ISOLATION OF ISOFLAVONOIDS POSSESSING ANTIOXIDANT ACTIVITY FROM THE FERMENTATION BROTH OF *STREPTOMYCES* SP.

KANKI KOMIYAMA, SHINJI FUNAYAMA, YUMI ANRAKU, AKIRA MITA, YŌKO TAKAHASHI and SATOSHI ŌMURA*

The Kitasato Institute, and School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

HIROYUKI SHIMASAKI

Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan

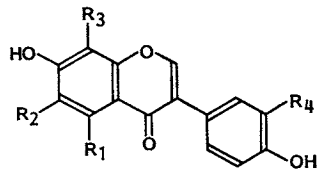
(Received for publication April 17, 1989)

Three antioxidant isoflavonoids characterized as 4',7,8-trihydroxyisoflavone (**1**), 3',4',7-trihydroxyisoflavone (**2**) and 8-chloro-3',4',5,7-tetrahydroxyisoflavone (**3**) were isolated from the cultured broth of *Streptomyces* sp. OH-1049. Among them, **3** is a novel isoflavonoid possessing a chlorine atom in the molecule.

In *in vitro* studies, these antibiotics were found to possess antioxidant activity whereas showed almost no cytocidal activities against HeLa S_3 cells.

In the course of a screening program for novel antibiotics showing antioxidant activity, a fraction of fermentation broth of *Streptomyces* sp. OH-1049 which had been isolated from a soil sample collected in Kanagawa Prefecture, Japan showed potent antioxidant activity and three active components, 4',7,8-trihydroxyisoflavone (**1**), 3',4',7-trihydroxyisoflavone (**2**) and 8-chloro-3',4',5,7-tetrahydroxyisoflavone (**3**), were isolated.

The present paper deals with the taxonomy of the producing strain together with the production, isolation and biological properties of these antibiotics. Chemical characterization and structural studies of these compounds will be reported in a separate paper[1].



1 $R_1=R_2=R_4=H$ $R_3=OH$
2 $R_1=R_2=R_3=H$ $R_4=OH$
3 $R_1=R_4=OH$ $R_2=H$ $R_3=Cl$
4 $R_1=R_3=R_4=H$ $R_2=OH$
5 $R_1=R_2=R_3=R_4=H$

Materials and Methods

Taxonomic Studies

The type of diaminopimelic acid (DAP) in the microorganisms was determined by the method of HASEGAWA *et al.*[2]

To investigate the cultural and physiological characteristics, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[3] and those recommended by WAKSMAN[4] were used. Cultures were observed after incubation at 27°C for 2 weeks. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th Ed.)[5]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon source at 27°C.

Antioxidant Activity Tests

Male Wistar rats were sacrificed by decapitation and exsanguination. To prepare a microsomal fraction, livers were collected and homogenized with cold 1.15% KCl and centrifuged at 10,000×*g* for 30 minutes. The microsomal fraction was sedimented by ultracentrifugation at 78,000×*g* for 1 hour. The microsomal fraction was diluted with 150 mM KCl - 50 mM Tris-HCl buffer, pH 7.5 to make the concentration of protein to be 1.7 mg/ml. To this microsomal solution (1.0 ml) was added 0.0425 M NADPH (0.1 ml), 2.0 mg/ml doxorubicin (ADM) and a sample solution (0.1 ml) and the total volume was adjusted to be 1.7 ml with KCl-Tris buffer (pH 7.4) and incubated for 1 hour at 37°C.

After the incubation, lipid peroxide level was determined by the method of UCHIYAMA and MIHARA[6] slightly modified as follows. The solution (0.5 ml) was taken from each test tube and was mixed with 1% phosphoric acid (3.0 ml) and 0.67% thiobarbital (TBA) solution (1.0 ml). The mixture was heated on a boiling water for 45 minutes. After cooling, 4.0 ml of BuOH was added and mixed vigorously. The butanol phase was separated by centrifugation and absorbance was measured at 520 and 535 nm. The diffference was used as the TBA value. As a standard solution, 10 nmol of 1,1,3,3-tetraethoxypropane was used.

