

# INDUCTION OF STRESS METABOLITE FORMATION IN SUSPENSION CULTURES OF *VIGNA ANGULARIS*

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**Key Word Index**—*Vigna angularis*, Leguminosae, red bean, lignan, isoflavone glucoside, stress metabolite, cell suspension culture, induction, actinomycin D

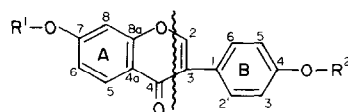
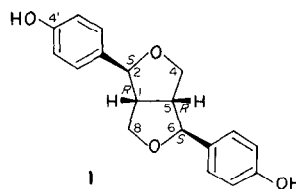
**Abstract**—Three isoflavone glucosides were isolated from cell suspension cultures of red bean (*Vigna angularis*) treated with actinomycin D and identified as daidzin, daidzein 7,4'-di-O-β-D-glucoside, and a new compound, 2'-hydroxydaidzein 7,4'-di-O-β-D-glucoside. These compounds were also accompanied by a new lignan, (+)-(1R,2S,5R,6S)-2,6-di(4'-hydroxyphenyl)-3,7-dioxabicyclo(3,3,0)octane.

## INTRODUCTION

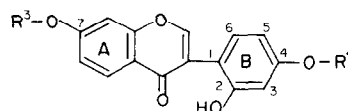
Stress metabolites are inductively formed by plant tissues in response to a wide variety of stresses such as infection with micro-organisms, mechanical wounding, UV irradiation, dehydration, or treatment with phytotoxic chemicals [1]. Among these metabolites, phytoalexins which accumulate in the tissue through contact with a fungus exhibit inhibitory activities against certain potential plant pathogens [2, 3], and have been shown to be produced in response to treatment with both biotic and abiotic elicitors [2–6]. The use of chemical elicitors, such as actinomycin D, may provide a simple model for the study of stress metabolism observed in plant tissue treated with biotic elicitors. Cell suspension cultures have also been employed in studies aimed at elucidating the regulatory mechanism of the biosynthesis of such stress metabolites. There are, however, only a few studies [7, 8] on phytoalexin production using a combination of suspension-cultured cells and abiotic elicitors. This paper reports the isolation and characterization of four compounds produced by cell suspension cultures of red bean (*Vigna angularis* Ohwi et Ohashi, 'azuki') in response to a stress caused by the administration of actinomycin D. Two are known compounds, daidzin (daidzein 7-O-β-D-glucoside, 2) and daidzein 7,4'-di-O-β-D-glucoside (3), whereas the other two are new, namely (+)-(1R,2S,5R,6S)-2,6-di(4'-hydroxyphenyl)-3,7-dioxabicyclo(3,3,0)octane (1) and 2'-hydroxydaidzein 7,4'-di-O-β-D-glucoside (4).

## RESULTS AND DISCUSSION

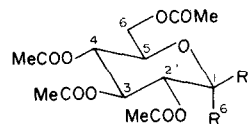
Culture medium (800 ml) and cells (170 g fr. wt) of 30-day-old actinomycin D treated cell suspensions of red bean were separated by filtration and extracted with ether and methanol, respectively. HPLC analysis of each extract showed that some metabolites accumulated in response to the administration of actinomycin D. Repeated chromatography (HPLC and Si gel CC) of the extract from the culture medium gave 590 μg 1. On the other hand, the defatted methanol extract of cells was roughly separated into two fractions by chromatography on a



- 2 R<sup>1</sup> = β-Glc, R<sup>2</sup> = H  
 3 R<sup>1</sup> = R<sup>2</sup> = β-Glc  
 5 R<sup>1</sup> = R<sup>2</sup> = H  
 8 R<sup>1</sup> = H, R<sup>2</sup> = Me



- 4 R<sup>3</sup> = R<sup>4</sup> = β-Glc  
 9 R<sup>3</sup> = R<sup>4</sup> = H



- 6 R<sup>5</sup> = OMe, R<sup>6</sup> = H  
 7 R<sup>5</sup> = H, R<sup>6</sup> = OMe

Sephadex LH-20 column Droplet counter-current chromatography of one fraction yielded 12.4 mg **2**, whereas chromatography of the other fraction (on Sephadex LH-20 and Si gel) gave 7.2 mg **3** and 17.6 mg **4**.

