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Flavonol Caffeoylglycosides as α-Glucosidase Inhibitors from *Spiraea cantoniensis* Flower

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In the screening experiments for rat intestinal α-glucosidase inhibitors in 218 plants cultivated in the Japanese temperate region, potent maltase-inhibiting activity was found in the extract of flowers of *Spiraea cantoniensis*. The enzyme assay guided fractionation of the extract led to the isolation of three flavonol caffeoylglycosides, quercetin 3-*O*-(6-*O*-caffeoyl)-β-galactoside (**1**), kaempferol 3-*O*-(6-*O*-caffeoyl)-β-galactoside (**2**), and kaempferol 3-*O*-(6-*O*-caffeoyl)-β-glucoside (**3**), as rat intestinal maltase inhibitors. This is the first report on the α-glucosidase-inhibitory activity of those flavonol caffeoylglycosides. Comparison in the activity of the isolates indicated the importance of caffeoyl substructures in the molecule for the α-glucosidase-inhibiting activity. The relatively high contents of the active isolates in the plant suggest that *S. cantoniensis* could be physiologically useful for treatment of diabetes.

KEYWORDS: ***Spiraea cantoniensis*; α-glucosidase inhibitor; flavonol acylglycoside; caffeoyl group**

INTRODUCTION

Diabetes mellitus is one of the most serious chronic diseases that is developing along with an increase in both obesity and aging in the general population (*1*). One of the therapeutic approaches for decreasing postprandial hyperglycemia is to retard absorption of glucose by the inhibition of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive organs (*2*). In recent years, many efforts have addressed the search for effective α-glucosidase inhibitors from natural sources in order to develop a physiological functional food or lead compounds for use against diabetes (*3*). In the course of our search for rat intestinal α-glucosidase-inhibiting principles from plants, we have isolated and identified several active compounds from a variety of plants grown not only in Japan (*4–6*) but also in Thailand (*7, 8*), China (*9, 10*), and Nepal (*11*). In this paper, we present results of a screen of temperate plants in Japan for α-glucosidase inhibition. In the screening experiments for rat intestinal maltase and/or sucrase inhibitors in 218 plants cultivated in Tsukuba, Japan, potent maltase-inhibiting activity was found in extracts of flowers of *Spiraea cantoniensis* (Rosaceae), an ornamental deciduous shrub. There have been only a few reports on the chemical constituents of this plant (*12–14*), but no medicinal usage is known, although some rosaceous plants are known to contain anthocyanins and soluble tannins showing antiglucosidase and antiamylase activities (*15*). Hence, the promising screening result prompted us to isolate and elucidate the structure of active compounds from this plant species.

MATERIALS AND METHODS

Materials. Two hundred and eighteen species of Japanese temperate plants were cultivated and collected in the experiment field in Tsukuba, Japan. All voucher specimens are deposited in Tsukuba Division, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, Tsukuba, Japan. All chemicals used were of reagent grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan) unless otherwise stated. All solvents were distilled before use.

General Procedure. NMR spectra were recorded on a Bruker AMX500 instrument (^{1}H, 500 MHz; ^{13}C, 125 MHz). Chemical shifts were determined relative to residual signals of methanol-d_4 as a solvent (δ_H 3.3 ppm, δ_C 49.0 ppm). Field desorption (FD) and fast atom bombardment (FAB) mass spectra were determined by a JEOL SX102A instrument. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. Melting points were measured on a hot stage and are uncorrected.

Intestinal α-Glucosidase Inhibitory Activity Determination. The maltase- and sucrase-inhibitory activities designating an inhibition of maltose- and sucrose-hydrolyzing activities, respectively, in rat intestinal glucosidase complexes were measured as described previously (*10*). The crude enzyme solution prepared from rat intestinal acetone powder (Sigma-Aldrich Japan Co., Tokyo, Japan) was used as the small intestinal α-glucosidases, maltase and sucrase, showing specific activities of 0.70 and 0.34 U/mL, respectively. The reaction mixture consisted

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Table 1. NMR Assignments of **1**−**3** (Methanol-d_4)

