TMC-260, a New Inhibitor of IL-4 Signal Transduction Produced by *Acremonium kiliense* Grütz TC 1703

MASAAKI SAKURAI*, HIROYUKI HOSHINO, JUN KOHNO, MAKI NISHIO, NOBORU KISHI, TORU OKUDA†, KIMIO KAWANO and TETSUO OHNUKI

Discovery & Pharmacology Research Laboratories, Tanabe Seiyaku Co., Ltd., 2-50 Kawagishi-2-chome, Toda-shi, Saitama 335-8505, Japan

(Received for publication May 9, 2003)

Interleukin-4 (IL-4) signal transduction *via* IL-4 receptor, Janus kinase 1 and 3 (JAK1 and JAK3), and signal transducer and activator of transcription 6 (STAT6) are essential for the production of IgE. IL-4 receptor associated JAK1 and JAK3 phosphorylate a tyrosine residue ^{641}Tyr of STAT6 after IL-4 stimulation. The phosphorylated STAT6 forms a homodimer, translocates to the nucleus, and binds to the STAT6 recognition sequences (TTCCCAAGAA) within the promoter region of germline Cε[1,2]. Expression of germline Cε mRNA plays a critical role in the immumogloblin isotype switching from IgM to IgE in B cells[3,4]. IgE induces release of inflammatory mediators in mast cells to cause allergic diseases such as asthma, nasal inflammation and atopic dermatitis. Thus, the IL-4 signal transduction might be a therapeutic target for allergic diseases. As a result of screening for inhibitors of IL-4 signal transduction using an IL-4 driven luciferase assay system, we found new naphtho-γ-pyrones (TMC-256A1 and C1) and a novel tricyclic polyketide (TMC-264) from the fermentation broth of *Aspergillus niger* var. *niger* TC 1629[5] and *Phoma* sp. TC 1674[6,7], respectively. Further screening identified a new inhibitor of IL-4 signal transduction designated as TMC-260 (Fig. 1) from the fermentation broth of *Acremonium kiliense* Grütz TC 1703. This report describes the taxonomy, fermentation, isolation, structure determination and biological activities of TMC-260.

Materials and Methods

Materials

Dulbecco's modified Eagles' medium (DMEM) and RPMI1640 medium were purchased from SIGMA (St. Louis, MO). Human recombinant IL-4 and IFN-γ were from R&D systems (Minneapolis, MN).

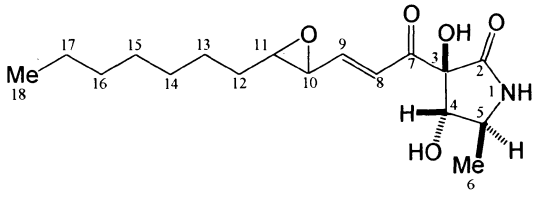
Taxonomic Studies

For identification of the strain TC 1703, cultures grown on malt extract agar (MEA) and oatmeal agar (OA) were used. The color name used in this study was taken from Munsell color system[8]. Taxonomic studies were based on GRAMS[9].

Fermentation

A slant culture of the producing strain, *Acremonium kiliense* Grütz TC 1703 was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of the seed medium consisting of 1.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co.; Osaka, Japan), 0.5% dried yeast (Asahi Brewery Co.; Tokyo, Japan), 20% V-8 juice (Campbells; New Castle, CO), 20% apple juice (Kirin Tropicana Inc.; Tokyo, Japan), and 0.5% $CaCO_3$, adjusted at pH 6.0 before autoclaving. The inoculated flask was incubated at 27°C for 4 days on a rotary shaker (220 rpm). Fifteen milliliters of the seed culture were transferred into a 500-ml Erlenmyer flask containing the solid medium composed of 0.02 g of pressed barley (Nagakura Seibaku Co.; Shizuoka, Japan), 0.04 g of yeast extract (Asahi Brewery Co.; Tokyo, Japan), 0.02 g of Na-tartrate, 0.02 g of KH_2PO_4, and 20 ml of deionized water. The fermentation was conducted under static conditions at 25°C for 12 days.

Fig. 1. Structure of TMC-260.



† Present address: Tamagawa University Research Institute. 1-1 Tamagawa Gakuen-6-chome, Machida, Tokyo 194-8610, Japan.

* Corresponding author: sakura@tanabe.co.jp

Cells

Stable transfectants HeLa IL-4/STAT6 (2-21)[5], HeLa IFN-γ/STAT1 (1-26)[5], and HeLa H-144[10] cells were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS) and 200 μg/ml hygromycin. DND39 (human Burkitt lymphoma B-cell line) cells were kindly provided by Fujisaki Cell Center (Hayashibara Biochemical Laboratories; Okayama, Japan) and were cultured in RPMI1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 0.1 g/liter streptomycin and 100,000 U/liter penicillin G.

