VOL. 54 NO. 9, SEPT. 2001 THE JOURNAL OF ANTIBIOTICS pp. 697 — 702

Cytokine Production Inhibitors Produced by a Fungus, *Oidiodendron griseum*

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(Received for publication May 9, 2001)

A series of diterpenes were isolated from the fermentation broth of a fungus, *Oidiodendron griseum* CL37215. The diterpenes were identified as LL-Z1271α, LL-Z1271γ, CJ-14,445, PR 1388, CJ-14,604 and a new diterpene, CJ-14,515. They inhibited both lipopolysaccharide-induced interleukin-1β and tumor necrosis factor-α production in human whole blood with IC_{50}s of the range from 0.049 to 100 μM.

Cytokines are produced by a variety of different cell types and act on nearly every tissue and organ[1]. Among identified cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) share many properties of immunoregulation and other physiological conditions such as inflammation. Antagonism of IL-1β and TNF-α with the use of natural IL-1 antagonists, soluble receptors, neutralizing antibodies and cytokine production inhibitors has been proven to be beneficial to treatment of inflammatory diseases in animal models and human[1~9]. However, their protein-based modulators are unlikely to feasible because of development of immunogenicity and lack of oral availability. Currently available low molecular inhibitors have adverse side effects and poor bioavailability. Consequently, there is still a need for safe and oral active compounds having excellent antagonism of IL-1β and TNF-α such as their receptor antagonists, production inhibitors and signal transduction inhibitors.

In a screening program designed to discover new inhibitors of cytokine production, a fungus *Oidiodendron griseum* CL37215 was found to produce a series of diterpenes showing inhibitory activities for IL-1β and TNF-α production. In this paper, we describe the taxonomy of the producing microorganism, and the fermentation, isolation, structure elucidation and biological activities of the diterpenes.

Results

Taxonomy of the Producing Microorganism

The cultural characteristics of the strain CL37215 are shown in Table 1. Sporulation was good on all of the media used. The morphological properties were observed on malt extract and potato dextrose agar plates 14 days after inoculation. On malt extract agar, the vegetative mycelium was olive-gray to olive-brown, septate, branched, and measured 1.5 to 4.0 μm in diameter. The conidiophores were macronematous, semi-macronematous, micronematous or mononematous, septate, brown to olivaceous brown, smooth, monopodially or verticillately branched, measuring 40~230×2.0~3.5 μm. They may branch up to four levels, with each level two to three subbranches, primary branches being 20~40×2.0~3.0 μm, secondary branches being 15~30×2.0~2.5 μm, and tertiary branches being 10~12×2.0 μm. Conidia were olivaceous to olivaceous green, smooth

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or slightly roughened, one-celled, oval, elliptical, barrel-shaped to elongated, measuring 3.0~7.0×2.0~4.0 μm. They were produced basipetally, maturing from top to bottom, arthrosporic in conidiogenesis. Chlamydospores were not produced. On potato dextrose agar, the morphological properties were similar to those on malt extract agar except that the conidiophores were generally shorter, and the conidia were shorter and narrower, measuring 3.0~6.0×1.6~3.0 μm. The strain could grow well at 20 and 28°C but not at 37, 45 and 50°C.

The strain CL37215 was characterized by the slow growth; the olive-gray to dark olive-gray colonies; the olivaceous black to black colony reverse; and the smooth to finely roughened, one-celled, olivaceous conidia which were arthrosporic in nature. It grew well at 20 and 28°C but not between 37 and 50°C. These features fitted into the description of *Oidiodendron griseum* Robak in the general characteristics of morphologies and the cultural properties[10]. Minor differences were noted. Some conidiophores were taller and wider, and some conidia were barrel-shaped in addition to being oval to elliptical and were slightly larger than those of the strains of *O. griseum*. As most species of *Oidiodendron* exhibited a wide range in conidiophore dimensions and conidial shapes, these differences were considered as minor variations. Thus, the strain CL37215 was considered as a new strain of *O. griseum* Robak. It was deposited as FERM BP-5778 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Tsukuba, Japan).

