Terpendoles, Novel ACAT Inhibitors Produced by *Albophoma yamanashiensis*

I. Production, Isolation and Biological Properties

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A series of new acyl-CoA: cholesterol acyltransferase (ACAT) inhibitors termed terpendoles were isolated from the culture broth of a fungal strain FO-2546 which was proposed to belong to a new genus designated as *Albophoma yamanashiensis*. Among four structurally related terpendoles, terpendole C showed the most potent ACAT inhibitory activity with an IC_{50} value of 2.1 μM in an *in vitro* enzyme assay, followed by terpendoles D (IC_{50}: 3.2 μM), A (15.1 μM) and B (26.8 μM). Evaluation of their ACAT inhibition in the cell assay using J774 macrophages indicated that terpendole D exhibited the highest specificity (cytotoxicity *vs.* ACAT inhibition) among microbial ACAT inhibitors we discovered so far.

During our continuous screening for acyl-CoA: cholesterol acyltransferase (ACAT) inhibitors of microbial origin[1~5], a soil-isolated fungal strain FO-2546 was found to produce a new series of ACAT inhibitors. From the taxonomic study, a new genus, named *Albophoma*, has been proposed for this strain[6]. Six structurally related active compounds were isolated from the fermentation broth of the producer. Two compounds were identified as paspaline[7] and emindole SB[8], which were previously reported as fungal metabolites, but the others appeared to be new compounds and are named terpendoles (Fig. 1).

In this paper, the fermentation, isolation and biological properties of terpendoles are described.

Materials and Methods

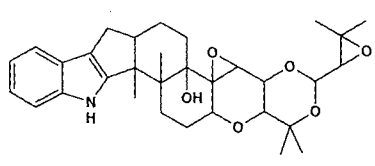
General Experimental Procedures

Fungal strain FO-2546 isolated from a soil sample was used for production of terpendoles. Kieselgel 60 (E. Merck) was used for column chromatography. HPLC was carried out using JASCO system (TRI ROTAR V) and an ODS packed column (YMC-pack D-ODS-5, 20 × 250 mm).

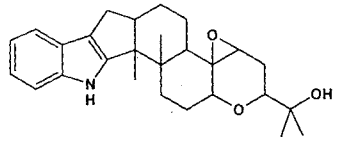
ACAT Activity

ACAT activity was assayed in an enzyme assay using rat liver microsomes[2]. Cholesteryl ester formation and

Fig. 1. Structures of terpendoles A, B, C and D, paspaline and emindole SB.



Terpendole A



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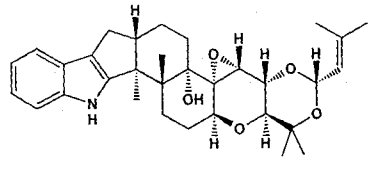
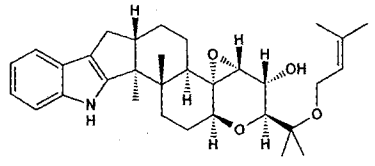
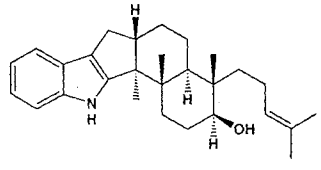


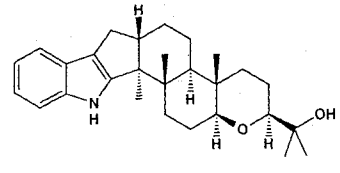
Terpendole C



Terpendole D



Emindole SB



Paspaline

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cell viability were determined in a cell assay using J774 macrophages as reported previously[2].

Antimicrobial Activity

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

Results

Producing Organism

Fungal strain FO-2546 was isolated from a soil sample collected at Shosenkyo, Koufu-shi, Yamanashi, Japan. From the taxonomic studies including cultural, physiological and morphological characteristics, the strain was found to be a new genus member of Nectrioidacese, Coelomycetes. As a result, a new genus *Albophoma* has been proposed, and the strain FO-2546 is named *Albophoma yamanashiensis*[6]. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4406.