Compound **1** ( $M^+$  298,  $C_{18}H_{18}O_4$ ),  $[\alpha]_D + 25.3^\circ$ , was identified as the lignan, 2,6-di(4'-hydroxyphenyl)-3,7-dioxabicyclo(3,3,0)octane by UV, IR, NMR and mass spectral data which were essentially identical with the published data for natural (–)-ligballinol [9],  $[\alpha]_D - 7.1^\circ$ , and its synthetic racemate or '(±)-*p*-coumarylresinol' [10]. Thus, although there is a disparity in the rotation values, it is clear that **1** is the enantiomer of ligballinol. Since all (+)-sesamin type lignans so far isolated have the 1*R*,2*S*,5*R*,6*S* configuration [11, 12], the structure shown in **1** is reasonable. The positive CD Cotton effects at 226 nm  $\Delta\epsilon + 0.47$  and 280 nm  $\Delta\epsilon + 0.02$  are also consistent with this conclusion [13]. This compound is a rare example of a naturally occurring lignan with mono-oxygenated aromatic rings.

The  $^1H$  NMR (Table 1), UV and mass spectra of 2-peracetate (Ac-2) showed **2** to be an isoflavone monoglycoside. Hydrolysis of **2** with  $\beta$ -glucosidase (EC 3.2.1.21) yielded daidzein (7,4'-dihydroxyisoflavone, **5**) [14] as the aglycone, which was identified by UV and mass spectra, the sugar moiety was determined as D-glucose by comparison of the HPLC retention time with that of an authentic sample. The  $^{13}C$  NMR data (Table 2) of Ac-2, in addition to the fact that **2** can be hydrolysed by  $\beta$ -

glucosidase, indicate that the anomeric configuration of the glucosyl moiety is  $\beta$ , since the chemical shifts of C-3'' and C-5'' are similar to those of methyl  $\beta$ -D-(tetra-*O*-acetyl) glucopyranoside (**6**) [15] but different from those of the  $\alpha$ -isomer (**7**) [15] (Table 2). Enzymatic hydrolysis of the methylated derivative of **2** gave formononetin (7-hydroxy-4'-methoxyisoflavone, **8**), whose mass spectrum showed a fragment ion at  $m/z$  132 caused by a retro-Diels–Alder cleavage (wavy line in **8**), whereas that of the 7,4'-dihydroxy compound, **5**, showed the corresponding ion at  $m/z$  118. These results indicate that the 7-hydroxyl group in **2** is glucosylated and that **2** is daidzin (daidzein-7-*O*- $\beta$ -D-glucoside) [14].

The  $^1H$  NMR spectrum (Table 1) of 3-peracetate (Ac-3) showed **3** to be a daidzein derivative in which both hydroxyl groups are glycosylated. Enzymatic hydrolysis of **3** gave **5** and D-glucose as the sole sugar; furthermore, the  $^{13}C$  NMR data (Table 2) indicate the configurations at C-1'' and C-1''' to be  $\beta$ . Hence, **3** is daidzein 7,4'-di-*O*- $\beta$ -D-glucoside.

