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Inhibition of Prostaglandin Biosynthesis by the Constituents of Medicinal Plants

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Hot aqueous extracts of medicinal plants used in oriental medicine were tested for inhibitory effect on prostaglandin biosynthesis in order to detect biologically active compounds present therein. Of 162 samples tested, 35 showed significant inhibition. Inhibitors of prostaglandin biosynthesis contained in *Cyperus rotundus* were identified as sesquiterpenes. α-Cyperone, the main inhibitor of *C. rotundus*, showed competitive-type inhibition with respect to the substrate, arachidonic acid.

Keywords——prostaglandin; biosynthesis; inhibitor; oriental medicine; *Cyperus rotundus*; α-cyperone

Medicinal drugs based on traditional medicines have been widely used in Asian countries. The elucidation of the real mode of action and active principles of the crude drugs is a very interesting and challenging subject of study. Although a large number of compounds have been isolated from these drugs, only a few are recognized as active principles. An effective method for the isolation of biologically active constituents of crude drugs is to carry out the isolation of compounds in parallel with bioassay tests; some reports with this methodology have recently appeared in the literature.[1-5]

Previously we introduced a random screening method using an enzyme inhibition test in our investigation on biologically active constituents contained in Chinese medicinal drugs. The enzyme inhibition test provides high sensitivity and efficiency, which are prerequisite to our investigation, and its effectiveness in the isolation of biologically active compounds was shown in our previous studies using c-AMP phosphodiesterase.[6-8] In this study we introduce a prostaglandin synthesizing enzyme system for screening.

Prostaglandins (PGs) are biosynthesized from C_{20} unsaturated fatty acids such as arachidonic acid by a complex of microsomal enzymes.[9,10] In recent years, PGs have drawn much attention because of their potent and remarkably wide variety of physiological actions. PGs are the most prevalent autacoids and have been detected in almost every tissue and body fluid.[11,12] Since PGs show many different biological functions, compounds that alter the metabolism of PGs are expected to have various physiological actions. Non-steroidal anti-inflammatory drugs such as aspirin are representative examples of drugs whose mode of action is ascribed to the inhibition of PG biosynthesis.[13,14] In fact, the inhibitors of PG biosynthesis obtained from *Zingiber officinale* and *Alpinia officinarum*[15,16] have been shown to exhibit various pharmacological activities.[17]

In this report we describe the results of screening using a PG-synthesizing enzyme system prepared from rabbit kidney, and the identification of inhibitors of PG biosynthesis contained in *Cyperus rotundus*.

Experimental

All melting points are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were determined on a JEOL PS-100 spectrometer; infrared (IR) spectra were determined on a JASCO DS-701G spectrometer; mass spectrum (MS) were measured on a JEOL JMS-DX300 spectrometer.

Materials——Ethylendiaminetetraacetic acid (EDTA), diethyl dithiocarbamate and tryptophan were purchased from Wako Co., Tokyo. Hemoglobin (Type I) and arachidonic acid (Grade I) were obtained from Sigma and reduced glutathione from P-L Biochemicals. [1-^{14}C]-Arachidonic acid (55.5 mCi/mol) was obtained from the Radiochemical Centre, Amersham.

Samples of Medicinal Plants——Medicinal plants used in this study were purchased from Uchida Pharmacy for Oriental Medicine (Tokyo), Kinokuniya Pharmacy for Chinese Medicine (Tokyo) and Mayuzumi Pharmacy for Chinese Medicine (Tokyo). A sample of each medicinal plant (10—15 g) was extracted with water (100—150 ml) at 90—100 °C for 6 h and the extract was concentrated and lyophilized. A powder-like sample obtained by lyophilization was tested for its inhibitory effect on PG synthesis at a concentration of 750 μg/ml.