	δ_C, ppm			δ_H, ppm (mult, J in Hz)		
	1	2	3	1	2	3
2	158.4	159.2	159.4			
3	135.6	135.6	135.2			
4	179.1	179.6	179.4			
5	162.6	162.9	162.9			
6	99.7	100.1	100.1	6.26(d, 2.1)	6.14(d, 1.7)	6.14(d, 2.0)
7	165.2	166.2	166.0			
8	94.7	94.9	94.9	6.51(d, 2.1)	6.32(d, 1.7)	6.31(d, 2.0)
9	157.9	158.5	158.4			
10	105.3	105.5	105.6			
1′	122.7	122.6	122.7			
2′	117.4	132.3	132.2	7.87(d, 2.2)	8.05(d, 9.0)	7.98(d, 8.9)
3′	145.3	116.2	116.1		6.84(d, 9.0)	6.81(d, 8.9)
4′	149.3	161.6	161.5			
5′	115.7	116.2	116.1	6.92(d, 8.5)	6.84(d, 9.0)	6.81(d, 8.9)
6′	123.2	132.3	132.2	7.70(dd, 8.5, 2.2)	8.05(d, 9.0)	7.98(d, 8.9)
1″	105.3	105.2	104.1	5.22(d, 7.9)	5.07(d, 7.9)	5.20(d, 7.4)
2″	72.5	72.9	75.8	3.83(dd, 9.6, 7.9)	3.80(dd, 9.9, 7.9)	3.47(m)
3″	74.6	74.9	78.0	3.66(dd, 9.6, 3.3)	3.56(dd, 9.9, 3.3)	3.47(m)
4″	69.5	70.2	71.7	3.90(dd, 3.3, 1.2)	3.80(dd, 3.3, 1.5)	3.34(m)
5″	74.2	74.8	75.7	3.81(ddd, 7.1, 4.9, 1.2)	3.73(ddd, 8.0, 4.4, 1.5)	3.44(m)
6″	63.8	64.3	64.3	4.21(dd, 11.9, 7.1)	4.12(dd, 11.3, 8.0)	4.18(dd, 11.9, 6.7)
				4.26(dd, 11.9, 4.9)	4.32(dd, 11.3, 4.4)	4.28(dd, 11.9, 2.2)
1‴	127.5	127.7	127.7			
2‴	115.1	115.2	115.2	7.10(d, 2.0)	6.92(d, 1.6)	6.95(d, 1.7)
3‴	146.2	147.0	147.0			
4‴	148.7	149.5	149.5			
5‴	116.3	116.5	116.5	6.85(d, 8.1)	6.76(d, 8.1)	6.76(d, 8.4)
6‴	122.7	123.0	123.1	6.88(dd, 8.1, 2.0)	6.76(dd, 8.1, 1.6)	6.79(dd, 8.4, 1.7)
7‴	145.9	146.7	146.7	7.37(d, 16.0)	7.32(d, 16.0)	7.34(d, 15.8)
8‴	115.1	114.6	114.7	6.08(d, 16.0)	6.00(d, 16.0)	6.03(d, 15.8)
9‴	167.2	168.8	168.9			

of crude enzyme solution (0.05 mL of maltase and 0.2 mL of sucrase), substrate (maltose, 3.5 mM, 0.35 mL; sucrose, 56 mM, 0.2 mL) in 0.1 M potassium phosphate buffer (pH 6.3), and the test sample in 50% aqueous dimethyl sulfoxide (0.1 mL). After incubation for 15 min at 37 °C, the reaction was stopped by adding 0.75 mL of 2 M Tris-HCl buffer (pH 7.0). The reaction mixture was passed through a short column of basic alumina (ICN Alumina B, grade I, ICN Biomedical GmbH, Eschwege, Germany) to remove phenolic compounds, which might interfere with enzymatic glucose quantification in the following step. The amount of liberated glucose was measured by the glucose oxidase method, using a commercial test kit (Glucose B-test Wako, Wako Pure Chem. Co., Osaka, Japan).

Screening Experiment. The screening experiments for rat intestinal sucrase and maltase inhibition were carried out with extracts of 509 plant parts from 218 species. Each dried plant part was extracted with 50% aqueous methanol. The extracts were evaporated, redissolved in 50% aqueous dimethyl sulfoxide, and subjected as the test sample to the assay for rat intestinal α-glucosidase inhibitory activity at the final concentration of the extractable constituents obtained from 50 mg of plant material in 1 mL of solution.

