Communications to the Editor

METACYTOFILIN, A NOVEL IMMUNOMODULATOR PRODUCED BY *Metarhizium* sp. TA2759

Sir:

In the course of screening for substances that modulate immune responses, we found a novel immunosuppressive substance named metacytofilin (MCF) in the culture filtrate of strain TA2759 which was isolated from a soil sample collected in Enzan city, Yamanashi Prefecture, Japan. The strain was identified as *Metarhizium* sp. TA2759. The structure of MCF was determined by X-ray crystallographic analysis to be 3α-hydroxy-6β-methylamino-6α-(2-methylpropyl)-3β-phenylmethyl-4*H*-2,3,5,6-tetrahydro-1,4-oxazine-2,5-dione. Although MCF exhibited an immunosuppressive effect on the mixed lymphocyte culture reaction and immune responses in mice, it had low toxicity and no antimicrobial activity. In this communication, the production, isolation, physicochemical properties, structure and biological activity of MCF are reported.

The activity of MCF was evaluated in the mixed lymphocyte culture reaction (MLCR) according to the method described previously[1]. Spleen cells (2×10^5) taken from F344 rat (10~13 weeks old) used as a responder were mixed with stimulator spleen cells (2×10^5) taken from WKY rat. Responder cells were passed through a nylon wool column and stimulator cells were treated with 50 μg/ml mitomycin C (Kyowa Hakko, Co., Inc.) at 37°C for 20 minutes. The mixed cells were cultured with test samples in RPMI-1640 medium containing 5% fetal calf serum at 37°C for 5 days in 5% CO_2 in air. MLCR was determined by measuring the incorporation of [^{3}H]-thymidine ([^{3}H]-TdR) into the cultured cells by liquid scintillation counting ([^{3}H]-TdR pulsed 16 hours before assay). Triplicate determinations were made.

The strain was inoculated to 500-ml Erlenmeyer flasks containing 100 ml of a medium consisting of soluble starch 2%, glucose 1%, yeast extract 0.5%, Tripticase peptone 0.5%, $CaCO_3$ 0.4%, Jamarin S 25% v/v (Jamarin Laboratory, Co., Ltd.), pH 7.2 before sterilization for seed culture and was cultured on a rotary shaker at 27°C for 3 days. For production of MCF, 2 ml of the seed cultured broth was transferred to 100 ml of the same medium and was cultured on a rotary shaker at 27°C for 4 days.

The culture filtrate (8 liters) was adjusted to pH 8.0 with 4% $NaHCO_3$ and extracted with BuOAc. The extract was concentrated to an oily residue (0.6 g) *in vacuo*, and it was applied to a silica gel column (Wakogel C-200). After washing with $CHCl_3$, the active substance was eluted with $CHCl_3$-MeOH (20:1). The active eluate was concentrated *in vacuo* to afford a crude powder (0.2 g) that was dissolved in $CHCl_3$-MeOH-H_2O (2:2:1). The solution was subjected to centrifugal partition chromatography (Sanki Engineering, Co., Ltd.). The chromatography was performed with a solvent system of $CHCl_3$-MeOH-H_2O (2:2:1), (lower phase stationary). The active fractions were concentrated *in vacuo* and MCF was crystallized from aq MeOH. MCF was obtained as colorless prisms (30 mg).

The physico-chemical properties of MCF are shown in Table 1. The molecular formula $C_{16}H_{22}N_2O_4$ was determined by FAB-MS and elemental analysis. The ^{1}H NMR spectrum (CD_3OD, 400 MHz) of MCF showed the signals of two methyls, two methylenes, one methine, one *N*-methyl and five aromatic protons. The ^{13}C NMR spectrum (CD_3OD, 100 MHz) exhibited sixteen carbons shown in Table 2. The structure of MCF in Fig. 1 (excluding stereochemistry) was proposed from the analysis of its NMR spectra. The position

Table 1. Physico-chemical properties of MCF.

