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A *Penicillium* sp. F33 metabolite and its synthetic derivatives inhibit acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (a key enzyme in platelet-activating factor biosynthesis) and carrageenan-induced paw edema in mice

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ABSTRACT

Acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) acetyltransferase is a key enzyme in the biosynthesis of 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) in inflammatory cells. Substances which inhibit this enzyme are of therapeutic interest. In this study, we screened for new inhibitors of lyso-PAF acetyltransferase with anti-inflammatory effects. In a metabolite from *Penicillium* sp. F33, we isolated an acetyltransferase inhibitor identified as dihydrofumigatin (2-methoxy-1,3,4-trihydroxy-5-methylbenzene) from high resolution mass spectrometer and NMR data. Dihydrofumigatin had strong acetyltransferase inhibitory activity, but was not stable in aqueous solution. Thus, we chemically synthesized its oxidized form fumigatin (3-hydroxy-2-methoxy-5-methyl-1,4-benzoquinone) and derivatives thereof, and evaluated their inhibitory effects. Strong inhibitory activity was observed for saturated fatty acid esters of fumigatin; the order of inhibition was 3-decanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone (termed FUD-7, IC_{50} = 3 μM) > 2-methoxy-5-methyl-3-tetradecanoyloxy-1,4-benzoquinone (termed FUD-8, IC_{50} = 20 μM) > 3-hexanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone (IC_{50} = 139 μM). Interestingly, these compounds also significantly suppressed the gene expression of lyso-PAF acetyltransferase/LPCAT2 in mouse bone marrow-derived macrophages stimulated by lipopolysaccharide (LPS). We further evaluated the effect of these substances on anti-inflammatory activity in vivo using the carrageenan-induced mouse paw edema test. FUD-7 and FUD-8 at 2.5 mg/kg showed significant, 47.9–51.7%, inhibition stronger than that of prednisolone at 10 mg/kg (41.9%). These results suggest that FUD-7 and FUD-8 are potent inhibitors with anti-inflammatory activity.

1. Introduction

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) has been shown to function as a potent chemical mediator in a diverse range of both normal and pathophysiological responses including anaphylactic, inflammatory and allergic responses [1–3] through its G protein-coupled receptor (PAF receptor) [4,5]. Accordingly, many PAF antagonists

Abbreviations: PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; c-PAF, 1-hexadecyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; LPCAT2, lysophosphatidylcholine acyltransferase 2; HPLC, high-performance liquid chromatography; HRMS, high resolution mass spectrometer; DART, direct analysis in real time; TLC, thin layer chromatography; TCA, trichloroacetic acid; LPS, lipopolysaccharide; BMDMs, bone marrow-derived macrophages; NDGA, nordihydroguaiaretic acid; FUD-1, 3,4-dihydroxy-1,2-dimethoxy-5-methylbenzene; FUD-2, 2-methoxy-5-methyl-1,3,4-triacetoxybenzene; FUD-3, 1,4-diacetoxy-2-methoxy-5-methyl-3-tetradecanoyloxybenzene; FUD-4, 3-hydroxy-2-methoxy-5-methyl-1,4-benzoquinone (fumigatin); FUD-5, 3-acetoxy-2-methoxy-5-methyl-1,4-benzoquinone; FUD-6, 3-hexanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone; FUD-7, 3-decanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone; FUD-8, 2-methoxy-5-methyl-3-tetradecanoyloxy-1,4-benzoquinone; FUD-9, 2-methoxy-5-methyl-3-octadecanoyloxy-1,4-benzoquinone.

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have been developed, and several have been reported to inhibit vascular disease [6], asthma [7], renal ischemia [8], pancreatic disease [9], and the systemic inflammatory response syndrome [10]. However, inhibitors of PAF synthesis as anti-allergic and/or anti-inflammatory drugs have until now not been commercially available.

PAF activates numerous types of cells that may be involved in inflammatory and allergic processes, such as neutrophils, basophils, eosinophils, monocytes/macrophages, mast cells and endothelial cells. These cells also synthesize PAF in response to extracellular stimuli via a remodeling pathway. In this pathway, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF), the precursor of PAF, is synthesized from 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (1-alkyl phosphatidylcholine; PC) by the actions of phospholipase A_2. Lyso-PAF is subsequently converted to PAF by acetyl-CoA:lyso-PAF acetyltransferase, a key enzyme in PAF biosynthesis. Recently, two endogenous lyso-PAF acetyltransferases were identified: the constitutively expressed enzyme LPCAT1 (lysophosphatidylcholine acyltransferase 1) [11,12], and the inducible enzyme, LPCAT2 [13,14]. In these studies, the endogenous lyso-PAF acetyltransferase in inflammatory cells was activated by prophlogistic stimuli [15], and the LPCAT2 in mouse peritoneal macrophages and RAW264.7 cells was indeed activated by lipopolysaccharide (LPS) through toll-like receptor 4 [13,14]. Thus, in addition to the development of specific PAF antagonists, substances which are capable of inhibiting these enzymes selectively would be useful for inhibiting the pathological functions of PAF under inflammatory conditions.

We previously developed a simple and reproducible method (the TCA precipitation assay) for screening substances which inhibit acetyltransferase activity, and showed that major components from the cortex of Magnoliae (magnolol and honokiol), and the polyphenols from green tea and black tea (catechins and theaflavins) were potent inhibitors of PAF biosynthesis [16,17]. By using this method, we newly found strong inhibitory activity for an acetyltransferase in the culture broth of a fungus from among 8000 species of actinomyces and fungi. In this study, we isolated an inhibitor from *Penicillium* sp. F33, and identified it as dihydrofumigatin (2-methoxy-1,3,4-trihydroxy-5-methylbenzene). The substance was unstable in aqueous solution, and easily interconverted to its oxidized and more stable form fumigatin (3-hydroxy-2-methoxy-5-methyl-1,4-benzoquinone). Therefore, we chemically synthesized fumigatin and its derivatives, and examined their effect on the activity of lyso-PAF acetyltransferase in rat spleen microsomes and the enzyme induction by LPS in mouse bone marrow-derived macrophages. Further, to evaluate the efficacy of these compounds in vivo, we performed the carrageenan-induced mouse paw edema test.

2. Materials and methods

2.1. Chemicals

1-Hexadecyl-*sn*-glycero-3-phosphocholine (lyso-PAF) was obtained from Cayman Chemical (Ann Arbor, MI), 1-hexadecyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine (c-PAF) from Merck Millipore (Billerica, MA), WEB2086 from Boehringer Ingelheim (Ingelheim am Rhein, FRG), [^{3}H]Acetyl-CoA (92.5 GBq/mmol) from Perkin Elmer (Boston, MA), acetyl-CoA, sodium fluoride (NaF), trichloroacetic acid (TCA), trifluoroacetic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, hexanoic anhydride, zinc, and sodium acetate from Wako Chemicals (Tokyo, Japan), 4-dimethylaminopyridine, nordihydroguaiaretic acid, 6-p-toluidino-2-naphthalene sulfonic acid, prednisolone and BSA (fractionV, essentially fatty acid free) from Sigma (St Louis, MO), ficoll–paque from GE Healthcare (Uppsala, Sweden), pyridine and 2,6-lutidine from Kishida Chemical (Osaka, Japan), decanoyl chloride, tetradecanoyl chloride, and octadecanoic anhydride from TCI (Tokyo, Japan), acetic anhydride from Kanto Chemical (Tokyo, Japan), λ-carrageenan from Nacalai Tesque (Kyoto, Japan), bacto malt extract, bacto yeast extract, bacto peptone from BD (Sparks, MD) and lipopolysaccharide (LPS) *Escherichia coli* 0111:B4 from Difco Laboratories (Detroit, MI).

2.2. Animals

All the experiments followed protocols approved by the Institutional Animal Care and Use Committee, University of Shizuoka. Male Wistar rats, ddY mice and C57BL/6 mice were obtained from Nihon SLC (Hamamatsu, Japan) and acclimatized for 1 week before the experiments. The animals were housed in stainless-steel hanging cages with free access to food and water, and were maintained on a 12 h light/dark cycle.

2.3. Cultivation of the strain of Penicillium sp. F33

The ITS-5.8S rDNA sequence of a fungus in which lyso-PAF acetyltransferase inhibitory activity had been detected in the culture supernatant was identical to that of *Penicillium* sp. F33 (GenGank accession No. JF439500) identified by TechnoSuruga Laboratory Co.,Ltd. The culture medium used had the following composition: Bacto malt extract, 20 g; Bacto yeast extract, 1 g; Bacto peptone, 1 g; glucose, 20 g in 1000 ml of distilled water, and the pH was adjusted to 6.0 with NaOH. This medium was distributed in 400 ml portions, sterilized, inoculated with spores and cultivated at 30 °C.

2.4. Isolation of PAF synthesis inhibitors

The culture broth (about 48 l in total) was separated by filtration from the mycelium with four layers of gauze. The culture filtrate was extracted with ethyl acetate by shaking at least five times. The ethyl acetate solution was evaporated and the reddish brown residue (20.4 g) was dissolved with chloroform. The chloroform solution was applied to a column packed with 230 g of PSQ100B silica gel (Fuji silysia, Japan), then eluted with chloroform–methanol (98.5:1.5). A total of 49 fractions (200 ml each) were collected. Similar fractions were combined after TLC analysis to yield seven fractions (A1: 0.1 g, A2: 0.4 g, A3: 1.3 g, A4: 5.3 g, A5: 2.6 g, A6: 3.3 g, A7: 0.6 g) (Fig. 2). The subfraction A5 (0.1 g), which had the highest specific activity for inhibition of PAF synthesis, was redissolved in 50% acetonitrile in water and injected onto the COSMOSIL 5PE-MS HPLC column (20 × 250 mm, Nacalai tesque, Japan). The HPLC column was eluted with 4% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 6.5 ml/min. The UV detector was set at 250 nm. Fractions were collected as indicated in Fig. 2C, dried in vacuo, and then subfractions B1 (2.5 mg), B2 (59.7 mg), and B3 (30.5 mg) were obtained.

2.5. Stability of fumigatin derivatives

The stability of fumigatin and dihydrofumigatin was analyzed by HPLC separation. The sample solution dissolved in 50% acetonitrile in water (1.25 mg/ml) was stored in sealed glass containers at room temperature for 0, 24 and 72 h. The stored samples (10 μl) were injected onto the Mightysil RP-18GP column (4.6 × 250 mm, Kanto reagent, Japan). The HPLC column was eluted with 20% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 1.0 ml/min. The UV detector was set at 250 nm.

