FUNGAL METABOLITES. Part 11†. A POTENT IMMUNOSUPPRESSIVE ACTIVITY FOUND IN *Isaria sinclairii* METABOLITE

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A potent immunosuppressive activity was found in the culture broth of the fungus *Isaria sinclairii* (ATCC 24400). The metabolite, ISP-I ((2*S*,3*R*,4*R*)-(*E*)-2-amino-3,4-dihydroxy-2-hydroxymethyl-14-oxoeicos-6-enoic acid, myriocin = thermozymocidin) suppressed the proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reaction, but had no effect on the growth of human tumor cell lines. It also suppressed the appearance of plaque-forming cells in response to sheep red blood cells and the generation of allo-reactive cytotoxic T lymphocytes in mice after intraperitoneal or oral administration. The metabolite was 10- to 100-fold more potent than cyclosporin A as an immunosuppressive agent of the immune response *in vitro* and *in vivo*, and appears to be a candidate for clinical application as a powerful immunosuppressant.

The success of human organ transplantations is dependent upon the use of potent immunosuppressive drugs. Cyclosporin A (CsA)[2], a fungal cyclic peptide, is one such drug with powerful immunosuppressive properties in experimental and clinical organ transplantations. Recently, FK-506[3,4], a novel macrolide from *Streptomyces tsukubaensis*, was found to be 10- to 100-fold more potent than CsA as an immunosuppressant and is currently under clinical trial. These two compounds appear to have very similar immunosuppressive properties, but higher doses of both compounds induce renal dysfunction and other side effects. Therefore, improved drugs for the prevention of graft rejection with fewer side effects are needed.

CsA was initially isolated from *Trichoderma polysporum*[5,6] (the producing fungus has since been redesignated as *Tolypocladium inflatum*). On the other hand, FUJITA *et al.*[7,8] isolated a cyclic depsipeptide named trichospolide as an antibiotic from a strain of *T. polysporum* (Link ex Pers) Rifi. A strain of *Isaria* also produces a similar cyclic depsipeptide[9,10]. We therefore thought it worthwhile to screen these fungi for the production of novel immunosuppressive agents.

As a result of the screening, we have found strong activity in the culture broth of *Isaria sinclairii* (ATCC 24400), which is the imperfect stage of *Cordyceps sinclairii*. *Cordyceps* is a genus of fungus which belongs to Hypocreaceae, in the family of Ascomycetes, and is parasitic on insects such as *Lepidoptera adonata* and so on. *Cordyceps sinensis* Sacc. (Chinese name: Dong Chong Xia Cao) has been used in a

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Chinese traditional medicine as a drug for eternal youth[11]. In this paper we describe the isolation, structure elucidation and some chemical and biological properties of an immunosuppressive metabolite (Fig. 1), produced by *I. sinclairii*.

Materials and Methods

Fermentation of *Isaria* and *Trichoderma* Species for Screening

The microorganisms and the media used are listed in Tables 1 and 2. *Trichoderma* species were provided by the Tottori Mycological Institute. Erlenmeyer flasks (500-ml) containing 100 ml of the medium was sterilized at 121°C for 20 minutes. A portion of slant culture of each strain was inoculated into the medium and cultured at 30°C for 10 days on a reciprocal shaker (145 strokes/minute).

Fractionation of the Culture Broths

Each culture broth was filtered and the mycelial cake was extracted with MeOH (50 ml × 2, Fr. 1). The filtrate was passed through an Amberlite XAD-2 column (2 i.d. × 15 cm) eluting with water 200 ml (Fr. 2), MeOH 200 ml (Fr. 3) and acetone 200 ml (Fr. 4). Each fraction was concentrated to dryness *in vacuo*.

Fermentation of *Isaria sinclairii* (ATCC 24400)

The seed medium and a production medium were of the same composition as the screening medium (Table 2). An Erlenmeyer flask (500-ml) containing 100 ml of the seed medium was sterilized at 121 °C for 20 minutes. A portion of slant culture of *I. sinclairii* (ATCC 24400) was inoculated into the medium and cultured at 30°C for 7 days on a reciprocal shaker (145 strokes/minute). The resultant culture was transferred to the same medium (5 liters) in a 10-liter jar fermentor which had been sterilized at 121°C for 30 minutes. The mixture was cultured at 30°C for 10 days with agitation at 150 rpm and with an air flow of 5 liters per minute.

