Prenylflavonoids: A New Class of Non-Steroidal Phytoestrogen (Part 1). Isolation of 8-Isopentenylnaringenin and an Initial Study on its Structure-Activity Relationship

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Abstract: **Bioassay-guided fractionation of a methanolic extract of a Thai crude drug, derived from heartwood of *Anaxagorea luzonensis* A. Gray (Annonaceae), resulted in the isolation of 8-isopentenylnaringenin (1) as an estrogen agonist with an activity of about an order of magnitude greater than genistein. Various flavonoids possessing isopentenyl side chains in the A-ring have been prepared and evaluated for their ability to bind estrogen receptor. In addition, enantiomers of 1 were separated and the respective enantiomers were assayed. These studies have demonstrated that the presence of an 8-isopentenyl group is an important factor for binding. Flavones, flavanones and flavonols having an isopentenyl substituent at C-8 exhibited an appreciable affinity for estrogen receptor. Conversely, isoflavones possessing an 8-isopentenyl substituent at C-8 did not show this activity. Movement of the isopentenyl group from position 8 to 6 resulted in loss of the activity. No significant difference was observed between 2(*S*)- and 2(*R*)-enantiomers of 1 in their binding affinity. Prenylflavonoids are reported to possess a wide range of biological activities; however, estrogenic activity has not been described.**

Key words: **Anaxagorea luzonensis A. Gray, Annonaceae, prenylflavonoid, non-steroidal estrogen agonist, phytoestrogen, isopentenylnaringenin, isopentenylapigenin.**

Introduction

The female sex hormone estrogen has a variety of beneficial and detrimental effects in women. On the one hand, it seems to protect women against heart attacks and other cardiovascular problems, osteoporosis, and possibly Alzheimer's disease. On the other hand, estrogen is thought to contribute to the development of breast and uterine cancer (1). Recently, mounting evidence suggests that plant-derived estrogens (phytoestrogens), such as genistein, may exert beneficial effects on the above-mentioned chronic diseases (2). Thus, the findings regarding a new class of non-steroidal estrogen may open the way to designing drugs that selectively block estrogen's unwanted effects and that mimic its beneficial ones.

We engaged in a random screening of various plant extracts in the hope of isolating such compounds that serve as models for discovering new estrogen alternatives. In a previous paper we reported that certain homoisoflavones and retrodihydrochalcones, isolated from a Thai medicinal plant, were found to exhibit appreciable estrogenic activities (3). This observation prompted us to continue the investigation by searching for specific estrogen agonists.

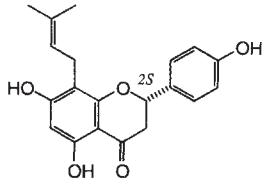
The MeOH extract of the heartwood of *Anaxagorea luzonensis* A. Gray (Annonaceae) (4), was found to inhibit [^3H]-17β-estradiol binding to estrogen receptor. When subjected to activity-guided fractionation, we obtained 8-isopentenylnaringenin (**1**) as an active ingredient. The estrogenic activity of **1** was twice as high as that of genistein as evaluated by receptor binding assay and was estimated as 25 times more potent than that of genistein as measured by the ability to stimulate the growth of estrogen-sensitive cells in culture. Furthermore, **1** has been shown to act as an estrogen agonist on uterus as well as bone metabolism (5).

Numerous phytoestrogens with a diversity of structures have now been recognized, and most of these compounds are isoflavones, e.g., genistein (6). 8-Isopentenylnaringenin (**1**) belongs to a class of prenylflavonoid which is distributed widely in the plant kingdom. Prenylflavonoids are reported to possess a wide range of biological activities; however, estrogenic activity has not been described (7). We therefore examined whether other types of prenylflavonoids exhibiting appreciable estrogenic activity exist. This report describes the isolation of **1** as an active ingredient from a plant source as well as its estrogenic activities tested *in vitro*. The structure-activity relationships of the initial series of 8-prenylflavonoids are also described.

