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Radiolytic cyclization products of phloridzin as potent anti-glycation agents

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Abstract: The current research examined for radiolytic structure modification and improved bioefficacy of phloridzin by gamma-ray, subsequent to a 50 kGy irradiation dose. Structures of the unusual degraded products phlorocyclin (**2**), isophlorocyclin (**3**), and radiophlorisin (**4**) were determined spectroscopically, by detailed nuclear magnetic resonance (NMR) and mass spectrometry (MS). Additionally, absolute stereochemistry of the novel cyclized phlorocyclin (**2**) and isophlorocyclin (**3**) were proposed by circular dichroism (CD) spectrum analysis. Among the compounds tested, phlorocyclin (**2**) and isophlorocyclin (**3**) exhibit potent anti-diabetic complication capacities toward advanced glycation end products (AGEs) formation inhibition assay, with IC_{50} values of 9.1±0.5 and 13.8±0.7 μM, respectively. Furthermore, the predominantly formed products phlorocyclin (**2**) and isophlorocyclin (**3**) exerted significantly enhanced DPPH radical scavenging activity compared to the parent phloridzin. These results indicate that gamma-ray mediated cyclization of phloridzin exerts a positive influence on the bioactivity.

Keywords: gamma-irradiation • neolignan • phloridzin • DPPH radical • AGEs formation

Introduction

Major diabetic complications include overexpression of protein kinase C, increased polyol pathway metabolic flux, and production of advanced glycation end products (AGEs).[1,2] Of these, the increased production and accumulation of AGEs are associated with prominent pathogenesis in the progression of diabetes related complications.[3] The glycation reaction is initiated by the nonenzymatic reaction of free amino groups with reducing sugars to form Amadori products. This reaction is accelerated by reactive oxygen species (ROS).[4-6] Thus, suppressing the generation of AGEs and oxidative stress is recognized as an effective therapeutic strategy for diabetic complications in humans. Aminoguanidine is a nucleophilic hydrazine agent and has attracted attention as a promising anti-glycation drug. This compound prevents generation of AGEs by blocking carbonyl groups on the Amadori products, and also exerts antioxidant activity at high concentrations. However, aminoguanidine has displayed undesirable side effects in clinical trials, including flu-like symptoms, anemia and gastrointestinal disturbances.[7] In recent years, the availability of abundant natural resources and known biological activities of natural products has resulted in considerable focus being shifted to natural products for discovering effective AGE inhibitors with antioxidant properties .[8]

Phloridzin is a representative element of the class dihydrochalcone, and is described as a natural bitter phenolic compound.[9] This naturally occurring secondary metabolite is abundant in leaves, seeds, and skin of the apple tree.[10,11] Dihydrochalcone glucoside possesses a diverse range of biological efficacies such as antioxidant, whitening, and anti-diabetic effects.[12-14] Structure modification studies of phloridzin using enzyme and intestinal bacteria were previously achieved to create new valuable analogues.[15,16] Our recent study has verified that nonthermal cold plasma technique is an effective green synthetic technology for providing chemically modified phloridzin with reinforced anti-adipogenic capacity.[17]

Gamma-ray treatment is recognized as an alternative method in food processing, and is also known to play a significant role in the inactivation of pathogenic microorganisms due to the enhanced generation of reactive oxygen species and free radicals.[18-20] Although gamma irradiation is widely used for microbial inactivation, very few studies have determined the influence of gamma irradiation on naturally occurring polyphenols extensively present in vegetables and fruits.[21-24] Continuous investigations related to gamma-ray treatment of major natural products with diverse backgrounds are still very limited. We report here the unusual structural modification of phloridzin mediated by gamma irradiation. The present study resulted in isolation of the novel cyclized products **2**, **3**, and alkylphenol **4**, having significantly increased bioefficacies toward AGE formation and DPPH radical scavenging compared to the original phloridzin, as determined by *in vitro* bioassays.