Fig. 1. Structure of ISP-I (myriocin = thermozymocidin).

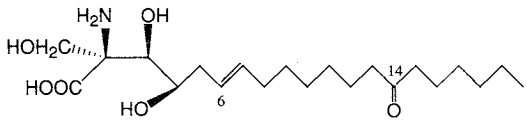


Table 1. Microorganisms screened and activity of culture filtrate fractions.

Microorganism	Activity			
	Fr. 1	Fr. 2	Fr. 3	Fr. 4
Isaria atypicola IFO 31160	−	−	−	−
I. japonica IFO 31161	−	−	−	−
I. felina ATCC 26680	−	−	−	−
I. sinclairii ATCC 24400	−	−	+++	−
I. sulfurea ATCC 22280	−	−	−	−
Trichoderma polysporum				
TMI 60146	++	−	++	+
TMI 60289	±	−	±	−
TMI 60471	−	−	++	±
TMI 60868	±	−	++	−
TMI 60901	±	−	++	+
TMI 60902	+	±	−	+

Immunosuppressive activity was evaluated by mouse allogeneic mixed lymphocyte reaction as described in Materials and Methods.

−; ≦50% inhibition at 10 μg/ml. +; >50% inhibition at 10 μg/ml, ≦50% inhibition at 1 μg/ml. ++; >50% inhibition at 10 μg/ml, 50~80% inhibition at 1 μg/ml. +++; >80% inhibition at 10 μg/ml, 80% inhibition at 1 μg/ml.

Fermentation of *Myriococcum albomyces* (ATCC 16425) and Isolation of Myriocin

A seed medium (100 ml) containing glucose 3%, yeast extract (Difco) 0.5%, K_2HPO_4 0.03% and $MgSO_4 \cdot 7H_2O$ 0.05% at pH 5.5 was poured into a 500-ml Erlenmeyer flask and sterilized at 121°C for 20 minutes. A portion of slant culture of *M.*

Table 2. Medium composition for the fermentation of *Isaria* and *Trichoderma* species.

Component	Content (%)	
	Isaria	*Trichoderma*
Glucose	3	2.5
Bacto peptone	0.5	
Yeast extract	0.3	
KH_2PO_4	0.03	0.2
K_2HPO_4	0.03	
$MgSO_4 \cdot 7H_2O$	0.03	0.1
$FeCl_2 \cdot 6H_2O$		0.01
Ammonium tartrate		0.4
pH	5.5	3.5

albomyces (ATCC 16425) was inoculated into the medium and cultured at 40°C for 4 days on a reciprocal shaker (145 strokes/minute). The resultant culture was transferred to the same medium (5 liters) in a 10-liter jar fermentor which had been sterilized at 121°C for 30 minutes. The mixture was cultured at 40°C for 10 days with an agitation at 150 rpm and with an air flow of 5 liters per minute.

The culture broth was filtered and the mycelial cake was extracted with MeOH (500 ml × 3). The MeOH extract from the mycelium and the filtrate (4.5 liters) were combined and applied to a column of Amberlite XAD-2 (4.5 i.d. × 75 cm). The column was washed with water (4 liters) and 50% aqueous MeOH (2 liters), then eluted with 80% aqueous MeOH (4 liters). The eluate was evaporated under reduced pressure to give crude myriocin. Recrystallization from MeOH gave pure myriocin (645 mg).

Fermentation of *Mycelia sterilia* (ATCC 20349) and Isolation of Thermozymocidin

M. sterilia (ATCC 20349) was cultured in the same manner as *M. albomyces*. Thermozymocidin (800 mg) was obtained from the culture broth in the same way as described above for myriocin.

Drugs

CsA (Sandimmun) was purchased from Sandoz Co., Ltd.

Animals

Male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from Japan Charles River Laboratories, Kanagawa, Japan. All animals were used at 6 to 10 weeks of age.