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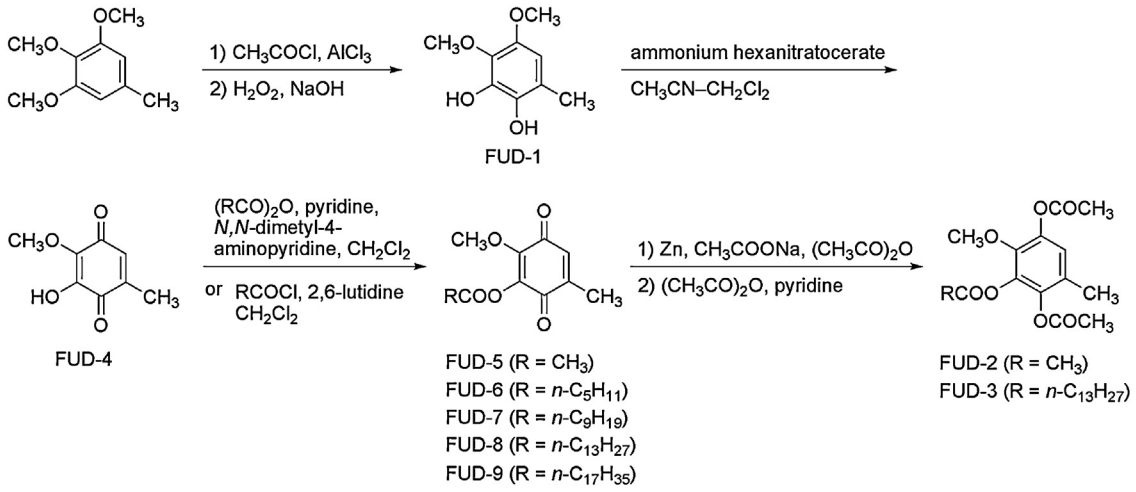

Scheme 1.

2.6. NMR

^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-α-400 FT-NMR or JEOL JNM-ECA 500 spectrometer and chemical shifts were given as δ values with TMS as the internal standard in acetone-d_6 or $CDCl_3$.

2.7. Preparation of rat spleen microsomal fractions

Spleens were obtained from male Wistar rats. The tissue was minced in 9 volumes of a 0.25 M sucrose solution, 1 mM ethylenediamine tetraacetic acid (EDTA) and 25 mM NaF, and homogenized with four strokes of a glass–Teflon homogenizer. The homogenization and subsequent fractionation procedures were carried out at 4 °C. Nuclei and cell debris were removed by centrifugation at 900*g* for 10 min. Microsomes were pelleted from the 9000*g* (10 min) supernatant by centrifugation at 105,000*g* for 60 min. The pellet was suspended in homogenization medium containing no EDTA. The microsomal preparations were stored at −80 °C.

2.8. Assay for acetyl-CoA: Lyso-PAF acetyltransferase activity (TCA precipitation assay)

The activity of the acetyltransferase in rat spleen microsomes was measured as follows. Rat spleen microsomes (2.5 μg protein) were incubated with 300 μM [^{3}H]acetyl-CoA and 40 μM lyso-PAF in 100 μl of 0.1 M Tris–HCl buffer (pH 6.9) containing 25 mM NaF and 0.1% BSA in the presence of various concentrations of inhibitors dissolved in dimethyl sulfoxide (final concentration 5%) or dimethyl sulfoxide alone as a control at 37 °C for 15 min as described before [16]. The reaction was stopped by the addition of 25 μl of a 5.2% BSA saline solution. After 75 μl of a 30% trichloroacetic acid (TCA) solution was added, the reaction mixture was centrifuged at 750*g* for 2 min to obtain [^{3}H]acetyl-PAF bound to the denatured BSA. The resulting pellet was dissolved in 200 μl of 0.1 M sodium phosphate buffer containing 1% SDS (pH 8.0), and mixed with 4 ml of scintillation cocktail (clearsol I, Nacalai Tesque, Japan). Radioactivity was measured in an Alloka LSC-3100 liquid scintillation counter. The IC_{50} value was determined from a regression analysis of the concentration-PAF production curve. Protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

2.9. Synthesis of fumigatin and its derivatives

3,4-Dihydroxy-1,2-dimethoxy-5-methylbenzene (FUD-1), 3-hydroxy-2-methoxy-5-methyl-1,4-benzoquinone (fumigatin, FUD-4), and 3-acetoxy-2-methoxy-5-methyl-1,4-benzoquinone (FUD-5) were synthesized according to procedures (Scheme 1) described previously, and the spectroscopic data were in good agreement with those in the literature, except for the ^{13}C NMR data for 3-acetoxy-2-methoxy-5-methyl-1,4-benzoquinone (FUD-5). There seems to be a typographical error in the reported data [18,19]. FUD-2, 3, and 6–9 were prepared as shown in Scheme 1, whose details are given below.

2.9.1. 3-Acetoxy-2-methoxy-5-methyl-1,4-benzoquinone (FUD-5)

A brown solid, mp 89–91 °C (lit. mp 92–94 °C) [19]. IR (KBr) ν 2926, 1778, 1663, 1618 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.05 (d, 3H, J = 1.5 Hz), 2.34 (s, 3H), 4.08 (s, 3H), 6.50 (d, 1H, J = 1.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.5, 20.4, 60.4, 131.3, 134.9, 144.7, 147.6, 168.1, 181.2, 182.8; high resolution mass spectrometer (HRMS) (direct analysis in real time. DART) m/z calculated for $C_{10}H_{11}O_5$ $[M + H]^+$: 211.0601, found: 211.0588.

2.9.2. Preparation of 3-hexanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone (FUD-6) using fumigatin and hexanoic anhydride

Hexanoic anhydride (0.14 ml, 0.60 mmol) and pyridine (0.10 ml, 1.2 mmol) were added to a mixture of fumigatin (50.0 mg, 0.30 mmol) and 4-dimethylaminopyridine (1.8 mg, 0.015 mmol) in CH_2Cl_2 (1 ml) at room temperature. After stirring for 1.5 h at room temperature, the reaction mixture was quenched with saturated aq. $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate = 5:1) to give FUD-6 (51.0 mg, 64%). A brown oil. IR (KBr) ν 2958, 2932, 1769, 1663, 1618 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.92 (t, 3H, J = 7.0 Hz), 1.32–1.44 (m, 4H), 1.72–1.80 (m, 2H), 2.06 (s, 3H), 2.60 (t, 2H, J = 7.5 Hz), 4.07 (s, 3H), 6.50 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.8, 15.5, 22.2, 24.3, 31.0, 33.7, 60.3, 131.2, 135.0, 144.6, 147.6, 171.0, 181.3, 182.8; HRMS (DART) m/z calculated for $C_{14}H_{19}O_5[M + H]^+$: 267.1227; found: 267.1242.

2.9.3. Preparation of 3-decanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone (FUD-7) using fumigatin and decanoyl chloride

A mixture of fumigatin (52.0 mg, 0.31 mmol), decanoyl chloride (0.090 ml, 0.45 mmol), and 2,6-lutidine (0.080 ml, 0.69 mmol) in CH_2Cl_2 (6 ml) was stirred for 45 min at 0 °C. After quenching with H_2O, the reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated in

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vacuo. The residue was purified by column chromatography (hexane/ethyl acetate = 5:1) to give FUD-7 (61.0 mg, 61%). A yellow solid, mp 53–54 °C. IR (KBr) ν 2928, 2857, 1769, 1663, 1618 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 7.0 Hz), 1.22–1.45 (m, 12H), 1.71–1.78 (m, 2H), 2.06 (d, 3H, J = 1.5 Hz), 2.60 (t, 2H, J = 7.5 Hz), 4.07 (s, 3H), 6.50 (d, 1H, J = 1.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 15.5, 22.7, 24.7, 29.0, 29.19, 29.22, 29.4, 31.8, 33.8, 60.4, 131.2, 135.0, 144.7, 147.6, 171.1, 181.3, 182.9; HRMS (DART) m/z calculated for $C_{18}H_{27}O_5$ $[M + H]^+$: 323.1853; found: 323.1851.

2.9.4. Preparation of 2-methoxy-5-methyl-3-tetradecanoyloxy-1,4-benzoquinone (FUD-8)

Similarly to the preparation of FUD-7, FUD-8 (93.0 mg, 62%) was prepared from fumigatin (66.6 mg, 0.40 mmol), tetradecanoyl chloride (0.12 ml, 1.0 mmol), and 2,6-lutidine (0.12 ml, 0.87 mmol). A yellow solid, mp 63–65 °C. IR (KBr) ν 2926, 2855, 1769, 1663, 1618 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 7.0 Hz), 1.22–1.45 (m, 20H), 1.71–1.79 (m, 2H), 2.06 (d, 3H, J = 1.5 Hz), 2.60 (t, 2H, J = 7.5 Hz), 4.08 (s, 3H), 6.50 (d, 1H, J = 1.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 15.5, 22.7, 24.7, 29.0, 29.2, 29.36, 29.42, 29.59, 29.63, 29.67, 31.9, 33.8, 60.4, 131.3, 135.1, 144.7, 147.6, 171.1, 181.3, 182.9; HRMS (DART) m/z calculated for $C_{22}H_{35}O_5[M + H]^+$: 379.2479; found: 379.2458.

2.9.5. Preparation of 2-methoxy-5-methyl-3-octadecanoyloxy-1,4-benzoquinone (FUD-9)

Similarly to the preparation of FUD-6, FUD-9 (67.5 mg, 52%) was prepared from fumigatin (48.6 mg, 0.29 mmol), octadecanoic anhydride (246 mg, 0.45 mmol), pyridine (0.070 ml, 0.87 mmol), and 4-dimethylaminopyridine (6.1 mg, 0.050 mmol). A yellow solid, mp 74–75 °C. IR (KBr) ν 2926, 2855, 1769, 1663, 1618 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 7.0 Hz), 1.22–1.45 (m, 28H), 1.71–1.79 (m, 2H), 2.06 (d, 3H, J = 1.0 Hz), 2.60 (t, 2H, J = 7.5 Hz), 4.07 (s, 3H), 6.50 (d, 1H, J = 1.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 15.5, 22.7, 24.7, 29.0, 29.2, 29.37, 29.42, 29.58, 29.63, 29.65, 29.69, 31.9, 33.8, 60.4, 131.3, 135.0, 144.7, 147.6, 171.1, 181.3, 182.9; HRMS (DART) m/z calculated for $C_{26}H_{43}O_5[M + H]^+$: 435.3105; found: 435.3091.

2.9.6. Typical procedure for synthesis of triacyl compounds

Zinc (20 equiv) and sodium acetate (3 equiv) were added to a solution of 3-acetoxy- or 3-tetradecanoyloxy-2-methoxy-5-methyl-1,4-benzoquinone (1 equiv) in acetic anhydride (0.1 M) at room temperature. After stirring for 1 h at room temperature, the reaction mixture was quenched with H_2O and extracted with CH_2Cl_2. The organic layer was washed with saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated in vacuo. The residue was diluted with pyridine (100 equiv) and acetic anhydride (250 equiv), and the reaction mixture was stirred at room temperature overnight. After quenching with saturated aq. $NaHCO_3$, the reaction mixture was extracted with diethyl ether. The organic layer was washed with saturated aq. $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified by column chromatography (hexane/ethyl acetate) to give the desired products.

