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Constituents of the Roots of *Pueraria lobata* Inhibit Formation of Advanced Glycation End Products (AGEs)

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Two isoflavone *C*-glucosides, puerarin (**1**) and PG-3 (**2**), a but-2-enolide, (±)-puerol B (**3**), two isoflavone *O*-glucosides, daidzin (**4**) and genistin (**5**), and three pterocarpans, (–)-medicarpin (**6**), (–)-glycinol (**7**) and (–)-tuberosin (**8**), were isolated from a MeOH extract of the roots of *Pueraria lobata*, using an *in vitro* bioassay based on the inhibition of the formation of advanced glycation end products (AGEs) to monitor chromatographic fractionation. The structures of **1-8** were determined by spectroscopic data interpretation, particularly by 1D- and 2D-NMR studies, and by comparison of these data with values in the literature. All of the isolates (**1-8**) were evaluated for their inhibitory activity on AGEs formation *in vitro*. Of these, puerarin (**1**), PG-3 (**2**), and (±)-puerol B (**3**) exhibited more potent inhibitory activity than the positive control aminoguanidine.

Key words: *Pueraria lobata*, Leguminosae, Puerarin, PG-3, (±)-Puerol B, Advanced glycation end products (AGEs), Diabetic complications

INTRODUCTION

Persistent hyperglycemia induces various abnormal biochemical changes, including increases in the formation of advanced glycation end products (AGEs) (Makita *et al.*, 1993), increase of polyol pathway activation (Shinohara *et al.*, 1998) and activation of protein kinase C isomers (Larkins and Dunlop, 1992). Ever-increasing evidence identifies the formation of AGEs as the major pathogenic link between hyperglycemia and the long term complications of diabetes, namely nephropathy, neuropathy and retinopathy (Bucala and Vlassara, 1995; Kalousova *et al.*, 2004). Nonenzymatic glycation occurs through a complex series of reactions between the free amino group in proteins and the carbonyl group of reducing sugars, which lead to the formation of fructosamines via a Schiff's base followed by Amadori rearrangement. Both the resulting Schiff's base and Amadori product undergo a further series of reactions through dicarbonyl intermediates to form AGEs (Pickup and Williams, 2003; Forbes *et al.*, 2003).

Thus, the discovery and design of inhibitors of the glycation cascade offer a promising therapeutic approach for the prevention of diabetic or other pathogenic complications (Forbes *et al.*, 2003). Although, the synthetic glycation inhibitor, aminoguanidine, attenuates the development of a range of diabetic vascular complications, some toxicity problems have been encountered in clinical trials with this drug (Kalousova *et al.*, 2004). On the other hand, some herbal extracts and natural products have proven to be somewhat effective for inhibiting AGEs formation (Matsuda *et al.*, 2003; Yokozawa *et al.*, 2001).

In our ongoing efforts to discover preventive agents for diabetic complications from herbal medicines (Kim *et al.*, 2002), we chose to investigate Puerariae Radix in more detail, as its MeOH extract showed a significant *in vitro* inhibitory effect on AGEs formation.

Puerariae Radix, the root of *Pueraria lobata* (Willd.) Ohwi [Leguminosae], is an important Oriental medicine that has been widely used as an antipyretic, migraine, and antispasmodic agent (Bae, 2000). The constituents and bioactive substances of Puerariae Radix have been studied extensively (Kinjo *et al.*, 1987; Ohshima *et al.*, 1988; Lee *et al.*, 1994). However, to date, the active components of

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Puerariae Radix that exhibit the inhibitory effect on AGEs formation have not been identified. In the present study, bioassay-guided fractionation of the MeOH extract of the roots of *Pueraria lobata*, using an *in vitro* AGEs inhibition assay, led to the purification of isoflavone *C*-glucosides (**1** and **2**) and a but-3-enolide (**3**) as the active constituents, along with five inactive compounds (**4-8**). The structure elucidation of the active compounds (**1-3**) and the biological evaluation of **1-8** are described herein.

MATERIALS AND METHODS

General experimental procedures

Melting points were measured on an IA9100 melting point apparatus (Barnstead International, U.S.A.) and were quoted uncorrected. Optical rotations were obtained using a digital polarimeter (Jasco, Japan) at 25°C. LRESI was recorded on a Mariner mass spectrometer (Perspective Biosystem, U.S.A.). NMR experiments were conducted on a DRX-300 or DRX-600 FT-NMR (Bruker, Germany), and the chemical shifts were referenced to the residual solvent signals. TLC analysis was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness); compounds were visualized by dipping plates into 10% (v/v) H_2SO_4 reagent (Aldrich) and then heat treating at 110°C for 5-10min. Silica gel (Merck 60A, 70-230 or 230-400 mesh ASTM), Sephadex LH-20 (Amersham Pharmacia Biotech), and reversed-phase silica gel (YMC Co., ODS-A 12 nm S-150, S-75 μm) were used for column chromatography. All solvents used for the chromatographic separations were distilled before use.