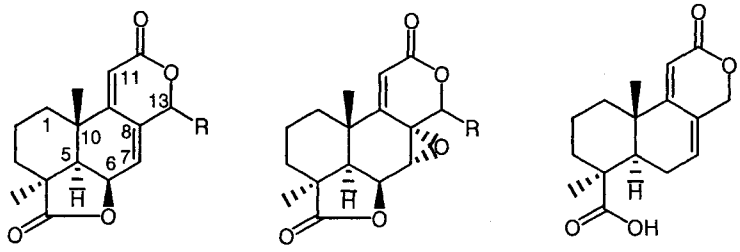
Isolation

The fermentation broth (12 liters) treated with the same volume of EtOH was filtered with the aid of celite. The filtrate was concentrated to an aqueous solution (1 liter), and extracted 3 times with the equal volume of EtOAc. The combined organic layers (3 liters) were evaporated under reduced pressure to yield an oily residue (2.8 g). The residue was dissolved in small volume of MeCN and centrifuged at 2,000 rpm. The precipitate and supernatant portions were individually applied to preparative HPLC on an ODS column (YMC-pack ODS AM-343, 20×250 mm,

Table 1. Cultural characteristics of *Oidiodendron griseum* CL37215.

Medium	Growth	Texture	Colony Surface	Colony Reverse	Soluble Pigment
Malt extract	Moderate	Velvety, thin, smooth	Olive-gray to deep olive-gray (LI)	Blackish mouse gray, olivaceous black (3) (LI) to black	None
Cornmeal	Poor to moderate	Velvety, thin, smooth	Olive-gray to deep olive-gray (LI)	Olivaceous black (1) (XLVI) to black	None
Czapek-Dox	Moderate	Velvety to slight floccose, thin, smooth	Andover green (XLVII), light olive-gray to olive-gray (LI)	Olivaceous black (3) (LI) to black	None
Potato dextrose	Good	Velvety, moderately raised, radiately wrinkled	Andover green (XLVII), olive-gray to deep olive-gray (LI) with a pale olive-gray (LI) edge	Iron gray (LI) to black	None
Glucose	Moderate	Velvety to slightly floccose, thin, smooth	Light olive-gray, olive-gray to deep olive-gray (LI)	Iron gray (LI) to black	None
Oatmeal	Poor to moderate	Velvety, thin, smooth	Olive-gray, deep olive-gray to dark olive-gray (LI)	Same as surface	None
Phytone yeast extract	Excellent	Velvety, highly raised, radiately wrinkled	Andover green (XLVII), light olive-gray, olive-gray to deep olive-gray (LI)	Dark mouse gray (LI) to black	Capucine yellow (III)
V-8 Juice	Good	Velvety to funiculose, thin to slightly raised, smooth	Light olive-gray, olive-gray to deep olive-gray (LI)	Dark olive-gray to olivaceous black (3) (LI)	None

Fig. 1. Structures of IL-1β and TNF-α production inhibitors produced by a fungus, *Oidiodendron griseum* CL37215.



	R		R	
LL-Z1271α	····OMe	PR 1388	····OMe	CJ-14,604
LL-Z1271γ	····OH	CJ-14,515	∿OH	
CJ-14,445	····H			

YMC Co. Ltd., Kyoto, Japan). Three diterpenes, LL-Z1271α (0.8 mg), CJ-14,445 (2.4 mg) and PR 1388 (101.8 mg), were isolated from the precipitate portion by eluting with MeCN - 0.1% TFA in H_2O (40 : 60) at a flow rate of 8 ml/minute. Other three diterpenes, LL-Z1271γ (20.8 mg), CJ-14,515 (17.6 mg) and CJ-14,604 (13.7 mg), were from the supernatant one with MeOH - H_2O (40 : 60) at a flow rate of 10 ml/minute.

Structural Elucidation

The structure of CJ-14,515 was speculated by the comparison of its spectral properties with those of PR 1388. The UV spectrum showed maximum absorption at 225 nm, suggesting the presence of α,β-unsaturated lactone. The IR absorption bands at 1,770 and 1,694 cm^{-1} implied the presence of γ- and δ-lactone, respectively. The HRFAB-MS gave a parent ion peak at *m/z* 305.104 [$(M-H)^-$; calcd. for $C_{16}H_{17}O_6$, 305.099], indicating the loss of CH_2. The ^1H and ^{13}C NMR spectra showed that its structure was similar to that of PR 1388[11], except for the absence of CH at C-13 and CH_3 at C-17. On the hypothesis that the proton and carbon signals of hemiacetal methine were not observed in the NMR measurement, we proposed the hemiacetal structure of CJ-14,515 as shown in Fig. 1. This structure was proved by the hydrolysis of PR 1388. PR 1388 was hydrolyzed in a 3 : 1 : 1 mixture of AcOH, H_2O and THF at 70 °C and the isolated compound was compared with CJ-14,515. Both compounds gave identical ^1H NMR spectra. Thus, the structure of CJ-14,515 was determined as shown in Fig. 1, although the configuration of the hydroxyl group remains to be elucidated. The physico-chemical properties of CJ-14,515 were previously disclosed in our patent literature[12].

