

XANTHOCILLIN X MONOMETHYL ETHER, A POTENT INHIBITOR OF PROSTAGLANDIN BIOSYNTHESIS

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(Received for publication August 29, 1981)

Xanthocillin X monomethyl ether, known as an antiviral antibiotic, was isolated as a potent inhibitor of *Dichotomomyces ceipii*. The compound inhibited prostaglandin synthesis from ^{14}C -arachidonic acid in rabbit kidney microsomes by 50% at a concentration of $0.2\ \mu\text{M}$, while prostaglandin synthesis by microsomes of ram seminal vesicle was inhibited by 50% at $20\ \mu\text{M}$. The inhibition by xanthocillin X monomethyl ether was reversible. Of the enzymatic steps involved in the synthesis of prostaglandins and thromboxanes tested, conversion of arachidonic acid into prostaglandin H_2 was specifically inhibited by xanthocillin X monomethyl ether. Anti-inflammatory activity of this antibiotic on carrageenan-induced oedema in the rat foot was, however, not detectable.

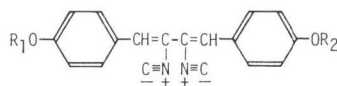
Prostaglandin biosynthesis involves a series of reaction that are probably catalyzed by enzymes acting sequentially. In the events leading to the formation of prostaglandins from arachidonic acid, the precursor is first converted to the prostaglandin endoperoxide G_2 , which is then oxidized to the second endoperoxide prostaglandin H_2 .¹⁻³⁾ The latter endoperoxide is then converted to the classical prostaglandins (*i.e.* E_2 and $\text{F}_{2\alpha}$).¹⁻³⁾

Recent studies from several laboratories^{1,4-7)} have shown that these classical prostaglandins are not the important triggering agents in acute inflammation, in platelet aggregation and in rabbit aorta contraction. Rather, prostaglandin G_2 and/or H_2 or a non-prostaglandin products derived from these endoperoxides seem to be the major inflammatory agent formed during the enzymatic oxidation of arachidonic acid.

During a search for specific inhibitors of prostaglandin synthesis of microbial origin, the fungus *Dichotomomyces ceipii* was found to produce an inhibitory principle. The metabolite was isolated and identified to be xanthocillin X monomethyl ether (Fig. 1), previously described as an antiviral antibiotic.^{8,9)}

The present paper reveals that xanthocillin X monomethyl ether is a potent inhibitor of prostaglandin synthesis and that the inhibition is specific for the conversion of arachidonic acid into prostaglandin H_2 .

Fig. 1. Structures of xanthocillin X mono- and dimethyl ether.



Xanthocillin X monomethyl ether $\text{R}_1 = \text{H}$, $\text{R}_2 = \text{CH}_3$

Xanthocillin X dimethyl ether $\text{R}_1 = \text{CH}_3$, $\text{R}_2 = \text{CH}_3$

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Materials and Methods

Chemicals

$1\text{-}^{14}\text{C}$ -Arachidonic acid (61.0 Ci/mole) was obtained from New England Nuclear and unlabeled arachidonic acid (grade 1), prostaglandins $F_{2\alpha}$, E_2 and A_2 , glutathione (reduced) and L-tryptophan from Sigma. Acetylsalicylic acid (aspirin) and indomethacin were kindly given by Dr. E. MISAOKA and xanthocillin X dimethyl ether was kindly supplied by Dr. A. OGISO. ^{14}C -Prostaglandin H_2 was enzymatically prepared from ^{14}C -arachidonic acid as described by UBATUBA.¹⁰⁾