Plant materials

The roots of *Pueraria lobata* (Willd.) Ohwi [= *Pueraria thunbergiana* (Sieb. *et* Zucc.) Bentham; Leguminosae] were collected in Jeonmin-dong, Yuseong-gu, Daejeon, Korea, in March 2005 and identified by Prof. K. H. Bae. A voucher specimen (no. KIOM-K042) has been deposited at the Herbarium of the Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine, Korea.

Extraction and isolation

The dried plant material (4.9 kg) was extracted with 20 L of MeOH three times by maceration. The extracts were combined and concentrated *in vacuo* at 40°C to yield the MeOH extract (665 g). The MeOH extract showed a significant *in vitro* inhibitory effect on AGEs formation with an IC_{50} value of 13.4 μg/mL. Thus, the MeOH extract (300 g) was chromatographed over silica gel (ϕ 12×50 cm, 70-230 mesh) as the stationary phase with the following solvent system: $CHCl_3$/MeOH/H_2O (9:1:0.1 → 4:1:0.1 → 7:3:0.2 v/v, each 6 L) and MeOH (6 L) to afford 12 pooled fractions (F01-F012). Of these, fractions F02, F03, and F07 exhibited the most potent AGEs inhibitory activities. Based on this biological testing, fraction F02 [eluted with $CHCl_3$/MeOH/H_2O (9:1:0.1 v/v); 8.9 g] was chromatographed over silica gel (ϕ 7×36 cm, 230-400 mesh; $CHCl_3$-MeOH gradient from 1:0 to 0:1 v/v) resulting in ten subfractions (F0201-F0210). (–)-Medicarpin (**6**, 15 mg), (–)-tuberosin (**8**, 20 mg), and (±)-puerol B (**3**, 5 mg) were obtained from fraction F0202 (3.83 g), F0203 (1.23 g), and F0204 (690 mg), respectively, by repeated chromatography. Next, fraction F03 [eluted with $CHCl_3$/MeOH/H_2O (9:1:0.1 v/v); 1.14 g] was further fractionated through Sephadex column chromatography (ϕ 3.6×47 cm, $CHCl_3$–MeOH = 1:1 v/v) to afford six subfractions (F0301-F0306). Fraction F0304 (210 mg) was subjected to reversed-phase column chromatography (ϕ 3.6×35 cm, 12 nm S-150 μm; MeOH–H_2O = 7:3) to give additional (±)-puerol B (**3**, 13 mg) and (–)-glycinol (**7**, 15 mg). Fraction F07 [eluted with $CHCl_3$/MeOH/H_2O (5:4:1 v/v); 11.6 g] was chromatographed over silica gel (ϕ 7×45 cm, 230-400 mesh; $CHCl_3$/MeOH/H_2O = 6:2:0.5) resulting in seven subfractions (F0701-F0707). The three isoflavone glycosides, PG-3 (**2**, 30 mg), daidzin (**4**, 75 mg), and genistin (**5**, 40 mg), were obtained from fraction F0704 (1.58 g) by reversed-phase column chromatography (ϕ 3.6×40 cm, 12 nm S-150 μm; MeOH–H_2O = 4:6). Finally, fraction F0706 (3.14 g) was further fractionated through Sephadex LH-20 column chromatography (ϕ 3.6×47 cm, MeOH) to give puerarin (**1**, 900 mg).

Puerarin (1)

White powder: mp 198-200°C; $[\alpha]_D^{25}$ +9.8° (c 0.2, DMSO); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.28-4.05 (m, glucosyl-H), 4.82 (1H, d, *J* = 9.9 Hz, glucosyl H-1'), 6.80 (2H, d, *J* = 8.4 Hz, H-3'/H-5'), 6.99 (1H, d, *J* = 8.7 Hz, H-6), 7.39 (2H, d, *J* = 8.4 Hz, H-2'/H-6'), 7.94 (1H, d, *J* = 9.0 Hz, H-5), 8.34 (1H, s, H-2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 61.4 (glc-6), 70.5 (glc-4), 70.9 (glc-3), 73.4 (glc-2), 78.8 (glc-1), 81.8 (glc-5), 112.7 (C-8), 115.0 (C-6, C-3'/C-5'), 116.8 (C-10), 122.5 (C-1'), 123.1 (C-3), 126.2 (C-5), 130.2 (C-3'/C-5'), 152.6 (C-2), 157.1 (C-9), 157.2 (C-4'), 161.1 (C-7), 174.9 (C-4); LRESIMS *m/z*: 439 ([M+Na]$^+$), 417 ([M+H]$^+$).