Isolation of Quercetin 3-*O*-(6-*O*-Caffeoyl)-*β*-galactoside (1), Kaempferol 3-*O*-(6-*O*-Caffeoyl)-*β*-galactoside (2), and Kaempferol 3-*O*-(6-*O*-Caffeoyl)-*β*-glucoside (3) from *S. cantoniensis* Flower. Dried flowers (100 g) of *S. cantoniensis* were extracted with 50% aqueous methanol and the extracts were concentrated and partitioned between ethyl acetate and water. The inhibitory activity assay was carried out at the concentration of each fraction obtained from 0.1 g of plant material in 1 mL of reaction solution throughout the fractionation. The ethyl acetate fraction showed a strong inhibitory activity of 62% against maltase, whereas the sucrase-inhibitory activity was relatively low (36%). In contrast, the aqueous phase showed inconspicuous activities for both maltase (37%) and sucrase (26%). Hence, further fractionation was performed for isolating maltase inhibitors from the ethyl acetate fraction. This active fraction (6 g of dry weight) was charged onto a silica gel column and eluted with a chloroform−methanol gradient. The maltase-inhibitory activity was eluted in the chloroform−methanol (6:1) eluate (2.1 g of dry weight, 50% inhibition). The eluate was further purified by preparative HPLC (column, Inertsil PREP-ODS, 20 × 250 mm, GL-Science Co.; mobile phase, 27.5% MeCN in water; flow rate, 5.0 mL/min; detection, UV 254 nm). Eight principal peaks were detected in the region of t_R = 15−50 min. Among them, a peak eluted at t_R = 19.4 min, showing the highest activity of 38%, was rechromatographed under the same condition except for using 23% MeCN in water as the mobile phase to give **1** (33 mg, 0.03%, t_R = 39.2 min). A peak cluster that eluted at t_R = 22.5−25.4 min in the first HPLC, showing the second highest activity of 31%, was rechromatographed (column, Inertsil PREP-ODS, 20 × 250 mm × 2 (serial connection); mobile phase, 20% MeCN in water; flow rate, 7.0 mL/min (0−30 min) and 5.0 mL/min (30−120 min); detection, UV 254 nm) to give **2** (26 mg, 0.03%, t_R = 89.0 min) and **3** (29 mg, 0.03%, t_R = 101.8 min). **1**: yellow powder; mp 190−195 °C; $[\alpha]_D^{23}$ −11° (*c* 0.22, MeOH); FD-MS *m/z* 626 ($[M]^+$), 464 (M − 162), 302 (M − 162 × 2); FAB-HR-MS (negative) *m/z* 625.1185 ($[M - H]^-$, calcd for $C_{30}H_{25}O_{15}$, 625.1193). **2**: yellow powder; mp 210−212 °C; $[\alpha]_D^{23}$ −15° (*c* 0.22, MeOH); FD-MS *m/z* 611 ($[M + H]^+$), 448 (M − 162), 286 (M − 162 × 2); FD-HR-MS *m/z* 611.1423 (calcd for $C_{30}H_{27}O_{14}$, 611.1400). **3**: yellow powder; mp 205−208 °C; $[\alpha]_D^{23}$ −41° (*c* 0.24, MeOH); FD-MS *m/z* 611 ($[M + H]^+$), 448 (M − 162), 286 (M − 162 × 2); FD-HR-MS *m/z* 611.1397 (calcd for $C_{30}H_{27}O_{14}$, 611.1400). For ^1H and ^{13}C NMR data, see **Table 1**.

RESULTS AND DISCUSSION

In the screening experiment, 73 and 40 samples showed more than 50% enzyme−inhibitory activity for maltase and sucrase, respectively, out of 509 samples from 218 plant species. Among them, notable inhibitory activity (>90%) against rat intestinal maltase was observed in *Cicuta virosa* (leaf, 97%), *Akebia trifoliate* (berry, 97%), *Punica granatum* (seed, 95%; fruit skin, 100%), *Quercus myrsinaefolia* (seed, 90%), *Wisteria floribunda* (leaf, 95%), *S. cantoniensis* (flower, 91%), *Paeonia suffruticosa* (flower, 96%; leaf, 98%), *Paeonia lactiflora* (fruit, 97%; leaf, 94%), *Rheum unduratum* (root, 92%), and *Lythrum salicaria*

(leaf, 90%), and activity against sucrase was seen for *Akebia trifoliate* (berry, 96%), *Trichosanthes rostrata* (stem, 91%), *Chaenomeles sinensis* (leaf, 93%; stem, 99%), *Elaegnus umbellata* var. *rotundifolia* (fruit, 97%), and *Lythrum salicaria* (leaf, 93%). Among these promising species, we first chose extracts of *S. cantoniensis* flower for identifying active principles, since only a limited number of studies (*12–14*) have been carried out concerning chemical constituents of this species.

Dried flowers of *S. cantoniensis* were extracted with 50% aqueous methanol. After evaporation, the crude extracts were partitioned between ethyl acetate and water. The maltase-inhibiting activity was found principally in the ethyl acetate-soluble part. The active part was chromatographed on silica gel followed by HPLC purification to yield three major compounds, **1**−**3**.

