TMC-256A1 and C1, New Inhibitors of IL-4 Signal Transduction Produced by *Aspergillus niger* var *niger* TC 1629

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New inhibitors of IL-4 signal transduction, designated as TMC-256A1 and C1, were discovered together with TMC-256B1, a previously known dihydronaphthopyrone, from the fermentation broth of *Aspergillus niger* var *niger* TC 1629 by using an IL-4 driven reporter gene assay. Based on spectroscopic analyses, TMC-256A1 and C1 were found to be new members of the naphthopyrone antibiotics. TMC-256A1, B1 and C1 inhibited the IL-4 driven luciferase activity with IC_{50} values of 25 μM, 30 μM and 1.7 μM, respectively in this assay system. Furthermore, these compounds inhibited the expression of germline $C\varepsilon$ mRNA with IC_{50} values of 6.6 μM , 34 μM and 0.31 μM, respectively.

Interleukin-4 (IL-4) mediates class switching of an immumoglobulin isotype IgM to IgE in B cells. IL-4 binding to its receptor activates two members of the Janus family of tyrosine kinases, JAK1 and JAK3. Subsequent to JAK activation, STAT6 (signal transducers and activators of transcription 6) is activated by phosphorylation of a tyrosine residue ^{641}Tyr of STAT6 by JAKs.[1,2]. The activated STAT6 homodimerizes, translocates to the nucleus, and induces the germline $C\varepsilon$ transcription through binding to the STAT6 responsive element[3,4]. The IL-4-driven germline $C\varepsilon$ transcription is enhanced by binding of CD40 ligand to CD40 on B cells, which induces the deletional switch recombination to lead to maturation of the $C\varepsilon$ transcripts and IgE synthesis[5]. IgE triggers the release of inflammatory mediators in mast cells.

The importance of IL-4 signal transduction for IgE production and development of allergy and inflammation has been suggested by a number of experiments. Serum level of IgE is elevated in IL-4 transgenic mice[6], whereas strongly reduced in IL-4 deficient mice[7]. STAT6-deficient ($STAT6^{-/-}$) mice abrogate IL-4 mediated functions including Th2 differentiation, expression of cell surface markers, and Ig class switching to IgE[8~10]. The $STAT6^{-/-}$ mice are resistant to bronchial eosinophilic inflammation and airway hyperactivity *in vivo*[11,12]. Therefore, it is possible that selective inhibitors of IL-4 signal transduction prevent allergic diseases.

We have screened microbial metabolites for inhibitors of IL-4 signal transduction using a reporter assay and discovered three active naphtho-γ-pyrones designated as TMC-256A1 (**1**), B1 (**3**) and C1 (**5**), together with their inactive analogs, TMC-256A2 (**2**), B2 (**4**) and C2 (**6**) (Fig. 1). Among them, TMC-256A1 (**1**) and C1 (**5**) were found to be a new member of naphtho[2,3-*b*]pyran-4-one and naphtho[1,2-*b*]pyran-4-one, respectively. This report describes the taxonomy, fermentation, isolation, structure determination and biological activities of TMC-256s.

Materials and Methods

Materials

Dulbecco's modified Eagles'medium (DMEM) and RPMI1640 medium were purchased from SIGMA. Human recombinant IL-4 and IFN-γ were from R&D systems. pGL3-basic was purchased from Promega, and pREP4 was

