Erabulenols, Inhibitors of Cholesteryl Ester Transfer Protein Produced by *Penicillium* sp. FO-5637

I. Production, Isolation and Biological Properties

HIROSHI TOMODA, NORIKO TABATA, ROKURO MASUMA, SHU-YI SI and SATOSHI ŌMURA*

Research Center for Biological Function, The Kitasato Institute and Graduate School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

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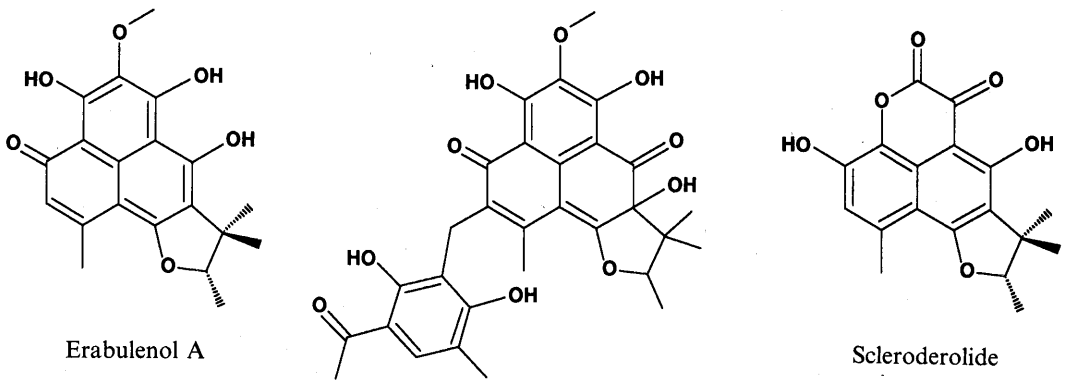
Penicillium sp. FO-5637, a soil isolate, was found to produce a series of inhibitors of cholesteryl ester transfer protein (CETP). Novel active compounds, designated erabulenols A and B, were isolated from the fermentation broth of the producing strain by solvent extraction, ODS column chromatography and HPLC. Erabulenols A and B inhibit human CETP activity with IC_{50} values of 47.7 and 58.2 μM in an *in vitro* assay system containing 200 μM BSA, respectively.

The cholesteryl ester transfer protein (CETP) promotes exchange and transfer of neutral lipids such as cholesteryl ester (CE) and triacylglycerol between plasma lipoproteins[1~3].

Evidence is accumulating for involvement of CETP in atherosclerosis; 1) CETP decreased cholesterol concentration in high density lipoprotein (HDL) *in vitro*[4] and *in vivo*[5], 2) rats and mice deficient in CETP activity have high plasma HDL and are resistant to atherosclerosis, 3) human subjects with a genetic defficiency of CETP have very high HDL and low LDL cholesterol levels and are resistant to atherosclerosis[6,7], and 4) human CETP introduced transgenic mice have a redistribution of cholesterol from HDL to LDL[5,8], leading to diet-induced atherosclerosis[9]. Therefore, CETP is expected as a new target of inhibition for development of effective anti-atherosclerotic drugs.

During the course of our screening program for CETP inhibitors of microbial origin, we discovered and isolated a series of CETP inhibitors from the culture broth of a fungal strain FO-5637. Three structurally related active compounds were isolated from the culture broth. One compound was identical with scleroderolide[10] previously isolated as a fungal metabolite, but the other two were

Fig. 1. Structures of erabulenols A and B and scleroderolide.



found to be new compounds designated erabulenols A and B (Fig. 1)[11]. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of erabulenols are described.

Materials and Methods

General Experimental Procedures

Fungal strain FO-5637 isolated from a soil sample was used for production of erabulenols. SSC-ODS-7515-12 (Senshu Sci. Co.) was used for column chromatography. HPLC was carried out using Waters 600E system.

Taxonomic Studies

For the identification of the fungus, Czapek yeast extract agar, malt extract agar, 25% glycerol nitrate agar and potato dextrose agar (Difco) were used. Morphological observations were done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (Hitachi S-430).

