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Isolation and Properties of Antitumor Antibiotic YS-1649 from *Penicillium* sp. strain 1649

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An antitumor antibiotic named YS-1649 was isolated from the culture filtrate of a newly isolated fungus identified as *Penicillium sp.*. The fermentation yield reached about 40 mg per liter of the broth. YS-1649, a γ-lactone-structured antibiotic, has the molecular fomular of $C_7H_6O_4$. Its structure was determined to be patulin by spectral analysis. It is active against some bacteria and showed cytotoxic effect on the proliferation of human breast cancer cell line, MCF-7, at concentrations of more than 0.048 μg/ml. This compound also showed strong cytotoxic effect on the proliferation of human cancer cell lines, A549 and ACHN.

Several antitumor antibiotics from natural products and synthetic compound which have α-methylene-γ-lactone structure were reported(1, 2, 4, 7, 9, 17). Recently, novel antibiotics such as terrecyclic acid (10), oxaspirol(3), myrocin C(5) and methylenolactocin(12) were found from the culture filtrate of microbial sources. These compounds have antitumor activity and the activity was lost by the addition of glutathione to the microbial culture broth. Also α,β-unsaturated-γ-lactone, such as sultricin (11), exhibited potency *in vivo* antitumor activity against p388 and L1210 leukemias.

In our continuing search for new antitumor antibiotics having α,β-unsaturated-γ-lactone from microbial metabolites, we found that *Penicillium* sp. strain 1649 produces an active substance after being isolated from the soil picked up in a farm yard near Chunchon city.

The assay for antitumor antibiotics was based on the Michael addition reaction (6, 8) and the antibiotics whose activity was reversed by the addition of glutathione by the use of a *Bacillus cereus* IFO 3514 as a test organism, was chosen. The chemical investigation revealed that the antibiotic was patulin (13).

The present paper deals with the new screening method of producing organism,the taxonomy of the producing organism,fermentation, isolation, structure determination and biological properties including antitumor activity of YS-1649.

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Key words: Antitumor antibiotic, *Penicillium sp.* YS-1649, patulin lactone compound, glutathione reversal

MATERIALS AND METHODS

Screening Method

Isolation of microorganisms was carried out using a medium composed of the following in 1000 ml of distilled water; glucose 30 g; polypeptone 2.0 g; yeast extract 0.5 g; KH_2PO_4 1.0 g; $MgSO_4$ $7H_2O$ 1.0 g; NaCl 0.5 g; $CaCl_2$ $2H_2O$ 0.5 g; $FeCl_3$ $6H_2O$ 2.0 mg and $ZnSO_4$ $7H_2O$ 3.0 mg. It was adjusted to pH 5.5 before sterilization. A loopful of spores from the stock culture of selected mold strain was inoculated in a 500 ml Erlenmyer flask containing of 100 ml liquid medium. The medium composition was the same as above except that 2.5 g of soy bean meal was added instead of 2.0 g of polypeptone being added as nitrogen source. Fermentation was carried out at 28°C for 5 days on a reciprocal shaker. First screening was carried out by determining an antibacterial activity of the culture broth against *Bacillus cereus* IFO 3514 as a test strain.

The selected cultured broths were those showing antibacterial activity. Among them, the strain which showed antibacterial activity and whose activity was reversed by the addition of glutathione was chosen during the second screening. Antibacterial activity was assayed by the paper-disc agar diffusion method and glutathione concentration was found to be 1mg/ml in reversal test.

Taxonomy of the Strain.

Morphological and cultural studies were carried out using the following media.

Czapek's agar ; $NaNO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4$ $7H_2O$

0.5 g, KCl 0.5 g, $FeSO_4$ $7H_2O$ 0.01 g, sucrose 30 g, agar 15 g, distilled water 1000 ml. Potato-dextrose agar ; 39 g of Bacto potato-dextrose agar, distilled water 1000 ml. Malt extract agar ; malt extract 20 g, polypeptone 1 g, glucose 20 g, agar 15 g, distilled water 1000 ml. Sabouraud agar ; glucose 40 g, polypeptone 10 g, agar 15 g, distilled water 1000 ml

After 10 days of incubation at 28°C, observations were made using a microscope.

