Flavan-3-ols Having a γ-Lactam from the Roots of *Actinidia arguta* Inhibit the Formation of Advanced Glycation End Products *in Vitro*

Dae Sik Jang,[a] Ga Young Lee,[a] Yun Mi Lee,[a] Young Sook Kim,[a] Hang Sun,[b] Dong-Hee Kim,[c] and Jin Sook Kim*,[a]

[a] *Department of Herbal Pharmaceutical Development, Korea Institute of Oriental Medicine; Daejeon 305–811, Korea:* [b] *Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences; Kunming, Yunnan 650204, Peoples Republic of China: and* [c] *College of Oriental Medicine, Daejeon University; Daejeon 300–716, Korea.* Received October 13, 2008; accepted January 5, 2009; published online January 6, 2009

Two new flavan-3-ols, 6-(2-pyrrolidinone-5-yl)-(−)-epicatechin (1) and 8-(2-pyrrolidinone-5-yl)-(−)-epicatechin (2), as well as five known compounds, (−)-epicatechin (3), (+)-catechin (4), proanthocyanidin B-4 (5), (+)-pinoresinol, and *p*-hydroxybenzoic acid, were isolated from an EtOAc-soluble extract of the roots of *Actinidia arguta*. The structures of compounds 1 and 2 were elucidated by spectroscopic data interpretation, particularly by extensive 1D- and 2D-NMR studies. All the isolates were evaluated *in vitro* for inhibitory activity on the formation of advanced glycation end products (AGEs). Of these, compounds 1—5 exhibited significant inhibitory activity against AGEs formation with IC_{50} values ranging from 10.1 to 125.2 μM.

Key words *Actinidia arguta*; Actinidiaceae; flavan-3-ol; advanced glycation end product; diabetic complication

Hyperglycaemia has an important role in the pathogenesis of diabetic complications by increasing protein glycation and the gradual build-up of advanced glycation end products (AGEs) in body tissues.[1)] The formation of AGEs progressively increases with normal aging, even in the absence of disease. However, they are formed at accelerated rates in diabetes.[2)] AGEs are not only markers but also important causative factors for the pathogenesis of diabetes,[1)] cataracts,[3)] atherosclerosis,[4)] diabetic nephropathy,[5)] and neurodegenerative diseases, including Alzheimer's disease.[6)] Thus, the design and discovery of inhibitors of AGEs formation can offer a promising therapeutic approach for the prevention of diabetic or other pathogenic complications. Aminoguanidine, a hydrazine-like small molecule, is the first AGEs inhibitor explored in clinical trials. However, the drug was not ultimately approved for commercial production because side effects were observed in phase III clinical trials in patients with diabetes.[7)]

In our ongoing project directed toward the discovery of preventive agents for diabetic complications from the herbal medicines,[8)] the roots of *Actinidia arguta* were chosen for more detailed investigation, since its EtOAc-soluble extract showed a significant *in vitro* inhibitory effect on the formation of AGEs. *Actinidia arguta* Planchon (Actinidiaceae) is a smooth-skinned grape-sized kiwifruit native to northern China, Korea, Siberia, and Japan.[9)] This species has a long history of human consumption, and have been used in traditional Chinese medicine to improve general health.[10)] Previous phytochemical investigations on *A. arguta* have resulted in the isolation of flavonoids,[11)] phenolic compounds,[12)] triterpenes,[13)] and lignans.[13)] In the present study, repeated chromatography of the EtOAc-soluble extract of the roots of *A. arguta*, led to the purification of two new flavan-3-ols (**1**, **2**) as well as five known compounds which were subjected to an *in vitro* bioassay to evaluate their inhibitory activities on the formation of AGEs. The structure elucidation of **1** and **2** and the biological evaluation of the isolates are described herein.