Xanthocillin X Monomethyl Ether

Dichotomomyces cejpil SANK 23575 was aerobically grown in a medium (500 ml) containing 5% glucose, 2% Polypepton (Daigo Eiyo Kagaku Co.) and 0.3% corn steep liquor (Corn Products Co., U.S.A.) in a 2-liter fermentation flask at 27°C for 4 days. The culture was transferred to 15 liters of the same medium in a 30-liter fermentor and grown at 27°C and aeration rate of 15 liters/minute. After 71 hours of growth, the time when xanthocillin X monomethyl ether was produced maximally (monitored by inhibitory activity), mycelia were harvested by filtration. The mycelial mat (1,900 g) obtained from 2 cultures in the 30-liter fermentor was extracted with 12 liters of acetone. The extract obtained by filtration was concentrated to 1,500 ml *in vacuo* and then extracted with 5 liters of ethyl acetate. The solvent layer (4 liters) was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The oily residue obtained was applied to a column of silica gel (Wako C-50, Wako Pure Chemical Industries Ltd., Osaka, 4.6×25 cm) equilibrated with benzene - *n*-hexane (1:1). After washing with 2 liters of benzene, the column was developed with benzene - ethyl acetate (95:5) and the active eluate (3.5 liters) obtained was concentrated to 50 ml and kept at 4°C overnight. The yellowish crystals formed (5.0 g) were further purified by recrystallization from benzene. The active compound isolated was identified as xanthocillin X monomethyl ether by comparing its molecular formula ($C_{19}H_{14}N_2O_2$), ultraviolet and infrared absorption spectra and NMR and mass spectra with those reported.^{8,9)}

Enzyme Preparations

The microsomal fraction of rabbit kidneys was obtained from male white rabbits (3~5 kg) essentially as described by BLACKWELL *et al.*¹¹⁾ The final pellet obtained after centrifugation at $105,000 \times g$ for 60 minutes was suspended in 100 mM tris-HCl buffer, pH 7.5, and used. The microsomal fraction of ram seminal vesicle was isolated as described by NUGTEREN *et al.*²⁾ and stored at -20°C until use. Microsomal fraction of bovine platelets was prepared according to the method of YOSHIMOTO *et al.*¹²⁾

Synthesis of Prostaglandin E_2 and $F_{2\alpha}$ from Arachidonic Acid

Reaction mixture for rabbit kidney microsomes contained in a final volume of 0.2 ml: 100 mM tris-HCl buffer, pH 7.5; 60 μM $1\text{-}^{14}\text{C}$ -arachidonic acid (2.2×10^5 dpm); 10 mM L-tryptophan; 4 mM reduced glutathione; 0.3 mg of microsomal protein. After incubation at 37°C for 10 minutes with shaking, the reaction was terminated with 0.15 ml of 1 N HCl and prostaglandins formed were extracted with diethyl ether and submitted to thin-layer chromatography on 60 F_{254} plates (E. Merck). The plates were developed in either of the following two solvent systems: (A) chloroform - methanol - acetic acid (18:1:1) and (B) benzene - dioxane - acetic acid (20:10:1). The zones containing prostaglandins E_2 and $F_{2\alpha}$ were scraped off, placed into scintillation vials and counted in a toluene-based counting fluid. Values obtained were corrected for recoveries through thin-layer chromatography and counting efficiency. In some experiments the radioactivity on the silica gel plates was monitored by a Packard radiochromatogram scanner model 7201.

In the experiments using microsomes of ram seminal vesicle, the reaction mixture contained in a total volume of 0.2 ml: 100 mM tris-HCl buffer, pH 8.0; 20 μM $1\text{-}^{14}\text{C}$ -arachidonic acid (1.2×10^5 dpm); 2.5 μM hemoglobin; 10 mM L-tryptophan; 2 mM reduced glutathione; 1 mg of microsomal protein. After incubation at 37°C for 2 minutes, the reaction was terminated and prostaglandins formed were determined as described above.

Conversion of Arachidonic Acid to Prostaglandin H_2

The reaction mixture for the synthesis of prostaglandin H_2 from arachidonic acid was the same as that for the conversion of arachidonic acid into prostaglandin E_2 and $F_{2\alpha}$ by ram seminal enzyme system

except that reduced glutathione was excluded. After incubation at 24°C for 2 minutes, the reaction was terminated by adding 0.6 ml of diethyl ether - methanol - 0.2 M citric acid (3: 0.4: 0.1) precooled at -20°C to the incubation mixture.¹³⁾ The solvent layer was collected and evaporated to dryness *in vacuo* at 0°C. The residue was dissolved in 50 μ l of diethyl ether and applied to silica gel plates, which were developed at -16°C in the solvent system (C) diethyl ether - petroleum ether - glacial acetic acid (85: 15: 0.1). The chromatography was started within 30 minutes after incubation. The reaction products on the plates were located by iodine vapor and/or by the Packard radiochromatogram scanner.