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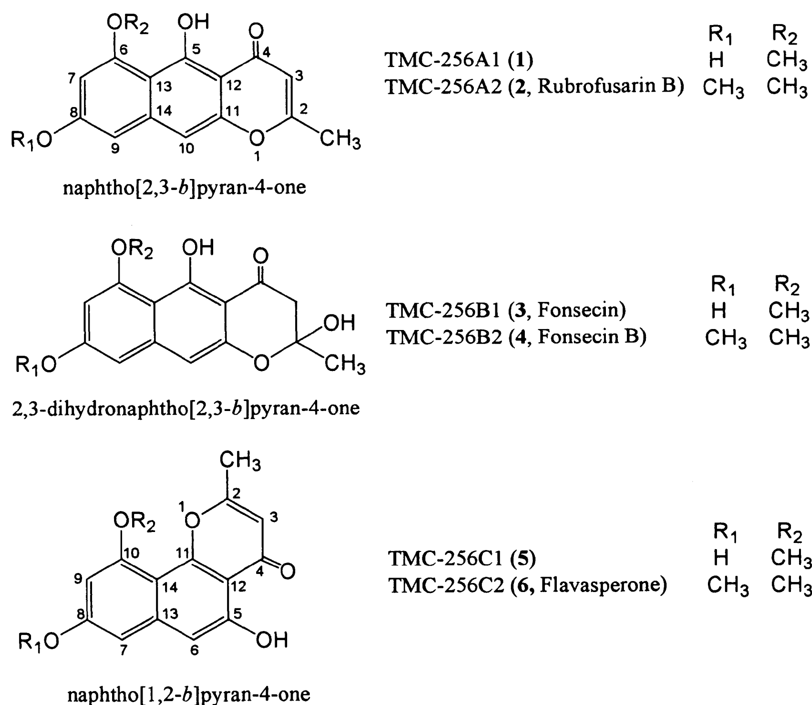
Fig. 1. Structures of TMC-256s.



from Invitrogen.

Microorganism

The producing strain, *Aspergillus niger* var *niger* TC 1629, was isolated from a soil sample collected at Machida-shi, Tokyo, Japan. *Aspergillus niger* var *niger* TC 1629 was maintained on an agar slant of malt extract agar consisting of malt extract 2%, Soytone (Difco) 0.2%, yeast extract 0.2%, glucose 2%, and agar 1.5% at 4°C.

Taxonomic Studies

For identification of the strain TC 1629, Czapek-yeast extract agar and malt extract agar were used. Morphological observation was done under a microscope (Olympus BH-2). The color name used in this study was taken from Munsell color system[13]. Taxonomic studies were based on RAPER & FENNELL[14] and KLICH & PITT[15].

Media Used for Seed Culture and Production

The liquid medium used for seed culture contained 10 g of glucose, 5 g of Polypepton (Nihon Pharmaceutical Co), 5 g of dried yeast (Wako Pure Chemical), 200 ml of V-8 juice (Campbells Japan), 200 ml of apple juice (Kirin Tropicana Inc.), and 5 g of $CaCO_3$ in 1 liter of deionized water, adjusted pH to 6.0 before autoclaving. The solid medium for production consisted of 20 g of rolled barley (Nagakura Seibaku), 0.04 g of yeast extract (Asahi Beer), 0.02 g of Na-tartrate, 0.02 g of KH_2PO_4, and 20 ml of deionized water.

Cells and Culture Conditions

HeLa (human cervix adenocarcinoma) cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1 g/liter streptomycin and 100,000 U/liter penicillin G. Stable transfectants HeLa IL-4 / STAT6 (2-21), HeLa IFN-γ STAT1 (1-26), and HeLa H-144 cells were cultured in DMEM containing 10% FBS and 0.2 g/liter of hygromycin.

DND39 (human Burkitt lymphoma B-cell line) cells were kindly provided by Fujisaki Cell Center (Hayashibara Biochemical Laboratories, Okayama, Japan), and cultured in RPMI1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 0.1 g/liter streptomycin and 100,000 U/liter penicillin G.

Plasmids

STAT-binding elements, and thymidine kinase minimal promoter were chemically synthesized by Amersham

Pharmacia. The DNA sequences of the nucleotides are as follow: STAT6-binding element, 5′-gttcgtacaactgctagctgc-cttagtcaacttcccaagaacagatgccttagtcaacttcccaagaacagatgcctta gtcaacttcccaagaacagatgccttagtcaacttcccaagaacagactcgaggtg aaacggtat-3′; STAT1-binding element, 5′-gttcgtacaactgct-agcgatccgtatttcccagaaaaggaacagatccgtatttcccagaaaaggaacag atccgtatttcccagaaaaggaacagatccgtatttcccagaaaaggaacactcga ggtgaaacggtat-3′; thymidine kinase minimal promoter, 5′-cgaattcgaacacgcagatgcagtcggggcggcgcggtcccaggtccacttcgc atattaaggtgacgcgtgtggcctcgaacaccgagcgaccctgcagcgacccgct taacagcgtcaacagcgtgccgca-3′. These oligonucleotides were amplified by PCR, and each of STAT-binding elements was ligated with thymidine kinase minimal promoter. pGL3-STAT6 and pGL3-STAT1 were constructed by subcloning the ligated DNA fragments into *NheI*/*Hin*dIII sites of pGL3-basic.