Compound **1** showed a molecular ion at *m*/*z* 626 in FD-MS and the molecular formula was determined as $C_{30}H_{26}O_{15}$ from the high resolution FAB-MS analysis. The characteristic mass spectral fragments at *m*/*z* 464 (M − 162) and 302 (M − 162 × 2), resulting from successive loss of the 162 mass unit, suggested **1** to be a quercetin caffeoylglycoside. The ^{1}H NMR spectrum strongly supported this indication. The aromatic proton region (δ 6.0−8.0) contained 10 protons assignable to H-6, -8, -2′, -5′, and -6′ of quercetin and H-2‴, -5‴, -6‴, -7‴, and -8‴ of caffeic acid. These assignments were supported by the COSY cross peaks of two 1,2,4-trisubstituted benzenes and an isolated *trans*-olefin. The proton signals of a sugar unit appeared at δ 3.6−5.6 and could be fully correlated by COSY. The small coupling constants of 3.3 Hz between H-3″ (δ 3.66) and H-4″ (δ 3.90) and 1.2 Hz between H-4″ and H-5″ (δ 3.81) strongly supported the presence of galactose. In the HMBC spectrum, interunit cross peaks between the anomeric proton of the sugar (H-1″, δ 5.22) and C-3 (δ 135.6) of the aglycon and between the nonequivalent methylene protons (H-6″, δ 4.21 and 4.26) and the ester carbonyl (C-9‴, δ 167.2) were observed, which showed that the galactose was connected to the C-3 of quercetin and that caffeic acid was esterified with 6-OH of the galactose. Thus, **1** was concluded to be quercetin 3-*O*-(6-*O*-caffeoyl)-β-D-galactoside.

Compound **2** showed a pseudomolecular ion ($[M + H]^+$) at *m*/*z* 611 in FD-MS and the high-resolution analysis indicated the molecular formula of $C_{30}H_{26}O_{14}$. The mass spectral fragments at *m*/*z* 448 (M − 162) and 286 (M − 162 × 2) again indicated the presence of a caffeoylglycoside unit like **1**. The difference in molecular formula of **2** and **1** was one oxygen. Hence, **2** was suggested to be the kaempferol analog of **1**. The NMR spectrum of the aromatic region contained a pair of two-proton doublets (J = 9.0 Hz) at δ 6.84 and 8.05, being characteristic of a 4-hydroxyphenyl group in place of one of the 3,4-dihydroxyphenyls in **1**. The sugar unit was determined to be galactose in the similar manner as **1**, that is, J (H-3″/H-4″) = 3.3 Hz and J (H-4″/H-5″) = 1.5 Hz. The HMBC correlations confirmed the connectivity of each unit to be the same as that of **1**. The structure of **2** was determined to be kaempferol 3-*O*-(6-*O*-caffeoyl)-β-galactoside.

Compound **3** gave a similar spectral pattern to **2**, except for the sugar protons and carbons in the NMR spectra. Overlapping of H-2″, -3″, and -5″ at δ 3.44−3.47 and higher field resonance of H-4″ indicated the presence of glucose in **3** in place of galactose in **2** (*16, 17*). In addition, relatively higher chemical shifts of the sugar carbons also support the glucoside structure (*17, 18*). The structure of **3** was thus determined to be kaempferol 3-*O*-(6-*O*-caffeoyl)-β-glucoside.

Table 2. Inhibitory Activity of **1**−**3** and Related Compounds against Rat Intestinal Glucosidases

compd	IC_{50} (mM)	enzyme activity	ref
1	0.085	maltase	this work
2	0.35	maltase	this work
3	0.47	maltase	this work
4	(19%)[a]	maltase	(*29*)
5	(3%)[a]	maltase	(*29*)
6	(28%)[a]	maltase	(*29*)
7	(8%)[a]	maltase	(*29*)
8	0.029	sucrase	(*31*)
9	0.038	sucrase	(*31*)
10	1.89	maltase (immobilized)	(*3*)
11	1.91	maltase (immobilized)	(*3*)
12	18.9	maltase (immobilized)	(*3*)
13	0.024	maltase (immobilized)	(*3*)

[a] Percent inhibition at 0.5 mM.

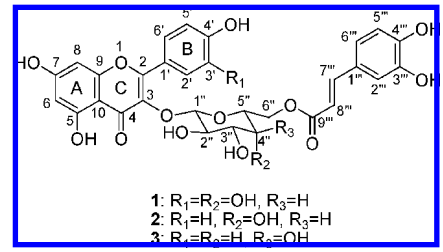

Figure 1. Structures of **1**−**3**.

