VOL. 54 NO. 9, SEPT. 2001 THE JOURNAL OF ANTIBIOTICS pp. 703 — 709

Novel Cytokine Production Inhibitors Produced by a Basidiomycete, *Marasmiellus* sp.

KATSUOMI ICHIKAWA*, HIDEO HIRAI, MASARU ISHIGURO, TAKAHITO KAMBARA†, YOSHINAO KATO, YOON JEONG KIM, YASUHIRO KOJIMA, YASUE MATSUNAGA, HIROYUKI NISHIDA, YUKIO SHIOMI††, NOBUJI YOSHIKAWA and NAKAO KOJIMA†††

Exploratory Medicinal Sciences, PGRD, Nagoya Laboratories, Pfizer Pharmaceuticals Inc., 5-Gochi, Taketoyo-cho, Chita-gun, Aichi 470-2393, Japan

(Received for publication May 9, 2001)

New cytokine production inhibitors, CJ-14,877 (I) and CJ-14,897 (II), were isolated from the fermentation broth of a basidiomycete, *Marasmiellus* sp. CL21624. Their structures were determined to be methyl-(7*R*,8*S*)-5-(7,8-dihydroxypropyl)pyridine-2-carboxylate and methyl-(7*R*,8*S*)-5-(8-acetoxy-7-hydroxypropyl)pyridine-2-carboxylate, respectively, by spectroscopic analyses. These compounds showed inhibitory activities for lipopolysaccharide-induced production of interleukin-1β and tumor necrosis factor-α in human whole blood with IC_{50} values of the range from 0.059 to 2.6 μM.

Proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are secreted proteins produced by a variety of cell types (*e.g.*, monocytes and macrophages) in response to many inflammatory stimuli[1~3]. These cytokines are known to play a central role in inflammatory responses, because the administration of inhibitors and protein antagonists, such as the interleukin-1 receptor antagonist (IL-1Ra) and monoclonal antibodies to TNF-α, block various acute and chronic responses in animal models of inflammatory diseases[1,4~9]. Significant progress in developing IL-1β or TNF-α modulators has been achieved though the use of recombinantly derived proteins, such as IL-1Ra, a chimeric TNF monoclonal antibody and a recombinant human TNF receptor (p75)-Fc fusion protein[9,10]. However, these modulators, which are polypeptides, are needed to be administered intravenously and are easily metabolized in the bloodstream with a short half life. Thus, active research has been carried out to develop stable long-acting agents that are taken by oral administration or by parenteral injections rather than by intravenous infusion.

In a screening program designed to discover novel inhibitors of cytokine production, a basidiomycete, *Marasmiellus* sp. CL21624 was found to produce two novel methyl-5-substituted pyridine-2-carboxylates, CJ-14,877 (**I**) and CJ-14,897 (**II**) having inhibitory activities for IL-1β and TNF-α production. In this paper, we report the fermentation, isolation, structure elucidation and biological activities of these compounds. In addition, we describe the structure-activity relationship (SAR) study of the methyl-5-substituted pyridine-2-carboxylates.

Results

Isolation

The fermentation broth (4 liters) was filtered after the addition of 2 liters of EtOH and concentrated to an aqueous solution (1 liter). The solution was extracted 3 times with the same volume of *n*-BuOH. The combined extracts were evaporated to afford an oily residue. The residue (3.5 g) was applied to a Sephadex LH-20 column (40×500 mm,

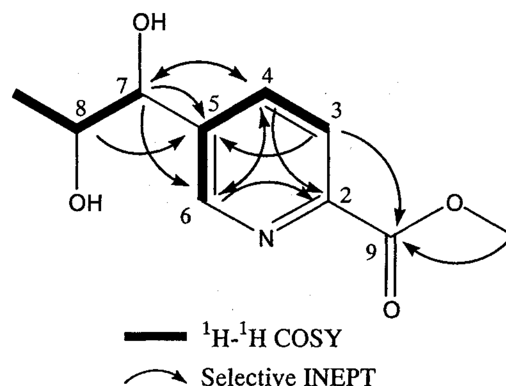
Present address: † The Queen's Veterinary School Hospital, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK.
†† Business Intelligence Department, Pfizer Pharmaceuticals Inc., 2-1-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 163-0461, Japan.
††† Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan.
* Corresponding author: katsuomi.ichikawa@japan.pfizer.com

Table 1. Physico-chemical properties of CJ-14,877 (**I**) and CJ-14,897 (**II**).

