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Structure-activity Relationships for α-Glucosidase Inhibition of Baicalein, 5,6,7-Trihydroxyflavone: the Effect of A-Ring Substitution

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In order to estimate the effects of the A-ring hydroxyl group of baicalein (5,6,7-trihydroxyflavone, 1) on rat intestinal α-glucosidase inhibition, flavone, monohydroxyflavones, dihydroxyflavones, and methylated derivatives of 5,6,7-trihydroxyflavone were used for the structure-activity relationship (SAR) study. The importance of the 6-hydroxyl group of baicalein was validated for an exertion of the activity. And also, the tested flavones which lacked a hydroxyl substituent on any of positions 5, 6, or 7, showed no activity. Hence, the 5,6,7-trihydroxyflavone structure was concluded to be crucial for the potent inhibitory activity. In addition, an introduction of electron-withdrawing or electron-donating groups at position 8 of baicalein led to a dramatic decrease for activity, except for 8-fluoro-5,6,7-trihydroxyflavone, which carried a less bulky substituent on position 8. Hence, this result suggested that a sterically bulky substituent on C-8 of baicalein was detrimental for the activity regardless of its electronic nature. Through examining the inhibitory mechanism of baicalein against rat intestinal α-glucosidase, it was suggested to be a mixed type inhibition.

Key words: 5,6,7-trihydroxyflavone; α-glucosidase inhibitor; structure-activity relationship

Mammalian digestive α-glucosidases, membrane-bound enzymes at the epithelium of the small intestine, hydrolyze disaccharides and oligosaccharides to liberate glucose. Inhibitors of the α-glucosidases can retard the decomposition and absorption of dietary carbohydrates to suppress postprandial hyperglycemia.[1] In the course of our screening study for rat intestinal α-glucosidase-inhibiting substances from natural resources, baicalein (5,6,7-trihydroxyflavone, **1**) was isolated from *Scutellaria baicalensis*,[2] and also five 6-hydroxyflavonoids from marjoram (*Origanum majorana*).[3] Baicalein is a unique flavone bearing a 6-hydroxyl group together with ordinary 5,7-dihydroxyl substituents. In the assay for rat intestinal α-glucosidase inhibitory activity on hydroxyflavones, apigenin (4′,5,7-trihydroxyflavone) and luteolin (3′,4′,5,7-tetrahydroxyflavone), which lack a 6-hydroxyl substituent, did not show any inhibitory activity, while **1**, 6-hydroxyapigenin (4′,5,6,7-tetrahydroxyflavone) and 6-hydroxyluteolin (3′,4′,5,6,7-pentahydroxyflavone) showed high activity. Hence, this result suggested that the 6-hydroxyl substituent on 5,6,7-trihydroxyflavones was crucial for the α-glucosidase inhibitory activity.[3]

We are interested in the structure-activity relationship of **1** and the related compounds against rat intestinal α-glucosidase. In order to estimate the effects of the hydroxyl and other groups on A-ring of **1** for inhibition of the enzyme, we synthesized a series of hydroxyflavones and examined their α-glucosidase inhibitory activity.

Materials and Methods

General experimental procedures. NMR spectra were recorded with a Bruker AMX500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) instrument. Chemical shifts were calculated from the residual solvent signals of δ_H 3.30 ppm in methanol-d_4, δ_H 7.24 ppm in chloroform-d, and δ_H 2.49 and δ_C 39.5 ppm in dimethyl sulfoxide-d_6. Field desorption (FD), FD-high resolution (HR), electron ionization (EI), and EI-HR mass spectra (MS) were obtained on a Jeol JMS-SX102A instrument. Melting points were measured on a hot stage and were uncorrected. The following experimental conditions were used for chromatography: ordinary phase column chromatography; Silica gel Wakogel C-300 (Wako Pure Chem. Co., Osaka, Japan, 40-64 mesh), reverse phase silica gel column chromatography; Cosmosil 75C_{18}-OPN (Nacalai Tesque, Inc., Kyoto, Japan). TLC, precoated TLC plates with Silica gel 60 F_{254} (Merck, 0.25 mm or 0.5 mm thickness, normal phase) and Silica gel RP-18 F_{254s} (Merck, 0.2 mm thickness, reverse phase). Detection was done by UV lamp (254 nm). Preparative HPLC was done with an Inertsil PREP-ODS column (20.0×250 mm, GL-Science). The detailed analytical conditions are mentioned in each section.

