GLISOPRENINS, NEW INHIBITORS OF ACYL-CoA: CHOLESTEROL ACYLTRANSFERASE PRODUCED BY *Gliocladium* sp. FO-1513

I. PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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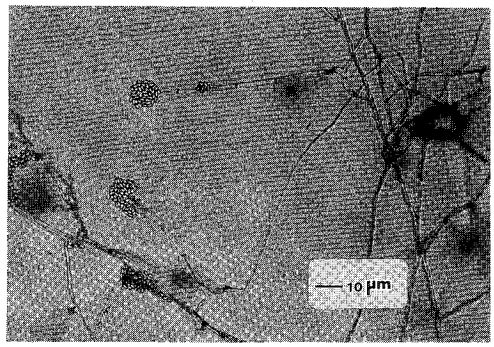
Gliocladium sp. FO-1513 was found to produce novel inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT). Two active compounds, designated glisoprenins A and B, were isolated from the culture broth of the producing strain by a conventional method. The IC_{50} values of glisoprenins A and B for ACAT activity were 46 and 61 μM in an enzyme assay using rat liver microsomes, and 1.2 and 0.57 μM in a J774 macrophage assay, respectively.

During our screening program for microbial inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT) (EC 2.3.1.26), a fungal strain FO-1513 was found to produce a series of new ACAT inhibitors. Among them, two main compounds named glisoprenins A and B were isolated from the fermentation broth. In this paper, the taxonomy of the fungal strain, fermentation, isolation, and physico-chemical and biological properties of glisoprenins A and B are described.

Taxonomy of Producing Organism

Strain FO-1513 was originally isolated from a soil sample. For the identification of the fungus, potato-glucose agar, malt extract agar, corn meal agar, CZAPEK's agar and yeast extract-soluble starch (YpSs) agar were used. After a slant culture of the isolate was inoculated on these media and incubated at 25°C for 14 days, the cultural and physiological characteristics of the organism were observed. The organism grew rather rapidly on various agar media, and formed white to pale pink or pale yellowish brown colonies (60~80 mm in colony diameter). The colony surface was convolutedly floccose or tomentose. The conidial structures were abundantly produced on various agar media. The reverse color was pale pink or pale yellowish brown. At 37°C the growth was nil.

Fig. 1. Micrograph of *Gliocladium* sp. FO-1513.



The morphological observation (Fig. 1) was done under a microscope (Olympus, NEW VANOX, model AH2-NIC). Conidiophores were erect or suberect, septate, nearly smooth and 80~200×2~3 μm in size. Penicilli were asymmetrical to symmetrical. The number of metulae per penicilli was 2~4, the shape was cylindrical and the size

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was $15\sim20\times2\sim3\,\mu m$. Phialides were slender like a nib. Conidia, not forming a chain but aggregated to form gelatinous balls or masses, were mostly oval and $3\sim7\times3\,\mu m$ in size. Neither sclerotium nor chlamydospores was observed under the conditions used.

From the above characteristics, strain FO-1513 was considered to belong to the genus *Gliocladium*[1] and named to *Gliocladium* sp. FO-1513. The culture was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology Japan, as FERMP-12220.

Fermentation and Isolation

A slant culture of strain FO-1513 grown on YpSs agar was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4\cdot7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1%, agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 2 days at 27°C. Seven hundred milliliters of the seed culture were transferred into 70 liters of a production medium (soluble starch 3.0%, glycerol 1.0%, soy bean meal 2.0%, dried yeast 0.3%, KCl 0.3%, $CaCO_3$ 0.2%, $MgSO_4\cdot7H_2O$ 0.05% and KH_2PO_4 0.05%, pH 6.5) in a 100-liter jar fermenter. The fermentation was carried out at 27°C and the time course of the fermentation is shown in Fig. 2. The production of glisoprenins was measured by HPLC under the following conditions: Column; Chemcosorb 5 ODS-H (4.6×150 mm), solvent; 90% aq CH_3CN, detection; UV at 225 nm, flow rate; 1.0 ml/minute. Under these conditions, glisoprenins A and B were eluted with a Rt at 10.3 and 8.4 minutes, respectively. After 48 hours, the production of glisoprenin A was increased rapidly and appeared to reach a maximum (3.3 μg/ml) at about 76 hours after inoculation.

