Identification of a Naturally-occurring 8-[α-D-Glucopyranosyl-(1→6)-β-D-glucopyranosyl] daidzein from Cultivated Kudzu Root

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ABSTRACT:
Introduction – Kudzu root (Radix puerariae) is a rich source of isoflavones that are effective in preventing osteoporosis, heart disease and symptoms associated with menopause. The major isoflavonoids in kudzu root extracts were reported as puerarin, daidzin and daidzein. Recently, an unknown isoflavonoid (compound 1) was detected from one-year-old kudzu root cultivated in Vietnam.
Objective – To identify a novel compound 1 in kudzu root extract and determine the structure of the compound by ESI⁺ TOF MS-MS, ¹H-, ¹³C-NMR and enzymatic hydrolysis.
Methodology – Samples were prepared by extraction of one-year-old kudzu root with 50% ethanol and the isoflavonoids were purified using recycling preparative HPLC. Unknown compound 1 was detected using UV-light at 254 nm in TLC and HPLC analyses. The molecular weight of 1 was determined using a TOF mass spectrometer equipped with an electrospray ion source. The structure of 1 was determined from the ¹³C and ¹H NMR spectra recorded at 100.40 and 400.0 MHz, respectively.
Results – ESI⁺ TOF MS-MS analysis shows that 1 is a puerarin diglycoside. The interglycosidic linkage of diglycoside determined by ¹H-, ¹³C-NMR, and enzymatic hydrolysis suggests that 1 has a glucosyl residue linked to puerarin by an α-1,6-glycosidic bond. This compound is the first naturally-occurring 8-[α-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]daidzein in kudzu root. The concentration of glucosyl-α-1,6-puerarin in kudzu root was 2.3 mg/g as determined by HPLC.
Conclusion – The results indicate that puerarin diglycoside is one of the major isoflavonoids in kudzu root and has a significant impact on the preparation of highly water-soluble glycosylated puerarin. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: glucosyl-puerarin; puerarin; isoflavone; transglycosylation; Radix puerariae

Introduction

Kudzu (*Pueraria lobata*), a perennial leguminous plant native to many countries, is used in traditional Asian medicine. Recently, it has been shown to contain various isoflavones and their derivatives. Isoflavones are prescribed to prevent and treat osteoporosis (Mori *et al.*, 2004), heart disease (Powles, 2004), alcoholism (Benlhabib *et al.*, 2004a), prostate cancer (Messina and Wood, 2008) and symptoms associated with menopause (Sugihara and Watanabe, 2002). Isoflavones specifically inhibit the activity of various growth-regulating enzymes (Shertzer *et al.*, 1999), have inhibitory effects on cancer cell growth and act against non-hormone-related cancers, such as colon, rectum, lung and stomach cancers (Yanagihara, 1993; Li *et al.*, 1999; Guo *et al.*, 2004). A recent study on the metabolism of isoflavones by gut bacteria suggested that daidzein and genestein are converted via 5-hydroxyl-equol to equol (Matthies *et al.*, 2008). Owing to their structural similarity to mammalian oestrogen, isoflavones are effective against hormone-dependent diseases.

 Puerarin (daidzein 8-*C*-glucoside), daidzin (daidzein 7-*O*-glucoside) and daidzein are the major isoflavonoids of kudzu root extracts (Guo *et al.*, 2001; Kim *et al.*, 2003; Benlhabib *et al.*, 2004b;

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He *et al.*, 2004; Prasain *et al.*, 2004, 2007; Sibao *et al.*, 2007; Xu and He, 2007a, b). Even if the isoflavonoids have a glucose residue linked to the parent structure, their water solubility is very low due to their low hydrophilicity. Therefore, applications of the iso-flavonoids in the food and pharmaceutical industries are very limited. In our previous studies, transglycosylated puerarins, such as glucosyl-puerarin and maltosyl-puerarin, were synthesised *in vitro* using various enzymes (Li *et al.*, 2004a, b). Among the enzymes employed for transglycosylation, maltogenic amylase from *Bacillus stearothermophilus* (BSMA) was more effective than maltosyl transferase from *Thermotoga maritima* (TMMT; Li *et al.*, 2004a), 4-α-glucanotransferase from *Thermus scotoductus* (TSαGTase; Li *et al.*, 2004b) and cyclodextrin glucanotransferase from *Bacillus* sp. I-5 (BSCGTase; Li *et al.*, 2004b). The major transfer products of the BSMA reaction were identified as α-D-glycosyl-(1,6)-puerarin and α-D-maltosyl-(1,6)-puerarin. Overall, the trans-glycosylated puerarins, glucosyl-puerarin and maltosyl-puerarin, showed approximately 14 and 168 times higher relative solubility than puerarin, respectively (Li *et al.*, 2004b).

