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A New Anti-influenza Virus Antibiotic, 10-Norparvulenone from *Microsphaeropsis* sp. FO-5050

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Influenza virus infection is widespread and sometimes critical for patients with respiratory diseases, immunosuppressive syndromes such as AIDS, aged persons with cardiopulmonary diseases and so on[1]. Although several anti-influenza virus agents such as amantadine, rimantadine and zanamivir have been used, they are not always effective. The side-effects and rapid development of viral resistance cause limited medical application[2,3]. For these reasons, new effective anti-influenza viral drugs are required. Recently, we screened anti-influenza virus compounds from culture broths of actinomycetes and fungi. The screening was carried out by estimating the inhibition of the replication of influenza virus A/PR/8/34 in Madin-Darby canine kidney (MDCK) cells, and we isolated a new antibiotic, 10-norparvulenone (**1**, Fig. 1).

In this paper, we report the fermentation, isolation, physico-chemical properties, structure determination and biological activities of **1**, a new inhibitor of influenza virus replication. The taxonomy of the producing microorganisms was previously reported[4].

Materials and Methods

Materials

2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-NeuAc) was purchased from Sigma Co. Ltd. Influenza virus A/PR/8/34 was grown in allantoic sacs of 10-day-old embryonated eggs for 48 hours at 34°C.

Antimicrobial Activity

Antimicrobial activity was tested for 14 species of microorganisms. Agar plates inoculated with each microorganism and on which were placed 6 mm paper disks containing 10 μg of test samples were incubated for 24~48 hours at 27°C or 37°C, as appropriate. The antimicrobial activity was determined by the diameter of the inhibitory zone.

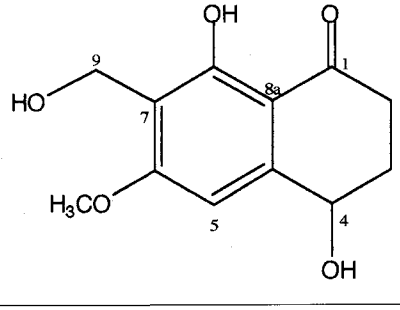
Anti-influenza Virus Activity[5]

Confluent monolayers of MDCK cell cultures in 96-well culture plates (Becton Dickinson) were infected with mouse-adapted influenza virus A/PR/8/34 at a multiplicity of infection of 0.0035 PFU/cell in 0.25 ml of EAGLE's minimun essential medium containing 0.2% bovine serum albumin, acetyltrypsin (3 μg/ml) and antibiotics (maintenance medium). Then the sample solution (10 μl) was added to the well of the culture plate. The plate was incubated at 37°C for 72 hours under 5% CO_2 atmosphere. Then the monolayers in the culture plate were separated from the medium, washed with PBS, pH 7.4, to remove the dead cells resulting from infection by the influenza virus. The viable cells were determined by a colorimetric method which is based on the *in situ* reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells. Anti-influenza virus activity was also estimated by the determination of virus in the medium as sialidase activity.

Sialidase Assay

The sialidase activity of influenza virus was assayed using modification of a previously described method[5]. In brief, the reaction mixture containing 0.1 mM 4-MU-NeuAc and mouse-adapted influenza virus A/PR/8/34 (which contains a sialidase) in citrate-phosphate buffer (pH 7.0) was incubated at 37°C for 10 minutes in a 96-well

Fig. 1. Structure of 10-norparvulenone (**1**).



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microtiter plate, and the released 4-methyumbelliferone was determined from fluoresence intensity (ex. 355 nm, em. 460 nm, Labsystems Fluoroskan II, Flow Laboratories Inc.).

Spectroscopic Studies

The melting point was measured using a Micro Melting Point Apparatus (Yanaco, Japan). The UV spectrum was recorded on a Hitachi U-2000 spectrophotometer. The IR spectrum was recorded on a Horiba FT-210 FT-IR spectrometer. Mass spectra were recorded on JMS-DX300 and JMS-AX505 HA mass spectrometers. NMR spectra were obtained on a Varian UNITY INOVA 600 MHz NMR Spectrometer System.