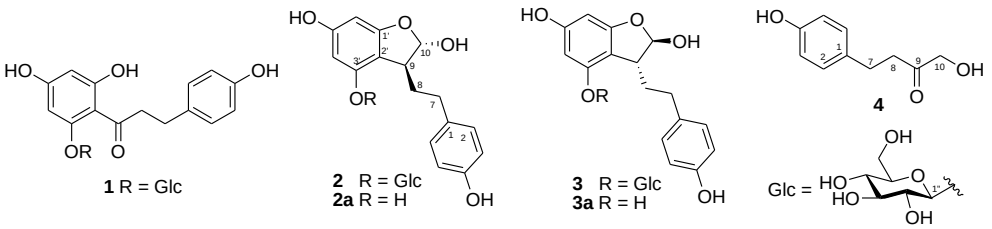

Figure 1. Structures of the newly formed compounds **2**–**4** from gamma-irradiated phloridzin (**1**).

Results and Discussion

Structure determination of the newly generated products

The natural dihydrochalcone phloridzin was dissolved in MeOH and directly irradiated with 50 kGy gamma-ray, after which the newly generated products were detected by HPLC. The irradiated phloridzin was fractionated using flash column chromatography over a Toyopearl HW-40 gel, and resultant fractions were further separated by column chromatography using YMC ODS AQ gel, which afforded two unusual stereoisomeric neolignans **2** and **3**, and one previously undescribed simple phenol **4**. The structural features of the new compounds are detailed below (**Figure 1**).

Compound **2** was obtained as a colorless oil with a negative optical rotation ($[\alpha]^{20}_{D}$= -45.3). The positive ion mode HRFABMS measurement of **2** indicated a pseudomolecular ion peak at *m/z* 450.1523 $[M]^+$ (calculated for $C_{22}H_{26}O_{10}$, 450.1526), consistent with the molecular formula of $C_{22}H_{26}O_{10}$. The typical absorbances at 224 and 279 nm in the UV spectrum suggested the presence of a dihydrobenzofuran framework.[25] The ^{1}H NMR measurement of **2** revealed one A_2B_2 type aromatic proton signals at δ_H 7.06 (d, *J* = 8.4, H-2, 6) and 6.70 (d, *J* = 8.4, H-3, 5), one *meta*-coupled AB type aromatic signals at δ_H 6.25 (d, *J* = 1.8, H-4') and 5.95 (d, *J* = 1.8, H-6') and two methylene protons at δ_H 2.53 (t, *J* = 7.8, H-7), 2.16–2.21 (m, H-8), and 1.67–1.73 (m, H-8). Andditional signals were observed as one doubly oxygenated methine signal at δ_H 5.72 (d, *J* = 1.2, H-10), and one methine proton at δ_H 3.18 (ddd, *J* = 8.4, 4.2, 1.2, H-9). Consistent with these ^{1}H NMR observations, the ^{13}C NMR, HSQC, and ^{1}H–^{1}H COSY experiments indicated the presence of a dihydrobenzofuran type neolignan.[25] The ^{1}H NMR spectrum also showed resonances for one characteristic anomeric proton at δ_H 4.88 (d, *J* = 7.8, H-1'') and six oxygen containing signal at δ_H 3.89-3.40, and the coupling constant of 7.8 Hz observed for the anomeric proton indicated a β-configuration of the glucose moiety (**Table 1**).[26] The linkage position of the dihydrobenzofuran core on the sugar moiety in **2** was determined unambiguously from the key HMBC cross peaks, which displayed H-1'' to C-3' (160.8) correlation (**Figure 2A**). The relative configuration of H-9 and H-10 of **2** was determined as *trans*-configuration, based on the small-coupling constant ($J_{2,3}$ = 1.2 Hz) of H-9 and H-10 [27,28] Enzymatic hydrolysis yielded β-glucose and aglycone **2a** from the new **2**, and structure of **2a** was also not previously undescribed.