	CJ-14,877 (**I**)	CJ-14,897 (**II**)
Appearance	White amorphous powder	White amorphous powder
$[\alpha]_D$ (24°C)	+20.0° (*c* 0.13, MeOH)	+27.1° (*c* 0.17, MeOH)
Molecular formula	$C_{10}H_{13}NO_4$	$C_{12}H_{15}NO_5$
Molecular weight	211	253
HRFAB-MS (*m/z*)		
Found :	212.0940 $(M+H)^+$	254.1051 $(M+H)^+$
Calcd. :	212.0923 (for $C_{10}H_{14}NO_4$)	254.1028 (for $C_{12}H_{16}NO_5$)
UV λ_{max} (nm, MeOH)	230 (ε 9500), 270 (ε 5800)	230 (ε 8200), 270 (ε 4400)
IR ν_{max} (cm^{-1}, KBr)	3325, 1736, 1437, 1309, 1257	3465, 1732, 1435, 1370, 1309
Solubility		
Soluble:	MeOH, DMSO	MeOH, DMSO
Insoluble:	Hexane	Hexane

Amersham Pharmacia Biotech, Piscataway, NJ, USA) with MeOH. The active fractions were concentrated and applied to preparative HPLC on an ODS column (YMC-pack ODS AM-343, 20×250 mm, YMC Co., Ltd., Kyoto, Japan) with MeOH-H_2O (15:85 to 70:30 for 45 minutes) at a flow rate of 10 ml/minute. The detection was made by UV absorbance at 220 nm. The eluted peaks showing the activity were collected and concentrated to yield **I** (76.7 mg) and **II** (10.2 mg) as white powder.

Fig. 1. ^1H-^1H COSY and selective INEPT experiments of CJ-14,877 (**I**).



Physico-chemical Properties

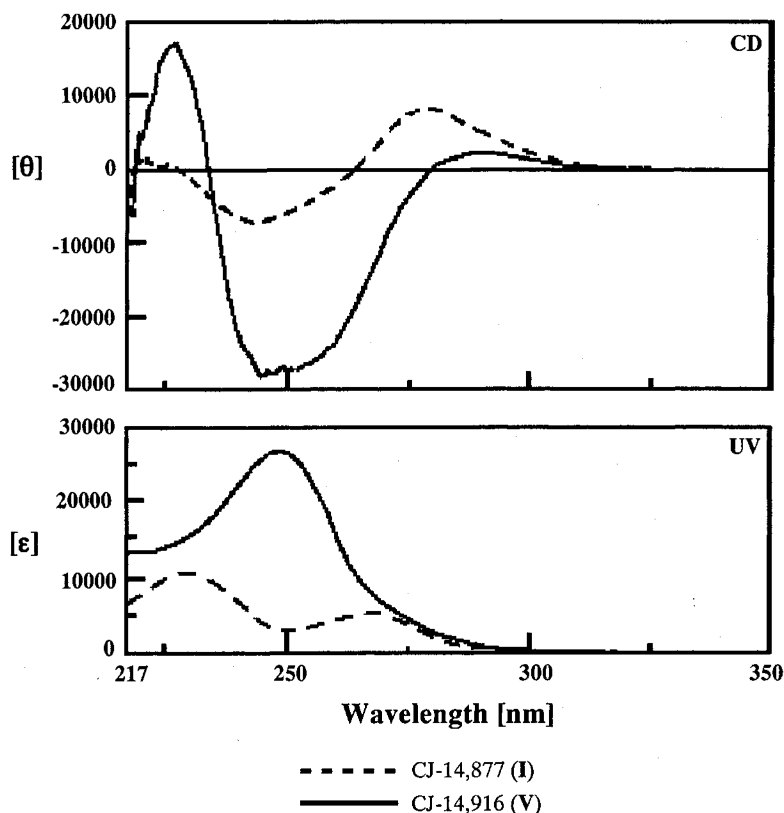
The physico-chemical properties of **I** and **II** are summarized in Table 1. They were obtained as amorphous white powder and were soluble in MeOH and DMSO, but insoluble in *n*-hexane. The IR spectra exhibited the presence of hydroxyl (**I**: 3325 and **II**: 3465 cm^{-1}) and carbonyl (**I**: 1736 and **II**: 1732 cm^{-1}) groups.

