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Roselipins, Inhibitors of Diacylglycerol Acyltransferase, Produced by *Gliocladium roseum* KF-1040

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Gliocladium roseum KF-1040, a marine isolate, was found to produce a series of new inhibitors of diacylglycerol acyltransferase (DGAT). Four active compounds, designated roselipins 1A, 1B, 2A and 2B, were isolated from the fermentation broth of the producing strain by solvent extraction, ODS column chromatography and preparative HPLC. The highest production of roselipins was observed when cultured in the medium containing natural sea water. Roselipins inhibit DGAT activity with IC_{50} values of 15~22 μM in an enzyme assay system using rat liver microsomes.

Triacylglycerol is the main storage form of energy, but too much accumulation of triacylglycerol in certain organs and tissues of the body causes high risk conditions of fatty liver, obesity, and hypertriglyceridemia, leading to serious diseases of atherosclerosis, diabetes, metabolic disorders and functional depression of some organs. Control of triacylglycerol synthesis is expected to be effective on treatment and prevention for these diseases. Diacylglycerol acyltransferase (acyl-CoA: 1,2-diacyl-*sn*-glycerol *O*-acyltransferase, abbreviated as DGAT) [EC 2.3.1.20] catalyzes the reaction of acyl residue transfer from acyl-CoA to diacylglycerol to form triacylglycerol[1]. The reaction is the final step of *de novo* triacylglycerol biosynthesis, and is the only pathway which is exclusively involved in triacylglycerol formation[2]. Therefore, DGAT is considered as a potential target of inhibition for specific control of such high triacylglycerol-induced disorders.

We have reported DGAT inhibitors, amidepsines[3~5] isolated from fungal strains having a tridepside linked with an amino acid, and xanthohumols[6] isolated from hop having a calcone skeleton. From our continuous screening for DGAT inhibitors, we discovered and isolated a series of DGAT inhibitors from the culture broth of a marine-isolated fungal strain KF-1040[7]. Four structurally related active compounds, designated roselipins 1A, 1B, 2A and 2B (Fig. 1), were isolated from the culture broth.

In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of roselipins are described. The structure elucidation of roselipins will be described in the accompanying paper[8].

