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Importance of the B Ring and Its Substitution on the α-Glucosidase Inhibitory Activity of Baicalein, 5,6,7-Trihydroxyflavone

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Hydroxychromones and B-ring-substituted 5,6,7-trihydroxyflavones were prepared to evaluate the contribution of the B ring of baicalein (5,6,7-trihydroxyflavone, 1) to its potent α-glucosidase inhibitory activity. Hydroxychromones, which lack 6-hydroxyl substitution, did not show any inhibitory activity, while 5,6,7-trihydroxy-2-methylchromone (5) showed high activity. Among the tested B-ring-substituted 5,6,7-trihydroxyflavones, the 4′-hydroxy-, 3′,4′-dihydroxy-, and 3′,4′,5′-trihydroxy-substituted derivatives were found to give more activity than that of 1. The methoxy-substituted derivatives, however, showed less activity than 1. The results suggest that the B ring of 1 was not essential, although advantageous to the activity; hydroxyl substitution on the B ring of 5,6,7-trihydroxyflavones was favorable to the activity, whereas methoxyl substitution was unfavorable; at least 4′-hydroxyl substitution of 5,6,7-trihydroxyflavones was required for enhanced activity, in which the number of hydroxyl groups did not take part.

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

The α-glucosidase enzyme catalyzes the final step in the digestive process of carbohydrates; hence, α-glucosidase inhibitors can retard the decomposition and absorption of dietary carbohydrates to suppress postprandial hyperglycemia.[1)] The results of the previous study on 6-hydroxylated flavonoids as α-glucosidase inhibitors indicate that the 5,6,7-trihydroxyflavone structure was crucial for exerting high activity.[2,3)] We have recently reported the effect of A-ring substitution of baicalein (**1**), 5,6,7-trihydroxyflavone, on the α-glucosidase inhibition.[4)] Structure-activity relationship (SAR) studies validated the importance of the 6-hydroxyl group of **1** to this activity. The 8-substituted derivatives of **1** tended to have less activity regardless of the electronic nature of the substituents, suggesting that excess steric bulkiness around position 8 was detrimental to the potent inhibitory activity.

We examined in this study a class of hydroxychromones and B-ring-substituted 5,6,7-trihydroxyflavones in order to evaluate the contribution of the B-ring of **1** toward the α-glucosidase inhibitory activity.

Materials and Methods

General experimental procedures. NMR spectra were recorded with a Bruker AMX500 instrument (^{1}H, 500 MHz; ^{13}C, 125 MHz). Chemical shift data were calculated from the residual solvent signals of δ_H 3.30 and δ_C 49.0 ppm in methanol-d_4, δ_H 2.04 and δ_C 29.8 ppm in acetone-d_6, δ_H 7.24 and δ_C 77.0 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 with a Jeol JMS-SX102A instrument. Melting point data were measured with a hot-stage apparatus and are uncorrected. The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Wakogel C-300 (Wako Pure Chem. Co., Osaka, Japan); reverse-phase ODS column chromatography, Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan); TLC, precoated TLC plates with silica gel 60 F_{254} (Merck, 0.25 mm or 0.5 mm thickness, normal phase) and RP-18 F_{254s} (Merck, 0.2 mm thickness, reverse phase). Spots were detected by a UV lamp (254 nm). Preparative HPLC was conducted with an Inertsil PREP-ODS column (20.0 × 250 mm, GL-Science). Detailed analytical conditions are mentioned in each section.

All chemicals used were of reagent grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan) unless otherwise stated. Biflorin (**2**) was isolated from dried flower buds of clove in our laboratory.[5)] Rat intestinal acetone powder, 2,3,4-trihydroxybenzaldehyde (**6**) and 2′,3′,4′-trihydroxyacetophenone (**7**), were supplied by Sigma Aldrich Japan Co. (Tokyo, Japan). Acetone and tetrahydrofuran were dried by storing over 3A molecular sieves. All solvents were distilled before use.

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Assay for rat intestinal α-glucosidase inhibitory activity. The α-glucosidase inhibitory activity was measured as described previously.[4] The concentration of an inhibitor required to inhibit 50% of the α-glucosidase activity under the assay conditions is defined as the IC_{50} value. The activity of each test compound is presented as the relative value (RA) defined as $IC_{50\ (baicalein)}/IC_{50\ (test\ compound)}$.[6]

*5,7-Dihydroxy-2-methylchromone (**3**).* A mixture of biflorin (**2**, 52 mg, 0.15 mmol), phenol (0.22 g, 2.34 mmol) and hydriodic acid (1.7 g/ml specific gravity, 1 ml) was refluxed for 6 hr. The reaction mixture was cooled, and an aqueous solution of $NaHSO_3$ (10%, 5 ml) was added to it. The precipitate appearing was collected by filtration and washed with water. The solid was dissolved in a small amount of methanol and subjected to normal-phase silica gel column chromatography, using chloroform–methanol (20:1) as the eluent, to give **3** (14 mg, 50%) as colorless needles: mp 118–119 °C (lit.[7] mp 117–118 °C); FD-HR-MS m/z 192.0450 (calcd. for $C_{10}H_8O_4$, 192.0422).