Conversion of Prostaglandin H₂ to Prostaglandin E₂

In the experiments for the synthesis of prostaglandin E₂ from prostaglandin H₂, the reaction mixture was the same as that for the conversion of arachidonic acid into prostaglandin E₂ and F_{2 α} by ram seminal enzyme system except that ¹⁴C-arachidonic acid was replaced by 10 μ M ¹⁴C-prostaglandin H₂ (1.8 \times 10⁴ dpm). After incubation at 24°C for 2 minutes, the reaction was terminated by adding 0.6 ml of diethyl ether - methanol - 0.2 M citric acid (3: 0.4: 0.1) precooled, and the mixture was then evaporated to dryness *in vacuo* at 0°C. The residue was dissolved in 50 μ l of diethyl ether and applied to silica gel plates, which were then developed at -16°C in the solvent system (D) diethyl ether - methanol - acetic acid (90: 2: 0.1). The reaction products on the plates were located by the Packard radiochromatogram scanner.

Thromboxane Synthesis

The incubation mixture for the synthesis of thromboxanes contained in a final volume of 0.2 ml: 100 mM tris-HCl buffer, pH 8.0; 10 μ M ¹⁴C-prostaglandin H₂; 200 μ g of microsomes of bovine platelets. After incubation at 24°C for 2 minutes the reaction was terminated by adding 0.6 ml of diethyl ether - methanol - 0.2 M citric acid (3: 0.4: 0.1) (cold). The mixture was evaporated to dryness, dissolved in 50 μ l of diethyl ether and submitted to the thin-layer chromatography at -16°C in the solvent system (E) ethyl acetate - acetic acid (99: 1). The reaction products on the plates were located by the Packard radiochromatogram scanner.

Anti-inflammatory Activity

Male Wistar-Imamichi rats (110~140 g body weight) were used in groups of 5 each. Compounds to be tested were given by stomach tube in water solution of 0.5% tragacanth used as a suspending medium (0.5 ml/100 g body weight). After 30 minutes, 0.05 ml/100 g body weight of 1% carrageenan suspension was injected subcutaneously under the plantar surface of the right hind foot. The volumes of the foot were determined at the time of carrageenan injection and 3 hours later by measuring the volume of water displaced. The difference between the two volumes was the swelling. Drug effects were calculated as percentage inhibition of the swelling, taking the swelling of the control group as 100%. Where indicated, test compounds were given intravenously in a saline solution.

Results and Discussion

Under the standard assay conditions, prostaglandin E₂ was the major products formed from arachidonic acid by rabbit kidney microsomes, and prostaglandin F_{2 α} was produced in a smaller amount (Fig. 2). These products were identified by comparing with the authentic reference compounds their R_f values in different solvent systems (A and B). Peaks I and II in the solvent system A were not further identified but the former may be prostaglandin D₂ as judged from its R_f values.²⁾

Xanthocillin X monomethyl ether inhibited the formation of prostaglandins E₂ and F_{2 α} by rabbit enzyme system to the same extent at various concentrations tested (data not shown). The inhibition by this antibiotic was 50% and 80% at concentrations of 0.2 and 0.5 μ M, respectively, indicating that xanthocillin X monomethyl ether was 5 times more potent than indomethacin (Fig. 3). Under the same conditions, xanthocillin X dimethyl ether inhibited the synthesis of prostaglandin E₂ plus F_{2 α} 50% at 30 μ M (data not shown).

Fig. 2. Thin-layer chromatograms of products formed from ^{14}C -arachidonic acid by the microsomal fraction of rabbit kidney.

PGA_2 , PGE_2 , and $\text{PGF}_{2\alpha}$ represent prostaglandin A_2 , E_2 , and $\text{F}_{2\alpha}$, respectively.