The mass spectrum of **4** showed a cationized molecular ion peak at  $m/z$  617  $[M + Na]^+$  (FDMS) and that of 4-peracetate (Ac-4) showed a  $M^+$  peak at  $m/z$  972 (EIMS). This indicates the presence of an additional hydroxyl group in **4** in comparison to **3**. The chemical shifts ( $\delta$  6.88, 6.95 and 7.28) and the coupling constants ( $J_{3-4} = 2.6$  Hz and  $J_{5-6} = 8.8$  Hz) in the spectrum of Ac-4 suggested a 2',4'-dioxxygenated structure for ring B. The  $^{13}C$  NMR

Table 1  $^1H$  NMR data for peracetylated isoflavone glucosides Ac-2, Ac-3 and Ac-4

Proton	Ac-2		Ac-3		Ac-4	
	$\delta$	$J$ (Hz)	$\delta$	$J$ (Hz)	$\delta$	$J$ (Hz)
H-2	7.97 s	—	7.94 s	—	7.84 s	—
H-5	8.25 d	8.8	8.24 d	8.9	8.22 d	9.1
H-6	7.06 dd	8.8, 2.4	7.06 dd	8.9, 2.5	7.06 dd	9.1, 2.3
H-8	7.04 d	2.4	7.04 d	2.5	7.04 d	2.3
H-2'	7.58 d	8.9	7.49 d	9.1	—	—
H-3'	7.17 d	8.9	7.07 d	9.1	6.88 d	2.6
H-5'	7.17 d	8.9	7.07 d	9.1	6.95 dd	8.8, 2.6
H-6'	7.58 d	8.9	7.49 d	9.1	7.28 d	8.8
H-1''	5.24 m	—	5.24 m	—	5.23 m	—
H-2''	5.34 m	—	5.33 m	—	5.34 m	—
H-3''	5.34 m	—	5.33 m	—	5.34 m	—
H-4''	5.19 m	—	5.19 m	—	5.18 m	—
H-5''	3.96 ddd	10.0, 5.7, 2.5	3.96 ddd	10.3, 6.0, 2.6	3.96 ddd	10.3, 6.0, 2.5
H-6''b	4.22 dd	12.4, 2.5	4.22 dd	12.6, 2.6	4.22 dd	12.6, 2.5
H-6''a	4.30 dd	12.4, 5.7	4.30 dd	12.6, 6.0	4.29 dd	12.6, 6.0
H-1'''	—	—	5.12 m	—	5.13 m	—
H-2'''	—	—	5.31 m	—	5.23 m	—
H-3'''	—	—	5.31 m	—	5.23 m	—
H-4'''	—	—	5.18 m	—	5.18 m	—
H-5'''	—	—	3.87 ddd	10.3, 6.0, 2.6	3.88 ddd	10.3, 6.0, 2.5
H-6'''b	—	—	4.18 dd	12.6, 2.6	4.18 dd	12.6, 2.5
H-6'''a	—	—	4.29 dd	12.6, 6.0	4.28 dd	12.6, 6.0
Ac (Me)	2.06 s	—	2.05 s	—	2.04 s	—
	2.07 s	—	2.06 s ( $\times 2$ )	—	2.06 s ( $\times 2$ )	—
	2.08 s	—	2.07 s ( $\times 2$ )	—	2.08 s ( $\times 2$ )	—
	2.10 s	—	2.08 s	—	2.09 s ( $\times 2$ )	—
			2.09 s	—	2.11 s	—
			2.10 s	—	2.12 s	—

Spectra were measured at 360 MHz in  $CDCl_3$  (TMS reference)

Table 2  $^{13}\text{C}$  NMR data for peracetylated isoflavone glucosides Ac-2, Ac-3 and Ac-4