2.9.7. 2-Methoxy-5-methyl-1,3,4-triacetoxybenzene (FUD-2)

Yield: 81%, a brown oil. IR (KBr) ν 2931, 1775 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 2.13 (s, 3H), 2.31 (s, 6H), 2.32 (s, 3H), 3.79 (s, 3H), 6.85 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.8, 20.2, 20.3, 20.7, 61.1, 121.6, 126.5, 136.8, 139.6, 141.3, 142.9, 167.6, 167.8, 168.7; HRMS (DART) m/z calculated for $C_{14}H_{17}O_7$ $[M + H]^+$: 297.0969; found: 297.0974.

2.9.8. 1,4-Diacetoxy-2-methoxy-5-methyl-3-tetradecanoyloxybenzene (FUD-3)

Yield: 83%, a brown oil. IR (KBr) ν 2927, 2855, 1775 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 7.0 Hz), 1.22–1.44 (m, 20H), 1.71–1.78 (m, 2H), 2.12 (s, 3H), 2.29 (s, 3H), 2.31 (s, 3H), 2.55 (t, 2H, J = 7.5 Hz), 3.79 (s, 3H), 6.85 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 15.9, 20.3, 20.7, 22.7, 25.1, 29.1, 29.2, 29.3, 29.4, 29.56, 29.60, 29.63, 31.9, 33.8, 61.1, 121.6, 126.5, 136.8, 139.7, 141.2, 142.9, 167.6, 168.7, 170.6; HRMS (DART) m/z calculated for $C_{26}H_{41}O_7[M + H]^+$: 465.2847; found: 465.2836.

2.10. Assay for cyclooxygenase-1, and -2, and lipoxygenase activity

The effect of FUD-7 and NDGA on the activity of the cyclooxygenase-1 and -2, and 15-lipoxygenase were measured enzymatically with commercial kits from Cayman Chemical (Ann Arbor, MI).

2.11. Preparation of bone marrow-derived macrophages

Primary bone marrow-derived macrophages (BMDMs) were harvested from the femurs (8 to 9-week old mice) using standard procedures [20]. These cells were cultured with RPMI/10%FCS/penicillin and streptomycin. Thirty percent cultured supernatant from a mouse fibroblast cell line (L929, RIKEN BRC, Ibaraki, Japan) was added to the medium as a source of M-CSF. Seven-day-cultured BMDMs were used for functional assays.

2.12. Cell culture

BMDMs (6×10^5 cells) seeded onto six-well plates and cultured for 24 h were pretreated with compounds or vehicle for 30 min and stimulated with 50 ng/ml of LPS. At 24 h after the stimulation, total RNA was extracted.

2.13. Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent from Invitrogen (Carlsbad, CA), and cDNAs were synthesized with a PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. cDNA synthesized from 0.5 μg of total RNA was subjected to quantitative real-time PCR with an Mx300P Sequence Detector (Stratagene, La Jolla, CA) using SYBR Premix Ex Taq reagent (Takara Bio, Ohtsu, Japan) for LPCAT2 (GenBank accession number NM173014) and 18S rRNA (NR003278). The primers for mouse LPCAT2 were 5′-GTCCAGCAGACTACGATCAGTG-3′ (forward) and 5′-CTTATTGGATGGGTCAGCTTTTC-3′ (reverse). Those for 18S rRNA were 5′-TTCTGGCCAACGGTCTAGACAAC-3′ (forward) and 5′-CCAGTGGTCTTGGTGTGCTGA-3′ (reverse). The thermal profile was as follows: hold for 10 s at 95 °C, then two-step PCR for 40 cycles of 95 °C for 5 s followed by 60 °C for 30 s. 18S rRNA was used to normalize gene expression in all samples. Fold induction values were calculated by subtracting the mean difference of LPCAT2 and 18S rRNA cycle threshold Ct numbers for each treatment group from the mean difference of LPCAT2 and 18S rRNA Ct numbers for the vehicle group and raising the difference to the power of 2 (2^{-xxCt}).

2.14. Biosynthesis and quantitation of PAF in BMDMs

BMDMs (6×10^6 cells) were pretreated with compounds or vehicle for 30 min and stimulated with 50 ng/ml of LPS. At 15 min after the stimulation, the reaction was stopped by the addition of methanol and chloroform. After centrifugation for 5 min at 1000*g*, total lipids were extracted from the samples by the method of Bligh and Dyer and separated on a TLC plate (TLC plates silica gel 60,

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Merck, Whitehouse Station, NJ) using a solvent system of cloroform:methanol:H_2O (65:35:6, by volume). The plate was exposed to 6-*p*-toluidino-2-naphthalene sulfonic acid, and PAF, located on the TLC plate between the areas corresponding to sphingomyelin and lysophosphatidylcholine, was scraped off and extracted by the method of Bligh and Dyer. The sample obtained after TLC was used to determine the PAF concentration. PAF was quantified by bioassay using rabbit platelet aggregation [16,17], i.e., by comparison through platelet suspension stimulated by experimental samples with that through a suspension stimulated by a known quantity of 1-hexadecyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine (c-PAF). Platelet aggregation activity was measured as the change in light transmission using an aggregometer (NBS Hematracer 601, Nikko Bioscience, Tokyo, Japan).

2.15. Carrageenan-induced mouse foot edema

The anti-inflammatory activity of acetyltransferase inhibitors was determined using a carrageenan-induced paw edema test. Seven-week-old male ddY mice were divided into groups (n = 6–9 each group) and anti-inflammatory reagents dissolved in saline containing 10% ethanol were administered intravenously for three (prednisolone) or one (other agents) hour before the subcutaneous injection of carrageenan. Each group of animals were anesthetized with diethyl ether before, receiving a subplantar administration of 15 μl of saline to the left paw or 15 μl of carrageenan 2% (w/v) in saline to the right paw. The paw thickness was measured with a micrometer caliper immediately before the subplantar injection and at 1, 2, 3, 4 h after the injection. The increase in paw volume was calculated as the difference between the right paw thickness (carrageenan) and the left paw thickness (saline) measured at each point. The assessment of paw thickness was performed always in a triple blind manner by the same operator.

2.16. Statistical analysis

The data were analyzed by means of Student's t test for unpaired samples with $p < 0.05$ considered significant.

3. Results

3.1. Isolation and structural determination of a acetyl-CoA:lyso-PAF acetyltransferase inhibitor from Penicillium sp. F33 metabolites

To discover new inhibitors for lyso-PAF acetyltransferase, we isolated and identified target substances from metabolites of *Penicillium* sp. F33. This microorganism was cultivated on a malt extract medium at 30 °C. The incubation period was determined by examining the culture supernatant for a change in color and inhibitory activity for PAF synthesis with a microsomal fraction from rat spleen. As shown in Fig. 1, the inhibitory activity declined at 25 d or later, at which a large change in the absorbance of the supernatant at 550 nm occurred. In consideration of these results, we cultivated this fungus until the absorbance at 550 nm exceeded 0.2.

Fig. 2 shows the method, used to separate the acetyltransferase inhibitor. The culture filtrate (48 l) was extracted with ethyl acetate and the solvent was evaporated. The ethyl acetate extract (20.4 g) was chromatographed on a silica gel column. Based on TLC analysis (Fig. 2B), the eluate was separated into seven fractions, A1 to A7. The inhibitory effect on PAF synthesis by the fractions was determined using the TCA precipitation method described in the Section 2. Fraction A5 (100 mg), in which the strongest activity was detected, was further chromatographed using a preparative HPLC system equipped with the 5PE-MS HPLC column using a solvent system of 4% acetonitrile in water containing 0.05% trifluoroacetic acid with a flow rate of 6.5 ml/min, yielding subfraction B2 (Fig. 2C).

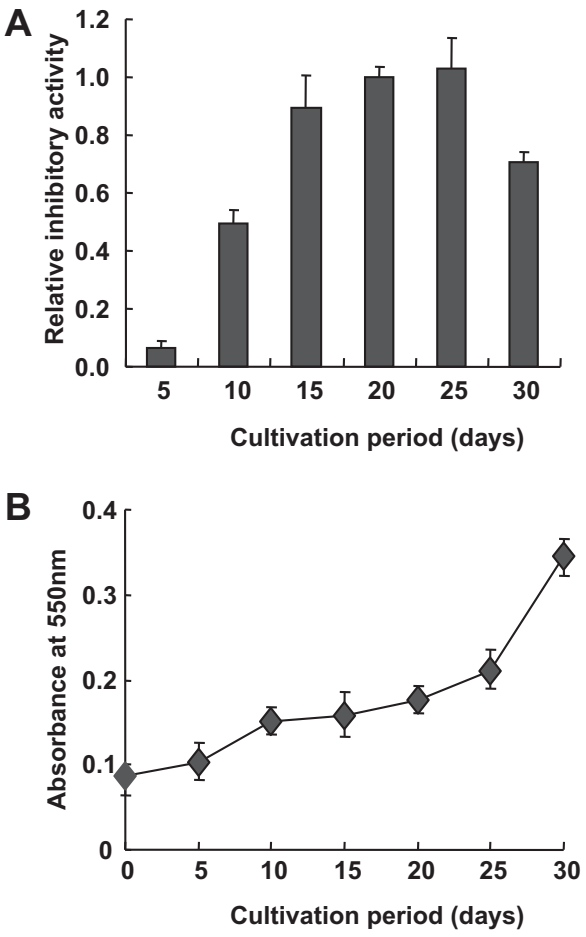

Fig. 1. Relationship between production of PAF acetyltransferase inhibitors and changes in color of culture broth. The fungus was cultivated on a malt extract medium at 30 °C for 30 days. The culture broth (10 ml) was removed at intervals of 5 days. (A) Time course of changes in the inhibitory activity toward PAF acetyltransferase in culture broth. The culture broth was extracted with ethyl acetate to obtain the PAF acetyltransferase inhibitor. Acetyltransferase activity was measured by the TCA precipitation method as described in Section 2. Relative inhibitory activities are expressed taking the control value obtained for the culture broth of 20 days' cultivation as 1. (B) Changes in absorbance of the culture broth at 550 nm. The results are expressed as the mean ± S.E. for three independent experiments.

On an octadecyl silica HPLC column (Mightysil RP-18GP column, Kanto reagents) using a solvent system of 20% acetonitrile in water containing 0.05% trifluoroacetic acid with a flow rate of 1.0 ml/min, the fraction B2 was eluted as a single peak at 5.1 min (Fig. 2D). The component of the fraction was identified as dihydrofumigatin (2-methoxy-1,3,4-trihydroxy-5-methylbenzene), which had inhibitory effects on the acetyltransferase activity in rat spleen microsomes (Table 1). Its structure was elucidated by HRMS analysis (m/z calculated for $C_8H_{11}O_4[M + H]+$: 171.0657; found: 171.0681) as well as ^1H and ^{13}C NMR spectroscopic assignments using heteronuclear multiple bond correlation and heteronuclear multiple quantum correlation experiments (Table 2). More solid evidence was obtained by the direct comparison of its ^1H NMR chemical shifts with those of the compound synthesized by reduction of fumigatin (3-hydroxy-2-methoxy-5-methyl-1,4-benzoquinone) with sodium thiosulfate (Table 2).