The 76-hour cultured broth (70 liters) was centrifuged to obtain mycelial part, which was extracted with 30 liters of acetone. After filtration and concentration of the acetone extracts, the resulted aqueous solution (8 liters) was extracted twice with 4 liters of chloroform. The extracts were concentrated *in vacuo* to dryness to give brown oily materials (26 g). The material dissolved in chloroform was applied to a silica gel column (E. Merck, Kieselgel 60, 500 g). Total materials were successively eluted with each 4 liters of chloroform, chloroform-methanol (99:1), chloroform-methanol (98:2), chloroform-methanol (95:

Fig. 2. Time course of glisoprenin A production in a 100-liter jar fermenter.

●; Glisoprenin A production, □; packed cell volume, ■; pH.

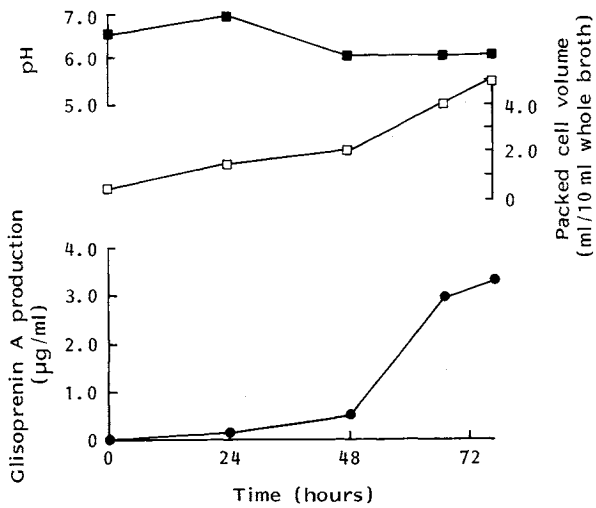


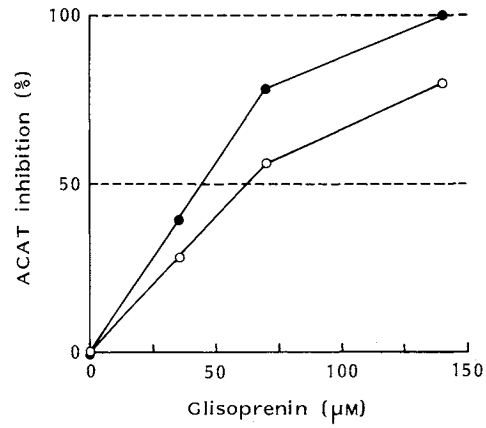
Table 1. Physico-chemical properties of glisoprenins A and B.