All reagents were of reagent grade and were purchased from Wako Pure Chem. Co., Osaka, Japan,

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unless otherwise stated. Rat intestinal acetone powder, flavone (**2**), 5-hydroxyflavone (**3**), 6-hydroxyflavone (**4**), and 7-hydroxyflavone (**5**) were supplied by Sigma Aldrich Japan Co., Tokyo, Japan. 5,7-Dihydroxyflavone (**7**) and 7,8-dihydroxyflavone (**10**) were purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan. Acetone and tetrahydrofuran were dried by storage over 3A molecular sieves. All solvents were distilled before use. All nonaqueous reactions were done in dry glassware.

Assay for rat intestinal α-glucosidase inhibitory activity. The α-glucosidase inhibitory activity was measured as described previously.[4,5] The crude rat intestinal α-glucosidase solution prepared from rat intestinal acetone powder contained 526 mg of protein, and its relative α-glucosidase activity against sucrose was 0.385 unit/mg protein or 0.352 unit/ml enzyme solution. The reaction mixture consisted of crude enzyme solution (0.2 ml), 56 mM sucrose in 0.1 M potassium phosphate buffer (pH 6.3, 0.2 ml), and the test sample in 50% aqueous dimethyl sulfoxide (DMSO, 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 glucose measurement.[2] 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). The concentration of inhibitors required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value. The IC_{50} value was measured graphically by a plot of percent inhibition versus log of the test compound.

*5,6-Dihydroxyflavone (**6**) and 5,8-dihydroxyflavone (**8**).* To a stirred solution of 5-hydroxyflavone (**3**) (4.76 g, 20 mmol) in water (100 ml) containing sodium hydroxide (4 g, 100 mmol) was added dropwise during 4 hr a solution of potassium persulfate (6 g, 22 mmol) in water (200 ml), the temperature being kept at 15–20 °C. After 24 hr, the solution was acidified to pH 4–5 and filtered. The filtrate was extracted twice with ether. To the aqueous phase was added concentrated hydrochloric acid (20 ml), and this was refluxed for 2 hr. The resulting mixture was cooled and extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate. After the ethyl acetate was removed, the resulting oil was put through silica gel preparative thin layer chromatography using chloroform-methanol (100:4) as the eluent to give **6** (R_f 0.46, 254 mg, 5%) as yellow crystalline solid: mp 193–195 °C (lit.[6] mp 189–190 °C); FD-MS *m/z* (%): 254 (100, $[M]^+$); and **8** (R_f 0.56, 305 mg, 6%) as yellow powders: mp 230–232 °C (lit.[6] mp 230–231 °C); FD-MS *m/z* (%): 254 (100, $[M]^+$).

*6,7-Dihydroxyflavone (**9**).* A mixture of 1,2,4-trihydroxybenzene (5 g, 40 mmol) and ethyl benzoylacetate (13.5 g, 70 mmol) in diphenyl ether (25 ml) was heated under reflux for 4 hr. After cooling, the mixture was put through silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to give **9** (3.25 g, 32%) as yellow powders: mp 254–256 °C (lit.[6] mp 254 °C); EI-HR-MS *m/z* 254.0617 (calcd. for $C_{15}H_{10}O_4$, 254.0579).

*5-Hydroxy-6,7-dimethoxyflavone (**11**) and 5,7-dihydroxy-6-methoxyflavone (**12**).* A mixture of **1** (100 mg, 0.37 mmol) and methyl iodide (0.2 ml, 2.5 mmol) in dry acetone (15 ml) and anhydrous potassium carbonate (0.35 g, 2.5 mmol) was well stirred under reflux for 2 hr. The reaction mixture was cooled and filtered. The filtrate was concentrated and the resulting oil was put through preparative thin layer chromatography using chloroform-methanol-formic acid (50:2:1) as the eluent to yield **11** (R_f 0.78, 60 mg, 60%) as pale yellow powders: mp 157–159 °C (lit.[7] 146–148.7 °C); EI-HR-MS *m/z* 298.0827 (calcd. for $C_{17}H_{14}O_5$, 298.0841); and **12** (R_f 0.54, 15 mg, 15%) as yellow powders: mp 181–184 °C (lit.[7] 184–186 °C); EI-HR-MS *m/z* 284.0695 (calcd. for $C_{16}H_{12}O_5$, 284.0685).