4′,6,7-Trihydroxyisoflavone (**4**) and daidzein (**5**) were purchased from Funakoshi Pharmaceutical Co., Ltd., Tokyo, Japan.

Antimicrobial Activity Test

The antimicrobial activities of **1**, **2** and **3** were tested using 6 mm paper discs (Toyo Seisakusho Co., Ltd.) and Mueller-Hinton agar medium (Difco) for bacteria and potato broth agar medium for fungi or yeasts. Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Anti HeLa S_3 Activity Tests

HeLa S_3 cells were maintained in monolayers in EAGLE's minimum essential medium (MEM) supplemented with 10% bovine serum and an antibiotic (60 μg/ml of kanamycin) at 37°C.

To determine the cytotoxicity of **1**, **2** and **3**, HeLa S_3 cells (5×10^4) in 2 ml of medium were placed in a 30-mm Petri dish and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture dish was filled with fresh medium containing a different concentration of the antibiotic. After further 72 hours incubation, the HeLa S_3 cells were counted in hemocytometer.

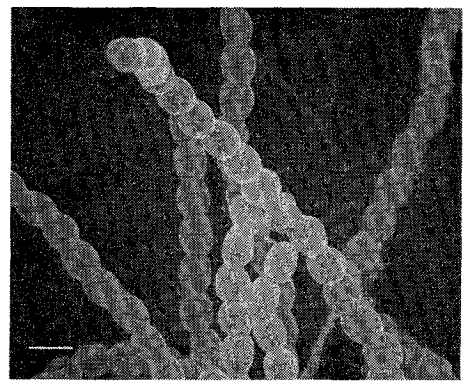
Results

Taxonomy of the Producing Strain OH-1049

The vegetative mycelia grow abundantly on both synthetic and complex agar media, and do not show fragmentation into coccoid or bacillary elements. The velvety aerial mycelia grow abundantly on inorganic salts - starch agar and glycerol - asparagine agar. The mature sporophores were of the *Rectiflexibiles* type and had more than 20 spores per chain. The spores were oval in shape, 1.1×0.7 μm in size and had a smooth surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

Fig. 1. Scanning electron micrograph of spore chains of strain OH-1049 grown on inorganic salts - starch agar for 14 days.

Bar represents 1.0 μm.



The type of DAP in the cell wall was determined as LL by the method of HASEGAWA *et al.*[2].

The cultural and the utilization of carbon sources of OH-1049 are shown in Tables 1, 2 and 3, respectively.

Table 1. Cultural characteristics of strain OH-1049.

Medium	Cultural characteristics
Yeast extract - malt extract agar[a]	G: Moderate, light ivory (2ca) R: Light mustard tan (2ie) AM: Moderate, velvety, ashes (5fe) SP: None
Oatmeal agar[a]	G: Moderate, penetrant, light ivory (2ca) R: Light mustard tan (2ie) AM: Moderate, velvety shadow gray (5ih) SP: None
Inorganic salts - starch agar[a]	G: Good, light wheat (2ea) R: Light mustard tan (2ie) AM: Abundant, velvety, ashes (5fe) SP: None
Glycerol - asparagine agar	G: Good, light ivory (2ca) R: Covert tan (2ge) AM: Abundant, velvety, ashes (5fe) SP: None
Glucose - asparagine agar	G: Good, light ivory (2ca) R: Light mustard tan (2ie) AM: Abundant, velvety, silver gray (3fe) SP: None
Peptone - yeast extract - iron agar[a]	G: Good, rose beige (4gc) R: Light amber (3ic) AM: Moderate, velvety, cream (1 1/2ca) SP: Maple (4le)
Tyrosine agar[a]	G: Good, ivory (2db) R: Clove brown (3pl) AM: Moderate, velvety, covert gray (2fe) SP: None
Sucrose - nitrate agar[a]	G: Poor, colorless R: Light ivory (2ca) AM: Poor, powderly, light beige (3ec) SP: None
Glucose - nitrate agar[a]	G: Poor, colorless R: Pearl (3ba) AM: Poor, sand (3cb) SP: None
Glycerol - calcium malate agar[b]	G: Good, light ivory (2ca) R: Sand (3cb) AM: Moderate, velvety, ashes (5fe) SP: None
Glucose - peptone agar[b]	G: Good, light ivory (2ca) R: Light wheat (2ea) AM: Moderate, velvety, white (a) or pearl gray (13dc) SP: None
Nutrient agar[b]	G: Good, light wheat (2ea) R: Bamboo (2gc) AM: Abundant, velvety, pussywillow gray (5dc) SP: None

Abbreviations: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

[a] Medium recommended by ISP.

