) chromatography using 80% aqueous acetone as the developing solvent. The active fractions were further purified by reversed phase HPLC, using a column of YMC Pack ODS (2 × 25 cm, flow rate 5 ml/minute)

Fig. 1. Structures of cytovaricin B (**1**) and cytovaricin (**2**).



Cytovaricin B (**1**) : R = CH_3
Cytovaricin (**2**) : R = H

Table 1. Physico-chemical properties of cytovaricin B (**1**).

Appearance	Colorless amorphous powder
Optical rotation	$[\alpha]_D^{23}$ −9° (*c* 0.1, $CHCl_3$)
MP (°C)	210~213
Formula	$C_{48}H_{82}O_{16}$
HR-FAB-MS (*m/z*)	915.5688 $(M+H)^+$
Calcd.	915.5681
UV λ_{max} nm (ε) in MeOH	217 (11,000)
IR ν_{max} (KBr) cm^{-1}	3400, 1720, 1270

with elution using 65% aqueous acetonitrile to give two fractions. The fraction containing **1** was evaporated *in vacuo* to give 8.0 mg of pure **1** as a colorless amorphous powder, and the other fraction yielded a colorless powder (10.2 mg) of cytovaricin (**2**)[10,11].

Physico-chemical properties of **1** are summarized in Table 1. **1** was soluble in $CHCl_3$, EtOAc, Me_2CO, MeOH and DMSO, but insoluble in *n*-hexane and H_2O. The molecular formula of **1** was established as $C_{48}H_{82}O_{16}$ from the HRFAB-MS and NMR spectral analyses. The IR spectrum of **1** showed the presence of an α,β-unsaturated ester at 1720 cm^{-1}. NMR data of **1** are summarized in Table 2. The structure of **1** was determined by the analyses of DQF-COSY, HMQC and HMBC spectral data. All assignments were made based on comparison with **2**. An additional methoxy proton (17-OMe, 3.20 ppm) only observed in **1** was long-range coupled to a quaternary carbon C-17 (101.1 ppm) in the HMBC spectrum of **1**. Therefore, the tertiary hydroxyl group at C-17 in **2** is concluded to be substituted by a methoxy group in **1**. Thus, the structure of **1** was established as a lactol methyl ether of **2**. In order to determine the stereochemistry of **1**, **2** was treated with methanol in the presence of Dowex 50 (H^+) at 0°C for 2 hours to give a lactol methyl ether of **2**[12]. Comparison of the spectral data for the purified product from the reaction mixture and **1** showed complete agreement. Thus, the structure including the absolute stereochemistry of **1** was determined as shown in Fig. 1. Since the purification of **1** from the cultured broth was made in the absence of methanol, **1** is believed to be a natural product isolated from the nature for the first time.

The inhibitory effect of **1** in our system used for the screening was 32 μM (IC_{50}). **2** was also active at the same concentration. In the evaluation system we employed[3,4,13], **1** directly inhibited STAT5 phosphorylation in naive Ba/F3 cells without affecting JAK2 phosphorylation. Further studies on the mechanism of inhibition for transcription of STAT5 and other biological activities are now under way.

Table 2. ^{13}C (125 MHz) and ^1H (500 MHz) NMR data of cytovaricin B (**1**) in MeOH-d_4.

No.	δ_C	δ_H
1	167.14	
2	120.86	6.03 d
3	153.34	6.93 d
4	76.85	
5	80.40	3.70 m
6	36.78	1.75 m
7	77.56	3.73 dd
8	82.75	3.80 dd
9	73.88	3.08 br s
10	75.84	
11	41.75	1.32 m, 1.72 m
12	25.29	1.32 m
13	35.15	1.89 m, 2.12 m
14	133.44	5.33 ddd
15	132.87	5.41 ddd
16	47.12	2.33 m
17	101.09	
18	37.29	1.78 m, 1.90 m
19	68.15	3.94 m
20	37.24	2.12 m
21	72.12	5.27 ddd
22	36.34	1.64 m, 1.71 m
23	98.75	
24	30.74	1.40 m, 1.63 m
25	27.44	1.40 m, 2.13 m
26	31.93	2.13 m
27	70.27	4.06 ddd
28	42.37	1.62 m, 1.39 m
29	71.94	3.70 m
30	32.00	1.46 m, 1.62 m
31	10.72	1.00 t
32	68.15	3.15 t, 3.54 dd
33	31.35	1.78 m
34	36.78	1.42 m, 1.80 m
35	26.85	1.36 s
36	7.13	0.98 d
37	24.31	1.10 s
38	5.79	0.86 d
39	11.68	0.95 d
40	17.34	0.79 d
1′	100.71	4.90 dd
2′	34.79	1.53 ddd, 2.38 ddd
3′	78.96	3.59 br dd
4′	74.27	3.18 dd
5′	72.00	3.80 m
6′	18.53	1.25 d
3′OMe	57.79	3.40 s
17OMe	47.44	3.20 s