Table 1. ^{1}H and ^{13}C NMR chemical shits of compounds **2**–**4**.[a]

	2 (acetone-d_6)		**3** (acetone-d_6)		**4** (CD_3OD)	
Position	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.
1	—	133.5	—	133.7	—	133.0
2	7.06 (d, 8.4)	130.1	7.04 (d, 8.4)	130.0	6.99 (d, 8.4)	130.3
3	6.70 (d, 8.4)	115.7	6.72 (d, 8.4)	115.8	6.66 (d, 8.4)	116.2
4	—	156.0	—	156.1	—	156.7
5	6.70 (d, 8.4)	115.7	6.72 (d, 8.4)	115.8	6.66 (d, 8.4)	116.2
6	7.06 (d, 8.4)	130.1	7.04 (d, 8.4)	130.0	6.99 (d, 8.4)	130.3
7	2.53 (t, 7.8)	32.5	2.57 (t, 7.8)	32.6	2.78 (t, 7.2)	29.8
8	2.16–2.21 (m), 1.67–1.73 (m)	34.1	2.03–2.08 (m), 1.69–1.72 (m)	34.9	2.67 (t, 7.2)	41.1
9	3.18 (ddd, 8.4, 4.2, 1.2)	48.3	3.24 (ddd, 8.4, 4.2, 1.2)	48.2	—	211.8
10	5.72 (d, 1.2)	107.2	5.71 (d, 1.2)	107.4	4.12 (s)	69.0
1'	—	155.7	—	155.7		
2'	—	109.6	—	109.3		
3'	—	160.8	—	160.8		
4'	6.25 (d, 1.8)	96.2	6.23 (d, 1.8)	96.0		
5'	—	159.7	—	159.7		
6'	5.95 (d, 1.8)	92.6	5.95 (d, 1.8)	92.6		
1''	4.88 (d, 7.8)	101.7	4.88 (d, 7.8)	101.5		
2''	3.47–3.49 (m)	74.3	3.48–3.49 (m)	74.4		
3''	3.52 (t, 8.4)	78.0	3.52 (t, 8.4)	77.6		
4''	3.39–3.41 (m)	71.1	3.38 (t, 8.4)	71.1		
5''	3.44–3.45 (m)	77.5	3.46–3.47 (m)	77.5		
6''	3.89 (dd, 12.0, 2.4)	62.4	3.89 (dd. 12.0, 2.4)	62.5		
	3.67 (dd, 12.0, 6.0)		3.67 (dd, 12.0, 6.0)			

[a] Measured at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR. Assignments are based on HSQC and HMBC NMR spectroscopic data. *J* values (Hz) are given in parentheses.

The absolute structures at H-9 and H-10 of compound **2** using hydrolysate **2a** was determined on the basis of the CD spectroscopic data.[29] The CD spectroscopic measurement of **2a** showed a negative Cotton effects at 234 nm (Δε -24.5) and 275 nm (Δε -6.5), representing that the absolute stereochemistry of **2** was the 9*R*, 10*S*-configuration (**Figure 2B**). Also, the absolute stereostructure of the sugar moiety was established as D-glucose by

HPLC analysis of the arylthiocyanate analogous by applying the previously reported UV detection method.[30] Based on the above verification, the dihydrobenzofuran-type neolignan structure of the new compound **2** was designated the trivial name phlorocyclin.

The molecular formula for **3** was determined as $C_{22}H_{26}O_{10}$ (*m/z* 450.1529 $[M]^+$, calculated for *m/z* 450.1523), which was identical to that of **2**. The 1D NMR and UV spectroscopic data of **3** were also almost similar to **2** indicating that both **2** and **3** structures are isomers. Also, relative and absolute stereochemistries of **3** were established as *trans*-configuration based on the small-coupling constant ($J_{2,3}$ = 1.2 Hz) of H-9 and H-10 [27,28] and D-glucose by applying the convenient sugar analytical method.[30] The CD spectrum of **3a** displayed a positive Cotton effects at 235 nm (Δε +14.6) and 275 nm (Δε +2.9), suggesting that the absolute structure of **3** was the 9*S*, 10*R*-stereochemistry (**Figure 2B**).[29] Accordingly, the structure of **3** was assigned as isophlorocyclin, which is a new radiolysis product (**Figure 1**).