Compound **1** was obtained as a 5 : 3 mixture of two diastereomers and produced a molecular ion $[M-H]^+$ at *m/z* 372.1085 by high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), which is consistent with an elemental formula $C_{19}H_{19}NO_7$. Its IR (KBr) spectrum showed hydroxyl (3340 cm^{-1}), amide CO (1644 cm^{-1}), and aromatic ring (1513 cm^{-1}) absorptions.[14)] The ^1H- and ^{13}C-NMR spectra of the major isomer of **1** (Table 1) were very similar to those of (−)-epicatechin (**3**), except for the disappearance of a ^1H-signal due to one of the ring A protons and the appearance of a methine (C-1″), two methylenes (C-2″, C-3″), and an amide signals (C-4″). The ^1H–^1H correlative spectroscopy (COSY) correlation of these methine and methylenes revealed the presence of a partial structure of $–CH_2–CH_2–CH–$. The methine was shown to be attached to a nitrogen atom, based on the ^1H- and ^{13}C-NMR chemical shift (δ_H 5.38, δ_C 50.89), suggesting that **1** may be an epicatechin derivative possessing a 2-pyrrolidinone-5-yl group at the 6- or 8-position. The chemical shifts of the 2-pyrrolidinone-5-yl group in **1** were consistent with those of davalliosides, flavan-3-ols with pyrrolidin-2-one from *Davallia mariesii*,[14)] and dracocephins, flavanones with pyrrolidin-2-one from *Dracocephalum rupestre*,[15)] strongly supporting the suggestion. The three-bond heteronuclear multiple bond connectivity

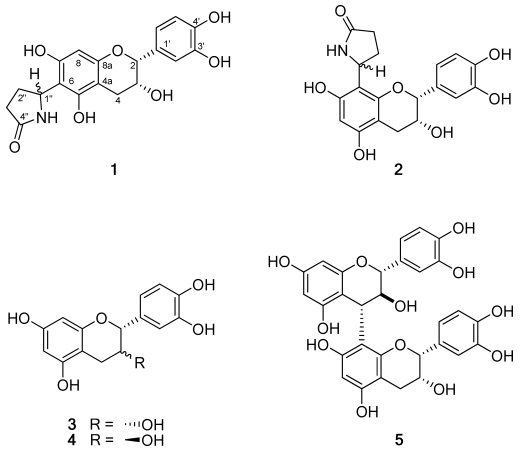

Fig. 1. Structures of **1**—**5** Isolated from the Roots of *A. arguta*

* To whom correspondence should be addressed. e-mail: jskim@kiom.re.kr

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1**—**3** (in CD_3OD)[a)]

Position	**1** (Major)		**2** (Major)		**3**	
	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)	δ_C
2	4.81[b)]	79.79 d	4.84 d (3.0)	80.24 d	4.82[b)]	80.00 d
3	4.20 br t (3.0)	67.43 d	4.16 br td (3.0)	67.06 d	4.18 ddd (4.2, 3.0, 1.2)	67.62 d
4	2.88 dd (16.5, 4.5)	29.65 t	2.88 dd (16.8, 4.5)	29.56 t	2.88 dd (16.8, 4.5)	29.39 t
	2.69 dd (16.5, 3.0)		2.76 dd (16.8, 3.0)		2.76 dd (16.8, 3.0)	
4a	—	100.87 s	—	100.21 s	—	100.22 s
5	—	156.17 s	—	157.37 s	—	158.13 s
6	—	110.22 s	6.01 s	96.62 d	5.92 d (2.1)	96.04 d
7	—	156.60 s	—	156.43 s	—	157.80 s
8	6.01 s	97.00 d	—	107.90 s	5.95 d (2.1)	96.55 d
8a	—	156.37 s	—	155.64 s	—	157.51 s
1′	—	132.20 s	—	132.33 s	—	132.43 s
2′	6.97 d (1.8)	115.48 d	6.98 d (1.5)	115.53 d	6.98 d (1.8)	115.46 d
3′	—	145.99 s	—	146.03 s	—	145.91 s
4′	—	146.12 s	—	146.12 s	—	146.07 s
5′	6.76 d (8.1)	116.06 d	6.77 d (8.1)	116.14 d	6.77 d (8.1)	116.04 d
6′	6.80 dd (8.4, 1.8)	119.54 d	6.81 dd (8.4, 1.8)	119.67 d	6.81 dd (8.4, 1.8)	119.55 d
1″	5.38 dd (9.0, 5.1)	50.89 d	5.42 dd (8.1, 5.7)	50.50 d	—	—
2″	2.39 m	27.34 t	2.37 m	26.82 t	—	—
	2.34 m		2.33 m			
3″	2.47 m	32.44 t	2.47 m	32.29 t	—	—
	2.28 m		2.28 m			
4″	—	181.81 s	—	181.46 s	—	—

a) The assignments were based on DEPT, COSY, HMQC, and HMBC experiments. *b*) Overlapping with other signals.

