Puerariafuran, a New Inhibitor of Advanced Glycation End Products (AGEs) Isolated from the Roots of *Pueraria lobata*

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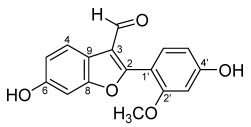
A new 2-arylbenzofuran, puerariafuran (1), as well as three known compounds, coumestrol (2), daidzein (3), and genistein (4), were isolated from a MeOH extract of the roots of *Pueraria lobata* as active constituents, using an *in vitro* bioassay based on the inhibition of advanced glycation end products (AGEs) to monitor chromatographic fractionation. The structure of 1 was determined by spectroscopic data interpretation, particularly by extensive 1D and 2D NMR studies. All the isolates (1—4) were evaluated for the inhibitory activity on AGEs formation *in vitro*.

Key words puerariafuran; coumestrol; advanced glycation end product (AGE); *Pueraria lobata*; Leguminosae

Persistent hyperglycemia induces abnormal changes such as increase of advanced glycation end-products (AGEs) formation,[1)] increase of polyol pathway flux,[2)] and over-activation of protein kinase C isoforms.[3)] Among them, enhanced formation and accumulation of AGEs have been implicated as a major pathogenesis process in the development of diabetes related complications and health complications associated with the normal aging process.[4,5)] Thus, the design and discovery of inhibitors of the glycation cascade can offer a promising therapeutic approach for the prevention of diabetic or other pathogenic complications.[1,6,7)] In our ongoing project directed toward the discovery of preventive agents for diabetic complications from the herbal medicines,[8)] the roots of *Pueraria lobata* were chosen for more detailed investigation, since a MeOH extract showed a significant *in vitro* inhibitory effect on AGEs formation.

Pueraria lobata (WILLD.) OHWI [Leguminosae] is a deciduous woody vine and is widely distributed in temperate regions of Far Eastern Asia including Korea, Japan, Taiwan, NE China, and FE Russia. Traditionally this species has been used for medicinal plants in Korea, China, and Japan according to the parts such as seeds (Semen Puerariae), roots (Radix Puerariae), and flowers (Flos Puerariae).[9)] The roots of *P. lobata* have been widely used as an antipyretic, migraine, and antispasmodic agent and studied extensively on the constituents and bioactive substances.[10—13)] However, the active principles in the roots of *P. lobata* that exhibit the inhibitory effect on AGEs formation have not been identified up to date. In the present study, bioassay-guided fractionation of the MeOH extract of the roots of *P. lobata*, using an *in vitro* AGEs inhibition assay, led to the purification of a new 2-arylbenzofuran derivative (**1**) and three known compounds (**2**—**4**) as the active constituents. The structure elucidation of **1** and the biological evaluation of **1**—**4** are described herein.

Compound **1** was obtained as a pale yellowish powder and produced a molecular ion $[M+Na]^+$ at *m/z* 307.0577 by HR-ESI-MS, consistent with an elemental formula of $C_{16}H_{12}O_5Na$. The presence of a CHO group was demonstrated by means of IR (1638 cm^{-1}), ^{1}H-NMR (δ 9.89), and ^{13}C-NMR (δ 187.1). The ^{1}H-NMR spectrum of **1** showed resonances for two sets of ABX-type signals [δ 7.83 (1H, d, J=8.5 Hz), 6.99 (1H, d, J=2.0 Hz), 6.85 (1H, dd, J=8.5, 2.0 Hz); δ 7.46 (1H, d, J=8.0 Hz), 6.61 (1H, d, J=2.0 Hz), 6.55 (1H, dd, J=8.0, 2.0 Hz)], indicating that compound **1** has two 1,2,4-trisubstituted benzene moieties and one methoxyl group [δ 3.77 (3H, s)]. The ^{13}C- and DEPT-NMR spectra (see Experimental) of compound **1** showed 15 carbon signals including a relatively more intense signal at δ 116.5. With this observation and the molecular formula ($C_{16}H_{12}O_5$) of **1** obtained by HR-MS data, it was suggested that two aromatic quaternary carbons (C-3, C-9) have the same chemical shifts and their carbon signals were overlapped at δ 116.5 in the ^{13}C-NMR spectrum of **1**. On the basis of the above results and by the careful analysis of the ^{1}H–^{1}H correlation



