YM-92447 (Spinosulfate A), a Neuraminidase Inhibitor Produced by an Unidentified Pycnidial Fungus

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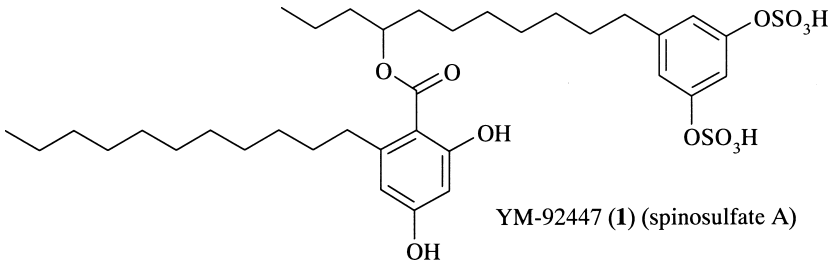
Neuraminidase (EC 3.2.1.18, exo-α-sialidase, α-*N*-acetylneuraminosyl glycohydolase) is a glycosidase, which catalyzes the cleavage of the α-2,3- and α-2,6-ketosidic linkages between terminal sialic acid and adjacent galactose on glycoproteins and glycolipids[1]. Influenza virus neuraminidase is located on the surface of virus particles and plays an important role in the elution of newly formed viruses from infected cells by digesting sialic acids in the haemagglutinin receptor[2,3] and the maintenance of mobility of progeny viruses by the prevention of self-aggregation[4]. Neuraminidase may also assist in the movement of viruses through the mucous lining of the respiratory tract, enhancing viral infectivity[3,5]. Therefore, neuraminidase has been thought to be a promising target for antiviral agents against influenza viruses[6]. Now zanamivir and oseltamivir, neuraminidase inhibitors, are approved for treatment of influenza and effective against both influenza A and B viruses[7~9]. Although the emergence of viruses resistant to these drugs is less frequent at present, increasing use may make it more common[10]. Therefore, we tried to screen neuraminidase inhibitors with a new class of structure.

In the course of our screening program for neuraminidase inhibitors from microbial metabolites, we found a hydroxyphenylundecane derivative, YM-92447 (spinosulfate A, **1**), from the culture extract of an unidentified pycnidial fungus. Herein, we report fermentation, isolation, and biological activity of **1** with brief description of the producing strain.

The strain (JCM 12827, =G. Okada OFC 4343, =Yamanouchi Q29340) was isolated from a yellowish pycnidium of an unidentified coelomycete growing on dead branches of a tree (probably *Cornus controversa* Hernsl.) collected in Sugadaira, Nagano Pref., Japan in 5 July 1993. The dried herbarium specimen was deposited in the Kanagawa Prefectural Museum of Natural History (KPM-NC0012304; =G. Okada GO 1530). The strain exhibited good growth at 24°C on potato dextrose agar (PDA). Colonies growing on PDA were floccose, velvety, and grayish white to yellowish orange. The fungus abundantly formed pycnidia in the center of the colonies. Pycnidia were yellowish brown to orange in color and mostly globose to subglobose with a single ostiole. Conidiogenous cells were phialides rising from the cells of pycnidial inner wall, flask-shaped, and 8~12 μm long. Conidia were single-celled, hyaline, smooth, ellipsoidal, and 4~6× 1.5~2.5 μm. As the taxonomical position of this strain is unclear at the moment, we treat it in this paper as an unidentified pycnidial fungus JCM 12827. Further taxonomic studies are now in progress on this fungus.

Loopful of mycelia of the strain grown on an agar slant

Fig. 1. Structure of YM-92447 (**1**), identical with spinosulfate A.



YM-92447 (**1**) (spinosulfate A)

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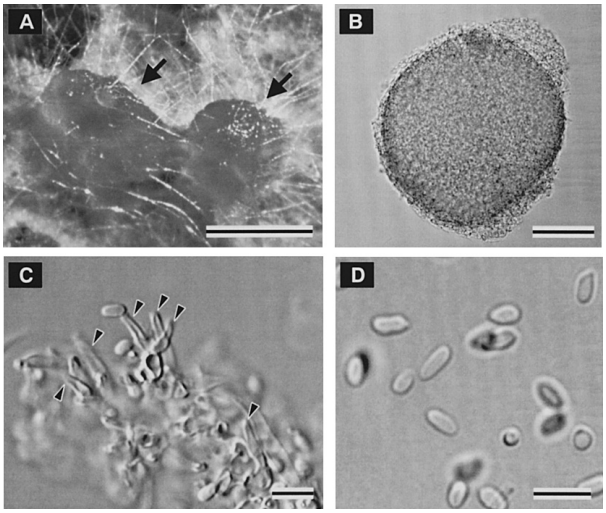
Fig. 2. Optical micrographs of an unidentified pycnidial fungus JCM 12827 on PDA.

