Structures of 4-Aryl-coumarin (Neoflavone) Dimers Isolated from *Pistacia chinensis* BUNGE and Their Estrogen-like Activity

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Activity-guided fractionation of twigs of *Pistacia chinensis* resulted in the isolation and characterization of two novel ingredients as potent estrogen agonists. On the basis of spectral analysis and comparison with a related compound their structures were elucidated as 3,3″-dimers of 4-aryldihydrocoumarins (3,4-dihydro-4-(4′-hydroxyphenyl)-7-hydroxycoumarin) differing only in the stereochemical disposition of the linkage between the two 4-arylcoumarin moieties. These compounds are the first examples of bis-flavonoids which have been proven to possess estrogen-like activity.

Key words *Pistacia chinensis*; Anacardiaceae; phytoestrogen; neoflavone; dimer; 4-aryldihydrocoumarin

In recent years there has been increasing interest in estrogen replacement therapy, since estrogen is suggested to have beneficial effects for women in preventing heart attacks and other cardiovascular problems, osteoporosis and possibly Alzheimer's disease.[1)] Higher plants are known to contain a structurally diverse array of non-steroidal constituents that represent estrogenic activities (phytoestrogens).[2)] It is also becoming clear that phytoestrogens exert a variety of beneficial effects in prevention of diseases caused by estrogen deficiency.[3)] Previously we reported the isolation and characterization of several phytoestrogens belonging to various structural types which include homoisoflavone,[4)] prenylflavone,[5,6)] diphenylpropanoid,[7)] and iboga alkaloid.[8)]

Forest products continue to be a rich source of phytochemical diversity and are, therefore, important sources of new drugs as exemplified by the anticancer agents paclitaxel and camptothecin.[9)] Tracts of forest still cover a considerable portion of Japan and a recent environmental profile estimated that approximately 60 percent of the country could be classified as forestland. We have embarked on a program to discover potential therapeutic agents from forest plant species. Plant collections were conducted in forests in Japan as well as several botanical gardens and a total of 221 plants representing 71 families have been collected, extracted and screened.

The MeOH extract of the twigs of *Pistacia chinensis* BUNGE was found to have noteworthy inhibitory activity against [^{3}H]estradiol binding to estrogen receptors. *P. chinensis* is a tree belonging to the Anacardiaceae family, and is indigenous to southern China and cultivated in gardens in Japan. *P. chinensis* has not been extensively investigated in the past, and studies of phenolic compounds and terpenes from its leaves[10)] are the only publication in recent years. In this paper we wish to report on an activity-guided isolation and the structure determination of two novel dimeric 4-arylcoumarines (neoflavones) in addition to their estrogen-like activity.

Results and Discussion

Approximately 800 g of fresh twigs of *P. chinensis* were chopped into small pieces and an extract was prepared using MeOH. The extract was then partitioned between H_2O and EtOAc. Activity was concentrated in the organic fraction. This fraction was further separated by silica gel and reversed-phase column chromatography to afford two active ingredients, **1a** and **2**, in good yield.

Compound **1a** was obtained as a colorless, optically active solid ($[\alpha]_D = +237°$). The FAB-MS of **1a** gave a *pseudo*-molecular ion at *m/z* 511 $(M+H)^+$ corresponding to the molecular formula of $C_{30}H_{22}O_8$, which was confirmed by high resolution (HR) FAB-MS [Calcd 511.1393, Found *m/z* 511.1358 $(M+H)^+$]. The UV spectrum showed an absorption maximum at 278 nm and the IR spectrum displayed absorption bands for hydroxyl (3384 cm^{-1}) and lactone (1752 cm^{-1}) groups. The NMR spectra of **1a** indicated a dimeric structure with an asymmetrical coupling of molecules comprised of $C_{15}H_{11}O_4$, since doubling up of signals from the two moieties was clearly observed in its ^{1}H and ^{13}C resonances (Table 1). The spectral data of **1a** was very similar to those of diphysin ($C_{30}H_{22}O_{10}$, 3-3″-dimer of 3,4-dihydro-4-(4′-hydoxyphenyl)-5,7-dihydroxycoumarin) (**3**),[11)] indicating **1a** to be a 3-3″-dimeric 4-phenyldihydrocoumarin, with two notable differences. A total of 15 carbon signals were observed for **3** in the ^{13}C-NMR spectrum, while 30 signals were clearly distinguished for **1a**. Another major difference was observed in the ^{1}H resonance in which a pair of AMX coupling systems with signals at δ 6.97 (2H, m (overlapped)), 6.50 (1H, dd, J=9.8, 2.4 Hz) and 6.60 (1H, dd, J=8.3, 2.4 Hz), and 6.49 (1H, br s) and 6.55 (1H, d, J=2.4 Hz) was present. These results suggest that **1a** differs from **3** by lacking hydroxy groups at C-5 and C-5″ positions.

