Chemical Constituents of the Style of *Zea mays* L. with Glycation Inhibitory Activity

Ryuichiro SUZUKI, Miki IIJIMA, Yoshihito OKADA, and Toru OKUYAMA*

Department of Natural Medicine and Phytochemistry, Meiji Pharmaceutical University; 2–522–1 Noshio, Kiyose, Tokyo 204–8588, Japan. Received September 8, 2006; accepted October 24, 2006; published online October 31, 2006

A new sesquiterpene along with 15 known compounds were isolated from the style of *Zea mays* L. These structures were elucidated on the basis of spectroscopic analysis. The ability of these compounds to inhibit glycation *in vitro* was analyzed. This allowed determination of chemical functional groups required for the inhibition of glycation.

Key words style; *Zea mays*; sesquiterpene; glycation inhibitory activity

The style of *Zea mays* L. (Gramineae) is used in Chinese traditional medicine for the treatment of dropsy and hypertension. In previous studies, we isolated novel flavone *C*-glycosides from this material[1,2] and we showed that a water extract of this herbal drug has favorable effects in streptozotocin-induced diabetic nephropathy in rats.[3] In the current studies, we describe the isolation and characterization of a new sesquiterpene as well as several known compounds (two flavonoids, five steroids, five *N*-containing compounds, and three vanillin derivatives). We confirmed the structures of isolates by MS, two-dimensional (2D) NMR techniques including ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC), ^{1}H–^{13}C heteronuclear multiple bond coherence (HMBC) experiments, and comparison of spectral data to previously reported data. These isolates were also tested for inhibitory activity on glycation. And then, structure–activity relationship for the inhibition of glycation was examined. The carbonyl carbon of reducing sugars nonenzymatically reacts with amino residues of proteins to form advanced glycation end products (AGEs) through a process known as glycation. Formation of AGEs is known to cause diabetic complications such as nephropathy and retinopathy.[4]

The water extract of *Z. mays* was extracted with ethyl acetate followed by *n*-butanol. The portion soluble in ethyl acetate was purified by a combination of gel permeation chromatography on Sephadex LH-20 and reversed-phase HPLC to afford compounds **8**—**10**. Compounds **5**—**7** were isolated from the *n*-butanol-soluble portion by reversed-phase HPLC. Separation of the methanol extract of *Z. mays* by chromatography on Diaion HP-20 followed by reversed-phase HPLC gave compounds **13** and **14**. To obtain sparsity compounds, a large amount of style of *Z. mays* was extracted with methanol and water, and the methanol extract was subjected to column chromatography on Diaion HP-20 and then Toyopearl HW40F, followed by reversed-phase HPLC to give compounds **1**, **2**, **4**, and **11**. An aqueous extract of this material was extracted with ethyl acetate. The ethyl acetate-soluble portion was separated by silica gel chromatography, followed by reversed-phase HPLC to afford compounds **12**, **15**, and **16**.

Compounds **2**—**16** were identified as lumichrome (**2**),[5] chrysoeriol (**3**),[6] genistein (**4**),[7] adenosine (**5**),[8] guanosine (**6**),[9] uracil (**7**),[10] acetovanillone (**8**),[11] vanillin (**9**),[11] vanillic acid (**10**),[12] 6-methoxy-benzoxazolinone (**11**),[13] stigmast-4-en-3-one (**12**),[14] β-sitosterol (**13**),[15] stigmasterol (**14**),[16] stigmastanone (**15**),[17] and 7α-hydroxysitosterol (**16**)[18] based on comparison of their NMR spectral data with published spectral data.

Compound **1**, a white gum, had a molecular formula $C_{15}H_{18}O_3$ as determined from its high resolution (HR)-electron ionization (EI)-MS, ^{13}C-NMR, and ^{13}C-distortionless enhancement by polarization transfer (DEPT) spectral data. The ^{1}H-NMR spectrum of **1** had AA′XX′-type aromatic proton signals at δ 7.49 (2H, d, J=8.8 Hz) and δ 7.86 (2H, d, J=8.8 Hz), and a signal at δ 6.15 (1H, t, J=3.8 Hz) attributed to an olefinic proton. Furthermore, there were ^{1}H-NMR spectral signals for two methyl protons at δ 0.83 (3H, s) and δ 0.96 (3H, s), an aliphatic methylene proton at δ 2.18 (2H, m), two aliphatic methine protons at δ 1.64 (1H, m) and δ 1.21 (1H, m), and an oxymethine proton at δ 3.90 (1H, s). ^{13}C and DEPT spectra indicated 15 carbon signals, which arose from 2 methyl, 2 sp^3-hybridized methylene, 1 sp^3- and 5 sp^2-hybridized methines, 1 sp^3- and 3 sp^2-hybridized quarternary carbon, 1 carbonyl carbon. The HMQC spectrum signals at δ 1.64 (1H, m) and δ 1.21 (1H, m) correlated with a signal at δ 30.3. The resonances for δ 6.15 (1H, t, J=3.8 Hz) and δ 2.18 (2H, m) were coupled in the COSY spectrum. The latter signal at δ 2.18 (2H, m) was further coupled in the COSY spectrum to nonequivalent geminal protons at δ 1.64 (1H, m) and δ 1.21 (1H, m). Meanwhile, HMBC correlations of H-3 with C-2 and C-3 and the correlations of H-1 with C-2, C-6, and C-1′ as well as the methyl protons with C-5 demonstrated that compound **1** is a previously unknown sesquiterpene (Fig. 1).