Chemical shifts were determined in ppm based on TMS as an internal standard.

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References

1) ARAI, K.; F. LEE, A. MIYAJIMA, S. MIYATAKE, N. ARAI & T. YOKOTA: Cytokines: Coordinators of immune and inflammatory responses. Annu. Rev. Biochem. 59: 783~836, 1990
2) IHLE, J. N.; B. A. WITTHUHN, F. W. QUELLE, K. YAMAMOTO, W. E. THIERFELDER, B. KREIDER & O. SILVENNOINEN: Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem. Sci. 19: 222~227, 1994
3) QUELLE, F. W.; N. SATO, B. A. WITTHUHN, R. C. INHORN, M. EDER, A. MIYAJIMA, J. D. GRIFFIN & J. N. IHLE: JAK2 associates with the bc chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol. Cel. Biol. 14: 4335~4341, 1994
4) MIU, A. L.-F.; H. WAKAO, A.-M. O'FARRELL, N. HARADA & A. MIYAJIMA: Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signal through two STAT5 homologs. EMBO J. 14: 1166~1175, 1995
5) GRIMALDI, J. C. & T. C. MEEKER: The t (5;14) chromosomal translocation in a case of acute lymphocytic leukemia joins the interleukin-3 gene to the immunoglobulin heavy chain gene. Blood 73: 2081~2085, 1989
6) DADI, H.; S. KE & C. M. ROIFMAN: Interleukin 7 receptor mediates the activation of phosphatidylinositol-3 kinase in human B-cell precursors. Biochem. biophys. Res. Commun. 192: 459~464, 1993
7) BURKE, T. R.; B. LIM, V. E. MARQUEZ, Z. Li, J. B. BOLEN, I. STEFANOVA & I. D. HORAK: Bicyclic compounds as ring-constrained inhibitors of protein-tyrosine kinase $p56^{lck}$. J. Med. Chem. 36: 425~432, 1993
8) CUSHMAN, M.; H. ZHU, R. L. GEAHLEN & A. J. KRAKER: Synthesis and Biochemical evaluation of a series of aminoflavones as potential inhibitors of protein-tyrosine kinase $p56^{lck}$, EGFr, and $p60^{v\text{-}src}$. J. Med. Chem. 37: 3353~3362, 1994
9) MEYDAN, N.; T. GRUMBERGER, H. DADI, M. SHAHAR, E. ARPAIA, Z. LAPIDOT, J. S. LEEDER, M. FREEMAN, A. COHEN, A. GRAZIT, A. LEVITZKI & C. M. ROIFMAN: Inhibition of acute lymphoblastic leukaemia by a JAK-2 inhibitor. Nature 379: 645~648, 1996
10) KIHARA, T.; H. KUSAKABE, G. NAKAMURA, T. SAKURAI & K. ISONO: Cytovaricin, a novel antibiotic. J. Antibiotics 34: 1073~1074, 1981
11) KIHARA, T.; M. UBUKATA, J. UZAWA & K. ISONO: Biosynthesis and ^{13}C NMR assignment of cytovaricin, a neutral macrolide antibiotic. J. Antibiotics 42: 919~925, 1989
12) EVANS, D. A.; S. W. KALDOR, T. K. JONES, J. CLARDY & T. J. STOUT: Total synthesis of the macrolide antibiotic cytovaricin. J. Am. Chem. Soc. 112: 7001~7031, 1990
13) MIU, A. L.-F.; H. WAKAO, T. KINOSHITA, T. KITAMURA & A. MIYAJIMA: Suppression of interleukin-3-induced gene expression by a *C*-terminal truncated Stat5: role of Stat5 in proliferation. EMBO J. 15: 2425~2433, 1996