*6-Acetyl-5,7-dihydroxy-2-methylchromone (**4**).* A solution of 2′,4′,6′-trihydroxyacetophenone (7 g, 41.7 mmol) and ethyl acetoacetate (21 ml, 224 mmol) in 20 ml of diphenyl ether was refluxed for 3 hr. After cooling, diethyl ether (40 ml) was added, and the insoluble solid formed was filtered and washed twice with diethyl ether. This solid was recrystallized from methanol and chloroform to give **4** (1 g, 10%) as yellow plates: mp 206–208 °C (lit.[8] 205–207 °C); HR-FD-MS m/z 234.0524 (calcd. for $C_{12}H_{10}O_5$, 234.0528).

*5,6,7-Trihydroxy-2-methylchromone (**5**).* To an ice-cooled solution of **4** (117 mg, 0.5 mmol) in 4% NaOH (4 ml, 4 mmol), was added dropwise a mixture of 30% hydrogen peroxide (200 μl, 1.5 mmol) and 4% NaOH (2 ml, 2 mmol) in an atmosphere of argon over 3 hr. After acidifying with 1 M HCl, the solid formed was extracted with ethyl acetate (3 × 50 ml). The ethyl acetate layer was washed with water and dried over sodium sulfate. After removing the solvent, the residue was dissolved in a small amount of methanol and subjected to reverse-phase column chromatography, using water–methanol–formic acid (60:40:0.1) as the eluent, to give crude **5**. This was further purified by preparative HPLC (mobile phase, water-methanol-formic acid (45:65:0.1); flow rate, 4.0 ml/min; detection, UV 254 nm) to give **5** (t_R 13 min, 12 mg, 12%) as yellow powder: mp 286–287 °C (lit.[8] 285–287 °C); FD-HR-MS m/z 208.0394 (calcd. for $C_{10}H_8O_5$, 208.0371).

*5,7-Dihydroxy-3′,4′,5′-trimethoxyflavone (**8a**).* To a solution of 2′,4′,6′-trihydroxyacetophenone (3.36 g, 20 mmol) in dry acetone (100 ml) was added anhydrous potassium carbonate (13.8 g, 100 mmol), and the mixture was stirred at room temperature for 10 min. 3,4,5-Trimethoxybenzoyl chloride (5.52 g, 24 mol) was then added, and the mixture was refluxed for 24 hr. After cooling to room temperature, water was added, and the acetone was removed. The aqueous mixture was extracted with ethyl acetate (3 × 100 ml). The extract was washed with water and dried over sodium sulfate. After removing the solvent, the residue was crystallized from ethyl acetate and hexane to give **8a** (2.61 g, 38%) as yellow powder: mp 276–277 °C; FD-HR-MS m/z 344.0879 (calcd. for $C_{18}H_{16}O_7$, 344.0896); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 3.74 (3H, s, 4′-OMe), 3.90 (6H, s, 3′ and 5′-OMe), 6.21 (1H, d, J = 1.7, 6-H), 6.57 (1H, d, J = 1.7, 8-H), 7.06 (1H, s, 3-H), 7.33 (2H, s, 2′ and 6′-H), 10.85 (1H, s, 7-OH), 12.85 (1H, s, 5-OH).

*6-Acetyl-5,7-dihydroxy-3′,4′,5′-trimethoxyflavone (**8b**).* Zinc chloride (a 1.0 M solution in diethyl ether, 0.5 ml, 0.5 mmol) was mixed with glacial acetic acid (2 ml), and the diethyl ether was then removed. After adding acetic anhydride (60 μl, 0.64 mmol) and **8a** (0.22 g, 0.64 mmol), the temperature was maintained at 140–150 °C for 2 hr while frequently stirring. The reaction mixture was cooled to room temperature and poured into ice-cooled water. The solid material was separated and purified by silica gel preparative thin-layer chromatography, using chloroform-methanol (10:0.05) as the eluent, to give **8b** (R_f 0.76, 27 mg, 11%) as yellow powder: mp 237 °C; FD-HR-MS m/z 386.0993 (calcd. for $C_{20}H_{18}O_8$, 386.1001); ^{1}H-NMR δ (chloroform-d) ppm: 2.78 (3H, s, 12-H), 3.91–3.94 (9H, m, 3′, 4′ and 5′-OMe), 6.44 (1H, s, 8-H), 6.58 (1H, s, 3-H), 7.05 (2H, s, 2′ and 6′-H), 14.12 (1H, s, 7-OH), 15.50 (1H, s, 5-OH); ^{13}C-NMR δ (chloroform-d) ppm: 33.2 (4′-OMe), 56.4 (3′ and 5′-OMe), 61.1 (12-C), 95.1 (8-C), 103.6 (10-C), 103.9 (2′ and 6′-C), 105.1 (3-C), 106.9 (6-C), 125.6 (1′-C), 141.9 (4′-C), 153.7 (3′ and 5′-C), 160.4 (9-C), 164.1 (2-C), 167.4 (5-C), 169.7 (7-C), 182.6 (4-C), 204.9 (11-C).