Structure Elucidation

Structure Elucidation of CJ-14,877 (I)

The molecular formula of **I** was determined to be $C_{10}H_{13}NO_4$ [*m/z* found: 212.0940 $(M+H)^+$, calcd. 212.0923 for $C_{10}H_{14}NO_4$] by HRFAB-MS. The ^1H and ^{13}C NMR spectra showed 11 protons and 10 carbons, indicating the presence of two D exchangeable protons in **I**. The carbon signals were classified into two $-CH_3$, two $-O-CH-$, three $-CH=$, two $-C=$ and one carbonyl carbons by the analysis of the DEPT spectra. The degree of unsaturation from molecular formula was five: three were assigned to double bonds including one imine (five sp^2 carbons at δ 150.6, 148.2, 144.8, 138.6 and 126.5), one to a carbonyl group (δ 167.4) and the remainder to the one ring of **I**. The structure of **I** was elucidated as shown in Fig. 1, based on the results of ^1H-^1H COSY and selective INEPT[11] experiments. The ^1H-^1H COSY experiment revealed two proton sequences, $-C^3H=C^4H-$ and $-C^7H(O)-C^8H(O)-CH_3$. The olefinic proton of H-4 (δ 7.99) was coupled with H-3 (δ 8.12) in 8.1 Hz and also showed allylic coupling with olefinic proton of H-6 (δ 8.66) in 1.9 Hz. The

Fig. 2. UV and CD spectra of CJ-14,877 (I) and CJ-14,916 (V).



chemical shifts and the coupling constants of three olefinic protons were very similar to those of methyl fusarate[12] (H-3: δ 8.04, J=7.8 Hz; H-4: δ 7.62, J=8.1, 1.9 Hz; H-6: δ 8.54, J=1.8 Hz). This indicated the presence of a 2,5-disubstituted pyridine ring, which was also suggested by the long-range couplings from H-3 to C-5 (δ 144.8), from H-4 to C-2 (δ 148.2) and C-6 (δ 150.6), and from H-6 to C-2 and C-4 (δ 138.6) in the selective INEPT. The proton sequence, $-C^7H(O)-C^8H(O)-CH_3$, should be attached to the C-5 position of the pyridine ring by the long-range couplings from H-7 (δ 4.55) to C-4, C-5 and C-6, from H-8 (δ 3.86) to C-5, and from H-4 to C-7 (δ 77.5) in the selective INEPT. The presence of the methyl ester group was suggested by the long-range coupling from methyl proton (δ 3.96) to the carbonyl carbon (C-9: δ 167.4). This was also proved by the formation of the corresponding acid, CJ-15,335 (**III**) by the hydrolysis of **I** in the presence of LiOH. The attachment of the methyl ester group to the 2 position of the pyridine ring was suggested by the observation of long-range couplings from H-3 to C-9. Accordingly, the remained two D exchangeable protons should be attributed to the proton of two hydroxy groups at C-7 and C-8. Thus, the plain structure of **I** was determined as methyl-5-(7,8-dihydroxypropyl)pyridine-2-carboxylate.

The stereochemistry of **I** was elucidated by the exciton chirality method[13]. Treatment of **I** with *p*-bromobenzoyl chloride afforded the di-benzoate, CJ-14,916 (**V**). The UV and CD spectra are shown in Fig. 2. The CD spectrum of **V** did not show the split Cotton effect between two *p*-bromobenzoyl groups at C-7 and C-8, but clearly exhibited negative first and positive second Cotton effects (240 and 225 nm) between the *p*-bromobenzoyl group at C-8 and the pyridine ring. Therefore, **V** gave a negative split CD, suggesting that the possible configuration of **V** was 7*R*, 8*S* or 7*R*, 8*R* (Fig. 3). In considering with the coupling constant between H-7 and H-8 (J=4.4 Hz), the absolute configuration of **V** was deduced to be 7*R*, 8*S*. From the above data, the structure of **I** was determined to methyl-(7*R*,8*S*)-5-(7,8-dihydroxypropyl)pyridine-2-carboxylate as shown in Fig. 4.