Assay for CETP Activity

The assay for CETP activity was carried out essentially according to the method of KATO *et al.*[12] with some modification[13]. In brief, the assay mixtures consisted of 25 μl of reconstituted [^{14}C]CE-HDL (22.5 nmol of egg phosphatidylcholine, 5.63 nmol of cholesterol, 0.47 nmol (10 μCi) of cholesteryl [1-^{14}C]oleate and 15.4 μg of apo A-I) as the donor for cholesteryl ester, 10 μl (32.5 μg as protein) of LDL as the acceptor, 0.21 μmol of 5,5′-dithiobis-2-nitrobenzoic acid, 200 μM BSA, 5 μl of partially purified CETP and 5 μl of a sample (MeOH solution) in a final volume of 150 μl in 26 mM phosphate buffer (pH 7.4) containing 45.6 mM NaCl and 0.017% EDTA in eppendorf tubes (1.5 ml). The assay incubation was carried out at 37°C. After a 30-minute incubation, the assay tubes were immediately placed in an ice bath, to which was added 30 μl of a LDL-precipitation solution (5 μl of 60 mM $MgCl_2$, 5 μl of 0.1% dextran sulfate and 20 μl of 39 mM phosphate buffer (pH 7.4) containing 68 mM NaCl and 0.025% EDTA). After standing for 20 minutes in an ice bath, the assay mixtures were centrifuged at 13,000 $\times g$ for 15 minutes at 4°C. The 140 μl of supernatant solutions containing [^{14}C]CE-HDL were carefully transferred into scintillation vials to analyze for radioactivity. The CETP mediated cholesteryl ester transfer activity was computed by subtracting the blank values, which include the spontaneous transfer, from the total cholesteryl ester transfer obtained in the presence of CETP.

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (i.d. 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

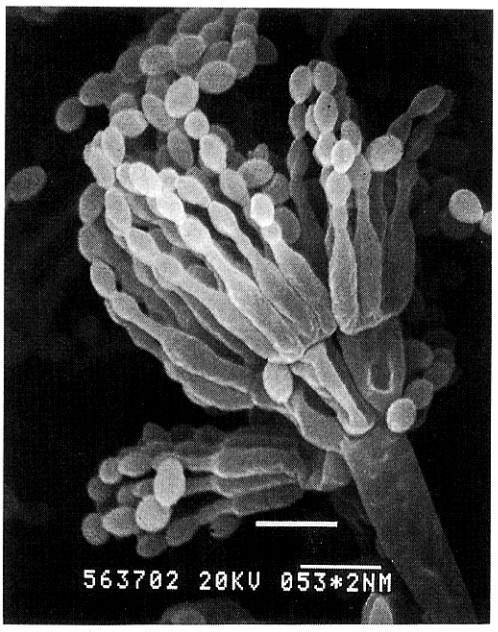
Characteristics of the Producing Strain

The fungal strain FO-5637 was originally isolated from a soil sample collected at Okinoerabu Island, Okinawa, Japan. This strain grew rapidly to form olive gray to dull yellow colonies with a diameter of 7~30 mm after incubation for 7 days at 25°C. At 5°C and 37°C, growth is nil. The colony surface was velvety. Reverse of the colonies was olive gray to dark yellowish gray. Pale yellow or dark yellow soluble pigments were produced on Czapek yeast extract agar, malt extract agar and potato dextrose agar. Conidia formation was abundant when observed on Czapek yeast extract agar, malt extract agar and potato dextrose agar. In contrast, conidia formation was repressed on 25% glycerol nitrate agar.

When the strain FO-5637 was grown on Czapek yeast extract agar at 25°C for 7 days, the conidiophores were

Fig. 2. Photomicrograph of penicillia of strain FO-5637.

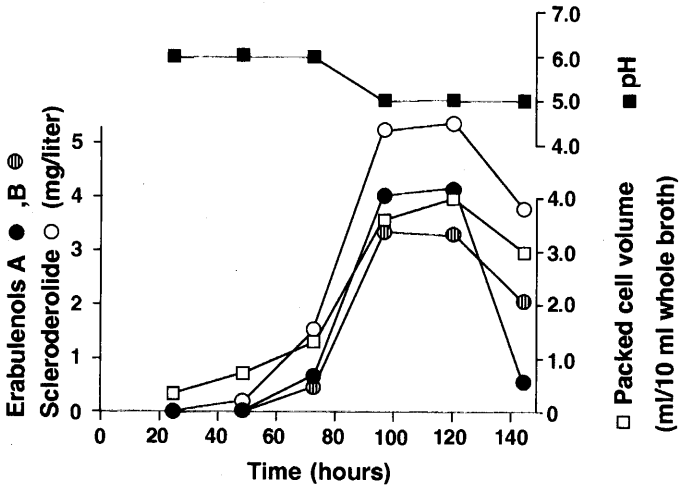
Bar represents 5 μm.