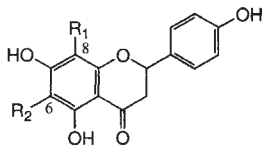
Materials and Methods

Plant materials

The traditional Thai medicine ("Kam-lang-wua-talerng" in Thai) was purchased in Chiang-Mai (Thailand) from a commercial supplier in 1985 and the voucher sample (code number EAR-0028) was placed in our plant depository. "Kam-lang-wua-talerng" has the botanical name *Anaxagorea luzonensis* A. Gray (Annonaceae) and is a tree indigenous to Thailand (4). Its heartwood is available in chips of varying size, mostly 5 – 10 cm in length, consisting of reddish-brown heartwood to which a little of the whitish bark still adheres. The wood is hard but easily split.



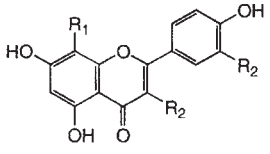
1a: 2(*S*)-8-isopentenylnaringenin



1: R_1 = -CH_2CH=$C(CH_3)_2$, R_2 = H 8-isopentenylnaringenin (racemic)

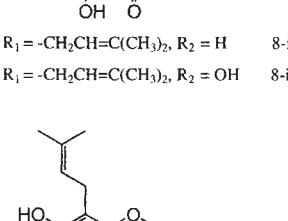
2: R_1 = H, R_2 = -CH_2CH=$C(CH_3)_2$ 6-isopentenylnaringenin (racemic)

3: R_1 = R_2 = -CH_2CH=$C(CH_3)_2$ 6,8-diisopentenylnaringenin (racemic)



4: R_1 = -CH_2CH=$C(CH_3)_2$, R_2 = H 8-isopentenylapigenin

5: R_1 = -CH_2CH=$C(CH_3)_2$, R_2 = OH 8-isopentenylquercetin



6: 8-isopentenylgenistein

Instrumentation

The NMR experiments were conducted on a Bruker AMX-360 in acetone-d_6 at 360 and 90.8 MHz for ^{1}H- and ^{13}C-NMR, respectively. Circular dichroism (CD) measurements were carried out using a Jasco J-500C spectrometer, in MeOH solution, and scanning from 420 to 200 nm. Centrifugal partition chromatography (CPC) (8) was carried out with a Sanki model CPC-LNN (Sanki Engineering Ltd.) with 12 cartridges.

Isolation of active ingredient

The dried and chopped heartwood (5 kg) of *Anaxagorea luzonensis* was extracted three times with MeOH under reflux. After filtration, the MeOH extract was concentrated under reduced pressure and the residue was then partitioned between 90% aqueous MeOH and *n*-hexane. The *n*-hexane extract was rejected. Evaporation of the 90% aqueous MeOH layer yielded a reddish brown syrup which was further partitioned between EtOAc and H_2O. The EtOAc fraction, after removal of the solvent, afforded an EtOAc extract (756 g). A portion of EtOAc extract (10 g) was treated with 50 ml of the mixture of upper and lower phases of the developing solvent (see below) and filtered. The filtrate was evaporated, and dissolved into the upper layer of the developing solvent (50 ml). The solution was subjected to CPC (cartridge 1000E, 700 rpm, $CHCl_3$-MeOH-H_2O [40 : 33 : 27] descending mode, 10 ml/min, 85 ml/fraction). Fractions 3 and 4 were combined, filtered, and the solvent was evaporated. The residue (2.33 g) was further subjected to CPC (cartridge 250W, 300 rpm, $CHCl_3$-MeOH-H_2O [13 : 3 : 4], descending mode, 2.5 ml/min, 10 ml/fraction). Fractions 1 through 3 were combined and evaporated (1.66 g). Further purification using CPC (cartridge 250W, 700 rpm, ascending mode, 2.5 ml/min, 10 ml/fraction) was undertaken using a solvent system of *n*-hexane-ether-MeOH-H_2O (2 : 5 : 5 : 2). The residue was dissolved into 20 ml of the lower layer of the solvent and then applied to the instrument. A total of 92 fractions were collected. Fractions 34–57 (320 mg) were further fractionated by CPC using the same solvent system under similar conditions (600 rpm, 1.0 ml/min, 5 ml/fraction) to afford 125 mg of crude active ingredient. Further purification by repeated flash chromatography over SiO_2 (20 × 150 mm, solvent: $CHCl_3$-MeOH [98 : 2] and 100% $CHCl_3$) yielded 17 mg of the active compound in essentially pure form. Final purification was achieved by reversed phase HPLC (6 mg × 3, Asahipack ODP-90 (octadecyl polymer column, 300 × 28 mm, 10 μm, Asahi Chemical Industry Co., Ltd./Japan), CH_3CN-H_2O [45 : 55], 20 ml/min, t_R: 54 min), and furnished the active ingredient (10 mg). The compound was identified as 8-isopentenylnaringenin (**1**) by comparing ^{1}H- and ^{13}C-NMR spectra to published data (9). CD (in MeOH): $[\theta]_{312}$ +2,000; $[\theta]_{288}$ −10,600; $[\theta]_{235}$ +1,800; $[\theta]_{214}$ +6,400.