*5,6,7-Trihydroxy-3′,4′,5′-trimethoxyflavone (**8**).* To a stirred mixture of boric acid (66 mg, 1.1 mmol) and 30% hydrogen peroxide (54 μl, 0.47 mmol) in dry THF (5 ml) was added conc. H_2SO_4 (22 μl), and the reaction mixture was further stirred at room temperature for 30 min. A solution of **8b** (82 mg, 0.21 mmol) in dry THF (5 ml) was added, and the reaction mixture was stirred at 60 °C for 36 hr. After cooling to room temperature, the reaction mixture was extracted with ethyl acetate (3 × 25 ml). The extract was washed with water and concentrated to dryness. The residue was dissolved in a small amount of methanol and subjected to reverse-phase column chromatography, using water–methanol–formic acid (40:60:0.1) as the eluent, to give crude **8**. This was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (30:70:0.1); flow rate, 5.0 ml/min; detection, UV 254 nm) to give **8** (t_R 22 min, 16 mg, 21%) as pale yellow powder: mp 246–248 °C; FD-HR-MS m/z 386.0815 (calcd. for $C_{18}H_{16}O_8$,

360.0845); ^{1}H-NMR δ (DMSO-d_6) ppm: 3.73 (3H, 4′-OMe), 3.89 (6H, 3′ and 5′-OMe), 6.64 (1H, s, 8-H), 7.01 (1H, s, 3-H), 7.31 (2H, s, 2′ and 6′-H), 12.67 (1H, s, 5-OH).

*3′,4′,5,5′,6,7-Hexahydroxyflavone (**9**).* A mixture of **8** (75.6 mg, 0.21 mmol), 47% aqueous hydrobromic acid (1.4 ml, 12.6 mmol) and acetic acid (11.2 ml) was heated under reflux for 23 hr. After cooling to room temperature, the reaction mixture was extracted with 1-butanol (2 × 50 ml). The organic phase was washed with water and concentrated to dryness. The residue was dissolved in a small amount of methanol and subjected to reverse-phase column chromatography, using water–methanol–formic acid (50:50:0.1) as the eluent, to yield crude **9**. This was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (50:50:0.1); flow rate, 5.0 ml/min; detection, UV 254 nm) to give **9** (t_R 18 min, 52 mg, 82%) as pale yellow powder: mp > 300 °C; FD-HR-MS m/z 318.0389 (calcd. for $C_{15}H_{10}O_8$, 318.0375); ^{1}H-NMR δ (DMSO-d_6) ppm: 6.48 (1H, s, 3-H), 6.49 (1H, s, 8-H), 6.94 (2H, s, 2′ and 6′-H), 12.78 (1H, s, 5-OH).

*5,7-Dihydroxy-3′,4′-dimethoxyflavone (**10a**).* 3,4-Dimethoxybenzoyl chloride (4.8 g, 24 mmol) was reacted with 2′,4′,6′-trihydroxyacetophenone (3.36 g, 20 mmol) in a similar way to that described for **8a** to give **10a** (1.88 g, 30%) as yellow crystals: mp 288–289 °C; FD-HR-MS m/z 314.0789 (calcd. for $C_{17}H_{14}O_6$, 314.0790); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 3.84 and 3.87 (3H each, s, 3′ and 4′-OMe), 6.20 (1H, d, J = 1.3, 6-H), 6.52 (1H, d, J = 1.3, 8-H), 6.96 (1H, d, J = 0.5, H-3), 7.12 (2H, d, J = 8.4, 5′-H), 7.56 (1H, d, J = 1.4, 2′-H), 7.67 (1H, dd, J = 1.4, 8.4, 6′-H), 10.82 (1H,s,7-OH), 12.92(1H,s, 5-OH).

*5,6,7-Trihydroxy-3′,4′-dimethoxyflavone (**10**).* By the same method as that used for **8b**, **10a** (1.9 g, 6 mmol) was acetylated to 6-acetyl-5,7-dihydroxy-3′,4′-dimethoxyflavone, this in turn being oxidized to **10** by the same method as that used for **5**. Crude **10** was purified by preparative HPLC (mobile phase, water–methanol–formic acid (30:70:0.1); flow rate, 4.5 ml/min; detection, UV 254 nm) to give **10** (t_R 16 min, 63 mg, 21%) as yellow powder: mp 254 °C; FD-HR-MS m/z 330.0728 (calcd. for $C_{17}H_{14}O_7$, 330.0739); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 3.84 and 3.87 (3H each, s, 3′ and 4′-OMe), 6.61 (1H, s, 8-H), 6.92 (1H, s, 3-H), 7.11 (1H, d, J = 8.6, 5′-H), 7.55 (1H, d, J = 2.0, 2′-H), 7.66 (1H, dd, J = 8.6, 2.0, 6′-H), 12.75 (1H, s, 5-OH).

*6-Hydroxyluteolin (**11**).* Compound **10** (105 mg, 0.32 mmol) was treated with 47% aqueous hydrobromic acid (1.08 ml, 9.6 mmol) and acetic acid (9 ml) as described for the formation of **9** from **8**. The resulting solid was subjected to reverse-phase column chromatography, using water–methanol–formic acid (50:50:0.1) as the eluent, to yield crude **11**. This was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (40:60:0.1); flow rate, 4.5 ml/min; detection, UV 254 nm) to give **11** (t_R 18 min, 79 mg, 82%) as yellow powder: mp > 300 °C; FD-HR-MS m/z 302.0400 (calcd. for $C_{15}H_{10}O_7$, 302.0426); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 6.51 (1H, s, 3-H), 6.55 (1H, s, 8-H), 6.88 (2H, d, J = 8.9, 5′-H), 7.36–7.37 (2H, m, 2′ and 6′-H).

