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Immunosuppressive Characteristics of Oligomycin Derivatives Produced by *Streptomyces lydicus* MCY-524

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A strain producing immunosuppressive substances was isolated from a soil in Cheju island. By morphological, cultural, and physiological studies, the strain was identified as *Streptomyces lydicus* MCY-524. Cultured broth was purified by silica gel, sephadex LH-20 and preparative HPLC and gave two immunosuppressive compounds, MCH-22 and MCH-32. They dramatically suppressed the B cell activation with lipopolysaccharide, T cell activation by mixed lymphocyte response, and primary T-dependent antibody response at a final concentration of 1 μg/ml. They also markedly suppressed the proliferation of lymphocytes induced by lipopolysaccharide, pokeweed mitogen, and concanavaline A at the same concentration. Their suppressive activities, which were comparable to those of cyclosporin A, suggested that they were potent and broad immunotoxic agents on the immune functions of murine lymphocytes.

An immunosuppressive agent can be used in therapeutic procedures such as organ transplant where rejection is likely and in mechanism studies of immune responses. Owing to these potential applications, much research has been undertaken to find better immunosuppressant compounds and several immunosuppressants such as FK-506, cyclosporin A and rapamycin have been discovered from microbial resources. These compounds showed inhibitory activities of B cell and T cell activation (22). Especially cyclosporin A and FK-506 are known to inhibit the early signal transduction pathway from the T cell receptor, which is responsible for the gene expression of many cytokines including IL-2 (21). Rapamycin prevents the rejection of transplanted organs by blocking the activation of helper T cells in analogy with cyclosporin A and FK 506.

The discovery of a new type of immunomodulator will contribute to the clarification of the immune responses and related diseases. Immunomodulating drugs, such as FK506, cyclosporin A, and streptonigrin are expected not only to be useful for the study of cellular and biological events of immune responses but also to present clinical drugs for immunologic diseases.

In the course of our screening program for novel biological active substances from microorganisms, we found oligomycin analogues, MCH-22 and MCH-32, which showed potent immunosuppressant activities. Oligomycin, a macrolide antibiotic, is known to bind non-covalently mitochondrial type proton ATPases, inhibiting the transfer of protons through the Fo site and inhibiting both ATP hydrolysis and oxidative phosphorylation (8). Oligomycin F is reported to be an extremely potent suppressive agent for various immunological systems (7) like bafilomycin (3) and concanamycin B (11). The present paper describes the taxonomy of the producer microorganisms as well as isolation, and physicochemical and immuosuppressive activities of MCH-22 and MCH-32 following fermentation.

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MATERIALS AND METHODS

Organism

The strain MCY-524 was isolated from a soil sample collected in Cheju-island, Korea. The isolation medium of MCY-524 was Humic acid-Vitamin (HV) agar medium (Humic acid 0.1%, Na_2HPO_4 0.05%, KCl 0.17%, $MgSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CaCO_3$ 0.002%, and agar 2%, pH 7.2) and Bennett's agar medium (glucose 1%, yeast extract 0.1%, bacto peptone 0.1%, beef extract 0.1%, and agar 2%, pH 7.2) supplemented with 50 μg/ml cycloheximide and 50 μg/ml nalidixic acid. The strain was cultured on Bennett's agar medium

at 28°C. To investigate the morphological properties of the strain, the International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb were employed (18). Morphological observations were made with light and scanning electron microscopes. Diaminopimelic acid in the whole cell hydrolysates was analyzed according to the method of Lechevalier and Lechevalier (9, 10) The utilization of carbon sources was examined according to the method of Pridham and Gottlieb (16).

Fermentation

A loopful of *S. lydicus* MCY-524 on modified Bennett's agar slant was inoculated in two 500 ml baffled flasks each containing 100 ml of the medium consisting of soluble starch 2%, glucose 1%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.005%, $CaCO_3$ 0.2%, and soybean meal 2.5%. The inoculated flasks were cultured at 28°C for 3 days on a rotary shaker. Each 100 ml of the cultured broth was transferred into 7.5 liter jar fermenters containing 5 liter of the same medium as used for the seed culture. Fermentation was carried out for 96 h at 28°C, 200 rpm and aeration of 200 l/h. This procedure was repeated four times.