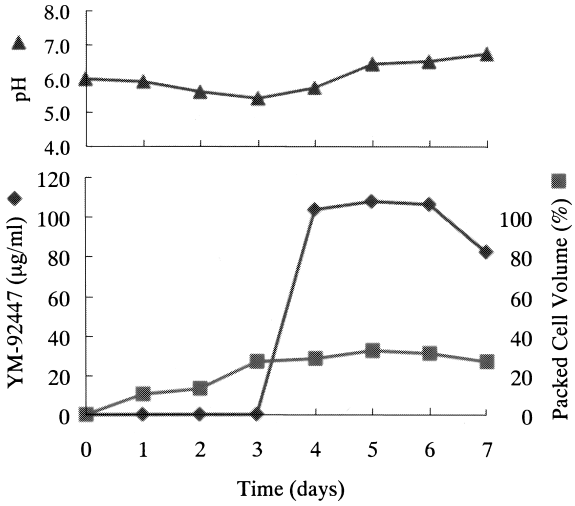


A. Pycnidia (arrows) under stereomicroscope. B. Pycnidium. C. Phialides (arrowheads). D. Conidia. Bars in A = 1 mm, in B = 100 μm, in C and D = 10 μm.

was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of seed medium consisting of glucose 1%, potato starch 2%, yeast extract 0.5%, Polypepton (Nihon Pharmaceuticals) 0.5% and $CaCO_3$ 0.4%. The pH of the medium was adjusted to 7.0 before sterilization. The seed culture was incubated at 24°C for 72 hours on a rotary shaker at 200 rpm. Two ml of the seed culture was transferred to each of fifty 500-ml Erlenmeyer flasks containing 100 ml of production medium consisting of glucose 1%, sucrose 2%, corn steep liquor 1%, meat extract 0.5%, agar 0.1%, $CaCl_2$ 0.000055%, $CaCO_3$ 0.3%, $CoCl_2 \cdot 6H_2O$ 0.000016%, $FeSO_4 \cdot 7H_2O$ 0.000016%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $MnCl_2 \cdot 4H_2O$ 0.00005%, and $ZnSO_4 \cdot 7H_2O$ 0.000022%. The pH of the medium was adjusted to 6.0 before sterilization. After the inoculation the flasks were incubated at 24°C for 6 days at 200 rpm. Typical time course of the fermentation is shown in Fig. 3. The growth of the strain reached a plateau after 3-day cultivation. The production of **1** was monitored by HPLC analysis under the following conditions: column, L-column ODS (4.6×250 mm, Chemicals Evaluation and Research Institute); mobile phase, 80% MeCN in 10 mM ammonium acetate buffer, pH 5.5; flow rate, 1 ml/minute; detection, UV 210 nm. The production started at the early stationary phase and immediately reached the maximum. **1** was mainly obtained from the mycelium fraction.

The mycelium was obtained from 5 liters of the culture broth by filtration and then extracted with 80% aqueous

Fig. 3. Typical time course of production of YM-92447.



acetone. The acetone extract was separated from the mycelium by filtration and concentrated to remove acetone *in vacuo*. The aqueous solution was adjusted to pH 8.0 with 1 N NaOH and extracted twice with EtOAc. The aqueous layer was further extracted with *n*-BuOH. The organic layer was concentrated to dryness *in vacuo*. The *n*-BuOH extract was subjected to silica gel flash chromatography (Kieselgel 60, 0.040～0.063 mm, MERCK), and eluted with a step

Fig. 4. Lineweaver-Burk plots of inhibition of virus neuraminidase by YM-92447.