In the present study, an unknown puerarin derivative was identified as one of the two major components of kudzu root isoflavonoids and determined the structure of the novel compound, a puerarin diglycoside containing an interglycoside linked by an α-1,6-bond, was determined.

Experimental

Extraction of puerarin from kudzu root

The kudzu root (Radix puerariae) of 1-year-old Vietnamese *P. lobata* was harvested. The root was cleaned, peeled, steamed, sliced and dried in a dryer at 60°C to about 5% moisture content and then ground to powder. Ten grams of kudzu root powder and 100 mL of 50% ethanol were mixed and shaken (50 rpm) at room temperature for 4 h. The extract was filtered through a filter paper (Whatman, Maidstone, UK) and centrifuged (5000 rpm, 10 min) at room temperature. After the solution had been filtered through a 0.45 µm membrane (GyroDisc Syr. CA-PC 30 mm, Orange Scientific, Belgium), the solution was passed through a Sep-Pak C$_{18}$ cartridge (Waters, Milford, MA, USA) that had been previously activated using ethyl acetate, methanol, and water. Compound **1** was eluted from the Sep-Pak cartridge using methanol and was further purified using recycling preparative high-performance liquid chromatography (LC-918, JAI Co. Ltd, Tokyo, Japan) equipped with a JAI UV-detector 3702 and a polymeric gel filtration column (W-251). For HPLC, the mobile phase was 80% methanol at 2 mL/min, and the injection volume was 2 mL. The fraction containing **1** was collected and concentrated using a rotary vacuum evaporator. Purified compound **1** was analysed by thin-layer chromatography, MALDI-TOF MS, and nuclear magnetic resonance (NMR) spectroscopy. The samples were kept at −20°C until required for analysis. The isoflavonoid content was determined by HPLC using a calibration curve of the standards, puerarin and glucosyl-α-1,6-puerarin (Li *et al.*, 2004b).

Preparation of standard isoflavonoids

The puerarin diglycoside containing the α-1,6-linkage was prepared as described previously (Li *et al.*, 2004b); 1% puerarin and 4% maltotriose in 50 mM sodium citrate buffer (pH 6.0) were incubated with BSMA for 5 h at 55°C. The reaction mixture was purified further to obtain puerarin-α-1,6-glycoside by GPC (Bio-Gel P2 column, 20 × 1000 mm, Bio-Rad, Richmond, CA, USA) fractionation and freeze-drying. Puerarin di-and tri-glycosides containing the α-1,4 linked glycoside were prepared by incubating with 1% puerarin, 5% maltotriose and CGTase. The compounds were then purified by polymeric gel filtration (W-251) using recycling preparative HPLC as described previously (Li *et al.*, 2004b).

Thin-layer chromatography

The extract was spotted onto Whatman K5F silica gel plates that had been activated at 105°C for 10 min. The developing solution, *n*-butanol:acetic acid:water (5:3:1; v/v/v), was used for isoflavones and 1-propanol:ethyl acetate:water (3:1:1; v/v/v) for the analysis of carbohydrates. The developed TLC plate was dried completely by hot air. Compound **1** on the TLC plate was visualised using UV light (CAMAG Repro-star 3, Muttenz, Switzerland) at 254 nm. The carbohydrates were visualised by dipping the TLC plate into methanol solution containing 3 g of *N*-(1-naphthyl)-ethylenediamine and 50 mL of concentrated sulphuric acid per litre.

Analysis of isoflavonoids by HPLC

The extracted solution was diluted with double-distilled water and filtered through a 0.22 µm Millipore filter prior to chromatographic analysis. A Waters 600E HPLC system connected with a Nova-Pak C$_{18}$ column (150 × 3.9 mm i.d.) and a UV detector (SLC 200, Samsung, Seoul, Korea) set at 254 nm were used to quantify the amounts of isoflavonoids. The gradient mobile phases were composed of solvent A (water:formic acid, 100:0.1 v/v) and solvent B (HPLC-grade acetonitrile). The gradient started with 85% solvent A and 15% solvent B, and then solvent B was increased gradually from 15 to 70% over a period of 10 min. The flow rate was 1 mL/min. The concentration of each isoflavonoid in kudzu root was calculated from the standard curve of each pure compound in the range of 0.1–0.02 mg/mL (see Supporting information).

