Isoflavone Dimers from Yeast Extract-Treated Cell Suspension Cultures of *Pueraria lobata*

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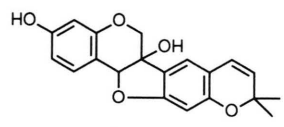
Isoflavone, *Pueraria lobata*, Dimer, Lignin, Phenol Oxidative Coupling

Two new isoflavone dimers, kudzuisoflavone A and B have been isolated from yeast extract-treated cell suspension cultures of *Pueraria lobata* (Leguminosae). Their structures were characterized by spectral data, and they were considered to be formed by phenol oxidative coupling of two isoflavone moieties through C–C *ortho-ortho* and *ortho-para* couplings, respectively.

Introduction

The accumulation of phytoalexins against microbial attacks is one of the important defense responses in higher plants [1, 2]. The induction of the phytoalexins which is triggered after the recognition of signal materials "elicitors" by plant cells is initiated at the transcription level of specific genes. Synthesis of specific enzymes results in the *de novo* activation of a secondary metabolic pathway leading to phytoalexins. Therefore, elicitor-treated plant tissues and cell cultures are suitable model systems for studying the signal recognition mechanisms by plant cells, the regulation mechanisms of specific gene expression and the enzyme reaction mechanisms in the biosynthesis of secondary metabolites [3, 4]. In the course of our enzymatic studies on isoflavonoid biosynthesis in cell suspension cultures of *Pueraria lobata* (Japanese name kudzu), we attempted elicitor induction strategy to obtain cells of high activity in isoflavonoid biosynthesis. The treatment of *P. lobata* cells with a glycoprotein elicitor prepared from the fungus *Phytophthora megasperma* f. sp. *glycinea* (Pmg) resulted in the rapid accumulation of a pterocarpan tuberosin (**1**) which was reported as a phytoalexin of *P. lobata* leaves [5] and stems [6]. Similar responses were observed in experiments using $CuCl_2$ treatment. However, cells obtained by Pmg elicitor or $CuCl_2$ treatment were not suitable for enzymatic studies, because these conditions led to rapid death of *P. lobata* cells probably due to hypersensitive response of cells to glycoprotein elicitor and $CuCl_2$ (these results will appear elsewhere). Among all elicitors tested, the most desirable results were obtained with an endogenous elicitor which was prepared by the hydrolysis of



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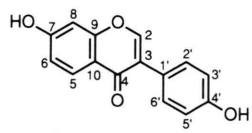

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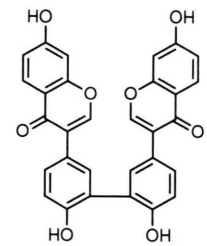

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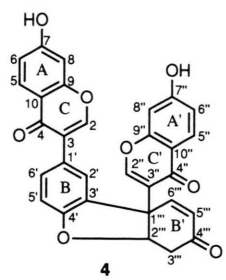

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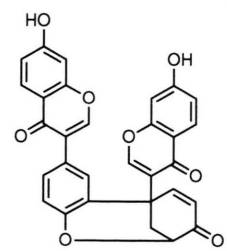

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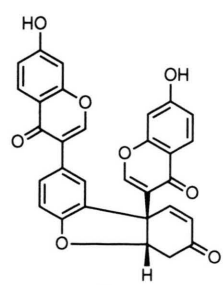

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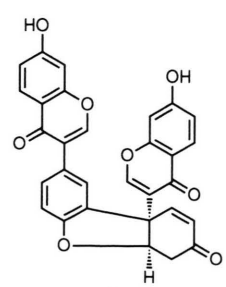

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P. lobata cell walls with a fungal endopolygalacturonase. In the endogenous elicitor-treated cells, we have already reported the detection and characterization of deoxychalcone synthase [7] and isoflavone synthase [8–10], key enzymes in the biosynthesis of 5-deoxyisoflavonoids. Later, a commercially available yeast extract (YE, from Difco) was found to have the same effects as the endogenous elicitor [11]. The treatment of *P. lobata* cells with the endogenous elicitor and the YE elicitor resulted in the activation of isoflavone biosynthesis and the accumulation of the isoflavone daidzein (**2**), one of the main constituents of *P. lobata* cells. Although tuberosin (**1**) was not accumulated, two new compounds were formed along with daidzein (**2**). This report deals with the isolation and the structure determination of these induced metabolites, kudzuisoflavone A (**3**) and B (**4**), which were identified as isoflavone dimers (biisoflavones).