Isolation and Purification of the Active Compounds

The isolation procedures are summerized in Fig. 1. The mycelium, collected by centrifugation from the cultured broth (20 l), was extracted with acetone. After removal of acetone *in vacuo*, the extracts were partitioned with 4 l of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to give brown oily residue (4.7 g). This oily material was chromatographed with silica gel column chromatography eluted with $CHCl_3$-MeOH (9 : 1), using sephadex LH-20 column chromatography developed with MeOH, and preparative HPLC on a DELTA PAK column (ø 19 mm × 300 mm) with 70% MeOH. Immunosuppressive materials, MCH-22 (485 mg) and MCH-32 (440 mg), were obtained as white powders.

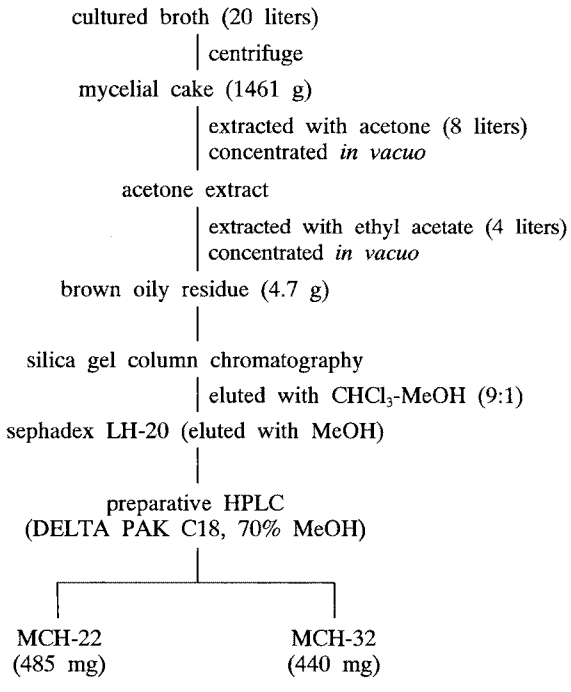


Fig. 1. Isolation and purification of MCH-22 and MCH-32.

T-dependent Primary Antibody Response to Sheep Red Blood cell (SRBC)

A single cell suspension was prepared from normal spleens and the number of cells was adjusted to 1×10^7 cells/ml in 10% fetal calf serum supplemented-RPMI 1640 medium (5). The cell suspension was loaded onto two 48 well microplates (0.5 ml/well) and cultured for 5 days with T-dependent antigen, SRBC (1.3×10^7 cells/ml). All cell cultures were incubated at 37°C in a humidified atmosphere of 7% O_2, 10% CO_2, and 83% N_2 with rocking (7-10 cycles/min). After incubation, the cell suspensions were used in the determination of antibody production by plaque forming cell (PFC) assay.

Polyclonal B Cell Activation with LPS

In the case of polyclonal B cell activation, the number of cells was adjusted to 5×10^6 cells/ml and cultured for 2 days with LPS (25 μg/ml). The other steps were the same as in the method for T-dependent antibody response.

Plaque Forming Cell (PFC) Assay

The number of antibody forming cells was determined by PFC assay (4). The cell suspensions (100 μl) were mixed with 25 μl complement (50%, v/v Earle's Balanced Salt Solution (EBSS): $CaCl_2$ 0.2 mg/ml, KCl 0.4 mg/ml, $MgSO_4$ 0.2 mg/ml, NaCl 6.8 mg/ml, $NaHCO_3$ 2.2 mg/ml, NaH_2PO_4 0.14 mg/ml, GIBCO BRL, Grand Island, NY, U.S.A.), 350 μl agarose (0.85%, w/v EBSS), and 25 μl target cells (25%, v/v EBSS) in a test tube. Then, the mixture was poured on to a petridish, covered with a coverglass, and incubated for 2 h in a CO_2 incubator. After enumerating the plaques, the number of antibody forming cells (AFCs) was expressed as AFCs/10^6 cells. In the case of T-dependent antibody responses, SRBC was used as the target cell. For polyclonal B cell activation, trinitrophenol-conjugated SRBC was used (17).

Primary Induction of T Cell by Mixed Lymphocyte Response (MLR)

Primary activation of T lymphocytes was determined by MLR (1, 20). The number of cells, which were isolated from B6C3F1 and BDF1 mouse supplied by Korea Research Institute of Bioscience and Biotechnology, were adjusted to 2.5×10^6 cells/ml and each 100 μl was loaded onto individual wells of plates. After incubation for 3 days, the degree of proliferation was measured by the incorporation of 1 μCi/well [^{3}H]-thymidine for the

last 18 h.