The chiral HPLC analysis was performed using a column packed with cellulose carbamate (4.6 mm × 25 cm, 10 μm, OC; tris-phenylcarbamate, Chiralcel®, Daicel Chemical Industries, Japan). Two peaks were observed [t_R, 20.3 min and 22.9 min, *n*-hexane/n-propanol (9 : 1), 1.0 ml/min].

Syntheses of 8-isopentenylnaringenin (**1**), *6-isopentenylnaringenin* (**2**), *6,8-diisopentenylnaringenin* (**3**), *8-isopentenylapigenin* (**4**), *8-isopentenlyquercetin* (**5**), *and 8-isopentenylgenistein* (**6**)

These compounds were prepared by the published procedures (10, 11, 12, 13) with some modifications and their structures were confirmed by comparing ^{1}H- and ^{13}C-NMR spectra with those of respecitve compounds described in the literature (9, 14, 15).

Separation of the enantiomers of 8-isopentenylnaringenin (**1a**) *and* (**1b**)

The enantiomers of **1** were separated semi-preparatively by liquid chromatography on a cellulose carbamate column (2 × 25 cm, 10 μm, OC; tris-phenylcarbamate, Chiralcel®, Daicel Chemical Industries, Japan) using a mixed solvent of *n*-hexane : 1-propanol (9 : 1) as eluent (5 ml/min). The chromatogram showed an incomplete separation of peaks for the two isomers (t_R = 81.5 min and t_R = 92.0 min). Repeated separation of racemate (**1**, 10 mg) resulted in optically pure (**1a**, 2.4 mg) and **1b**, 2.4 mg). CD (**1a**, in MeOH): $[\theta]_{312}$ +6,600; $[\theta]_{288}$ −37,100; $[\theta]_{233}$ +7,200; $[\theta]_{215}$ +25,700. (**1b**, in MeOH): $[\theta_{312}$ −6,800; $[\theta]_{288}$ +35,000; $[\theta]_{235}$ −6,700; $[\theta]_{213}$ −25,600.

Estradiol receptor binding assay

Competitive binding assay to measure the affinity for uterine estrogen receptor followed the method described previously (3).

Effect on MCF-7 cell proliferation

Full details of the experimental method used for the cell culture and the determination of cell number using the MTT method have been published (3).

Results and Discussion

Isolation of the active ingredient and its biological activity

Methanol extracts of traditional Thai medicines, purchased at Chiang-Mai market in Thailand, were subjected to an evaluation of estrogenic activity. The extract of heartwood of *Anaxagorea luzonensis* (Annonaceae) appreciably inhibited [^3H]-estradiol binding to estrogen receptor. An active ingredient was purified by subjecting the crude extract to a bioassay-monitored fractionation and was identified as the known 8-isopentenylnaringenin (**1**) (9). Compound **1** represents a prenylflavone with an asymmetric carbon atom at C-2. The CD spectrum of **1** exhibited a positive Cotton effect in the region of 310–360 nm and a negative Cotton effect in the region of 260–310 nm, which is characteristic of a 2(*S*) configuration (16). However, relatively low values of molecular ellipticity compared with that of 2(*S*)-naringenin implied that **1** was obtained as a partially racemic mixture. The composition was then checked using a chiral HPLC column [Chiralpak OC(+)]. Two peaks were clearly observed at retention times of 20.3 and 22.9 minutes at an approximate ratio of 56 : 44, with *n*-hexane/*n*-propanol (9 : 1) as the mobile phase at a flow rate of 1.0 ml/min. From the above data, we concluded that **1** is as illustrated in structure **1a** and partially racemized at C-2.