Carbon	Ac-2 $\delta$	Ac-3 $\delta$	Ac-4 $\delta$	6¶ $\delta$	7¶ $\delta$
C-2	152.82	152.47	153.67	—	—
C-3	124.71	124.71	122.13	—	—
C-4	175.28	175.43	174.67	—	—
C-5	128.22	128.10	128.13	—	—
C-6	115.41	115.32	115.47	—	—
C-7	160.60	160.51	160.63	—	—
C-8	104.32	104.23	104.38	—	—
C-8a	157.32	157.29	157.41‡	—	—
C-4a	120.23	120.09	119.88§	—	—
C-1'	129.24	126.64	119.74§	—	—
C-2'	130.00	130.18	149.81	—	—
C-3'	121.67	116.99	112.01	—	—
C-4'	150.71	156.83	157.56‡	—	—
C-5'	121.67	116.99	114.62	—	—
C-6'	130.00	130.18	132.11	—	—
C-1'', C-1'''	98.35	99.06, 98.24	98.91, 98.29	101.70	96.95
C-2'', C-2'''	71.00	71.09, 70.92	71.06 (× 2)	71.40	70.95
C-3'', C-3'''	72.52*	72.64, 72.35†	72.67, 72.47	73.05	70.25
C-4'', C-4'''	68.17	68.22, 68.08	68.22, 68.14	68.60	68.75
C-5'', C-5'''	72.44*	72.35, 72.03†	72.47, 72.14	71.95	67.30
C-6'', C-6'''	61.91	61.85 (× 2)	61.91 (× 2)	62.00	62.05
Ac (C=O)	170.43	170.49	170.52 (× 2)	—	—
	170.11	170.37	170.40	—	—
	169.35 (× 2)	170.11 (× 2)	170.11 (× 2)	—	—
	169.17	169.32 (× 3)	169.35 (× 2)	—	—
	—	169.14	169.14	—	—
			168.79	—	—
Ac (Me)	21.13	—	20.90	—	—
	20.57 (× 4)	20.52 (× 8)	20.57 (× 8)	—	—

Spectra were measured at 25 MHz in  $\text{CDCl}_3$  ( $\text{CDCl}_3$  reference)

\*, †, ‡, §, || Values with the same sign are interchangeable

¶ Ref. [15]

data established the substitution position of the additional hydroxyl group. Thus, the chemical shifts (Table 2) of C-2–C-4 in Ac-4 differed from those in Ac-2 and Ac-3, a fact which can be explained only by the steric effects caused by the presence of the additional hydroxyl group linked to C-2' and not to C-3' in ring B. This conclusion was also supported by good agreement between observed and calculated  $^{13}\text{C}$  NMR shifts [16, 17] for Ac-4 but not between observed shifts and calculated values [16, 17] for the 3'-acetoxy isomer. Compound 4 is thus 2'-hydroxydaidzein 7,4'-di-O- $\beta$ -D-glucoside.

The presence of these isoflavone glucosides in the intact plant, in callus grown on agar medium, and in cells cultured in the liquid medium without actinomycin D was also examined. Although no significant amounts were detected in the intact plant and callus of red bean, a small amount was shown to accumulate in the suspension-cultured cells; treatment with actinomycin D greatly enhanced this accumulation (Fig. 1). Thus, these compounds must be stress metabolites produced by the cells in response to the administration of actinomycin D. Daidzin (2) is known as one of the allelochemicals of red clover (*Trifolium pratense* L.) [18], as well as 5 and 8, whereas 3 has recently been isolated [19] from a Chinese drug, the

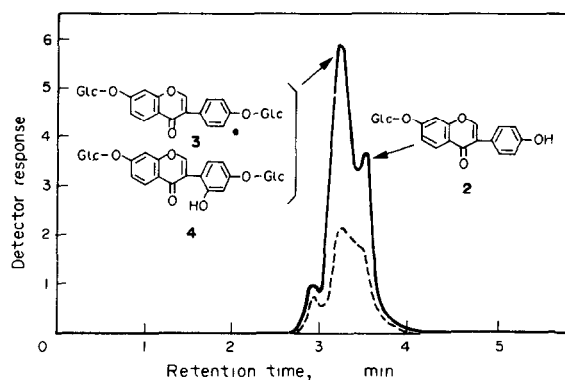


Fig. 1 Comparison of accumulation of isoflavone glucosides on day 14 in actinomycin D treated (—) and untreated (----) cell suspensions. Extracts from both cell suspensions were analysed by HPLC on a Partisil-10 ODS-2 column (4 mm i.d. × 25 cm) using  $\text{MeOH-H}_2\text{O}$  (4:1) as solvent (flow rate 0.5 ml/min), and the eluate was monitored with a UV detector (250 nm). The difference in accumulation was further enhanced on day 30, the day of harvest.