Blastogenesis of B and T Cells

The number of cells was adjusted to 1×10^6 cells/ml and 200 μl was loaded onto individual wells of plates. Various mitogens, such as lipopolysaccharide (LPS), pokeweed mitogen (PWM), and concanavaline A (Con A) were added to culture medium at a final concentration of 5 μg/ml. After incubation for 3 days, the degree of proliferation was measured by the incorporation of 1 μCi/well [^{3}H]-thymidine for the last 18 h (23).

RESULTS AND DISCUSSION

Taxonomic Characterization of the Strain MCY-524

The aerial mycelium formed spiral chains of spores with 10-50 spores per chain. The spores were cylindrical (0.5-0.7×0.6-1.3 μm), and their surface was smooth (Fig. 2). Sclerotic granules, sporangia, and flagellated spores were not observed. The cultural characteristics of strain MCY-524 grown on various agar media are summarized in Table 1. All cultures were incubated at 28°C for 21 days. Media ISP 2,3,4, and 7 supported the best growth and abundant sporulation. The aerial mass color was gray. Pigmentation of the substrate mycelia and soluble pigment were not observed.

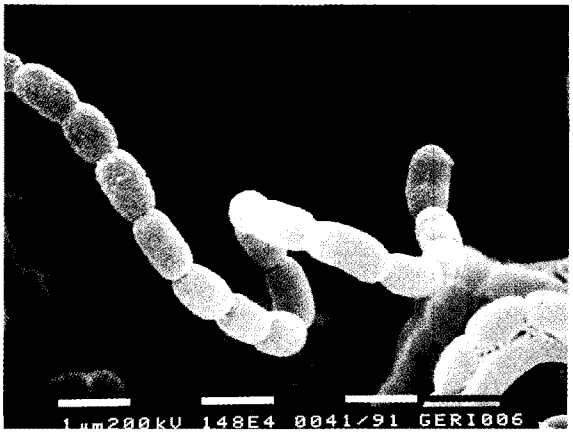


Fig. 2. Scanning electron micrograph of spore chains (×14,800) of the isolate MCY-524 cultured on ISP4 medium for 2 weeks at 28°C.

Physiological characteristics and the utilization of carbohydrates are shown in Tables 2 and 3, respectively. Lipolysis, pectin hydrolysis and H_2S production were positive. Starch hydrolysis and proteolysis were negative. Growth was observed on 7% NaCl. The strain was sensitive to streptomycin at 100 μg/ml and rifampicin at 50 μg/ml. The temperature range for growth was 10~45°C.

Whole cell hydrolysates of strain MCY-524 contained L,L-diaminopimelic acid. Glycine was detected in the cell wall. Based on the taxonomic characteristics described above, we assigned strain MCY-524 to the genus *Streptomyces*. Among the known species of this genus, strain MCY-524 showed the closest resemblance to *Streptomyces lydicus* (12, 19). The only difference is enzyme production. Therefore, we designated this strain as *S. lydicus* MCY-524.

Physicochemical Properties

Oligomycin analogues, MCH-22 and MCH-32 were isolated from the fermentation extract of *S. lydicus* MCY-524. Each compound was separated by reversed-phase HPLC (Fig. 3). *S. lydicus* MCY-524 produced only two oligomycin compounds in contrast to the other oligomycin producers such as *Streptomyces diastatochromogenes* which produced oligomycin A, B and C (13), and *Streptomyces bottropensis* which produced 44-Homooligomycins A and B (24).

They were soluble in methanol, chloroform, ethylacetate,

Table 2. Utilization of carbohydrates.

D-Glucose	+	Xylitol	+
L-Arabinose	+	Cellobiose	+
D-Xylose	+	Cellulose	−
Inositol	+	Inulin	+
D-Mannitol	+	Melibiose	+
D-Fructose	+	Maltose	+
L-Rhamnose	+	Raffinose	+
Sucrose	+	D-Galactose	+

Table 1. Cultural characteristics of strain MCY-524*.