*5,6-Dihydroxy-7-methoxyflavone (**13**).* To a solution of **1** (0.78 g, 3 mmol) in *N*,*N*-dimethylformamide (15 ml) were added lithium carbonate (0.8 g, 8 mmol) and methyl iodide (6 mmol). The resulting suspension was stirred at 55 °C for 17 hr. The reaction mixture was cooled, poured into water (20 ml) containing concentrated hydrochloric acid (10 ml), and extracted with ethyl acetate (50 ml×3). The extracts were washed with water and dried over anhydrous sodium sulfate. After the ethyl acetate was removed, the residue was recrystallized from 70% acetic acid to give **13** (0.61 g, 72%) as yellow powders: mp 223–224 °C (lit.[8] 225–228 °C); EI-HR-MS *m/z* 284.0670 (calcd. for $C_{16}H_{12}O_5$, 284.0685).

*5-Hydroxy-6,7-dimethoxy-8-nitroflavone (**14a**).* Sodium nitrate (0.85 g, 10 mmol) and lanthanum nitrate (44 mg, 0.1 mmol) were dissolved in water (10 ml). To this solution was added **11** (2.98 g, 10 mmol) dissolved in chloroform (40 ml). The reaction mixture was stirred for 12 hr at room temperature and then extracted with chloroform. The organic phase was washed with water and dried over anhydrous sodium sulfate. After removal of the chloroform, the resulting oil was put through silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to yield **14a** (1.85 g, 54%) as pale yellow powders: mp 213–214 °C; EI-HR-MS *m/z* 343.0664 (calcd. for $C_{17}H_{13}O_7N$, 343.0692); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.95 (3H, s, OMe-6), 3.97 (3 H, s, OMe-7), 6.75 (1H, s, H-3), 7.50–7.57 (3H, m, H-3′, 4′ and 5′), 7.83–7.85 (2H, m, H-2′ and 6′), 13.28 (1H, s, OH-5).

*5,6,7-Trihydroxy-8-nitroflavone (**14**).* **14a** (1 mmol, 343 mg) was dissolved in dichloromethane (30 ml) and cooled to −80 °C and 4 ml of 1 M boron tribromide in dichloromethane (4 mmol) was added dropwise with stirring. The mixture was stirred for 1 hr at −80 °C and for 12 hr at room temperature. The reaction was stopped by adding ice-water (20 ml). To this mixture was added 1-butanol (50 ml), and the organic phase was separated, washed with water, and evaporated to dryness. The residue was taken up in methanol and put through reverse phase silica gel column chromatography using water-methanol-formic acid (30:70:0.1) as the eluent to yield the crude solid of **14**. The solid was further purified by preparative HPLC (mobile phase, water-acetonitrile-formic acid (60:40:0.1); flow rate, 5.0 ml/min; t_R 19 min; detection, UV 254 nm) to give **14** (201 mg, 63%) as yellow powders: mp 266 °C; FD-HR-MS *m/z* 315.0361 (calcd. for $C_{15}H_9O_7N$, 315.0379); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 7.15 (1H, s, H-3), 7.51–7.62 (3H, m, H-3′, 4′ and 5′), 7.98 (2H, d, *J*=5.1, H-2′ and 6′), 13.13 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 102.6 (C-10), 105.2 (C-3), 129.5 (C-6), 126.3 (C-2′ and 6′), 129.3 (C-3′ and 5′), 146.5 (C-7), 130.2 (C-1′), 132.3 (C-4′), 121.8 (C-8), 142.8 (C-5), 149.1 (C-9), 162.9 (C-2), 181.6 (C-4).