dried root of *Pueraria lobata* (Willd.) Ohwi, together with 2 and 5. The free isoflavones, such as daidzein (5) or 7,2',4'-trihydroxyisoflavone (9), have been detected in fungus-infected plants and designated as possible intermediates in the biosynthesis of pterocarpin phytoalexins [20]. On the other hand, 2–4 are, as far as we know, the first examples of glycosylated isoflavones produced by plant material under stressed conditions including infection with microorganisms. These glycosylated metabolites may be involved in the storage or transport of phytoalexins or their precursors.

## EXPERIMENTAL

**Suspension cultures and administration of actinomycin D.** The cell suspension culture of red bean (*Vigna angularis* Ohwi et Ohashi) used was that established by Mr Kenji Katoh of this Institute and maintained for more than 4 years by subculturing at 15-day intervals in Murashige-Skoog medium containing 3% sucrose and 1 mg/l 2,4-dichlorophenoxyacetic acid. Cultures were grown at 25 °C in 500-ml Sakaguchi flasks containing 100 ml of the medium and 10 ml of inoculative cell suspension on a reciprocal shaker (110 strokes/min). In order to induce production of stress metabolites, 10 ml of an aq. soln. of actinomycin D was added to a 10-day-old cell suspension, to give a final concn of 10 mg/l, and cultures were continued for a further 20 days, in control expts  $H_2O$  (10 ml) was added instead of a soln. of actinomycin D.

**Isolation and purification of stress metabolites.** Cultured cell suspension was separated into culture medium (800 ml) and cells (170 g fr. wt) by filtration, which were extracted with  $Et_2O$  and MeOH, respectively. Both extracts were analysed by HPLC on a Partisil-10 ODS-2 column (Whatman, 4 mm i.d.  $\times$  25 cm) using MeOH– $H_2O$  (4:1) as solvent (flow rate 0.5 ml/min). The eluate was monitored with a UV detector (250 nm) and only peaks whose intensities enlarged when actinomycin D was administered to the cell suspension were checked. For prep. purposes, the  $Et_2O$  extract of the culture medium was chromatographed on a HPLC column, under the same conditions as mentioned above, and the fractions which showed increased peak height on actinomycin D treatment were collected. This fraction was repeatedly chromatographed on Si gel columns eluted with *n*-hexane–EtOAc (1:3 and 1:1) to yield 1. The defatted MeOH extract of suspension-cultured cells was chromatographed on a Sephadex LH-20 column (Pharmacia) eluted with MeOH– $H_2O$  (1:1) to give two portions, as monitored by UV absorption at 250 nm. The minor portion was purified by droplet counter-current chromatography using both aq. and organic layers of  $CHCl_3$ –MeOH– $H_2O$  (7:13:8) as solvents to yield 2. The major portion containing a large amount of sugars was chromatographed twice with Sephadex LH-20, eluting with MeOH– $H_2O$  (1:1 and 3:2) and once with Si gel, eluting with  $CHCl_3$ –MeOH– $H_2O$  (6:4:1), to give 3 and 4.