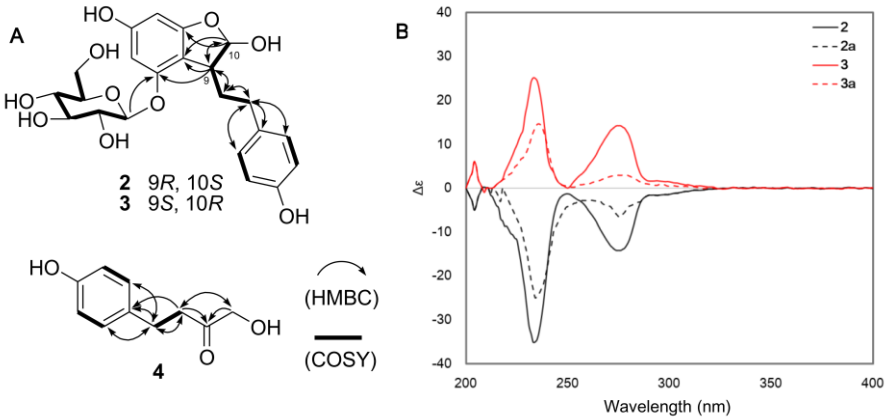

Figure 2. Key HMBC and ^1H-^1H COSY correlations (**A**) and CD spectra (**B**) of compounds **2**–**4**.

Compound **4** was obatained as a minor product. The molecular formula of **4** was determined to be $C_{10}H_{12}O_3$ by positive HRFABMS exhibition $[M]^+$ at *m/z* 180.0786 (calculated for *m/z* 180.0786). The 1D-and 2D-NMR spectra of **4** showed A_2B_2 type aromatic proton signals at δ_H 6.99 (d, *J* = 8.4, H-2, 6) and 6.66 (d, *J* = 8.4, H-3, 5), two methylene proton signals at δ_H 2.78 (t, *J* = 7.2, H-7), 2.67 (t, *J* = 7.2, H-8) and one ketone signal at δ_C 211.8 (C-9) (**Table 1**). The linkage positions of a alkyl chain in **4** were unambigously determined by the key HMBC relationships (**Figure 2A**). Although compound **4** was reported previously as a raspberry metabolite in rats identified using GC-MS data,[31] this is the first report of structural characterization using spectroscopic interpretation.

HPLC analysis revealed that contents of the most active compounds **2** and **3** were present in samples that were gamma-ray treated at 10, 25, and 50 kGy (Supporting information). With increased irradiation dose, the production of the relatively potent compounds **2** and **3** increased remarkably, and reached a maximum at the 50 kGy irradiation. Simultaneously, a complete decrease in the phloridzin content was detected by gamma irradiation at the 50 kGy dose. Methanolic radiolysis is recognized to mediate specific chemical changes associated with sufficient production of reactive oxygen species in the natural products of chalcone, anthraquinone, and flavonoids.[32-35] Our results support that the hydroxymethyl radical may also be implicated in the generation of **2** and **3** during gamma-irradiation of phloridzin in MeOH. This is the first example of a new radiolytic cyclization of dihydrochalcone phloridzin.

Suppression of AGEs formation and DPPH radical scavenging capacities of newly generated products

The ability of cyclized products obtained from phloridzin to inhibit AGE production was estimated using the bovine serum albumin (BSA) - glucose assay (**Table 2**). Results revealed that compared to other irradiation doses, the 50 kGy gamma-ray irradiated phloridzin had highest inhibitory activity with IC_{50} value of 21.5±1.2 μg/mL. Among the products isolated from the 50 kGy irradiated phloridzin, the dihydrobenzofuran-type neolignan derivative, phlorocyclin (**2**) having a 9*R*, 10*S*-stereostructure at the chiral center, was the most potent in this bioassay, with IC_{50} value of 9.1±0.5 μM. Interestingly, isophlorocyclin (**3**) with 9*S*, 10*R*-absolute configuration was found to show relatively weaker AGE inhibition than compound **2**. In addition, hydrolysates **2a** and **3a** revealed slightly lower inhibitory activities with IC_{50} values of 29.2±1.3 and 39.9±1.5 μM, respectively, than glucosides **2** and **3**. The previously undescribed alkylphenol derivative, radiophlorisin (**4**) displayed AGE inhibition with an IC_{50} value of 47.9±1.4 μM.