The purified active compound **1** was then evaluated for its *in vitro* estrogenic activity by a competitive binding assay using [^3H]-17β-estradiol as tracer and bovine uterine cytosol as the receptor source. The dextran-coated charcoal method was used. Genistein, the best known of the naturally occurring non-steroidal estrogens, was used as a reference compound. The semi-logarithmic plot of bound radioactivity vs. molar concentration of compounds exhibited curves parallel to those of 17β-estradiol, suggesting a common binding site for the compounds tested. Concentrations required for 50% displacement of specifically bound radioactivity (IC_{50} values) were calculated from the plotted data and are shown in Table **1**. 8-Isopentenylnaringenin (**1**) was proven to exhibit estrogenic activity with IC_{50} of 140 nM, and this value indicated that **1** possesses an activity two times greater than that of genistein.

In order to examine if **1** functioned as an estrogen receptor agonist or antagonist, we tested its ability to stimulate the growth of estrogen-dependent MCF-7 cells (derived from human breast cancer) in culture (17). Test compounds were incubated for 3 days with cells which had been brought into log phase during a 5-day preculture period. The number of cells was assessed by MTT assay. Figure **1** shows the dose-dependency of MCF-7 cell proliferation on 17β-estradiol, **1** and genistein. These compounds stimulated the cell proliferation in a concentration dependent manner and caused 1.6- to 1.7-fold increases in cell numbers compared to the control, suggesting that **1** is an estrogen receptor agonist. It was observed that maximum stimulation of the cell growth occurred at concentrations of 10^{-10} M for 17β-estradiol, 10^{-8} M for **1** and 10^{-6} M for genistein. The EC_{50} values (concentration of a compound required to increase the cell number to 50% of plateau level) were calculated, and in Table **2** the activities measured in this study are compared.

Compound **1** has been reported to occur in plants (9); however, no indication of biological activities attributable to **1**

Table 1 Binding affinities of the compounds for uterine estrogen receptor[a].

Compounds	IC_{50} (nM) mean	SEM[b]	n[c]
Estradiol	1.0	0.3	11
genistein	320	15	2
1 (natural)	140	33	4
1 (synthetic)	110	41	4
2	> 5,000		
3	> 5,000		
4	160	40	2
5	650	50	2
6	> 5,000		

[a] The binding affinity was determined in a competitive binding assay with [^3H]-estradiol (1.1 nM).
[b] Standard error of mean.
[c] Number or experiments.

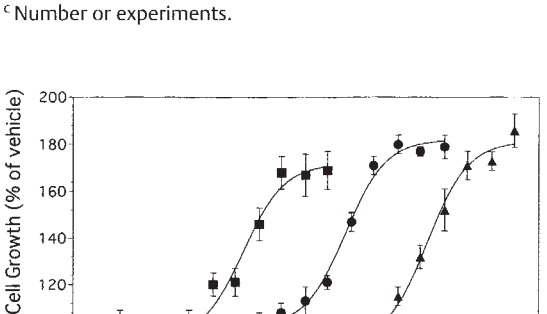


Fig. 1 Dose-response effect of the compounds on the proliferation of MCF-7 cells. Cells were grown for 3 days in 96-well plates containing 10% dextran-coated charcoal-treated calf serum at the indicated concentrations of the compounds. Growth was monitored by the MTT method and was expressed by the increase in the absorbance at 550 nm. Points are mean values, and vertical bars represent standard errors of the mean (n = 8). ■: 17β-estradiol, ●: 8-isopentenylnaringenin, ▲: genistein.