(+)-(1R, 2S, 5R, 6S)-2,6-Di-(4'-hydroxyphenyl)-3,7-dioxabicyclo (3,3,0)octane (1). Colorless needles. UV  $\lambda_{max}^{MeOH}$  nm 227, 276, 283 (sh). IR  $\nu_{max}^{film}$   $cm^{-1}$  3300 (br, OH), 1610, 1512, 823 (1,4-disubstituted aromatic ring).  $^1H$  NMR (360 MHz,  $Me_2CO-d_6$ , TMS)  $\delta$  3.07 (2H, m, H-1 and H-5), 3.78 (2H, dd,  $J_{4ax,4eq} = J_{8ax,8eq} = 9.4$  Hz,  $J_{4ax,5} = J_{8ax,1} = 3.8$  Hz, H-4ax and H-8ax), 4.18 (2H, dd,  $J_{4ax,4eq} = J_{8ax,8eq} = 9.4$  Hz,  $J_{4eq,5} = J_{8eq,1} = 7.4$  Hz, H-4eq and H-8eq), 4.67 (2H, d,  $J_{1,2} = J_{5,6} = 4.7$  Hz, H-2 and H-6), 6.80 (4H, d,  $J = 9.2$  Hz, H-3' and H-5' of both aromatic rings), 7.21 (4H, d,  $J = 9.2$  Hz, H-2' and H-6' of both aromatic rings), 8.32 (2H, br s, phenolic protons). EIMS (70 eV),  $m/z$  (rel. int.) 298 [ $M$ ] $^+$  (33.0), 267 [ $M - CH_2O - H$ ] $^+$  (11.5), 175 [ $M - HOC_6H_4CHO - H$ ] $^+$  (26.3), 133 [ $HOC_6H_4CH$

$=CHCH_2$ ] $^+$  (62.0), 121 [ $HOC_6H_4CO$ ] $^+$  (100.0), 107 [ $HOC_6H_4CH_2$ ] $^+$  (54.7). High resolution MS  $m/z$   $M^+$  298.1195 ( $C_{18}H_{18}O_4$ ) [ $\alpha$ ] $^+$  + 25.3 (MeOH,  $\epsilon$  0.06). CD  $\Delta\epsilon_{226} + 0.47$ ,  $\Delta\epsilon_{280} + 0.02$  (MeOH,  $\epsilon$  0.016).

**Daidzin (daidzein-7-O- $\beta$ -D-glucoside) (2).** Colorless needles.  $^1H$  NMR (360 MHz,  $CD_3CN$ , TMS)  $\delta$  3.34 (1H, m, H-4'), 3.43 (2H, m, H-2' and H-3'), 3.52 (1H, ddd,  $J_{4',5'} = 9.7$  Hz,  $J_{5',6'a} = 6.1$  Hz,  $J_{5',6'b} = 2.6$  Hz, H-5'), 3.61 (1H, dd,  $J_{6'a,6'b} = 12.0$  Hz,  $J_{5',6'a} = 6.1$  Hz, H-6'a), 3.80 (1H, dd,  $J_{6'a,6'b} = 12.0$  Hz,  $J_{5',6'b} = 2.6$  Hz, H-6'b), 5.07 (1H, d,  $J_{1,2'} = 7.5$  Hz, H-1'), 6.86 (2H, d,  $J_{2',3'} = J_{5',6'} = 8.7$  Hz, H-3' and H-5'), 7.11 (1H, dd,  $J_{5,6} = 8.4$  Hz,  $J_{6,8} = 2.3$  Hz, H-6), 7.13 (1H, d,  $J_{6,8} = 2.3$  Hz, H-8), 7.40 (2H, d,  $J_{2,3} = J_{5,6} = 8.7$  Hz, H-2' and H-6'), 8.08 (1H, s, H-2), 8.11 (1H, d,  $J_{5,6} = 8.4$  Hz, H-5). FDMS (70 eV)  $m/z$  (rel. int.) 439 [ $M + Na$ ] $^+$  (16.7), 416 [ $M$ ] $^+$  (90.0), 254 [ $M - glucosyl + H$ ] $^+$  (100.0), 163 [ $glucosyl$ ] $^+$  (10.0), 127 [ $C_6H_7O_3$ ] $^+$  (11.1).