Results and Discussion

A combination of silica gel and Sephadex LH-20 column chromatography and preparative reverse-phase HPLC of acetone extracts of YE-treated *P. lobata* cells afforded two newly induced metabolites, kudzuisoflavone A (**3**) and B (**4**).

The UV spectrum (MeOH) of kudzuisoflavone A (**3**) showed an intense maximum at 248 nm with a weak absorption at 299 nm, which is characteristic of an isoflavone [12]. This skeleton was also supported by a low-field singlet at δ 8.34 in the ^{1}H NMR spectrum (d_6-acetone) which was assignable to isoflavone H-2. The ^{1}H NMR signals of an aromatic ABX system at δ 8.07 (d, J = 8.8 Hz), 6.99 (dd, J = 2.2, 8.8 Hz) and 6.86 (d, J = 2.2 Hz) resembled those of ABX system of **2** (H-5, H-6 and H-8, respectively, see Table I), suggesting **3** to be a 5-deoxy type isoflavone. Furthermore, another aromatic ABX system at δ 7.62 (d, J = 2.2 Hz), 7.57 (dd, J = 2.2, 8.4 Hz) and 7.07 (d, J = 8.4 Hz) showed the presence of a trisubstituted isoflavone B ring. The molecular weight of **3** was determined as 506 from FAB-MS analysis. The high molecular weight and the simple pattern of ^{1}H NMR spectrum indicated that **3** had a symmetrical homodimer structure. Comparison of the molecular weight of **3** with that of daidzein (m/z 254), which is the simplest 5-deoxyisoflavone, suggested that the monomer unit of the homodimer **3** was daidzein (**2**). The position to form the dimer was assigned at C-3′ of B ring from the comparison of the ^{1}H NMR chemical shifts of **3** with those of **2** (Table I). The ^{13}C NMR spectrum of **3** was similar to that of **2** except for the down-field-shifted signal at δ 125.7 which was assignable to C-3′. The assignment of ^{13}C chemical shifts in d_6-DMSO is shown in Table I which was deduced from the 2D ^{1}H-detected one-bond heteronuclear correlation (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments, and also from the comparison with ^{13}C spectral data of **2.**

Table I. ^{13}C NMR (in d_6-DMSO) and ^{1}H NMR (in d_6-acetone) data for daidzein (**2**), kudzuisoflavone A (**3**) and B (**4**).

	2			**3**			**4**			**4** (continued)			
Atom	^{13}C	^{1}H	J [Hz]	^{13}C	^{1}H	J [Hz]	^{13}C	^{1}H	J [Hz]	Atom ^{13}C	^{1}H	J [Hz]	
2	152.5 d	8.11 s		153.1 d	8.24 s		155.6 d	8.16 s		2″ 153.3 d	8.19 s		
3	123.4 s			123.6 s			125.0 s			3″ 123.0 s			
4	174.5 s			174.8 s			174.4 s			4″ 174.4 s			
5	127.1 d	8.07 d	8.8	127.4 d	8.07 d	8.8	126.9 d	7.95 d	8.8	5″ 127.2 d	8.05 d	8.8	
6	114.9 d	6.99 dd	2.2, 8.8	115.2 d	6.99 dd	2.2, 8.8	115.3 d	6.99 dd	2.2, 8.8	6″ 115.1 d	6.98 dd	2.2, 8.8	
7	162.3 s			162.6 s			162.8 s			7″ 162.5 s			
8	101.9 d	6.90 d	2.2	102.2 d	6.91 d	2.2	102.0 d	6.92 d	2.2	8″ 102.0 d	6.88 d	2.2	
9	157.0 s			157.6 s			157.4 s			9″ 157.3 s			
10	116.6 s			116.8 s			116.6 s			10″ 116.5 s			
1′	122.5 s			122.7 s			120.0 s			1‴ 49.3 s			
2′	129.8 d	7.47 d	8.4 (2H)	132.0 d	7.62 d	2.2	124.2 d	7.57 d	1.8	2‴ 83.7 d	5.52 ddd	1.8, 2.2, 4	
3′	114.8 d	6.89 d	8.4 (2H)	125.7 s			130.3 s			3‴ 37.9 t	2.88 dd	2.2, 17.2	
											3.18 dd	4.0, 17.2	
4′	157.3 s			154.6 s			158.4 s			4‴ 195.9 s			
5′	114.8 d			115.6 d	7.07 d	8.4	109.3 d	6.89 d	8.4	5‴ 126.5 d	6.06 d	9.2	
6′	129.8 d			128.9 d	7.57 dd	2.2, 8.4	129.5 d	7.48 dd	1.8, 8.4	6‴ 145.1 d	6.82 dd	1.8, 9.2	