*5,7-Dihydroxy-4′-methoxyflavone (**12a**).* 4-Methoxybenzoyl chloride (4.08 g, 24 mmol) was reacted with 2′,4′,6′-trihydroxyacetophenone (3.36 g, 20 mmol) in a similar way to that described for **8a** to give **12a** (2.56 g, 45%) as yellow crystals: mp 260–261 °C; FD-HR-MS m/z 284.0666 (calcd. for $C_{16}H_{12}O_5$, 284.0685); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 3.85 (3H, s, 4′-OMe), 6.19 (1H, d, J = 2.0, 6-H), 6.49 (1H, d, J = 2.0, 8-H), 6.86 (1H, s, 3-H), 7.10 (2H, d, J = 8.9, 3′ and 5′-H), 8.02 (2H, d, J = 8.9, 2′ and 6′-H), 10.88 (1H, s, 7-OH), 12.92 (1H, s, 5-OH).

*5,6,7-Trihydroxy-4′-methoxyflavone (**12**).* By the same method as that used for **10**, **12a** (1.7 g, 6 mmol) was converted to **12**. After separating by reverse-phase column chromatography, using water–methanol–formic acid (60:40:0.1) as the eluent, the crude material was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (30:70:0.1); flow rate, 4.5 ml/min; detection, UV 254 nm) to give **12** (t_R 26 min, 84 mg, 28%) as yellow powder: mp 274 °C; FD-HR-MS m/z 300.0644 (calcd. for $C_{16}H_{12}O_6$, 300.0634); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 3.85 (3H, s, 4′-OMe), 6.59 (1H, s, 8-H), 6.82 (1H, s, 3-H), 7.10 (2H, d, J = 8.8, 3′ and 5′-H), 8.02 (2H, d, J = 8.8, 2 and 6′-H), 12.75 (1H, s, 5-OH).

*6-Hydroxyapigenin (**13**).* Compound **12** (125 mg, 0.42 mmol) was treated with 47% aqueous hydrobromic acid (4.2 ml, 4.2 mmol) and acetic acid (4 ml) as described for the formation of **9** from **8**. After separating by reverse-phase column chromatography, using water–methanol–formic acid (50:50:0.1) as the eluent, the crude material was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (40:60:0.1); flow rate, 4.5 ml/min; detection, UV 254 nm) to give **13** (t_R 15 min, 98 mg, 82%) as yellow powder: mp > 300 °C; FD-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): 6.56 (1H, s, 8-H), 6.55 (1H, s, 3-H), 6.91 (2H, d, J = 8.7, 3′ and 5′-H), 7.90 (2H, d, J = 8.7, 2′ and 6′-H), 12.78 (1H, s, 5-OH).

*5,7-Dihydroxy-2′-methoxyflavone (**14a**).* 2-Methoxybenzoyl chloride (4.08 g, 24 mmol) was reacted with 2′,4′,6′-trihydroxyacetophenone (3.36 g, 20 mmol) in a similar way to that described for **8a** to give **12a** (1.82 g, 32%) as yellow powder: mp 279–282 °C (lit.[9] 281–

283 °C); FD-HR-MS *m/z* 284.0704 (calcd. for $C_{16}H_{12}O_5$, 284.0685).

*5,6,7-Trihydroxy-2′-methoxyflavone (**14**).* By the same method as that used for **10**, **14a** (1.7 g, 6 mmol) was converted to **14**. After separating by reverse-phase column chromatography, using water–methanol–formic acid (60:40:0.1) as the eluent, the crude material was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (35:65:0.1); flow rate, 5 ml/min; detection, UV 254 nm) to give **14** (t_R 28 min, 76 mg, 25%) as yellow powder: mp 291–293 °C; EI-HR-MS *m/z* 300.0625 (calcd. for $C_{16}H_{12}O_6$, 300.0634); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 3.91 (3H, s, 2′-OMe), 6.54 (1H, s, 8-H), 6.79 (1H, s, 3-H), 7.14 (1H, dt, $J = 8.4$, 0.8, 5′-H), 7.24 (1H, d, $J = 8.4$, 3′-H), 7.56 (1H, dt, $J = 7.9$, 1.7, 4′-H), 7.86 (1H, dd, $J = 7.9$, 1.7, 6′-H), 12.64 (1H, s, 5-OH).

*2′,5,6,7-Tetrahydroxyflavone (**15**).* Compound **14** (120 mg, 0.4 mmol) was treated with 47% aqueous hydrobromic acid (4 ml) and acetic acid (4 ml) as described for the formation of **9** from **8**. After separating by reverse-phase column chromatography, using water–methanol–formic acid (50:50:0.1) as the eluent, the crude material was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (40:60:0.1); flow rate, 5 ml/min; detection, UV 254 nm) to give **15** (t_R 15.4 min, 84 mg, 74%) as yellow powder: mp > 300 °C; FAB-HR-MS (negative) *m/z* 285.0374 ($[M - H]^-$, calcd. for $C_{15}H_9O_6$, 285.0374); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.54 (1H, s, 8-H), 6.97 (1H, d, $J = 7.5$, 4′-H), 6.99 (1H, s, 3-H), 7.04 (1H, d, $J = 7.5$, 5′-H), 7.38 (1H, ddt, $J = 8.0$, 1.6, 3′-H), 7.84 (1H, dd, $J = 8.0$, 1.6, 6′-H), 12.69 (1H, s, 5-OH); ^{13}C-NMR δ (DMSO-d_6) ppm: 93.8 (8-C), 104.0 (10-C), 108.4 (3-C), 117.0 (3′-C), 117.5 (1′-C), 119.4 (5-C), 128.5 (6′-C), 129.1 (6-C), 132.6 (4′-C), 146.9 (5-C), 149.9 (7-C), 153.6 (9-C), 156.5 (2′-C), 261.0 (2-C), 182.1 (4-C).