Stable Transfection and Luciferase Assay

HeLa H-144 expressing the luciferase activity constitutively under the SV40 promoter was described previously[16]. Transfection of HeLa cells with pGL3-STAT6 plus pREP4, or pGL3-STAT1 plus pREP4 were carried out by using SuperFect™ Transfection Reagent (QIAGEN) as described by the manufacturer's instructions. Among the hygromycin resistant clones obtained, the transfectants that expressed the IL-4 driven luciferase or the IFN-γ driven luciferase activities were selected, and named as HeLa IL-4/STAT6 (2-21) and HeLa IFN-γ STAT1 (1-26), respectively.

Cells were seeded into 96-well white cell culture microplates (2×10^4 cells per well) and incubated for 24 hours, and then samples were added to the cell culture at 10% (v/v). The cells were treated with IL-4 (10 ng/ml) or IFN-γ (10 ng/ml) for 6 hours. The cells were washed twice with 200 μl of PBS, and added to 20 μl of cell lysis buffer (Promega) and 50 μl of luciferase assay buffer (Promega). The luminescence was measured using a luminometer (Berthold Microlumat LB96P).

Measurement of Germline Cε mRNA Expression

DND39 cells were seeded into 24-well cell culture plates (1×10^6 cells per well) in RPMI1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol and 25 mM HEPES. The cells were treated with samples and IL-4 (10 ng/ml) for 4 hours. Total RNA (50 μl) was isolated from the cultured cells (5×10^5 cells) using RNeasy Mini (QIAGEN) according to the manufacturer's instructions. Primer and probe sequences of germline Cε expression used in this experiment were as follows: forward primer, 5′-ctgggagctgtccaggaacc-3′; reverse primer, 5′-gcagcagcgg-gtcaagg-3′; probe, 5′-ccacaccatccacaggcacca-3′. Human glyceraldhyde-3-phosphate dehydrogenase (GAPDH) was used for standardizing the amount of the RNAs. Primers and a probe of GAPDH were obtained from Applied Biosystems. RT-PCR was performed in a 50 μl reaction volume by using TaqMan EZ RT-PCR kit (Applied Biosystems) according to the manufacturer's instructions. Amplification and detection were carried out with an ABI7700 sequence detection system (Applied Biosystems). The relative intensity (germline Cε mRNA/GAPDH mRNA) was calculated and represented by percentage of the relative intensity of DND39 cells treated by IL-4 as 100%.

General

UV spectra were measured on a Shimadzu model UV-2200A spectrophotometer. IR spectra were recorded on a JASCO model 100 infrared spectrophotometer. Mass spectra were obtained using MStation 700 tandem type mass spectrometer (JEOL, Japan) equipped with an electrospray ionization source. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL GSX-400 NMR spectrometer at 30°C. The chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as an internal standard.

HPLC Analysis

Analytical HPLC was carried out on a Hewlett-Packard HP-1090 equipped with a diode array detector. The condition for HPLC analysis was as follows: column, YMC-Pack ODS-AM-301-3 (4.6 i.d.×100 mm); mobile phase, 45% aqueous CH_3CN; flow rate, 1.2 ml/minute; detection, UV at 254 nm. Under these conditions, compounds **1**~**6** were eluted at 2.9, 8.7, 1.8, 2.8, 3.2 and 7.1 minutes, respectively.

Preparative Chromatography

Silica gel column chromatography was performed on a column (40 i.d.×440 mm, packed with Wako gel C-200) with a mobile phase of CH_2Cl_2-MeOH (100:0, 1.0 liter; 98:2, 1.5 liters; 95:5, 1.5 liters). The presence of TMC-256s was monitored by TLC and HPLC analyses. Combined fractions eluting between 1.68~2.37 liters and a second set eluting between 2.89~3.53 liters yielded fraction (fr)-1 and fr-2, respectively. Preparative HPLC separations were carried out using Gilson HPLC system and YMC D-ODS-5B: column size, 30 i.d.×250 mm; mobile phase, 50% aq CH_3CN for separation of **1**, **2**, **4**, **5** and **6**, and 40% aq CH_3CN for isolation of **3**; flow rate, 25 ml/minute; detection, UV light at 254 nm. Under these

conditions, compounds **1**~**6** were eluted at 10.4, 31.2, 9.8, 13.5, 12.0 and 38.3 minutes, respectively.

