# STUDIES ON LIPOXYGENASE INHIBITORS

## I. MY3-469 (3-METHOXYTROPOLONE), A POTENT AND SELECTIVE INHIBITOR OF 12-LIPOXYGENASE, PRODUCED BY *STREPTOVERTICILLIUM HADANONENSE* KY11449

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*Streptoverticillium hadanonense* KY11449 was found to produce a 12-lipoxygenase inhibitor MY3-469. The compound was purified by chromatography on Diaion HP-10, charcoal, Sephadex LH-20 and crystallization. The chemical structure of MY3-469 was determined to be 3-methoxytropolone on the basis of its physico-chemical properties.

The half maximal inhibitory concentration ($IC_{50}$) of MY3-469 against bovine platelet 12-lipoxygenase was $1.8\times10^{-6}$ M. The compound did not inhibit bovine platelet cyclooxygenase at $10^{-3}$ M and showed weak inhibition ($IC_{50}$ $2.8\times10^{-4}$) against 5-lipoxygenase of rat basophilic leukemia cells. The results indicate that MY3-469 is a potent and selective inhibitor of 12-lipoxygenase.

Arachidonate 12-lipoxygenase is one of the key enzyme of arachidonic acid cascade and widely distributed in mammalian tissues and cells such as platelet, neutrophil and lung[1,2]. This enzyme converts arachidonic acid into 12-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE), which is easily reduced enzymatically to 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). Although 12-HETE has been reported to be involved in platelet aggregation[3,4] or to possess a potent chemotactic activity for smooth muscle cells[5], the physiological function of 12-lipoxygenase is still obscure.

Several inhibitors of 12-lipoxygenase, such as nordihydroguaiaretic acid (NDGA)[6] and 15-HETE[7], have been reported, but these are not selective to 12-lipoxygenase. In the course of searching for ipoxygenase inhibitors from microbial metabolites, we found a potent and selective inhibitor of 12-lipoxygenase, designated MY3-469 from the culture broth of *Streptoverticillium hadanonense* KY11449. In this communication, fermentation, isolation, and biological properties of MY3-469 are described.

### Materials and Methods

#### Materials

[1-$^{14}$C]Arachidonic acid (55.4 mCi/mmol), [5,6,8,9,11,12,14,15-$^{3}$H]-5-HETE (60.0 mCi/mmol), and [5,8,6,9,11,12,14,15-$^{3}$H]thromboxane $B_2$ (150 mCi/mmol) were obtained from New England Nuclear (Boston). 12-HETE was a generous gift from Dr. D. H. NUGTEREN (Uniliver Research Laboratorium). Rat basophilic leukemia (RBL-1) cells (ATCC CRL1378) were obtained from American Type Culture Collection (Rockville). Silica gel precoated TLC (Fertigplatten) was obtained from E. Merck (Darmstadt). Soybean lipoxygenase (Type IV) was obtained from Sigma (St. Louis).

#### Microorganism, Medium and Culture Condition

*S. hadanonense* KY11449, isolated by NARA *et al.*[8] from a soil in Kanagawa Prefecture, Japan, was used in this experiment. The stock medium contained soluble starch 1%, N-Z Amine Type A

(Sheffield) 0.2%, beef extract 0.1%, yeast extract 0.1%, and agar 2%, pH 7.2. The seed medium contained glucose 1%, soluble starch 1%, beef extract 0.5%, yeast extract 0.5%, Bacto Tryptone (Difco) 0.5%, and $CaCO_3$ 0.2%, pH 7.2. The production medium contained dextrin 3%, soybean meal 2%, corn steep liquor 0.25%, $K_2HPO_4$ 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03%, and $CaCO_3$ 0.3%, pH 7.8. A slant culture grown on the stock medium was inoculated into large test tube (2.5×19 cm) containing 10 ml of the seed medium and incubated with shaking at 30°C for 3 days. Fifty milliliters of the first seed culture was transferred to five 2-liter flasks containing 450 ml of the seed medium each. The flasks were incubated at 30°C for 24 hours (220 rpm). The content of the flasks was transferred into 15 liters of the production medium in a 30-liter jar fermentor. Fermentation was conducted for 5 days at 30°C by stirring at 300 rpm with aeration of 15 liters per minute.

The growth was monitored by packed cell volume (PCV). The production of MY3-469 was measured by bovine 12-lipoxygenase inhibitory activity, which was indicated by percent inhibiton when the cultured broth was added at 5% volume of the reaction mixture.