*5,7-Dihydroxy-3′-methoxyflavone (**16a**).* 3-Methoxybenzoyl chloride (4.08 g, 24 mmol) was reacted with 2′,4′,6′-trihydroxyacetophenone (3.36 g, 20 mmol) in a similar way to that described for **8a** to give **12a** (2.23 g, 40%) as yellow powder: mp 232–235 °C (lit.[10] 234 °C); HR-FD-MS *m/z* 284.0680 (calcd. for $C_{16}H_{12}O_5$, 284.0685).

*5,6,7-Trihydroxy-3′-methoxyflavone (**16**).* By the same method as that used for **10**, **16a** (1.1 g, 4 mmol) was converted to **16**. After separating by reverse-phase column chromatography, using water–methanol–formic acid (60:40:0.1) as the eluent, the crude material was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (30:70:0.1); flow rate, 5 ml/min; detection, UV 254 nm) to give **16** (t_R 24 min, 65 mg, 21%) as yellow powder: mp 265 °C; FD-HR-MS *m/z* 300.0632 (calcd. for $C_{16}H_{12}O_6$, 300.0634); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 3.88 (3H, s, 3′-OMe), 6.61 (1H, s, 8-H), 6.72 (1H, s, 3-H), 7.13 (1H, dd, $J = 8.4$, 1.7, 2′-H), 7.44–7.48 (2H, m, 4′ and 5′-H), 7.54 (1H, d, $J = 7.4$, 6′-H).

*3′,5,6,7-Tetrahydroxyflavone (**17**).* Compound **16** (135 mg, 0.45 mmol) was treated with 47% aqueous hydrobromic acid (6 ml) and acetic acid (6 ml) as described for the formation of **9** from **8**. After separating by reverse-phase column chromatography, using water–methanol–formic acid (50:50:0.1) as the eluent, the crude material was further purified by preparative HPLC (mobile phase, water–methanol–formic acid (30:70:0.1); flow rate, 4 ml/min; detection, UV 254 nm) to give **17** (t_R 18 min, 91 mg, 81%) as yellow powder: mp > 300 °C; FD-HR-MS *m/z* 286.0483 (calcd. for $C_{15}H_{10}O_6$, 286.0477); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 6.58 (1H, s, 8-H), 6.64 (1H, s, 3-H), 6.97 (1H, dd, $J = 6.6$, 1.0, 2′-H), 7.32–7.36 (2H, m, 4′ and 5′-H), 7.41 (1H, d, $J = 8.1$, 6′-H).

*6-Acetyl-5,7-dihydroxy-4′-nitroflavone (**18a**).* By the same method as that described for **4**, **18a** was obtained from 4-nitrobenzoylacetic acid ethyl ester (5 g, 27 mmol) and 2′,4′,6′-trihydroxyacetophenone (11 g, 46 mmol) in boiling diphenyl ether (25 ml). Recrystallization from chloroform and hexane gave **18a** (4 g, 43%) as yellow powder: mp 232 °C; FD-HR-MS *m/z* 341.0507 (calcd. for $C_{17}H_{11}O_7N$, 341.0535); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 2.79 (3H, s, 12-H), 6.47 (1H, s, 3-H), 6.75 (1H, s, 8-H), 8.04 (2H, d, $J = 9.1$, 2′ and 6′-H), 8.38 (2H, d, $J = 9.1$, 3′ and 5′-H).

*5,6,7-Trihydroxy-4′-nitroflavone (**18**).* Compound **18a** (5 mmol, 1.71 g) was dissolved in 12 ml of 4% NaOH and 12 ml of pyridine. The mixture was cooled in an ice bath, and hydrogen peroxide (20%, 12 ml) was added dropwise while stirring during 5 min. The reaction mixture was kept stirred for 1 hr under argon gas. Acidification with 2 M HCl resulted in the separation of **18** as a yellow precipitate. Recrystallization from ethyl acetate and hexane gave **18** (0.96 g, 61%) as yellow powder: mp > 300 °C; FD-HR-MS *m/z* 315.0359 (calcd. for $C_{15}H_9O_7N$, 315.0379); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.65 (1H, s, 3-H), 7.13 (1H, s, 8-H), 8.33 (2H, d, $J = 9.1$, 2′ and 6′-H), 8.36 (2H, d, $J = 9.1$, 3′ and 5′-H).