(HMBC) correlations (Fig. 2) from the methane (H-1″) to C-5, C-6, and C-7 determined the position of the 2-pyrrolidinone-5-yl at C-6.

Compound **2** was also obtained as a 2 : 1 mixture of two diastereomers. The same molecular formula ($C_{19}H_{19}NO_7$) as **1** was obtained by HR-FAB-MS measurement (*m*/*z*: 374.1239 $[M+H]^+$) of **2**. The ^{1}H- and ^{13}C-NMR spectra of **2** were closely resembled to those of **1**, led us to consider that **2** may be also an epicatechin derivative possessing a 2-pyrrolidinone-5-yl group at the 6- or 8-position. The position of 2-pyrrolidinone-5-yl in **2** was determined to be C-8 by the analysis of the HMBC spectrum (Fig. 2).

In the circular dichroism (CD) spectra, compounds **1** and **2** showed a negative Cotton effect at *ca.* 280 nm. Flavanols with a (2*R*)-configuration show a negative Cotton effect at 280 nm that is independent of the configuration at C-3.[16)] In combination with the 2,3-*cis* configuration determined by ^{1}H-NMR ($J_{2,3}$=3.0 Hz, Table 1), it was demonstrated that **1** and **2** has a (2*R*,3*R*)-configuration. Therefore, the structure of the new compounds **1** and **2** were elucidated as 6- and 8-(2-pyrrolidinone-5-yl)-(−)-epicatechin, respectively. Nitrogen-containing flavonoids are very small class of natural products, which have been isolated only rarely from nature. The few examples comprise davalliosides A and B from *Davallia mariesii*,[14)] dracosephins A—D from *Dracocephalum rupestre*,[15)] prolinalins A and B from *Bombyx mori*,[17)] aquiledine and isoaquiledine from *Aquilegia ecalcarata*,[18)] ficine and isoficine from *Ficus pantoniana*,[19)] and ethylpyrrolididinonyl theasinensin A from commercial black tea.[20)] In a previous work, Kouno group[20)] obtained 6-(*N*-ethyl-2-pyrrolidinone-5-yl)-epicatechin-3-*O*-gallate by condensation between epicatechin-3-*O*-gallate and 1-ethyl-5-hydroxy-2-pyrrolidinone, which was spontaneously produced from the Strecker aldehyde of theanine, in acidic aqueous solution. 5-Hydroxypyrrolidin-2-one has been isolated from several higher plants

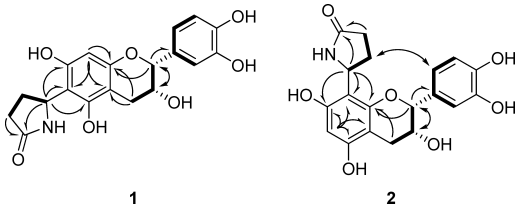

Fig. 2. Selected Correlations Observed in the COSY (▬), HMBC (→), and NOESY (↔) Spectra of **1** and **2**

including *Jatropha curcas*[21)] and *Pteridium aquillinum*.[22)] Thus we suggest that compounds **1** and **2** may be produced by condensation between (−)-epicatechin and 5-hydroxypyrrolidin-2-one in an acidic condition.

The known compounds were identified as (−)-epicatechin (**3**),[23)] (+)-catechin (**4**),[23)] proanthocyanidin B-4 (**5**),[24)] (+)-pinoresinol,[13)] and *p*-hydroxybenzoic acid[25)] by physical and spectroscopic data (mp, ^{1}H-, ^{13}C-NMR, and MS) measurement and by comparison with published values. This is the first report on the isolation of **5** from *Actinidia arguta*.

All isolates obtained in the present study were evaluated for their potential to inhibit the formation of AGEs (Table 2). Among them, proanthocyanidin B-4 (**5**) and (+)-catechin (**4**) exhibited the most potential inhibitory activity against AGEs formation, with IC_{50} values of 5.8 (10.1 μM) and 4.0 μg/ml (13.6 μM), respectively. The new compounds (**1**, **2**) and (−)-epicatechin (**3**) also showed a significant activity in the assay with IC_{50} values of 36.0, 47.8, and 125.2 μM, respectively, better than the well known glycation inhibitor, aminoguanidine (IC_{50} value=961 μM), while (+)-pinoresinol and *p*-hydroxybenzoic acid were not active in this experiment. This observation is well consistent with a recent report that some catechin and epicatechin derivatives and proanthocyanidin B-2 exert inhibitory activity against the formation of AGEs.[26)] There is also a recent report that flavonoids including (+)-