Structure Elucidation of CJ-14,897 (II)

The structure of **II** was determined by a comparison of its spectral properties with those of **I** (Fig. 4). The UV and

Fig. 3. Elucidation of stereochemistry of CJ-14,916 (V) by the exciton chirality method.

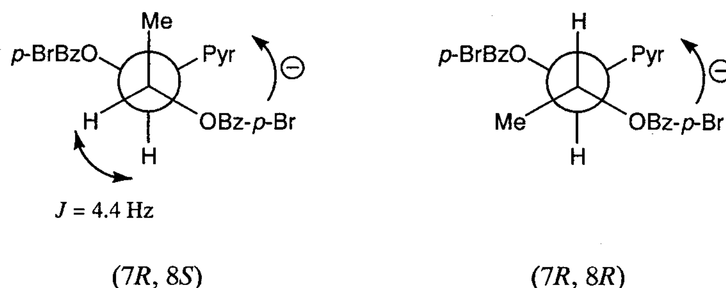
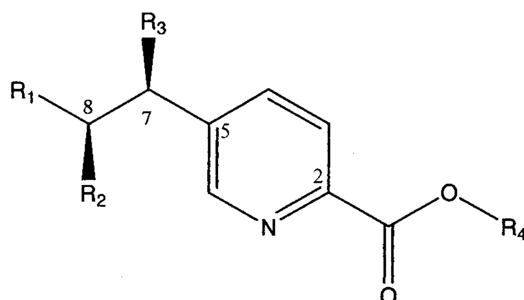


Fig. 4. Structures of CJ-14,877 (I) and its derivatives.



	R_1	R_2	R_3	R_4
CJ-14,877 (I)	Me	OH	OH	Me
CJ-14,897 (II)	Me	OAc	OH	Me
CJ-15,335 (III)	Me	OH	OH	H
CJ-15,336 (IV)	Me	OAc	OAc	Me
CJ-14,916 (V)	Me	OBz-*p*-Br	OBz-*p*-Br	Me
Methyl fusarate	Et	H	H	Me
Fusaric acid	Et	H	H	H

IR spectra of **II** were very similar to those of **I**. The ^{1}H NMR spectrum of **II** was similar to that of **I**, except for the presence of one methyl proton at δ 1.95 in **II**. The molecular formula of **II** was determined to be $C_{12}H_{15}NO_5$ [*m/z* found: 254.1051 $(M+H)^+$, calcd. 254.1028 for $C_{12}H_{16}NO_5$] by HRFAB-MS. The comparison of the molecular formula with that of **I** indicated the presence of one acetyl group in **II**. The lower chemical shift of H-8 (δ 5.03) revealed that **II** was the 8-*O*-acetyl derivative of **I**. From the above data, the structure of **II** was determined as shown in Fig. 4.