The FAB mass spectrum of kudzuisoflavone B gave an ion $[M+H]^+$ at *m/z* 507. In the ^1H NMR spectrum (d_6-acetone) two low-field singlet signals at δ 8.19 and 8.16 corresponding to H-2 of isoflavone were observed. Twenty-eight signals, two of which were overlapped with two independent signals, appeared in ^{13}C NMR spectrum (d_6-DMSO). These data suggested that kudzuisoflavone B was also an isoflavone dimer which was fused asymmetrically. The ^1H NMR spectrum contained two sets of aromatic ABX systems at δ 7.95 (d, *J* = 8.8 Hz), 6.99 (dd, *J* = 2.2, 8.8 Hz) and 6.92 (d, *J* = 2.2 Hz): at δ 8.05 (d, *J* = 8.8 Hz), 6.98 (dd, *J* = 2.2, 8.8 Hz) and 6.88 (d, *J* = 2.2 Hz) which were similar to ABX systems on A ring of **2** and **3.** Another aromatic ABX system which was assignable to B ring protons was observed at δ 7.57 (d, *J* = 1.8 Hz), 7.48 (dd, *J* = 1.8, 8.4 Hz) and 6.89 (d, *J* = 8.4 Hz). Furthermore, kudzuisoflavone B possessed the functional groups of (i) one methine proton near an oxygen atom at δ 5.52 (ddd, *J* = 1.8, 2.2, 4.0 Hz), and two diastereotopic methylene protons at δ 3.18 (dd, *J* = 4.0, 17.2 Hz) and 2.88 (dd, *J* = 2.2, 17.2 Hz), (ii) two *cis*-coupled olefinic protons at δ 6.82 (dd, *J* = 1.8, 9.2 Hz) and 6.06 (d, *J* = 9.2 Hz). ^{13}C NMR spectral data in d_6-DMSO indicated the presence of the non-aromatic quaternary carbon (δ 49.3) and the carbonyl carbon at δ 195.9 in addition to the C-4 carbonyl at δ 174.4 (overlapped with C-4″). $^1H-^1H$ 2D long range COSY NMR spectra showed the cross peaks between the methine proton and the olefinic proton at δ 6.82, and between the methylene protons and the olefinic proton at δ 6.06. These data suggested that another B ring (B′ ring) had lost its aromaticity and the structure of kudzuisoflavone B could be represented by **4** or **5.** A decision in favour of **4** rather than **5** was made by nuclear Overhauser effect (NOE) experiments (Fig. 1). When the methine proton at δ 5.52 was irradiated, NOE was observed at a low-field singlet proton at δ 8.19 (1.31%) and a methylene proton at δ 2.88 (7.63%). The irradiation of the methylene proton at δ 2.88 and the olefinic proton at δ 6.82 resulted in NOE enhancement of the low-field singlet at δ 8.19 (3.75% and 8.27%, respectively). In the structure of **5,** steric restriction required two bridge-forming bonds, which were attached to a quaternary and a methine carbon of cyclohexenone B′ ring, to have a 1,3-*cis*-diaxial orientation. Consequently both of

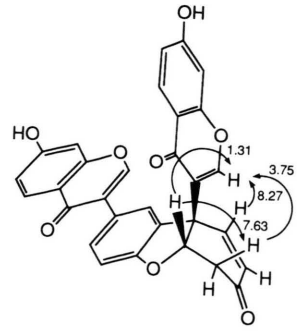


Fig. 1. NOE experiments of kudzuisoflavone B (%). This model corresponds to structure **7.**