Next, we examined the inhibition of the isolated compounds on *N*-ε-(carboxymethyl)lysine (CML) formation using an enzyme-linked immunosorbent assay (ELISA). CML is the best characterized AGE and is referred to as a glycoxidation product.[19] We tested the inhibitory activity of flavonoids **3**, **4**, **17** (chrysoeriol 6-*C*-β-boivinopyranosyl-7-*O*-β-glucopyranoside), **18** (alternanthin), **19** (chrysoeriol 6-*C*-β-fucopyranoside), and **20** (ax-4″-OH-3′-methoxymaysin); sterols **12**—**16**; *N*-containing compounds **2**, **5**, **6**, **7**, and **11**; vanillin derivatives **8**—**10**; and new sesquiterpenoid **1** (Table 2). We found that alternanthin (**18**), chrysoeriol 6-*C*-β-fucopyranoside (**19**), and genistein (**4**) inhibited glycation, but none of the isolated sterols affected glycation. Of the *N*-containing compounds, only guanosine (**6**) inhibited glycation. Vanillin (**9**), which possesses an aldehyde group, and its de-

* To whom correspondence should be addressed. e-mail: okuyama@my-pharm.ac.jp © 2007 Pharmaceutical Society of Japan

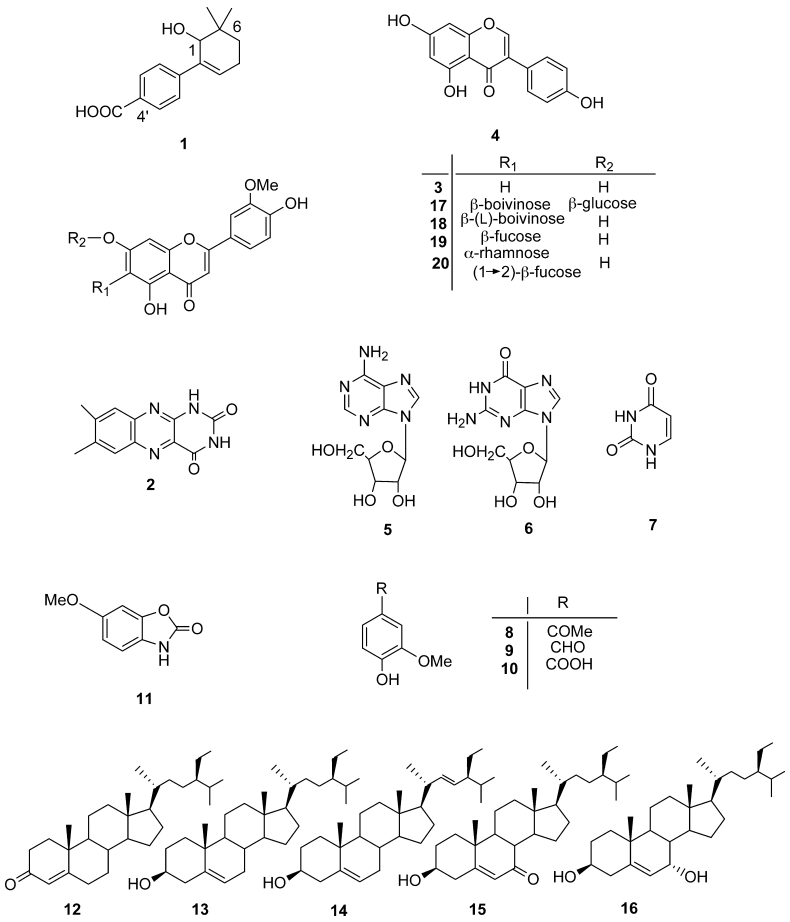

Chart 1

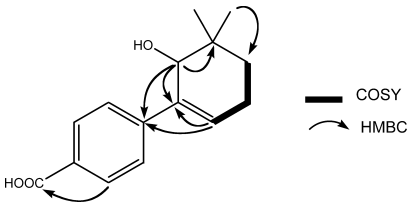

Fig. 1

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for **1** in CD_3OD

H-1	3.90 (1H s)	C-1	74.4 (d)
H-3	6.15 (1H t 3.8)	C-2	147.5 (s)
H-4	2.18 (2H m)	C-3	129.9 (d)
H-5_a	1.21 (1H m)	C-4	24.9 (t)
H-5_b	1.64 (1H m)	C-5	30.3 (t)
H-2′, 6′	7.49 (2H d 8.8)	C-6	35.1 (s)
H-3′, 5′	7.86 (2H d 8.8)	C-1′	138.9 (s)
6-Me_a	0.83 (3H s)	C-2′, 6′	126.8 (d)
6-Me_b	0.96 (3H s)	C-3′, 5′	130.5 (d)
		C-4′	129.7 (s)
		6-Me_a	26.4 (q)
		6-Me_b	24.4 (q)
		COOH	169.6 (s)

Proton resonance integral, multiplicity, and coupling constant (*J* in Hz) are in parentheses. NMR data were measured at 400 MHz for proton and at 100 MHz for carbon.

rivatives did not exhibit inhibitory activity. On the basis of these results, we consider the structure–activity relationship of the isolated compounds for inhibition of glycation. All of the isolated flavones have a chrysoeriol skeleton. Although chrysoeriol (**3**) did not inhibit glycation, 6-*C*-glycosylated chrysoeriols, alternanthin (**18**) and chrysoeriol 6-*C*-*β*-fucopyranoside (**19**) were inhibitors. *C*- and *O*-glycosylated chrysoeriols, chrysoeriol 6-*C*-*β*-boivinopyranosyl-7-*O*-*β*-glucopyranoside (**17**) and chrysoriol 6-*C*-*α*-rhamnopyranosyl-(1→2)-*β*-fucopyranoside (**20**) did not inhibit glycation. Therefore, mono 6-*C*-glycosylation on chrysoeriol contributes to the glycation inhibitory activity.