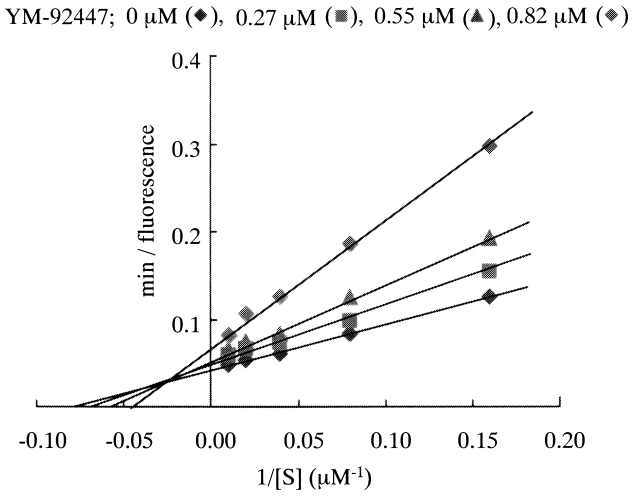


Table 1. Inhibition of neuraminidases by YM-92447 and Neu5Ac2en.

Enzymes	IC_{50} (μM)	
	YM-92447	Neu5Ac2en[a)]
influenza virus A/WSN/33	0.5	0.9
Clostridium perfringens	3	11

[a)] 2-deoxy-2,3-dehydro-N-acetylneuraminic acid

gradient of $CHCl_3$ - MeOH. The active fraction eluted with $CHCl_3$ - MeOH (1 : 1) was evaporated to dryness (1.1 g). The residue was subjected to ODS flash chromatography (YMC-GEL ODS-A, 120-130/70, YMC) and eluted with a step gradient from 50% to 100% MeOH. The active fraction eluted with 90% MeOH was evaporated to dryness (169 mg). The aliquot of the residue was further purified by preparative HPLC on L-column ODS, eluted with 80% MeCN in 10 mM ammonium acetate buffer, pH 5.5 and desalted by HP-20 (Mitsubishi Chemical) to yield 20 mg of **1**. By the analysis of the spectral data including ^{1}H NMR, ^{13}C NMR, UV and MS, the structure was determined to be identical with spinosulfate A[11], which was recently reported as an inhibitor of spinophillin and protein phosphatase-1 interaction.

Influenza virus A/WSN/33 (H1N1) was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs and purified by linear sucrose gradient centrifugation. Neuraminidase from *Clostridium perfringens* was purchased from Sigma Chemicals. Neuraminidase activity was determined by the fluorometric assay described by POTIER *et al.*[12] and HOLZER *et al.*[13] with some modifications. The reaction mixture contained 25 μM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (Sigma Chemicals), 50 mM sodium acetate buffer (pH 5.5), 6 mM $CaCl_2$, neuraminidase and a test compound in a total volume of 200 μl. The mixture was incubated at 37°C for 30 minutes and the reaction was terminated by the addition of 50 μl of stop solution (400 mM sodium monochloroacetate, 120 mM sodium acetate and 280 mM acetic acid). The fluorescence of released 4-methylumbelliferone was measured using an MTB-32 (Hitachi) microplate reader (excitation wavelength, 365 nm; emission wavelength, 450 nm).

Inhibitory activity by **1** is shown in Table 1, contrasted with that of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en)[13]. **1** inhibited the neuraminidases from influenza virus A/WSN/33 and *Clostridium perfringens* with IC_{50} values of 0.5 and 3 μM, respectively. Kinetic studies were carried out to determine the mechanism of viral neuraminidase inhibition by **1**. The Lineweaver-Burk plots showed that **1** inhibited the neuraminidase in a mixed manner. The *Ki* and *Km* values were calculated to be 0.7 and 13 μM, respectively. Cytotoxicity against HeLa S3 cells was determined by a cell counting kit (Wako Pure Chemical Industries, Ltd.). HeLa S3 cells were exposed to the compound for 3 days at 37°C. The cytotoxicity of **1** was an IC_{50} of 77 μM and 10^2-fold weaker than its inhibitory activity against neuraminidase.

This is the first report of spinosulfate A (YM-92447) as an inhibitor of neuraminidase.

Panosialins, also microbial metabolites[14] have been reported as neuramindase inhibitors. Panosialins and YM-92447 contain similar structure in sulfated benzenediol and alkyl chain. They might inhibit neuraminidases *via* the same mechanism. Panosialins also have been reported to show inhibitory activity against various glycosidases[14]. Studies on the activity against these glycosidases by YM-92447 are in progress.

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