Luciferase Assay

HeLa IL-4/STAT6 (2-21) and HeLa IFN-γ/STAT1 (1-26) cells expressed IL-4 driven luciferase and IFN-γ driven luciferase activities under the promoter having the binding sequences of STAT6 and STAT1, respectively, were treated with TMC-260 in the presence of IL-4 (10 ng/ml) and IFN-γ (10 ng/ml) for 6 hours, respectively. H-144 cells, which expressed constitutively the SV40 driven luciferase activity, were treated with TMC-260 for 6 hours. The luciferase reporter assays were carried out as described previously[5,10].

Measurement of Germline Cε mRNA Expression

DND39 cells were treated with TMC-260 in the presence of IL-4 (10 ng/ml) for 4 hours. The assay for analysis of germline Cε mRNA expression was performed as described previously[5].

General

^{1}H and ^{13}C NMR spectra were obtained in DMSO-d_6 with a JEOL GSX-400 NMR spectrometer and chemical shifts were given in ppm (δ) relative to tetramethylsilane as an internal standard. Optical rotation was determined on a Horiba model SEPA-200 high sensitive polarimeter using the sodium D line. UV and IR spectra were recorded on a Hitachi U-3000 and a Perkin Elmer PARAGON 1000 FT-IR spectrometer, respectively. A mass spectrum was obtained by using a MStation 700 tandem type mass spectrometer (JEOL, Japan) equipped with an electrospray ionization source. The melting point was determined on a Yanaco MP-500D micro melting point apparatus and was uncorrected.

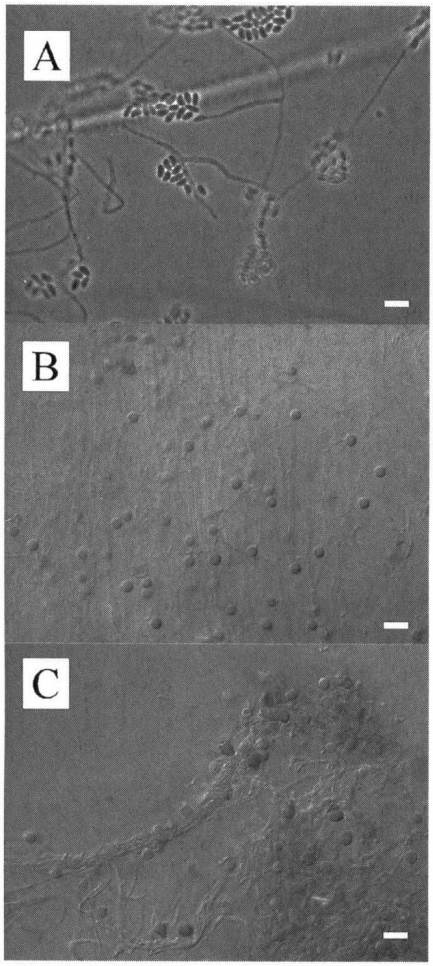
Results and Discussion

Colonies on MEA grew very slowly, attaining a diameter of 8~9 mm after 7 days and 11 mm after 14 days at 25°C. They were irregularly sulcate, moist, tough and convoluted, cream to brownish orange (Munsell 10YR9/2 to 10YR7/4-6) with pale yellow to cream (Munsell 10YR9/2) irregular margins. The reverse was brownish orange. No pigment was produced in the agar. Colonies on OA grew also slowly, attaining 10 mm in diameter after 7 days and 19~20 mm after 14 days at 25°C. They were plane, tough, velutinous with white floccose mycelial tufts, light brown to light grayish brown (Munsell 5YR6/4 to 7/4) with pale yellow to grayish orange (Munsell 5-10YR9/2) partly immersed margins. The reverse was light grayish brown (Munsell 5YR7/4) to pink (Munsell 10R7/6) or grayish orange (Munsell 10YR8/6) in older cultures. Ocher red (Munsell 10R5/8) pigment and crystals were produced. The growth temperature was between 10°C and 32°C, and optimal temperature for growth was between 25°C and 32°C. Conidiophores were mainly born from the hyphal strands creeping on the surface of tough mycelial mat, phalacrogenous to plectonematogenous. Phialides are mostly solitary and rarely in pair or verticils, flexuous, 27.5~45.5×1.5~2.0 μm, with indistinct collarette. Conidia were not often observed, were aggregated in mass on the apex of phialides, 1-celled, mostly reniform, curved or ellipsoidal, but sometimes almost globose, 3.5~5.5×2.2~3.5 μm (Fig. 2A). Chlamydospores were abundant, directly attached on the hyphal strands or born on a thin hypha of 1.0~11.0×0.5~1.5 μm. They were 1-celled, globose to obovate, 3.5~5.5×3.5~4.5 μm (Fig. 2B), smooth when young, but often became distinctively verrucose with irregular chromophilic spins of 1.1 μm in diameter (Fig. 2C).

