VOL. 54 NO. 6, JUNE 2001 THE JOURNAL OF ANTIBIOTICS pp. 489 — 493

F13459, a New Derivative of Mycophenolic Acid

I. Taxonomy, Isolation, and Biological Properties

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(Received for publication November 1, 2000)

In the course of screening for inhibitors of intracellular trafficking of glycoprotein, a new inhibitor, F13459 was isolated from the culture broth of a *Penicillium* sp. It was purified using solvent extraction, silica gel, Sephadex LH-20 and ODS column chromatography. From structural analysis, F13459 was a derivative of mycophenolic acid, an inhibitor of inosine 5′-monophosphate dehydrogenase. F13459 inhibited hemagglutinin synthesis of NDV at concentrations more than 25 μg/ml. However, syncytium formation as a result of cell surface expression of F-glycoprotein of NDV was inhibited at concentrations of F13459 lower than those required for appreciable inhibition of glycoprotein synthesis.

Glycoproteins destined to the cell surface are synthesized on the ER membrane and translocated to the cell surface *via* the Golgi.[1] Intracellular trafficking of glycoprotein is mediated by vesicles. Many essential components in glycoprotein trafficking have been revealed using biochemical and genetic approaches.[2] Specific inhibitors of intracellular trafficking, such as brefeldin A, have also contributed in analyses of glycoprotein trafficking.[3]

To find inhibitors of intracellular trafficking of glycoproteins, we have searched for those affecting cell surface expression of viral glycoproteins and reported that brefeldin A,[4] concanamycin A,[5] leucinostatin A,[6] and effrapeptins[7] inhibit intracellular trafficking of viral glycoproteins. In continued screening, we have discovered a new inhibitor which we have named F13459. From structural analysis described in an accompanying paper,[8] F13459 is 3,4-dihydro-3,4,6,8-tetrahydroxy-3-methyl-1*H*-2-benzopyran-1-one 4-*O*-mycophenolate, a derivative of mycophenolic acid. The structures of mycophenolic acid (**1**) and F13459 (**2**) are shown in Fig. 1. In this paper, the taxonomy of the producing strain, isolation and biological properties of F13459 are described. The physico-chemical properties and structure elucidation of F13459 are described in the accompanying paper.[8]

Materials and Methods

Materials

Penicillium sp. F13459 was isolated from a soil sample and was used for production of F13459. The strain was deposited at the National Institute of Bioscience and Human-Technology, Japan, and at the Japan Collection of Microorganisms, RIKEN, under the accession numbers FERM P-18077 and JCM 10949, respectively. Newcastle disease virus (NDV, the Miyadera strain) was obtained from

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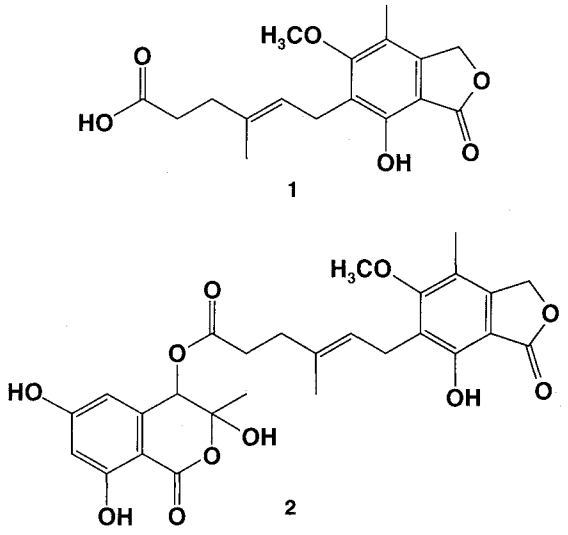
Fig. 1. Structures of mycophenolic acid (**1**) and F13459 (**2**).



National Institute of Animal Health of Japan. Silica gel 60 (Merck, Darmstadt, Germany), Sephadex LH-20 (Pharmacia biotech, Uppsala, Sweden), and Senshu Pak ODS-N (Senshu Sci. Co., Tokyo, Japan) were used for column chromatographies.