Results and Discussion

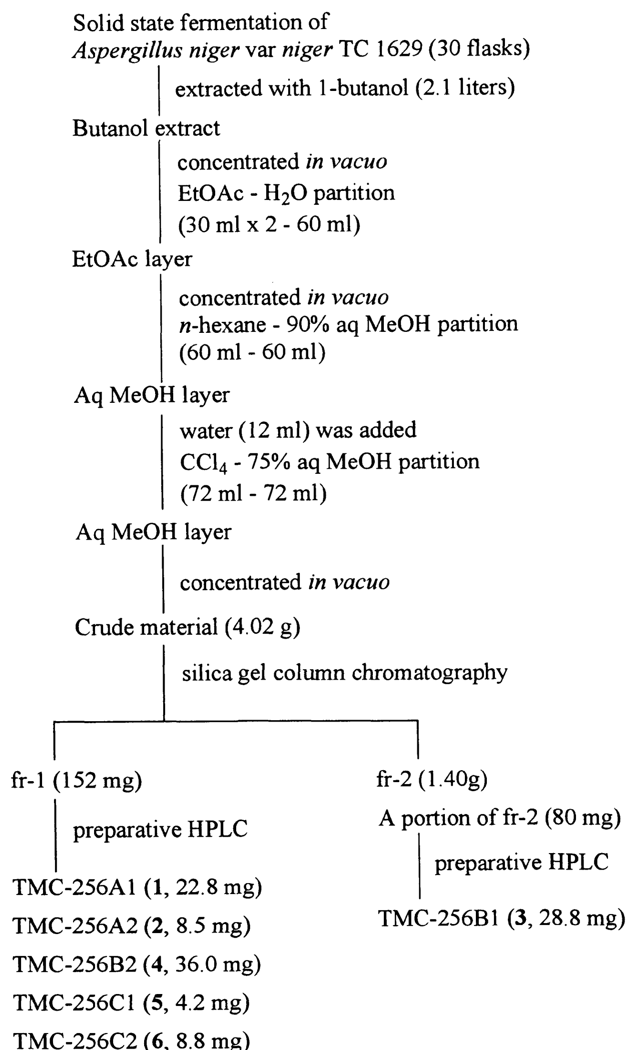
Taxonomy

The producing strain, TC 1629 was identified as *Aspergillus niger* van Tieghem var. *niger* based on the following cultural and morphological characteristics. Colonies on Czapek-yeast extract agar (CYA) and malt extract agar (MEA) exceeding 60 mm after 7 days at 25°C showed almost black (Munsell 5Y2/2 to 5YR2/2) and velutinous appearance. Reverse was pale yellow (5Y9/4-8/4). No exudates or pigments were produced. Conidial heads were characteristically dark brown, and splitting into divergent columns. Stipes were 480~970×24~28 μm, hyaline to pale yellow, and smooth-walled. Vesicles were 54~69 μm in diameter, and globose. Aspergilla were biseriate; metulae covering the entire surface of the vesicle, 16~18×5~6.5 μm. Phialides were 7~10×3.5~4 μm. Conidia were 4.0~4.5×3.5~4.0 μm, subglobose to globose, and roughened with irregular ridges.

Fermentation

A slant culture of the producing strain was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of the seed medium. The culture was incubated at 27°C for 4 days on a rotary shaker (220 rpm). One milliliter of the seed culture was transferred into a 500-ml Erlenmeyer flask containing the production medium, and incubated under static conditions at 27°C for 12 days.

Fig. 2. Isolation and purification procedure for TMC-256s.