The fungus produced simple unbranched conidial structures with phialides bearing small unicellular conidia in mass, which are characteristic to the genus *Acremonium*. It was further characterized by its slow growth, rather dirty colored moist colonies, solitary phialides, ellipsoidal or curved conidia in slimy droplets, and presence of distinctive chlamydospores. On the basis of these morphological characteristics, production of distinctively roughened chlamydospores in particular, the fungus was identified as *Acremonium kiliense* Grütz.

The resultant culture of one hundred flasks was extracted with 1-butanol (6.0 liters), and concentrated to dryness *in vacuo*. The residual solid was suspended in water (500 ml), and extracted twice with ethylacetate (500 ml). The ethylacetate extract was concentrated *in vacuo*, and the residue was partitioned between *n*-hexane (300 ml) and 90% aqueous methanol (300 ml). The methanol layer was concentrated *in vacuo* to yield amorphous powder (6.4 g), which was applied to a reversed-phase column (YMC ODS A60, 40 i.d.×200 mm), and developed with acetonitrile-water (20 : 80~30 : 70, gradient). Active fractions were

Fig. 2. Photomicrographs of producing strain, *Acremonium kiliense* Grütz TC 1703.



A, phialoconidia aggregated on the apex of phialides; B, young and smooth chlamydospores; C, older verrucose chlamydospores.
Bar represents 10 μm.

Table 1. Physico-chemical properties of TMC-260.

Appearance	Colorless powder
Melting point	140～144 °C
$[\alpha]_D^{20}$ (c 0.096, MeOH)	-29 °
Molecular formula	$C_{17}H_{27}NO_5$
HRESI-MS(*m/z*)	
Found	324.1814 $[M-H]^-$
Calcd for $C_{17}H_{26}NO_5$	324.1811
UV λ_{max}(MeOH) nm (ε)	245 (14,050)
IR ν(KBr) cm^{-1}	3483, 3279, 2924, 2855, 1713, 1675, 1616, 1252

Table 2. ^1H and ^{13}C NMR data for TMC-260.

Position	^1H	^{13}C
1	8.05 (s)	
2		171.5 (s)
3		86.3 (s)
4	3.67 (dd 7.33,5.13)	83.7 (d)
5	3.21 (dq 6.53, 6.53)	51.4 (d)
6	1.23 (s)	18.2 (q)
7		197.3 (s)
8	6.98 (d 15.63)	127.6 (d)
9	6.39 (dd 15.75, 7.45)	142.1 (d)
10	3.39 (dd 7.32, 1.83)	55.9 (d)
11	2.97 (ddd 5.86, 5.37, 1.95)	60.7 (d)
12	1.53 (m)	31.1 (t)
13	1.38 (m)	25.3 (t)
14	1.26 (m)	28.5 (t)*
15	1.26 (m)	28.6 (t)*
16	1.26 (m)	31.1 (t)
17	1.26 (m)	22.0 (t)
18	0.86 (t 6.96)	13.8 (q)
3-OH	6.32 (s)	
4-OH	5.77 (d 5.12)	

* May be interchangeable.

combined and concentrated to give 1.6 g of a brown solid. The solid was purified by preparative HPLC (column, YMC-Pack D-ODS-5-B, 30 i.d.×250 mm; mobile phase, 50% aqueous acetonitril; flow rate, 25 ml/minute; detection, UV absorption at 210 nm; Rt, 16.5 minutes). The semi-pure product was crystallized from ethylacetate-hexane to yield pure TMC-260 (640 mg) as a colorless powder.

TMC-260 was soluble in methanol, dimethylsulfoxide and chloroform but practically insoluble in *n*-hexane and water. Physico-chemical properties of TMC-260 are summarized in Table 1. The UV absorption maximum at 245 nm along with the IR absorption band at 1675 cm^{-1} suggested the presence of an α,β-unsaturated carbonyl group. The IR spectrum also indicated the presence of an epoxy (1252 cm^{-1}) and another carbonyl (1713 cm^{-1}) groups. The molecular formula was established to be $C_{17}H_{27}NO_5$ on the basis of HRESI-MS and NMR data.