Confirmation of the proposed structure of 4-aryldihydrocoumarin for **1a** was provided by a heteronuclear multiple bond connectivity (HMBC) experiment in which the proton at C-4 (δ 3.97) was found to couple to a carbonyl carbon at C-2 (δ 169.5) in addition to aromatic doublet carbons at C-5 (δ 129.5) and C-2′ (δ 130.6). The connection between the dihydrocoumarin moieties was also supported by long-range correlation of an H-3 proton (δ 2.99) to a C-2″ carbon (δ 168.5). The proof of the relative stereochemistry was given by the coupling constants observed in the ^{1}H-NMR spectrum of **1a**. The double doublet at δ 2.99 (H-3) had a coupling constant of 5.3 Hz to the signal at δ 3.97 (H-4), supporting a *cis-gauche* relationship, while the coupling constant of 2.2

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Table 1. ^{1}H- and ^{13}C-NMR Data for **1a**, **1b** and **2**

Carbon	**1a**[a]		**1b**[b]		**2**[c]	
	δ_C	δ_H (mult. J(Hz))	δ_C	δ_H (mult. J(Hz))	δ_C	δ_H (mult. J(Hz))
2	169.5		166.8		171.1	
3	46.0	2.99 (dd, 10.9, 5.3)	43.8	3.13 (dd, 11.6, 5.2)	44.5	3.09 (d, 3.4)
4	45.3	3.97 (d, 5.3)	44.4	4.14 (d, 5.2)	43.2	4.41 (d, 3.4)
4a	120.2		124.5		119.7	
5	129.5	6.97[d]	128.8	7.11 (d, 8.4)	129.7	7.00 (d, 7.1)
6	113.2	6.50 (dd, 9.8, 2.4)[d]	118.4	6.82 (dd, 8.4, 2.3)	113.1	6.51 (dd, 7.1, 2.4)[d]
7	159.2		150.9		159.4	
8	104.5	6.49 (br s)[d]	111.0	6.89 (d, 2.3)	104.6	6.52 (br s)[d]
8a	152.5		150.7		152.8	
1'	130.3		133.9		130.9	
2′, 6′	130.6	7.01 (d, 8.5)	129.7	7.27 (d, 8.6)	129.7	7.12 (d, 8.7)
3′, 5′	116.7[d]	6.66[d]	122.2	7.03 (d, 8.6)	117.0	6.72 (d, 8.7)
4′	158.0		150.5		158.3	
2″	168.5		165.6			
3″	49.6	3.20 (dd, 10.9, 2.2)	47.1	3.32 (dd, 11.6, 1.6)		
4″	44.8	4.73 (d, 2.2)	43.7	5.03 (br s)		
4a″	114.8		119.2			
5″	131.8	6.97[d]	130.7	7.16 (d, 8.3)		
6″	113.7	6.60 (dd, 8.3, 2.4)	118.8	6.93 (dd, 8.3, 2.3)		
7″	159.7		151.3			
8″	104.3	6.55 (d, 2.4)	110.6	6.97 (d, 2.3)		
8a″	153.4		151.3			
1‴	133.3		137.5			
2‴, 6‴	129.2	6.82 (d, 8.5)	128.3	7.06 (d, 8.6)		
3‴, 5‴	116.6[d]	6.66[d]	122.3	6.98 (d, 8.6)		
4‴	157.7		150.1			
			$\underline{C}H_3CO$ 21.0	2.25		
			3×21.1	2.26		
				2.28		
				2.30		
			$CH_3\underline{C}O$ 168.8			
			169.0			
			169.1			
			169.2			

a) In acetone-d_6. *b*) In $CDCl_3$. *c*) In CD_3OD. *d*) Overlapping.