Biological Properties

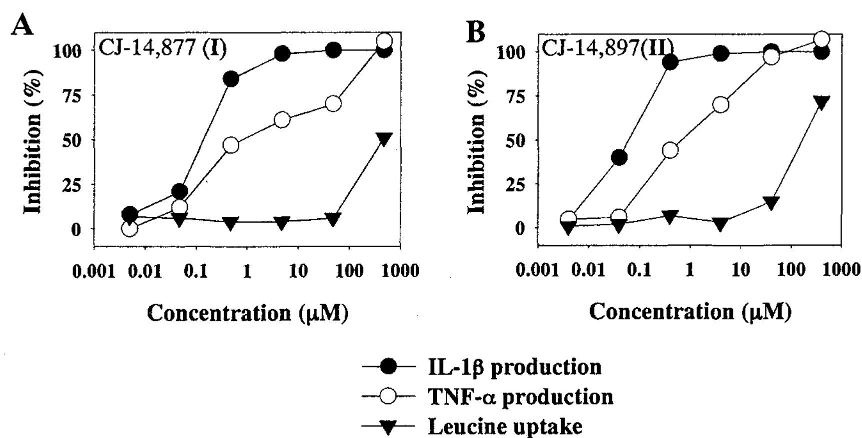
Compounds **I** and **II** were evaluated for inhibitory activities of lipopolysaccharide (LPS)-stimulated IL-1β and TNF-α production, and general protein synthesis in human whole blood. As shown in Fig. 5, both of the compounds dose-dependently inhibited LPS-stimulated IL-1β and TNF-α production. Compound **I** inhibited IL-1β and TNF-α production with IC_{50} values of 0.11 μM and 2.6 μM, respectively (Table 2). On the other hand, **II** exhibited somewhat more potent activities than those of **I** (IC_{50} values of 0.059 and 0.59 μM for IL-1β and TNF-α, respectively). With regard to leucine uptake, both **I** and **II** showed rather weak inhibitory potencies with IC_{50} values of 470 and 180 μM, respectively.

To examine the SAR of the methyl-5-substituted pyridine-2-carboxylates, two derivatives (**III** and **IV**) were prepared, and then evaluated for the inhibitory activities for IL-1β and TNF-α production (Table 2). Acetoxy groups at C-7 and C-8 (**IV**) had little effect on potency and selectivity, whereas a carboxylic acid at C-2 (**III**) had weaker inhibitory activities for IL-1β and TNF-α production. On the other hand, fusaric acid[14] (a *n*-butyl group at C-5 and a carboxylic acid at C-2) and its methyl ester, methyl fusarate, showed no inhibition for IL-1β and TNF-α.

Discussions

Two novel methyl-5-substituted-pyridine-2-carboxylates, **I** and **II**, were isolated from the fermentation broth of a basidiomycete, *Marasmiellus* sp. CL21624. These compounds showed inhibitory activities for LPS-induced production of interleukin-1β and tumor necrosis factor-α in

Fig. 5. Effects of CJ-14,877 (**I**, A) and CJ-14,897 (**II**, B) on IL-1β production, TNF-α production and leucine uptake in human whole blood.



Data are from a typical experiment and represent the mean of triplicate determinations.

Table 2. IC_{50} values of methyl-5-substituted pyridine-2-carboxylates for IL-1β production, TNF-α production and leucine uptake.

Compound	IC_{50} (μM)		
	IL-1β production	TNF-α production	Leucine uptake
CJ-14,877 (**I**)	0.1	2.6	470
CJ-14,897 (**II**)	0.059	0.59	180
CJ-15,335 (**III**)	89	510	220
CJ-15,336 (**IV**)	0.059	0.51	58
Methyl fusarate	>520	>520	>520
Fusaric acid	>520	>520	>520

human whole blood with IC_{50} values of the range from 0.059 to 2.6 μM. They inhibited both IL-1β and TNF-α production with no inhibition of leucine uptake at concentrations lower than approximately 50 μM, indicating that their inhibition is not due to effects on general protein synthesis.

The SAR study on the methyl-5-substituted-pyridine-2-carboxylates suggests the followings: 1) both the methylcarboxylate moiety and the 7,8-dihydroxy group in the C-5 side chain are essential for their inhibitory activities of IL-1β and TNF-α production, 2) the acetoxy group in the C-5 side chain does not influence their inhibitory activities for IL-1β and TNF-α production, and 3) there is no apparent SAR between inhibition of IL-1β and TNF-α production and that of general protein synthesis. The understanding of the SAR on the methyl-5-substituted-pyridine-2-carboxylates may provide useful information for the design of a new type of inhibitors for IL-1β and TNF-α production.

Identification of the target of the methyl-5-substituted-pyridine-2-carboxylates can be expected to lead to the discovery of a critical molecule in IL-1β and TNF-α production. Detailed studies on the mode of action of the methyl-5-substituted-pyridine-2-carboxylates are in progress.