The ^{13}C and ^1H NMR spectra of TMC-260 are summarized in Table 2. The ^{13}C NMR displayed 17 signals composed of two methyl, six methylene, six methine including two olefinic carbons, and three quaternary carbons including two carbonyls. The ^1H NMR spectrum showed three D_2O exchangeable protons at δ 8.05, 6.32 and

Fig. 3. DQF-COSY and HMBC correlations of TMC-260.

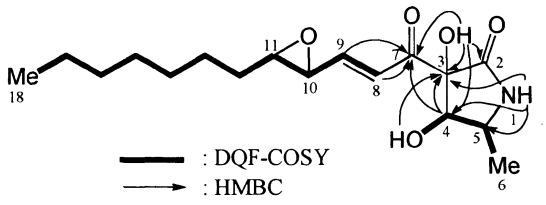
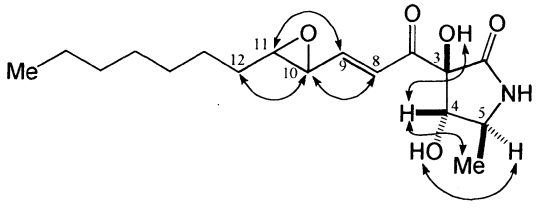


Fig. 4. Key NOE correlation (arrows) of TMC-260.



5.77. In the DQF-COSY experiment, the cross peaks, observed unambiguously, constructed a sequential proton network from H-8 to H-18 as shown in Fig. 3. The ^{13}C chemical shifts and the characteristic ^{1}H-^{13}C coupling constants at C-10 (δ 55.9, $^{2}J_{C10,H10}$=178.6 Hz) and C-11 (δ 60.7, $^{2}J_{C11,H11}$=173.7 Hz) revealed the presence of the epoxide. The configuration of the double bond at C-8 was assigned as *trans* based on the large vicinal coupling constant, $J_{8,9}$=15.7 Hz, and NOE correlation between H-8 and H-10 (Fig. 4). The geometrical configuration of the epoxide was determined to be *trans* from the small ^{1}H-^{1}H coupling constant, $J_{10,11}$=1.9 Hz, and NOE correlations between H-9 and H-11, and between H-10 and H-12 (Fig. 4). These results and HMBC correlations from H-8 and H-9 to C-7 (δ 197.3) clarified the structure of (2*E*)-3-(*trans*-3-heptyloxiranyl)-1-oxo-2-propenyl moiety.

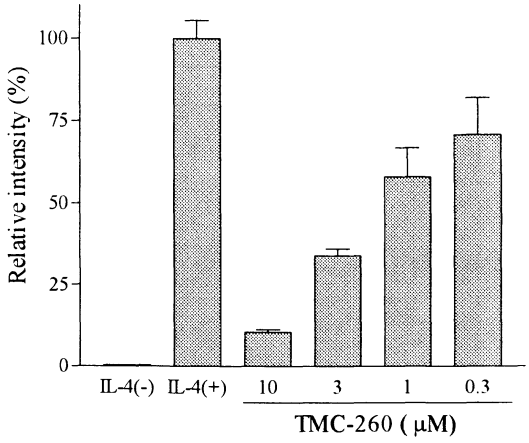
The chemical shifts of the carbonyl carbon at C-2 (δ 171.5) and the NH proton (δ 8.05), and the presence of two unsaturations deduced from the molecular formula ($C_5H_8NO_3$) of the remaining partial structure suggested the presence of the lactam ring, which was supported by the IR absorption at 1713 cm^{-1}. In addition, the HMBC spectrum showed the clear ^{1}H-^{13}C correlation network of the NH proton (δ 8.05) and the two hydroxyl protons (δ 6.32 and 5.77) (Fig. 3). The structure of the lactam ring moiety was thus determined as 3,4-dihydroxy-5-mehtyl-2-pyrrolidinone. The relative stereochemistry of the lactam ring was determined as shown in Fig. 4 by strong NOE correlations between H-4 and OH-3, H-4 and Me-6, and OH-4 and H-5. The attachment of the 3-(3-heptyloxiranyl)-1-oxo-2-propenyl moiety to the lactam ring was confirmed by HMBC correlations from H-4 and OH-3 to C-7.

The structure of TMC-260 was thus determined as dihydroxy-3-[(2*E*)-3-(*trans*-3-heptyloxiranyl)-1-oxo-2-propenyl]-5-mehtyl-2-pyrrolidinone, and which was a new analog of pramanicin[11]. The absolute stereochemistry and the relative stereochemistry of the epoxide to the lactam stereocenters of TMC-260 were remained to be determined.