Fermentation Procedure

A loopful of conidia from *Penicillium sp.* strain YS-1649 was inoculated in 100 ml of the medium in a 500 ml Erlenmyer flask. The medium composition was same as the one used in screening method. Glucose and soybean meal were the best carbon and nitrogen source, respectively. Fermentation was carried out at 30°C for 5 days on a reciprocal shaker at 108 rpm. Scale-up fermentation was carried out by inoculating 50 ml of the seed culture (1% glucose, 0.5% polypeptone, 0.5% yeast extract, 0.003% $FeSO_4$ $7H_2O$) in 2 liter of the same medium in a 5 liter Erlenmyer flask for 5 days at 30°C on a rotary shaker at 167 rpm. Antimicrobial activity occurring during fermentation was assayed by the paper disc agar diffusion method using *Bacillus cereus* IFO 3514 as a test organism.

Physico-chemical Measurements

The melting point was determined on a microscope hot plate and uncorrected. Elementary analysis was carried out by Elemental analyzer Model 240 C (Perkin-Elmer). The IR spectrum was obtained with a Pye Unicam SP-300IR (Philips). The UV spectrum was measured on a U-3210 spectrophotometer (Hitachi). The mass spectrum was obtained on a MS-Engine 5989A mass spectrometer (Hewlett Packard). The ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Unity 300 spectrophotometer (Varian).

Antimicrobial Assay

The diameter of the inhibition zone was determined by the paper disc agar diffusion method, specifically using Boullion agar for bacteria and malt extract agar for fungi. Following the inoculation of the test organism, observations on the bacteria were made after another 18 hours at 30°C whereas fungi were studied after 48 hours at 30°C

Antitumor Activity

To determine the antitumor effect of YS-1649, inhibition of proliferation of MCF-7 (human breast cancer cell line) was examined. The sample solution was serially diluted with phosphate buffer saline (PBS). The media used for the culture of MCF-7 was RPMI-1640 containing 10% fetal bovine serum (FBS). The cell line was precultured in 96-well microplate(7×10^3 cells/well) with 100 μl of RPMI 1640 for 24 hours. This was then added to dilute sample solution with a final volume of 200 μl and cultured under 5% CO_2 at 37°C for 72 hours. Inhibition of the proliferation of the cell line was determined after 48 hours of culture with SRB method (15). After fixing with 10% trichloroacetic acid, cells were stained using 0.4% surforhodamine B after which the dye was extracted from the stained cells with 10 mM Tris (hydroxymethyl)-amino-methane solution. The absorbance of the extracts were read at 540 nm. Antitumor effects against A549 (lung cancer cell line) and ACHN (renal cancer cell line) were also tested. Cancer cell lines used in this study were purchased from Korean Cell Line bank.

RESULTS AND DISCUSSION

Results of Screening Test

Of about 700 strains isolated, 45 strains were active against test organism. Among these strains, 5 strains were chosen because their antibacterial activity was lost by the addition of glutathione and in particular the strain labeled YS-1649 showed potent activity which was selectively reversed by glutathione. Thus,we chose this strain for further studies.

Taxonomy of the Producing Organism

On Czapek's agar, colonies grew to 30~35 mm in diameter after 10 days of incubation at 28°C. The colonies were floccose and white to yellowish in colors, when reversed the color turned pale yellow and no soluble pigment was observed. Sporulation on Czapek's agar was scant however it was good on malt extract and PDA, and grew more rapidly than on Czapek's agar. The colonies on malt extract agar and PDA were also floccose and white to yellow to yellowish blue. On Sabouraud agar, the colonies were predominantly white and when reversed, the color turned pale yellow. Conidiophores were unbranched and perpendicular to funicular hyphae. In the marginal area, they were formed directly from the surface hyphae, bearing biverticillate asymmetrical penicilli with colorless conidial heads. Conidiophore's length and diameter,below the penicillus, ranged from 90 to 200 μm and 2.5 to 3.5 μm, respectively. The conidia as well as conidiophore were smooth. Conidia formed chains at the top of phialides. Conidia were ovoid to subglobose in shape and 2.5×3.5 μm in size. Both scelerotia and cleistothecia were absent. The above characteristics serve to identify the organism as the genus *Penicillium* (16), although the species of the strain should be revealed in further studies.

Production and Isolation of YS-1649.