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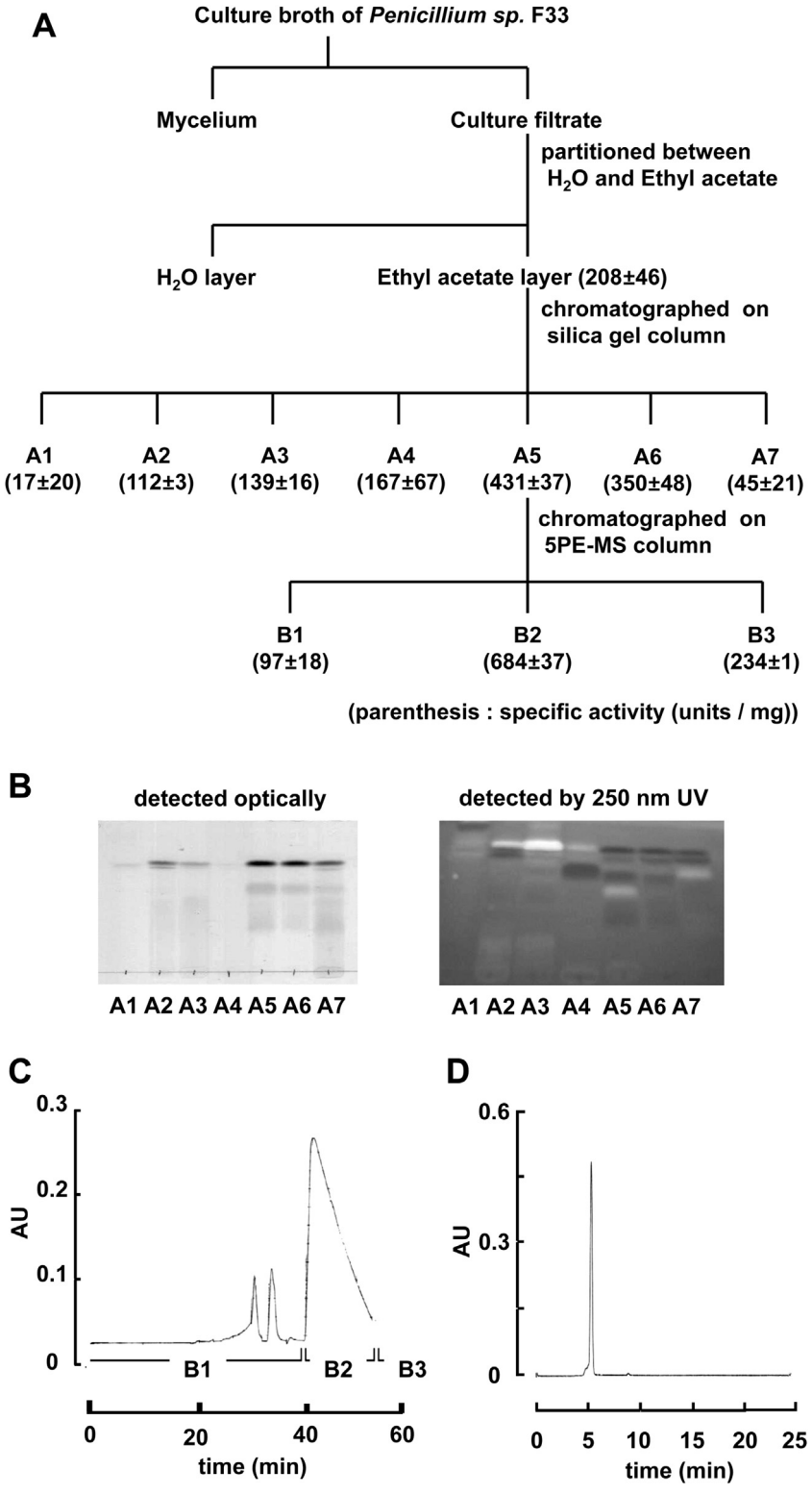

Fig. 2. Isolation of the acetyl-CoA:lyso-PAF acetyltransferase inhibitors from the metabolites of *Penicillium* sp. F33. (A) Schematic diagram of the isolation of inhibitors. One unit is defined as the amount of sample that inhibited 50% of PAF synthesis in the TCA precipitation assay described in Section 2. The specific activity is expressed as the mean ± S.E. for three independent experiments. (B) The eluate from the silica gel column chromatography was monitored by TLC, and separated into A1–A7 fractions. (C) HPLC chromatogram of subfraction A5. The subfraction was applied to a COSMOSIL 5PE-MS HPLC column (20 × 250 mm). The column was eluted with 4% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 6.5 ml/min. The eluate was separated into B1–B3 fractions. (D) HPLC chromatogram of subfraction B2. The subfraction was applied to a Mightysil RP-18GP column (4.6 × 250 mm). The column was eluted with 20% acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 1.0 ml/min. The UV detector was set at 250 nm.

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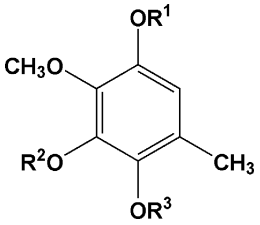
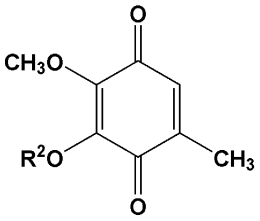
Table 1
Effect of inhibitors on acetyl-CoA: lyso-PAF acetyltransferase activity in rat spleen microsomes.

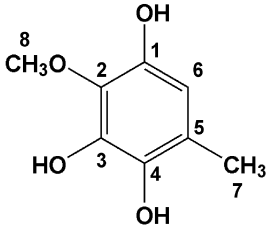
Compound			Inhibition of PAF synthesis IC_{50} (μM)
	Dihydrofumigatin	$R^1=R^2=R^3=H$	101 ± 7
	FUD-1	$R^1=CH_3$, $R^2=R^3=H$	166 ± 8
	FUD-2	$R^1=R^2=R^3=CH_3CO$	>500
	FUD-3	$R^1=R^3=CH_3CO$, $R^2=n\text{-}C_{13}H_{27}CO$	>500
	FUD-4 (fumigatin)	$R^2=H$	109 ± 11
	FUD-4 + decanoic acid	IC_{50}	108 ± 17
	FUD-5	$R^2=CH_3CO$	121 ± 5
	FUD-6	$R^2=n\text{-}C_5H_{11}CO$	139 ± 0
	FUD-7	$R^2=n\text{-}C_9H_{19}CO$	3 ± 0
	FUD-8	$R^2=n\text{-}C_{13}H_{27}CO$	20 ± 0
	FUD-9	$R^2=n\text{-}C_{17}H_{35}CO$	391 ± 53

The acethyltransferase activity was measured by the TCA precipitation method as described in Section 2. Rat spleen microsomes (2.5 μg protein) were incubated with various concentrations of inhibitors dissolved in dimethyl sulfoxide (final concentration, 5%) in 100 μl of 0.1 M Tris buffer (pH 6.9) containing 300 μM [^{3}H]acetyl-CoA, 40 μM lyso-PAF, 25 mM NaF and 0.1% bovine serum albumin at 37 °C for 15 min. FUD-4 + decanoic acid means equimolar mixture of these compounds. The results are expressed as the mean ± S.E. for three separate experiments.

Table 2
NMR chemical shifts of fraction B2 and reduced fumigatin.

	Fraction B2		Reduced fumigatin
	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
1	–	143.6	–
2	–	135.0	–
3	–	137.3	–
4	–	139.0	–
5	–	120.1	–
6	6.18	108.4	6.15
7	2.10	15.7	2.08
8	3.77	60.7	3.75

^{1}H and ^{13}C NMR measurements were performed in acetone-d_6 at 35 °C.



3.2. Fumigatin, an oxidized and highly stable form of dihydrofumigatin, has a similar level of lyso-PAF acetyltransferase inhibitory activity as dihydrofumigatin

The stability test of purified dihydrofumigatin was carried out as described in Section 2. The chromatographs show that the peak at 5.1 min corresponding to dihydrofumigatin decreased dramatically (18.8% and 17.4% after 24 h and 72 h storage, respectively). Alternatively, a main additional peak at 8.8 min, a moderate peak at 2.9 min, and several minor other peaks were detected (Fig. 3A and D). The decrease in the inhibitory activity of the stored sample was small (Fig. 3B). These observations suggest the altered substances to be potent inhibitors.

Since dihydrofumigatin is reported to readily interconvert to its oxidized form fumigatin [21], we chemically synthesized fumigatin (termed FUD-4, Table 1) and evaluated its inhibitory activity and stability. The peak at 8.8 min in the chromatograph of dihydrofumigatin was identified as fumigatin. Actually, the stability test under the conditions used for dihydrofumigatin showed that the peak corresponding to fumigatin remained unchanged and other peaks could not be detected after 72 h storage (Fig. 3C and 3D). As shown in Fig. 4 and Table 1, the inhibitory activity of fumigatin toward the acetyltransferase was equivalent to that of dihydrofumigatin. These results indicate that fumigatin has a very stable conformation, and a similar inhibitory effect on lyso-PAF acetyltransferase as dihydrofumigatin.

3.3. Inhibition of acetyl-CoA:lyso-PAF acetyltransferase by synthetic fumigatin derivatives

Using fumigatin as a starting material, we further synthesized derivatives chemically, and evaluated them. Modifying the hydroxyl moieties of dihydrofumigatin resulted in three compounds, FUD-1, FUD-2, and FUD-3 (Table 1). As shown in Fig. 4 and Table 1, FUD-1 had a slightly weaker inhibitory effect on acetyl-CoA:lysoPAF acetyltransferase than dihydrofumigatin, while FUD-2 and FUD-3 did not have a significant effect. These results indicate that the C-1 and/or C-4 hydroxy groups are essential to inhibit the acetyltransferase activity.

We found that several commercially available saturated fatty acids (Table 3) exhibited inhibitory activity toward the acetyltransferase. This prompted us to examine whether fatty acid esters of fumigatin, termed FUD-5, FUD-6, FUD-7, FUD-8, and FUD-9 (Table 1), inhibit the acetyltransferase activity. As shown in Fig. 4 and Table 1, esterification of the C-3 hydroxyl group of fumigatin with decanoic acid and tetradecanoic acid remarkably enhanced the inhibitory effect. FUD-5, FUD-6, and equimolar mixture of fumigatin and decanoic acid showed similar levels of activity to fumigatin, whereas FUD-9 exhibited weak activity. Nordihydroguaiaretic acid (NDGA) has been reported to be the first potent inhibitor for acetyl-CoA:lyso-PAF acetyltransferases [22], and was used as a standard substance in our previous reports [16,17]. In our assay, the IC_{50} value of NDGA against the acetyltransferase activity in rat spleen microsomes was 123 ± 6 μM, quite a bit larger than that of FUD-7 or FUD-8 (Fig. 4 and Table 1).