Taxonomic Studies

The identification procedure and system used for the *Penicillium* strain were basically those of PITT.[9,10] RAMIREZ[11] was also referred to as a guide. Colors of morphological structures and colonies were determined using the charts of KORNERUP and WANSCHER[12] (showing numeric-alphabetic-numeric codes in the form 26A2). For light microscopy, a Nikon Biophot microscope was used with differential interference contrast (DIC). For scanning electron microscopy (SEM), sporulating material from agar media was fixed and dehydrated using the methods of NAKAGIRI.[13] After critical-point drying with a Hitachi HCP-2, the materials were coated with Pt-Pd (*ca.* 100~200 Å thick) in an Eiko ion coater (IB-3) and observed with a Hitachi scanning electron microscope (S-2400) at 20 kV. Microphotographs were taken using digital camera systems under DIC and SEM.

Fermentation

Strain F13459 was inoculated from agar slants into twenty 3000-ml flasks containing 500 ml of a medium consisting of 5% glucose, 0.5% yeast extract, 0.05% KCl, 0.05% $MgSO_4$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4 \cdot 7H_2O$, and 0.0005% $CuSO_4 \cdot 7H_2O$. The inoculated flasks were incubated as stationary phase cultures at 25°C for 14 days.

Syncytium Formation and Hemagglutinin Titration of NDV-infected Cells

Baby hamster kidney (BHK) cells were cultured using Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% newborn bovine serum (JRH Biosciences, Leexa, KS) at 37°C. BHK cells in 96-well culture plates (Corning, NY) were infected with NDV at a hemagglutination unit (HAU/ml), and samples were added at 1 hour after infection. Syncytium formation of the cells incubated for 18 hours at 37°C was observed under an optical microscope. Cells were disrupted by sonication, and HAU was determined as described previously.[5,6]

Results

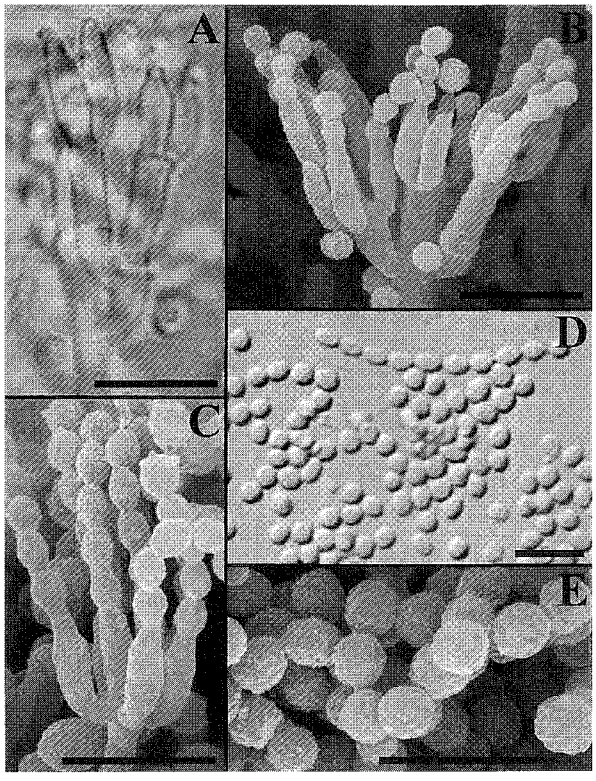
Taxonomy of the Strain F13459

Cultural and morphological characteristics of *Penicillium* strain F13459 are described below with Fig. 2 A~E.

Colonies on Czapek Yeast Extract Agar (CYA) for 7 days at 25°C were 17~21 mm in diameter, radially sulcate, protuberant at the center, with velutinous texture, and dull green (26E4-27E4) with white edges; soluble pigment was not produced; the reverse side was dark blonde (5D4) and radially sulcate. Colonies on Malt Extract Agar (MEA) for 7 days at 25°C were 12~14 mm in diameter and almost the same to those on CYA, but showing much restricted growth; soluble pigment was not produced; the reverse side was clay (5D5) and radially sulcate. Colonies on 25% Glycerol Nitrate Agar (G25N) for 7 days at 25°C were 14~17 mm in diameter, dull green (26E3-27E4) with broad white edges, and similar to those on CYA; soluble pigment was not produced; the reverse side was champagne (4B4) and radially sulcate. Microcolonies/colonies at 5°C were up to 5~7 mm in diameter on CYA, 4~6 mm in diameter on MEA, and 0 mm on G25N (conidia germinated, but did not grow). Colonies did not grow at 37°C on all media (conidia did not germinate).

