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Aromatase Inhibitors from *Isodon excisus* var. *coreanus*

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The diethyl ether extract of *Isodon excisus* var. *coreanus* exhibited significant inhibitory activity in aromatase assay. Bioactivity-guided fractionation of the extract led to the isolation of three active compounds: inflexin (*ent*-1α-hydroxy-3β,6a-diacetoxykaur-16-en-11,15-dione) **(1)**, ursolic acid **(2)**, and ursolic acid 3-*O*-acetate **(3)**.

Key words: Aromatase inhibition, Inflexin, Ursolic acid

INTRODUCTION

One third of female breast cancers are known to occur hormone-dependently and regress by anti-hormonal therapy (Santen *et al.*, 1990). Therefore, one of therapeutic approaches is to use antiestrogens that interact with estrogen receptors and inhibit receptor-mediated gene transcription (Yue *et al.*, 1996). In addition, however, using antibodies capable of binding to aromatase, Santen *et al.* demonstrated significant amounts of aromatase in breast tumors as well as the stroma surrounding breast tumors (Santen *et al.*, 1994). Estrogens are biosynthesized from androgens by the microsomal cytochrome P-450 enzyme complex system, and, therefore, the ability of breast tumors to synthesize estrogen *in situ* through aromatization may represent an important mechanism of autocrine and paracrine growth. This presents a therapeutic opportunity relevant to the control of breast cancer. In particular, effective agents for breast cancer may be developed to lower plasma estrogen levels by virtue of inhibiting synthetic procedure of estrogen *in situ* (Harvey *et al.*, 1996). Since estrogen production is the last step in the biosynthetic sequence of steroid formation, selective inhibition of aromatase should not interfere with the biosynthesis of other steroids. Thus, aromatase inhibitors developed from a natural source could be a new class of cancer chemopreventive agents with the potential for palliative therapy of hormone-dependent metastatic breast cancer, especially in post-menopausal women (Santen *et al.*, 1994).

In order to isolate novel cancer chemopreventive agents from natural products, we have evaluated 50 plant extracts for their potential to inhibit partially purified aromatase. Of these, the diethyl ether extract of *Isodon excisus* var. *coreanus* Nakai (Labiatae) was found to be active in this assay and selected for bioassay-directed fractionation. *Isodon excisus* var. *coreanus* is one of the endemic plants in Korea, and has been used for the treatment of the anorexia, indigestion, stomachache, inflammation, and esophageal carcinoma in Korean folk medicine (Lee, 1989 and Yook, 1981). In the previous paper, new *ent*-kaurene derivatives from this plant were isolated (Kim *et al.*, 1997). As a result, three active compounds [inflexin **(1)**, ursolic acid **(2)**, and ursolic acid 3-*O*-acetate **(3)**] were isolated and found to exhibit significant aromatase inhibitory activity.

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MATERIALS AND METHODS

Chemicals and apparatus

Androstenedione, aminoglutethimide, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). [1, 2-^{3}H]Androstenedione (42.0 Ci/mmol, 1.0 mCi/mL) was obtained from Dupont/NEN, Inc. (Boston, MA).

Isolation of active compounds

Whole plants of *Isodon excisus* var. *coreanus* Nakai (Labiatae) were collected from Mt. Chii, Korea in August 1994. A voucher specimen was deposited in the College of Pharmacy, Chung-Ang University, Seoul, Korea. Fresh plant material (1.3 kg) was extracted three times for 3 h with MeOH at room temperature, to afford 96 g of an extract upon removal of solvent *in vacuo*. This extract was suspended in hot water, and partitioned between

diethyl ether and water. The diethyl ether fraction was dried *in vacuo* to yield the extract (27.0g), showed significant aromatase inhibitory activity (IC_{50} 13.7 mg/mL), was chromatographed on a silica gel column (500 g), and eluted with a stepwise gradient from $CHCl_3$-MeOH (30:1) to MeOH to give eight fractions. Of these, fraction 4 (IC_{50}: 12.7 μg/mL) showed the most potent inhibitory activity and was chromatographed on a silica gel (60 g) column, using mixtures of $CHCl_3$ and MeOH (50:1) for elution. According to differences in composition indicated by TLC, six subfractions were obtained. Of the 6 subfractions, only subfractions 2-4 were found to be bioactive. Further purification of subfraction 2 (IC_{50}: 11.3 μg/mL) by silica gel (20 g) column chromatography, and elution with a stepwise gradient of $CHCl_3$-acetone (15:1 to 9:1), afforded pure compound **1** (11 mg). Subfraction 3 (IC_{50}: 20.8 μg/mL) was chromatographed on a silica gel (30 g) with $CHCl_3$ and MeOH (12:1), to yield compounds **2** (26.6 mg) and **3** (4.0 mg).

Inflexin (1): $[\alpha]_{22}^{D}$ -9.2°(*c*=0.09, $CHCl_3$); UV (MeOH) λ_{max} nm (log e): 239 (3.87); EIMS *m/z* 432.221 $[M]^+$ (calc. for $C_{24}H_{32}O_7$ 432.215); ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm): δ 0.87, 1.16, 1.30 (each 3H, s, 3 × OMe), 1.77, 2.13 (3H each, s, 3β-, 6α-OAc), 2.11 (1H, d, *J*=12.5, H-14α), 2.73 (1H, brs, H-5β), 2.73 (1H, dd, *J*=12.5, H-7α), 3.12 (1H, m, H-13α), 4.01 (1H, dd, *J*=12, 3.4, H-11), 4.62 (1H, t, *J*=4.4, H-3α), 5.39 (1H, brs, H-17β), 5.97 (1H, dd, *J*=3.6, 3.6, 2.8, H-1), 6.00 (1H, brs, H-17α); ^{13}C-NMR ($CDCl_3$, 125.8 MHz, ppm): δ 75.0 (C-1), 32.2 (C-2), 78.2 (C-3), 35.9 (C-4), 58.0 (C-5), 210.2 (C-6), 50.2 (C-7), 54.6 (C-8), 60.2 (C-9), 51.0 (C-10), 70.3 (C-11), 37.5 (C-12), 36.4 (C-13), 36.6 (C-14), 205.8 (C-15), 148.4 (C-16), 115.4 (C-17), 26.3 (C-18), 21.8 (C-19), 14.2 (C-20), 171.7, 21.3 (3b-$COCH_3$), 170.3, 21.5 (6a-$COCH_3$). Spectroscopic data consistent with literature values (Fujita *et al.*, 1982 and Takeda *et al.*, 1988).