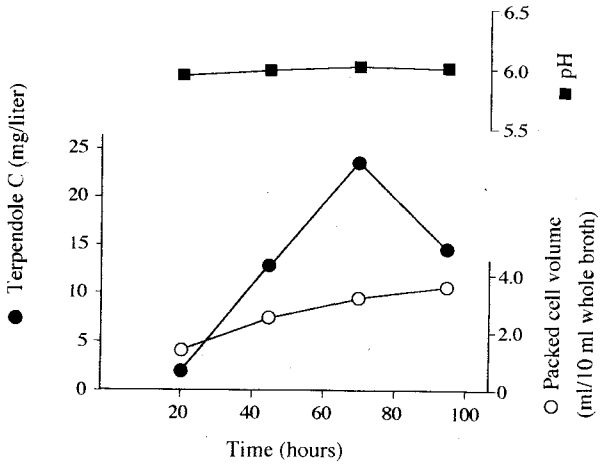
Fermentation

A slant culture of strain FO-2546 grown on 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) 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 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 2 days at 27°C. Two hundred milliliters of the seed culture were transferred into 20 liters of the production medium (soluble starch 3.0%, glycerol 1.0%, soybean meal 2.0%, dry yeast 0.3%, KH_2PO_4 0.05%, $CaCO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, and KCl 0.3%, pH 6.5) in a 30-liter jar fermentor. The fermentation was carried out at 27°C. The production of terpendoles was measured by HPLC under the same conditions described below. A typical time course of the fermentation is shown in Fig. 2. The production of terpendole C started on day 2 and the concentration reached a maximum (20 μg/ml) on day 4 after inoculation.

Isolation

The 96-hour old cultured broth (20 liters) was centrifuged to obtain the mycelium, which was extracted with 20 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution

Fig. 2. Time course of terpendole C production by *Albophoma yamanashiensis*.



(1.5 liters) was extracted with 2 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brownish oil (19.3 g). The material was dissolved in chloroform and applied on a silica gel column (Kieselgel 60, 400 g) previously equilibrated with $CHCl_3$. After washing the column with 1.6 liters of $CHCl_3$, the active components were eluted with 1.6 liters of $CHCl_3$-CH_3OH (99:1), and 400 ml fractions were eluted and collected successively. The 1st fraction rich in terpendoles was concentrated *in vacuo* to give a brownish powder (1.0 g). Terpendoles were finally purified by preparative HPLC (YMC-pack D-ODS-5 (20 × 250 mm); solvent, 70% CH_3CN (0~20 minutes), a linear gradient from 70% CH_3CN to 90% CH_3CN (20~40 minutes) and 90% CH_3CN (40~50 minutes); UV at 225 nm; 8.0 ml/minute). Under this condition, terpendoles A, B, C and D were eluted with retention times of 16.3, 18.5, 22.5 and 30.5 minutes, respectively, and emindole SB (31 minutes) and paspaline (36 minutes) were also eluted. The active fractions were concentrated and extracted with ethyl acetate to give pure terpendoles A (3.4 mg), B (1.5 mg), C (129 mg) and D (24 mg), paspaline (10 mg) and emindole SB (21 mg) as white powders.

Biological Properties

Effect of Terpendoles on ACAT Activity in Microsomes

As shown in Fig. 3, terpendoles inhibited ACAT activity dose-dependently in the enzyme assay. Terpendole C showed the most potent inhibitory activity with an IC_{50} value of 2.1 μM, followed by terpendoles D (IC_{50}: 3.2 μM), A (15.1 μM) and B (26.8 μM). Emindole SB (IC_{50}: 41 μM) and paspaline (134 μM) were less potent ACAT inhibitors than terpendoles.

Fig. 3. ACAT inhibitory activity by terpendoles, paspaline and emindole SB in the enzyme assay using rat liver microsomes.

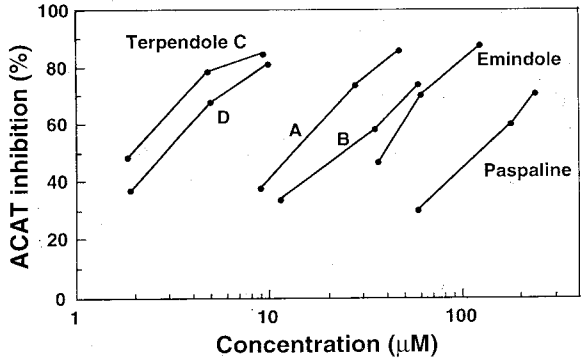
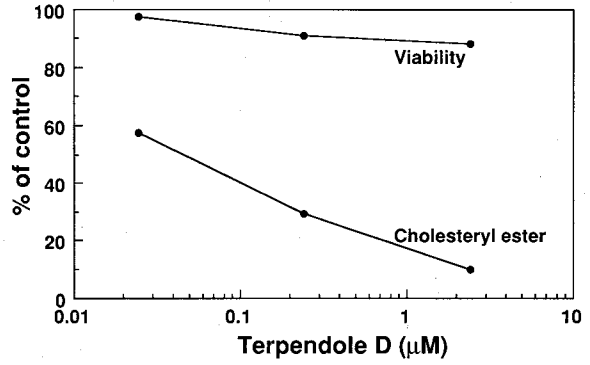


Fig. 4. Effects of terpendole D on cholesteryl ester formation and cell viability in the cell assay using J774 macrophages.