Isolation

Isolation and purification procedures for TMC-256s (**1**~**6**) are summarized in Fig. 2. TMC-256s were extracted with 1-butanol from the production media (solid state) of *Aspergillus niger* var *niger* TC 1629. The extract was purified by a combination of solvent partition and silica gel column chromatography to afford two bioactive fractions. Each fraction was further purified by preparative HPLC on ODS column to give six pure components (**1**~**6**) as yellowish powders.

Structure Determination

TMC-256A2 (**2**), B1 (**3**), B2 (**4**) and C2 (**6**) were identified as known naphtho[2,3-*b*]pyran-4-one (rubrofusarin B[17~21]), 2,3-dihydronaphtho[2,3-*b*]pyran-4-ones (fonsecin[21~23] and fonsecin B[19,21,23]) and naphtho[1,2-*b*]pyrane-4-one (flavasperone[17,18,20]), respectively, by their spectral data (NMR, MS, UV and IR).

TMC-256A1 (**1**) and C1 (**5**) were soluble in DMSO, methanol, acetone, ethyl acetate, chloroform and diethyl ether, but practically insoluble in *n*-hexane and water. These compounds gave positive color reaction to iodine vapor and ammonium molybdate-sulfuric acid reagent, but negative to ninhydrin. In the Gibbs test for detecting an unsubstituted position *para* to a phenolic group, both **1** and **5** gave violet colors. Additional physico-chemical properties of **1** and **5** are summarized in Table 1.

The characteristic UV and IR spectra of **1** and **5** closely resembled those of **2** (rubrofusarin B[17~21]) and **6**

Table 1. Physico-chemical properties of **1** and **5**.

	1	**5**
Appearance	Yellow powder	Pale yellow powder
ESI-MS (*m/z*)	273 $(M+H)^+$, 295 $(M+Na)^+$	273 $(M+H)^+$, 295 $(M+Na)^+$
	271 $(M-H)^-$	271 $(M-H)^-$
HRESI-MS (*m/z*)		
Found	271.0602 $(M-H)^-$	271.0609 $(M-H)^-$
Calcd.	271.0606 for $C_{15}H_{11}O_5$	271.0606 for $C_{15}H_{11}O_5$
Molecular formula	$C_{15}H_{12}O_5$	$C_{15}H_{12}O_5$
UV λ_{max} nm (log ε)		
in MeOH	224 (4.42), 253 (4.42), 276 (4.61)	241 (4.59), 283 (4.30)
	326 (3.40), 405 (3.81)	320 (sh, 3.83), 372 (3.68)
IR ν_{max} (KBr) cm^{-1}	3370, 1660, 1620, 1580, 1525, 1475	1665, 1615, 1575, 1545, 1450, 1430
	1435, 1375, 1320, 1270, 1200, 1170	1395, 1375, 1340, 1275, 1255, 1200
	1160, 1110, 1090, 1025, 975, 950	1175, 1155, 1135, 1080, 1025, 980
TLC, Rf Value*		
CH_2Cl_2 - MeOH (95:5)	0.47	0.43

* Merck Kieselgel 60 F_{254} (Art. 5719)

(flavasperone[17,18,20]), respectively. The molecular formulas ($C_{15}H_{12}O_5$) of **1** and **5** determined by high-resolution ESI-MS, and ^1H and ^{13}C NMR spectral data, differed from those ($C_{16}H_{14}O_5$) of **2** and **6** by loss of CH_2 unit. The ^{13}C and ^1H NMR data of **1**, **2**, **5** and **6**, obtained from DEPT, pulsed field gradient (pfg)-HMQC and pfg-HMBC spectra are shown in Tables 2 and 3, respectively. The ^{13}C NMR spectra of **1** and **5** were almost identical to those of **2** and **6**, respectively, except for the absence of one *O*-methyl signal and the observation of upfield shift at C-8 and downfield shift at C-9 in **1** and at C-7 in **5**. In the ^1H NMR spectra of **1** and **5**, additional signals (**1**: δ 10.27 and **5**: δ 10.26) of phenolic hydroxyl protons were observed, which had NOE correlations with aromatic protons at C-7 and C-9 (Fig. 3). From these results along with unambiguous HMBC correlations, the structures of **1** and **5** were determined to be 8-*O*-demethyl analogs of **2** and **6**, respectively.

Table 2. ^{13}C NMR data for **1**, **2**, **5**, and **6** in DMSO-d_6.