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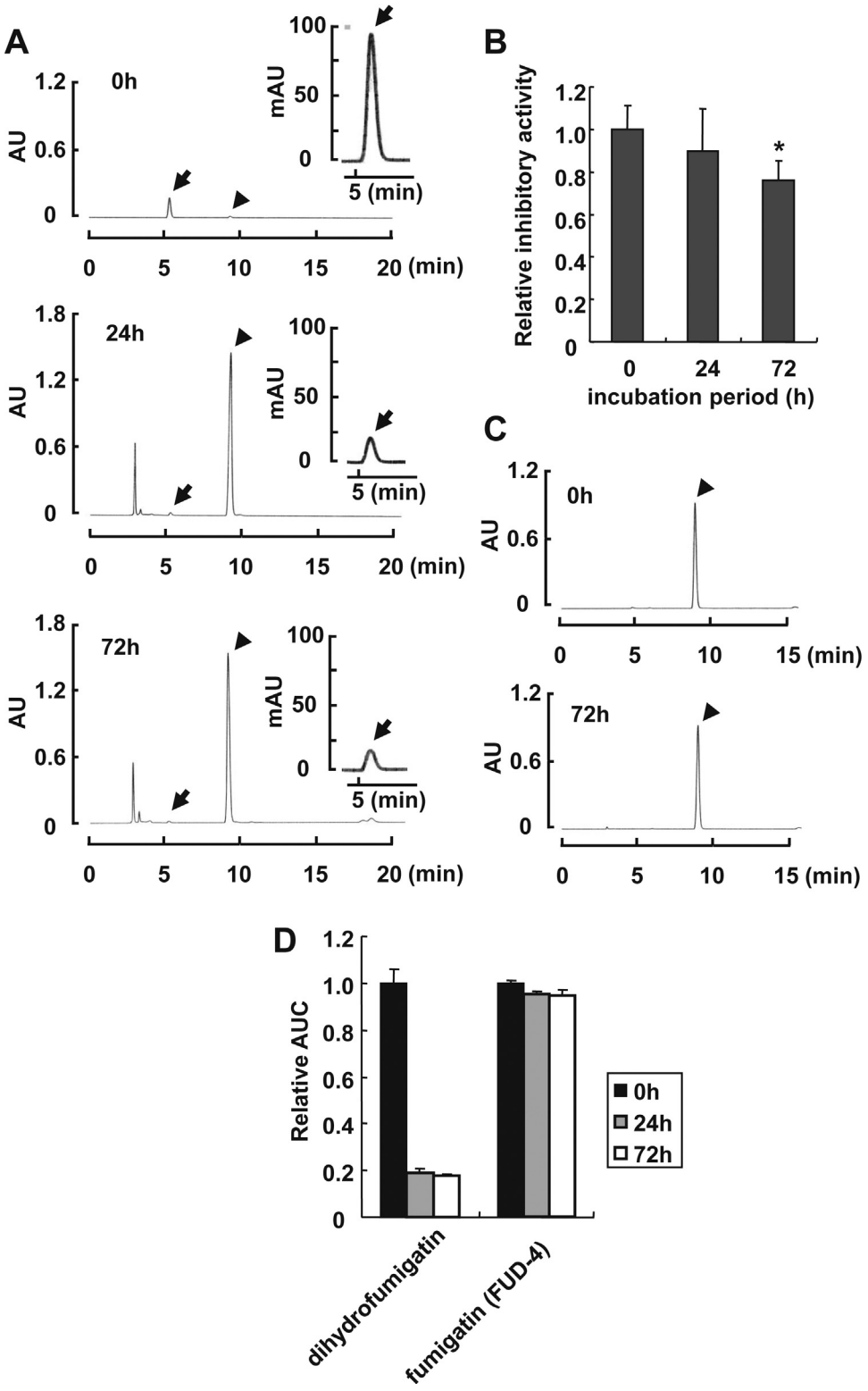

Fig. 3. Stability of fumigatin derivatives. Fumigatin derivatives (1.25 mg/ml) were dissolved in acetonitrile-H_2O (50:50, v/v). After 0, 24 and 72 h storage at room temperature, each sample was analyzed as described in Section 2. (A) HPLC chromatogram of purified dihydrofumigatin. Arrows (5.1 min) and arrowheads (8.8 min) indicate the peak of dihydrofumigatin and fumigatin, respectively. Right upper images are enlargements of the peak of dihydrofumigatin (5.5 min). (B) Acetyltransferase activity of each sample was measured by the TCA precipitation method. Relative inhibitory activity is expressed taking the control value obtained for the sample at 0 h as 1. Data are shown as the mean ± S.E. for three independent experiments. *$p < 0.05$. (C) HPLC chromatogram of synthesized fumigatin. Arrowheads (8.8 min) indicate the peak of fumigatin. (D) Surviving samples were calculated from the area under the curve (AUC) of the corresponding peaks. Relative AUCs are expressed taking the control value obtained for the sample at 0 h as 1. Data are shown as the mean ±S.E. for three independent experiments.

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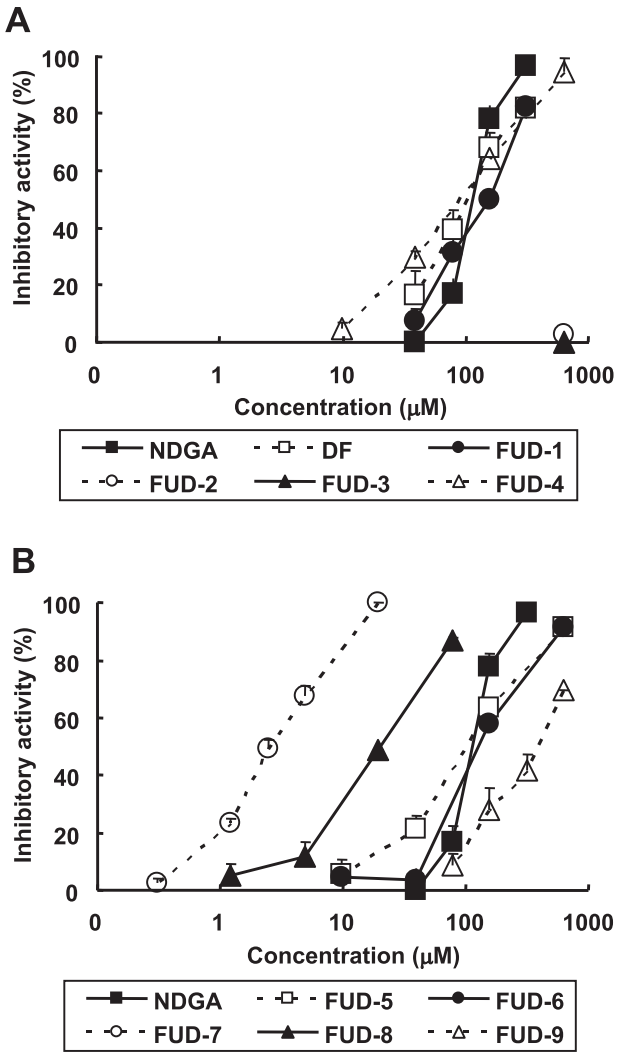

Fig. 4. Effects of PAF acetyltransferase inhibitors on acetyltransferase activity in rat spleen microsomes. Acetyltransferase activity was measured by the TCA precipitation method as described in Section 2. Rat spleen microsomes (2.5 μg protein) were incubated with various concentration of inhibitors; nordihydroguaiaretic acid (NDGA) (A, B, ■), dihydrofumigatin (DF) (A, □), FUD-1 (A, ●), FUD-2 (A, ○), FUD-3 (A, ▲), fumigatin (FUD-4) (A, △), FUD-5 (B, □), FUD-6 (B, ●), FUD-7 (B, ○), FUD-8 (B, ▲), FUD-9 (B, △) dissolved in dimethyl sulfoxide (final concentration, 5%) in 100 μl of 0.1 M Tris buffer (pH 6.9) containing 300 μM [^{3}H]acetyl-CoA, 40 μM lyso-PAF, 25 mM NaF and 0.1% BSA at 37 °C for 15 min. Values are expressed as the mean ± S.E. for one of three experiments, in triplicate, that gave similar results.

We further evaluated the effect of FUD-7 on the inhibitory activity toward 15-lipoxygenase and cyclooxygenases. As shown in Table 4, FUD-7 did not have significant effects.

3.4. Inhibition of lipopolysaccharide (LPS)-induced PAF production and gene expression of lysophosphatidylcholine acyltransferase (LPCAT2)/lyso-PAF acetyltransferase by synthetic fumigatin derivatives

Bone marrow-derived macrophages (BMDMs) were pretreated with the inhibitors or vehicle for 30 min and stimulated by 50 ng/ml of LPS. The expression of LPCAT2 mRNA was then measured 24 h later. As shown in Fig. 5A, FUD-7 significantly inhibited the expression at 3 μM. The inhibitory activity of FUD-7 was stronger than that of NDGA at 25 μM or WEB2086 at 5 μM. Furthermore, we evaluated the effect of other fumigatin derivatives. Fig. 5B shows the inhibitory effect of FUD-6, FUD-7, and FUD-8 on LPCAT2 mRNA expression, indicating that it correlated with the lyso-PAF

Table 3
Effect of saturated fatty acids on acetyl-CoA: lyso-PAF acetyltransferase activity in rat spleen microsomes.

Compound	Inhibition of PAF synthesis IC_{50} (μM)
n-$C_7H_{15}COOH$	>500
n-$C_9H_{19}COOH$	>500
n-$C_{11}H_{23}COOH$	419 ± 13
n-$C_{13}H_{27}COOH$	183 ± 5
n-$C_{15}H_{31}COOH$	>500

The acethyltransferase activity was measured by the TCA precipitation method as described in Section 2. Rat spleen microsomes (2.5 μg protein) were incubated with various concentrations of fatty acids dissolved in dimethyl sulfoxide (final concentration, 5%) in 100 μl of 0.1 M Tris buffer (pH 6.9) containing 300 μM [^{3}H]acetyl-CoA, 40 μM lyso-PAF, 25 mM NaF and 0.1% bovine serum albumin at 37 °C for 15 min. The inhibitory activity of palmitic acid was measured dispersed in solution because of poor solubility. The results are expressed as the mean ± S.E. for three separate experiments.