Hz between H-3″ (δ 3.20) and H-4″ (δ 4.73) indicated a *syn-periplanar* arrangement. A larger coupling constant (10.9 Hz) suggested a *trans-diaxial* relationship between H-3 and H-3″. To establish the relative stereochemistry of the molecule, gradient enhanced nuclear Overhauser effect (NOE) spectroscopy (GOESY)[12] was applied. Although many of the proton signals representing the two "halves" of **1a** were nearly overlapping, its acetate **1b** gave a well-resolved ^{1}H-NMR spectrum using $CDCl_3$. It was shown that irradiation of H-3″ resulted in NOEs of H-4″ and H-4. NOEs were also measured between H-3″ and phenolic protons arising from H-2′ (H-6′) (δ 7.27) in addition to H-2‴ (H-6‴) (δ 7.06), indicating the spatial vicinity of these protons as depicted in Fig. 1. Consequently, **1a** is thus represented by the relative stereostructure illustrated in Chart 1.

Compound **2** was also isolated as a colorless, optically active solid ($[\alpha]_D$=+267°) and had a FAB-MS [*m/z* 511 $(M+H)^+$] similar to that of compound **1a**, indicating that it was an isomer of **1a**. The presence of only 15 resonances in the ^{13}C-NMR spectrum of **2** indicated that the two components were equivalent. Compounds **1a** and **2** showed nearly identical chemical shifts and coupling constants. These results clearly indicated that **2** was a symmetrical 3,3″-dimer of 3,4-dihydro-4-(4′-hydroxyphenyl)-7-dihydroxycoumarin.

Compounds **1a** and **2** were then evaluated for their estrogenic activity. The primary *in vitro* assay measured their ability to compete with [^{3}H]estradiol for binding to the bovine uterine estrogen receptor by the method described earlier[4] using genistein as the reference standard for comparison. The apparent IC_{50} value of **1a** was 50 nM which was approximately 7 times greater than genistein, while 500 nM was observed for **2**. Compound **1a** was next tested for its ability to stimulate the growth of estrogen-dependent T47D cells in culture to examine if it functioned as an estrogen receptor agonist or antagonist using the procedures described in our previous paper.[7] Compound **1a** stimulated the cell proliferation

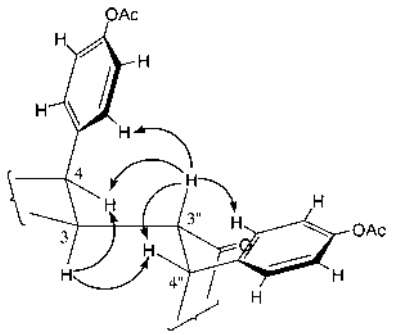
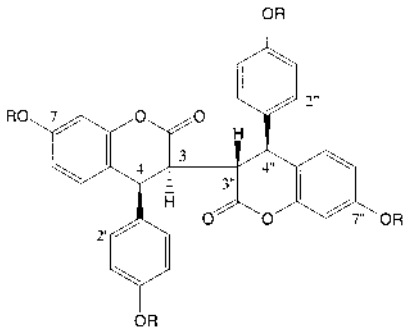


Fig. 1. Important NOE Difference Observed in GOESY NMR Spectrum of **1b**



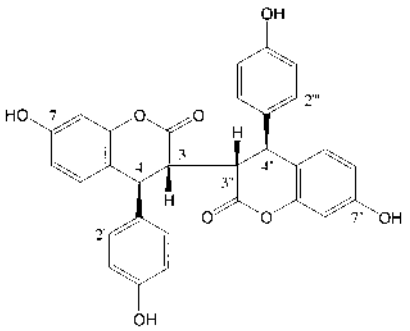


Chart 1

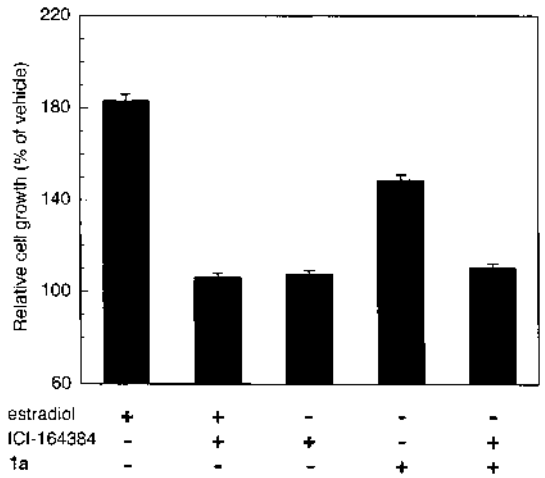


Fig. 2. The Effect of ICI-164384 on the Ability of **1a** to Stimulate the Growth of T47D Cells