Materials and Methods

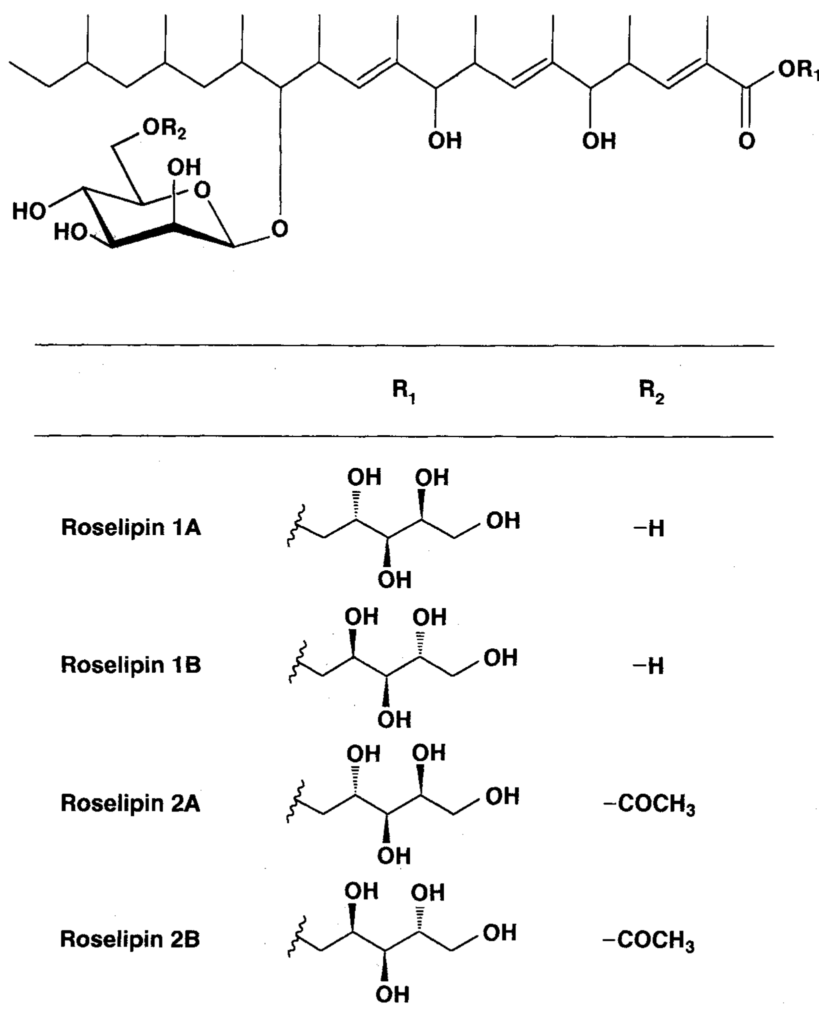
General Experimental Procedures

Fungal strain KF-1040 was isolated from a sea weed sample collected at Yap island, Federated State of Micronesia, and was used for production of roselipins. HPLC was carried out using a JASCO (TRI ROTAR V) system.

Taxonomic Studies

For the identification of the fungus, potato dextrose agar (Difco), malt extract agar, CZAPEK's agar, corn meal agar (Difco) and YpSs agar (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 2.0%, pH 6.0) were used. The morphological properties were

Fig. 1. Structures of roselipins 1A, 1B, 2A and 2B.



observed with a scanning electron microscope (model JSM-5600, JEOL).

DGAT Activity

DGAT activity was assayed in an enzyme assay using rat liver microsomes[1,3].

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (6 mm, ADVANTEC). Bacteria were grown on Müeller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

Results and Discussion

Characteristics of the Producing Strain KF-1040

Morphological properties were examined after incubation at 25°C for 14 days on potato dextrose agar (PDA, Difco), malt extract agar (MEA), corn meal agar (CMA, Difco) and Miura's medium (LcA). This strain grew moderately to form white to yellowish brown colonies with a diameter of 20~30 mm. The reverse of the colonies was yellow to pale yellow brown. On each medium the colony was granular to floccose. Two types of conidiophores erected; (A) the primary conidiophores with divergent, *Verticillium*-like branches, phialides in whorls of 3 to 5, $17 \sim 25 \times 3.0 \sim 5.0\,\mu m$ in size, and conidial heads discrete (Fig. 2A) and (B) the secondary conidiophores, densely penicillate,

phialides in closely appressed whorls of 4 to 7, 10~23×2.5~3.0 μm, and stipate conidia cohering in slimy masses (Fig. 2B). Conidia from the both types of conidiophores elongated, and were slightly asymmetrical, smooth-walled, and 4.0~7.0×2.5~3.0 μm in size.

From the above characteristics, strain KF-1040 was identified as *Gliocladium roseum*[9] and named *Gliocladium roseum* KF-1040. The culture was deposed at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-6251.

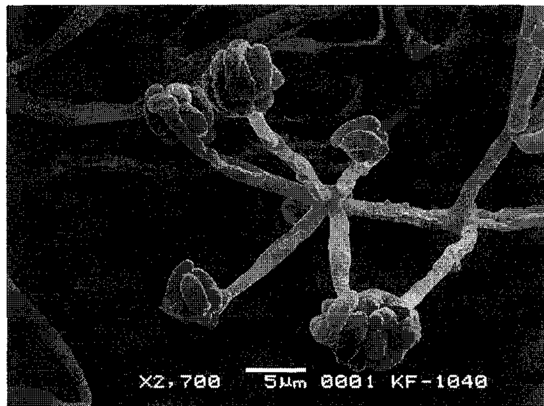
Fermentation

A slant culture of strain KF-1040 grown on YpSs agar was used to inoculate a 500-ml Erlenmeyer flasks contain-

Fig. 2. SEM photomicrograph of strain KF-1040.

(A) The primary *Verticillium*-type and (B) the secondary penicillate conidiophores, phialides and conidia. Bar represents 5 (A) and 10 (B) μm.