	Glisoprenin A	Glisoprenin B
Appearance	Colorless oil	Colorless oil
$[\alpha]_D^{18}$ (c 1.0, $CHCl_3$)	+0.4	−0.8
Molecular formula	$C_{45}H_{82}O_5$	$C_{45}H_{82}O_6$
HREI-MS (m/z)		
Calcd:	630.57359 for $C_{45}H_{74}O$ $(M-4H_2O)^+$	646.56850 for $C_{45}H_{74}O_2$ $(M-4H_2O)^+$
Found:	630.57212	646.56867
EI-MS (m/z)	702 $(M)^+$, 684 $(M-H_2O)^+$, 666 $(M-2H_2O)^+$, 648 $(M-3H_2O)^+$, 630 $(M-4H_2O)^+$, 612 $(M-5H_2O)^+$	718 $(M)^+$, 700 $(M-H_2O)^+$, 682 $(M-2H_2O)^+$, 664 $(M-3H_2O)^+$, 646 $(M-4H_2O)^+$, 628 $(M-5H_2O)^+$
FAB-MS (m/z)	704 $(M+2H)^+$, 726 $(M+H+Na)^+$, 632 $(M+2H-4H_2O)^+$, 614 $(M+2H-5H_2O)^+$	720 $(M+2H)^+$, 742 $(M+H+Na)^+$, 648 $(M+2H-4H_2O)^+$, 630 $(M+2H-5H_2O)^+$
FD-MS (m/z)	704 $(M+2H)^+$	720 $(M+2H)^+$
UV λ_{max}^{MeOH} (nm)	208	208
IR $\nu_{max}^{CCl_4}$ (cm^{-1})	3400 (OH)	3400 (OH)
Solubility		
Soluble:	MeOH, $CHCl_3$, EtOAc	MeOH, $CHCl_3$, EtOAc
Insoluble:	H_2O	H_2O
Color reaction		
Positive:	50% aq H_2SO_4	50% aq H_2SO_4
Negative:	$FeCl_3$, ninhydrin	$FeCl_3$, ninhydrin

5), chloroform - methanol (90 : 10) and finally eluted with 2 liters of MeOH and each 500 ml was continuously collected (total 44 fractions). Glisoprenins A and B were recovered from the 29th to 34th fractions. The fractions enriched with glisoprenins A and B were concentrated *in vacuo* to yield pale brown oily materials (650 mg). Further purification was carried out by HPLC (column; YMC pack ODS (20 × 250 mm), solvent; 90% aq CH_3CN, detection; UV at 225 nm, flow rate; 8 ml/minute). The fractions corresponding to glisoprenins A and B were separately pooled and concentrated to aqueous solutions, which were extracted with ethyl acetate to give clear oils (200 and 63 mg, respectively).

Fig. 3. Inhibition of ACAT activity by glisoprenins A and B in an enzyme assay using rat liver microsomes.

●; Glisoprenin A, ○; glisoprenin B.



Physico-chemical Properties

Glisoprenins A and B were isolated as colorless oils. The physico-chemical properties of glisoprenins are summarized in Table 1. The molecular formula were determined to be $C_{45}H_{82}O_5$ for glisoprenin A and $C_{45}H_{82}O_6$ for glisoprenin B on the basis of HREI-MS. These data indicated that glisoprenins A and B are new compounds. Their structures are described in the near future.[2)]

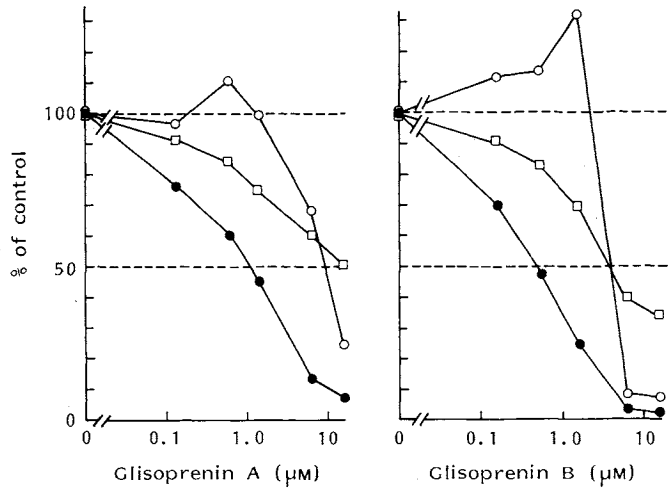
Biological Properties

Inhibitory Effect on ACAT Activity

ACAT activity was assayed in an enzyme assay using rat liver microsomes and in a cell assay using

Fig. 4. Effects of glisoprenins A and B on cholesterol ester and triacylglycerol formations in J774 macrophages and J774 cell viability.

○; Triacylglycerol, □; cell viability, ●; cholesteryl ester.