Enzyme Preparation——The microsomal fractions of rabbit kidneys and sheep seminal vesicles were prepared and used for assay as described previously.[18]

Rabbit Renal Microsomes: A rabbit (Japanese white, male, 3—5 kg) was sacrificed and the kidneys were immediately removed and chilled on ice. The renal medulla was isolated and homogenized with a Waring blender for 1 min in 4 times its weight of 0.1 M sodium phosphate buffer (pH 7.5). The homogenate was centrifuged at 9000 × *g* for 15 min and the supernatant was filtered through linen. The filtrate was centrifuged at 105000 × *g* for 60 min and precipitated microsomes were resuspended in 0.1 M phosphate buffer (pH 7.5; the same weight as that of renal medulla) with a glass homogenizer. The microsomal suspension (5—15 mg protein/ml) could be stored at −80 °C for a month without appreciable loss of activity. The frozen suspension was thawed at 0 °C before use.

Sheep Vesicular Gland Microsomes: Frozen sheep vesicular glands were thawed at 0—4 °C. After the removal of free fat and connective tissue, sheep vesicular glands were homogenized with a Waring blender for 1 min in 4 times their weight of 0.05 M Tris-HCl buffer (pH 8.0) containing EDTA (10 mM) and diethyl dithiocarbamate (1 mM). The crude homogenate was centrifuged at 9000 × *g* for 15 min and the supernatant was filtered through linen. The filtrate was centrifuged at 105000 × *g* for 60 min. The precipitate was resuspended with a glass homogenizer in 0.1 M Tris-HCl buffer (pH 8.0; the same weight as that of sheep vesicular glands). The microsomal suspension (5—10 mg protein/ml) could be stored at −80 °C for 2—3 months without appreciable loss of activity.

Assay Methods——Radioisotope Method: The assay mixture (200 μl) contained [1-^{14}C]-arachidonic acid (20 μM; 3×10^4 dpm), reduced glutathione (4.0 mM), tryptophan (10 mM), hemoglobin (0.25 μM), sodium phosphate buffer (0.1 M; pH 7.5) and rabbit renal microsomes (100 μg protein). Test samples were dissolved in distilled water or acetone (less than 8 μl) and added before the addition of microsomal suspension. The reaction was initiated by the addition of microsomes, carried out at 37 °C for 30 min and terminated by the addition of 1 N HCl (50 μl). The reaction mixture was extracted with ether and the ether layer was transferred to another test tube. After removal of the ether, the residue was dissolved in a small volume of acetone and applied to a thin layer chromatography (TLC) plate (Merck Kieselgel 60F_{254} with a concentration zone). Standard solutions of PGE_2 and $PGF_{2\alpha}$ were spotted on the same zone and the TLC plate was developed with a solvent mixture ($CHCl_3$: MeOH : AcOH = 18 : 1 : 1). The zone corresponding to PGE_2 was visualized with iodine vapor, and scraped from the plate. Radioactivity of PGE_2 was determined with a scintillation counter (Aloka LSC-670) in toluene scintillation cocktail.

Oxygen Absorption Method: Oxygen absorption of a reaction mixture was measured with a 1.3 ml reaction vessel (Kimura Rikagaku Seisakusho, Tokyo) equipped with a Clark oxygen electrode (Yellow Springs Instrument Co.; Model 5331). A constant temperature of 30 °C was maintained with a circulator (Neslab, Endocal RTE-5B). The assay mixture (1.3 ml) contained tryptophan (10 mM), hemoglobin (0.25 μM), Tris-HCl buffer (0.1 M; pH 8.0), sheep vesicular gland microsomes (600 μg) and arachidonic acid. The reaction was initiated by the addition of arachidonic acid. A test sample dissolved in acetone or ethanol was added simultaneously with the addition of arachidonic acid. Initial reaction rate was determined from the linear portion of the oxygen absorption curve on a recorder.

Isolation of Active Principles from *Cyperus Rotundus*——Dried rhizomes (10 g) of *Cyperus rotundus* L. (Cyperaceae) were successively extracted with *n*-hexane, chloroform, methanol and water. The inhibitory activities of these extracts at a concentration of 750 μg/ml were 80, 90, 48 and −19%, respectively.