*4′-Amino-5,6,7-trihydroxyflavone (**19**).* A solution of **18** (200 mg) in THF (150 ml) was hydrogenated for 4 hr in the presence of 10% palladium on charcoal (100 mg). The catalyst was removed by filtration, and the solution was concentrated. The residue was crystallized from methanol and water to give crude **19** as a yellow solid. Recrystallization from ethyl acetate and hexane gave **19** as yellow powder (130 mg, 72%): mp > 300 °C; FD-HR-MS *m/z* 285.0611 (calcd. for $C_{15}H_{11}O_5N$,

285.0637); ^{1}H-NMR δ (DMSO-d_6) ppm (J in Hz): 6.02 (2H, s, 4′-NH_2), 6.52 (1H, s, 3-H), 6.58 (1H, s, 8-H), 6.64 (2H, d, $J = 8.4$, 3′ and 5′-H), 7.73 (2H, d, $J = 8.4$, 2′ and 6′-H).

*4′-Fluoro-5,7-dihydroxy-6-propionylflavone (**20a**).* By the same method as that described for **4**, **20a** was obtained from 4-fluorobenzoylacetic acid ethyl ester (5 g, 23.8 mmol) and 2′,4′,6′-trihydroxypropiophenone (21.7 mmol, 4 g) in boiling diphenyl ether (15 ml). Recrystallization from chloroform and hexane gave **20a** (4 g, 56%) as yellow powder: mp 201–202 °C; FD-HR-MS m/z 328.0751 (calcd. for $C_{18}H_{13}O_5F$, 328.0747); ^{1}H-NMR δ (chloroform-d) ppm (J in Hz): 1.21 (3H, t, $J = 6.9$, 13-H), 3.22 (2H, q, $J = 6.9$, 12-H), 6.44 (1H, s, 3-H), 6.61 (1H, s, 8-H), 7.22–7.24 (2H, m, 2′ and 6′-H), 7.87–7.89 (2H, m, 3′ and 5′-H), 14.29 (1H, s, 7-OH), 15.16 (1H, s, 5-OH).

*4′-Fluoro-5,6,7-trihydroxyflavone (**20**).* By the same method as that described for **19**, **20** was obtained from **20a** (3.28 g). Recrystallization from ethanol gave **20** (1.87 g, 65%) as yellow powder: mp 279–280 °C; FD-HR-MS m/z 288.0405 (calcd. for $C_{15}H_9O_5F$, 288.0434); ^{1}H-NMR δ (DMSO-d_6) ppm: 6.61 (1H, s, 3-H), 6.93 (1H, s, 8-H), 7.39–7.42 (2H, m, 2′ and 6′-H), 8.12–8.15 (2H, m, 3′ and 5′-H).

Results and Discussion

Biflorin (**2**) was deglucosylated with hydriodic acid to give **3**.[7] Treatment of 2′,4′,6′-trihydroxyacetophenone with ethyl acetoacetate in boiling diphenyl ether led to **4**, whose acetyl group was oxidized by Dakin hydrogen peroxide oxidation to afford **5**.[8] 3′,4′,5,5′,6,7-Hexahydroxyflavone (**9**) was obtained *via* a four-step synthesis starting with the condensation of 2′,4′,6′-trihydroxyacetophenone and 3,4,5-trimethoxybenzoyl chloride to give the corresponding flavone **8a** in moderate yield.[11] In the next step, **8a** was acetylated to give **8b**, which in turn was oxidized with a hydrogen/boric acid system to yield **8**.[8,12] In the final step, the methoxyl groups of **8** were hydrolyzed by hydrobromic acid in glacial acetic acid to give **9** in a high yield.[13] Compounds **10**–**17** were obtained according to the procedures used for the preparation of **8** and **9**, except that the acetyl group was oxidized by Dakin hydrogen peroxide oxidation.

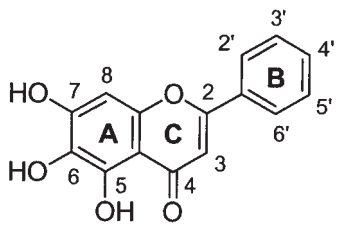
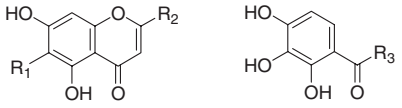


Fig. 1. Chemical Structure of Baicalein (5,6,7-trihydroxyflavone, **1**).

Table 1. Rat Intestinal α-Glucosidase Inhibition of Baicalein (**1**), Hydroxychromones (**2**–**5**), 2,3,4-Trihydroxybenzaldehyde (**6**) and 2′,3′,4′-Trihydroxyacetophenone (**7**)



Compound	R_1	R_2	R_3	RA[a]
1	OH	Ph		1
2	Glc	Me		NI[b]
3	H	Me		NI
4[c]	Ac	Me		NI
5	OH	Me		0.220
6			H	0.110
7			Me	0.086

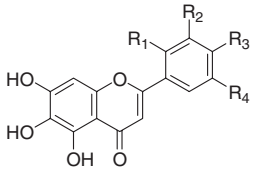
[a]The relative activity is defined as $IC_{50\ (baicalein)}/IC_{50\ (test\ compound)}$. [b]Less than 30% inhibition at a concentration of 500 μM. [c]The sample was dissolved in DMSO in this assay.