ESI$^+$ TOFMS-MS analysis

The molecular weights of the transfer products were determined using a TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source. Spectra were obtained in the positive-ion mode. Product ion spectra were gained by selecting protonated or deprotonated ions for collision (energy = 32eV) using argon as the collision gas (Prasain *et al.*, 2003).

NMR analysis

The ^{13}C NMR and ^{1}H NMR spectra were recorded at 100.40 and 400.0 MHz, respectively with an NMR spectrometer (LA-400 MHz; JEOL JNM, Tokyo, Japan). Samples were dissolved in D$_2$O at 24°C.

Results and Discussion

Analysis of isoflavonoids in kudzu root extracts by TLC

In order to analyse isoflavonoids in kudzu root, the sample was extracted with 50% ethanol and analysed by TLC (Fig. 1) and HPLC (Fig. 2). The root extract was resolved into two spots (Fig. 1, 1 and 2 in lane **a**) by TLC and was represented by one major peak [Fig. 2(d), peak 1], one intermediate peak [Fig. 2(d), peak 2] and one minor peak [Fig. 2(d), peak 6] in HPLC analysis. Comparing the mobility, the major peak was likely to be puerarin [Fig. 1, spots 2; Fig. 2(a), peak 1′]. One of the minor peaks [Fig. 2(b), peak 6] was eluted at the same time with the standard, daidzin. The interglycosidic linkage in **1** was deduced by TLC analysis: **1** had the same mobility as a glucosylated puerarin possessing an α-1,6 glucosidic linkage that was prepared as a standard reagent by BSMA transglycosylation reaction (Li *et al.*, 2004b). Compound **1** in spot **1** (Fig. 1, 1′ in lane **c**) had the same elution time as the standard compound, glucosyl-α-1,6-puerarin [Fig. 2(c), peak 1′]. Therefore, **1** was postulated as α-D-glucosyl-α-(1,6)-puerarin. The amount of **1** was 2.3 mg/g kudzu root (dry weight). Interestingly, the glucosyl puerarin containing α-1,4-glucosidic
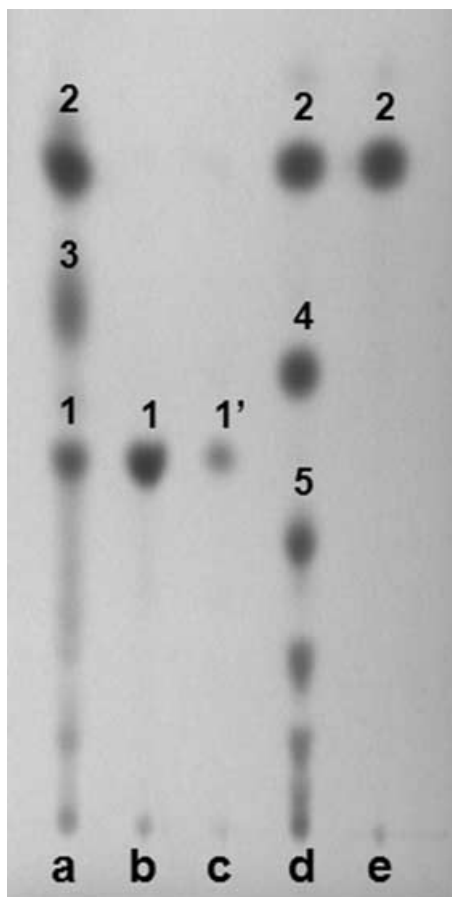
Figure 1. Thin-layer chromatography analysis of **1** isolated from kudzu root. The spots were visualised using UV at 254 nm: (lane a) kudzu root extract; (lane b) purified **1**; (lane c) glucosyl-α-1,6-puerarin standard; (lane d) puerarin transfer product by CGTase; (lane e) puerarin standard. 1, compound **1**; 1′, glucosyl-α-1,6-puerarin standard; 2, puerarin; 3, unknown component; 4, glucosyl-α-1,4-puerarin; 5, diglucosyl-α-1,4-puerarin.

linkages exclusively (Fig. 1, spots 4 and 5) showed mobility different from that of **1**, indicating that **1** does not possess an α-1,4-interglucosidic linkage.