PG-3 (3'-methoxypuerarin; 2)

White powder, mp 212-213°C; $[\alpha]_D^{25}$ +15.9° (c 0.1, DMSO); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.28-4.05 (m, glucosyl-H), 3.80 (3H, s, OCH_3-3), 4.82 (1H, d, *J* = 9.9 Hz, glucosyl H-1'), 6.81 (1H, d, *J* = 8.1 Hz, H-5'), 7.01 (1H, d, *J* = 8.7 Hz, H-6'), 7.04 (1H, dd, *J* = 8.1, 1.8 Hz, H-6), 7.17 (1H, d, *J* = 1.8 Hz, H-2'), 7.94 (1H, d, *J* = 9.0 Hz, H-5), 8.40 (1H, s, H-2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 55.7 (OCH_3-3'), 61.4 (glc-6), 70.5 (glc-4), 70.8 (glc-3), 73.5 (glc-2), 78.8 (glc-1), 81.9 (glc-5), 112.6 (C-8), 113.0 (C-2'), 113.1 (C-5'),

115.2 (C-6), 116.8 (C-10), 121.5 (C-6'), 122.9 (C-3), 123.0 (C-1'), 126.3 (C-5), 146.4 (C-3'), 147.2 (C-4'), 152.9 (C-2), 156.1 (C-9), 161.2 (C-7), 174.9 (C-4); LRESIMS *m/z*: 469 ([M+Na]$^+$), 447 ([M+H]$^+$).

(±)-Puerol B (3)

White powder, mp 270-271°C; $[\alpha]_D^{25}$ +0.1° (c 0.05, MeOH); ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 2.63 (1H, dd, *J* = 8.1, 1.8 Hz, H-6'), 7.04 (1H, dd, *J* = 8.1, 1.8 Hz, H-6'), 3.28-4.05 (m, glucosyl-H), 3.80 (3H, s, OCH_3-3'), 4.82 (1H, d, *J* = 9.9 Hz, glucosyl H-1'), 6.81 (1H, d, *J* = 8.1 Hz, H-5'), 7.01 (1H, d, *J* = 8.7 Hz, H-6), 7.04 (1H, dd, *J* = 8.1, 1.8 Hz, H-6'), 7.17 (1H, d, *J* = 1.8 Hz, H-2'), 7.94 (1H, d, *J* = 9.0 Hz, H-5), 8.40 (1H, s, H-2); ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 55.7 (OCH_3-3'), 61.4 (glc-6), 70.5 (glc-4), 70.8 (glc-3), 73.5 (glc-2), 78.8 (glc-1), 81.9 (glc-5), 112.6 (C-8), 113.0 (C-2'), 113.1 (C-5'), 115.2 (C-6), 116.8 (C-10), 121.5 (C-6'), 122.9 (C-3), 123.0 (C-1'), 126.3 (C-5), 146.4 (C-3'), 147.2 (C-4'), 152.9 (C-2), 156.1 (C-9), 161.2 (C-7), 174.9 (C-4); LRESIMS *m/z*: 335 ([M+Na]$^+$), 313 ([M+H]$^+$).

Determination of AGEs formation

According to the established method for measuring the formation of AGEs (Vinson and Howard, 1996), 10 mg/mL of bovine serum albumin (Sigma, St Louis, MO, U.S.A.) in 50 mM phosphate buffer (pH 7.4), with 0.02% sodium azide to prevent bacterial growth, was added to 0.2 M fructose and glucose. The reaction mixture was then mixed with the isolated compounds or aminoguanidine (Sigma, St Louis, MO, U.S.A.). After incubating at 37°C for 14 days, the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350 nm, Em: 450 nm). The AGEs assay was performed in quadruplicate. The concentration that resulted in 50% inhibition of the activity (IC_{50}) was estimated for each test sample from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

RESULTS AND DISCUSSION

Two isoflavone *C*-glucosides (**1** and **2**), a but-2-enolide (**3**), two isoflavone *O*-glucosides (**4** and **5**), and three pterocarpans (**6-8**), were isolated from a MeOH extract of the roots of *Pueraria lobata*. Chromatographic fractionation was monitored through an *in vitro* bioassay based on the inhibition of the formation of advanced glycation end products (AGEs). The structures of **4-8** were identified as daidzin (**4**) (Kinjo *et al.*, 1987), genistin (**5**) (Kinjo *et al.*, 1987), (–)-medicarpin (**6**) (Yoon *et al.*, 2004), (–)-glycinol (**7**) (Lyne and Mulheirn, 1978), and (–)-tuberosin (**8**) (Shirataki *et al.*, 1990), respectively, by measurement of physical and spectroscopic data (mp, MS, $[\alpha]_D$, ^{1}H- , ^{13}C-, and 2D-NMR) and by comparison with published values.