Effect of Terpendoles on Cholesteryl Ester Formation in J774 Macrophages

The ACAT inhibitory activity of terpendoles, paspaline and emindole SB was evaluated in the cell assay using J774 macrophages. Fig. 4 shows the effects of terpendole D on cholesteryl ester (CE) formation and cell viability. CE formation was inhibited by terpendole D in a dose-dependent fashion with an IC_{50} value of 0.048 μM, indicating its inhibition of ACAT activity in the cells. On the other hand, it showed very low cytotoxicity with only about 10% decrease in viability of the cells even at 24.8 μM. The IC_{50} and CD_{50} (a drug concentration causing 50% cell damage) values in the cell assay are summarized in Table 1. Among the compounds isolated, terpendole D exhibited the most potent inhibition of CE formation and the highest specificity (CD_{50}/IC_{50}: >520 under the present condition). Terpendoles A and C also had good specificity. However, terpendole B, paspaline and emindole SB, all lacking the additional prenyl moiety in their structures, showed one to two magnitude higher IC_{50} values and increased cytotoxicity, resulting in low specificity especially for paspaline and emindole SB.

Table 1. ACAT inhibitory activity (IC_{50}) and cytotoxicity (CD_{50}) of terpendoles.

Compound	Rat liver microsomes	J774 macrophages		
	IC_{50} (μM)	IC_{50} (μM)	CD_{50} (μM)	CD_{50}/IC_{50}
Terpendole A	15.1	0.29	>23.4	>81
Terpendole B	26.8	1.80	>29.7	>17
Terpendole C	2.1	0.46	>24.1	>52
Terpendole D	3.2	0.048	>24.8	>520
Paspaline	134.0	2.85	29.0	10
Emindole SB	41.0	6.48	16.0	2.5

Other Biological Activities

No antimicrobial activity was observed at a concentration of 1 mg/ml (10μg/paper disk) for terpendoles A, B, C and D against the following microorganisms; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces sake*, *Pyricularia oryzae*, *Mucor racemosus and Aspergillus niger*.

No acute toxicity was observed when terpendoles C or D was injected intraperitoneally into ddY mice at 100 mg/kg.

Discussion

Terpendoles, paspaline[7] and emindole SB[8] produced by *Albophoma yamanashiensis* have the common indole and diterpene moieties as described in detail in the following paper[9]. Two ACAT inhibitors, synthetic SaH 57-118[10] and fungal gypsetin[11], also possess one and two indole moieties in their structures, respectively. It was reported that they inhibit ACAT activity competitively with respect to acyl-CoA. The mechanism of action of ACAT inhibition might be different between these two known inhibitors and terpendoles since terpendoles possess a steroid-like structure and are likely to compete with the substrate cholesterol.

The order of ACAT inhibitory activity in the microsomal assay shown in Fig. 4 and Table 1 indicated that terpendoles C and D are the most potent, followed by terpendoles A and B. Emindole SB and paspaline are weak inhibitors. This result suggests that a non-epoxidated prenyl moiety attached to the diterpene *via* oxygen atom(s) in terpendoles C and D is responsible for their potent ACAT inhibition.

Many indoloditerpenes reported to be produced by fungi[12] are classified into three groups from a biosynthetic point of view[13], that is, nominine, emindole and paspaline groups. The paspaline group includes aflatrem, paxilline, paspalinine, paspaline, emindole SB and terpendoles. The first three compounds possessing

a hydroxy residue at the C-13 position of the diterpene moieties[9] were demonstrated to show tremorgenic activity, but the biological activity of paspaline and emindole SB has not been reported. In this paper, we demonstrated that terpendoles, paspaline and emindole SB have ACAT inhibitory activity (Fig. 4). It would be interesting to study the relationship between tremorgenic and ACAT inhibitory activities of these indoloditerpenes.

Acknowledgments

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