*8-Amino-5-hydroxy-6,7-dimethoxyflavone (**21a**).* A solution of **14a** (172 mg, 0.5 mmol) in tetrahydrofuran (30 ml) was hydrogenated for 12 hr in the presence of 10% palladium on charcoal (20 mg). The catalyst was removed by filtration to afford **21a** (148 mg, 95%) as pale yellow powders: mp 134 °C; EI-HR-MS *m/z* 313.0981 (calcd. for $C_{17}H_{15}O_5N$, 313.0950); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 3.85 (3H, s, OMe-6), 3.96 (3H, s, OMe-7), 7.05 (1H, s, H-3), 7.56–7.64 (3H, m, H-3′, 4′, and 5′), 8.27 (2H, d, *J*=7.6, H-2′ and 6′).

*8-Amino-5,6,7-trihydroxyflavone (**21**).* By the same method described in **14** from **14a**, compound **21** was obtained from **21a** (0.4 mmol, 125 mg). Crystallization from hexane-ethyl acetate to give **21** (81 mg, 71%) as yellow powders: mp 204–205 °C; FD-HR-MS *m/z* 285.0652 (calcd. for $C_{15}H_{11}O_5N$, 285.0637); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 6.68 (1H, s, H-3), 7.55–7.56 (3H, m, H-3′, 4′ and 5′), 8.06 (2H, d, *J*=7.9, H-2′ and 6′).

*8-Formyl-5-hydroxy-6,7-dimethoxyflavone (**15a**).* A mixture of **11** (2.98 g, 10 mmol), hexamethylenetetramine (1.68 g, 12 mmol), and trifluoroacetic acid (50 ml) was heated under reflux for 24 hr. The mixture was poured into 4 M hydrochloric acid (50 ml), stirred for 1 hr, and extracted with chloroform (200 ml×2). The combined organic extracts were washed successively with water, and sat. brine, then dried over anhydrous sodium sulfate. After removal of the chloroform, the resulting oil was put through silica gel column chromatography using chloroform as the eluent to give **15a** as yellow powders: mp 205–206 °C ; FD-HR-MS *m/z* 326.0810 (calcd. for $C_{18}H_{14}O_6$, 326.0790); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.95 (3H, s, OMe-6), 4.19 (3H, s, OMe-7), 6.81 (1H, s, H-3), 7.54–7.55 (3H, m, H-3′, 4′ and 5′), 8.13–8.15 (2H, m, H-2′ and 6′), 10.43 (1H, s, CHO-8), 13.69 (1H, s, OH-5).

*8-Formyl-5,6,7-trihydroxyflavone (**15**).* By the same method as **14** and **21**, **15a** (1 mmol, 326 mg) was demethylated to **15**. Crystallization from hexane-ethyl acetate to give **15** (223 mg, 75%) as yellow powders: mp 274–276 °C; EI-HR-MS *m/z* 298.0471 (calcd. for $C_{16}H_{10}O_6$, 298.0477); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 7.19 (1H, s, H-3), 7.58–7.62 (3H, m, H-3′, 4′, and 5′), 8.21 (2H, d, *J*=7.4, H-2′ and 6′), 10.46 (1H, s, CHO-8), 13.69 (1H, s, OH-5).

*5,6,7-Trihydroxy-8-methylflavone (**19**).* To a solution of **15** (200 mg, 0.5mmol) and sodium cyanoborohydride (1.5 mmol, 95 mg) in 30 ml of tetrahydrofuran was added methyl orange (0.1 mg) as an indictor, giving the solution a yellow color. The mixture was stirred for 3 hr at room temperature. Hydrochloric acid (1 M) was occasionally added to the reaction mixture in order to keep the color of the solution red. The mixture was diluted with water (50 ml), and extracted with ethyl acetate (50 ml×3). After removing the ethyl acetate, the residue was dissolved in a small amount of methanol and put through preparative HPLC (mobile phase, water-acetonitrile-formic acid (40:60:0.1); flow rate, 5.0 ml/min; t_R 21 min; detection, UV 254 nm) to give **19** (92 mg, 65%) as yellow powders: mp 286 °C (lit.[9] 286–288 °C); FD-HR-MS *m/z* 284.0706 (calcd. for $C_{16}H_{12}O_5$, 284.0685).