A)



B)

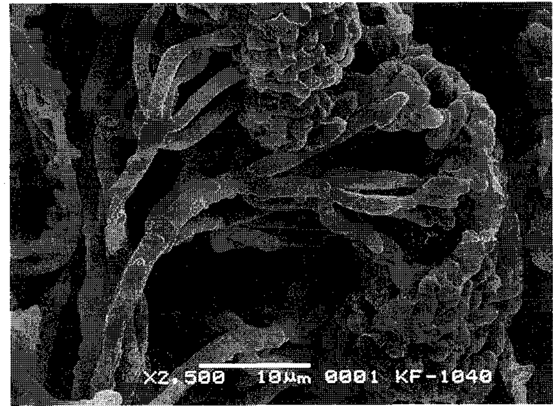
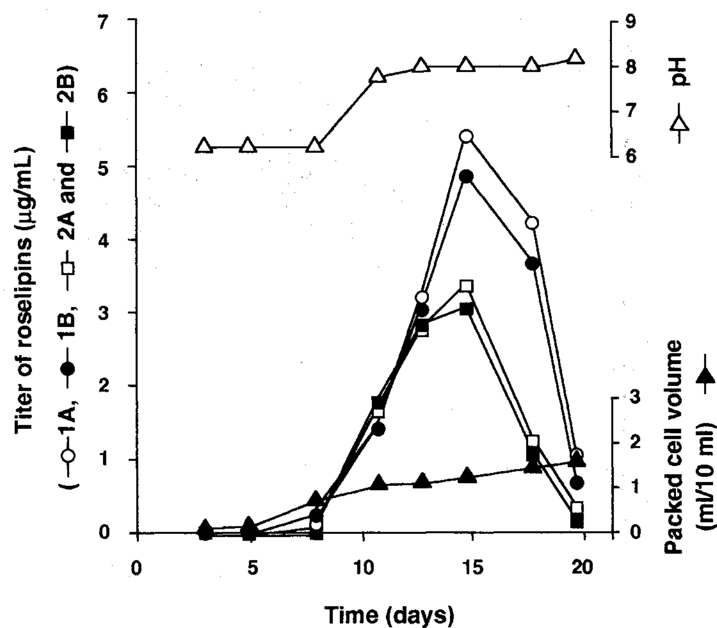


Fig. 3. A typical time course of roselipins 1A, 1B, 2A and 2B production by *Gliocladium roseum* KF-1040.

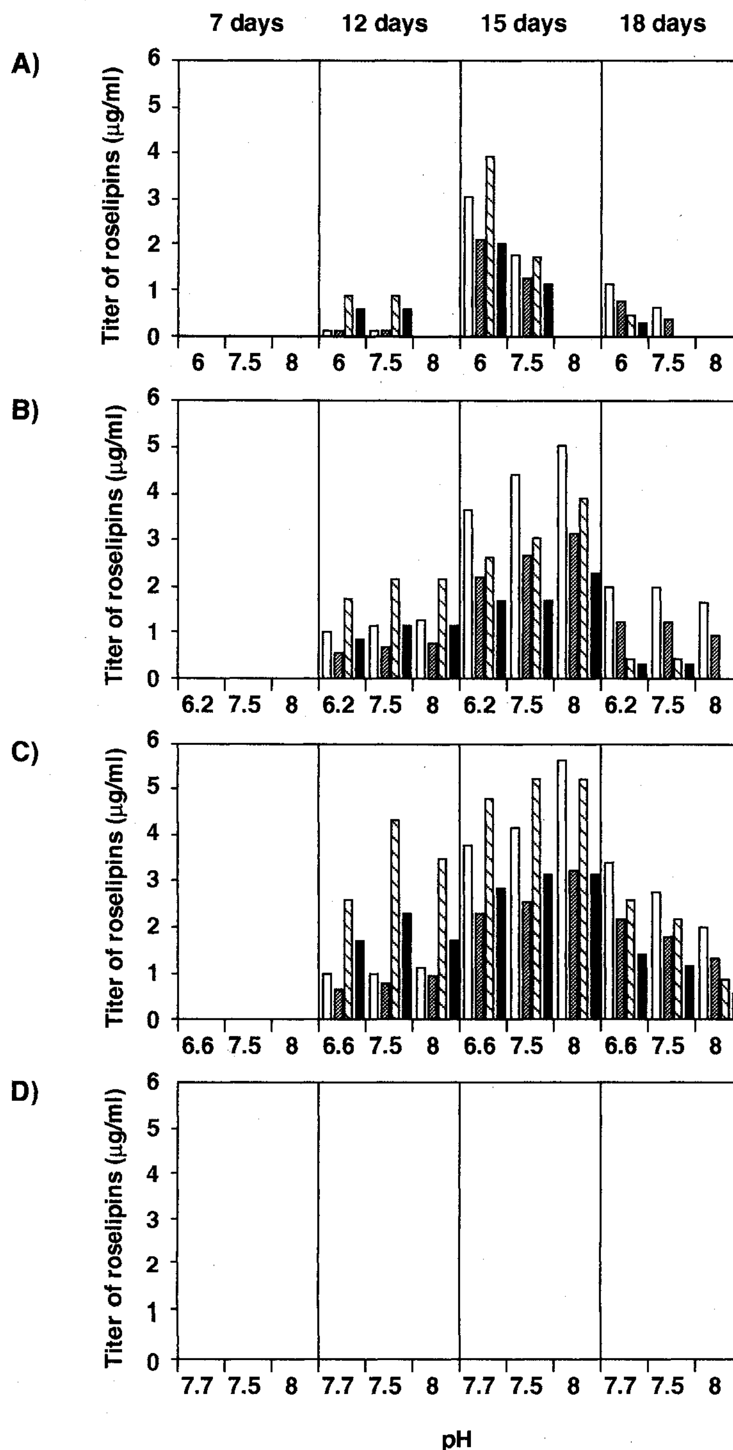
○ Roselipin 1A, ● roselipin 1B, □ roselipin 2A, ■ roselipin 2B, ▲ packed cell volume, and △ pH.