Determination of molecular weight of compound 1 by ESI⁺TOFMS-MS

Molecular weight and fragmentation pattern of the purified **1** were determined by ESI⁺TOFMS-MS (Fig. 3). The molecular ion peak appearing at *m/z* 579.9 corresponded to the molecular mass of puerarin diglycoside, in which a glucose molecule is attached to puerarin. The peaks of **1** at *m/z* 417.1, 399.1, 381.2, 351.3 and 297.3 coincided with typical puerarin peaks (Prasain *et al.*, 2003) and the overall pattern of **1** was exactly the same as that of glucosyl-α-1,6-puerarin synthesised enzymatically (Li *et al.*, 2004b).

Enzymatic analysis of compound 1

The structure of **1** (Fig. 4) was investigated further using α- and β-glucosidase. β-Glucosidase specifically hydrolyses the β-

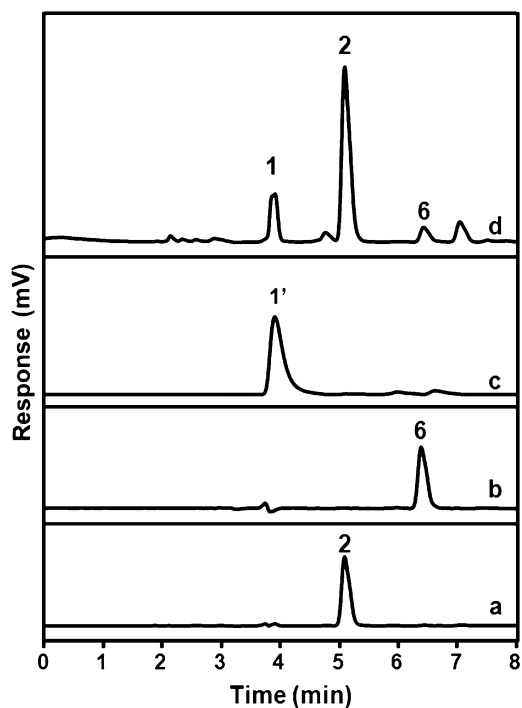**Figure 2.** HPLC chromatogram of analysed kudzu root extract. Chromatograms: a, standard puerarin; b, standard daidzin; c, standard glucosyl-α-1,6-puerarin; d, kudzu root extract. Peaks: 1, **1**; 2, puerarin; 6, daidzin.
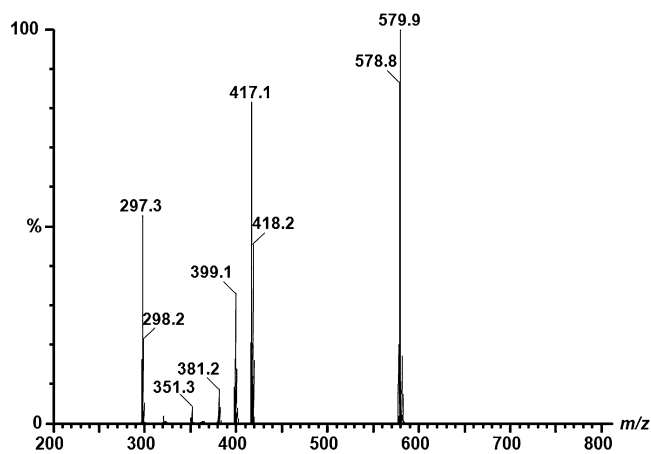

Figure 3. ESI⁺TOFMS-MS spectra of the compound **1** from kudzu root.

glucosyl linkage of glucans, but **1** was not hydrolysed by the enzyme [Figs 5(b) and 6(B)]. It appeared that **1** did not contain a β-glucosyl linkage, implying that the glucose moiety of diglycoside puerarin is not attached via β-1,4-linkage at the 4′-position of the puerarin aglycon as in puerarin 4′-O- β-glucoside (Xu and He, 2007b).

