VOL. 52, NO. 1, JAN. 1999 THE JOURNAL OF ANTIBIOTICS pp. 20 — 24

IC202A, a New Siderophore with Immunosuppressive Activity Produced by *Streptoalloteichus* sp. 1454-19

I. Taxonomy, Fermentation, Isolation and Biological Activity

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(Received for publication September 2, 1998)

IC202A, a new immunosuppressive compound, was isolated from the culture filtrate of *Streptoalloteichus* sp. 1454-19. It showed a suppressive effect on mixed lymphocyte culture reaction with an IC_{50} value of 3.6 μg/ml and mitogen induced lymphocyte blastogenesis *in vitro*.

In the course of our screening program for low molecular weight immunomodulators in microbial products, we have found several new compounds[1] which are immunosuppressants or cytokine inducers. Among these is the novel compound, metacytofilin[2], a new immunosuppressant, which was detected using mixed lymphocyte culture reaction (MLCR) as a primary screening method. Proceeding with the same screening method, we have found another new compound, IC202A in the cultured broth of *Streptoalloteichus* sp. 1454-19. In this report, we present the taxonomy of the producing strain, fermentation, isolation and biological activities of IC202A. The structure determination of IC202A is reported in the accompanying paper[3].

Materials and Methods

Chemicals

Deferoxamine mesylate was purchased from Sigma Chemical Co. Concanavalin A (Con A, Pharmacia AB.) and lipopolysaccharide (LPS, *Escherichia coli* 0127, Difco Laboratories) were employed as mitogens.

Microorganism

The strain 1454-19 was isolated from a soil sample collected in Karuizawa-cho, Nagano Prefecture, Japan.

Taxonomic Studies

For the morphological observations, the strain was grown at 27°C for 3 and 3~4 weeks on ISP-4 and glucose-asparagine agar, respectively. The medium and procedures used for the cultural and physiological characterization of the strain were those recommended by the ISP[4]. The color designation of mycelium and soluble pigment was determined by Color Harmony Manual (4th. ed, 1958, Container Corporation of America, Chicago, Illinois). Whole-cell analysis was performed by the method reported by BECKER *et al.*[5]. The temperature range for growth was determined on an ISP-4 agar slant using a temperature gradient incubator (Model TN3, Toyo Kagaku Sangyo Co., Ltd.). NaCl tolerance was tested on ISP-4 agar medium supplemented with various concentrations of NaCl.

Fermentation

Strain 1454-19 was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of medium composed of dextrin 2%, polypepton (Nippon Seiyaku Co., Inc.) 1%, corn steep liquor (Ajinomoto Co., Inc.) 0.5%, glycerin 1%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% (adjusted to pH 7.4 before sterilization). The flask was incubated at 27°C for 2 days on a rotary shaker (180rpm). For production of IC202A, the seed culture (2 ml) was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of the same medium, and cultivated for 5 days.

Mixed Lymphocyte Culture Reaction (MLCR)

MLCR was carried out according to the method described previously[6]. Briefly, spleen cells (2×10^5) taken from C3H/He mice ($8 \sim 12$ weeks old, H-2^k) as a responder were mixed with stimulator spleen cells (2×10^5) taken from BALB/c mice ($8 \sim 12$ weeks old, H-2^d). Responder cells were passed through a nylon wool column and stimulator cells were treated with mitomycin C at 37°C for 20 minutes. The mixed cells were cultured with test samples in RPMI-1640 medium containing 5% fetal calf serum (FCS) at 37°C under 5% CO_2 in air. The culture was pulsed with 0.5 μCi per well of ^{3}H-TdR 16 hours before assay, followed by counting with a Packard Matrix 9600 beta-counter apparatus.

Lymphocyte Blastogenesis

Mitogen induced lymphocyte blastogenesis was examined by the following procedure[7]. Murine spleen cells (1×10^6/ml) were cultured with ConA (0.5 μg/ml) or LPS (2 μg/ml) in the presence of various concentrations of the test samples in RPMI-1640 medium containing 1% FCS at 37°C for 72 hours under 5% CO_2 in air. Antiproliferation activities were examined by measuring the incorporation of ^{3}H-TdR into the culture cells using a beta-counter.