[b] Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain OH-1049.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (21~22°C)	±
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	10~37°C

+: Active, ±: weakly active, −: inactive.

Table 3. Utilization of carbon sources by strain OH-1049.

Utilized:	D-Glucose, D-fructose, D-mannitol, L-arabinose, D-xylose
Weakly utilized:	Sucrose
Not utilized:	L-Rhamnose, *i*-inositol, raffinose, melibiose

Table 4. Antioxidant activity of **1**~**5** and α-tocopherol.

Sample	Inhibitory percent of malondialdehyde generation			
	20[a]	4	0.8	0.16
1	100	100	100	40
2	100	100	70	41
3	100	100	80	32
4	100	100	90	70
5	77	20	6	6
α-Tocopherol	100	100	31	34

[a] Concentration of sample (μg/ml).

The strain exhibits the following properties. Sporophore, *Rectiflexibiles*; spores, oval and smooth surface; color of vegetative mycelia, light ivory; color of aerial mycelia, gray; soluble pigment, maple; DAP isomer in cell wall, LL-type.

Based on the taxonomic properties described above, strain OH-1049 is considered to belong to the genus *Streptomyces* and to be a strain of the gray series of the PRIDHAM and TRESNER's system[7]. The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. OH-1049 and the accession No. is FERM P-9858.

Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 80 ml seed medium consisting of starch 1.5%, glucose 0.2%, peptone 0.25%, yeast extract 0.15%, meat extract 0.3% and $CaCO_3$ 0.25% (pH 7.0 before sterilization). The flasks were incubated at 27°C for 72 hours on a reciprocal shaker. Then 240 ml of the resulting culture were transferred to a 30-liter fermenter containing 20 liters of medium consisting of glycerol 2.0%, soybean meal 2.0% and NaCl 0.3% (pH 7.0 before sterilization). The fermentation was carried out at 27°C for 72 hours using an agitation rate of 160 rpm and an aeration rate of 60 liters/minute. The 20 liters of the resulting culture were transferred to a 400-liter fermenter containing 200 liters of the same medium described above, and fermentation was carried out at the same conditions described above.

The whole broth of *Streptomyces* sp. OH-1049 (200 liters) was extracted with EtOAc (200 liters) and the EtOAc layer was concentrated *in vacuo* to about 10 liters, washed with H_2O (5 liters) and dried over Na_2SO_4 (anhydrous). Concentration of the EtOAc layer resulted in a brown oil.

The brown oil was chromatographed over Silica gel 60 (Merck) using $CHCl_3$ - MeOH as solvent. Fractions exhibiting antioxidant activity were collected and rechromatography of the active fractions over Sephadex LH-20 column chromatography using MeOH as solvent gave crude mixture of active components. Finally, three active components were purified through the preparative TLC using $CHCl_3$ - MeOH (9 : 1) as solvent and/or preparative HPLC using a column of YMC A-303 (Yamamura Chemical Laboratory; 4.6 i.d. × 250 mm) eluted with MeOH - H_2O (39 : 11) as solvent.

Antimicrobial Activity Test

Isoflavonoids **1**~**3** showed no antimicrobial activities at the concentration of 100 μg/ml against *Xanthomonas oryzae* KB 88, *Candida albicans* KF 1, *Saccharomyces sake* KF 26, *Mucor racemosus* KF 223 (IFO 4581), *Piricularia oryzae* KF 180, *Aspergillus niger* KF 103 (ATCC 6275), *Staphylococcus aureus* KB 34 (FDA 209P), *Bacillus subtilis* KB 27 (PCI 219), *Escherichia coli* KB 8 (NIHJ), *E. coli* KB 176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB 105 (P3), *Micrococcus luteus* KB 40 (PCI 1001), *Bacteroides fragilis* KB 169, *Mycobacterium smegmatis* KB 42 (ATCC 607) and *Acholeplasma laidlawii* PG 8 KB 174.