*8-Bromo-5,6,7-trihydroxyflavone (**16**).* To a suspension of **1** (0.18 g, 0.67 mmol) in methanol (20 ml), 30% H_2O_2 (1 ml) was added dropwise at 0–5 °C and then 47% aqueous hydrobromic acid (0.5 ml) was added dropwise for 20 min. After this was stirred for 4 hr at 0–5 °C, the mixture was warmed to 20 °C and further stirred for 1 hr. The reaction mixture was poured into water (200 ml). The precipitate was collected by suction and washed with water. The solid was further purified by preparative HPLC (mobile phase, water-methanol-formic acid (20:80:0.1); flow rate, 5.0 ml/min; t_R 24 min; detection, UV 254 nm) to give **16** (95 mg, 41%) as yellow powders: mp 239 °C; FD-HR-MS *m/z* 347.9655 (calcd. for $C_{15}H_9O_5Br$, 347.9633); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 7.07 (1H, s, H-3), 7.60–7.61 (3H, m, H-3′, 4′ and 5′), 8.12 (2H, dd, *J*=7.4, 1.1, H-2′ and 6′), 12.76 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 86.9 (C-8), 104.4 (C-10), 104.6 (C-3), 126.3 (C-2′ and 6′), 129.2 (C-3′ and 5′), 129.6 (C-6), 130.7 (C-1′), 132.1 (C-4′), 146.2 (C-5), 146.6 (C-7), 151.7 (C-9), 162.9 (C-2), 182.1 (C-4).

*8-Chloro-5,6,7-trihydroxyflavone (**17**) and 8-fluoro-*

*5,6,7-trihydroxyflavone (**18**).* A solution of **11** (1 mmol, 298 mg) in 5 ml of 1,1,2-trichloroethane was treated with N-fluoropyridinium triflate (1.2 mmol, 293 mg) under reflux for 32 hr. The reaction mixture was directly put through silica gel column chromatography using hexane-ethyl acetate (3:7) as the eluent to obtain a mixture of 8-chloro-5-hydroxy-6,7-dimethoxyflavone and 8-fluoro-5-hydroxy-6,7-dimethoxyflavone (R_f 0.62, 192 mg).

By the same method as **14**, the crude mixture was demethylated by boron tribromide to give a mixture of two main products. The resultant solid was separated by preparative HPLC (mobile phase, water-acetonitrile-formic acid (40:60:0.1); flow rate, 5.0 ml/min; detection, UV 254 nm) to give 21 mg of **17** (t_R 15.6 min) as yellow powders: mp 264–265 °C; EI-HR-MS *m/z* 304.0126 (calcd. for $C_{15}H_9O_5Cl$, 304.0139); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 7.04 (1H, s, H-3), 7.58–7.59 (3H, m, H-3′, 4′ and 5′), 8.08 (2H, d, *J*=6.9, H-2′ and 6′), 9.53 (1H, s, OH-7), 10.91 (1H, s, OH-6), 12.69 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 98.0 (C-8), 104.2 (C-10), 104.7 (C-3), 126.2 (C-2′ and 6′), 129.2 (C-3′ and 5′), 129.7 (C-6), 130.7 (C-1′), 132.1 (C-4′), 145.6 (C-5), 145.7 (C-7), 150.5 (C-9), 162.8 (C-2), 182.1 (C-4); and 86 mg of **18** (t_R 20.1 min) as yellow powders: mp 251 °C; EI-HR-MS *m/z* 288.0446 (calcd. for $C_{15}H_9O_5F$, 288.0434); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.97 (1H, s, H-3), 7.56–7.62 (3H, m, H-3′, 4′ and 5′), 8.02–8.03 (2H, m, H-2′ and 6′), 9.38 (1H, s, OH-7), 10.85 (1H, s, OH-6), 12.26 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 102.4 (C-10), 104.8 (C-3), 126.3 (C-2′ and 6′), 129.2 (C-3′ and 5′), 130.2 (C-6), 132.1 (C-4′), 134.1 (C-7), 132.3 (C-8), 142.6 (C-9), 142.3 (C-5), 162.8 (C-2), 181.9 (C-4).