Experimental

General

Spectral and physico-chemical data were obtained by the following instruments: UV, JASCO Ubest-30; CD, JASCO J-720WI; IR, Shimazu 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 strain, the basidiomycete *Marasmiellus* sp. CL21624, was obtained from University of Tennessee, USA. It was deposited on October 29, 1996, under the accession number FERM BP-5735 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology.

Fermentation

Marasmiellus sp. CL21624 was maintained on a plate of malt agar medium (malt extract 2.5% and agar 1.5%) for 10~21 days. A cell suspension from the plate (in 2 ml of sterile H_2O) was used to inoculate two 500-ml flasks containing 100 ml of seed medium (glucose 2%, malt extract 2%, yeast extract 0.18%, maltose 0.24% and agar 0.1%). The flasks were shaken at 26°C for 7 days on a rotary shaker with 7-cm throw at 220 rpm in order to obtain a seed culture. The seed culture was used to inoculate forty 500-ml flasks containing 100 ml of production medium (potato dextrose broth 2.4%). These flasks were shaken at 26°C for 14 days on a rotary shaker with 7-cm throw at 250 rpm.

Preparation of CJ-15,335 (**III**)

To a solution of **I** (5 mg) in water (100 ml), 1 M LiOH (50 ml) was added. After stirring for 1 hour at room temperature, the reaction mixture was neutralized with 1 M HCl. The solution was applied to a Diaion HP20SS column (MITSUBISHI CHEMICAL CORPORATION, Tokyo, Japan), and eluted with 50% aqueous MeOH to give **III** (5 mg) as amorphous white powder. Molecular formula $C_9H_{11}NO_4$; LRFAB-MS *m/z* 196 $(M-H)^-$; ^1H NMR (D_2O) δ 8.74 (1H, d, J=2.2 Hz), 8.47 (1H, d, J=8.1 Hz), 8.28 (1H, dd, J=8.1 and 2.2 Hz), 4.88 (1H, d, J=4.3 Hz), 4.12 (1H, dq, J=6.5 and 4.3 Hz), 1.21 (3H, d, J=6.5 Hz).

Preparation of CJ-15,336 (**IV**)

To a solution of **I** (6 mg) in pyridine (100 ml), acetic anhydride (50 ml) was added. After stirring for 1 hour at room temperature, the reaction mixture was evaporated under N_2 gas. The residue was applied to a silica gel plate (Kiesselgel GF_{254}, 10×10 cm, Merck & Co., Inc. Whitehouse Station, NJ, USA), and developed with chloroform - MeOH (95 : 5) to give **IV** (4 mg) as amorphous white powder. Molecular formula $C_{14}H_{17}NO_6$; LRFAB-MS *m/z* 296 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 8.68 (1H, d, J=2.2 Hz), 8.16 (1H, d, J=8.1 Hz), 8.03 (1H, dd, J=8.1 and 2.2 Hz), 5.95 (1H, d, J=4.3 Hz), 5.28 (1H, dq, J=6.5 and 4.3 Hz), 3.97 (3H, s), 2.12 (3H, s), 1.99 (3H, s), 1.18 (3H, d, J=6.5 Hz).

Preparation of CJ-14,916 (**V**)

To a solution of **I** (4.1 mg) and a catalitic amount of 4-(*N,N*-dimethylamino)pyridine in pyridine (1 ml), *p*-bromobenzoyl chloride (10 mg) was added at room temperature. After stirring at 90°C for 3 days, the reaction mixture was evaporated under N_2 gas. The residue was applied to a silica gel plate (Kiesselgel GF_{254}, 10×10 cm, Merck) and developed with chloroform - methanol (95 : 5) to give **V** (1.03 mg) as amorphous white powder. Molecular formula $C_{24}H_{19}Br_2NO_6$; LREI-MS *m/z* 577 $(M)^+$; ^1H NMR ($CDCl_3$) δ 8.88 (1H, d, J=2.0 Hz), 8.16 (1H, d, J=8.4 Hz), 7.94 (1H, dd, J=8.4 and 2.2 Hz), 7.89 (2H, d, J=8.4 Hz), 7.80 (2H, d, J=8.4 Hz), 7.61 (2H, d, J=8.4 Hz), 7.58 (2H, d, J=8.4 Hz), 6.27 (1H, d, J=4.4 Hz), 5.68 (1H, dq, J=6.6 and 4.4 Hz), 4.01 (3H, s) and 1.41 (3H, d, J=6.6 Hz).