Penicillium sp. FO-5637

Fig. 3. A typical time course of erabulenols A and B and scleroderolide production by *Penicillium* sp. FO-5637.

● Erabulenol A, ◍ erabulenol B, ○ scleroderolide, □ packed cell volume, ■ pH.



borne from substrate hyphae, and the penicillia were biverticillate as shown in Fig. 2. The phialides were 7.5~10.2×2.0~3.0 μm. The conidia were globose to subglobose and 2.5~3.0 μm in diameter, and its surface was smooth. From the above characteristics, the strain FO-5637 was identified as a member of the genus *Penicillium*[14,15].

The strain was deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Penicillium* sp. FO-5637 with the accession number FERM-P 16101.

Fermentation

A slant culture of the strain FO-5637 grown on YpSs agar was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton (Nippon Seiyaku) 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 4 days at 27°C. Two hundred milliliters of the seed culture was transferred into 20 liters of the production medium (sucrose 2.0%, glucose 1.0%, corn steep liquor 1.0%, meat extract 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, trace elements containing in g/liter: $FeSO_4 \cdot 7H_2O$ 1.0, $MnCl_2 \cdot 4H_2O$ 1.0, $ZnSO_4 \cdot 7H_2O$ 1.0, $CuSO_4 \cdot 5H_2O$ 1.0 and $CoCl_2 \cdot 2H_2O$ 1.0 (200 ml), $CaCO_3$ 0.3% and agar 0.1%, pH 6.0) in a 30-liter jar fermentor. The fermentation was carried out at 27°C. The production of erabulenols was measured by HPLC under the following conditions : Senshu pak ODS-H-1251, 4.6×250 mm; a linear gradient 30% CH_3CN in 0.05% H_3PO_4 to 80% CH_3CN in 0.05% H_3PO_4 for 60 minutes; UV at 220 nm; 0.7 ml/minute. Under this conditions, erabulenols A and B and scleroderolide were eluted as a peak with retention times of 18.6, 40.8 and 24.3 minutes, respectively. A typical time course of the fermentation is shown in Fig. 3. The production of erabulenols and scleroderolide started by 72 hours, and the concentrations reached maximal levels at 96~120 hours after inoculation. The maximal concentrations were 4.1 μg/ml for erabulenol A and 5.2 μg/ml for scleroderolide at 120 hours, and 3.2 μg/ml for erabulenol B at 96 hours.

Isolation

The 96-hour old whole broth (20 liters) was adjusted to pH 7.0 and then extracted with ethyl acetate (18 liters). The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a dark green powder (5.12 g). The powder (2.4 g) was distributed in a solution of *n*-hexane - methanol - H_2O (900 ml, 40 : 19 : 1, v/v). Then the lower layer was concentrated *in vacuo* to dryness to yield a dark green powder (1.4 g, IC_{50} 110 μg/ml). The powder suspended in 30% CH_3CN was subjected to an ODS column (Senshu SSC-ODS-7515-12, 240 ml). The materials were eluted by a linear gradient from 30% CH_3CN in 0.05% H_3PO_4 (1657 ml) to 100% CH_3CN (2000 ml) and each 12 ml of the elution was collected. From the CETP inhibitory activity of each elute, three fractions, A (the 81st~94th fractions), S (the 107th~149th fractions), and B (the 196th~213th fractions), were

pooled.

The fraction A was concentrated and extracted with ethyl acetate to give a brown powder (102 mg, IC_{50} 98 μg/ml), which was purified by preparative HPLC (YMC-pack D-ODS-5, 20 × 250 mm; 45% CH_3CN in 0.05% H_3PO_4; UV at 225 nm; 6.0 ml/minute) (Fig. 4 (1)). The active material was eluted as a peak with a retention time of 43.0 minutes. After concentration of the fraction, the material was extracted with ethyl acetate to give an orange powder (19.1 mg, IC_{50} 34 μg/ml) but it still contained impurity. Therefore, the fraction was re-purified by preparative HPLC (YMC-pack D-ODS-5, 20 × 250 mm; 35% CH_3CN in 0.05% H_3PO_4; UV at 225 nm; 6.0 ml/minute). The peak at a retention time of 110 minutes was extracted in a similar way to give pure erabulenol A (9.45 mg, IC_{50} 17 μg/ml) as an orange powder.