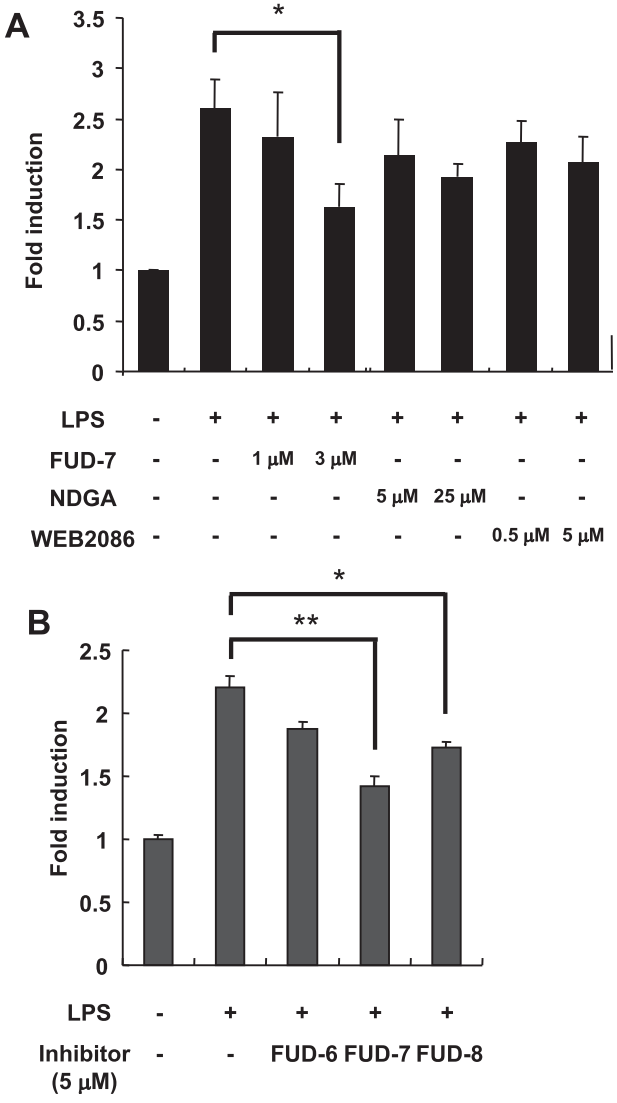

Fig. 5. Effects of the fatty acid esters of fumigatin on LPS-stimulated transcription of lyso-PAF acetyltransferase/LPCAT2 mRNA in bone marrow-derived macrophages (BMDMs).
(A, B) LPCAT2 mRNA levels were measured by quantitative RT-PCR. The y-axis shows relative levels expressed by taking the control vehicle only values obtained from non-stimulated BMDMs as 1. Data are shown as the mean ± S.E. for three to four independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ compared with LPS-stimulated control.

Table 4
Effect of FUD-7 and nordihydroguaiaretic acid (NDGA) on lipoxygenase and cyclooxygenases.

	Inhibition of enzymes IC_{50} (μM)	
	FUD-7	NDGA
15-Lipoxygenase	>100	8.8 ± 0.3
Cyclooxygenase-1	>100	44.5 ± 4.2
Cyclooxygenase-2	>100	9.1 ± 0.7

The enzyme activities were measured with commercially available kits as described in Section 2. The results are expressed as the mean ± S.E. for three separate experiments.

acetyltransferase inhibitory activity in rat spleen microsomes. LPCAT1 mRNA was not significantly induced by LPS in BMDMs, indicating that the effect of FUD-7 on the gene expression of LPCAT1 was not significant (data not shown).

The production of PAF 15 min after the LPS-stimulation was quantified by bioassay using rabbit platelet aggregation. As shown in Fig. 6B, the sample extracted from LPS-stimulated BMDMs caused the shape transforming response of rabbit platelet similar to that of c-PAF at 2.5×10^{-11} M, and pre-treatment of a PAF antagonist WEB2086 suppressed this effect. Pre-incubation of BMDMs with FUD-7 at concentration of 5 μM for 30 min resulted in a significant inhibition of PAF biosynthesis at the level of non-stimulated BMDMs (Fig. 6C and D).

3.5. Inhibition of carrageenan-induced mouse paw edema by fatty acid esters of fumigatin

To examine the anti-inflammatory effect of dihydrofumigatin and the synthetic fumigatin derivatives in vivo, the carrageenan-induced paw edema test was performed (Fig. 7). Prednisolone has been generally employed for carrageenan-induced paw edema test as a standard substance. To confirm this assay work properly and to compare the anti-edematous effect of fumigatin derivatives with those of other anti-inflammatory reagents, we used prednisolone as a positive control. Prednisolone at 10 mg/kg significantly reduced paw thickness compared to the control vehicle only. Mice were administered various doses of dihydrofumigatin or the fumigatin derivatives (1–10 mg/kg) intravenously 1 h before the carrageenan administration. Dihydrofumigatin at 10 mg/kg significantly decreased paw thickness, while at 5 mg/kg it had a significant effect from at 3 h compared to the control. The anti-edematous effect of the synthetic fatty acid esters of fumigatin was examined at a dose of 2.5 or 1 mg/kg because of poor solubility. FUD-7 and FUD-8 significantly ($p < 0.05$) suppressed paw edema at 2.5 mg/kg, and tended to do so at 1 mg/kg, whereas FUD-6 at both concentrations and co-administration of fumigatin and decanoic acid at equimolar amount of 2.5 mg/kg as FUD-7 had no effect. The maximum inhibition of edema by FUD-7 and FUD-8 at 2.5 mg/kg was 51.7% and 47.9%, respectively, which was greater than that of prednisolone at 10 mg/kg (41.9%). The efficacy of fumigatin derivatives was dose-dependent and consistent with the

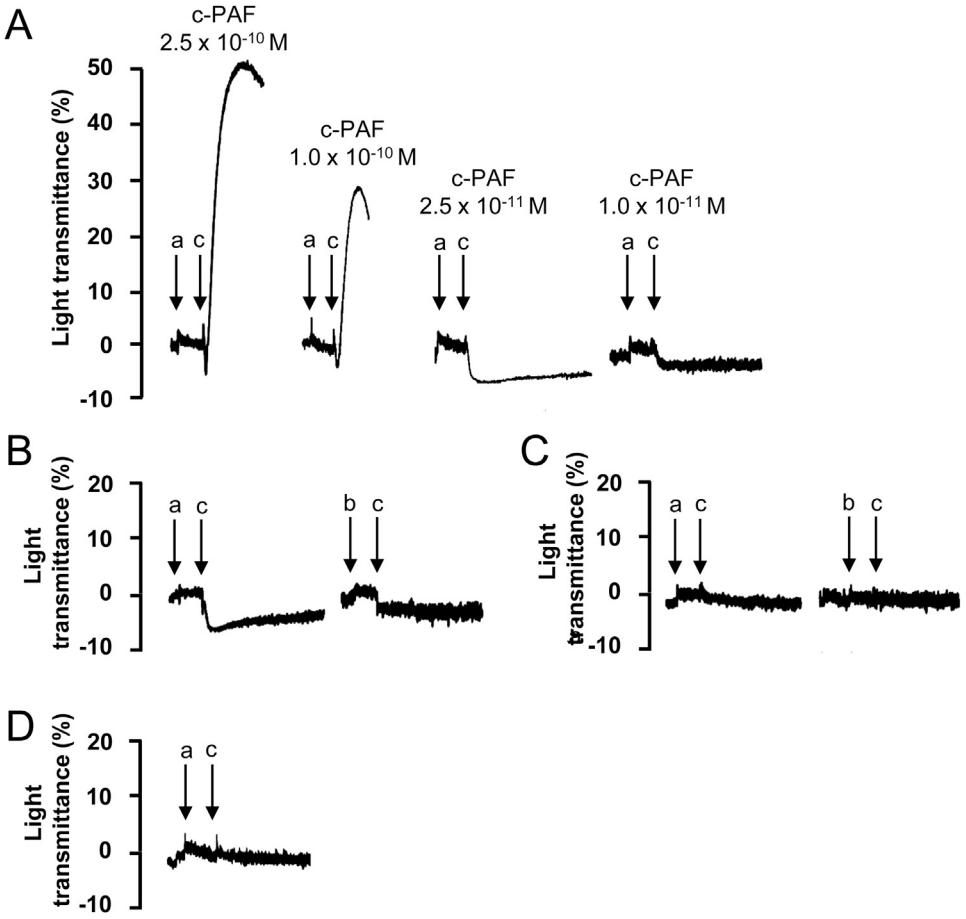

Fig. 6. Effect of FUD-7 on PAF biosynthesis in mice bone marrow-derived macrophages (BMDMs) stimulated with LPS. BMDMs (6×10^6 cells) seeded onto dishes and cultured for 24 h were pretreated with FUD-7 at 5 μM (C) or vehicle (B) for 30 min and stimulated with 50 ng/ml of LPS. At 15 min after the stimulation, the amount of PAF produced was determined by bioassay using rabbit platelet aggregation as described in Section 2. PAF biosynthesis in non-stimulated BMDMs (D) was also examined. Platelets were preincubated with vehicle (0.1% BSA/saline) or WEB2086 for 1 min and then exposed to a known quantity of c-PAF (A) or tenth part of the samples (B–D). a = vehicle (0.1% BSA in saline); b = WEB2086; c = c-PAF or the samples.