the benzopyranone ring and the methine proton should have equatorial orientations which are not close enough to show NOE. The structure **4** with *cis* configuration of the benzopyranone ring and the methine proton could only explain the results from the NOE experiments. The biosynthetic consideration of kudzuisoflavone B which is discussed below strongly supported the structure **4.** The results of NOE experiments also indicated that a low-field singlet proton at δ 8.19 should be located on C′ ring and consequently a proton at δ 8.16 could be assigned to H-2 on ring C. On $^1H-^1H$ 2D long-range COSY the correlation of a singlet proton at δ 8.19 (H-2″) with a doublet proton at δ 8.05 was observed. Therefore, ABX systems of protons at δ 8.05, 6.98 and 6.88 were assigned to H-5″, H-6″ an H-8″ on A′ ring, respectively. From these observations all signals of ^1H NMR spectra could be assigned as shown in Table I. And the analyses of DEPT, 2D ^1H-detected HMQC and HMBC spectra of **4** led us to assign the ^{13}C chemical shifts as shown in Table I. The quaternary carbons possessing similar chemical shifts, such as C-3 and C-3″, C-7 and C-7″, C-9 and C-9″, and C-10 and C-10″, could be distinguished by the long-range $^1H-^{13}C$ correlation in the HMBC spectra (Table II). In spite of two chiral centers at 1‴ and 2‴, **4** was an optically inactive ($[\alpha]_D = 0°$). Combining the results of NOE experiments mentioned above, **4** is a racemate consisting of **6** and **7.**

As described above two types of isoflavone dimer were isolated from YE-treated *P. lobata* cell suspension cultures. Biflavonoids are produced by a variety of plants and considered to be useful tool of chemotaxonomic studies [13]. On the contrary,

Table II. H–C correlations in the HMBC spectra of **4**.

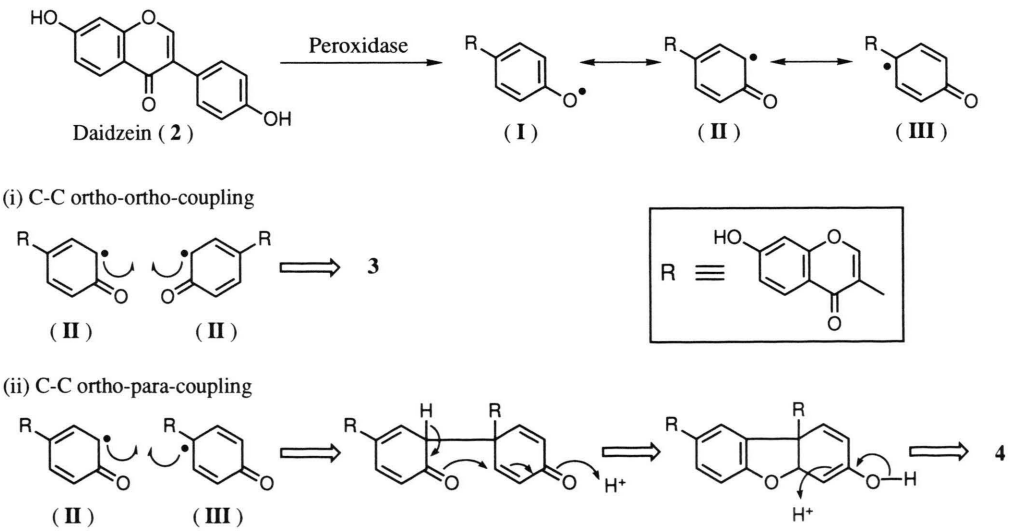
H	C
2	3, 4, 9, 1′
5	7, 8, 9
6	8, 10
8	6, 7, 9, 10
2′	4′, 5′, 6′
5′	2′, 3′, 4′
6′	2′, 4′
2″	3″, 4″, 9″
5″	7″, 8″, 9″
6″	8″, 10″
8″	6″, 7″, 9″, 10″
2‴	4‴, 6‴
3‴	2‴, 4‴
6‴	3′, 2‴, 4‴

C-1‴ was out of measurement region of the HMBC spectra. No correlations with H-5‴ were observed.

occurrence of biisoflavonoids is limited. To our knowledge, only a few cases of isoflavone–isoflavan [14] or isoflavan–isoflavan dimers [15–17] have been reported. This is the first report on the isolation of isoflavone–isoflavone dimers as natural products.

The role of isoflavone dimers **3** and **4** in the cultured cells is not clear. Although the antifungal activities of the isoflavone dimers could not be examined due to their low yields, one possibility is that they are produced as phytoalexins. But we prefer that they are the artificial by-products of peroxidase-related reaction such as lignification. In response to YE treatment it is plausible for *P. lobata* cells to induce peroxidase (phenol oxidase) for lignin formation. The induced peroxidase may oxidize the isoflavone daidzein, which is not a true substrate, and resulting phenoxy radicals (such as I, II and III in Scheme 1) can non-enzymatically dimerize to produce **3** and **4** through the combinations described in Scheme 1. The formation of kudzuisoflavone B (**4**) is analogous to the oxidation reaction yielding Pummerer's ketone [18]. The isolation of **4** as racemate supports the assumption that the coupling reactions proceed in the non-enzymatic manner which is usually observed in lignin formation. On the contrary, most of enzyme-catalyzed phenol oxidative coupling reactions have been reported to advance in a stereospecific manner, *e.g.*, the P-450-dependent formation of salutaridine from reticuline in morphine biosynthesis in *Papaver somniferum* [19], and the conversion of dihydrogeodin to (+)-geodin catalyzed by a blue copper protein in *Aspergillus terreus* [20].