Table 2 Effects of the compounds on the growth of estrogen-dependent MCF-7 cells.

Compunds	EC_{50} (nM)[a]		
estradiol	0.0032	± 0.0002	(19)[b]
genistein	47	± 6	(3)[b]
8-isopentenylnaringenin (**1**)	1.9	± 0.4	(19)[b]

[a] The EC_{50} value represents the concentration of the compound required to increase the cell number to 50% of the respective plateau level.
[b] Mean ± standard error. Parentheses indicate the number of experiment.

has been reported. The results described above demonstrate that another class of flavonoid, 8-isopentenylnaringenin (**1**), which belongs to the prenylflavans, has an appreciable estrogen-receptor affinity. Prenylflavans are known to exhibit a wide range of biological properties, such as antifungal and antibacterial activities, in addition to the inhibitory activities against several enzymes. However, the literature contains no indication of its estrogenic activity (7). The affinity of **1** was twice as high as that of genistein when bovine uterine cytosol was used as a receptor source. The results with estrogen-sensitive MCF-7 (cultured cells derived from breast cancer) showed that the estrogen agonist activity of **1** could be estimated to be 25 times more potent than that of genistein. One explanation of the higher activity of **1** in cell proliferation experiment compared with the activity observed for a receptor binding assay is that substitution of the flavan ring system with a prenyl group increases its lipophilicity and, consequently, enhances its cell proliferation activity through increased permeability of cellular membranes.

Structure-activity relationship

8-Isopentenylnaringenin (**1**) displays a range of principal-structure variation possibilities, including position of the prenyl group, configurative change at stereogenic center and further functionalization of the aromatic rings. The influence of these structural variations on the binding affinity for estrogen receptor was studied.

In a first approach to structure-activity investigation, we focused our attention on the role of the isopentenyl group for the estrogenic activity. Recently, Miksicek reported (18) that a variety of hydroxylated flavonoids (flavones, flavanones and chalcones) which lack an alkyl side chain exhibited weak estrogenic activity when assessed by a transient gene expression assay. These findings prompted us to examine a series of flavonoids (5 flavones: acacetin, apigenin, baicalein, chrysin, wogonin; 4 flavanones: eriodictyol, hesperetin, homoeriodictyol, naringenin; and 6 flavonols: datiscetin, fisetin, kaempferol, myricetin, quercetagetin, quercetin) for their ability to inhibit [^3H]-estradiol binding to the estrogen receptor. The results were negative: none of the commonly occurring flavonoids were detected to bind appreciably to the estrogen receptor at a concentration of 10 μM (data not shown). The result clearly indicated the prenyl side chain in **1** to be essential for the binding activity for estrogen receptor.

We then synthesized isopentenylnaringenins (**1**), (**2**) and (**3**) starting from commercially available naringenin in racemic form. These compounds which differ in the position(s) of prenylation in the A-ring were assessed for their estrogenic activity by the measurement of their ability to compete with [^3H]-estradiol for binding to estrogen receptor. Table **1** compares the binding properties of these analogs. Synthetic **1** showed identical affinity for the receptor with that of naturally occurring **1** within experimental error. No significant activity was observed in **2** nor in **3**, as demonstrated by their IC_{50} of greater than 5 μM. In other words, movement of the isopentenyl group from position 8 to 6 resulted in loss of activity and additional introduction of an isopentenyl group into position 6 of **1** produced a dramatic reduction in the binding affinity. These results indicate that the isopentenyl group in **1** plays a crucial role in the interaction with the receptor.

The interaction of drugs with biochemical targets such as enzymes or receptors is often characterized by a high degree of stereospecificity. Two enantiomers of **1** were expected to show different binding affinity for the estrogen receptor. Therefore, we separated the respective enantiomers by HPLC using cellulose carbamate as chiral stationary phase and mixed solvent of hexane and 1-propanol as eluent. Both enantiomers (**1a** and **1b**) were obtained and were characterized by their CD spectra. The CD spectrum of **1a** was nearly identical to that for 2(*S*)-naringenin (14), while the CD spectrum of **1b** appeared to be the mirror image of that observed for **1a**. These results clearly show that **1a** possesses 2(*S*) configuration and **1b** is assigned to 2(*R*). Interestingly, we were unable to observe a significant difference in the dose-dependent effects of the respective enantiomers (data not shown) along with the synthetic **1** (racemate) on the inhibition of [^3H]-estradiol to estrogen receptor.