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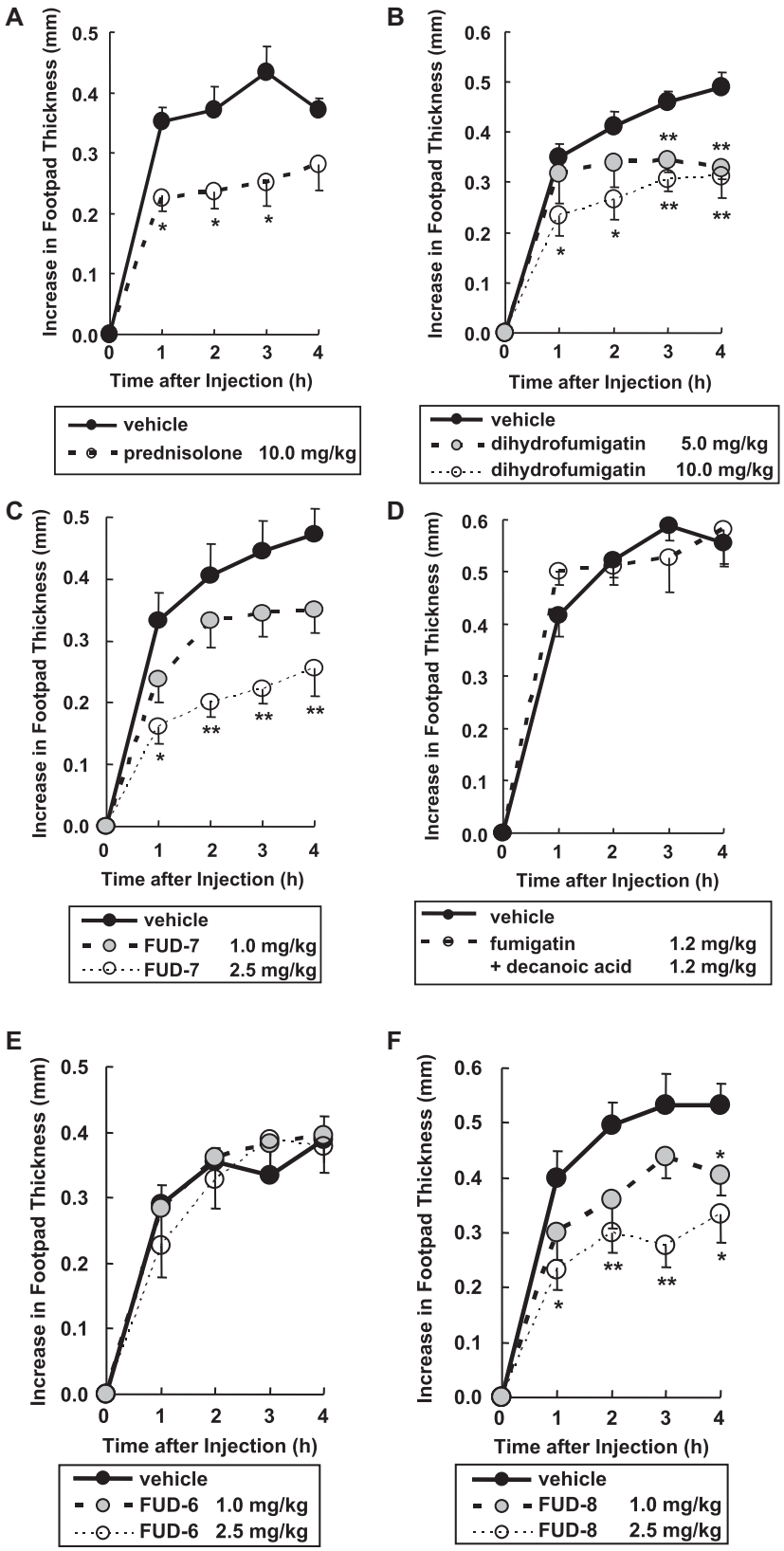

Fig. 7. Effect of fumigatin derivatives on carrageenan-induced paw edema. Mice were pretreated with prednisolone ((A) dissolved in saline containing 10% ethanol, *i.p.*, $n = 9$), dihydrofumigatin ((B) dissolved in saline, *i.v.*, $n = 6$), fatty acid esters of fumigatin ((C) FUD-7, (D) co-administration of fumigatin and decanoic acid, E:FUD-6, F:FUD-8 dissolved in saline containing 10% ethanol, *i.v.*, $n = 6$), or vehicle (saline containing 10% ethanol or saline, *i.p.* or *i.v.*, $n = 6$–7) 3 h (prednisolone) or 1 h (other substances) before the local injection of carrageenan (300 μg/paw) at the indicated dose. Edema was expressed as increases in paw thickness (mm) 0 to 4 h following the carrageenan injection. Data are expressed as the mean ± S.E. *,** indicate $p < 0.05$ or $p < 0.01$ vs. vehicle, respectively.

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results of the TCA precipitation assay. These results indicated that fumigatin derivatives have a strong anti-edematous effect on carrageenan-induced paw edema which correlated with the inhibitory activity for lyso-PAF acetyltransferase in rat spleen microsomes.

4. Discussion

In inflammatory and allergic processes, PAF is biosynthesized in specific enzymatic reactions, which involve acetyl-CoA:lyso-PAF acetyltransferases (remodeling pathway). Inhibitors of these acetyltransferases are expected to be of therapeutic interest. Here, we screened for such inhibitors with anti-inflammatory effects.

There are several reports of substances which inhibit PAF synthesis in intact cells such as human neutrophils, rat peritoneal macrophages and human endothelial cells. Calmodulin antagonists (trifluoperazine and *N*-6-aminohexyl-5-chloro-1-naphthalene sulfonamide) [23], antiflammins and luffariellolide which interfere with the activation of phospholipase A2 [24,25], nitroprusside and 3-morpholinosydnonimine which stimulate the production of cGMP [26], and a protein kinase C inhibitor (D-sphingosine) [27] have been shown to inhibit PAF biosynthesis. However, these substances did not directly inhibit acetyltransferase activity. In addition, the quinoline-based compounds PF-5901 and Wy-50295 [28], ketotifen [29,30], the cyclooxygenase and 5-lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) [22], magnolol, honokiol [16], and galloyl compounds [17] are also reported to be potent acetyltransferase inhibitors. In this study, we found fumigatin and its derivatives to be novel inhibitors. The mould metabolic product fumigatin, $C_8H_8O_4$, and its reductive form dihydrofumigatin, $C_8H_{10}O_4$, were reported to be isolated from cultures of *Aspergillus fumigatus* [21,31]. Whereas it is suggested that these two substances act on the oxidation-reduction system in the vital processes of this mould, their physiological function against mammals remains to be clarified. Thus, this is the first report on the physiologic activity of fumigatin and its derivatives.

Fumigatin was more stable, and its inhibitory activity toward lyso-PAF acetyltransferase was similar to that of dihydrofumigatin (Fig. 3 and Table 1). In addition, some saturated fatty acids also had inhibitory activity (Table 3). These observations prompted us to produce fatty acid esters of fumigatin chemically as pro-drugs. Carboxylic acid conjugates of drugs such as indomethacin farnesil, flurbiprofen axetil, and docosahexaenoic acid ester of paclitaxel have been shown to improve the therapeutic potential of drugs by increasing absorption and half-lives, improving drug delivery, and reducing side effects [32–34]. Since it has been proposed that these compounds are cleaved by a carboxyesterase in vivo and converted to an active form, it was expected that fatty acid esters of fumigatin may be converted to two active compounds at inflamed sites and exert additive effects. Among the fatty acid esters of fumigatin, FUD-7 and FUD-8, the decanoic and tetradecanoic acid esters of fumigatin, respectively, had strong inhibitory activity in rat spleen microsomes (Fig. 4 and Table 1). Interestingly, the inhibitory activities for the acetyltransferase and for the carrageenan-induced paw edema were far stronger than the additive effect of fumigatin and corresponding fatty acid (Fig. 7 and Table 1), and the order of inhibitory activity of fatty acid esters did not correlate with that of the fatty acids themselves. These results imply that the enhancement of inhibitory activity does not result in the hydrolysis of the compounds to fumigatin and fatty acids by a carboxylesterase. The IC_{50} value of FUD-7, which had the strongest activity in this study, was 40 times lower than that of NDGA. Since IC_{50} values of honokiol, magnolol, theaflavins, and dimeric flavan-3-ols with a galloyl group at C-3 under our experimental conditions have been reported to be 2–10 times lower than that of NDGA [16,17], the inhibitory effect on PAF biosynthesis of FUD-7 was stronger than that of these compounds. Although it still remains to be clarified whether or not the inhibition of PAF synthesis by fumigatin derivatives is due to direct effects on the acetyltransferase enzyme, the inhibitory effect was not on the oxidation–reduction reaction because the inhibitory activity of fumigatin was similar to that of its reduction product dihydrofumigatin. In relation to the mode of action of fumigatin derivatives, the quinone or dihydroquinone structure appeared necessary for the inhibition of PAF synthesis because diester derivative of FUD-8, i.e., FUD-3, abolished the inhibitory effect (Fig. 4 and Table 1).

FUD-7 significantly inhibited not only the activity of acetyltransferase in rat spleen microsomes but also the PAF production in LPS-stimulated bone marrow-derived macrophages (BMDMs) (Fig. 6). In addition to the efficacy to inhibit the function of lyso-PAF acetyltransferase/LPCAT2, FUD-6, FUD-7, and FUD-8 also inhibited the expression of LPCAT2 mRNA (Fig. 5). Together with the data that a PAF antagonist WEB2086 weakly inhibited, this may be a secondary effect by attenuating PAF-induced PAF synthesis by blocking the initial PAF synthesis rather than a direct effect by inhibiting the signals which induce the LPCAT2 expression. It was reported that PAF itself stimulated the lyso-PAF acetyltransferase and PAF biosynthesis in rat and human neutrophils [35,36]. In macrophages, inflammatory cytokines are produced in response to PAF, and known to stimulate PAF synthesis [37]. Thus, the gene expression of LPCAT2 may be involved in such a positive feedback mechanism.

Carrageenan-induced paw edema is a suitable animal model for evaluating anti-edematous effects. Since the involvement of PAF with the formation of paw edema was reported previously [38,39], we employed this model in the present study. The edema that develops following the injection of carrageenan serves as an index of acute inflammatory changes, and can be determined from differences in paw thickness measured immediately after the injection and then every hour. The edema induced by carrageenan is believed to be biphasic: the first phase (mouse: 0–4 h) involves the release of serotonin and histamine and the second phase (mouse: over 24 h) is mediated by prostaglandins, cyclooxygenase products. Continuity between the two phases is provided by kinins [40–42]. PAF is thought to be involved in the first phase, because PAF production transiently increased 1 h after carrageenan injections in rats and PAF antagonists inhibited the edema more effectively in the first phase than the second phase [38,39]. Thus, we examined the effect of the fumigatin derivatives on the first phase (1–4 h) of carrageenan-induced paw edema in mice. All the fumigatin derivatives used in this study except for FUD-6 showed a significant reduction of paw edema at 1 h or more after the carrageenan injection, and the efficacy was dose-dependent. The order in terms of inhibition of carrageenan-induced edema was FUD-7 > FUD-8 > FUD-6, which was similar that for the inhibition of PAF synthesis in rat spleen microsomes (Figs. 4 and 7, and Table 1). These results suggest that fumigatin derivatives produce an anti-edematous effect during the first phase of carrageenan-induced paw edema through a reduction of PAF synthesis. To date, several PAF receptor antagonists have been reported to inhibit carrageenan-induced paw edema: for example, kadsurenone (50 mg/kg *i.p.*) and L-652731 (10 mg/kg *p.o.*) inhibited the first phase of the edema by 27% and 38%, respectively [39], and administration of WEB2086 (4–16 mg/kg, *i.p.*) and WEB2170 (2–8 mg/kg, *i.p.*) achieved 34–50% and 30–60% inhibition at 4 h after the injection, respectively [38]. The efficacy of FUD-7 and FUD-8, whose administration (2.5 mg/kg *i.v.*) resulted in 47.9–51.7% inhibition in the first phase (Fig. 7), was stronger than that of PAF antagonists, although the difference in the route of administration should be considered. Esterification of fumigatin with fatty acids might be contributed to this strong effect by improving

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membrane permeability at inflamed sites. Accordingly, it may be possible to develop new inhibitors of platelet activation based on fumigatin derivatives such as FUD-7 as anti-platelet drugs.