During the initial stage of glycation, a lone pair of electrons from an amino group of a protein react with the carbonyl carbon of a sugar to form a Schiff base. This Schiff base rearranges to form Amadori products, which are converted to AGEs through various processes. Given the reaction mechanisms for forming a Schiff base, it is possible that a compound with an aldehyde or amino group could competitively inhibit the formation of a Schiff base; however, vanillin (**9**), which has aldehyde group, and adenosine (**5**), which has amino group, did not inhibit glycation. Furthermore, although lumichrome (**2**) and uracil (**7**) have secondary or tertiary amino groups did not inhibit glycation, guanosine (**6**) inhibited the formation of AGEs. Guanosine have guanidine group, which is common structure of aminoguanidine used as a positive control. Thus, a guanidine group appears to be necessary for inhibition of glycation. Formation of some kinds of AGEs are thought to be due to free radical generation, and pyridoxamine and thiamine, which are

Table 2. Inhibitory Activity of Isolates from the Style of *Zea mays* on Glycation

Inhibition (%)		Inhibition (%)	
Flavonoids		Sterols	
3	n.e.	**12**	n.e.
4	95	**13**	n.e.
17	12	**14**	n.e.
18	69	**15**	n.e.
19	81	**16**	n.e.
20	2	Vanillin derivatives	
N containing		**8**	n.e.
2	n.e.	**9**	28
5	20	**10**	n.e.
6	63	Sesquiterpone	
7	5	**1**	2
11	41	AG (10 mM)	57.1

Each value represents the mean of duplicate assay at the concentration of 1 mM without AG. n.e.; activity was not detectable at the concentration indicated. AG; aminoguanidine is well known glycation inhibitor.

known as a free radical scavengers, inhibit the formation of AGEs.[20,21] Thus, inhibition of glycation could be related to radical scavenging. Okawa *et al.* reported that *C*-glycosylation of flavonoids decreases their radical scavenging and that flavones are better radical scavengers than isoflavones.[22] Similar results were reported by Picerno *et al.*[23] These reports do not agree with our experimental results. Hence, these isolates from the style of *Zea mays* may inhibit AGE formation by mechanisms other than radical scavenging.