Media	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract − malt extract agar (ISP 2)	Good	A[a] (gray)	Pale yellow	None
Oatmeal agar (ISP 3)	Good	A (gray)	Pale yellow	None
Inorganic salts − starch agar (ISP 4)	Good	A (gray)	Pale yellow	None
Glycerol − asparagine agar (ISP 5)	Moderate	A (White)	Pale yellow	None
Peptone − yeast extract iron agar (ISP 6)	Moderate	A (gray)	Pale yellow	None
Tyrosine agar (ISP 7)	Good	A (gray)	Pale yellow	None
Bennett's agar	Good	A (gray)	Pale yellow	None
Nutrient agar	Good	A (gray)	Pale yellow	None

*The strain was cultured in various kinds of media at 28°C for 21 days.
[a]A: Abundant.

but insoluble in hexane and water. The UV spectra showed absorption maxima at 225, 232, and 241 nm. IR spectra showed hydroxyl, carbonyl and conjugated diene moieties. The molecular formula was determined as $C_{46}H_{74}O_{13}$ and $C_{45}H_{74}O_{11}$ by HRFABMS and molecular weights were found to be 835 $[M+H]^+$ and 791 $[M+H]^+$ by FABMS spectra. The physico-chemical properties of MCH-22 and MCH-32 are summarized in Table 4. Intensive NMR analysis of MCH-22 and MCH-32 revealed their structures as a new oligomycin homologue, homooligomycin E and oligomycin A, respectively (Fig. 4). Their precise structural elucidation will be the due course.

Table 3. Physiological characteristics.

Growth with (%, w/v)		Enzyme production	
NaCl (7.0)	+	Lecithinase	−
Sodium azide (0.01)	−	Proteolysis	−
Potassium tellurite (0.001)	+	Lipolysis	+
Thallous acetate (0.001)	−	Pectin hydrolysis	+
Antibiosis		H_2S production	+
Streptomyces murinus ISP 5091	+	Hippurate hydrolysis	−
Bacillus subtilis NCIB 3610	+	Starch hydrolysis	−
Candida albicans CBS 562	−	Antibiotics resistance	
Saccharomyces cerevisiae CBS 1171	−	Rifampicin (50 μg/ml)	−
		Streptomycin (100 μg/ml)	−
Aspergillus niger LIV 131	+		

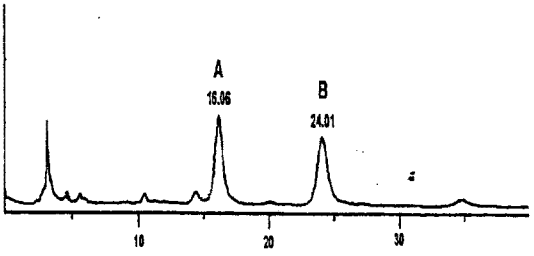
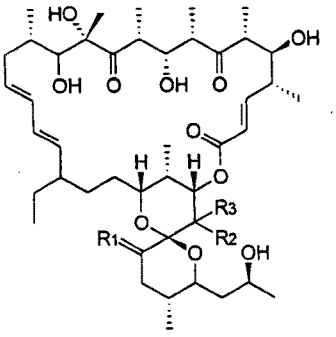


Fig. 3. Chromatograms of ethyl acetate extract of *S. lydicus* MCY-524.
A, MCH-22; B, MCH-32; Column, CAPCELL PAK (4.6 mm × 250 mm); Eluant, $CHCl_3$: CH_3OH=78 : 22; flow rate, 1 ml/min; Detection, absorbance at 225 nm.

Immunosuppresant Activity of MCH-22 and MCH-32

In the present studies, MCH-22 and MCH-32 were evaluated based on their immunosuppressive activities (Fig. 5). The results showed that MCH-22 exhibited 71.7% suppression of B cell activation, 95.5% of T cell activation and 80.8% of T-dependent IgM response at a final concentration of 1 μg/ml. MCH-32 also showed immunotoxic activity of about 70.1%, 96.9%, and 80.8% of B cell activation, T cell activation, and T-dependent antibody response, respectively. The induced proliferations of T and B cells by mitogens (LPS, PWM, ConA) were also dramatically suppressed up to about 95% of vehicle control by MCH-22 and MCH-32 at a final concentration of 1 μg/ml. The suppressive potency of MCH-22 and MCH-32 was comparable to that of cyclosporin A (1 μg/ml) which was used as a positive control. These results indicate that MCH-22 and MCH-32 are potent suppressive agents to the functions of mouse splenic lym-



Compound	R_1	R_2	R_3
MCH-22	O	CH_2CH_3	OH
MCH-32	H_2	CH_3	H

Fig. 4. Structures of MCH-22 and MCH-32.

Table 4. Physico-chemical properties of MCH-22, MCH-32.