ing 100 ml (50% sea water) of a seed medium (glucose 2.0%, yeast extract (Oriental Yeast Co.) 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Daigo Nutritive Chemicals) 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker (210 rpm) for 5 days at 27°C. Four ml of the seed culture was inoculated into a 1000-ml Ru-type flask containing 200 ml (50% sea water) of the production medium (potato 10% and glucose 1.0%). The fermentation was carried out under the static condition at 27°C. A typical time course of the fermentation is shown

Fig. 4. Effect of sea water and pH of the medium on the production of roselipins.

The concentration of sea water; (A) 0, (B) 50, (C) 100% and (D) 200%. □ Roselipin 1A, ▩ roselipin 1B, ▨ roselipin 2A, and ■ roselipin 2B.



in Fig. 3. The production of roselipins was measured by HPLC (CAPCELL PAK UG (4.6×250 mm), 50% aq CH_3CN, UV at 220 nm, 0.7 ml/minute). Under these conditions, roselipins 1A, 1B, 2A and 2B were eluted with retention times of 28.0, 29.0, 48.0 and 49.5 minutes, respectively. The production of roselipins was observed at day 8 after inoculation, and reached a maximum at day 15.

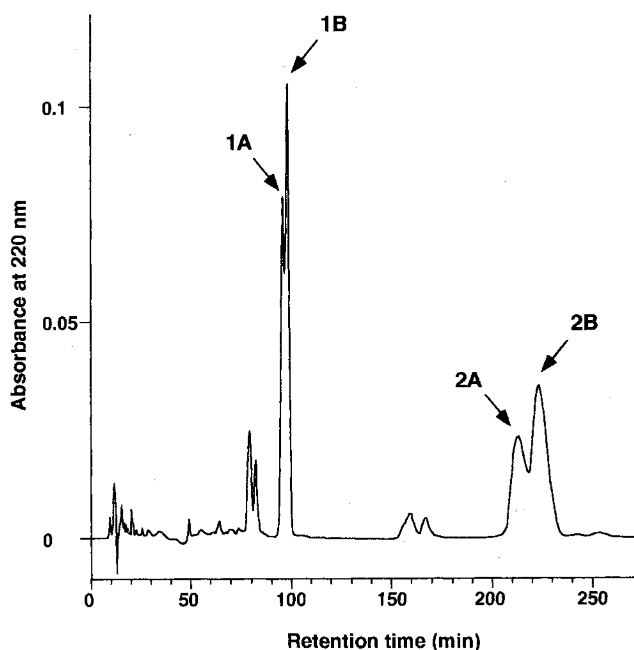
The producing organism was isolated from a sea weed sample. Therefore, the effects of sea water and pH in the medium were investigated on the roselipins production (Fig. 4). The production media were prepared in different concentrations of sea water (0, 50, 100% and 2-fold concentrated 200% sea water) at different pH (6.0~8.0). Among them, the culture condition (the medium in 100% sea water at pH 8.0 for 15 days) showed the best production of roselipins. However, no production of roselipins was observed in the media prepared in 200% sea water, probably due to the growth inhibition of the producing organism.