Cell Culture Medium

RPMI 1640 (Nissui Seiyaku, Tokyo, Japan) was supplemented with 2 mM L-glutamine, kanamycin at 60 μg/ml, penicillin at 100 U/ml, 10 mM HEPES and 0.1% $NaHCO_3$. Fetal calf serum (FCS, Hyclone, Laboratories Inc., Logan, Utah) was heat-inactivated at 56°C for 30 minutes and added to the medium as indicated.

Mouse Allogeneic Mixed Lymphocyte Reaction (MLR)[12]

Mouse allogeneic MLR was carried out by culturing BALB/c mouse spleen cells (5×10^5 cells, responder, H-2^d) and an equal number of C57BL/6 mouse spleen cells treated with mitomycin C at 40 μg/ml for 30 minutes at 37°C (stimulator, H-2^b) in 200 μl of RPMI 1640 medium containing 5×10^{-5} M 2-mercaptoethanol, 10% fetal calf serum and a variable amount of test substance. The cells were placed in a 96-well flat-bottomed microtest plate (No. 3072 Falcon, Becton Dickinson, Lincoln Park, NJ) and cultured for 4 days at 37°C in an atmosphere of 5% CO_2. After 92 hours, the cells in each well were pulsed for 4 hours at 37°C with ^{3}H-thymidine at 18.5 KBq/well and harvested by using a multiple cell harvester (Skatron A. S., Lierbyen, Norway). The radioactivity incorporated into the cells was measured with a liquid scintillation counter. Results were expressed as IC_{50} values.

Anti-SRBC Plaque-forming Cell Assay[13]

BALB/c mice were immunized intravenously with 10^7 sheep red blood cells (SRBC) and a test substance was administered intraperitoneally for 4 consecutive days thereafter. Four days after immunization, a single cell suspension of spleen cells was prepared from each mouse and used for the assay of direct plaque-forming cells (PFC) in the presence of SRBC and guinea-pig complement.

Generation of Allo-reactive Cytotoxic T Lymphocytes (CTL)[14] in Mice

BALB/c mice (H-2^d) were immunized intraperitoneally with 10^7 cells of EL4 mouse leukemia cell line derived from C57BL/6 mice (H-2^b). The test substance was administered intraperitoneally or orally for 5 consecutive days thereafter. Ten days after the immunization, a single cell suspension of spleen cells from each mouse was prepared and used as effector cells for CTL assay. *In vitro*-grown EL4 cells were prelabeled with $Na_2{}^{51}CrO_4$ (370 KBq) for 1 hour at 37°C and used as target cells. The radiolabeled EL4 cells (5×10^3) were incubated with various numbers of effector cells in a 96-well flat-bottomed microtest plate for 4 hours at 37°C. The radioactivity released from lysed target cells was determined as lytic unit (LU)[15] per spleen. One LU is defined as the number of effector cells required to cause 25% lysis of 5×10^3 target cells.

Effect on Growth of Human Leukemia Cell Lines

Human leukemia cell lines K562, MOLT4, U937 and HL60 were cultured at 10^4 cells in 200 μl of RPMI 1640 medium containing 10% fetal calf serum and a variable amount of test substance in 96-well flat-bottomed microtest plates (No. 3072 Falcon, Becton Dickinson, Lincoln Park, NJ) at 37°C in an atmosphere of 5% CO_2. After 68 hours, 20 μl of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide][16] solution was added to each well and the absorbance at 570 nm was measured with an automatic ELISA reader (Inter. Med. NJ-2000). Results were expressed as IC_{50} values.