Conidiophores on CYA were usually short, sometimes long, smooth-walled under SEM, sometimes very finely granulate under DIC, and bearing compact biverticillate (rarely terverticillate) penicilli; metulae were in divergent clusters and 9~14×2~3.2 μm. Phialides on CYA were usually ampulliform (7.5~12×2.5~3.2 μm) and sometimes cylindrical (12~15×2~3 μm). Conidia on CYA were

Fig. 2. *Penicillium* sp. F13459 on CYA.



A, B. Biverticillate (B) or terverticillate (A) penicilli. C. Ampulliform phialides producing ellipsoidal to subspherical conidia. D, E. Ellipsoidal, subspherical or spherical conidia with smooth (D) to finely rough (E) wall. DIC: A, D. SEM: B, C, E.
Scale bar: 10 μm in A~E.

ellipsoidal, subspherical or spherical, smooth-walled under DIC, smooth to finely rough-walled under SEM, 2.5~4.5× 2.5~3.8 μm, and borne in divergent short chains.

Isolation

The isolation procedure is outlined in Fig. 3. The culture whole broth was filtered through gauze to obtain a mycelial cake, which was extracted with 10 liter of acetone. The extract was evaporated *in vacuo* to remove acetone and then extracted with the same volume of EtOAc 3 times. The EtOAc extract was concentrated *in vacuo* and applied to a column of silica gel (50 g). The column was washed with benzene - MeOH (100 : 1) and eluted with benzene - MeOH (100 : 3) and (100 : 5). The eluted fractions were concentrated *in vacuo* and applied to a column of Sephadex LH-20. The column was eluted with MeOH and the eluted fractions were evaporated to give mycophenolic acid and crude F13459. 55 mg of F13459 was isolated by HPLC separation (Senshu-Pak ODS-N) as a pale brown oil.

Biological Properties

The minimum inhibitory concentration for the inhibition of syncytium formation was 0.4 and 3.2 μg/ml for mycophenolic acid and F13459, respectively (Table). Both antibiotics exerted a strong inhibition of viral glycoprotein synthesis, and more than 80% inhibition of glycoprotein synthesis expressed as HAU was observed with mycophenolic acid and F13459 at concentrations higher than 1.6 and 12.5 μg/ml, respectively. Therefore, the inhibition of syncytium formation at high concentrations is caused by inhibition of F-glycoprotein synthesis because syncytium formation depends on glycoprotein synthesis. Experiments with cycloheximide, a potent inhibitor of protein synthesis, indicate that glycoprotein synthesis needs to be inhibited by greater than 80% to prevent syncytium formation (data not shown). Therefore, the inhibition of syncytium formation at lower concentrations seems to be a result of their action other than *via* protein synthesis inhibition.

Mycophenolic acid is a potent inhibitor of inosine 5′-monophosphate dehydrogenase, a key enzyme in the synthesis of guanosine. The inhibition of syncytium formation and glycoprotein synthesis by mycophenolic acid could be reversed by the addition of guanosine (Table), indicating that these actions of mycophenolic acid are caused by the inhibition of guanosine synthesis. F13459 is a mycophenolic acid derivative having 3, 4, 6-trihydroxymelleine covalently bound through an ester linkage, and both are cleaved by a treatment with 0.1 N KOH/MeOH for 60 minutes at room temperature. Only the mycophenolic acid moiety exerted activity on NDV-infected cells, suggesting that this moiety is responsible for the action of F13459. In fact, the action of F13459 was lost in the presence of guanosine.

Discussion

In the course of screening for inhibitors of intracellular translocation of glycoprotein, we discovered a new compound, F13459, from the fermentation broth of a *Penicillium* sp.

General features of the morphology of strain F13459 are similar to those of *Penicillium brevicompactum* DIERCKX described by PITT.[9,10] Under SEM, similar rough-walled conidia were observed in *P. brevicompactum*.[11] However, characteristic terverticillate penicilli and soluble pigment

Fig. 3. Isolation procedure for F13459.