Biological Properties

As shown in Table 2, the diterpenes inhibited both IL-1β and TNF-α production with IC_{50}s of the range from 0.049 to 100 μM. They showed weaker inhibitory activity against leucine uptake than IL-1β and TNF-α production inhibition.

Discussion

We isolated 6 diterpenes, LL-Z1271α, LL-Z1271γ, CJ-14,445, PR 1388, CJ-14,604 and CJ-14,515 (a new analog) from the fermentation broth of a fungus, *O. griseum* CL37215. LL-Z1271α and LL-Z1271γ were originally isolated from a fungus, *Acrostalagmus* sp. as a potent antifungal agent useful for ringworm infection in guinea pigs[13]. CJ-14,445 synthesized from LL-Z1271γ by sodium borohydride reduction was reported as a plant growth inhibitor[14]. PR 1388 was isolated from a strain of *O. truncatum*[11]. CJ-14,604 was reported as an intermediate

Table 2. IC_{50} values of the diterpenes for IL-1β production, TNF-α production and leucine uptake.

Compound	IC_{50} (μM)		
	IL-1β production	TNF-α production	Leucine uptake
LL-Z1271α	0.049	3.0	11
LL-Z1271γ	69	11	120
CJ-14,445	1.2	1.3	5.5
PR 1388	1.5	4.7	78
CJ-14,515	9.8	11	160
CJ-14,604	82	100	>360

Each value is an average of triplicates.

during the total synthesis of LL-Z1271α[15]. These diterpenes inhibited IL-1β and TNF-α production and were less effective against general protein synthesis in human whole blood. LL-Z1271α showed the most potent and selective inhibition against IL-1β production (IC_{50}=0.049 μM, 60-fold *vs.* TNF-α production and 220-fold *vs.* leucine uptake). Epoxidation of the double bond between C-7 and C-8 in LL-Z1271α reduced the inhibitory activity against IL-1β production (PR 1388). The selectivity of LL-Z1271α against IL-1β production was changed by substitution at C-13 position (*e.g.*, LL-Z1271γ and CJ-14,515). Cleavage of the γ-lactone ring resulted in a remarkable decrease of the activities (CJ-14,604). The preliminary structure-activity relationships (SAR) study of isolated diterpenes suggests the followings: 1) the methoxy group at C-13 and the double bond between C-7 and C-8 critically participate in the potent inhibitory activity against IL-1β production, 2) the C-13 position is important for the selectivity between IL-1β and TNF-α production, and 3) the γ-lactone moiety is essential for the activities. The understanding of the SAR on the diterpenes may provide useful information on designing a new type of IL-1β and/or TNF-α production inhibitors.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: UV, JASCO Ubest-30; IR, Shimadzu IR-470; NMR, JEOL JNM-GX270 updated with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; FAB-MS, JEOL JMS-700; Optical rotations, JASCO DIP-370 with a 5-cm cell.

Producing Microorganism

The producing microorganism CL37215 was isolated from a soil collected in Tsu, Mie Prefecture, Japan. The culture was single-block or smear inoculated from a block or a spore suspension of malt extract agar slant onto plates of identification media. The plates were incubated at 25°C for 14 days under complete darkness. Then, the observations were made for cultural characteristics and temperature studies. The colors were determined by comparisons with color chips from Color Standards and Color Nomenclature[16]. For the identification of the fungus, the following media were used: cornmeal agar[17], Czapek-sucrose agar[18], malt extract agar[18], glucose agar (glucose 50 g, monobasic potassium phosphate 1 g, magnesium sulfate 0.5 g, potassium nitrate 2 g, agar 20 g and distilled water 1 liter), oatmeal agar (oatmeal 30 g, agar 15 g and distilled water 1 liter), phytone yeast extract agar (BBL Microbiology Systems), potato dextrose agar (peeled potato 100 g, dextrose 10 g, agar 20 g and tap water 1 liter), and V-8 juice agar (ATCC medium 343[19]). The growth range of temperature was determined on malt extract agar.