Ursolic acid (2): $[\alpha]_{23}^{D}$ +47.8° (*c*=0.5, pyridine); ^{1}H NMR, ^{13}C NMR, and EIMS data consistent with literature values (Lin *et al.*, 1987 and Budavari *et al.*, 1996).

Ursolic acid 3-*O*-acetate (3): $[\alpha]_{23}^{D}$ +58.8° (*c*=1.5, CH_3Cl); EIMS *m/z* 498 $[M^+]$, 456, 424, 218, 203, 175, 109, 91; ^{1}H NMR and ^{13}C NMR data consistent with literature values (Lin *et al.*, 1987 and Budavari *et al.*, 1996).

Preparation of partially purified human placental microsomes

Freshly delivered human term placenta was washed in cold 0.15 M KCl and the tissue was dissected free of adhering membranes and large blood vessels (Kellis *et al.*, 1987 and Hoffman *et al.*, 1980). The tissue was homogenized with a Polytron homogenizer,using three 20 sec bursts separated by 2 min cooling periods. The homogenate was centrifuged at 20,000 × g for 30 min to remove mitochondria, nuclei, and cell debris.

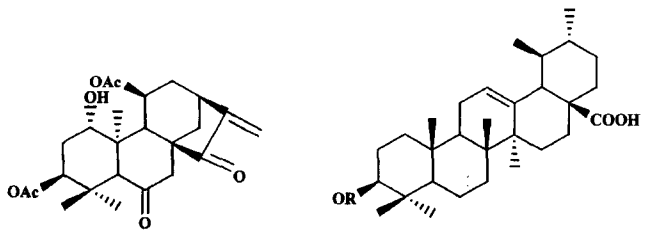
The postmitochondrial supernatant was then subjected to centrifugation at 148,000 × for 45 min to yield a microsomal pellet. The pellet was resuspended in 0.05 M potassium phosphate buffer (pH 7.4) and was centrifuged again at 148,000 × g for 45 min. This step was repeated twice, and microsomes were finally suspended in a minimal volume of buffer and stored frozen in plastic tubes at -75°C. Protein content was determined using the bichinchonic acid method with bovine serum albumin as a standard protein (Kellis *et al.*, 1987).

Assay for aromatase activity

Reaction mixtures were prepared in glass tubes containing 4 μL of placental microsomes (5 mg/mL), 0.3 μL of [1,2-^{3}H]androstenedione (42.0 Ci/mmol, 1.0 mCi/mL), 5 μL of unlabelled androstenedione (0.875 μM), 5 μL of NADPH (0.48 mM), and 10 μL of test sample dissolved in DMSO and 0.05 M potassium phosphate buffer, pH 7.4 (final volume, 500 μL). After an incubation period of 4 min at 37°C, reactions were terminated by adding 3 μL of chloroform. The test tubes were centrifuged at 2,000 × g for 10 min and 300 μL of each aqueous phase was transferred to tubes containing 300 μL of charcoal/dextrin solution (5%). Following another 10 min centrifugation at 2,000 × g, 500 μL of the supernatants were used for the determination of radioactivity (Thompson *et al.*,1974). Dose-response curves were prepared and the results were typically expressed as IC_{50} values. This assay was performed by triplet manner. Aminoglutethimide was used as a positive control (Rabe *et al.*, 1982).

RESULTS AND DISCUSSION

In searching for new cancer chemopreventive agents, we have evaluated plant extracts for their potential to inhibit partially purified aromatase. The diethyl ether extract of *Isodon excisus* var. *coreanus* Nakai (Labiatae) was found to be active in this assay (IC_{50}: 13.7 μg/ml) and was selected for bioassay-directed fractionation. As a result, inflexin **(1)** , ursolic acid **(2)**, and ursolic acid 3-*O*-acetate **(3)** (Fig. 1) were isolated, and these compounds were identified by comparison of their physical and spectroscopic data with literature values. These compounds were found to exhibit significant aromatase inhibitory activity (Table I). Of these, inflexin demonstrated the most potent activity. These three compounds are the first isolation from this plant. Specially, cytotoxicities of *ent*-kaurene derivatives have been reported (Qiu *et al.*, 1998, Fatope *et al.*, 1996, and Lee *et al.*, 1996). But, aromatase inhibitory effects of *ent*-kaurene derivatives have not been reported yet. At the outset of the study, it was not known if the



1 2 R=H 3 R=Ac

Fig. 1. Structure of compounds **1-3**

Table I. Aromatase inhibitory effects of compounds **1-3**

Compounds	IC_{50} (μg/mL)
1	9.2
2	14.0
3	42.7
Aminoglutethimide(positive control)	0.2

inhibitory activity observed with the plant extract was due to a highly active component in relatively low concentraion, or less active constituents present in greater abundance. From the results of this study, it appears the latter possibility applies, since the isolates **(1-3)** are of moderate potency in blocking the activity of aromatase. Thus, the studies on other effective constituents in this plant against aromatase should be performed.

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