*5,8-Dihydroxy-6,7-dimethoxyflavone (**20a**).* By the same method as **6** and **8**, compound **20a** was obtained from **11** by the oxidation with potassium persulfate. Silica gel column chromatography using hexane-ethyl acetate (10:1) as the eluent gave **20a** (378 mg, 12%) as a colorless solid: mp 230–231 °C (lit.[10] 232 °C); EI-HR-MS *m/z* 314.0762 (calcd. for $C_{17}H_{14}O_6$, 314.0790).

*5,6,7,8-Tetrahydroxyflavone (**20**).* A mixture of **20a** (157 mg, 0.5 mmol), 47% aqueous hydrobromic acid (0.7 ml, 6.2 mmol), and acetic acid (15 ml) was heated under reflux for 23 hr. After cooling to room temperature, the reaction mixture was extracted with 1-butanol (50 ml×2). The organic phase was washed with water and dried over anhydrous sodium sulfate. After the 1-butanol was removed, the residue was dissolved in a small amount of methanol and put through reverse phase silica gel column chromatography using water-methanol-formic acid (50:50:0.1) as the eluent to yield crude **20**. The solid was further purified with preparative HPLC (mobile phase, water-methanol-formic acid (70:30:0.1); flow rate, 4.0 ml/min; t_R 17.6 min; detection, UV 254 nm) to give **20** (117 mg, 82%) as yellow powders: mp 232–233 °C; EI-HR-MS *m/z* 286.0502 (calcd. for $C_{15}H_{10}O_6$, 286.0477); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.89 (1H, s, H-3), 7.56–7.61 (3H, m, H-3′, 4′ and 5′), 8.13–8.15 (2H, m, H-2′ and 6), 12.16 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 102.9 (C-10), 104.1 (C-3), 125.5 (C-8), 126.4 (C-2′ and 6′), 129.0 (C-3′ and 5′), 129.6 (C-6), 131.1 (C-1′), 131.8 (C-4′), 139.0 (C-7), 139.7 (C-5), 143.8 (C-9), 162.6 (C-2), 182.5 (C-4).

*5,6,7-Trihydroxy-8-piperidinomethylflavone (**22**).* To a stirred solution of **1** (0.54 g, 2 mmol) in ethanol (20 ml) was added a solution of methylene-bis-piperidine (0.73 g, 4 mmol) in ethanol (10 ml). The reaction mixture was refluxed for 1 hr. After cooling overnight at 4 °C, the solid formed was separated and washed twice with cold ethanol. Recrystallization from ethanol gave **22** as yellow powders: mp 220–221 °C; FD-HR-MS *m/z* 367.1400 (calcd. for $C_{21}H_{21}O_5N$, 367.1420); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 4.60 (2H, s, CH_2-8), 4.85 (10H, m, piperidine), 6.83 (1 H, s, H-3), 7.59–7.61 (3H, m, H-3′, 4′, and 5′), 8.03 (2H, dd, *J*=5.2, 1.7, H-2′ and 6′).

Results and Discussion

To embark upon the synthesis of 5,6-dihydroxyflavone (**6**) and 5,8-dihydroxyflavone (**8**), 5-hydroxyflavone (**3**) was chosen as the starting material, which was prepared by the method of Bois *et al.*[11] Compounds **6** and **8** were obtained from **3** by Elb's persulfate oxidation.[12] Treatment of 1,3,4-trihydroxybenzene with ethyl benzoylacetate in boiling diphenyl ether yielded 6,7-dihydroxyflavone (**9**) in 32% yield.[13] Methylation of **1** with methyl iodide and potassium carbonate gave 5-hydroxy-6,7-dimethoxyflavone (**11**) and 5,7-dihydroxy-6-methoxyflavone (**12**),[14] while 5,6-dihydroxy-7-methoxyflavone (**13**) was obtained selectively from **1** with methyl iodide and lithium carbonate.[15] UV shift tests for the position of 5 and 7-OH on hydroxyflavones, and comparison of mass and NMR spectra with reference data confirmed the structures of **3**, **6**, **8**, **9**, and **11**–**13**.