Results and Discussion

Fermentation

A 100-μl portion of stock spore suspension of *Microsphaeropsis* sp. FO-5050 (2×10^6/ml) was inoculated into a 50-ml test tube containing 10 ml of seed medium [glucose 2.0%, yeast extract 0.2%, $MgSO_4\cdot7H_2O$ 0.05%, Polypepton (Nippon Seiyaku) 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0]. The tube was shaken on a reciprocal shaker for 3 days at 27°C. One milliliter of the seed culture was transferred into each Erlenmeyer flasks containing 100 ml of the production medium consisting of soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4\cdot7H_2O$ 0.05% and KH_2PO_4 0.05%, pH 6.5. The fermentation was carried out at 27°C for 4 days on a rotary shaker (210 rpm).

Isolation

The whole broth of *Microsphaeropsis* sp. FO-5050 (6 liter) was extracted with the same volume of EtOAc. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give a brown oil. The oil was chromatographed on a silica gel column ($CHCl_3$-MeOH, 20:1) and an ODS MPLC column (C.I.G. Column System, i.d. 22×100 mm; detection, UV at 210 nm; flow rate 9 ml/minute; solvent system, CH_3CN-H_2O, 20:80). Compound **1** was finally purified by HPLC (Senshu Pak Pegasil-B ODS, i.d. 20×250 mm; detection UV at 210 nm; flow rate, 8 ml/minute; solvent system, CH_3CN-H_2O, 35:65), giving **1** as a white powder (3.2 mg).

Structure Determination

Physico-chemical properties of **1** are shown in Table 1. The molecular formula of this compound was determined to be $C_{12}H_{14}O_5$ by HR-EI-MS [*m/z* 238.0851 (M^+), error +1.0 mmu]. The IR spectrum indicated the presence of hydroxyl (3378 cm^{-1}) and carbonyl (1618 cm^{-1}) groups. ^1H and ^{13}C NMR spectral data in CD_3OD and DMSO-d_6 are summarized in Table 2. In the ^{13}C NMR spectrum, twelve signals were detected, and they were assigned to one methyl (C-δ 56.76), three methylene (δ 32.98, 36.28 and 56.76), two methine (δ 68.93 and 102.13) and six quarternary (δ 111.38, 115.99, 151.37, 163.98, 166.10 and 204.63) carbons by DEPT experiments. The direct connection of protonated carbons and the protons were assigned by ^{13}C-^1H COSY as shown in Table 2, and the partial structure of **1** was constructed by HMBC and ^1H-^1H COSY experiments as shown in Fig. 2. Three broad signals (δ 4.58, 5.80 and 13.1) observed in the ^1H NMR spectrum in DMSO-d_6 disappeared on addition of D_2O. These results indicated the presence of three hydroxyl groups. Therefore C-8 and C-8a should be connected through a double bond. The chemical shift of 8-OH (δ_H 13.10) indicated formation of a hydrogen bond to the C-1 carbonyl oxygen, which confirmed the

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

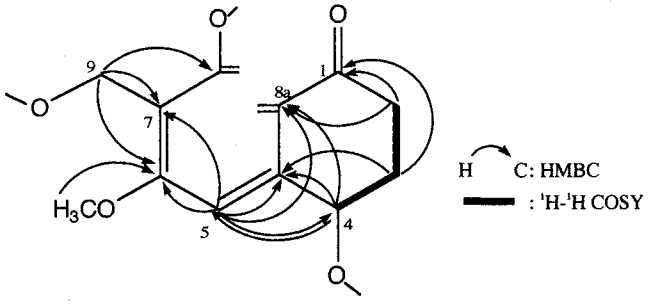
Appearance	White powder
Melting point (°C)	149-151
Molecular formula	$C_{12}H_{14}O_5$
FAB-MS	239 $(M+H)^+$, 237 $(M-H)^-$
HR EI-MS (m/z)	Found 238.0851 ($C_{12}H_{14}O_5$)
	Calcd. 238.0841
$[\alpha]_D^{25}$	-22.9°(*c* 0.5, MeOH)
UV λ max nm (log ε) (MeOH)	225 (4.61), 240 (sh, 4.41), 282 (4.50), 319 (4.12)
IR υmax (KBr) cm^{-1}	3378, 3205, 1618, 1209, 1215,1147, 1082, 991, 841

Table 2. NMR assignment of **1**.