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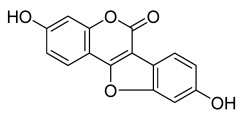

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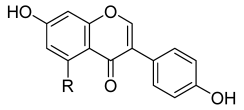

3 R = H
4 R = OH

Fig. 1. Structures of Compounds **1**—**4** Isolated from *P. lobata*

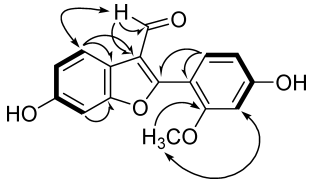

Fig. 2. Selected Correlations Observed in the COSY (—), HMBC (→), and NOESY (↔) NMR Spectra of **1**

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Table 1. The Inhibitory Effects of Compounds **1**—**4** from *P. lobata* on AGEs Formation

Compound	Inhibitory effect (IC_{50} value)[a]	
	μg/ml	μM
1	0.15	0.53
2	0.05	0.19
3	12.0	47.2
4	70.1	260
AG[b]	35.0	472

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

spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) NMR data of **1** (Fig. 2), it was proposed that compound **1** is a 2-aryl-3-formylbenzofuran derivative. Comparison of the ^{13}C-NMR spectrum of **1** with that of eryvarin P[14] revealed that **1** has the same 3-formylbenzofuran portion as eryvarin P but differs in substitution pattern of the B ring. Compound **1** displayed a 1,2,4-trisubstituted benzene ring bearing two oxygen atoms [δ_C 161.7 (C-4′), 158.6 (C-2′), 132.7 (C-6′), 107.8 (C-1′), 108.0 (C-5′), and 99.7 (C-3′)] in the ^{13}C-NMR spectrum whereas eryvarin P has a 1,2,4,5-tetrasubstituted benzene ring bearing three oxygen atoms [δ_C 153.0 (C-2′), 149.5 (C-4′), 141.1 (C-5′), 113.6 (C-6′), 108.8 (C-1′), 100.2 (C-3′)]. The positions of the formyl (C-3), methoxyl (C-2′), and hydroxyl groups (C-6, C-4′) were determined unambiguously by HMBC and nuclear Overhauser and exchange spectroscopy (NOESY) NMR experiments (Fig. 2). Therefore, the structure of this new arylbenzofuran, puerariafuran (**1**), was elucidated as 3-formyl-2-(4-hydroxy-2-methoxyphenyl)-6-hydroxybenzofuran. Puerariafuran (**1**) is a very rare 2-arylbenzofuran possessing a formyl group in plant kingdom.[14,15] To the best of our knowledge, this is the first isolation of a 2-aryl-3-formylbenzofuran from the genus *Pueraria*.

The known compounds were identified as coumestrol (**2**),[10] daidzein (**3**),[10] and genistein (**4**)[10] by physical and spectroscopic data (mp, ^{1}H-, ^{13}C-NMR, and MS) measurement and by comparison with published values.

All the isolates **1**—**4** were subjected to an *in vitro* bioassay to evaluate AGEs inhibitory activity (Table 1). The new compound **1** and coumestrol (**2**) exhibited a superior inhibitory activity against AGEs formation with IC_{50} values of 0.53 and 0.19 μM, respectively, compared to a well known positive control, aminoguanidine (IC_{50} value of 473 μM). While daidzein (**3**) and genistein (**4**) showed a significant inhibitory activity against AGEs formation with IC_{50} values of 47.2 and 260 μM, respectively. However, further investigations are needed to discuss their structure–activity relationship because of the limited number of compounds. Although a number of natural inhibitors of AGEs formation have been reported up to date,[16] compounds with very potent activity are rare. Thus, puerariafuran (**1**) and coumestrol (**2**) seem worthy of consideration as potential therapeutic agents for diabetic complications and related diseases though additional biological evaluation.