Fermentation

The strain CL37215 was maintained on a potato dextrose agar slant (Difco Laboratories, Detroit, MI, USA). A vegetative cell suspension from the slant was used to inoculate into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flask was shaken at 26°C for 4 days on a rotary shaker (7-cm throw at 210 rpm). Five ml of aliquots were inoculated into four 500-ml flasks containing 150 ml of the seed medium, and shaken at 26°C for 4 days. The second seed cultures were inoculated into four 6-liter jar fermentors containing 3 liters of a production medium (glucose 3%, malt extract 1.5%, yeast extract 0.5%,

$MgSO_4 \cdot 7H_2O$ 0.05% and KH_2PO_4 0.1%, pH 6.0). The fermentation was carried out at 26°C for 7 days with agitation at 1,700 rpm and aeration at a rate of 3 liters/minute.

Acid Hydrolysis of PR 1388

A mixture of PR 1388 (50.0 mg, 6.41 mmol) and AcOH-H_2O-THF (3 : 1 : 1, 10.0 ml) was stirred at 70°C for 75 hours. The reaction mixture was then concentrated *in vacuo* to give white powder: ^1H NMR (CD_3OD) δ 6.03 (1H, s), 5.06 (dd, J=4.3, 1.0 Hz), 4.02 (1H, d, J=1.0 Hz), 2.16 (1H, m), 1.92 (1H, d, J=4.3 Hz), 1.74 (3H, m), 1.53 (2H, m), 1.27 (3H, s), 1.11 (3H, m); LRFAB-MS *m/z* 307 $[M+H]^+$.

TNF-α Production Assay

Heparinized human whole blood diluted 4-fold with RPMI 1640 medium was dispensed into 80 μl per each well in 96-well microplates. Then, 10 μl of each sample was added into each well. The microplates were incubated with 10 μg/ml of lipopolysaccharide (Sigma, St. Louis, MO, USA) for 4 hours at 37°C in a humidified atmosphere containing 5% CO_2. TNF-α content was assessed with the L929 cytotoxicity assay. L929 cells (2.5×10^4 cells) in 90 μl of E-MEM medium containing 1% fetal calf serum and 0.5 μg/ml of actinomycin D (Sigma, St. Louis, MO, USA) were placed per each well in 96-well microplates. An aliquot (10 μl) of the supernatant was added to each well and incubated for 18 hours at 37°C in a humidified atmosphere containing 5% CO_2. After the incubation, the plates were rinsed with PBS(−) and stained for 10 minutes with 0.4% crystal violet in MeOH. The plates were washed with distilled water and dried. Fifty μl of methanol was added to each well to dissolve the crystal violet, and the plates were read on a microplate reader (Model 3550, BIO-RAD Laboratories, Hercules, CA, USA) at 595 nm.

IL-1β Production Assay

IL-1β levels in the supernatants prepared by the same method as the TNF-α assay were analyzed by an ELISA method using an IL-1β assay kit (Japan Immunoresearch Laboratories Co., Ltd., Takasaki, Japan).

Leucine Uptake Assay

Ten ml of heparinized human whole blood was centrifuged at 1,400 rpm for 5 minutes and then plasma was removed. After the human whole blood obtained were dispensed into a 250-ml tube, 90 ml of 0.83% NH_4Cl was added to the tube and then the tube was kept for 30 minutes on ice. After the tube was centrifuged at 1,400 rpm for 5 minutes, the supernatant was decanted. The cells were suspended in 30 ml of 0.83% NH_4Cl and kept for 10 minutes on ice. Again, the tube was centrifuged at 1,400 rpm for 5 minutes and then the supernatant was decanted. The cells were washed with PBS(−) followed by centrifugation at 1,400 rpm for 5 minutes, and then the supernatant was decanted. To the cell pellet, the plasma and leucine-free MEM supplemented with penicillin-streptomycin and L-glutamine were added (the total volume 9 ml). After phytohemagglutinin (Sigma) was added to the cell suspension (the final concentration of 10 μg/ml), the suspension was dispensed into 90 μl per each well in 96-well microplates. Then, 10 μl of each sample was added into each well. The microplates were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2 prior to the addition of 3.7 kBq of [^{3}H]leucine (New England Nuclear, Boston, MA, USA) diluted with leucine-free MEM. After incubation for 4 hours at 37°C, the cells in each well were transferred to glass filter membranes (Wallac, Turku, Finland) using a micro cell harvester (Micro96 Harvester, SKATRON Instruments AS, Lier, Norway). The radioactivity was counted in a scintillation counter (1205 Betaplate, Wallac).