In a large-scale extraction, dried rhizomes (2 kg) were extracted with chloroform to give 35 g of the extract, which was further fractionated into basic, acidic and neutral fractions in the usual manner. The inhibitory activities of these fractions at a concentration of 150 μg/ml were 60, 14 and 72%, respectively. The neutral fraction (23 g), the main fraction of the three, was applied to a silica gel column and successively eluted with *n*-hexane, *n*-hexane–benzene (1 : 1), benzene and benzene–acetone (*ca.* 5% acetone). Fractions eluted with benzene and benzene–acetone (99 : 1) were rechromatographed on silica gel repeatedly to give three inhibitors of PG biosynthesis, α-cyperone (I; 1.4 g; Rf = 0.37: benzene : acetone = 19 : 1), isocyperol (II; 65 mg; Rf = 0.23) and cyperenone (III; 230 mg; Rf = 0.31).

α-Cyperone (I) was obtained as a colorless oil and identified as its semicarbazone by direct comparison with an authentic sample[19] (mp 192—193 °C).

Isocyperol (II) was obtained as a colorless oil and identified by comparison of the spectral data with those reported.[20] $C_{15}H_{24}O$ (M^+, m/z: 220.1837, Calcd: 220.1847), IR ν_{max}^{KBr} cm^{-1}: 3360, 3100, 2940, 1647, 905, 890. ^1H-NMR ($CDCl_3$) δ: 0.73 (s, 3H), 1.77 (br s, 3H), 2.36 (br d, $J=12$ Hz, 1H), 4.30 (m, 1H), 4.60 (t, $J=1$ Hz, 1H), 4.72 (s, 2H), 4.92 (t, $J=1$ Hz, 1H).

Cyperenone (III) was identified as its 2,4-dinitrophenylhydrazone by direct comparison with an authentic sample (mp 232 °C).[21]

Results and Discussion

Hot aqueous extracts of 162 medicinal plants used in traditional medicines were tested for inhibitory effect on the PG-synthesizing enzyme system prepared from rabbit kidney. Among them, 35 samples significantly inhibited the conversion of arachidonic acid into PGE_2 (more than 30%, Table I).

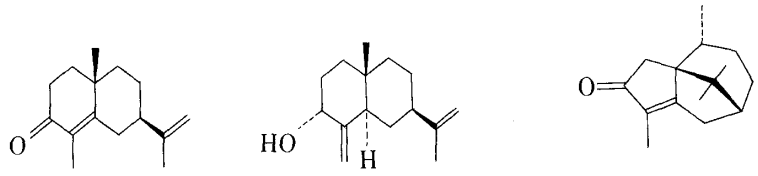
TABLE I. Inhibitory Activity of Aqueous Extracts of Medicinal Plants on Prostaglandin Biosynthesis

Chinese name	Plant name	Part	Inhibition % (750 μg/ml)
Xiebai	*Allium chinense*	Rhizome	32.7
Gaoliangjiang	*Alpinia officinarum*	Rhizome	32.7
Yizhi	*Alpinia oxyphylla*	Fruit	36.6
Sharen	*Amomun* sp.	Seed	48.1
Binglangzi	*Areca catechn*	Seed	83.1
Shuizaojiao	*Cassia nomame*	Seed	66.7
Guipi	*Cinnamomun cassia*	Bark	73.6
Guizhi	*Cinnamomun cassia*	Branch	53.6
Rougui	*Cinnamomun cassia*	Bark	61.6
Shanzha	*Cratagus* sp.	Fruit	49.9
Xiangfu	*Cyperus rotundus*	Rhizome	67.3
Yuanhuao	*Daphne genkwa*	Flower-bud	35.3
Shidi	*Diospyros kaki*	Calyx	35.1
Mahung	*Ephedra* sp.	Herb	72.0
Yinyanghuo	*Epimedium* sp.	Herb	63.7
Wuyao	*Lindera strychinifolia*	Root	34.2
Ruanzicao	*Macrotomia euchroma*	Root	40.0
Houpo	*Magnolia officinalis*	Bark	57.1
Kulianpi	*Melia* sp.	Bark	57.7
Roudoukou	*Myristica fragrans*	Seed	50.6
Sen kotsu[a]	*Nuphar japonicum*	Rhizome	67.8
Zhujieshen	*Panax japonicum*	Rhizome	36.7
Heshouwu	*Polygonum multiflorum*	Root	81.9
Oh hi[a]	*Purnus janasakura*	Bark	66.8
Shiliupi	*Punica granatum*	Pericarpium	47.0
Shiwei	*Pyrrosia* sp.	Herb	42.0
Dahuang	*Rheum* sp.	Rhizome	89.7
Wubeizi	*Rhus javanica*	Galla	90.2
Yingshi	*Rosa multiflora*	Fruit	53.7
Huangqin	*Scutellaria baicalensis*	Root	61.0
Tufuling	*Smilax glabra*	Rhizome	51.6
Ercha	*Uncaria gambier*	Leaf	92.5
Gouteng	*Uncaria rhynchophylla*	Prickle	67.3
Shanjiao	*Zanthoxylum piperitum*	Fruit	55.9
Shengjiang	*Zingiber officiale*	Rhizome	47.8