position	δ_C (mult.)	δ_H (No. of H, mult., *J* [Hz])
1	204.63 (s)	
2	36.28 (t)	2.65 (1H, ddd, 17.7, 10.5, 5.0)
		2.81 (1H, ddd, 17.7, 6.1, 4.7)
3	32.98 (t)	2.04 (1H, m)
		2.28(1H, m)
4	68.93 (d)	4.80 (1H, dd, 9.1, 3.9)
4a	151.37 (s)	
5	102.13 (d)	6.87 (1H, s)
6	166.10 (s)	
7	115.99 (s)	
8	163.98 (s)	
8a	111.38 (s)	
9	53.10 (t)	4.66 (2H, s)
6-OCH_3	56.76 (q)	3.95 (3H, s)
4-OH		5.80* (1H, brs)
8-OH		13.10* (1H, brs)
9-OH		4.58* (1H, brs)

^{13}C (125 MHz) and ^1H (600 MHz) NMR, δ from TMS in CD_3OD.
*: ^1H NMR (270 MHz), δ from TMS in DMSO-d_6.

Fig. 2. HMBC and ^1H-^1H COSY correlations of **1**.

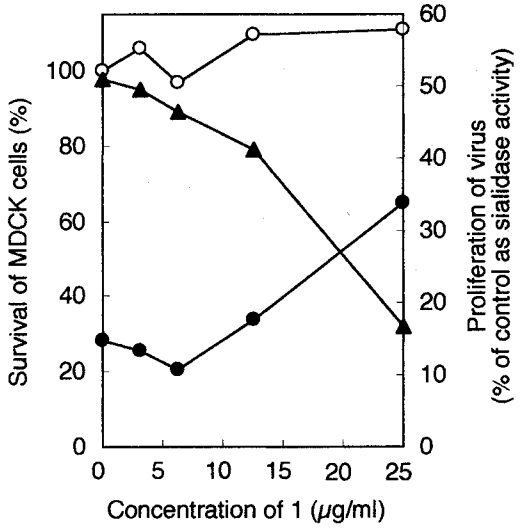


structure.

Thus the structure of the anti-influenza virus compound was determined to be 3,4-dihydro-4,8-dihydroxy-7-hydroxymethyl-6-methoxy-1(2*H*)-naphthalenone (**1**) and was named 10-norparvulenone. Although compound **1** is closely related to parvulenone[6] and *O*-methylasparvenone[7], previously isolated from *Aspergillus parvulus*, the compounds differ in the side chains. Since the optical rotation of *O*-methylasparvenone (+22° in methanol) and 10-norparvulenone (−22.9° in methanol) are quite similar in magnitude but opposite in sign and structural difference is remote from the chiral center, it

Fig. 3. Effect of **1** against infection of influenza virus on MDCK cells.

MDCK cell monolayers were infected with mouse-adapted influenza virus A/PR/8/34 at a MOI of 0.0035 PFU/cell and solutions of **1** were added to the wells. The cells were incubated for 72 hours in the presence of **1**. Viable cells were determined by MTT assay on infected (●) or mock-infected (○) conditions, and supernatant virus was quantified by its sialidase activity (▲).



seems that their absolute configurations are opposite. As the absolute configuration of *O*-methylasparvenone was reported to be *S*[8], the absolute configuration of 10-norparvulenone would be *R*. *O*-methylasparvenone was reported as a nitrogen-free serotonin antagonist[8].

Biological Activities

Compound **1** showed no antimicrobial activity at 10 μg/disk using the paper disk method against *Bacillus subtillis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Xanthomonas campestris* pv. *oryzae*, *Bacteroides fragilis*, *Acholeplasma laidlawii*, *Pyricularia oryzae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans* and *Saccharomyces cervisiae*.

The effect of **1** on replication of mouse-adapted influenza virus A/PR/8/34 in MDCK cells was studied by inoculating monolayers in wells of plastic plates and is shown in Fig. 3. In the absence of **1**, only 27.2% of the cells survived from the viral infection in comparison with the mock-infected solvent control. In the presence of **1** (25 μg/ml), 64.8% of

cells survived compared with the mock-infected solvent control. Dose dependent reduction of viral sialidase activity was also observed in the presence of **1** with 50% inhibitory dose at 21 μg/ml. These results indicate that **1** inhibits the replication of influenza virus A/PR/8/34 in MDCK cells. Compound **1** showed no effect on the viability of MDCK cells at 25 μg/ml by MTT assay.

The structure of **1** is different from that of known antiviral agents[9], such as amantadine, rimantadine and zanamivir, which are derivatives of adamantane or nucleic acid. Therefore, **1** is expected to be a new type of anti-influenza virus drug. Determination of the *in vivo* effects of **1** is now underway.

Acknowledgement

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