Table 3. Effects of TMC-260 on the IL-4 driven, IFN-γ driven, and SV40 promoter driven luciferase expressions.

compound	IC_{50} (μM)		
	IL-4 / STAT6	IFN-γ / STAT1	SV40
TMC-260	9.0	>100	>100

Fig. 5. Effect of TMC-260 on induction of germline Cε mRNA expression by IL-4.



DND39 cells were treated with TMC-260 in the presence of IL-4 (10 ng/ml) for 4 hours. Detection of mRNAs was carried out with quantitative RT-PCR as described previously[5]. The relative intensity (germline Cε mRNA/GAPDH mRNA) was calculated and represented by percentage of the relative intensity of DND39 cells treated with IL-4 as 100%.

TMC-260 suppressed the IL-4 driven luciferase activity with an IC_{50} value of 9.0 μM (Table 3). To investigate the selectivities of TMC-260, we measured the inhibitory activities of this compound towards the IFN-γ driven and the SV40 promoter driven expression of luciferase, respectively. TMC-260 did not affect these activities up to 100 μM. The above results indicated that TMC-260 selectively inhibited IL-4 signal transduction. Next, we investigated the effect of TMC-260 on IL-4-induced germline Cε mRNA expression in DND39 cells. TMC-260 dose-dependently inhibited the expression with an IC_{50} value of 1.2 μM (Fig. 5).

These data suggest that TMC-260 suppresses the production IgE and maybe useful in the treatment of allergic disease as germline Cε mRNA expression is essential for the production of IgE. Further studies are needed to clarify the molecular mechanism of the inhibitory nature of TMC-260.

Acknowledgments

We wish to thank Ms. NAOKO FUKUI for NMR measurements, Dr. NORIKO OHASHI for mass measurements.

References

1) JIANG, H.; M. B. HARRIS & P. ROTHMAN: IL-4/IL-13 signaling beyond JAK/STAT. J. Allergy Clin. Immunol. 105: 1063~1070, 2000
2) LAMB, P.; P. TAPLEY & J. ROSEN: Biochemical approaches to discovering modulators of the JAK-STAT pathway. Drug Discov. Today 3: 122~130, 1998
3) GAUCHAT, J. F.; D. A. LEBMAN, R. L. COFFMAN, H. GASCAN & J. E. DE VRIES: Structure and expression of germline epsilon transcripts in human B cells induced by interleukin 4 to switch to IgE production. J. Exp. Med. 172: 463~473, 1990
4) ICHIKI, T.; W. TAKAHASHI & T. WATANABE: Regulation of the expression of human C epsilon germline transcript. Identification of a novel IL-4 responsive element. J. Immunol. 150: 5408~5417, 1993
5) SAKURAI, M.; J. KOHNO, K. YAMAMOTO, T. OKUDA, M. NISHIO, K. KAWANO & T. OHNUKI: TMC-256A1 and C1, new inhibitors of IL-4 signal transduction produced by *Aspergillus niger* var *niger* TC 1629. J. Antibiotics 55: 685~692, 2002
6) SAKURAI, M.; M. NISHIO, K. YAMAMOTO, T. OKUDA, K. KAWANO & T. OHNUKI: TMC-264, a novel antiallergic heptaketide produced by the fungus *Phoma* sp. TC 1674. Org. Lett. 5: 1083~1085, 2003
7) SAKURAI, M.; M. NISHIO, K. YAMAMOTO, T. OKUDA, K. KAWANO & T. OHNUKI: TMC-264, a novel inhibitor of STAT6 activation produced by *Phoma* sp. TC 1674. J. Antibiotics 56: 513~519, 2003
8) Japan Color Enterprise Co.: Munsell color system, Tokyo, 1977
9) GRAMS, W.: Cephalosporium-artige Schimmelpilze (Hyphomycetes). pp. 262, Gustav Fischer Verlag, Stuttgart, 1971
10) SAKURAI, M.; J. KOHNO, M. NISHIO, K. YAMAMOTO, T. OKUDA, K. KAWANO & N. NAKANISHI: TMC-205 a new transcriptional up-regulator of SV40 promoter produced by an unidentified fungus. Fermentation, isolation, physico-chemical properties, structural determination and biological activities. J. Antibiotics 54: 628~34, 2001
11) SCHWARTZ, R. E.; G. L. HELMS, E. A. BOLESSA, K. E. WILSON, R. A. GIACOBBE, J. S. TKACZ, G. F. BILLS, J. M. LIESCH, D. L. ZINK, J. E. CUROTTO, B. PRAMANIK & J. C. ONISHI: Pramanicin, a novel antimicrobial agent from a fungal fermentation. Tetrahedron 50: 1675~1686, 1994