Compound **1** was obtained as a white powder and gave a protonated molecular ion at *m/z* 417 [M+H]$^+$ by ESIMS, consistent with an elemental formula of $C_{21}H_{20}O_9$. The ^{1}H-NMR spectrum of **1** showed a resonance for an isoflavone skeleton that exhibited a diagnostic vinylic singlet at δ 8.34 (H-2). Two *ortho*-coupled doublets centered at δ 6.80 (2H, *J* = 8.4 Hz) and δ 7.39 (2H, *J* = 8.4 Hz) were assigned to the protons of a *para*-di-substituted benzene ring (B ring). The doublet resonances at δ 6.99 (1H, *J* = 8.7 Hz, H-6) and δ 7.94 (1H, *J* = 9.0 Hz, H-5) were also observed in the ^{1}H-NMR spectrum of **1**. A β-anomeric doublet at δ 4.82 (*J* = 9.9, glc-1') and other multiplet resonances at δ 3.28~4.05 (glucosyl-H) in the ^{1}H-NMR spectrum implied that **1** is a daidzein *C*-glycoside. By means of ^{13}C-NMR data interpretation, the sugar attached to **1** was proved to be glucopyranoside. Careful analysis of the ^{1}H- and ^{13}C-NMR spectra and comparison of the data with those in the literature (Lee *et al.*, 1994) led to identification of **1** as puerarin.

Compound **2** was obtained as a white powder and gave a protonated molecular ion at *m/z* 447 [M+H]$^+$ by ESIMS, consistent with an elemental formula of $C_{22}H_{22}O_{10}$. The ^{1}H-NMR spectrum of **2** also displayed a resonance for an isoflavone skeleton that exhibited a diagnostic vinylic singlet at δ 8.40 (H-2). A set of ABX-type signals [δ 7.17 (1H, d, *J* = 1.8 Hz, H-2'), 6.81 (1H, d, *J* = 8.1 Hz, H-5'), and 7.04 (1H, dd, *J* = 8.1, 1.8 Hz, H-6')], two doublet signals [δ 7.01 (1H, *J* = 8.7 Hz, H-6) and δ 7.94 (1H, *J* = 9.0 Hz, H-5)], a methoxyl signal at δ 3.80 (3H, s), a β-anomeric doublet at δ 4.82 (*J* = 9.9, glc-1') and other multiplet resonances at δ 3.28~4.05 (glucosyl-H) indicated that **2** is also a daidzein *C*-glycoside with a tri-substituted benzene ring (B-ring). Thus, compound **2** was inferred to be PG-3 (3-methoxypuerarin); this was confirmed by

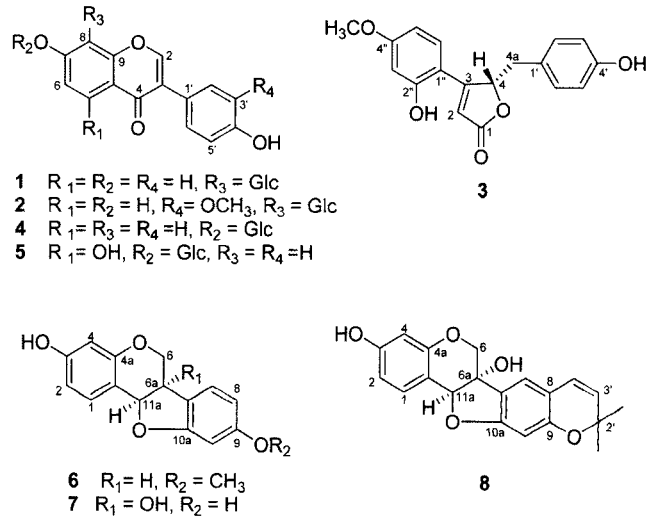

Fig. 1. Structures of compounds **1-8** isolated from *P. lobata*

comparison of its spectral data with those reported in the literature (Lee *et al.*, 1994).