Compounds **18** and **20** were obtained by oxidizing the acyl group, using hydrogen peroxide/4% NaOH-pyridine from compounds **18a** and **20a**, respectively, which had been prepared by the method described for **4**.[14] Hydrogenation of **18** in the presence of 10% Pd-C afforded **19**.[15]

In order to evaluate the importance of the B-ring of **1** on the inhibitory activity against α-glucosidase, B-ring-eliminated hydroxychromones (**2**–**5**), as well as B- and C-ring-eliminated 2,3,4-trihydroxybenzaldehyde (**6**) and 2′,3′,4′-trihydroxyacetophenone (**7**) were tested for their inhibition against rat intestinal α-glucosidase with sucrose as the substrate (Table 1). Hydroxychromones **2**–**4**, which lacked 6-hydroxyl substitution, did not show any inhibitory activity, while 5,6,7-trihydroxychromone **5** showed high activity, although the activity of **5** was a little weaker than that of **1**. This result suggested that the B-ring of **1** was not essential, although advantageous to the activity. Further evidence supporting this rationale is the surprising fact that **6** and **7**, having neither a B- nor C-ring, still retained the activity, even though their activity was considerably weaker than that of **1** or **5**. In consequence, it is suggested that 5,6,7-trihydroxyl substituents and presumably a 4-carbonyl group were important as a minimal structure for exerting the inhibitory activity and that the C-ring in chromones and B-ring in flavones effectively enhanced this activity.

It was indicated in the previous paper that 6-hydroxyluteolin (**11**, $IC_{50} = 10\ \mu$M) and 6-hydroxyapigenin (**13**, $IC_{50} = 12\ \mu$M), both of which have an additional hydroxyl group on the B-ring, showed higher activity than that of **1** ($IC_{50} = 32\ \mu$M).[3] In order to clarify the contribution of the position and number of hydroxyl groups on the B-ring of **1** to the inhibitory activity, we examined the inhibitory activity of a series of B-ring-hydroxylated 5,6,7-trihydroxyflavones (**9**, **11**, **13**, **15** and **17**) together with the corresponding methoxyl counterparts (**8**, **10**, **12**, **14** and **16**), as well as the nitro

Table 2. Rat Intestinal α-Glucosidase Inhibition of Baicalein (**1**) and Related B-Ring-substituted 5,6,7-Trihydroxyflavones (**8**–**20**)



Compound	R_1	R_2	R_3	R_4	RA[a]
1	H	H	H	H	1
8	H	OMe	OMe	OMe	0.223
10	H	OMe	OMe	H	0.308
12	H	H	OMe	H	0.616
14	OMe	H	H	H	0.481
16	H	OMe	H	H	0.550
9	H	OH	OH	OH	1.495
11	H	OH	OH	H	1.791
13	H	H	OH	H	1.375
15	OH	H	H	H	0.733
17	H	OH	H	H	0.770
18	H	H	NO_2	H	NI[b]
19	H	H	NH_2	H	0.581
20	H	H	F	H	0.310

[a]The relative activity is defined as $IC_{50\ (baicalein)}/IC_{50\ (test\ compound)}$. [b]Less than 30% inhibition at a concentration of 500 μM.

(**18**), amino (**19**) and fluoro (**20**) derivatives (Table 2). As shown in Table 2, all of the tested 5,6,7-trihydroxyflavones (**8**–**17**, **19** and **20**) were more or less active, except for **18** ($IC_{50} > 1$ mM), which validates the importance of the basic structure of 5,6,7-trihydroxyflavone for the activity.

Among the B-ring-hydroxylated 5,6,7-trihydroxyflavones, **9** (RA = 1.495), **11** (RA = 1.791), and **13** (RA = 1.375), all of which carried the 4′-OH group, were more active than **1** (RA = 1), although the inhibitory activity was independent upon the number of hydroxyl groups on the B-ring. The highest activity was observed for dihydroxylated **11**, compared to monohydroxylated **9** and trihydroxylated **13**. On the other hand, the compounds with a hydroxyl group at the 2′-(**15**, RA = 0.733) or 3′-(**17**, RA = 0.770) position all showed lower activity than that of **1**. These results indicate that only 4′-hydroxylation of the B-ring enhanced the inhibitory activity of **1**, although extra hydroxyl groups were not always advantageous, and that 2′- or 3′-hydroxylation was detrimental to the activity.

A comparison of the B-ring-methoxylated 5,6,7-trihydroxyflavones (**8**, **10**, **12**, **14** and **16**) with the corresponding B-ring-hydroxylated compounds (**9**, **11**, **13**, **15** and **17**) revealed that methylation of the hydroxyl groups on the B-ring obviously reduced the activity (**8** > **9**, **10** > **11**, **12** > **13**, **14** > **15**, and **16** > **17**). Hence, the methoxyl group substitution on the B-ring of **1** was unfavorable for the activity, unlike the hydroxyl group substitution.