Appearance	Colorless prisms
FAB-MS (*m/z*)	307 $(M+H)^+$, 305 $(M-H)^+$
Elemental analysis	
Calcd for $C_{16}H_{22}N_2O_4$:	C 62.73, H 7.24, N 9.14
Found:	C 62.35, H 7.22, N 8.90
MP	201~202°C
$[\alpha]_D^{27}$ (*c* 0.63, MeOH)	+5.1°
UV λ_{max}^{MeOH} nm (log ε)	End absorption 252 (sh, 2.26), 258 (sh, 2.30), 264 (sh, 2.18)
IR (KBr) cm^{-1}	3325, 2960, 1685, 1450, 1415, 1305, 1130, 1090, 895, 700
Solubility	
Soluble in:	MeOH, DMSO
Insoluble in:	$CHCl_3$, H_2O
Color reaction	Ninhydrin, Rydon-Smith
Rf value*	0.58

* Silica gel TLC (Merck Art No. 5715), hexane-EtOAc (3:7).

of each substituent in the oxazine ring was determined from the results of the HMBC spectrum and deuterium isotope effects[3] on ^{13}C chemical shifts of MCF. The deuterium isotope effects were observed by the use of concentric tubes (inner tube; 0.8 mg/0.91 ml of CD_3OH and 1.7 mg/0.40 ml of CD_3OD solutions). The isotope shift values are shown in Fig. 2.

The proposed structure of MCF was confirmed by X-ray crystallographic analysis. Crystals of MCF were grown in MeOH solution as colorless prisms. The lattice constants and intensity data were obtained from the data collected by a Philips PW1100 diffractometer using CuKα radiation

Table 2. ^1H and ^{13}C NMR data for MCF in CD_3OD.

Position	δ_C (100 MHz)	δ_H (400 MHz)
2	169.71	—
3	84.33	—
5	168.53	—
6	88.44	—
7	49.53	1.55 (dd, 6.0, 13.1)*, 1.82 (dd, 6.0, 13.1)
8	24.20	1.74 (m)
9	24.25	0.89 (d, 6.7)
10	24.60	0.92 (d, 6.7)
11	49.9	2.11 (s)
12	46.09	2.92 (d, 13.3), 3.56 (d, 13.3)
13	136.05	—
14, 18	132.18	7.29 (m)
15, 17	129.37	7.14~7.26 (m)
16	128.30	~7.16 (m)

Values relative to TMS=0 ppm.
* Proton signal multiplicity and coupling constant (J=Hz).

Fig. 1. Structure of metacytofilin.

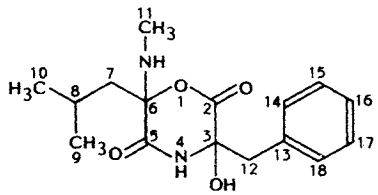

Fig. 2. Deuterium isotope effects on ^{13}C chemical shifts of MCF observed in CD_3OD and CD_3OH solutions.

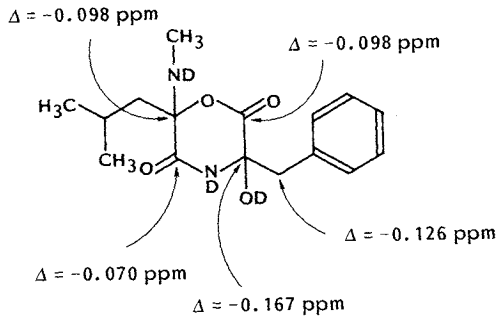

Table 3. Inhibitory effect of MCF on the MLC reaction.

MCF (μg/ml)	MLC reaction cpm±SD	Inhibition (%)
—	10,401±485	—
100	2,259±340	78***
10	4,863±828	53***
1	4,960±302	52***
0.1	9,241±642	11**

Spleen cells taken from F344 rat as the responder were mixed with mitomycin C-treated spleen cells of WKY rat as the stimulator and cultured with or without MCF for 5 days. The radioactivity of responder alone was 2,372±528 cpm.
** $P<0.01$, *** $P<0.001$.

Fig. 3. Molecular structure of MCF using PLUTO program.

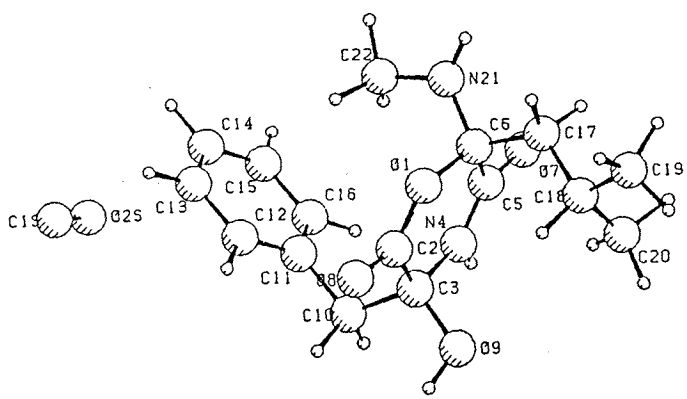

Table 4. Immunosuppressive effect of MCF on DTH and antibody formation to SRBC in mice.