Compound **3** was obtained as a white powder and gave a protonated molecular ion at *m/z* 313 $[M+H]^+$ by ESIMS, consistent with an elemental formula of $C_{18}H_{16}O_5$. The ^{1}H-NMR spectrum of **3** showed a set of ABX-type signals [δ 6.54 (1H, d, *J* = 2.4 Hz, H-3"), 7.46 (1H, d, *J* = 9.0 Hz, H-5"), and 6.55 (1H, dd, *J* = 9.0, 2.4 Hz, H-6")], a set of ABMX-type signals [δ 3.14 (1H, dd, *J* = 14.7, 3.2 Hz, H-4a), 2.63 (1H, dd, *J* = 14.7, 6.6 Hz, H'-4a), 5.88 (1H, ddd, *J* = 6.6, 3,3, 1.2 Hz, H-4), and 6.26 (1H, d, *J* = 1.2 Hz, H-2)], two *ortho*-coupled doublets signals [δ 6.80 (2H, *J* = 8.4 Hz) and 7.39 (2H, *J* = 8.4 Hz)], and an aromatic methoxyl signal at δ 3.80 (3H, s). The ^{13}C-NMR spectrum of **3** exhibited a carbonyl carbon (δ 172.9), an olefinic carbon (δ 112.3), an aliphatic tertiary carbon (δ 82.8) bearing an oxygen atom, an aliphatic secondary carbon (δ 38.4), and a methoxyl group (δ 55.2) in addition to two benzene ring units. Based on the above observations, compound **3** is predicted to be composed of a 4-methylene-but-2-en-4-olide moiety, a *para*-di-subsituted benzene ring, a tri-substituted benzene ring, and a methoxyl group. The linkage of the 4-methylene-but-2-en-4-olide moiety and two benzene rings and the position of the methoxyl group were deduced by NOESY NMR experiments. By analysis of these results and by comparison with those in the literature (Nohara *et al.*, 1993), compound **3** was identified as (±)-puerol B [3-(2-hydroxy-3-methoxyl phenyl)-4-(4-hydroxybenzyl)but-2-en-4-olide]. However, the stereochemistry at C-4 of **3** could not be determined, because it was isolated as a racemic mixture {$[\alpha]_D^{25}$ +0.1° (c 0.05, MeOH)} {lit. for (+)-puerol B $[\alpha]_D^{25}$ +81.2° (c 0.38, acetone); lit. for (–)-puerol B $[\alpha]_D^{25}$ -76.2° (c 0.50, acetone); Nohara *et al.*, 1993}.

Table I. The inhibitory effects of compounds **1-8** from *P. lobata* on AGEs formation

Compound	Conc. (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)[a]
1	2.5	18.2±2.8	8.7
	5	27.5±5.1	
	10	57.8±1.7	
2	2.5	5.5±3.4	24.9
	10	21.5±3.5	
	25	50.0±2.0	
3	10	14.0±3.5	28.6
	25	49.6±3.2	
	50	85.3±0.4	
AG[b]	37	27.4±1.5	71.1
	55.5	40.0±0.9	
	74	51.8±1.9	

[a] The concentration required for a 50% inhibition (IC_{50}) of the intensity of fluorescence relative to the negative control. IC_{50} values were calculated from the dose inhibition curve. Inhibitory effect was expressed as mean±S.D. of quadruplicate experiments. Compounds **4-8** were inactive (IC_{50} > 100 μg/mL) in this assay system.

[b] Aminoguanidine (AG) was used as a positive control.

The inhibitory effects of compounds **1-8**, which were isolated from the roots of *Pueraria lobata*, on AGEs formation is summarized in Table I. In a recent investigation (Matsuda *et al.*, 2003), some flavonoid derivatives were found to exhibit significant *in vitro* inhibitory activity against AGEs formation, while isoflavones, including puerarin, did not inhibit AGEs formation after incubation for 2 days at 60°C at a higher glucose concentration. However, in the present study, the isoflavone *C*-glucosides, puerarin (**1**) and PG-3 (**2**), and the but-2-enolide, (±)-puerol B (**3**) (IC_{50} values: 8.7, 24.9, and 28.6 μg/mL, respectively) showed more potent *in vitro* inhibitory activity against AGEs formation than the positive control, aminoguanidine (IC_{50} value of 71.1 μg/mL); these compounds were incubated for 14 days at 37°C at a lower glucose concentration to more closely replicate *in vivo* conditions. Puerarin (**1**) is a major component of Puerariae Radix, and in fact, is considered the marker substance of this root. Furthermore, in a recent clinical study, it was shown to play a positive therapeutic role in diabetic retinopathy (Ren *et al.*, 2000). Therefore, puerarin (**1**) is worthy of consideration as a therapeutic agent for diabetic complications or related diseases following additional biological evaluation.

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