Fermentation was performed as described in Materials and Methods. As most of the antibiotic activity was found in the broth filtrate, the filtrate was adjusted to pH 3.0 with 2N HCl, and active principles were extracted with

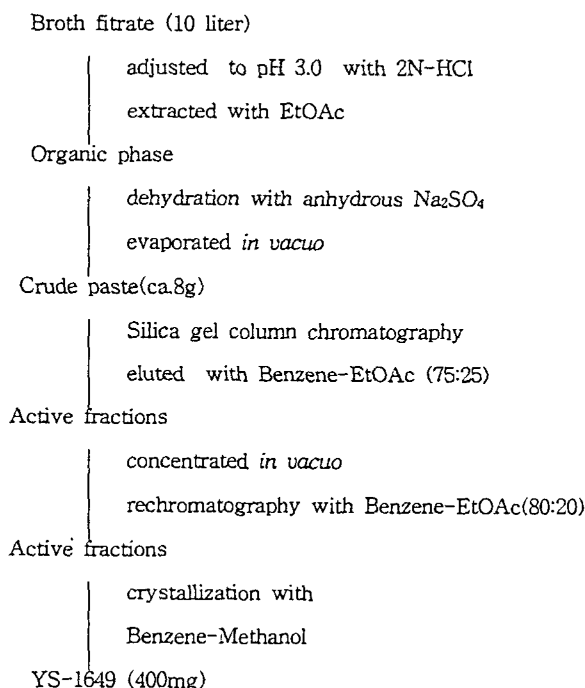

Fig. 1. Isolation procedure of YS-1649

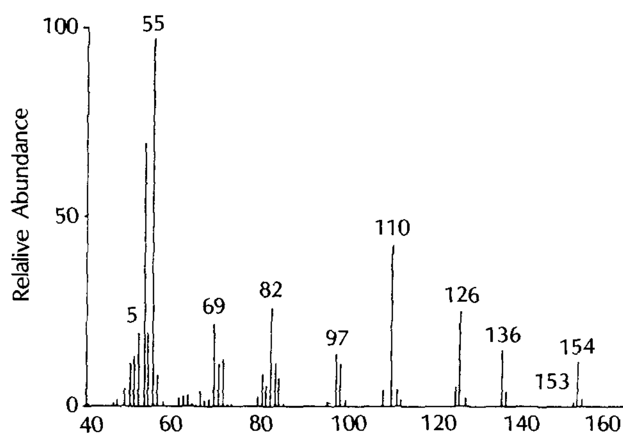

Fig. 2. Mass spectrum of YS 1649.

ethyl acetate with the same volume of broth. The combined ethyl acetate extract was dried with anhydrous sodium sulfate and concentrated *in vacuo* (at 40°C). The residue was subjected to silicagel column chromatography. (Silicagel 60, 70~230 mesh, Wako) The column (30 × 350 mm) was developed with a solvent system of benzene-ethyl acetate. The elution was done stepwise with a solvent ratio from 95:5 to 60:40. The elution volume was 200 ml each and fractionized to 10 ml per tube. The elutions were monitored by TLC and detected under UV lamps. Active fractions were combined, concentrated *in vacuo* and rechromatographed on silicagel column with a solvent system of benzene-ethyl acetate (80:20). Active fractions which showed one spot on TLC

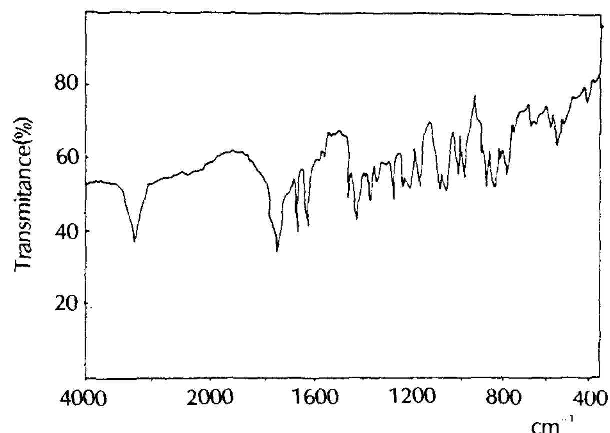

Fig. 3. IR spectrum of YS-1649(KBr).

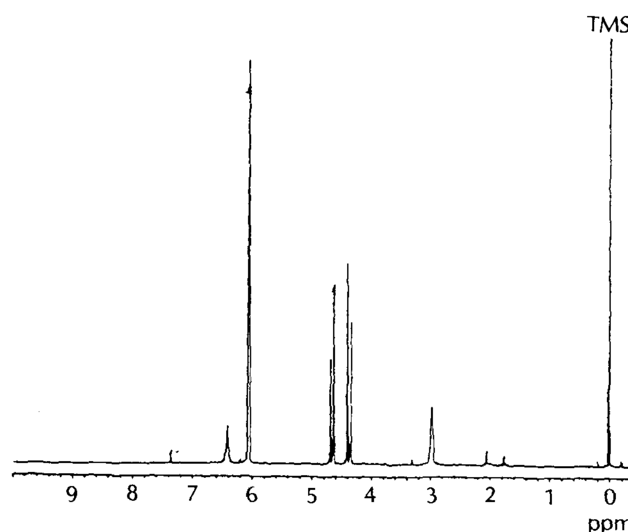

Fig. 4. ^{1}H NMR spectrum of YS 1649(300MH,, aceton-d6).

plate were combined and concentrated. After the crystallization from benzene-methanol, pure antibiotic was obtained as a colorless leaflet. The yield was about 40 mg from 1 liter of broth. Isolation prococedure is shown in Fig. 1.