Results

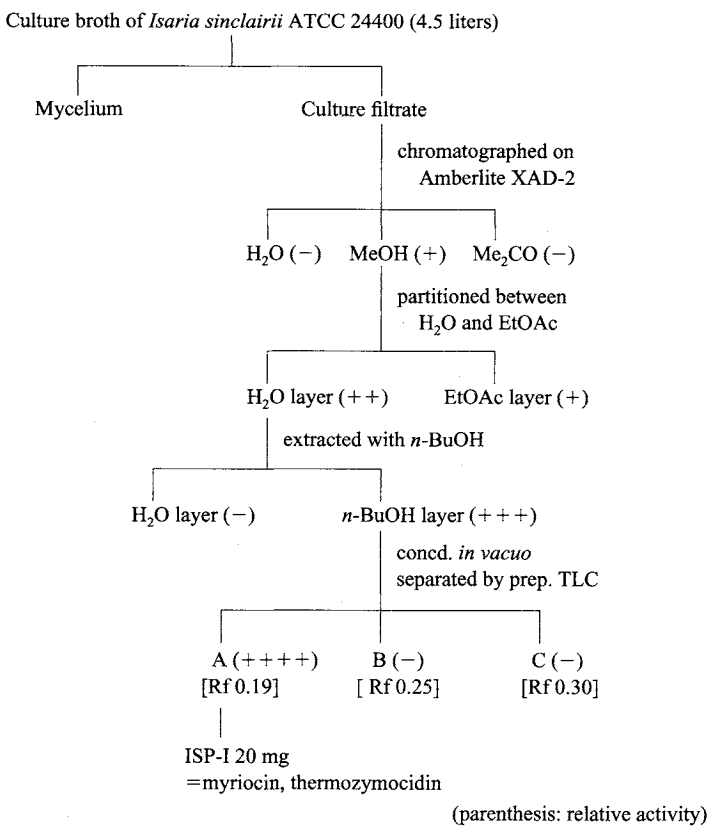
Screening for Immunosuppressive Activity

Table 1 showed the results of screening of five strains of *Isaria* and six strains of *Trichoderma*. Strong immunosuppressive activity was found in Fr. 3 of *I. sinclairii* and *T. polysporum*. As Fr. 3 of *I. sinclairii* showed the strongest activity, we purified the active substance from the culture broth of this fungus.

Isolation of Immunosuppressant

An immunosuppressant was isolated from the culture broth of *I. sinclairii* (ATCC 24400) through bioassay-directed fractionation as shown in Fig. 2. The activity was followed by means of the mouse allogeneic mixed lymphocyte reaction (MLR). The filtered broth (4.5 liters) was applied to an Amberlite XAD-2 column (4.5 i.d. × 75 cm) eluting with water (4 liters), MeOH (4 liters) and acetone (4 liters). The activity was found in the MeOH fraction, which was concentrated by evaporation and partitioned between

Fig. 2. Isolation of the immunosuppressively active principle, ISP-I.



water and EtOAc. Strong activity was found in the aqueous layer, which was extracted with *n*-BuOH. The organic layer, showing potent activity, was evaporated and the residue was purified by thin layer chromatography [Kieselgel 60F, Merck, 0.5 mm, $CHCl_3$ - MeOH (6 : 4, v/v)] followed by recrystallization from MeOH to give an immunosuppressive principle (20 mg), ISP-I.

Structure Elucidation of ISP-I

Physico-chemical properties of purified ISP-I were as follows. MP 169~171°C, $[\alpha]_D$ +4.8° (*c* 0.286, MeOH), FAB-MS: *m/z* 402 $(M+H)^+$, HREI-MS: Calcd $C_{21}H_{37}NO_5$: 383.2672 Found 383.2666 $(M-H_2O)^+$, IR ν_{max} (KBr) cm^{-1}: 3400, 3250, 3125, 1710, 1670, 1660, 1605 and 970. ^1H NMR (400 MHz, CD_3OD) δ: 5.52 (1H, dtt-like, J=15.2 and 6.5 Hz, 7-H), 5.38 (1H, dtt-like, J=15.2 and 7.0 Hz, 6-H), 3.99 (1H, d, J=11.0 Hz, 21-H), 3.86 (1H, d, J=11.0 Hz, 21-H), 3.83 (1H, td, J=7.0 and 1.0 Hz, 4-H), 3.77 (1H, d, J=1.0 Hz, 3-H), 2.43 (4H, t, J=7.0 Hz, 13- and 15-H_2), 2.26 (2H, t, J=7.0 Hz, 5-H_2), 2.00 (2H, br q, J=6.5 Hz, 8-H_2), 1.53 (4H, quintet, J=7.0 Hz, 12- and 16-H_2), 1.41~1.25 (12H, m, 9-~11- and 17-~19-H_2), 0.90 (3H, deformed t, J=7.0 Hz, 20-H_3). These data coincide with those of myriocin[17,18] (thermozymocidin)[19], which is an antifungal agent isolated from *M. albomyces* (*M. sterilia*). The absolute structure of myriocin (thermozymocidin) was determined by X-ray analysis[20] and asymmetric synthesis[21,22]. Table 3 shows the melting points and optical rotations of these three compounds. These data and the NMR spectral data indicate that the absolute structure of ISP-I is identical with that of myriocin.