Experimental

General Experimental Procedures Melting points were measured on an IA9100 melting point apparatus (Barnstead International, U.S.A.) and were quoted uncorrected. IR spectra were recorded using a FTS 165 FT-IR spectrophotometer (Bio-Rad, CA, U.S.A.), respectively. LR- and HR-ESI-MS were recorded on a Mariner mass spectrometer (Perspective Biosystem, U.S.A.), respectively. NMR experiments were conducted on a DRX-300 or AVANCE 500 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 treated at 110 °C for 5—10 min. 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 μm) were used for column chromatography. All solvents used for the chromatographic separations were distilled before use.

Plant Material The roots of *Pueraria lobata* (Willd.) Ohwi [=*Pueraria thunbergiana* (Sieb. *et* Zucc.) Bentham; Leguminosae] were collected in Jeonmin-dong, Yuseong-gu, Daejeon, Korea, in April 2005 and were identified by one of us, Professor J.-H. Kim. A voucher specimen (no. KIOM-P041) has been deposited at the Herbarium of 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 give a MeOH extract (665 g). The MeOH extract showed a significant *in vitro* inhibitory effect on AGEs formation with 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 stationary phase using a solvent system [$CHCl_3$/MeOH/H_2O (9 : 1 : 0.1→4 : 1 : 0.1→7 : 3 : 0.2 v/v, each 6 l), MeOH (6 l)] to afford 12 pooled fractions (F01—F012). Of these, fractions F02 and F03 showed the most potent AGEs inhibitory activities (both IC_{50}<5 μg/ml). Based on the above 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). Daidzein (**3**, 24 mg) and the new compound **1** (20 mg) were obtained from fraction F0204 (690 mg) and F0205 (270 mg) 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 coumestrol (**2**, 18 mg) and genistein (**4**, 35 mg).

Puerariafuran (**1**): Pale yellowish powder; mp 294—296 °C (dec.); IR (KBr) cm^{-1}: 3368, 3086, 2950, 2928, 1638, 1611, 1590, 1446, 1376, 1306, 1127, 1061, 960, 896; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 9.89 (1H, s, 3-C<u>H</u>O), 7.83 (1H, d, J=8.5 Hz, H-4), 7.46 (1H, d, J=8.0 Hz, H-6′), 6.99 (1H, d, J=2.0 Hz, H-7), 6.85 (1H, dd, J=8.5, 2.0 Hz, H-5), 6.61 (1H, d, J=2.0 Hz, H-3′), 6.55 (1H, dd, J=8.0, 2.0 Hz, H-5′), 3.77 (3H, s, 2′-OC<u>H</u>$_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 187.1 (<u>C</u>HO), 162.3 (C-2), 161.7 (C-4′), 158.6 (C-2′), 156.3 (C-6), 154.9 (C-8), 132.7 (C-6′), 121.6 (C-4), 116.5 (C-3/C-9), 113.6 (C-5), 107.8 (C-1′), 108.0 (C-5′), 99.7 (C-3′), 97.7 (C-7), 55.9 (OCH_3); LR-ESI-MS *m/z* (rel. int.): 591 ([2M+Na]$^+$), 307 ([M+Na]$^+$), 285 ([M+H]$^+$), 149; HR-ESI-MS *m/z*: 307.0577 ([M+Na]$^+$, Calcd for $C_{16}H_{12}O_5Na$: 307.0585).

Determination of AGEs Formation *in Vitro* According to the method of Vinson and Howard,[17] the reaction mixture, 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 compounds. After incubating at 37 °C for 7 d, the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350, Em: 450 nm). Measurements were performed in quadruplicate, and the concentration required for a 50% inhibition (IC_{50}) of the intensity of fluorescence was determined graphically. Aminoguanidine (Sigma, St. Louis, MO, U.S.A.) was used as a reference compound.

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