a) Japanese name.

The isolation of inhibitors was carried out from the rhizomes of *Cyperus rotundus* ("Kobushi"), which have been used as a Chinese medicine for women's diseases. Many sesquiterpenes such as cyperol, isocyperol,[20] cyperenone,[21,22] sugetriol,[23] kobusone,[24] *etc.* have been isolated from the steam-distilled oil of *C. rotundus* of Japanese origin. However little is known about the biological activities of these sesquiterpenes.

In a preliminary experiment by solvent extraction, it was found that inhibitory activity was concentrated in the *n*-hexane and chloroform extracts. To identify the active principles, dried rhizomes were extracted with chloroform on a large scale, and from the neutral fraction of this extract, three inhibitors of PG biosynthesis, α-cyperone (I),[19] isocyperol (II),[20] and cyperenone (III),[21,22] were obtained by chromatographic purification. These compounds were identified from spectral data and by direct comparison with authentic samples.



(I) (II) (III)

Chart 1

IC_{50} (50% inhibitory concentration) values of these compounds for the enzymes prepared from rabbit kidney were 13, 240 and 520 μM, respectively. (The IC_{50} value of indomethacin under the same assay conditions is 4.9 μM.)

It is well known that indomethacin, a representative cyclooxygenase inhibitor, shows time-dependent inhibition.[25] However, the inhibitory activity of α-cyperone was not affected by preincubation with the enzyme preparation (Fig. 1) and reversible nature of the inhibition was suggested by the results shown in Fig. 2. Moreover, α-cyperone prevented the inhibition of cyclooxygenase by indomethacin, indicating that indomethacin and α-cyperone bind to the same site of the enzyme (Fig. 1). Finally, the inhibition type of α-cyperone was characterized

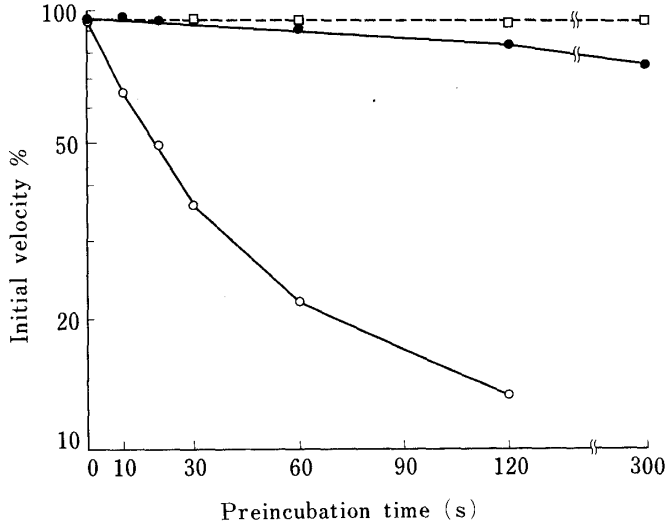


Fig. 1. Effect of Preincubation Time on the Inhibitory Activities of α-Cyperone and Indomethacin

In a 1.3 ml reaction cell equipped with an oxygen electrode, sheep seminal vesicle microsomes were preincubated with inhibitor(s) for various periods of time at 30 °C in the presence of hemoglobin (0.25 μM), tryptophan (10 mM) and Tris-HCl buffer (pH 8.0, 0.1 M). The cyclooxygenase reaction was initiated by the addition of arachidonic acid (200 μM) and the initial velocity of oxygen consumption was measured.

□, 10 μM α-cyperone; ○, 2 μM indomethacin; ●, 10 μM α-cyperone + 2 μM indomethacin.