**Acetylation of 2.** Compound 2 was acetylated with  $Ac_2O$  and pyridine to yield 4'-O-acetyldaidzein 7-O- $\beta$ -D-(2'',3'',4'',6'')-tetra-O-acetylglucoside (Ac-2). UV  $\lambda_{max}^{EtOH}$  nm 230, 251, 296, 304.  $^1H$  NMR, see Table 1.  $^{13}C$  NMR, see Table 2. EIMS (70 eV)  $m/z$  (rel. int.) 627 [ $M + H$ ] $^+$  (2.1), 584 [ $M - MeCO + H$ ] $^+$  (1.3), 331 [ $Ac_4glucosyl$ ] $^+$  (34.9), 297 [ $M + H - Ac_4glucosyl + H$ ] $^+$  (8.7), 271 [ $331 - HOAc$ ] $^+$  (8.7), 254 [ $297 - MeCO$ ] $^+$  (15.7), 211 [ $271 - HOAc$ ] $^+$  (4.4), 169 [ $211 - MeCO + H$ ] $^+$  (100.0), 109 [ $169 - HOAc$ ] $^+$  (47.4).

**Enzymatic hydrolysis of 2.** A mixture containing, in a final vol. of 5 ml, 500  $\mu$ mol acetate buffer, pH 4.7, 10  $\mu$ mol 2, and 25 units of  $\beta$ -glucosidase (emulsin) was incubated at 30 °C for 4 hr. The hydrolysate was extracted with EtOAc. The EtOAc extract was purified on a Si gel column eluted with *n*-hexane–EtOAc (1:3) to give daidzein (5). UV  $\lambda_{max}^{EtOH}$  nm 249, 258 (sh), 300, + NaOH 260, 291, 332. EIMS (70 eV)  $m/z$  (rel. int.) 254 [ $M$ ] $^+$  (100.0), 137 [ $M - 118 + H$ ] $^+$  (71.8), 118 [ $HC\equiv CC_6H_4OH$ ] $^+$  (40.2). The aq. layer in the EtOAc extraction of the hydrolysate was filtered through Sep-Pak  $C_{18}$  cartridge (Waters). The filtrate was analysed by HPLC on a MCI-CK08S column (Mitsubishi-Kasei, 4 mm i.d.  $\times$  25 cm,  $\times$  2) eluted with  $H_2O$  (flow rate 0.3 ml/min) at 65 °C. The eluate was monitored with a RI detector. Standard samples of D-glucose, D-galactose and D-mannose were eluted at 10.3, 11.1 and 11.4 min, respectively. The sugar component in the aq. layer had an identical  $R_f$  with that of D-glucose.

**Methylation and subsequent hydrolysis of 2.** After methylation of 2 with  $CH_2N_2$ , the resulting methyl ether was hydrolysed with  $\beta$ -glucosidase under similar conditions to those described above. The hydrolysate was extracted with EtOAc and purified on a Si gel column to yield formononetin (7-hydroxy-4'-methoxyisoflavone, 8). EIMS (70 eV)  $m/z$  (rel. int.) 268 [ $M$ ] $^+$  (100.0), 253 [ $M - Me$ ] $^+$  (14.5), 132 [ $HC\equiv CC_6H_4OMe$ ] $^+$  (76.8), 108 [ $M - 132 - CO$ ] $^+$  (32.2).

**Daidzein-7,4'-di-O- $\beta$ -D-glucoside (3).** Colorless needles. UV  $\lambda_{max}^{MeOH + H_2O}$  nm 231, 255, 294, 304 (sh), + NaOH 231, 254, 295, 304 (sh). IR  $\nu_{max}^{KBr}$   $cm^{-1}$  3420 (br, OH), 1620 (C=O of isoflavone). FDMS (70 eV)  $m/z$  (rel. int.) 601 [ $M + Na$ ] $^+$  (62.0), 439 [ $M - glucosyl + H + Na$ ] $^+$  (3.6), 108 [ $M - 2glucosyl + 2H - HC\equiv CC_6H_4OH - CO$ ] $^+$  (100.0).