MCF (mg/kg/day)	DTH[a]		Antibody formation[b]	
	Increase of footpad thickness (×0.1 mm)	Inhibition (%)	PFC/spleen (×1,000)	Inhibition (%)
—	14.10±0.55	—	255.50±22.25	—
100	1.00±0.06	93***	112.10±62.98	56**
25	5.60±0.48	61***	168.48±79.59	34
6	10.40±0.83	28*	199.26±28.68	22**

[a] CDF_1 mice (5 mice/group) were immunized with 10^5 SRBC iv MCF was given ip daily for 4 days after immunization.
[b] CDF_1 mice (5 mice/group) were immunized with 10^8 SRBC iv MCF was given ip daily for 3 days after immunization.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

monochromated by a graphite plate. Crystal data are: $C_{16}H_{22}N_2O_4 \cdot CH_3OH$, FW=338.4, monoclinic, space group $P2_1$, $Z=2$, lattice constants: $a=9.290(5)$ Å, $b=17.442(9)$ Å, $c=6.107(5)$ Å, $\beta=108.13(6)°$, V=940.4 Å^3, $D_x=1.195\,g\,cm^{-3}$, μ for CuK$\alpha=6.90\,cm^{-1}$.

The crystal structure was determined by direct method and refined by the method of least-squares with block-diagonal-matrix approximations. The final R value for 1,579 independent refrections was 0.069. The absolute configuration was not determined. A computer-generated drawing[4] of the structure is shown in Fig. 3.

The inhibitory effect of MCF on the MLCR is shown in Table 3. The IC_{50} of MCF in the MLCR was 1.0 μg/ml. The effects of MCF on delayed-type hypersensitivity (DTH) and antibody formation to sheep red blood cells (SRBC) in mice (CDF_1 mice, female 8~10 weeks old) were tested according to the method described previously[2]. Briefly, for testing DTH, mice were immunized by iv administration of 10^5 SRBC. After the immunization, MCF was given ip to mice daily for 4 days. Four days after the immunization, the DTH response was elicited by subcutaneous injection of 10^8 SRBC to the footpad, and 24 hours later, the footpad thickness was measured. For testing antibody formation, mice were immunized by intravenous injection of 10^8 SRBC, 4 days later, antibody formation was determined by enumerating the direct plaque forming cells (PFC) in spleens. MCF was given to mice ip on days 0 to 3 after immunization. Five mice were used in each group. As shown in Table 4, MCF suppressed DTH at 25 mg/kg to 100 mg and significantly suppressed antibody formation at 100 mg/kg.

MCF did not show toxicity at 200 mg/kg ip to ICR mice. MCF at 100 μg/ml exhibited no cytotoxicity against L1210, EL-4 and IMC carcinoma cells and had no antimicrobial activity against bacteria and fungi. The mechanisms of action are now under study.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

Masatomi Iijima
Tohru Masuda
Hikaru Nakamura†
Hiroshi Naganawa†
Shogo Kurasawa†
Yoshiro Okami†
Masaaki Ishizuka*
Tomio Takeuchi

Institute for Chemotherapy, M.C.R.F.,
18-24 Aza Motono, Miyamoto, Numazu-shi,
Shizuoka 410-03, Japan
†Institute of Microbial Chemistry, M.C.R.F.,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo
141, Japan

Yoichi Iitaka

Laboratory of Biophysics,
Faculty of Pharmaceutical Sciences,
Teikyo University,
Sagamiko-machi, Tsukui-gun,
Kanagawa 199-01, Japan

(Received April 3, 1992)

References

1) ISHIZUKA, M.; T. MASUDA, S. MIZUTANI, M. OSONO, H. KUMAGAI, T. TAKEUCHI & H. UMEZAWA: Induction of antitumor resistance to mouse leukemia L1210 by spergualins. J. Antibiotics 39: 1736~1743, 1986
2) ISHIZUKA, M.; T. MASUDA, N. KANBAYASHI, S. FUKASAWA, T. TAKEUCHI, T. AOYAGI & H. UMEZAWA: Effect of bestatin on mouse immune system and experimental murine tumors. J. Antibiotics 33: 642~652, 1980
3) HANSEN, P. E.: Isotope effects of nuclear shielding. *In* Annual Reports on NMR Spectroscopy. Vol. 15. *Ed.*, G.A. WEBB, pp. 105~234, Academic Press, 1983
4) MOTHERWELL, W. D. S. & W. CLEGG: A Program for Drawing Molecular and Crystal Structures. PLUTO 78. University of Cambridge, England, 1978