Antioxidant Activity Test

Antioxidant activities of **1**~**5** and α-tocopherol are shown in Table 4. The antioxidant activities of isoflavonoids **1**~**3** were comparable to those of **4** and α-tocopherol.

Anti HeLa S_3 Activity Test

Cytocidal activities (MIC) of compounds **1**~**5** against HeLa S_3 cells were 12.5, 6.3, 25.0, 12.5 and 3.2 μg/ml, respectively.

Discussion

A novel antibiotic, 8-chloro-3′,4′,5,7-tetrahydroxyisoflavone (**3**) was isolated from the cultured broth of *Streptomyces* sp. OH-1049 together with 4′,7,8-trihydroxyisoflavone (**1**) and 3′,4′,7-trihydroxyisoflavone (**2**).

Compounds **1**~**3** showed comparable antioxidant activity to those of α-tocopherol and 4′,6,7-trihydroxyisoflavone (**4**) whereas daidzein (**5**) showed only weak activity (Table 4). An isoflavone derivative, 4′,6,7-trihydroxyisoflavone (**4**) was isolated as an antioxidant component of Indonesian food "Tempeh" which was prepared by the action of *Rhizopus oryzae* on boiled soybeans[8,9] and daidzein (**5**) was obtained as an aglycone of daidzin (daidzein-7-*O*-glucoside) isolated from soybeans[10].

Compounds **1**~**3** were only obtained from the fermentation broth of *Streptomyces* sp. OH-1049 using a cultivation medium containing soybean meal, whereas these compounds could not been obtained from the untreated soybean meal. These facts indicated that both soybean meal and *Streptomyces* sp. OH-1049 are necessary for the production of **1**~**3**. The physico-chemical properties and structure elucidation procedures of compounds **1**~**3** are described in a subsequent paper[1].

We are now investigating the biological activities of these compounds and their related compounds further and results will be reported elsewhere.

Acknowledgment

This work was supported, in part, by Grants-in-Aid from the Ministry of Health and Welfare, the Ministry of Education, Science and Culture, Japan, and by funds from Japan Keirin Association.

References

1) Funayama, S.; Y. Anraku, A. Mita, K. Komiyama & S. Ōmura: Structural study of isoflavonoids possessing antioxidant activity isolated from the fermentation broth of *Streptomyces* sp. J. Antibiotics 42: 1350~1355, 1989
2) Hasegawa, T.; M. Takizawa & S. Tanida: A rapid analysis for chemical grouping of aerobic actinomycetes. J. Gen. Appl. Microbiol. 29: 319~322, 1983
3) Shirling, E. B. & D. Gottlieb: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) Waksman, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Description of Genera and Species. Williams & Wilkins Co., 1961

5) Color Harmony Manual, 4th Ed. Container Corporation of America, 1958
6) UCHIYAMA, M. & M. MIHARA: Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86: 271~278, 1978
7) PRIDHAM, T. G. & H. D. TRESNER: *Genus* I. *Streptomyces* Waksman and Henrici 1943, 339. *In* BERGEY's Manual of Determinative Bacteriology. 8th Ed. *Eds.*, R. E. BUCHANAN & N. E. GIBBONS, pp. 748~829, Williams & Wilkins Co., 1974
8) IKEHATA, H.; M. WAKAIZUMI & K. MURATA: Antioxidant and antihemolytic activity of a new isoflavone, "factor 2" isolated from Tempeh. Agric. Biol. Chem. 32: 740~746, 1968
9) GYÖRGY, P.; K. MURATA & H. IKEHATA: Antioxidants isolated from fermented soybeans (Tempeh). Nature 203: 870~872, 1964
10) WALZ, E.: Isoflavon- und Saponin-Glucoside in *Soja hispida*. Ann. Chem. 489: 118~155, 1931