The fraction S (326 mg, IC_{50} 105 μg/ml) was further purified by preparative HPLC using a solvent of 48% CH_3CN in 0.05% H_3PO_4 (Fig. 4 (2)). From the peak eluted with a retention time of 72.0 minutes, scleroderolide (23.1 mg, IC_{50} 31 μg/ml) was obtained as a yellow powder.

The fraction B was concentrated and extracted with ethyl acetate to give an orange powder (93.9 mg, IC_{50} 97 μg/ml), which was purified by preparative HPLC using a solvent of 72% CH_3CN in 0.05% H_3PO_4 (Fig. 4 (3)). Under the conditions, an active fraction was eluted as a peak with a retention time of 84.0 minutes. The fraction was concentrated and extracted with ethyl acetate to give erabulenol B (27.0 mg, IC_{50} 32 μg/ml) as an orange powder.

Biological Properties

Effect of Erabulenols on CETP Activity *In Vitro*

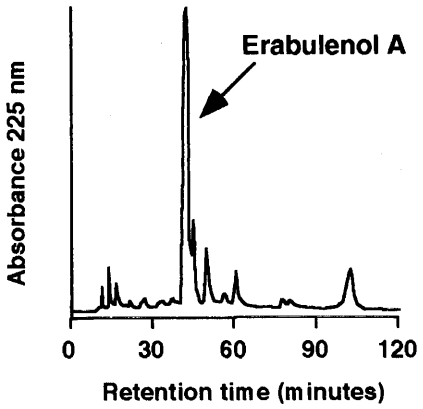
As shown in Fig. 5, erabulenols inhibited CETP activity dose-dependently in the *in vitro* assay. Erabulenol A showed the most potent inhibitory activity with an IC_{50} value of 47.7 μM (17 μg/ml), followed by erabulenol B (58.2 μM (32 μg/ml)) and scleroderolide (94.5 μM).

Other Biological Activities

Antimicrobial activity of the drugs at 1 mg/ml was tested against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Bacteroides fragilis*, *Pyricularia oryzae*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, *Mucor racemosus* and *Aspergillus niger* by paper disk (i.d. 6 mm) method. Erabulenol A showed no antimicrobial activity, and erabulenol B also showed no activity except against *S.*

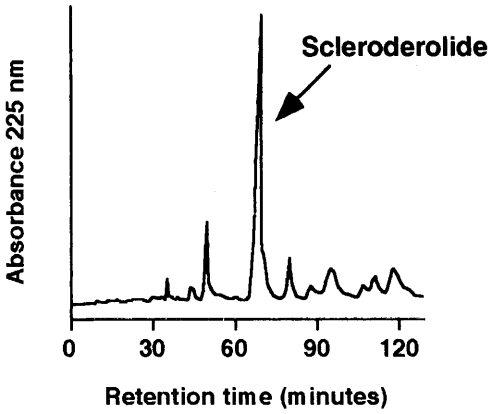
Fig. 4.

1) A chromatographic profile of erabulenol A separated by preparative HPLC.



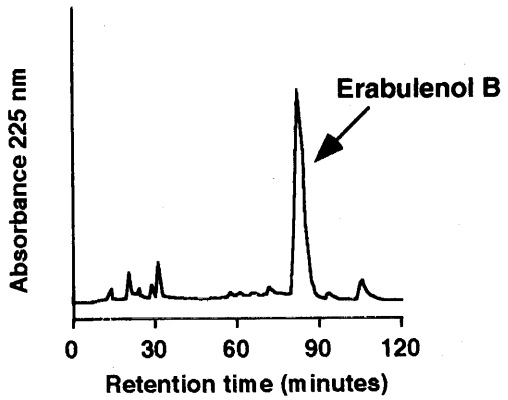
YMC-pack D-ODS-5 (20 × 250 mm); 45% CH_3CN in 0.05% H_3PO_4; UV at 225 nm; 6.0 ml/minute.