Isolation

The 15-day old whole broth (4.8 liters) was extracted with 9.0 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with 9.0 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brownish oil (2.20 g), which was then subjected to an ODS column (Senshu SS 1020T, 50 g). The materials were eluted stepwise with 30%, 50%, 70% and 100% CH_3CN (400 ml each), and each 50 ml of the elution was successively collected. The 17th to 22nd fractions containing roselipins were concentrated and extracted with ethyl acetate (300 ml). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to give a yellow powder (191 mg). The powder was further purified by preparative HPLC (YMC pack D-ODS-AM (20×250 mm), 50% aq CH_3CN, UV at 220 nm, 6.0 ml/minute). Under these conditions, roselipins 1A, 1B, 2A and 2B were eluted with retention times of 96, 100, 212 and 224 minutes, respectively (Fig. 5), each of which was concentrated and extracted with ethyl acetate to give pure roselipins 1A (2.10 mg) and 1B (3.92 mg) as white powders and roselipins 2A (3.43 mg) and 2B (10.1 mg) as colorless oils.

Fig. 5. A chromatographic profile of roselipins separated by preparative HPLC.

YMC-pack D-ODS-AM (20×250 mm); 50% aq CH_3CN; UV at 220 nm; 6.0 ml/min.



Biological Properties

Inhibition of DGAT Activity by Roselipins

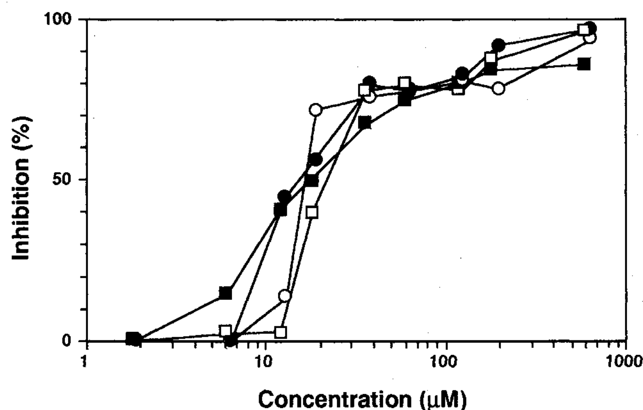
Roselipins 1A, 1B, 2A and 2B showed a dose-dependent DGAT inhibition with similar IC_{50} values of 17, 15, 22 and 18 μM, respectively (Fig. 6).

Other Biological Activities

Antimicrobial activity of roselipins was tested at a concentration of 1000 μg/ml (10 μg/disk). Roselipins 1A, 1B, 2A and 2B were active against *Aspergillus niger* KB103 (ATCC 6275) (diameter of inhibition zone: 11, 10, 0 and

Fig. 6. DGAT inhibition by roselipins in rat liver microsomes.

○ Roselipin 1A, ● roselipin 1B, □ roselipin 2A, and ■ roselipin 2B.



0 mm, respectively). No or very weak antimicrobial activity was observed against other microorganisms: *Bacillus subtilis* KB27 (PCI 219), *Staphylococcus aureus* KB210 (FDA 209P), *Xanthomonas campestris pv. oryzae* KB88, *Micrococcus luteus* KB40 (PCI 1001), *Mycobacterium smegmatis* KB42 (ATCC 607), *Escherichia coli* KB213 (NIHJ), *Escherichia coli* KB176 (NIHJJC-2 IFO 12734), *Pseudomonas aeruginosa* KB105, *Bacteroides fragilis* KB169 (ATCC 23745), *Acholeplasma laidlawii* KB174 (PG8), *Pyricularia oryzae* KB180, *Mucor racemosus* KF223 (IFO 4581), *Saccharomyces cerevisiae* KF-26 and *Candida albicans* KF1.

Roselipins showed cytotoxic effect on Raji cells[3] at 39 μM.

Acknowledgment

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