Compound **11** was treated by sodium nitrate in a two-phase system (water-chloroform) in the presence of a catalytic amount of lanthanum nitrate to give 5-hydroxy-6,7-dimethoxy-8-nitroflavone (**14a**) in 54% yield.[16] **14a** was demethylated by boron tribromide to yield 5,6,7-trihydroxy-8-nitroflavone (**14**).[17] Hydrogenation of **14a** afforded **21a**, which was then treated with boron tribromide to give 8-amino-5,6,7-trihydroxyflavone (**21**).[18] Compound **11** was formylated with Duff reaction conditions, using hexamethylenetetramine and TFA to yield 8-formyl-5-hydroxy-6,7-dimethoxyflavone (**15a**), which was demethylated to obtain 8-formyl-5,6,7-trihydroxyflavone (**15**).[19] Compound **15** was reduced with sodium cyanoborohydride to give 5,6,7-trihydroxy-8-methylflavone (**19**) in 65% yield.[20] Bromination of **1** with HBr-H_2O_2 gave 8-bromo-5,6,7-trihdyroxyflavone (**16**).[21] Treatment of **11** with *N*-fluoropyridinium triflate

in boiling 1,1,2-trichloroethane gave a mixture of 8-chloro-5-hydroxy-6,7-dimethoxyflavone and 8-fluoro-5-hydroxy-6,7-dimethoxyflavone, which was demethylated to give 8-chloro-5,6,7-trihdyroxyflavone (**17**) and 8-fluoro-5,6,7-trihydroxyflavone (**18**), respectively.[22] Compound **11** was converted to 5,8-dihydroxy-6,7-dimethoxyflavone (**20a**) by alkaline persulfate oxidation, and was then demethylated to give 5,6,7,8-tetrahydroxyflavone (**20**).[23] Condensation of **1** with methylene-bis-piperidine in refluxing ethanol gave 5,6,7-trihydroxy-8-piperidinomethylflavone (**22**) in good yield.[24] The structures of **14**–**22** were confirmed by their mass and NMR spectra.

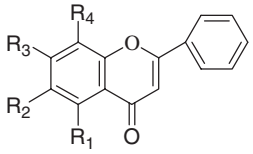
In order to estimate the effect of the A-ring hydroxyl group of **1** on α-glucosidase inhibitory activity, **1** and related compounds (**2**–**13**) were tested for inhibition of rat intestinal α-glucosidase with sucrose as the substrate (Table 1). As previously reported, **1** was a potent inhibitor of the α-glucosidase with IC_{50}=45 μM. On the other hand, **7**, which lacks a 6-hydroxyl group, did not show any activity. Hence, it was concluded that the 6-hydroxyl substituent of **1** was necessary for the activity. And also, removal of any hydroxyl group at positions 5,6,7 led to a dramatic loss of the inhibitory potency (**2**–**10**). This result indicated that the 5,6,7-trihydroxyflavone structure was crucial for high activity. Further evidence supporting this rationale is that replacement of the hydroxyl group with a methoxyl group at position 6 or 7 (**11**–**13**) appeared to be detrimental for the activity.

Having identified the importance of the 5,6,7-trihydroxyl group of **1**, we decided to explore incorporation of an additional hydroxyl group onto position 8 of **1** to afford **20**. Unfortunately, this also led to a reduction in potency. So, the question remains, why **20** decreases the activity despite having the 5,6,7-trihdyroxyflavone structure. Two possibilities are electronic and steric effects.

With the initial structure-activity relationship (SAR) results, we turned our attention to an electronic effect of substituents at position 8 of **1** on the activity. We used the *Hammett* substituent constant[25] to evaluate the electronic influence of **1** and **14**–**21** (Table 2). An introduction of an electron-withdrawing group (**14**–**17**) at position 8 of **1** resulted in disappearance of α-glucosidase inhibitory activity, though **18** was tolerated with a minor loss of potency. However, **19**–**21**, which have an electron-donating group at position of 8, were also inactive, although **20** (IC_{50}=960 μM) and **21** (IC_{50}=1000 μM) were very weak inhibitors. Except for **18**, an introduction of an electron-withdrawing or electron-donating group at position 8 of **1** resulted in a detrimental effect on the potent activity. Hence, it was considered that an electronic effect could not account for this reduction in potency. These 8-position substitutions (**14**–**17**, **19**–**21**) of **1**, which resulted in a disadvantage for α-glucosidase inhibitory activity, was then due possibly to a steric interference to the interaction between the 5,6,7-trihydroxyl group of **1** and the enzyme. Compound **22**, which has a large piperidinomethyl group at position 8, was also inactive, while **18** (IC_{50}=86 μM) which has a less bulky fluorine at position 8 showed moderate activity compared to other 8-substitued 5,6,7-trihydroxyflavones (**14**–**17**, **19**–**21**), suggesting that excess steric bulkiness around position 8 of **1** is detrimental for the potent inhibitory activity.