Fusaric Acid

Fusaric acid was purchased from Sigma, St. Louis, MO, USA.

Preparation of Methyl Fusarate

To a solution of fusaric acid (7.5 mg) in diethylether (100 ml), trimethylsilyldiazomethane (100 ml) was added. After stirring for 1 hour at room temperature, the reaction mixture was evaporated under N_2 gas. The residue was applied to a silica gel plate (Kiesselgel GF_{254}, 10×10 cm, Merck), and developed with chloroform - MeOH (95 : 5) to give methyl fusarate (5 mg) as amorphous white powder. Molecular formula $C_9H_{11}NO_4$; LRFAB-MS *m/z* 196 $(M-H)^-$; ^1H NMR (D_3O) δ 8.74 (1H, d, J=2.2 Hz), 8.47 (1H, d, J=8.1 Hz), 8.28 (1H, dd, J=8.1 and 2.2 Hz), 4.88 (1H, d, J=4.3 Hz), 4.12 (1H, dq, J=6.5 and 4.3 Hz), 1.21 (3H, d, J=6.5 Hz).

TNF-α Production, IL-1β Production and Leucine Uptake Assays

These assays were performed according to the methods as described previously[15].

Acknowledgments

We thank Mr. TAKASHI TAKAKUWA in Japan Spectroscopic Co., Ltd. for the CD measurements, and our colleague, Dr. TAISUKE INAGAKI for his 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) MÄNNEL, D. N.; R. N. MOORE & S. E. MERGENHAGEN: Macrophages as a source of tumoricidal activity (tumor-necrotizing factor). Infect. Immun. 30: 523~530, 1980
3) NISSEN-MEYER, J. & J. HAMMERSTROM: Physicochemical characterization of cytostatic factors released from human monocytes. Infect. Immun. 38: 67~73, 1982
4) 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
5) 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
6) 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
7) 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
8) KALDEN, J. R. & B. MANGER: Biologic agents in the treatment of inflammatory rheumatic diseases. Curr. Opin. Rheumatol. 10: 174~178, 1998
9) AREND, W. P.; M. MALYAK, C. J. GUTHRIDGE & C. GABAY: Interleukin-1 receptor antagonist: role in biology. Annu. Rev. Immunol. 16: 27~55, 1998
10) SEKUT, L. & K. CONNOLLY: AntiTNF-α agents in the treatment of inflammation. Exp. Opin. Invest. Drugs 7: 1825~1839, 1998
11) MCCORKINDALE, N. J.; S. A. HUTCHINSON, A. C. MCRITCHIE & G. R. SOOD: Lamellicolic anhydride, 4-*O*-carbomethoxylamellicolic anhydride and monomethyl 3-chlorolamellicolate, metabolites of *Verticillium lamellicola*. Tetrahedron 39: 2283~2288, 1983
12) RENSLO, A. R. & R. L. DANHEISER: Synthesis of substituted pyridines *via* regiocontrolled [4+2] cycloadditions of oximinosulfonates. J. Org. Chem. 63: 7840~7850, 1998
13) HARADA, N. & K. NAKANISHI: Circular dichroic spectroscopy—exciton coupling in organic stereochemistry—. Tokyokagaku-Dojin, Tokyo, 1982
14) WANG, H. & T. B. NG: Pharmacological activities of fusaric acid (5-butylpicolinic acid). Life sciences 65: 849~856, 1999
15) ICHIKAWA, K.; H. HIRAI, M. ISHIGURO, T. KAMBARA, Y. KATO, Y. J. KIM, Y. KOJIMA, Y. MATSUNAGA, H. NISHIDA, Y. SHIOMI, N. YOSHIKAWA, L. H. HUANG & N. KOJIMA: Cytokine production inhibitors produced by a fungus, *Oidiodendron griseum*. J. Antibiotics 54: 697~702, 2001