Table 2. The Inhibitory Effects of **1**—**5** from *A. arguta* on the Formation of Advanced Glycation End Products (AGEs) *in Vitro*[a)]

Compound	Inhibitory effect (IC_{50} value)[a)]	
	μg/ml	μM
1	13.5	36.0
2	17.9	47.8
3	36.1	125.2
4	4.0	13.6
5	5.8	10.1
AG[b)]	71.1	961

a) The concentration required for a compound required for a 50% inhibition (IC_{50}) of AGEs formation. IC_{50} values were calculated from the dose inhibition curve. Inhibitory effect was expressed as mean of triplicate experiments. (+)-Pinoresinol and *p*-hydroxybenzoic acid were not active in this assay system. *b*) Aminoguanidine (AG) was used as a positive control.

catechin inhibit the formation of the cross-linking AGEs pentosidine.[27)] However the inhibitory activities of proanthocyanidin B-4 (**5**) toward the formation of AGEs have not been previously reported up to date.

In summary, we found that flavan-3-ols (**1**—**4**) and proanthocyanidin B-4 (**5**) are responsible for inhibitory effect of the roots of *A. arguta* on AGEs formation. These compounds seem to be worthy of additional biological tests to more fully evaluate their potential as therapeutic agents for diabetic complications and related diseases.

Experimental

General 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 P-2000 digital polarimeter (Jasco, Japan). IR spectra were recorded using a Jasco FTIR-4100 spectrophotometer (Japan). LR/HR-ESI-MS and LR/HR-FAB-MS were recorded on a Marina mass spectrometer (Prospective Biosystem, U.S.A.) and JMS-700 Mstation (JEOL, Japan), respectively. CD spectra were measured on a Jasco J-715 spectropolarimeter (Japan). 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 analyses was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness); compounds were visualized by dipping plates into 20% (v/v) H_2SO_4 reagent (Aldrich) and then heated 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 *Actinidia arguta* Planchon (Actinidiaceae) were collected in Mt. Palbong, Palbong-myeon, Seosan-si, Chungcheongnam-do, Korea, in April 2006 and were identified by Prof. J.-H. Kim, Division of Life Science, Daejeon University, Daejeon 305–811, Korea. A voucher specimen (no. KIOM-ACAR1) 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 (16 kg) was extracted with 36 l of EtOH three times by maceration. The extracts were combined and concentrated *in vacuo* at 40 °C to give an EtOH extract (553 g). The EtOH extract (300 g) was suspended in H_2O (2 l) and then partitioned with *n*-hexane (3×2 l) to afford an *n*-hexane-soluble fraction (50.5 g) on drying. Next, the aqueous partition was partitioned again with EtOAc (3×2 l) to give an EtOAc-soluble fraction (60.4 g) and an aqueous residue. The EtOAc-soluble fraction (50 g) was chromatographed over silica gel (ϕ 6.5×47 cm, 70—230 mesh) as the stationary phase using a $CHCl_3$–MeOH gradient (from 20 : 1 to 0 : 1, v/v) to afford nine pooled fractions (F01—F09). Fraction F03 [eluted with $CHCl_3$–MeOH (8 : 1, v/v); 6.2 g] was subjected to silica gel column chromatography (CC) (ϕ 6.5×38 cm, 230—400 mesh; $CHCl_3$–MeOH gradient from 20 : 1 to 1 : 1, v/v) resulting in ten subfractions (F0301—F0310). (+)-Pinoresinol (50 mg) was purified from fraction F0308 by reversed-phase CC (ϕ 3.6×44 cm, 12 nm S-150 μm; MeOH–H_2O gradient from 1 : 1 to 4 : 1, v/v). Fraction F05 [eluted with $CHCl_3$–MeOH (8 : 1, v/v); 3.1 g] was fractionated by further silica gel CC (ϕ 6.5×40 cm, 230—400 mesh; $CHCl_3$–MeOH gradient from 10 : 1 to 1 : 1, v/v) to give *p*-hydroxybenzoic acid (5.7 mg). (−)-Epicatechin (**3**, 720 mg) and (+)-catechin (**4**, 50 mg) were obtained from fraction F06 [eluted with $CHCl_3$–MeOH (6 : 1, v/v); 4.7 g] by silica gel CC (ϕ 4.5×40 cm, 230—400 mesh; $CHCl_3$–MeOH–H_2O gradient from 7 : 3 : 0.2, v/v). Fraction F07 [eluted with $CHCl_3$–MeOH (2 : 1, v/v); 10.7 g] was purified over repeated CC, yielding the new compounds **1** (5.4 mg), **2** (18.6 mg), and proanthocyanidine B-4 (**5**, 189 mg). The new compounds **1** (5 : 3) and **2** (2 : 1) were isolated as a mixture of diastereomers and the portions were obtained by analyses of ^{13}C-NMR.