Physico-chemical Properties of YS-1649.

The YS-1649 (I) was obtained by recrystallization from benzene and methanol as crystals of mp. 98~100°C. The molecular formular of I was determined to be $C_7H_6O_4$ through the mass spectrometry (Fig. 2) and elementary analysis. The IR spectrum is shown in Fig. 3. Also, the ^{1}H-NMR and ^{13}C-NMR spectra are shown in Fig. 4 and Fig. 5, respectively. Physico-chemical properties including solubility and color reaction of I are summarized in Table 1.

Structure of YS-1649.

The YS-1649 (I) is a neutral substance and has a lactone structure judging from its IR spectrum (1740 cm^{-1}). UV spctrum of I revealed a maximum absorption at 275 nm in methanol. From data of the physico-chemical pro-

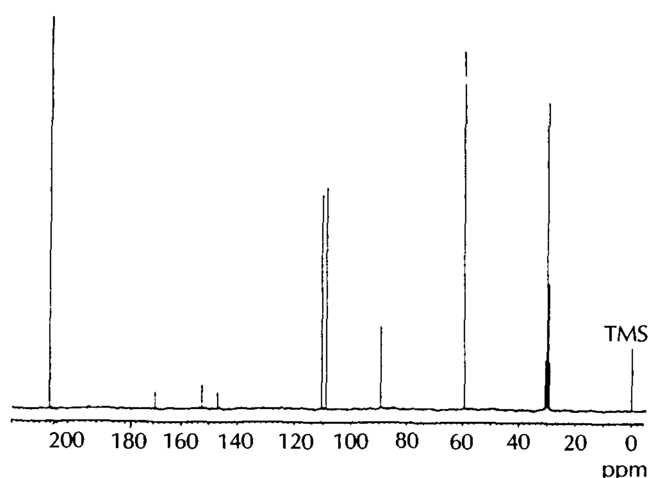

Fig. 5. ^{13}C-NMR spectrum of YS-1649(75MHz, aceton-d6).

Table 1. Physico-chemical properties of YS-1649.

Appearence	Neutral, Colorless leaflet
Mp(°C)	98-100 (decomposition)
Elementray analysis	Found. C:54.56% H:3.80% Calcd. C:54.55% H:3.92%
EI-MS(M^+)	m/z.154 for $C_7H_6O_4$
UV λmax nm(MeOH)	275
IR λmax cm^{-1}(MeOH)	3400, 1740, 1720, 1670, 1620, 1420, 1360, 1200
Solubility, soluble ;	water, EtOH, MeOH, EtOAc, acetone, $CHCl_3$.
insoluble;	n-Hexane.
Color reaction, positive;	$KMnO_4$, I_2 vapor,2,4-DNPH, o-tolidine,
negative;	Dragendorff, $FeCl_3$, 2,6-dichloroindophenol.

perties of I, I was found to be similar to patulin or isopatulin. When assessed by the melting point, UV spectrum and IR spectrum, I was found to have similarities with patulin (14). In order to determine whether I was in fact patulin, the NMR spectra of I was compared with that of patulin. In the ^{1}H-NMR spectrum of I, 6 proton signals appeared. Two of those signals at δ 4.38 ppm (H, dd) and δ 4.66 ppm were assigned to H5a and H5b of patulin, respectively. Another signal at δ 6.05 ppm (3H, m) may be correspond to H4, H6 and 4-OH protons of patulin. The remaining signal at δ 6.42 ppm (H, s) was assigned to H3 proton of patulin. The ^{13}C NMR spectrum of I showed the presence of seven carbons containing one methylene carbon adjacent to an oxygen function, one methine carbon also adjacent to an oxygen, two olefinic carbons, one carbonyl carbon and two singlet carbons. For reference, chemical shift of ^{13}C-NMR spectra of I and patulin are shown in Table 2. From the spectral data, especially that of IR, elementary analysis, mass spectra and ^{13}C-NMR described above, it was concluded that I was in actual patulin.

Table 2. Chemical shift of ^{13}C-NMR spectra of YS-1649 and patulin.