Position	1	2	5	6
2	168.3	168.5	167.4	167.6
3	106.5	106.7	109.6	109.7
4	183.5	183.6	182.0	182.1
5	162.2	162.0	155.4	155.1[a]
6	160.5	160.1	104.1	105.0
7	97.4	97.1	101.0	98.2
8	159.9	161.2	159.9	161.2
9	101.0	98.1	97.3	97.0
10	99.7	100.7	159.0	158.5
11	152.4	152.5	155.4	155.7[a]
12	102.8	103.4	107.4	107.8
13	106.5	107.4	140.7	140.6
14	140.8	140.7	103.0	104.0
2-CH_3	20.0	20.0	19.9	19.9
6-OCH_3	55.6	55.8		
8-OCH_3		55.3		55.4[b]
10-OCH_3			55.8	56.0[b]

[a, b] May be exchangeable.

Biological Properties

As shown in Table 4, **1**, **3** and **5** inhibited the IL-4 driven luciferase activity with IC_{50} values of 25 μM, 30 μM, and 1.7 μM, respectively, while **2**, **4** and **6** did not affect the activity up to 100 μM.

To investigate the selectivities of **1**, **3** and **5**, we tested the inhibitory activities of these compounds towards the

Table 3. ^{1}H NMR data for **1**, **2**, **5**, and **6** in DMSO-d_6.

Position	A1 (**1**)	A2 (**2**)	C1 (**5**)	C2 (**6**)
3	6.13 (1H, s)	6.18 (1H, s)	6.45 (1H, s)	6.47 (1H, s)
6			6.77 (1H, s)	6.91 (1H, s)
7	6.41 (1H, d, 2.2)	6.47 (1H, d, 2.2)	6.61 (1H, d, 2.1)	6.84 (1H, d, 2.2)
9	6.63 (1H, d, 2.2)	6.85 (1H, d, 2.2)	6.47 (1H, d, 2.1)	6.53 (1H, d, 2.2)
10	7.01 (1H, s)	7.16 (1H, s)		
2-CH_3	2.36 (3H, s)	2.39 (3H, s)	2.45 (3H, s)	2.49 (3H, s)
6-OCH_3	3.86 (3H, s)	3.88 (3H, s)		
8-OCH_3		3.88 (3H, s)		3.95 (3H, s)[a]
10-OCH_3			3.93 (3H, s)	3.88 (3H, s)[a]
5-OH	14.84 (1H, s)	14.81 (1H, s)	12.86 (1H, s)	12.91 (1H, s)
8-OH	10.27 (1H, brs)		10.26 (1H, brs)	

[a] May be exchangeable.

Fig. 3. HMBC and NOE correlations observed in **1** and **5**.