α-Glucosidases belong to a group of exo-type carbohydrases that release α-glucose from non-reducing sugars and glucans. These carbohydrases hydrolyse the α-1,6-glucosidic bond, albeit at a slower rate than the α-1,4-glucosidic bond. Compound
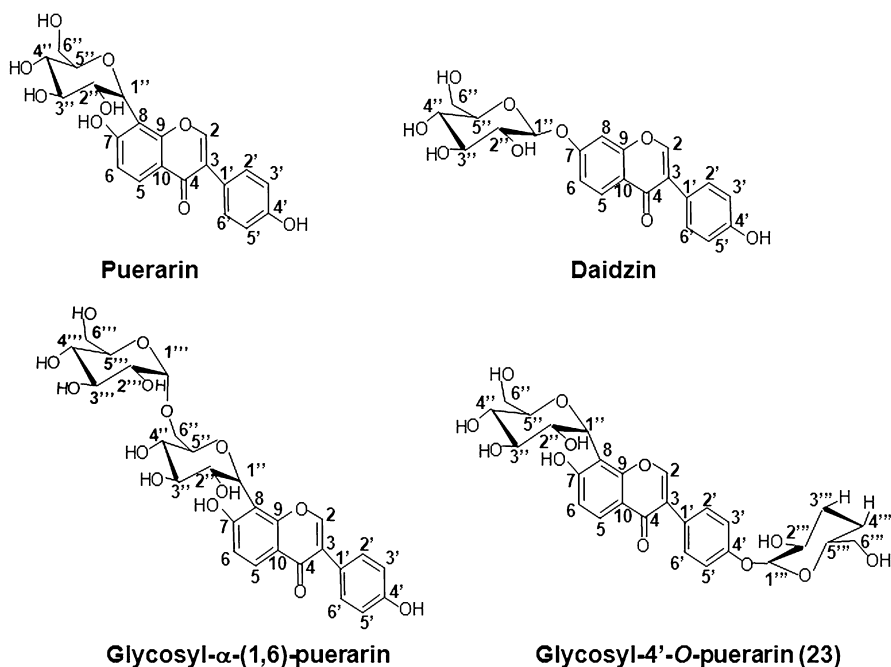
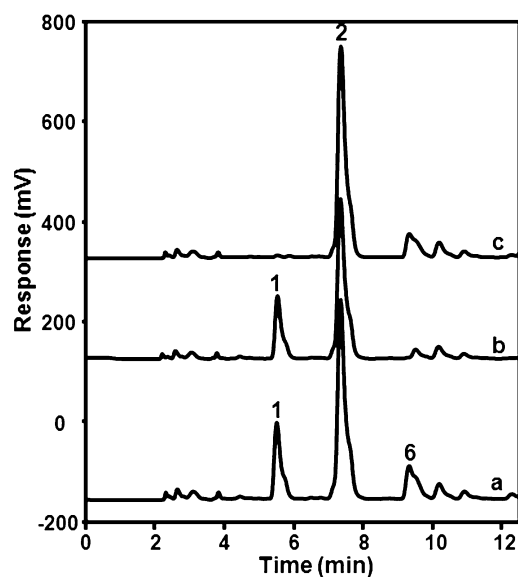**Figure 4.** Structure of puerarin and puerarin derivatives.

Figure 5. HPLC chromatogram of the enzymatically cleaved kudzu root extracts. Chromatograms: a, kudzu root extract; b, kudzu root extract hydrolysed by β-glucosidase; c, kudzu root extract hydrolysed by α-glucosidase. Peaks: 1, compound **1**; 2, puerarin; 6, daidzin.

1 was hydrolysed to puerarin and glucose by α-glucosidase [Figs 5(c) and 6(A)], indicating that **1** had a glucose molecule linked either by an α-(1,6)- or by an α-(1,4)-linkage to puerarin. However, the enzymatic hydrolysis patterns and the mobility on TLC indicated that **1** probably possesses a glucose linked by an α-(1,6) bond to the glucopyranose residue of puerarin. Furthermore, the hydrolysis product of **1** treated with α-glucosidase was analysed using high-performance anion exchange chromatography and the retention time of the hydrolysate

corresponded to glucose rather than other hexoses (see Supporting Information).