The results described in this paper suggest that fatty acid esters of fumigatin, especially FUD-7, are powerful anti-inflammatory agents with inhibitory activity for PAF biosynthesis. These compounds may have clinical potential for the treatment of allergies and inflammation.

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References

[1] Hanahan DJ. Platelet activating factor: a biologically active phosphoglyceride. Annu Rev Biochem 1986;55:483–509.
[2] Woodrad DS, Lee T, Synder F. The final step in the de Novo biosynthesis of platelet-activating factor. J Biol Chem 1987;262:2520–7.
[3] Venable ME, Zimmerman GA, Mcintyre TM, Prescott SM. Platelet-activating factor: a phospholipids autacoid with diverse actions. J Lipid Res 1993;34:691–702.
[4] Shimizu T. Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. Annu Rev Pharmacol Toxicol 2009;49:123–50.
[5] Prescott SM, Zimmerman GA, Mcintyre TM. Platelet-activating factor. J Biol Chem 1990;265:17381–84.
[6] Lehr HA, Weyrich AS, Saetzler RK, Jurek A, Arfors KE, Zimmerman GA, et al. Vitamin C blocks inflammatory platelet-activating factor mimetics created by cigarette smoking. J Clin Invest 1997;99:2358–64.
[7] Evans DJ, Barnes PJ, Cluzel M, O'Connor BJ. Effects of a potent platelet-activating factor antagonist, SR27417 on allergen-induced asthmatic responses. Am J Respir Cirt Care Med 1997;156:11–6.
[8] Kelly K, Tolkoff-Rubin NE, Rubin RH, Williams Jr WW, Meehan SM, Meschter CL, et al. An oral platelet-activating factor antagonist, Ro-24-4736, protects the rat kidney from ischemic injury. Am J Physiol 1996;271:F1061–67.
[9] Kingsnorth AN, Galloway SW, Formela LJ. Randomized double-blind phase II trial of Lexipafant, a platelet-activating factor antagonist, in human acute pancreatitis. Br J Surg 1995;82:1414–20.
[10] Dhainaut JFA, Tenaillon A, Le Tulzo Y, Schlemmer B, Solet JP, Wolff M, et al. Platelet-activating factor receptor antagonist BN52021 in the treatment of severe sepsis: a randomized, double-blind, placebo-controlled, multi-center clinical trial. BN52021 Sepsis Study Group. Crit Care Med 1994;22:1720–8.
[11] Nakanishi H, Shindou H, Hishikawa D, Harayama T, Ogasawara R, Suwabe A, et al. Cloning and characterization of mouse lung-type acyl-CoA:lysophosphatidylcholine acyltransferase 1 (LPCAT1): expression in alveolar type II cells and possible involvement in surfactant production. J Biol Chem 2006;281:20140–47.
[12] Harayama T, Shindou H, Ogasawara R, Suwabe A, Shimizu T. Identification of a novel noninflammatory biosynthetic pathway of platelet-activating factor. J Biol Chem 2008;283:11097–106.
[13] Shindou H, Hishikawa D, Nakanishi H, Harayama T, Ishii S, Taguchi R, et al. A single enzyme catalyzes both platelet-activating factor production and membrane biogenesis of inflammatory cells: cloning and characterization of acetyl-CoA:lyso-PAF acetyltransferase. J Biol Chem 2007;282:6532–9.
[14] Morimoto R, Shindou H, Oda Y, Shimizu T. Phosphorylation of lysophosphatidylcholine acyltransferase 2 at Ser^{34} enhances platelet-activating factor production in endotoxin-stimulated macrophages. J Biol Chem 2010;285:29857–62.
[15] Shindou H, Ishii S, Yamamoto M, Takeda K, Akira S, Shimizu T. Priming effect of lipopolysaccharide on acetyl-coenzyme A: lyso-platelet-activating factor acetyltransferase is MyD88 and TRIF independent. J Immunol 2005;175:1177–83.
[16] Yamazaki M, Sugatani J, Fujii I, Kuroyanagi M, Umehara K, Ueno A, et al. Development of a novel method for determination of acetyl-CoA:1-alkyl-sn-glycero-3-phsophocholine acetyltransferase activity and its application to screening for acetyltransferase inhibitors: Inhibition by magnolol and honokiol from magnoliae cortex. Biochem Pharmacol 1994;47:995–1006.
[17] Sugatani J, Fukazawa N, Ujihara K, Yoshinari K, Abe I, Noguchi H, et al. Tea polyphenols inhibit acetyl-CoA:1-alkyl-*sn*-glyceo-3-phosphocholine acetyltransferase (a key enzyme in platelet-activating factor biosynthesis) and platelet-activating factor-induced platelet aggregation. Int Arch Allergy Immunol 2004;134:17–28.
[18] Poon WW, Barkovich RJ, Hsu AY, Frankel A, Lee PT, Shepherd JN, et al. Yeast and rat Coq3 and *Escherichia coli* UbiG polypeptides catalyze both *O*-methyltransferase steps in coenzyme Q biosynthesis. J Biol Chem 1999;274:21665–72.
[19] van der Klei A, de Jong RLP, Lugtenburg J, Tielens AGM. synthesis and spectroscopic characterization of [1′-^{14}C]Ubiquinone-2, [1′-^{14}C]-5-Demethoxy-5-hydroxyubiquinone-2, and [1′-^{14}C]-5-demethoxyubiquinone-2. Eur J Org Chem 2002;3015–23.
[20] Weischenfeldt J, Porse B. Bone marrow-derived macrophages (BMM): isolation and applications, In: CSH Protoc 2008 Dec 1; 2008:pdb.prot5080.
[21] Anslow WK, Raistrick H. Studies in the biochemistry of micro-organisms: fumigatin (3-hydroxy-4-methoxy-2:5-toluquinone), and spinulosin (3:6-dihydroxy-4-methoxy -2:5-toluquinone), metabolic products respectively of *Aspergillus fumigatus* Fresenius and *Penicillium spinulosum* Thom. Biochem J 1938;32:687–96.
[22] White HL, Faison LD. Inhibition of lysoPAF:acetyl-CoA acetyltransferase by salicylates and other compounds. Prostaglandins 1988;35:939–44.
[23] Billah MM, Siegel MI. Calmodulin antagonists inhibit formation of platelet-activating factor in stimulated human neutrophils. Biochem Biophys Res Commun 1984;118:629–35.
[24] Tetta C, Camussi G, Bussolino F, Herrick-Davis K, Baglioni C. Inhibition of the synthesis of platelet-activating factor by anti-inflammatory peptides (antiflammins) without methionine. J Pharmacol Exp Ther 1991;257:616–20.
[25] Glaser KB, Lock YW, Chang JY. PAF and LTB4 biosynthesis in the human neutrophil: effects of putative inhibitors of phospholipase A2 and specific inhibitors of 5-lipoxygenase. Agents Actions 1991;34:89–92.
[26] Heller R, Bussolino F, Ghigo D, Garbarino G, Pescarmona G, Till U, et al. Nitrovasodilators inhibit thrombin-induced platelet-activating factor synthesis in human endothelial cells. Biochem Pharmacol 1992;44:223–9.
[27] Leyravaud S, Bossant MJ, Joly F, Bessou G, Benveniste J, Ninio E. Biosynthesis of paf-acether. X. Phorbol myristate acetate-induced paf-acether biosynthesis and acetyltransferase activation in human neutrophils. J Immunol 1989;143:245–9.
[28] Hogaboam CM, Donigi-Gale D, Shoupe TS, Bissonnette EY, Befus AD, Wallace JL. Platelet-activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds. Br J Pharmacol 1992;105:87–92.
[29] Joly F, Bessou G, Benveniste J, Ninio E. Ketotifen inhibits paf-acether biosynthesis and *b*-hexosaminidase release in mouse mast cells stimulated with antigen. Eur J Pharmacol 1987;144:133–9.
[30] Nakamura T, Kuriyama M, Ishihara K, Matsumura Y, Miyamoto T. Platelet-activating factor (PAF) in allergic disease: inhibitory effects of anti-allergic drugs, ketotifen and three kampo medicines on PAF production. Lipids 1991;26:1297–300.
[31] Yamamoto Y, Nitta K, Tango K, Saito T, Tsuchimuro M. Studies on the metabolic products of a strain of *Aspergillus fumigatus* (DH413). I. Isolation and chemical structures of metabolites. Chem Pharm Bull 1965;13:935–41.
[32] Kumakura S, Mishima M, Kobayashi S, Shirota H, Abe S, Yamada K, et al. Inhibition effect of indomethacin farnesil, a novel anti-inflammatory prodrug, on carrageenan-induced inflammation in rats. Agents Actions 1990;29:286–91.
[33] Karasawa F, Ehata T, Okuda T, Satoh T. Propofol injection pain is not alleviated by pretreatment with flurbiprofen axetil, a prodrug of a nonsteroidal anti-inflammatory drug. J Anesth 2000;14:135–7.
[34] Whelan J, Writer F. Targeted taxane therapy for cancer. Drug Discovery Today 2001;6:1153–64.
[35] Doebber TW, Wu MS. Platelet-activating factor (PAF) stimulates the PAF-synthesizing enzyme acetyl-CoA:1-alkyl-sn-glycero-3-phosphocholine-O^2-acetyltransferase and PAF synthesis in neutrophils. PNAS 1987;84:7557–61.
[36] Tessner TG, O'Flaherty JT, Wykle RL. Stimulation of platelet-activating factor synthesis by a nonmetabolizable bioactive analog of platelet-activating factor and influence of arachidonic acid metabolites. J Biol Chem 1989;264:4794–9.
[37] Hayashi H, Kudo I, Nojima S, Inoue K. Biological response of guinea pig peritoneal macrophages to platelet-activating factor. Lipids 1991;26:1193–9.
[38] Henriques MGMO, Weg VB, Martins MA, Silva PMR, Fernandes PD, Cordeiro RSB, et al. Differential inhibition by two heterazepine PAF antagonists of acute inflammation in the mouse. Br J Pharmacol 1990;99:164–8.
[39] Hwang SB, Lam MH, Li CL, Shen TY. Release of platelet activating factor and its involvement in the first phase of carrageenan-induced rat foot edema. Eur J Phrmacol 1986;120:33–41.
[40] Perianayagam JB, Sharma SK, Pillai KK. Anti-inflammatory activity of *Trichodesma indicum* root extract in experimental animals. J Ethnopharmacol 1969;104:410–4.
[41] Vinegar R, Scheirber W, Hugo R. Biphasic development of carrageenan edema in rat. J Pharmacol Exp Ther 1969;166:96–103.
[42] Henriques MGMO, Silva PMR, Martins MA, Flores CA, Cunha FQ, Assreuy-Filho J, et al. Mouse paw oedema. a new model for inflammation. Braz J Med Biol Res 1987;20:243–9.

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