Cytotoxicity

The cytotoxicity of IC202A against L1210, P388, LB32T, HL60, and RAW264.7 cells was determined using the MTT method. The cells were cultured in RPMI-1640 medium supplemented with 10% FCS for 48 or 72 hours in the presence of various concentrations of IC202A at 37°C under 5% CO_2 in air. The cytotoxicity was assessed by measuring O.D. at 570 nm.

Table 1. Taxonomical characteristics of the strain 1454-19.

Sugar pattern of whole mycelium	C
Cell wall type	III
Menaquinone	MK-9(H_6), MK-6(H_8)
Phospholipid type	P_{II}
Spore surface	Smooth
Aerial mycelium color	Grayish white to light gray
Substrate mycelium color	Grayish white to brownish gray
Soluble pigment	None
Melanin formation	None
Liquefaction of gelatin	Positive
Coagulation of skim milk	Positive
Peptonization of skim milk	Positive
Production of hydrogen sulfide	Negative
NaCl conc. tolerance	1.5%
Temperature range for growth	10 ~ 30°C
Hydrolysis of:	
Starch	Positive
Urea	Positive
Chitin	Negative
Esculin	Negative
Carbon utilization:	
D-Glucose	Positive
L-Arabinose	Negative
D-Xylose	Negative
D-Fructose	Negative
D-Mannitol	Positive
Inositol	Negative
L-Rhamnose	Negative
Raffinose	Positive
Sucrose	Negative

Results and Discussion

Taxonomic Studies

Aerial mycelium of the strain bore chains of 5~15 spores about 1 μm in diameter. The spore morphology resembled that of the genus *Streptomyces*. Fragmentation of the substrate mycelium was absent. The vegetative hyphae bore oval or spherical sporangium like vessels. Cell walls contained *meso* diamino pimelic acid. Whole cell hydrolysates contained galactose, but no madurose. Phospholipid-type and major menaquinones were P_{II} and MK-9(H_6), respectively. From these observations, the strain was identified as *Streptoalloteichus* sp.[8]. The taxonomical characteristics of the strain was shown in Table 1.

Isolation and Purification

IC202A was isolated according to the scheme as shown in Fig. 1. The culture filtrate (8 liters) was adjusted to pH 8.0 with 4% $NaHCO_3$ and was applied to a Diaion HP-20 column (800 ml). After washing with H_2O, the active substance was eluted with 50% MeOH. The crude material was dissolved in H_2O and applied to a CM-Sephadex C-25 column (80 ml). After washing with H_2O, the active substance was eluted with 0.2 M NaCl. The fractions containing IC202A were collected and applied to a Diaion HP-20 column to desalt them. Further purification was carried out using a Chromatorex gel (100~200 mesh, Fuji Silysia Chemical Ltd.) equilibrated with 5% MeOH. After the column (30 ml) was washed with 30% MeOH, the active substance was eluted with 30% MeOH containing 0.1 M NaCl. The fractions containing IC202A were concentrated and further purified by centrifugal partition chromatography (CPC, Sanki Engineering Co., Ltd.) using a solvent system of BuOH : MeOH : H_2O = 4 : 1 : 5 in ascending mode. Active fractions were concentrated to obtain a hygroscopic powder of IC202A (15 mg). IC202A is soluble in DMSO and H_2O, slightly soluble in MeOH, but insoluble in $CHCl_3$. The structure of IC202A is shown in Fig. 2. IC202A is a ferrioxamine-related compound

Fig. 1. Purification of IC202A.