Cells were grown in the presence (+) or absence (−) of 100 pM of estradiol, 1 μM of ICI-164384 or 10 μM of **1a**. Columns are mean values, and vertical bars represent standard errors of the mean (n=8).

in a concentration dependent manner and induced T47D proliferation to the same extent as estradiol, indicating that the compound was an estrogen agonist. The EC_{50} values (concentration of a compound required to increase the cell number to 50 percent of plateau level) were 800 nM for **1a** and 4 pM for estradiol. It has been shown that estrogen antagonists can inhibit estrogen agonist responses by forming complexes with high affinity to the estrogen receptor. We therefore examined the effect of the pure/complete estrogen antagonist ICI-164384 on the T47D cell proliferation induced by **1a**. As shown in Fig. 2, the stimulatory effect on the cell growth of estradiol and **1a** was clearly blocked by cotreatment with ICI-164384 in a similar manner. These results indicate that compound **1a** possesses estrogen agonist activity, although exact comparison with other phytoestrogens is somewhat hampered by the fact that the results derive from experiments with different values for the estradiol standard. This compound, however, is the first example of a bis-flavonoid which has been proven to be an estrogen agonist.

Recently the existence of two subtypes of estrogen receptor (ERα, ERβ) was discovered[13] and several phytoestrogens were reported to have higher affinity for ERβ.[14] The results presented here were obtained using bovine uterine extracts which involve mixtures of ERα and ERβ. Additional studies using these respective subtypes are critical to understanding the structure–activity relationship among various types of estrogen receptor ligands. In conclusion, we have now identified a bis-4-arylcoumarine (neoflavone) as a new type of phytoestrogen.

Experimental

Extraction and Isolation Fresh twigs (800 g) of *Pistacia chinensis* collected from Tama Forest Science Garden of the Forestry and Forest Products Research Institute were chopped into small pieces, and an extract was prepared using MeOH at 60 °C for 2 h. The extract was filtered and concentrated to dryness *in vacuo*. The residue was partitioned between EtOAc and H_2O to give a bioactive EtOAc fraction. The fraction (13.4 g) was subjected to column chromatography on silica gel with elution by $CHCl_3$–MeOH (90 : 10) to give three fractions after combination of similar fractions as judged by TLC. A biologically active fraction (2.81 g) was separated by flash chromatography on silica gel with (toluene–EtOAc–MeOH, 60 : 35 : 5). Compounds **1a** and **2** were obtained and each compound was further purified by RP18 silica gel column chromatography (MeOH–H_2O, 60 : 40) to give **1a** (920 mg) and **2** (33 mg).

Compound **1a**: Colorless powder, $[\alpha]_D$=+237° (c=0.1, MeOH). IR (KBr tablet) cm^{-1}: 3384, 1752. UV λ_{max} (EtOH) nm (ε): 278 (6600). HR-FAB-MS m/z: 511.1358 $(M+H)^+$: Calcd for $C_{30}H_{23}O_8$: 511.1393. The analysis of its NMR data including distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC), HMBC, and NOE spectra allowed for unambiguous assignment of all proton and carbon signals (Table 1).

Compound **2**: Colorless powder, $[\alpha]_D$=+267° (c=0.1, MeOH). IR (KBr tablet) cm^{-1}: 1752. UV λ_{max} (EtOH) nm (ε): 278 (7100). FAB-MS: 511 $(M+H)^+$, $C_{30}H_{23}O_8$. The analysis of its NMR data including DEPT, HMQC, HMBC, and NOE spectra allowed for an unambiguous assignment of all proton and carbon signals (Table 1).

Acetylation of 1a Compound **1a** was acetylated by acetic anhydride/pyridine by the usual method to give tetraacetate **1b** as a colorless powder. FAB-MS: 679 $(M+H)^+$, $C_{38}H_{31}O_{12}$. IR (KBr tablet) cm^{-1}: 1766.

Estradiol Receptor Binding Assay The competitive binding assay to measure the affinity for the estrogen receptor follows the method described previously.[4]

Effect on T47D Cell Proliferation Full details of the experimental method used for the cell culture and determination of cell number using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method have been published.[7]

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