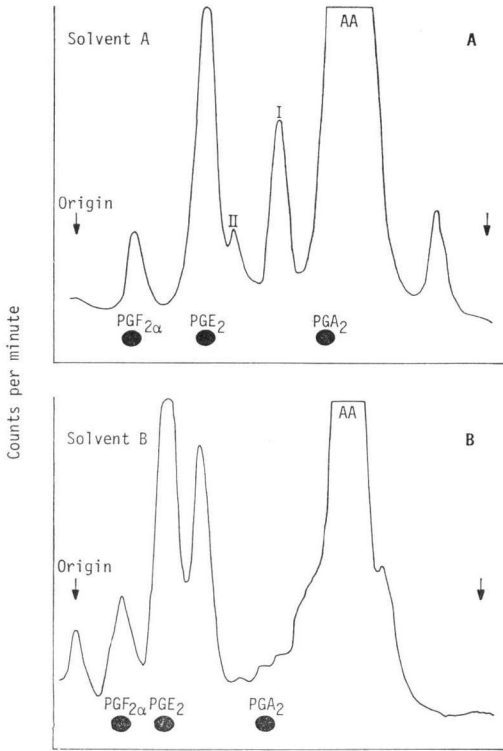


Fig. 3. Inhibition by indomethacin (IM) and xanthocillin X monomethyl ether (XM) at various concentrations of the synthesis of prostaglandin E_2 plus $\text{F}_{2\alpha}$ from arachidonic acid by rabbit kidney microsomes.

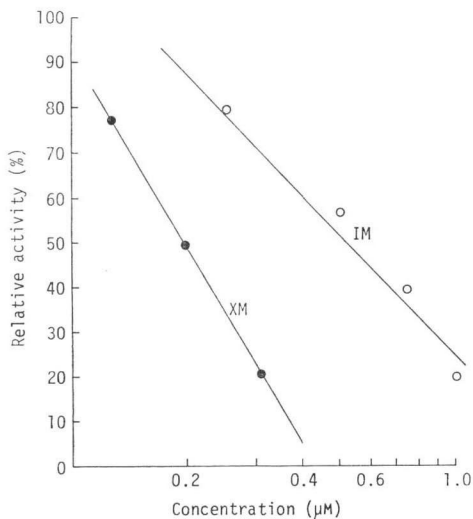
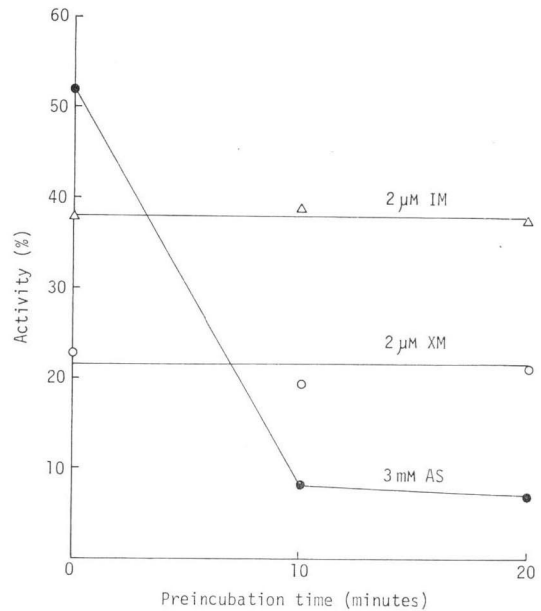


Fig. 4. Effects of preincubation of rabbit microsomes with indomethacin (IM), xanthocillin X monomethyl ether (XM) and aspirin (AS) on the prostaglandin synthesis.

Mixtures for preincubation were the same as that for the synthesis of prostaglandin E_2 and $\text{F}_{2\alpha}$ by rabbit kidney microsomes, replaced by inhibitors at the concentrations indicated. After preincubated at 37°C for the indicated times, the mixture was centrifuged at $105,000 \times g$ for 40 minutes. The resultant pellet was washed once with 0.5 ml of 100 mM tris-HCl, pH 7.5 and then assayed for the synthesis of prostaglandin E_2 plus $\text{F}_{2\alpha}$ from arachidonic acid.



Preincubation of aspirin with microsomes of rabbit kidney increased the greater inhibition as the time of exposure to the microsomes increased, while the time-dependent inhibition of this kind was not observed with either xanthocillin X monomethyl ether or indomethacin (Fig. 4). In the experiments shown in Fig. 5, rabbit kidney microsomes preincubated with inhibitors at the concentrations indicated were assayed for the activity to synthesize prostaglandin E_2 and $\text{F}_{2\alpha}$ either before washing the microsomes with buffer or after washing. As indicated, a marked restoration of the enzyme activity could be obtained by decreasing concentrations of xanthocillin X monomethyl ether at enzyme

Fig. 5. Effects on the restoration of enzyme activity of washing rabbit kidney microsomes preincubated with inhibitors of prostaglandin synthesis.