2) A chromatographic profile of scleroderolide separated by preparative HPLC.



YMC-pack D-ODS-5 (20 × 250 mm); 48% CH_3CN in 0.05% H_3PO_4; UV at 225 nm; 6.0 ml/minute.

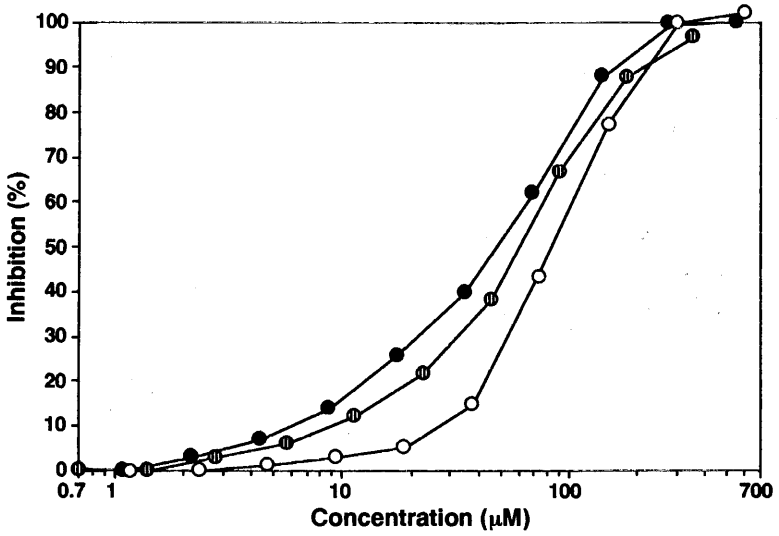
3) A chromatographic profile of erabulenol B separated by preparative HPLC.



YMC-pack D-ODS-5 (20 × 250 mm); 72% CH_3CN in 0.05% H_3PO_4; UV at 225 nm; 6.0 ml/minute.

Fig. 5. CETP inhibition by erabulenols A and B and scleroderolide in an *in vitro* assay system in the presence of 200 μM BSA.

● Erabulenol A, ◍ erabulenol B, ○ scleroderolide.



aureus (diameter of inhibition zone 6.5 mm). However, scleroderolide was active against *S. aureus* (7.4 mm), *M. luteus* (6.8 mm), *B. subtilis* (16.1 mm) *B. fragilis* (7.3 mm) and *P. oryzae* (8.9 mm).

Discussion

During our CETP screen, the *in vitro* CETP activity was found to be succeptible to certain fatty acids (IC_{50}; 55.0 μM for oleic acid and 71.9 μM for linolenic acid). To rule out such interferences, higher concentrations of BSA were added to the reaction mixture. Addition of BSA up to 200 μM showed no effect on CETP activity, but addition at 300 μM affected the activity slightly. Therefore, the concentration of BSA was set up at 200 μM in our assay, which is a very similar level to that in human plasma, resulting in almost no effect of oleic acid and linolenic acid on the CETP activity even at 180 μM. Thus, high levels of albumin in the CETP assay is recommended for evaluation of CETP inhibitors. Erabulenols were discovered under such conditions, and the IC_{50} values were essentially equivalent in the presence or absence of BSA. On the other hand, some CETP inhibitors such as wiedendiols[16], rosenonolactone derivatives[17], KRIBB-BP005m[18], PD 140195[19] and U-106305[20] have been reported. Their CETP inhibitory activities were assayed by the method using the Amersham Scintillation Proximity Assay (SPA) kit[16~18], whole plasma method[19] or LDL precipitation method[20] similar to ours in this report. The methods except the whole plasma method were carried out in the absence or in the presence of a low concentration of albumin. Therefore, it might be necessary that their inhibitory activities are evaluated in the presence of a high concentration of plasma or BSA.

Scleroderolide showed antimicrobial activity, while erabulenols showed almost no activity. The partial structure (–O–(C=O)–(C=O)–) in the tricyclic skeleton of scleroderolide might be responsible for exhibiting antimicrobial activity. Comparison with the inhibitory effects suggested no relationships between their CETP inhibition and antimicrobial activity.

Acknowledgments

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