In order to examine the types of inhibition of rat intestinal α-glucosidase by **1**, α-glucosidase solution

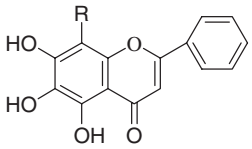
Table 1. Rat Intestinal α-Glucosidase Inhibition of Hydroxylated Flavone Derivatives



Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)
1	OH	OH	OH	H	45
2[a]	H	H	H	H	NI[b]
3[a]	OH	H	H	H	NI
4[a]	H	OH	H	H	NI
5[a]	H	H	OH	H	NI
6	OH	OH	H	H	NI
7	OH	H	OH	H	NI
8	OH	H	H	OH	NI
9	H	OH	OH	H	NI
10	H	H	OH	OH	NI
11[a]	OH	OMe	OMe	H	NI
12	OH	OMe	OH	H	NI
13	OH	OH	OMe	H	NI

[a] The sample was dissolved in 100% DMSO in this assay. [b] less than 30% inhibition at the concentration of 1000 μM.

Table 2. The *Hammett* Substituent Constant of 8-Substituted Groups of 5,6,7-Trihdyroxyflavones, and Their Inhibitory Activity against Rat Intestinal α-Glucosidase



Compound	R	δ_p[a]	IC_{50} (μM)
1	H	0	45
14	NO_2	0.81	NI[b]
15	CHO	ND[c]	NI
16	Br	0.26	NI
17	Cl	0.24	NI
18	F	0.15	86
19	CH_3	−0.14	NI
20	OH	−0.38	960
21	NH_2	−0.57	1000
22	$C_5H_{10}NCH_2$[d]	ND	NI

[a] *Hammett* substituent constant.[25] [b] less than 30% inhibition at the concentration of 1000 μM. [c] no data available. [d] piperidin-1-ylmethyl.

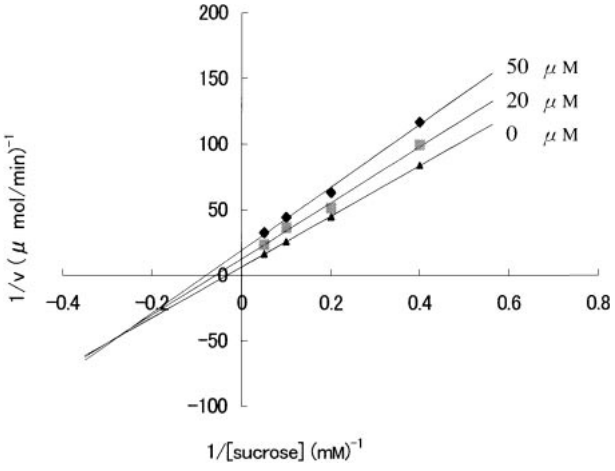


Fig. 1. Lineweaver-Burk Plots of the Inhibition of Rat Intestinal α-Glucosidase Activity by Baicalein (**1**).
Mixed inhibition, K_i=0.11 mM, K_m=12.3 mM.

was incubated with increasing concentration of sucrose (6.25 mM–50 mM). The result, plotted according to Lineweaver-Burk, revealed a fully mixed inhibiting type on the enzyme and K_i value of **1** on sucrose-hydrolyzing activity was 0.11 mM (Fig. 1).

In summary, **1** is a potent rat intestinal α-glucosidase inhibitor with mixed inhibitory mechanism. The SAR studies indicated that 5,6,7-trihdyroxyflavone structure was crucial for the activity, and validated the importance of the 6-hydroxyl substitution previously described. And also, 8-substituted derivatives of **1** tended to decrease the activity regardless of electronic nature of the substituents. Hence, it was suggested that possible excess steric bulkiness around position 8 was detrimental for the potent inhibitory activity.

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