J774 macrophages as previously reported.[3,4]

Glisoprenins A and B inhibited ACAT activity dose-dependently in an enzyme assay (Fig. 3). The IC_{50} values were calculated to be 46 μM for glisoprenin A and 61 μM for glisoprenin B. ACAT activity was also inhibited in a J774 macrophage assay (Fig. 4). At higher concentrations of the drugs, cytocidal effect on the macrophages was observed. IC_{50} and CD_{50} (drug concentration for 50% cell damage) values are summarized in Table 2. Glisoprenins showed a 10-fold specificity (CD_{50}/IC_{50}) for ACAT inhibition *versus* cell damage in this macrophage assay.

Table 2. ACAT inhibitory activity (IC_{50}) and cytotoxicity (CD_{50}) of glisoprenins A and B in assays using rat liver microsomes and J774 macrophages.

Compound	Rat liver microsomes	J774 macrophages		
	IC_{50} (μM)	IC_{50} (μM)	CD_{50} (μM)	CD_{50}/IC_{50}
Glisoprenin A	46	1.2	14.0	12.0
Glisoprenin B	61	0.57	5.2	9.1

Other Biological Activities

No antimicrobial activity was observed at 2,000 μg/ml of glisoprenins A nor B against *Candida albicans* and *Bacillus subtilis*.

No toxic effects were observed when glisoprenins A or B were intraperitoneally or orally administered to ddY mice at 100 mg/kg.

Discussion

Glisoprenins A and B were composed of nine isoprene units. The structure determination is described in detail in a future publication.[2] It was found that these fungal polyisoprene compounds inhibit ACAT activity, although the inhibition was not very potent. A preliminary test of *in vivo* efficacy was carried out using a hamster model. Inhibition of cholesterol absorption from intestines was observed (about 25% inhibition) when glisoprenin B was orally administered at 50 mg/kg (details to be published). Two types of synthetic isoprene-related compounds have been reported to inhibit cholesterol biosynthesis; isoprenoid phosphonates as inhibitors of squalene synthetase[5] and trisnorsqualene alcohol as a potent inhibitor of

vertebrate squalene epoxidase.[6] It might be interesting to see whether the glisoprenins inhibit these enzymes and whether the synthetic isoprenoids inhibit ACAT activity.

Acknowledgment

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References

1) BARRON, G. L. (*Ed.*): *Gliocladium* corda. *In* The Genera of Hyphomycetes from Soil. pp. 177~179, Williams & Wilkins Co., 1968
2) NISHIDA, H.; X.-H. HUANG, H. TOMODA & S. ŌMURA: Glisoprenins A and B, new inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Gliocladium* sp. FO-1513. II. Structure elucidation of glisoprenins A and B. J. Antibiotics 45 (10): 1992, in press
3) TOMODA, H.; H. NISHIDA, R. MASUMA, J. CAO, S. OKUDA & S. ŌMURA: Purpactins, new inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Penicillium purpurogenum*. I. Production, isolation and physico-chemical and biological properties. J. Antibiotics 44: 136~143, 1991
4) TOMODA, H.; X.-H. HUANG, J. CAO, H. NISHIDA, R. NAGAO, S. OKUDA, H. TANAKA, S. ŌMURA, H. ARAI & K. INOUE: Inhibition of acyl-CoA: cholesterol acyltransferase activity by cyclodepsipeptide antibiotics. J. Antibiotics 45 (10): 1992, in press
5) BILLER, S. A.; C. FORSTER, E. M. GORDON, T. HARRITY, W. A. SCOTT, C. P. CIOSEK, Jr.: Isoprenoid (phosphinylmethyl) phosphonates as inhibitors of squalene synthetase: J. Med. Chem. 31: 1869~1871, 1988
6) SEN, S. E. & G. D. PRESTWICH: Trisnorsqualene alcohol, a potent inhibitor of vertebrate squalene epoxidase: J. Am. Chem. Soc. 111: 1508~1510, 1989