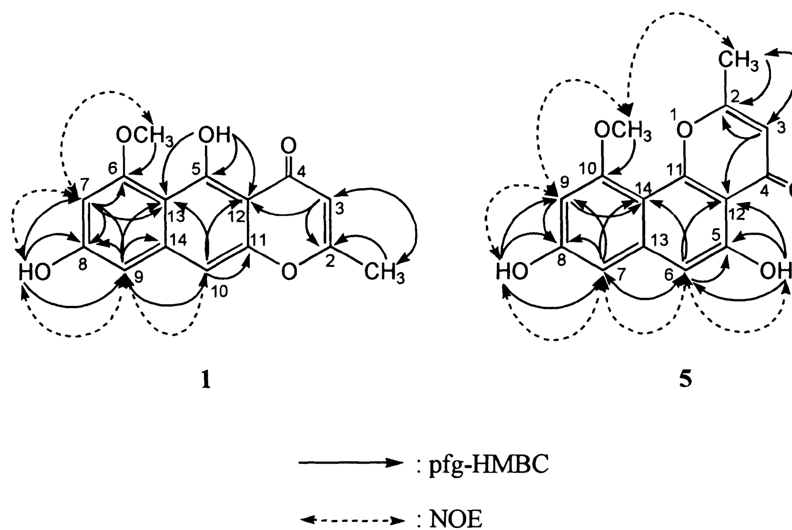


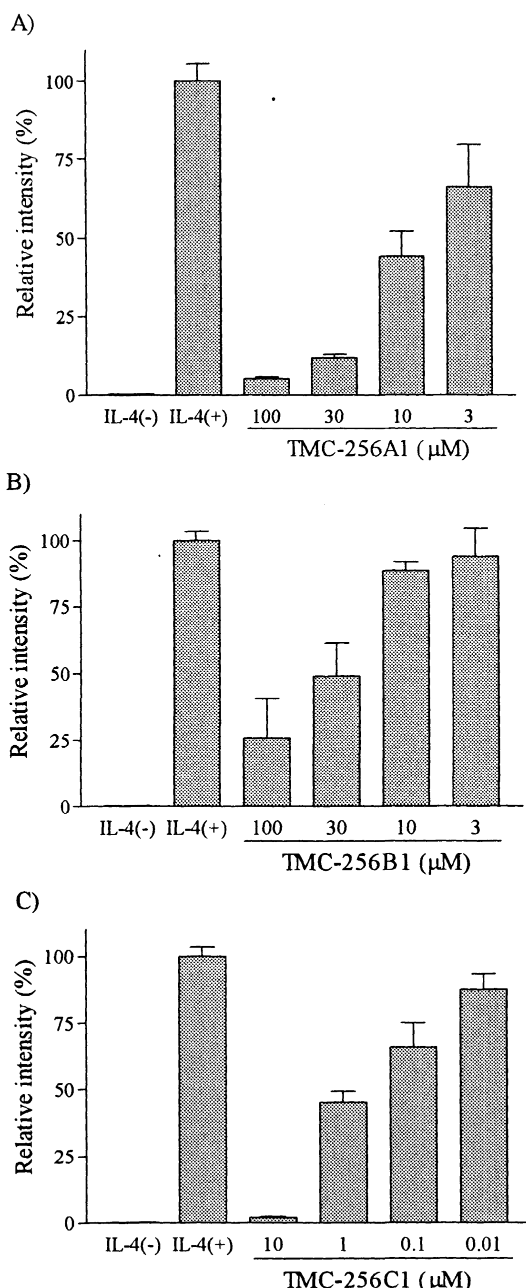
Table 4. Effects of **1**, **2**, **3**, **4**, **5**, and **6** on the IL-4 driven, IFN-γ driven, and SV40 promoter driven luciferase expressions.

compound	IC_{50} (μM)		
	IL-4 / STAT6	IFN-γ / STAT1	SV40
1	25	98	>100
2	>100	>100	ND*
3	30	>100	>100
4	>100	>100	ND*
5	1.7	7.5	>100
6	>100	>100	ND*

*ND: not determined

expression of IFN-γ driven and the constitutive SV40 promoter driven luciferase using HeLa IFN-γ STAT1 (1-26) cells and HeLa H-144 cells, respectively. Compounds **1** and **5** inhibited the IFN-γ driven luciferase activity with IC_{50} values of 98 μM and 7.5 μM, respectively. Compound **3** did not affect the IFN-γ driven luciferase activity up to 100 μM. Furthermore, **1**, **3** and **5** did not inhibit the SV40 driven luciferase activity up to 100 μM. These results suggested that **1**, **3** and **5** were the moderately selective inhibitors of IL-4 signal transduction.

Fig. 4. Effect of **1**, **3** and **5** on induction of germline Cε mRNA expression by IL-4.



DND39 cells were treated with TMC-256A1 (A), TMC-256B1 (B), or TMC-256C1 in the presence of IL-4 (10 ng/ml) for 4 hours. Detection of mRNAs was carried out with quantitative RT-PCR as described Materials and Methods. 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%.

Next, we tested the effect of **1**, **3** and **5** on induction of germline Cε mRNA expression by IL-4 in DND39 cells. As a result, **1**, **3** and **5** dose-dependently inhibited the expression, with IC_{50} values of 6.6 μM, 34 μM, and 0.31 μM, respectively (Fig. 4). This result demonstrated that **1**, **3** and **5** might suppress the production of IgE *via* inhibition of IL-4 signal transduction. Therefore, **1**, **3** and **5** might be useful in the treatment of allergic disease.

Further studies are needed to determine the molecular mechanism of inhibition by **1**, **3** and **5**.

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