Experimental

General Experimental Procedures UV spectra and optical rotation values were obtained using a Shimadzu UV 1600 spectrophotometer and a Horiba SEPA-300, respectively. FAB-MS was obtained with a JEOL JMS DX-302 mass spectrometer, and EI-MS was performed using a JEOL JMS-700 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were obtained using JNM-EX-270 (JEOL), JNM-AL-300 (JEOL), JNM-AL-400 (JEOL), and/or JNM-LA-500 (JEOL) spectrometers at room temperature with solvent signals as internal references. All chemical shifts (δ) are given in ppm. HPLC was performed using the following columns; Senshu Pak PEGASIL C-8 (20×250 mm i.d.), Senshu Pak PEGASIL ODS (20×250 mm i.d.), COSMOSIL 5C-18AR (10×250 mm i.d.), Wakopak Navi C22-5 (10×250 mm i.d.), Wakopak Navi C30-5 (10×250 mm i.d.), COSMOSIL 5SL (10×250 mm i.d.), COSMOSIL 5SL (20×250 mm i.d).

Plant Material The style of *Zea mays* L. (Gramineae) (Lot COFQO15) was purchased from Mikuni & CO., LTD. (Osaka, Japan). A voucher specimen (No. NP 021011) was deposited at the Department of Natural Medicine and Phytochemistry at Meiji Pharmaceutical University, Japan.

Extraction and Isolation The dried style of *Z. mays* L. (1 kg) was extracted twice in MeOH (12 l) and H_2O (12 l) for 2 h at 50 °C, to yield a methanol and aqueous extracts. The methanol extract (13.2 g) was separated by chromatography on Diaion HP-20 using steps of 50% aqueous MeOH, MeOH, and acetone. The acetone fraction (6.14 g) was subjected to reversed-phase (C-8) HPLC to afford **13** (35.7 mg) and **14** (19.6 mg). Aqueous extract (31.0 g) was resolved in a small amount of water, followed by partition with AcOEt followed by *n*-BuOH to obtain the corresponding layers. The AcOEt-soluble layer (422.3 mg) was successively purified by Sephadex LH-20 and reversed-phase HPLC (C-18, C-22) to give **8** (1.2 mg), **9** (6.5 mg), and **10** (9.1 mg). The *n*-BuOH soluble portion (2.12 g) was chromatographed on reversed-phased HPLC (C-18, C-22, C-30) to afford **5** (10.6 mg), **6** (11.1 mg), and **7** (2.2 mg).

To identify scarce compounds, large amounts of MeOH and H_2O extracts from the style of *Z. mays* L. were prepared by Koshiro Company Ltd. (Osaka, Japan). Style of *Z. mays* (30 kg) was extracted with MeOH and H_2O as described above. The MeOH-soluble portion (82.1 g) was subjected to Diaion HP-20, Toyopearl HW40F, and reversed-phase HPLC (C-8, C-18, C-22) to give **1** (10.1 mg), **2** (1.1 mg), **4** (0.9 mg), and **11** (1.7 mg). The AcOEt-soluble portion (100 g), which was prepared by extraction with AcOEt from an aqueous extract of the style of *Z. mays*, was separated by silica gel column chromatography. Eluted fractions were purified by normal- and reversed-phase HPLC to give **12** (3.6 mg), **15** (7.9 mg), and **16** (2.5 mg). A portion of the AcOEt-soluble fraction was subjected to Diaion HP-20, Toyopearl HW 40F, and reversed-phase HPLC (C-18) to afford **3** (3.1 mg).

Compound 1 (**1**): White gum. Formula: $C_{15}H_{18}O_3$. Molecular weight: 246, $[\alpha]_D^{25}$ −14.0° (c=1.01, MeOH). UV λ_{max} (MeOH) nm (log ε): 204 (4.15), 269 (4.13). EI-MS (70 eV) (rel. int.) *m/z*: 246 $[M]^+$ (49), 231 $[M-15]^+$ (23), 190 $[M-56]^+$ (100). HR-EI-MS *m/z*: 246.1260 (Calcd for $C_{15}H_{18}O_3$: 246.1256). ^{1}H- and ^{13}C-NMR: see Table 1.

Analysis of Inhibition of CML Formation *in Vitro* Bovine serum albumin (BSA) (4 mg/ml) was incubated with 200 mM glucose in the presence or absence of test compound for 7 d in 0.1 M phosphate buffer (pH 7.4) at 37 °C. After incubation, the level of CML was measured using a CML-specific ELISA based on the method described by Horiuchi.[24] The level of CML of each incubated mixture was the mean of duplicate experiments. The extent of inhibition was calculated as follows: inhibition (%)=$[1-(A_s-A_b)/(A_c-A_b)]\times100$, where A_s is the level of CML in the mixture containing the sample compound, A_c is the level of CML in the mixture lacking the test sample (positive control), and A_b is the level of CML in the mixture lacking test sample and glucose (blank).

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