Measurement of 12-Lipoxygenase Activity

12-Lipoxygenase was prepared from bovine platelet cytosol by ammonium sulfate fractionation according to the method of NUGTEREN[9]. Assay of 12-lipoxygenase was performed as follows by the method of SIEGEL *et al.*[10]. 12-Lipoxygenase was preincubated with inhibitors for 5 minutes at 30°C in 0.1 M Tris-HCl (pH 7.4) containing 2 mM reduced glutathione. Then 30 $\mu$M of [1-$^{14}$C]arachidonic acid (55.4 mCi/mmol; New England Nuclear) was added and the reaction mixture was incubated for 10 minutes. The reaction was terminated by acidification with 0.2 M citric acid and the radioactive products were extracted with ethyl acetate. The organic phase was chromatographed on silica gel TLC using ligroine - diethyl ether - acetic acid (50: 50: 1) as developing solvent. The radioactive spot of [1-$^{14}$C]-12-HETE on TLC was detected by autoradiography, scraped and counted by a liquid scintillation counter.

Measurement of Other Lipoxygenases and Cyclooxygenase Activities

Assessment of 5- and 15-lipoxygenase and cyclooxygenase activities was also based on the formation of each radioactive product from [1-$^{14}$C]arachidonic acid. The homogenate of RBL-1 cells was used as 5-lipoxygenase[11,12], bovine platelet microsome was used as cyclooxygenase by the method of YOSHIMOTO *et al.*[13] and soybean lipoxygenase was used as 15-lipoxygenase. Assay conditions of each enzyme were as follows. The reaction of 5-lipoxygenase was conducted in 0.1 M Tris-HCl (pH 7.4), 1 mM reduced glutathione, 1 mM $CaCl_2$, 2 mM ATP and 30 $\mu$M [1-$^{14}$C]arachidonic acid at 37°C for 5 minutes. The radioactive product, 5-HETE, was isolated by TLC using petroleum ether - diethyl ether - actetic acid (50: 50: 1)[14] as developing solvent. As for cyclooxygenase, the reaction was performed in 0.1 M Tris-HCl (pH 7.4), 5 mM L-tryptophan, 2 $\mu$M hemoglobin and 30 $\mu$M [1-$^{14}$C]arachidonic acid at 30°C for 10 minutes. The radioactive thromboxane $B_2$, a cyclooxygenase metabolite in this system, was isolated by TLC developed with petroleum ether - diethyl ether - acetic acid (15: 85: 0.1). The reaction of 15-lipoxygenase was conduced in 0.1 M Tris-HCl (pH 7.4) and 30 $\mu$M [1-$^{14}$C]arachidonic acid at 30°C for 5 minutes. The radioactive 15-HPETE was isolated by TLC developed with diethyl ether - *n*-hexane - acetic acid (60: 40: 1)[15]. The extraction, detection and quantification procedures of each radioactive product were the same as described those in the 12-lipoxygenase assay.

## Results

### Production of MY3-469 by *S. hadanonense* KY11449

A typical time course of MY3-469 in a 30-liter jar fermentor was shown in Fig. 1. The production of MY3-469 was increased accompanying with the cell growth. The inhibitory activity reached its maximum at 115 hours and then decreased.

### Isolation Procedure of MY3-469

MY3-469 was isolated from the fermentation broth as the following procedures (Fig. 2). The

Fig. 1. Time course of MY3-469 production.
● MY3-469 production, □ packed cell volume, △ pH.

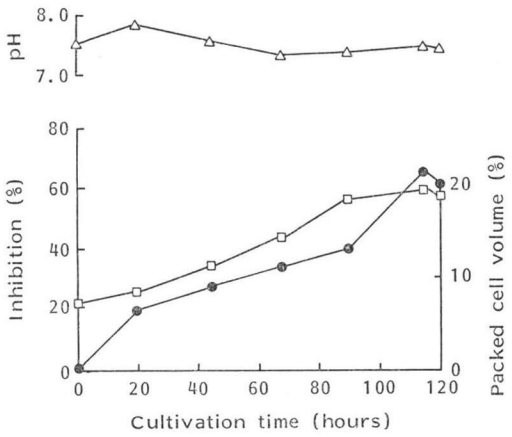



Fig. 2. Purification procedure for MY3-469.

Filtered broth
↓
Diaion HP-10 column chromatography
↓ washed with $H_2O$
eluted with 50% MeOH – 0.1 N $NH_4OH$ (1:1)
concd *in vacuo*
Charcoal
↓ washed with $H_2O$
eluted with 80% $Me_2CO$
concd *in vacuo*
Sephadex LH-20 column chromatography
↓ eluted with 50% MeOH
Active fractions
↓ concd *in vacuo*
Crystallization
↓
Recrystallization
↓
Pale yellow crystals of MY3-469

Fig. 3. Structure of MY3-469.