6-(2-Pyrrolidinone-5-yl)-(−)-epicatechin (**1**): White powder; mp 195—197 °C; $[\alpha]_D^{20}$ −16.3° (c=0.2, MeOH); IR (KBr) cm^{-1}: 3340 (br, OH), 2923, 2851, 1644 (amide CO), 1604, 1513 (aromatic ring), 1449, 1362, 1280, 1119, 1058; CD (MeOH, c=0.9×10^{-2} M) $\Delta\varepsilon$ (nm) : −4.47 (283), −3.66 (252). Major isomer: ^{1}H-NMR (CD_3OD, 300 MHz) and ^{13}C-NMR (CD_3OD, 75 MHz) data, see Table 1; Minor isomer: ^{1}H-NMR (CD_3OD, 300 MHz) signals were overlapped with corresponding signals of the major isomer; ^{13}C-NMR (CD_3OD, 75 MHz) δ: 181.81 (C-4″), 156.56 (C-7), 156.33 (C-8a), 156.19 (C-5), 146.12 (C-4′), 145.99 (C-3′), 132.20 (C-1′), 119.54 (C-6′), 116.06 (C-5′), 115.48 (C-2′), 110.18 (C-6), 100.83 (C-4a), 97.00 (C-8), 79.79 (C-2), 67.37 (C-3), 50.95 (C-1″), 32.44 (C-3″), 29.62 (C-4), 27.40 (C-2″); ESI-MS m/z: 372 $[M-H]^+$. HR-ESI-MS m/z: 372.1085 ($[M-H]^+$, Calcd for $C_{19}H_{18}NO_7$: 372.1083).

8-(2-Pyrrolidinone-5-yl)-(−)-epicatechin (**2**): White powder; mp 195—197 °C; $[\alpha]_D^{20}$ −1.0° (c=0.2, MeOH); IR (KBr) cm^{-1}: 3272 (br, OH), 2923, 2856, 1647 (amide CO), 1607, 1522 (aromatic ring), 1455, 1372, 1278, 1097, 1062; CD (MeOH, c=1.1×10^{-2} M) $\Delta\varepsilon$ (nm): −4.60 (279), −2.68 (249). Major isomer: ^{1}H-NMR (CD_3OD, 300 MHz) and ^{13}C-NMR (CD_3OD, 75 MHz) data, see Table 1; Minor isomer: ^{1}H-NMR (CD_3OD, 300 MHz) signals were overlapped with corresponding signals of the major isomer; ^{13}C-NMR (CD_3OD, 75 MHz) δ: 181.46 (C-4″), 157.43 (C-5), 156.24 (C-7), 156.05 (C-8a), 146.07 (C-4′), 145.99 (C-3′), 132.29 (C-1′), 119.48 (C-6′), 116.24 (C-5′), 115.31 (C-2′), 107.67 (C-8), 100.48 (C-4a), 96.56 (C-6), 80.24 (C-2), 67.46 (C-3), 50.69 (C-1″), 32.29 (C-3″), 29.85 (C-4), 27.49 (C-2″); FAB-MS m/z: 374 $[M+H]^+$. HR-FAB-MS m/z: 374.1239 ($[M+H]^+$, Calcd for $C_{19}H_{20}NO_7$: 374.1240).

Determination of the AGEs Formation According to well established method,[28)] 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 or aminoguanidine (Sigma, St. Louis, MO, U.S.A.). After incubating at 37 °C for 14 d, the fluorescent reaction products were assayed on a spectrofluorometric detector (BIO-TEK, Synergy HT, U.S.A.; Ex: 350, Em: 450 nm). AGEs assay was performed in quadruplicate. The concentration of each test sample giving 50% inhibition of the activities (IC_{50}) was estimated from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.

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