Determination of the interglucosidic linkage of compound 1 by NMR spectroscopy

The molecular structure of the interglucosidic linkage of **1** was identified using ^{13}C NMR spectroscopy. The chemical shifts in the ^{13}C NMR spectra were compared with those of puerarin standard. As shown in Table 1, a large downfield shift (bold figures in Table 1) was observed at C-6'' of the glucose moiety of puerarin from 61.0 to 66.6 ppm, implying that the glucose molecule was attached to C-6'' in the glucose moiety of puerarin. The ^{1}H NMR spectra of compound **1** is summarised together with ^{13}C NMR spectra in Table 1. Peak H-1'' was found at δ 4.91 with a J-value of 10.1, indicating that **1** has a β-configuration between C-1'' and C-8' linkage. The α-configuration of the additional glucose was confirmed by the ^{1}H NMR peak of H-1''' showing a J-value of 3.68 Hz at δ 4.76. Thus, **1** was found to be an 8-[α-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl]daidzein, the first to be found in nature. Prassain *et al.* (2003) identified novel naturally-occurring isoflavonoids by ESI-MS, and proposed the compound to be a puerarin-4'-O-glucoside, since ESI-MS analysis did not reveal the presence of an interglycosidic linkage. The discrepancy may be due to the different sample sources and cultivation years.

Our previous studies, in which glucosyl-α-1,6-puerarin was synthesised by the enzymatic transglycosylation reaction *in vitro*, indicated that glucosyl-α-1,6-puerarin was more water-soluble than puerarin (Li *et al.*, 2004b). Consequently, transglycosylated puerarin may be more applicable for cosmetic and pharmaceutical purposes. Given that a significant amount of glucosyl-α-1,6-puerarin was present in cultivated kudzu root, investigation of the biosynthetic pathway for glucosyl-α-1,6-puerarin in kudzu root would be very interesting. Studies
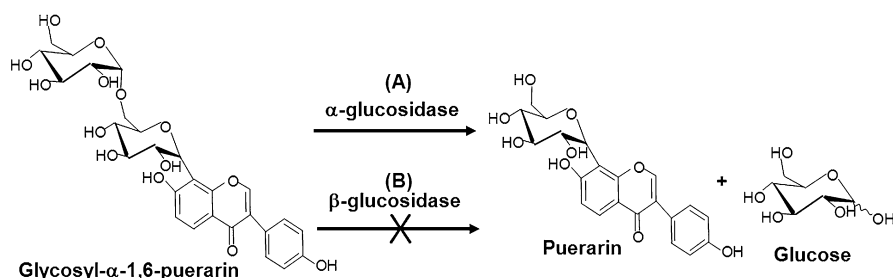
Figure 6. Summary of the reactions of α-glucosidase and β-glucosidase with isolated compound **1**. (A) Reaction of α-glucosidase with isolated **1**. (B) Reaction of β-glucosidase with isolated **1**.

Table 1. ^{1}H and ^{13}C NMR data for puerarin and glucosyl-puerarin isolated from cultivated kudzu root[a]

Ring	Carbon atoms	^{13}C NMR		^{1}H NMR
		Puerarin; δ	Glucosyl-α-(1-6)-puerarin; δ_1	Glucosyl-α-(1-6)-puerarin; δ_H, (J/Hz)[b]
Daidzein	2	154.3	154.4	7.87 (s)
	3	123.5	123.3	
	4	178.7	178.7	
	5	127.5	127.7	7.69 (d, 9.16)
	6	117.7	116.7	6.62 (d, 9.16)
	7	155.7	155.8	
	8	123.8	123.9	
	9	154.3	154.4	
	10	115.6	115.6	
	1'	123.5	123.3	
	2'	130.8	130.8	7.14 (d, 8.72)
	3'	115.6	115.6	6.75 (d, 8.72)
	4'	154.3	154.4	
	5'	115.6	115.6	6.75 (d, 8.72)
	6'	130.8	130.8	7.14 (d, 8.72)
β-D-glycosyl-(1–8)	1''	73.9	73.1	4.91 (d, 10.1)
	2''	70.9	71.5	4.28 (t, 9.16)
	3''	77.9	78.0	3.50–3.60 (m)
	4''	69.9	69.9	3.18 (m)
	5''	80.8	79.4	3.78 (m)
	6''	61.0	66.6	3.50–3.60 (m)
α-D-glycosyl-(1–6)	1'''		98.3	4.76 (d, 3.68)
	2'''		71.8	3.38–3.47 (m)
	3'''		73.1	3.50–3.60 (m)
	4'''		69.3	3.38–3.47 (m)
	5'''		73.1	3.50–3.60 (m)
	6'''		60.2	3.67 (m)

[a] Chemical shift were referred against TMS.
[b] s Singlet, d doublet, t triplet, m multiplet.

on the optimisation of cultivation conditions to maximise the yield of glycosyl puerarin should also be attempted when the biosynthesis pathway in the kudzu plant is understood.

Supporting information

Supporting information can be found in the online version of this article.

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