Quercetin 3-*O*-(6-*O*-caffeoyl)-β-galactoside (**1**) has been identified in several plants including *Hydrocotyle sibthorpioides* (*19*), *Scorzonera columnae* (*20*), *Polygonum viscosum* (*21*), *Blechnum novae-zelandiae* (*22*), *Monochaetum multiflorum* (*23*), and *Vaccinium corymbosum* (*24*), whereas kaempferol 3-*O*-(6-*O*-caffeoyl)-β-galactoside (**2**) was only isolated from *Conyza filaginoides* (*25*) and kaempferol 3-*O*-(6-*O*-caffeoyl)-β-glucoside from *Pteridium aquilinum* (*26*) and *Rubus ulmifolius* (*27*). However, this is the first report on the α-glucosidase-inhibitory activity of those compounds (**Table 2**). The similar extraction and fractionation of dried leaves of *S. cantoniensis* resulted in an isolation of **1** in 0.06% yield.

Among the isolates, the quercetin derivative **1** showed a higher activity (IC_{50} = 0.085 mM) than the kaempferol derivatives **2** (IC_{50} = 0.35 mM) and **3** (IC_{50} = 0.47 mM). The inhibition mode of **1** against rat intestinal maltase was determined to be mixed-inhibition type, as has been seen in other flavonoids (*29*) and the K_i value was calculated to be 110 μM. The moderate inhibitory activities of flavonols and their glycosides against mammalian intestinal glucosidases have been reported (*3, 30*). However, the maltase inhibitory activity (IC_{50} > 1 mM) of both quercetin and its glucoside was apparently lower than that of the caffeoylglycoside **1**. The substitution of the sugar moiety in flavonol glycosides by a phenolic acid, in particular, caffeic acid, could thus enhance their glucosidase inhibitory activity. In addition, the quercetin analogues having the caffeoyl substructure of C-2, -3, -4, and -1′−6′ in the B/C-rings of the flavonoid skeleton showed relatively higher glucosidase inhibitory activity than the corresponding kaempferol derivatives (*28, 29*). In the present case, the most potent compound, **1**, contains two caffeoyl substructures as the acyl substituent on the sugar and the B/C-rings of the aglycon, whereas **2** and **3** carry only one caffeoyl moiety. As shown in **Figures 1** and **2** and **Table 2**, the comparative study on the inhibitory activity of simple flavones and flavonols against rat intestinal glucosidase showed that luteolin (**4**) and quercetin (**6**) were more potent than apigenin (**5**) and kaempferol (**7**),

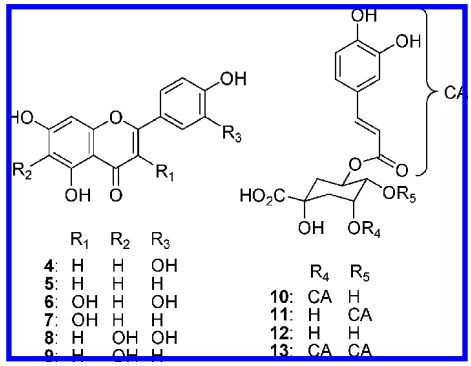

Figure 2. α-Glucosidase inhibitors having caffeoyl moieties.

respectively (*29*). This tendency was also seen in our structure−activity relationship study for baicalein (5,6,7-trihydroxyflavone) derivatives, in which 6-hydroxyluteolin (**8**) showed a higher activity than 6-hydroxyapigenin (**9**) (*31*). In any case, compounds possessing a caffeoyl substructure in the B/C ring of the flavone skeleton, **4**, **6**, and **8**, were more active than their *p*-coumaroyl counterparts, **5**, **7**, and **9**, respectively. In addition, in a series of caffeoylquinic acids, dicaffeoylquinic acids (**10**, **11**) showed a higher activity than monocaffeoyolquinic acid (chlorogenic acid, **12**), and a tricaffeoyl analogue (**13**) was much higher than those dicaffeoyl acids (*3*). These results strongly support the importance of a caffeoyl substructure in the molecule for exerting an effective glucosidase inhibitory activity. In contrast, the difference in sugar part, galactose and glucose, did not significantly affect the inhibitory activity.

In conclusion, the enzyme-assay guided fractionation of the extract from the dried flowers of *S. cantoniensis* led to the isolation of three flavonol caffeoylglycosides, **1**−**3**, as rat intestinal maltase inhibitors. The relatively high contents (0.03% each) of **1**−**3** in the plant compared to those in other plants (<0.002% *19, 21, 23–25*) suggest that *S. cantoniensis* could be physiologically useful for treatment of diabetes, although in vivo experiments are needed.

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