In order to obtain another line of evidence supporting the assumption above, we attempted the biomimetic synthesis of the isoflavone dimers. In the presence of H_2O_2 daidzein was incubated with horse-radish peroxidase (HRP, from Sigma). This resulted in the formation of **3** and **4** which were identified by the comparison of R_f values on silica gel TLC developed with different solvent systems and of retention times on reverse phase HPLC. Besides **3** and **4** several products with unknown struc-



Scheme 1. Proposed biosynthetic pathway of yeast extract-induced isoflavone dimers in *P. lobata*.

ture were formed in low yields. The investigation on the biological activities of the isoflavone dimers requires their preparation in a large scale. For this purpose, the chemical synthesis and the optimization of HRP-catalyzed *in vitro* synthesis of isoflavone dimers are in progress.

Materials and Methods

Daidzein was from our collection. Precoated silica gel TLC plate (60 F 254) and reverse phase TLC (RP-8, F 254) were obtained from Merck. Mass and NMR spectra were taken on a JEOL JMS-DX 300 mass spectrometer and on a JEOL GX-500 apparatus, respectively.

Cell cultures and yeast extract treatment

Cell suspension cultures of *P. lobata* were established from callus cultures which had been induced from its root as reported [11], and maintained in Murashige-Skoog's liquid medium containing 2,4-D (2 ppm), kinetin (0.1 ppm) and sucrose (3%) [8]. Yeast extract (YE, Difco) was dissolved in distilled water and sterilized by autoclaving. The YE solution was added to cell suspension cultures (7 days old) in a final concentration of 1 mg/ml. After incubation for 72 h cells were harvested with a suction filter.

Isolation of the isoflavone dimers

YE-treated cells (total 4 kg) were extracted by refluxing with acetone (16 l) for 3 h. The acetone extract was concentrated under reduced pressure below 40 °C. The aqueous concentrate was partitioned against total 16 l of EtOAc. The organic phase was dried over Na_2SO_4 and concentrated. The residue (14.2 g) was chromatographed on silica gel (500 g, ∅ 6.5 × 41 cm) with a mixture of benzene–EtOAc followed by chromatography on Sephadex LH-20 in MeOH. Finally, preparative reverse phase HPLC (TSK gel ODS-80TM, 7.8 × 300 mm, Tosoh) using MeOH–H_2O gradient gave kudzuisoflavone A (3 mg) and B (7 mg).

*Kudzuisoflavone A (**3**)*

Colorless powder, m.p. > 300 °C. UV λ_{max} MeOH nm: 210, 239 sh, 243, 246 sh, 248, 299. FAB-MS *m/z:* 507 $[M+H]^+$. Silica gel TLC: R_f 0.26 in CH_3Cl–MeOH (9:1); 0.13 in benzene–AcOEt (2:3). Reverse phase TLC: R_f 0.67 in 80% MeOH.

*Kudzuisoflavone B (**4**)*

Colorless powder, m.p. 252–253 °C (decomposed), $[\alpha]\ 25_D\ 0°$ (c = 0.093, DMSO). UV λ_{max} MeOH nm (log ε): 217 (4.63), 243 sh (4.62), 249 (4.63), 260 sh (4.51), 305 (4.30). FAB-MS *m/z:* 507 $[M+H]^+$. Silica gel TLC: R_f 0.51 in CH_3Cl–MeOH (9:1); 0.30 in benzene–AcOEt (2:3). Reverse phase TLC: R_f 0.61 in 80% MeOH.

In vitro synthesis of the isoflavone dimers

30 µg of daidzein (**2**) in acetone was added to 30 ml of 10 mM potassium phosphate buffer (pH 6.0) containing 300 µg of horse-radish peroxidase (Sigma) and 70 µmol H_2O_2. The mixture was incubated for 30 min at 20 °C and partitioned against EtOAc. EtOAc extract was concentrated and analyzed by TLC and reverse phase HPLC.

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