The DPPH radical scavenging capacity of the50 kGy irradiated phloridzin showed the most improved activity (IC_{50} value = 48.4±2.1 μg/mL). As summarized in **Table 2**, the novel neolignans **2** and **3**, having the enantiomer structure in the dihydrobenzofuran moiety, showed significantly potent scavenging activities in the DPPH radical scavenging assay, with IC_{50} values of 45.1±1.5 and 58.6±1.9 μM, respectively. Additionally, hydrolysates **2a** and **3a** also exhibit enhanced DPPH radical scavenging abilities (IC_{50} values = 16.1±0.7 and 20.8±0.9 μM, respectively), as compared to the glucosides **2** and **3**.

This investigation is well consistent with a recent study which reported that some neolignan derivatives exert inhibitory activity of AGE formation and DPPH radial scavenging capacities in the *in vitro* system.[36-38] In recent years, gamma-irradiation in methanolic solutions has attracted widespread attention owing to its potential to enhance the biological activities of polyphenolic compounds.[21-24] Our results indicate that cyclization induced by gamma-irradiation of phloridzin could be advantageous in blocking the progression of anti-diabetic complications and related diseases.

Table 2. Inhibition of AGEs production and DPPH radical scavenging capacities of the newly generated **2**–**4**.

Compounds	IC_{50} value (μM)[a]	
	Suppression of AGEs formation	DPPH radical scavenging
Phloridzin (**1**)	185.6±2.3	>500
Irradiated phloridzin (10 kGy)	73.9±2.5[b]	>500[b]
Irradiated phloridzin (25 kGy)	54.0±2.3[b]	120.8±3.9[b]
Irradiated phloridzin (50 kGy)	21.5±1.2[b]	48.4±2.1[b]
2	9.1±0.5	45.1±1.5
2a	29.2±1.3	16.1±0.7
3	13.8±0.7	58.6±1.9
3a	39.9±1.5	20.8±0.9
4	47.9±1.4	>500
Aminoguanidine[c]	978.3±4.7	—
(+)-Catechin[c]	—	16.6±0.3

[a] All compounds were tested in triplicate experiments. [b] Results revealed as IC_{50} values using μg/mL. [c] Positive control substances.

Conclusions

The present study evaluated various features of the 50 kGy gamma-irradiated phloridzin, which determined enhanced inhibition of AGE production and DPPH radical scavenging activity, as compared to the original phloridzin (**1**). The cyclized and unusual structures of the newly generated derivatives (compounds **2**–**4**) were determined by 1D-and 2D-NMR, HRFABMS, and CD spectra data interpretation. The absolute stereochemistry of dihydrobenzofuran-type neolignans **2** and **3** showed the most potent suppression of AGEs formation and DPPH radical scavenging property than the parent phloridzin. Importantly, the radiolysis products **2**–**4** of phloridzin, induced by gamma-irradiation, induce inhibition of AGE formation and DPPH radical scavenging activity *in vitro*, indicating the potential of compounds **2**–**4** as valuable for the prevention of diabetic complications and related diseases.

Experimental Section

General information

Phloridzin, aminoguanidine, (+)-catechin, bovine serum albumin (BSA), D-glucose, D-furctose, 1,1-diphenyl-2-picrylhydrazyl (DPPH), methanol (MeCH), acetonitrile (MeCN), and formic acid (HCOOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific rotation was measured using a JASCO P-2000 polarimeter and circular dichroism (CD) spectrum was run on a JASCO J-1500 spectrometer (JASCO, Tokyo, Japan). ^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY spectra were measured on a Varian VNS-600 MHz spectrometers (Varian, Palo Alto, CA, USA) equipment with CD_3OD (δ_H 3.35, δ_C 49.0) and acetone-d_6 (δ_H 2.04, δ_C 29.8). Fast atom bombardment mass spectrometer (FABMS) was conducted on a JMS-700 GC-HRMS spectrometer (JEOL, Tokyo, Japan) and ultraviolet (UV) spectrum was obtained on a T-60 spectrophotometer (PG Instrument, Leicestershire, UK).