YS-1649			Patulin		
Peak No.	ppm	Multiplicity	Peak No.	ppm	Multiplicity
1	59.7	(t)	C5	59.1	(t)
2	89.3	(d)	C4	88.4	(d)
3	108.9	(d)	C6	107.9	(d)
4	110.6	(d)	C3	109.8	(d)
5	147.2	(s)	C8	146.1	(s)
6	152.8	(s)	C7	151.5	(s)
7	169.3	(s)	C2	168.5	(s)

Table 3. Antimicrobial spectrum of YS-1649.

organism	zone diameter(mm)	
	1000μg/ml	500μg/ml
Staphylococcus aureus IFO 3060	20	10
Bacillus brevis IFO 3331	12	-
Bacillus cereus IFO 3514	14	8
Bacillus subtilis IFO 12210	14	8
Micrococcus luteus IFO 3333	14	8
Micrococcus roseus IFO 3764	24	16
Escherichia coli B IFO 13168	16	8
Escherichia coli K-12 IFO 3301	10	-
Proteus vulgaris IFO 3851	22	14
Pseudomonas putida IFO 3738	8	-
Klebsiella pneumoniae IFO 3317	14	-
Debaryomyces hansenii IFO 0794	10	8

*Zone diameter was determined by paper disc (φ 8mm) method

Biological Properties of YS-1649.

Results of the examination of the antimicrobial activity of YS-1649 are shown in Table 3. It shows moderate antimicrobial activity against *Staphylococus, Micrococcus* and *Proteus,* and also presents weak activity to gram negative bacteria including *Escherichia* and *Pseudomonas.* Fungi and yeast were not affected by concentration up to 1000 μg/ml except for some strains of *Debaryomyces.* In the cytotoxity assay, the concentration of YS-1649 required to inhibit the growth of MCF-7 cells by 50% (IC 50) was 0.048 μg/ml (Fig 6). YS-1649 also inhibited the growth of A549 (lung cancer line) and ACHN (renal cancer cell line) with the IC 50 value of 0.45 μg/ml and 0.38 μg/ml, respectively.

The screening of new leads to find more effective antitumor drugs are in process at present time. In the course of our new screening method, an active substance named YS-1649 has been isolated from the culture filtrate of *Penicillium sp.* strain YS-1649. It may be concluded that YS-1649 is indeed patulin from gathering the data of the spectral analysis although melting point and ^{1}H-NMR shift were slightly different from those of patulin. It is regrettable that newly isolated substance coincided with an already known compound and could not obtain a new compound to suit the purpose. But it is interesting that the activity of YS-1649 was reversed by adding

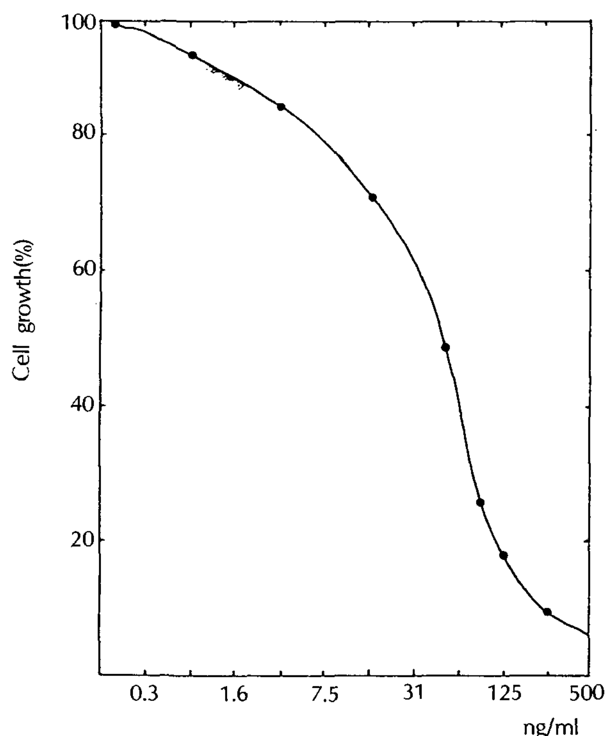

Fig. 6. Inhibition of YS-1649 on the growth of MCF-7cells.

glutathione. For SH compounds play an important role in a living body and have an effect on the reaction of living organism, it can easily be considered that the substance involved in Michael addition reactions is a merely toxic substance. But among them,several compounds such as α-methylene-γ-lactone compounds were known to have selective toxicity. YS-1649, identified as patulin, also possess an α-β-unsaturated-γ-lactone structure. We found that patulin has an antitumor activity against some human cancer cell lines. Although the secondary screening method used here were very basic, it was very effective for searching a new chemical structure, especially that of lactone compound during the screening stages.

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