Biological Properties of Myriocin

Effect of Myriocin on Mouse Allogeneic MLR

The effect of myriocin on mouse allogeneic MLR was examined in comparison with that of CsA. Table 4 shows the IC_{50} values of myriocin and CsA on mouse allogeneic MLR. These results indicate that myriocin is 5- to 10-fold more potent than CsA in its inhibition of lymphocyte proliferation in mouse allogeneic MLR.

Effect of Myriocin on Growth of Human Leukemia Cell Lines *In Vitro*

The effect of myriocin on growth of human leukemia cell lines was examined. As shown in Table 5, 10 μg/ml of myriocin had no growth-inhibitory activity, whereas the IC_{50} values of CsA were below

Table 3. Comparison of MP and $[\alpha]_D$ data of ISP-I, myriocin and thermozymocidin.

Compound	MP (°C)	$[\alpha]_D$	Microorganism
ISP-I	169~171	+4.8° (MeOH)	*Isaria sinclairii*
Myriocin	164~168	+5.2° (MeOH)	*Myriococcum albomyces*
Thermozymocidin	166~168	+4.7° (DMSO)	*Mycelia sterilia*

Table 4. Effect of myriocin and cyclosporin A (CsA) on mouse allogeneic mixed lymphocyte reaction.

Compound	IC_{50} (μg/ml)	
	Exp. 1	Exp. 2
Myriocin	0.0040	0.0071
CsA	0.028	0.032

Table 5. Effect of myriocin and cyclosporin A (CsA) on growth of human leukemia cell lines *in vitro*.

Compound	IC_{50} (μg/ml)			
	Cell line			
	MOLT4	U937	K562	HL60
Myriocin	>10	>10	>10	>10
CsA	6.3	3.5	>10	4.0

10 μg/ml for 3 leukemia cell lines. The anti-proliferative activity of myriocin, like that of CsA, is highly specific for antigen-stimulated lymphocytes, and is not due to nonspecific growth-inhibitory activity.

Effect of Myriocin on T Cell-dependent Antibody Production *In Vivo*

The influence of myriocin on T cell-dependent antibody production was examined by using SRBC as an antigen. After the immunization of 10^7 SRBC into BALB/c mice, myriocin and CsA were administered intraperitoneally from day 0 to 3. On day 4, spleen cells of each mouse were recovered and PFCs were determined. As shown in Table 6, intraperitoneal administration of myriocin led to a dose-dependent decrease of the PFC response and myriocin showed 10-fold greater potency than CsA for inhibiting T cell-dependent antibody production.

Table 6. Effect of myriocin and cyclosporin A (CsA) on T cell-dependent antibody production *in vivo*.

Compound	Dose (mg/kg)	PFC ($\times 10^4$/spleen)	Inhibition (%)
Control		1.4±0.8	
Myriocin	0.1	1.3±0.8	7
	0.3	0.9±0.3	36
	1	0.3±0.1	79
	3	0.1±0.0	93
CsA	3	1.5±0.8	0
	10	0.9±0.5	36
	30	0.1±0.0	93

Mice were immunized intravenously with 10^7 sheep red blood cells on day 0. Test substances were administered intraperitoneally on day 0~3. PFC were assayed on day 4. Results are shown as the mean±SD of 3 or 4 animals.