Gamma-irradiation of phloridzin and HPLC analysis

A sample solution of pure phloridzin (500 mg) in MeOH (2.5 L) was directly exposed to 10, 25, and 50 kGy in a cobalt-60 irradiator (point source AECL, IR-79, MDS Nordion International Co. Ltd, Otawa, ON, Canada) with a source strength of 320 kCi, at a dose rate of 10 kGy/h, at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (Jeongup, Korea).[39] Dosimeters were performed using 5 mm diameter alanine

dosimeters (BrukerInstruments, Rheinstetten, Germany) and calibrated against an International Standard Set by the International Atomic Energy Agency (Vienna, Austria). The methanolic sample solution of gamma-irradiated phloridzin was dried to remove the solvent. The Shimadzu HPLC instrument SPD-M20A (Shimadzu Co., Kyoto, Japan), equipped with a UV-diode array detector (280 nm), was used for chromatographic separation by gamma-irradiation from phloridzin. HPLC analysis was conducted using a YMC-Pack ODS A-302 column (4.6 mm i.d. × 150 mm, particle size 5 μm; YMC Co., Kyoto, Japan), and the mobile phase was composed of 0.1% HCOOH in H_2O (solvent A) and MeCN (solvent B). A gradient solvent system was performed with linear gradient from 8% to 100% solvent B for 30 min, with flow rate set at 1.0 mL/min. The newly generated by-products from phloridzin were monitored using their retention times (t_R), and compared with pure phloridzin.

Isolation of newly generated products

The dried irradiated phlorizin at 50 kGy exhibited the most enhanced inhibitory effect for AGE production, with an IC_{50} value of 21.5±1.2 μg/mL, as compared to the original phloridzin. The irradiated sample (490 mg) was chromatographed over the Toyopearl HW-40 column (2.5 cm i.d. × 40 cm, coarse grade), and eluted with a step-wise gradient of H_2O–MeOH of increasing polarity (80:20 to 0:100, followed by aqueous acetone) to produce four subfractions, PG01-PG04. The most potent subfraction PG03 was subjected to column chromatographic purification using the YMC GEL ODS AQ 120-50S gel column (1.0 cm i.d. × 39 cm, particle size 50 μm) with aqueous MeOH, to yield pure compounds **2** (35.5 mg, t_R 11.0 min), **3** (24.5 mg, t_R 11.2 min), and **4** (9.1 mg, t_R 9.3 min). Chemical structures of these generated products **2**–**4** were determined by 1D-, 2D-NMR, FABMS, and CD spectroscopic interpretations (**Figure 1**). IR data of the isolated **2**–**4** could not obtained because insufficient amounts for measurement.

New compounds information

Phlorocyclin (2): Colorless oil, $[\alpha]^{20}_D$= −45.3 (*c*=0.1, MeOH); UV λ_{max} MeOH (log ε): 224 (3.60), 279 (1.14) nm; CD (MeOH) Δε (nm): 233 (-35.1), 275 (-14.2) nm; ^{1}H and ^{13}C NMR, see **Table 1**; FABMS *m/z* 450 $[M]^+$, HRFABMS *m/z* 450.1523 $[M]^+$ (calcd for $C_{22}H_{26}O_{10}$, 450.1526).

Isophlorocyclin (3): Colorless oil, $[\alpha]^{20}_D$= −152.9 (*c*=0.1, MeOH); UV λ_{max} MeOH (log ε): 224 (3.64), 279 (1.19) nm; CD (MeOH) Δε (nm): 236 (+28.5), 275 (+13.9) nm; ^{1}H and ^{13}C NMR, see **Table 1**; FABMS *m/z* 450 $[M]^+$, HRFABMS *m/z* 450.1529 $[M]^+$ (calcd for $C_{22}H_{26}O_{10}$, 450.1526).