Based on these findings, there appeared to be value in investigating the estrogenic activity of a 2,3-dehydroflavonoid having an 8-isopentenyl group. 8-Isopentenylapigenin (**4**) and 8-isopentenylquercetin (**5**) were prepared. As shown in Table **1**, compound **4** displayed an estrogen response approximately as potent as that of **1**, while compound **5** inhibited specific binding of [^3H]-estradiol to the receptor with an IC_{50} value of 650 nM (Table **1**); addition of hydroxy groups at positions 3 and 2′ resulted in a slight decrease in activity.

A variety of isoflavones are known to show estrogenic activity *in vitro* as well as *in vivo*. In view of the positive influence of an isopentenyl substituent in flavonoids, we introduced an isopentenyl group into genistein in position 8 of the A-ring. No significant activity was observed in **6**, as demonstrated by the IC_{50} of greater than 5 μM (Table **1**).

The flavonoids examined in these experiments belong to the four different structural groups flavone, flavanone, flavonol and isoflavone. The comparison of molecular models shows that the distance between the two hydroxy functions in A- and C-rings is close to that of estradiol. The presence of phenolic functionalities on the flavonoid nucleus seems to be essential to possess estrogenic activity. Introduction of an isopentenyl group into position 8 of *inactive* flavonoids enhanced dramatically the estrogen binding affinity of the parent compound. Conversely, we observed the loss of binding ability when we introduced an isopentenyl group in the corresponding position of the A-ring in the *active* isoflavone structure. The substitution pattern in the A-ring seems to be critically important for the active site binding. The isopentenyl chain in the position 8 is probably necessary for hydrophobic interaction between the active compound and the receptor protein. Interestingly, the two enantiomers and the dehydro derivative (**4**) of **1** showed identical binding affinity for the receptor, suggesting that a part of the binding site of the receptor is very flexible. The loss of the activity caused by the introduction of a prenyl group into the isoflavone structure can be explained by the steric hindrance produced by the bulky group that prevents necessary interaction with the receptor site. This finding implies that a part of the binding site of the receptor is *not* flexible. These results described here may renew the structure-activity relationship in estrogen agonists.

In combination with the results described in our subsequent paper (5), **1** was found to function as estrogen agonist in *in vitro* and in *in vivo* experiments. We evaluated the effects of **1** on uterine weight gain as well as on bone metabolism in rats with estrogen-deficiency. Compound **1** was found to possess estrogen uterotropic effectiveness and also to prevent bone loss caused by ovariectomy (5). As is described above, several structurally related prenylflavonoids are shown to possess the ability to bind estrogen receptor. Compound **1**, therefore, represents a chemotype which can serve as a model for developing more effective synthetic analogs of high interest. Further chemical modification studies are underway to discover useful therapeutic agents for chronic diseases caused by estrogen deficiency.

Pharmacognostical significance

Estrogen has been shown to exert a variety of beneficial effects on men and women. It is recognized to provide protection against osteoporosis, heart attack, and other cardiovascular problems, and possibly Alzheimer's disease (1). The present study suggests that a variety of prenylflavonoids also possess such an activity. Compound **1** is an ingredient of a traditional Thai medicine that has been utilized in the treatment of various human ailments (4). 8-Prenylflavonoids are known to occur in traditional herbal medicines, e.g., *Glycyrrhiza inflata* (14), *Sophora flavescens* (19, 20) and other medicinal plants of the Leguminosae (21, 22) and Moraceae (23). Furthermore, a recent review described that prenylflavonoids are distributed widely in higher plants (7). In view of the variety of beneficial activities of estrogen, the occurrence of prenylflavonoids in traditional and folk medicines might be of value in preventing various chronic diseases.

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