		MCH-22	MCH-32
Nature		white powder	white powder
MP (°C)		114-116	109-111
$[\alpha]_D^{25}CH_3OH$		-38.17 (c 2.56)	-44.09 (c 0.88)
FAB-MS (m/z)		835 $[M+H]^+$	791 $[M+H]^+$
UV λ^{MeOH}max [log ε]		225(4.84), 232(4.81), 241(4.60)	225(5.06), 232(5.04), 241(4.83)
IR υ_{max} (KBr) cm^{-1}		3469, 2933, 1705, 1641, 1460, 1383, 991	3448, 2968, 1705, 1641, 1460, 1381, 987
MW		$C_{46}H_{74}O_{13}$	$C_{45}H_{74}O_{11}$
Solubility	soluble	CH_3OH, EtOAc, $CHCl_3$	CH_3OH, EtOAc, $CHCl_3$
	insoluble	H_2O, C_6H_{12}	H_2O, C_6H_{12}
TLC[a]		0.45	0.41
HPLC[b]		16.06	24.01

[a]solvent system ($CHCl_3$: CH_3OH=9 : 1). [b]column (CAPCELL PAK), solvent system ($CHCl_3$: CH_3OH=78 : 22), flow rate (1 ml/min).

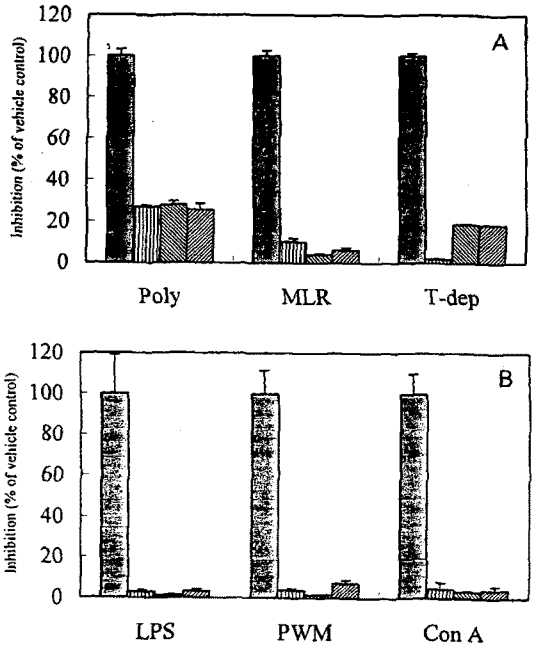


Fig. 5. Immunosuppressive activities of MCH-22 and MCH-32.

Lymphocytes were prepared from normal spleens. Compounds were added to culture medium at a final concentration of 1 μg/ml. Cyclosporine A (1 μg/ml) was used as positive control. A, The polyclonal B cell activation (Poly) with LPS, the primary activation of T cells by mixed lymphocyte response (MLR), and the primary T-dependent antibody response (T-dep) to SRBC were determined; B, The blastogenesis of T and B cells induced by LPS, PWM, and Con A were also determined. Results were calculated as percent inhibition to vehicle control. The data represent mean ± standard deviation of six determinations. ▒, control; ▓, Cyclosporin A; ▒, MCH-32; ▨, MCH-22.

phocytes.

Oligomycins have many kinds of biological activities including the inhibition of sodium, potassium ATPase (15) like *Escherichia coli* ATPase (14), mitochondrial oxidative phosphorylation on photosynthesis in protoplasts and leaves of barley (6), and blocking the lipolytic response of rat fat cells (2).

Recently the immunosuppressive effects of a homologue of oligomycin A, namely oligomycin F, were reported and also identified as a potent suppressive agent for human B-cell activation (7). These oligomycins exhibit a 50% suppression of the MLR at 0.1 μg/ml and 10^6 fold lower concentration is sufficient to suppress the IgG production of human spleen cells to 50%.

In this study, we found that MCH-22 and MCH-32 suppressed B and T cell proliferation induced by LPS and PWM or ConA. They also suppressed B cell activation, T cell activation, and the T-dependent IgM response at a final concentration of 1 μg/ml.

Many researchers have discovered and developed new immunosuppressive drugs. Among them, the useful human immunosuppressant are limited to cyclosporin A and FK506, an inhibitor of cytokine production. Therefore, it is necessary for us to develop more specific and less toxic immunosuppressant drugs and one possible route is through understanding lymphocyte cell biology and developing an awareness of immunological phenomena.

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