Radiophlorisin (4): Colorless oil, UV λ_{max} MeOH (log ε): 205 (4.10), 227 (sh), 279 (2.44) nm; ^{1}H and ^{13}C NMR, see **Table 1**; FABMS *m/z* 180 $[M]^+$, HRFABMS *m/z* 180.0786 $[M]^+$ (calcd for $C_{10}H_{12}O_3$, 180.0786).

Enzymatic hydrolysis of 2 and 3

Compounds **2** (9.1 mg) and **3** (8.3 mg) were enzymatically hydrolyzed with β-glucosidase (EC 3.2.1.21) (5 mg/mL) at 40°C for 12 h.[40] The EtOAc was used to extract the organic layer from each reaction mixture. The EtOAc portion was concentrated to yield pure compounds **2a** (3.5 mg, t_R 13.1 min) and **3a** (2.9 mg, t_R 13.1 min), respectively.

2a: Colorless oil, $[\alpha]^{20}_D$= +41.2 (*c*=0.1, MeOH); UV λ_{max} MeOH (log ε): 224 (3.70), 279 (1.49) nm; CD (MeOH) Δε (nm): 234 (-24.5), 275 (-6.5) nm; ^{1}H NMR (acetone-d_6, 600 MHz): δ 7.00 (d, *J* = 8.4, H-2, 6), 6.69 (d, *J* = 8.4, H-3, 5). 5.90 (d, *J* = 1.8, H-4), 5.79 (d, *J* = 1.8, H-6), 5.69 (d, *J* = 1.8, H-10), 3.13 (ddd, *J* = 9.0, 4.2, 1.8, H-9), 2.55 (t, *J* = 7.8, H-7), 2.03–2.05 (m, H-8), 1.63–1.66 (m, H-8); FABMS *m/z* 288 $[M]^+$, HRFABMS *m/z* 288.0994 $[M]^+$ (calcd for $C_{16}H_{16}O_5$, 288.0998).

3a: Colorless oil, $[\alpha]^{20}_D$= -59.0 (*c*=0.1, MeOH); UV λ_{max} MeOH (log ε): 224 (3.72), 279 (1.43) nm; CD (MeOH) Δε (nm): 235 (+14.6), 275 (+2.9) nm; ^{1}H NMR (acetone-d_6, 600 MHz): δ 7.00 (d, *J* = 8.4, H-2, 6), 6.69 (d, *J* = 8.4, H-3, 5). 5.90 (d, *J* = 1.8, H-4), 5.79 (d, *J* = 1.8, H-6), 5.69 (d, *J* = 1.8, H-10), 3.13 (ddd, *J* = 9.0, 4.2, 1.8, H-9), 2.55 (t, *J* = 7.8, H-7), 2.03–2.05 (m, H-8), 1.63–1.66 (m, H-8); FABMS *m/z* 288 $[M]^+$, HRFABMS *m/z* 288.0995 $[M]^+$ (calcd for $C_{16}H_{16}O_5$, 288.0998).

Determination of the absolute structures of sugars

The monosaccharides, obtained after the enzymatic hydrolysis of **2** and **3**, were added to pyridine (0.5 mL) and L-cysteine methyl ester hydrochloride (2.5 mg). The reaction mixture was heated at 60°C for 1 h, with subsequent addition of *o*-torylisothiocyanate (100 μL), followed by warming at 60°C for 1 h.[30] The standard D- and L-glucose were reacted via the same method. Analytical RP-HPLC was performed to analyze the reaction products on a Cosmosil 5C_{18}-MS-II column (4.6 mm i.d.×250 mm, particle size 5 μm) at 40°C with isocratic mode elution (50 mM H_3PO_4 – MeCN, 75:25) for 40 min at flow rate of 0.8 mL/min. D-Glucose (t_R 18.3 min) obtained from compounds **2** and **3** were identified as their glucosidic moiety, based on comparison with authentic samples of D-glucose derivative (t_R 18.3 min) and L-glucose derivative (t_R 16.4 min).