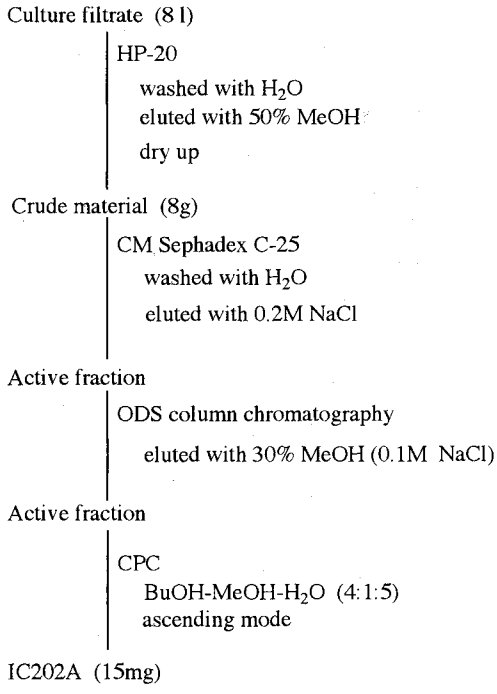

Fig. 2. Structure of IC202A.

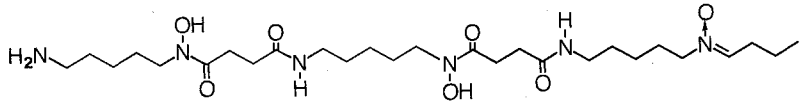

Table 2. Inhibitory effect of IC202A on MLCR and mitogen induced lymphocyte blastogenesis.

	IC_{50} (μg/ml)		
	MLCR	Lymphocyte blastogenesis	
		ConA	LPS
IC202A	3.6	9.6	11.3
Deferoxamine	2.3	4.7	5.9

Table 3. Reduction of immunosuppressive activity of IC202A by $FeCl_3$.

	Inhibition (%)		
	MLCR	Lymphocyte blastogenesis	
		ConA	LPS
IC202A 25μg/ml	100	84	60
IC202A 25μg/ml + 100μM $FeCl_3$	0	5	0
Deferoxamine 25μg/ml	100	93	80
Deferoxamine 25μg/ml + 100μM $FeCl_3$	0	0	0

Table 4. Cytotoxicity of IC202A on tumor cell lines.

Cells	IC_{50} (μg/ml)
L1210	>100
P388	20.0
LB32T	7.0
HL60	10.3
RAW264.7	>100

containing a butylidene *N*-oxide moiety. The details of the structural determination are given in the accompanying paper.

Biological Activities

The immunosuppressive activity of IC202A was evaluated by MLCR and mitogen induced lymphocyte blastogenesis. As shown in Table 2, the inhibitory activity (IC_{50}: 3.6 μg/ml) of IC202A on MLCR was comparable to that of deferoxamine, one of ferrioxamine family of compounds. Furthermore, IC202A showed a potent suppressive effect on the mitogen (ConA or LPS) induced lymphocyte blastogenesis.

As shown in Table 3, the inhibitory activities of IC202A and deferoxamine were remarkably reduced by cotreatment with a saturating dose of $FeCl_3$ (100 μM).

The cytotoxicity of IC202A was tested against L1210, P388, LB32T, HL60, and RAW264.7 cells. The results are shown in Table 4. IC202A inhibited the growth of LB32T, HL60, and P388 cells (IC_{50} range of 7.0~20.0 μg/ml). On the other hand, IC202A showed no significant cytotoxic activity against L1210 and RAW264.7 cells. Although IC202A showed cytotoxic activity against several cell lines, it inhibited MLCR at a lower dose. These cytotoxicities were also abrogated by cotreatment with $FeCl_3$ (data not shown).

Using MLCR as a primary screening method, we isolated IC202A from the culture filtrate of *Streptoalloteichus* sp. 1454-19. Although a number of ferrioxamine family compounds derived from microorganisms have been described, IC202A is structurally distinct from the others due to the presence of a terminal butylidene *N*-oxide function. It is known that some of these ferrioxamines exhibited an immunosuppressive effect[9]. Thus, the immunosuppressive activities of IC202A and deferoxamine were tested using MLCR and mitogen induced lymphocyte blastogenesis methods. As shown in Table 2 and Table 3, both compounds have almost the same immunosupressive effect. Further studies on the biological activities and biosynthesis of IC202A are in progress.

Acknowledgments

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

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