Effect of Myriocin on Generation of Allo-reactive Cytotoxic T Lymphocytes (CTL) *In Vivo*

The effect of myriocin on allo-reactive CTL induction was examined. BALB/c mice (H-2^d) were immunized with 10^7 EL4 leukemia cells derived from C57BL/6 mice (H-2^b). Myriocin and CsA were administered from day 0 to 4. On day 10, spleen cells from each mouse were recovered and allo-reactive (H-2^b restricted) CTL activities in the spleen cells were determined by using ^{51}Cr-labeled EL4

Table 7. Effect of myriocin and cyclosporin A (CsA) on generation of allo-reactive cytotoxic T lymphocytes (CTL) *in vivo*.

Compound	Dose (mg/kg)	Route	CTL activity	
			LU/Spleen	Inhibition (%)
Exp. 1				
Vehicle			4,213±1,674	
Myriocin	0.01	ip	5,632±2,829	0
	0.03	ip	2,516± 869*	40.3
	0.1	ip	739± 437**	82.5
	0.3	ip	340± 237**	91.9
	1.0	ip	69± 25**	98.4
Exp. 2				
Vehicle			1,956± 400	
Myriocin	0.01	po	2,201± 665	0
	0.03	po	2,035± 481	0
	0.1	po	1,353± 340*	30.8
	0.3	po	961± 332**	50.9
	1.0	po	772± 225**	60.5
	3.0	po	568± 182**	71.0
CsA	10	po	1,308± 740*	33.1
	30	po	792± 136**	59.5

BALB/c mice were immunized intraperitoneally with 10^7 EL-4 cells on day 0. Test substances were administered on day 0 to 4. CTL activity was determined by ^{51}Cr release assay on day 10. One lytic unit (LU) was defined as the number of effector cells required to cause 25% lysis of 5×10^3 target cells. Results are shown as mean±SD of 5 mice.

* $P<0.05$, ** $P<0.01$ (STUDENT's *t*-test).

target cells (H-2^b).

As shown in Table 7, myriocin suppressed allo-reactive CTL generation in a dose-dependent manner. The minimum effective concentrations of myriocin and CsA were estimated to be 0.1 mg/kg and 10 mg/kg, respectively. Thus, myriocin is 100-fold more potent than CsA in its inhibition of allo-reactive CTL generation in alloantigen-immunized mice.

Discussion

A screening study of fungi of *Isaria* and *Trichoderma* species for immunosuppressive activity on mouse allogeneic MLR revealed that culture broth of *Isaria sinclairii* (ATCC 24400), the imperfect stage of *Cordyceps sinclairii*, contained potent activity. Fractionation guided by bioassay led to the isolation of the active principle, ISP-I, which was identified as (2*S*,3*R*,4*R*)-(*E*)-2-amino-3,4-dihydroxy-2-hydroxymethyl-14-oxoeicos-6-enoic acid. This structure is identical with myriocin[17,18] or thermozymocidin[19], previously isolated as an antifungal agent. Nevertheless, we have found for the first time that myriocin is a potent immunosuppressant, being 10-fold more potent than CsA in terms of its inhibition of lymphocyte proliferation in mouse allogeneic MLR. The anti-proliferative activity of myriocin is specific for antigen-stimulated lymphocytes, and is not due to nonspecific growth-inhibitory activity, since myriocin had no effect on the growth of several human leukemia cell lines *in vitro*. Myriocin also suppressed allo-reactive CTL generation and T cell-dependent antibody production with a potency 10- to 100-fold greater than that of CsA *in vivo*.

In other experiments, we also found that myriocin inhibited interleukin 2 (IL-2)-induced T cell proliferation, but not IL-2 production from alloantigen-stimulated T cells (unpublished data). Since CsA and FK-506 were reported[2,23] to inhibit IL-2 production from antigen- or mitogen-stimulated helper T cells, the mechanism of immunosuppressive action of myriocin appears to be different from that of CsA and FK-506. Myriocin is an unusual lipophilic amino acid, whose biosynthetic pathway[24] may be similar to that of sphingosine. It is structurally quite different from the cyclic polypeptide CsA or the macrolide FK-506, has a smaller molecular weight.

The studies described in this paper suggest that myriocin is a powerful immunosuppressant with a novel mechanism. Thus, myriocin or its derivatives may have clinical potential for organ transplantation and for the treatment of autoimmune diseases. The structure-activity relationships of myriocin derivatives are described in the succeeding paper.

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