Rabbit kidney microsomes were preincubated with xanthocillin X monomethyl ether (XM), indomethacin (IM) or aspirin (AS) as described in Fig. 4. After preincubated at 37°C for 20 minutes, microsomes were separated by centrifugation at 105,000×*g* for 40 minutes and assayed for the activity to synthesize prostaglandin E₂ plus F_{2α} from ¹⁴C-arachidonic acid either before washing or after washing with 0.5 ml of 100 mM tris-HCl, pH 7.5.

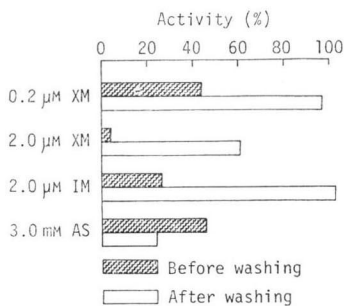
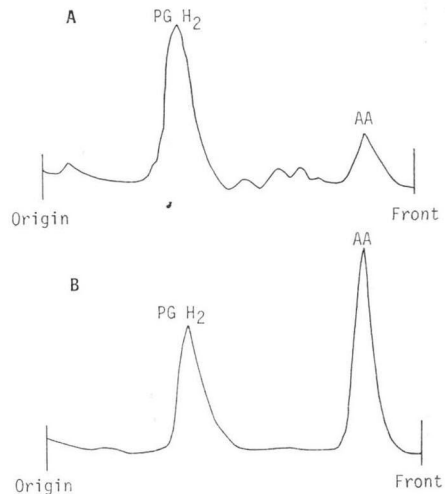


Fig. 6. Inhibition by xanthocillin X monomethyl ether of synthesis of prostaglandin H₂ (PGH₂) from ¹⁴C-arachidonic acid (AA) by microsomes of ram seminal vesicle.

Reaction mixtures were submitted for thin-layer chromatography, and after development the thin-layer plates were monitored for radioactivity.

(A) control, (B) 20 μM XM.



assay as well as indomethacin, while such restoration was not obtained with aspirin. The data indicate that the inhibition of prostaglandin synthesis by xanthocillin X monomethyl ether is, unlike that of aspirin, reversible. The synthesis of prostaglandin E₂ by microsomes of ram seminal vesicles was found to be far less sensitive to xanthocillin X monomethyl ether than that by rabbit enzyme system. Thus the concentration required for 50% inhibition was 20 μM.

The conversion of ¹⁴C-arachidonic acid into prostaglandin H₂ in the ram enzyme system was inhibited by xanthocillin X monomethyl ether (Fig. 6), being reduced by 50% at 20 μM, while the synthesis of both prostaglandin E₂ and thromboxanes from prostaglandin H₂ was not detectably inhibited by the antibiotic at a concentration of 100 μM. These results indicate that inhibitory activity of xanthocillin X monomethyl ether is, as that of indomethacin, specific for the conversion of arachidonic acid into the endoperoxide prostaglandin H₂. Xanthocillin X monomethyl ether showed no detectable anti-inflammatory effects on carrageenan-induced oedema in the rat foot both at an oral dose of 50 mg/kg and an intravenous dose of 10 mg/kg. Under the same conditions, indomethacin suppressed the oedema by approximately 30 and 70% at oral doses of 1 and 10 mg/kg, respectively.

The present study has shown that the antibiotic xanthocillin X monomethyl ether is a potent inhibitor of the biosynthesis of prostaglandin H₂ from arachidonic acid. The data that this compound has no detectable anti-inflammatory activity in rats even on intravenous administration cannot be explained at this moment and is to be studied further.

Acknowledgments

The authors are grateful to Dr. E. MISAKA for performing anti-inflammatory experiments, to Dr. S. TAKA-

HASHI for the identifying xanthocillin X monomethyl ether and to Dr. M. YAMAZAKI for valuable advice and discussion.

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