Inhibitory effects of AGEs formation assay

A previously reported method[41] with slight modification was adapted to evaluate inhibitory effects of the isolated compounds towards AGE formation. The reaction mixture (10 mg/mL BSA in 50 mM phosphate buffer (pH 7.4) containing 0.02% sodium azide) was added to sugar solution (200 mM D-fructose and 200 mM D-glucose). The reactant (800 μL) was then mixed with various concentrations of the test compounds (200 μL) or positive control (aminoguanidine), prepared in 5% DMSO. After incubation for 7 day at 37°C, the fluorescent reaction products were evaluated using an ELISA reader (Infinite F200; Tecan Austria GmBH, Grodig, Austria), with excitation and emission maxima at 350 and 450 nm, respectively. The concentration required for 50% inhibition (IC_{50} value) of the fluorescence intensity was determined by linear regression analysis; all measurements were obtained in triplicate.

DPPH radical scavenging activity assay

The DPPH radical scavenging activity was estimated using the previously reported method of Blois,[42] with minor modification. Briefly, 200 μM DPPH was prepared in EtOH, and 60 μL of this solution was added to 120 μL of each sample, assessed at varying concentrations (10 – 500 μM) in 70% EtOH. After 15 min incubation in the dark, the absorbance decrease of the reaction solution was recorded at 517 nm using an ELISA reader. The scavenging activity was calculated as %, using the following equation: DPPH radical scavenging activity (%) = (1-absorbance of sample/absorbance of control) × 100. The 50% inhibition (IC_{50} value) was evaluated by linear regression analysis of inhibitory effects under the assay conditions. (+)-Catechin was used as the positive control substance, and all measurements were obtained in triplicate.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

Gyeong Han Jeong and Tae Hoon Kim performed the experiments, and data and wrote the article. Tae Hoon Kim was the project leader conceived and designed the experiments.

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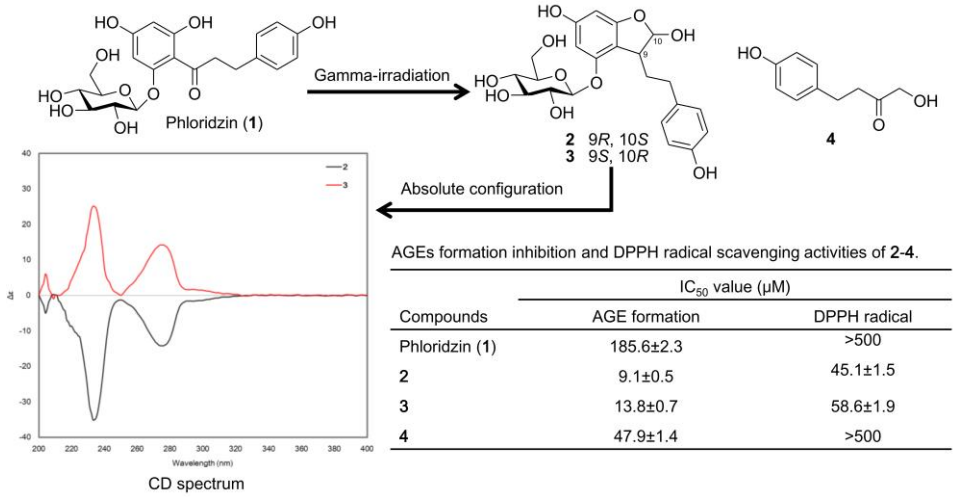

AGEs formation inhibition and DPPH radical scavenging activities of **2**-**4**.

Compounds	IC_{50} value (μM)	
	AGE formation	DPPH radical
Phloridzin (**1**)	185.6±2.3	>500
2	9.1±0.5	45.1±1.5
3	13.8±0.7	58.6±1.9
4	47.9±1.4	>500

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Radiolytic cyclization products of phloridzin as potent anti-glycation agents