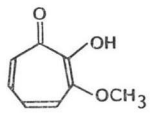
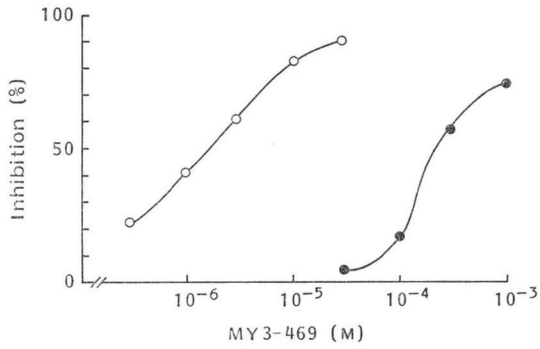



Fig. 4. Inhibition of 12- (○) and 5-lipoxygenase (●) *versus* the concentration of MY3-469.




filtered broth (15 liters) was passed through a Diaion HP-10 column and the active component was eluted with 50% methanol - 0.1 N ammonium hydroxide (1:1). The eluate was concentrated *in vacuo*, absorbed to charcoal (Wako Pure Chemical) and then eluted with 80% acetone. The active fractions in the eluate were collected and concentrated to dryness. Further purification was achieved by Sephadex LH-20 column chromatography. The elution was performed with 50% methanol. The active fractions were concentrated and kept standing at room temperature to crystallize. This crystalline material was recrystallized from methanol - water. The yield was about 680 mg. The crystalline MY3-469 gave one spot on a silica gel TLC developed with toluene - methanol - acetic acid (60: 35: 5).

## Physico-chemical Properties of MY3-469

MY3-469 thus obtained was pale yellow crystals, soluble in methanol, ethanol and water, and positive in $FeCl_3$ reaction. The compound showed the following physico-chemical properties: mp 113~114°C (dec); IR $\nu_{max}$ (KBr) $cm^{-1}$ 3260, 1598, 1553, 1428, 1260, 1080, 845, 792; UV $\lambda_{max}^{MeOH}$ nm 246, 325, (sh 269); HR-MS found 152.0470 (152.0473 calcd for $C_8H_8O_3$); $^1H$ NMR (100 MHz, $CDCl_3$) $\delta$ 4.03 (3H, s), 6.7~7.8 (4H, m), 8.50 (1H, br s). All data of MY3-469 were identical with those of 3-methoxytropolone, which had been found as an antimicrobial compound XK-100[8]. The structure is shown in

Fig. 3.

Biochemical Activities of MY3-469

MY3-469 was found to inhibit the bovine platelet 12-lipoxygenase in a dose-dependent manner as shown in Fig. 4. The concentration of the inhibitor required for 50% inhibition ($IC_{50}$) was $1.8\times10^{-6}$ M and a complete inhibition was obtained at approximately 10 $\mu$M. The effects of MY3-469 on 5-lipoxygenase and cyclooxygenase were also studied. Although MY3-469 inhibited 5-lipoxygenase of RBL-1, $IC_{50}$ for this enzyme ($2.8\times10^{-4}$ M) was two orders of magnitude higher than that for 12-lipoxygenase. On the other hand, cyclooxygenase was not inhibited but rather stimulated by MY3-469 at $10^{-3}$ M, and 15-lipoxygenase was not effected by the compound at this concentration. These data indicate that MY3-469 is a potent and selective inhibitor of 12-lipoxygenase.

## Discussion

MY3-469 was isolated as an inhibitor of bovine platelet 12-lipoxygenase from the cultured broth of *S. hadanonense* KY11449. The compound also inhibited human and rabbit platelet 12-lipoxygenases ($IC_{50}$ about $2\times10^{-6}$ M: data not shown). As shown in Fig. 4, MY3-469 was revealed to be a potent and quite selective inhibitor of 12-lipoxygenase. 15-HETE[7], baicalein[16] (5,6,7-trihydroxyflavon) and esculetin[17] (6,7-dihydroxycoumarin) were reported to be 12-lipoxygenase inhibitors. However, it has recently been reported that 15-HETE[14], baicalein[18] and esculetin[18] showed strong inhibition against 5-lipoxygenase. To our knowledge, this is the first report on the selective inhibitor of 12-lipoxygenase to be proved experimentally.

The availability of a potent and selective inhibitor of 12-lipoxygenase should facilitate studies on the biochemical properties and the physiological role of the enzyme. MY3-469 may be a useful tool for these investigations.

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