The fact that the α-glucosidase inhibitory activity of **1** was enhanced by hydroxyl substitution on the B-ring (except for **15** and **17**) and reduced by methoxyl substitution leads us to believe that the influence of the substituents was not due to their electronic properties, since both the hydroxyl and methoxyl groups were electron-releasing and had negative *Hammett* constants.[16] In addition, the result that the introduction of both strong electron-donating (**19**) and electron-withdrawing (**18** and **20**) groups at the 4′-position of **1** did not lead to any increase of the activity compared to **1** supports this rationale. Steric hindrance did not account for the unfavorable effect of methoxyl group substitution on the B-ring of **1** on the activity, since **20**, which carried a less bulky substituent at the 4′-position of **1**, showed weaker inhibition than **12**, **13** or **19** did. The question still remains, however, of why the 4′-OH substitution found in **9**, **11** and **13** enhanced the activity compared to that of **1**. After eliminating the electronic and steric effects of the substituents on the B-ring, the acidic hydroxyl group at the 4′-position might have interacted directly with a specific part of the enzyme by constructing an H-bond. The enhanced affinity to the enzyme could improve the inhibitory potency. On the other hand, the methoxyl group substituted on the B-ring (*e.g.*, as found in **8**, **10** or **12**) would simply have caused repulsion with the enzyme so as to reduce the inhibitory activity. In summary, this study has disclosed considerable information about the structure-activity relationship in respect of the number and relative position of the hydroxyl groups on the B-ring of **1** for enhancing the inhibitory activity against rat intestinal α-glucosidase. The combined results of our present and previous[4] studies would lead to the future design of more potent α-glucosidase inhibitors with a rational molecular structure, although the detailed three-dimensional structure of the rat enzyme remains obscured. It is well known that polyphenols efficiently interact with proteins and lead to inhibited enzyme activities.[17] However, our preliminary results show that baicalein specifically inhibited rat intestinal sucrase but had little effect on the activities of other glycosidases including α-amylase, β-glucosidase, α-mannosidase and α-galactosidase. Details of the enzyme specificity of the inhibitory activity of baicalein and its related compounds will be discussed elsewhere.

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References

1) Lebovitz, H. E., Alpha-glucosidase inhibitors. *Endocrin. Metab. Clin.*, **26**, 539–551 (1997).
2) Nishioka, T., Kawabata, J., and Aoyama, Y., Baicalein, an α-glucosidase inhibitor from *Scutellaria baicalensis.*

J. Nat. Prod., **61**, 1413–1415 (1998).
3) Kawabata, J., Mizuhata, K., Sato, E., Nishioka, T., Aoyama, Y., and Kasai, T., 6-Hydroxyflavonoids as α-glucosidase inhibitors from marjoram (*Origanum majorana*) leaves. *Biosci. Biotechnol. Biochem.*, **67**, 445–447 (2003).
4) Gao, H., Nishioka, T., Kawabata, J., and Kasai, T., Structure-activity relationships for α-glucosidase inhibition of baicalein, 5,6,7-trihydroxyflavone: the effect of A-ring substitution. *Biosci. Biotechnol. Biochem.*, **68**, 369–375 (2004).
5) Toda, M., Kawabata, J., and Kasai, T., α-Glucosidase inhibitors from clove (*Syzgium aromaticum*). *Biosci. Biotechnol. Biochem.*, **64**, 294–298 (2000).
6) Noguchi, T., Shimazawa, R., Nagasawa, K., and Hashimoto, Y., Thalidomide and its analogues as cyclooxygenase inhibitors. *Bioorg. Med. Chem. Lett.*, **12**, 1043–1046 (2002).
7) Ghosal, S., Kumar, Y., Singh, S., and Ahad, K., Biflorin, a chromone-*C*-glucoside from *Pancratium Biflorum. Phytochemistry*, **22**, 2591–2593 (1983).
8) Batawi, M. M., and Fayez, M. B. E., Natural chromones-1: A total synthesis of visnagin. *Tetrahedron*, **21**, 2925–2929 (1965).
9) Iinuma, M., and Mizuno, M., Natural occurrence and synthesis of 2′-oxygenated flavones, flavonols, flavanones and chalcones. *Phytochemistry*, **28**, 681–694 (1989).
10) Louis, L., Hypocholeretic acid spasmolytic 5,7-dihydroxyflavone derivatives. Ger. Offen., Ger. Patent DE2010505 (Sep. 24, 1970).
11) Bois, F., Beney, C., Mariotte, A., and Boumendjel, A., A one-step synthesis of 5-hydroxyflavones. *Synlett*, **9**, 1480–1482 (1999).
12) Roy, A., Leddy, K. R., Mohanta, P. K., Ila, H., and Junjappa, H., Hydrogen peroxide/boric acid: An efficient system for oxidation of aromatic aldehydes and ketones to phenols. *Synth. Commun.*, **29**, 3781–3791 (1999).
13) Landini, D., Montanari, F., and Rolla, F., Cleavage of dialkyl and aryl ethers with hydrobromic acid in the presence of phase-transfer catalyst. *Synth. Commun.*, **8**, 771–773 (1978).
14) Agasimundin, Y. S., and Rajagopal, Furano compounds. XII. Synthesis of furano[2,3-*b*]xanthones. *J. Org. Chem.*, **36**, 845–846 (1971).
15) Cushman, M., Zhu, H., Geahlen, R. L., and Kraker, A. J., Synthesis and biochemical evaluation of a series of aminoflavones as potential inhibitors of protein-tyrosine kinase $p56^{lck}$, EGFr, and $p60^{v\text{-}src}$. *J. Med. Chem.*, **37**, 3353–3362 (1994).
16) Smith, M. B., and March, J., Quantitative treatments of the effect of structure on reactivity. In "March's Advanced Organic Chemistry" 5th ed., Wiley Interscience, New York, pp. 368–375 (2001).
17) Haslam, E., Molecular recognition — phenols and polyphenols. Studies with peptides and proteins. In "Practical Polyphenols", Cambridge University Press, Cambridge, pp. 168–174 (1998).