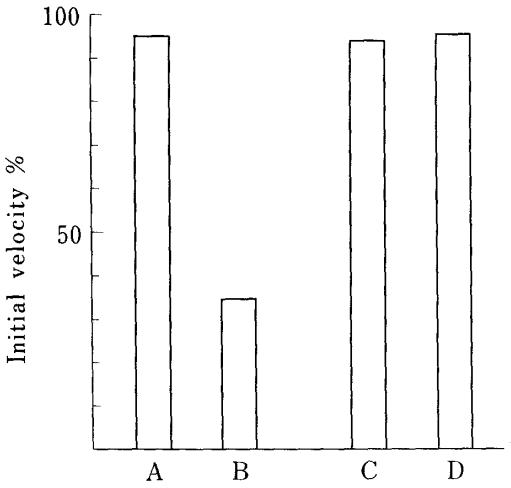


Fig. 2. Reversibility of Inhibition by α-Cyperone

Sheep seminal vesicle microsomes were incubated for 1 min with α-cyperone at a concentration of (A) 3 μM, (B) 200 μM under the same conditions as in Fig. 1. Sheep seminal vesicle microsomes were incubated at 30 °C with α-cyperone at a concentration of 200 μM for (C) 5 min or (D) 15 min. A 20 μl aliquot of microsomes was transferred to a reaction cell to give an α-cyperone concentration of 3 μM and incubated for 1 min under the same conditions as in Fig. 1. The cyclooxygenase reaction was initiated by the addition of arachidonic acid (200 μM) and the initial velocity of oxygen consumption was measured.

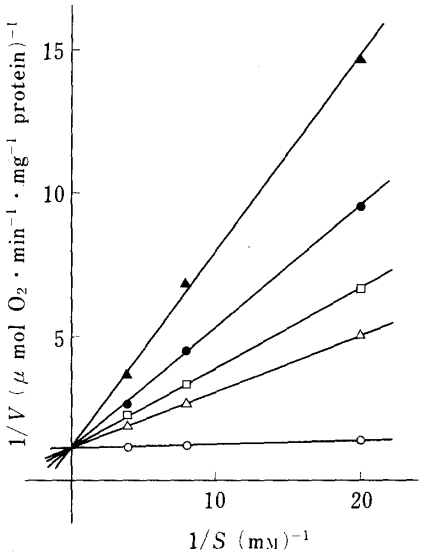


Fig. 3. Lineweaver–Burk Plot of Substrate Concentration Against the Initial Rate of Enzyme Reaction at Various Concentrations of α-Cyperone

The cyclooxygenase reaction was initiated by the simultaneous addition of arachidonic acid and α-cyperone, and the initial velocity of oxygen consumption was measured.

S, arachidonic acid concentration; *V*, initial rate of oxygen consumption; I, α-cyperone concentration.

○, I=0; △, I=26 μM; □, I=51 μM; ●, I=102 μM; ▲, I=204 μM.

by a kinetic study carried out by means of an oxygen electrode with sheep seminal vesicle microsomes. α-Cyperone showed competitive-type inhibition with respect to the substrate, arachidonic acid (Fig. 3), with a K_i value of 33 μM.

Inhibitors of PG biosynthesis are expected to have an anti-inflammatory effect and/or an anti-platelet aggregation effect.[26] α-Cyperone was ineffective on carrageenin edema, but it inhibited platelet aggregation completely at a concentration of 10 μM *in vitro*.[27]

In Chinese medicine, rhizomes of *C. rotundus* are mainly used as an analgesic for the pain of dysmenorrhea, which is considered to be a result of hyperactivity of the myometrium with accompanying uterine ischemia, which is caused by an elevated level of endometrial $PGF_{2\alpha}$.[28] Some PG synthesis inhibitors have been applied to the treatment of primary dysmenorrhea and have proved to be effective in the relief of pain.[29] The isolation of inhibitors of PG biosynthesis from an oriental medicine which is used for menorrhalgia provides a possible mode of action of this medicine, and it also gives an example of the effectiveness of using inhibitory effects on enzymes as a screening tool to detect biologically active principles contained in medicinal plants.

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