**Acetylation of 3.** Compound 3 was acetylated with  $Ac_2O$  and pyridine to yield daidzein 7,4'-di-O- $\beta$ -D-(2'',3'',4'',6'')-tetra-O-acetylglucoside (Ac-3). UV  $\lambda_{max}^{CHCl_3}$  nm 258, 297, 306.  $^1H$  NMR, see Table 1.  $^{13}C$  NMR, see Table 2. FDMS (70 eV),  $m/z$  (rel. int.) 915 [ $M + H$ ] $^+$  (33.2), 583 [ $M - Ac_4glucosyl$ ] $^+$  (12.8), 331 [ $Ac_4glucosyl$ ] $^+$  (100.0), 252 [ $M - 2Ac_4glucosyl$ ] $^+$  (24.6).

**Enzymatic hydrolysis of 3.** Compound 3 was hydrolysed with  $\beta$ -glucosidase under similar conditions to those described above. The aglycone was identified as daidzein (5) by cochromatography with an authentic sample [Si gel TLC, *n*-hexane–EtOAc–HOAc

(1.301),  $R_f$  0.57] and by HPLC [Partisil-10 ODS-2, MeOH-H<sub>2</sub>O (4:1)] The sugar component was identified as D-glucose by HPLC (MCI-CK08S, H<sub>2</sub>O, 65°)

**2'-Hydroxydaidzein 7,4'-di-O- $\beta$ -D-glucoside (4)** Colorless needles UV  $\lambda_{\text{max}}^{\text{MeOH}+\text{H}_2\text{O}}$  nm 240, 247, 261(sh), 286, 304(sh), + NaOH 224, 266, 296, 303(sh) IR  $\nu_{\text{max}}^{\text{film}}$  cm<sup>-1</sup> 3360 (br, OH), 1620 (C=O of isoflavone) FDMS (70 eV)  $m/z$  (rel. int.) 617 [M + Na]<sup>+</sup> (100.0), 595 [M + H]<sup>+</sup> (2.0), 455 [M - glucosyl + H + Na]<sup>+</sup> (1.0)

**Acetylation of 4** Compound 4 was acetylated with Ac<sub>2</sub>O and pyridine to yield 2'-acetyoxydaidzein 7,4'-di-O- $\beta$ -D-(2'',3'',4'',6''-tetra-O-acetyl)glucoside (Ac-4) UV  $\lambda_{\text{max}}^{\text{CHCl}_3}$  nm 248, 295, 305 <sup>1</sup>H-NMR, see Table 1. <sup>13</sup>C-NMR, see Table 2. EIMS (70 eV)  $m/z$  (rel. int.) 972 [M]<sup>+</sup> (0.4), 929 [M - MeCO]<sup>+</sup> (8.6), 641 [M - Ac<sub>4</sub>glucosyl]<sup>+</sup> (5.6), 600 [M - MeCO + H - Ac<sub>4</sub>glucosyl + H]<sup>+</sup> (100.0), 331 [Ac<sub>4</sub>glucosyl]<sup>+</sup> (4800)

**Enzymatic hydrolysis of 4** Compound 4 was hydrolysed with  $\beta$ -glucosidase, the hydrolysate was extracted with EtOAc and purified on a Si gel column to yield 7,2',4'-trihydroxyisoflavone (9) UV  $\lambda_{\text{max}}^{\text{MeOH}}$  nm 240, 248, 261(sh), 289, 306(sh), + NaOH 258, 302, 334. EIMS (70 eV)  $m/z$  (rel. int.) 270 [M]<sup>+</sup> (100.0), 137 [M - 134 + H]<sup>+</sup> (61.2), 134 [HC $\equiv$ CC<sub>6</sub>H<sub>3</sub>(OH)<sub>2</sub>]<sup>+</sup> (32.0)

**Cultivation of intact plant and callus culture, and analysis of metabolites** *Vigna angularis* plants were grown for 20 days at 20–27° in a phytotron with 15-hr photoperiod. Callus of *Vigna angularis* was grown for 30 days at 25° under continuous light on Murashige-Skoog agar medium containing 3% sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l kinetin. Metabolites of both plant materials were extracted with MeOH and analysed by HPLC [Partisil-10 ODS-2, MeOH-H<sub>2</sub>O (4:1)] and TLC [Si gel, CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (6:4:1)]

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