Acknowledgments

The authors are grateful to their colleagues, Dr. TAISUKE INAGAKI and Dr. ATSUSHI NAGAHISA for their helpful advice.

References

1) DINARELLO, C. A.: Inflammatory cytokines: interleukin-1 and tumor necrosis factor as effector molecules in autoimmune diseases. Curr. Opin. Immunol. 3: 941~948, 1991
2) GEIGER, T.; H. TOWBIN, A. COSENTI-VARGAS, O. ZINGEL, J. ARNOLD, C. RORDORF, M. GLATT & K. VOSVECK: Neutralization of interleukin-1β activity *in vivo* with a monoclonal antibody alleviates collagen-induced arthritis in DBA/1 mice and prevents the associated acute-phase response. Clin. Exp. Rheumatol. 11: 515~522, 1993
3) SCHORLEMMER, H. U.; E. J. KANZY, K. D. LANGNER & R. KURRLE: Immunomodulatory activity of recombinant IL-1 receptor (IL-1-R) on models of experimental rheumatoid arthritis. Agents Actions 39: C113~C116, 1993
4) TRACEY, K. J.; Y. FONG, D. G. HESSE, K. R. MANOGUE, A. T. LEE, G. C. KUO, S. F. LOWRY & A. CERAMI: Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 330: 662~664, 1987
5) SMITH, E. F. III; M. J. SLIVJAK, J. O. BARTUS & K. M. ESSER: SK&F 86002 inhibits tumor necrosis factor formation and improves survival in endotoxemic rats. J. Cardiovasc. Pharmacol. 18: 721~728, 1991
6) MURAKAMI, K.; F. KOBAYASHI, R. IKEGAWA, M. KOYAMA,

N. SHINTANI, T. YOSHIDA, N. NAKAMURA & T. KONDO: Metalloproteinase inhibitor prevents hepatic injury in endotoxemic mice. Eur. J. Pharmacol. 341: 105~110, 1998

7) KALDEN, J. R. & B. MANGER: Biologic agents in the treatment of inflammatory rheumatic diseases. Curr. Opin. Rheumatol. 10: 174~178, 1998

8) AREND, W. P.; M. MALYAK, C. J. GUTHRIDGE & C. GABAY: Interleukin-1 receptor antagonist: role in biology. Annu. Rev. Immunol. 16: 27~55, 1998

9) MORELAND, L. W.: Inhibitors of tumor necrosis factor for rheumatoid arthritis. J. Rheumatol. 26 suppl. 57: 7~15, 1999

10) BARRON, G. L.: New species and new records of *Oidiodendron*. Can. J. Bot. 40: 589~607, 1962

11) ANDERSEN, N. R.; P. R. RASMUSSE, C. P. FALSHAW & T. J. KING: The relative and absolute configuration of clerocidin and its cometabolites. Tetrahedron Lett. 25: 469~472, 1984

12) ICHIKAWA, K.; M. IKUNAKA, N. KOJIMA, H. NISHIDA & N. YOSHIKAWA (Pfizer Inc.): Terpenoid lactone compounds and their production process. EP 933373A1, Jan. 29, 1998

13) ELLESTAD, G. A.; R. H. EVANS, Jr. & M. P. KUNSTMANN: Structure of a C_{17} antifungal terpenoid from an unidentified *Acrostalagmus* species. J. Amer. Chem. Soc. 91: 2134~2136, 1969

14) SATO, M.; T.-I. RUO, T. HAYASHI, H. KAKISAWA, T. MIYAKI, H. YAMAMOTO & K. FUJISAWA: Structure of C_{16}-terpenes from *Acrostalagmus* NRRL-3481. Tetrahedron Lett. 25: 2183~2186, 1974

15) BARRERO, A. F.; J. F. SÁNCHEZ & J. ELMERABET: An efficient synthesis of the antifungal dilactone LL-Z1271α and of other biologically active compounds. Tetrahedron Lett. 36: 5251~5254, 1995

16) ROBERT, R.: Color Standards and Color Nomenclature, 1912

17) CARMICHAEL, J. W.: *Geotrichum candidum*. Mycologia 49: 820~830, 1957

18) RAPER, K. B. & D. I. FENNELL: The Genus *Aspergillus*, Baltimore, The Williams & Wilkins, pp. 36~38, 1965

19) ATCC Media Handbook, American Type Culture Collection, p. 17, 1984