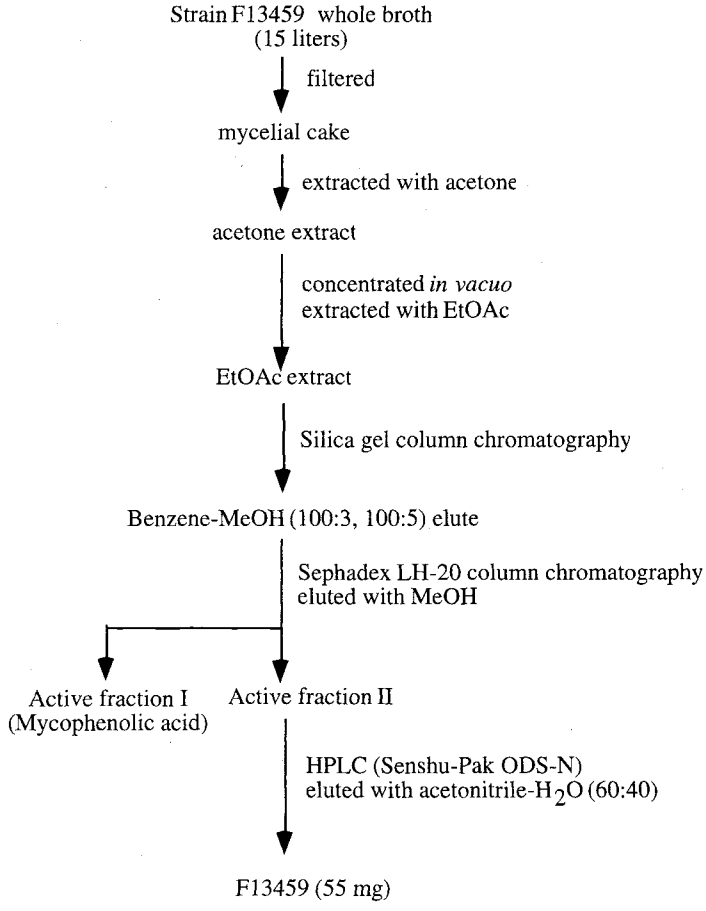


Table Effect of mycophenolic acid and F13459 on syncytium formation (SF) and hemagglutinin synthesis of NDV-infected BHK cells.

	Conc. (μg/ml)	50.0	25.0	12.5	6.25	3.2	1.6	0.8	0.4	0.2
Mycophenolic acid	SF	-	-	-	-	-	-	-	+/-	+
	%HAU	3	5	5	5	6	13	43	100	100
Mycophenolic acid	SF	+	+	+	+	+	+	+	+	+
+5 mM Guanosine	%HAU	100	100	100	100	100	100	100	100	100
F13459	SF	-	-	-	-	-	+	+	+	+
	%HAU	7	7	13	25	100	100	100	100	100
F13459	SF	+	+	+	+	+	+	+	+	+
+5 mM Guanosine	%HAU	100	100	100	100	100	100	100	100	100

BHK cells were infected with NDV. Indicated concentrations of mycophenolic acid or F13459 were added at 1 h after infection and cells were incubated for 18 h at 37°C in the absence or presence of 5 mM guanosine. Effects on SF and virus glycoprotein synthesis as quantified by HAU titration were determined. The degrees of SF are expressed as follows: -, no; +/-, slight; and +, severe.

production were not observed with strain F13459. Discrepancies in the description of a few characteristics of *P. brevicompactum* (*e.g.*, conidiophore surface structure), were found between PITT[9,10] and RAMIREZ.[11] For exact species identification of the strain F13459, further investigations are required using some authentic strains of *P. brevicompactum* and related species. In this report, therefore, we tentatively describe the strain only as a *Penicillium* sp.

F13459, 3,4-dihydro-3,4,6,8-tetrahydroxy-3-methyl-1*H*-2-benzopyran-1-one 4-*O*-mycophenolate, is a derivative of mycophenolic acid. A mycophenolic acid derivative, mycophenolate mofetil, is currently used as an immunosuppressive agent for organ transplant patients.[14,15] However, few naturally occurring derivatives of mycophenolic acid have been isolated so far. F13459 represents a new derivative of mycophenolic acid.

Mycophenolic acid is a potent inhibitor of guanosine synthesis at the level of inosine 5′-monophosphate dehydrogenase.[16] Inhibition by F13459 was completely reversed by addition of guanosine. In addition, after alkaline treatment, only the mycophenolic acid fraction had inhibitory activity. These results indicate the action of F13459 is the same as the action of mycophenolic acid. At lower concentrations of mycophenolic acid and F13459 not showing an appreciable inhibition of glycoprotein synthesis, syncytium formation as a result of cell surface expression of F-glycoprotein of NDV was inhibited. Some GTP binding proteins are known to participate in regulation of glycoprotein trafficking.[17] Further biological studies of F13459 on intracellular trafficking of glycoproteins are currently being undertaken.

Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and grants for Bioarchitect Research Program and Multibioprobe Research Program from RIKEN.

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