α-Glucosidase inhibition of 6-hydroxyflavones. Part 3: Synthesis and evaluation of 2,3,4-trihydroxybenzoyl-containing flavonoid analogs and 6-aminoflavones as α-glucosidse inhibitors

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Abstract—The SAR studies suggested that the C-ring of baicalein (**1)** was not necessary for the activity, and validated the importance of 2,3,4-trihydroxybenzoyl structure of **1**. Thus, a series of 2,3,4-trihydroxybenzoyl-containing flavonoid analogs were investigated for the α-glucosidase inhibitory activity. The results indicated that 5,6,7-trihydroxy-2-phenyl-4-quinolone (**2**) and 5,6,7-trihydroxyflavanone (**4**) showed the comparable activity to **1**, while 3,5,6,7-tetrahydroxyflavone (**7**), 5,6,7-trihydroxyisoflavone (**8**) and 6-hydroxygenistein (**9**) showed moderate α-glucosidase inhibitory activity. In addition, it was found that 6-amino-5,7-dihydroxyflavone (**16**) was a more potent and specific rat intestinal α-glucosidase inhibitor than **1**, and showed the comparable activity to acarbose. This is the first report on mammalian intestinal α-glucosidase inhibitory activity of 6-aminoflavones. Kinetic studies revealed that **16** inhibited both sucrose- and maltose-hydrolyzing activities of rat intestinal α-glucosidase un-competitively.

Key words α-glucosidase inhibitor, structure-activity relationship (SAR) study, 5,6,7-trihydroxyflavone, 6-hydroxyflavones, 6-aminoflavones.

1. Introduction

Mammalian α-glucosidases (α-D-glucoside glucohydrolase EC. 3.2.1.20) inhibitors, which interfere with enzymatic action in the brush-border of the small intestine, could slow the liberation of D-glucose from oligosaccharides and disaccharides, resulting in delaying glucose absorption and decreasing postprandial plasma glucose levels.[1] There are reports of established α-glucosidase inhibitors such as acarbose[2] and voglibose[3] form microorganisms, and nojirimycin[4], and 1-deoxynojirimycin[5] from plants and their effects on blood glucose levels after food uptake.[6-9] On the other hand, it is well known that polyphenols efficiently interact with proteins and lead to

inhibit enzyme activities.[10] Recent studies have indicated that phenonic compounds were potent class of α-glucosidase inhibitors.[11,12,13,14] But, most of these yeast's α-glucosidase (class **I**) inhibitors did not show any activity against mammalian α-glucosidase (class **II**) due to the difference of molecular recognition in the target binding-site of these enzymes.

In the course of our screening study on rat intestinal α-glucosidase-inhibiting principles from plant sources, we previously reported that baicalein (5,6,7-trihydroxyflavone, **1**) and the related 6-hydroxyflavonoids were a new class of α-glucosidase inhibitors.[15,16] As a continuing study, we evaluated the influences of A- and B-rings substitution for **1** on the α-glucosidase inhibitory activity.[17,18] From initial SAR studies, it was suggested that 5,6,7-trihydroxyl subsituents and presumably 4-carbonyl group of **1** (2,3,4-trihydroxybenzoyl moiety) were important to exert the inhibitory activity as a minimal structure, although the actual molecular interaction with the rat intestinal α-glucosidase is unknown.

Cushman and coworkers have reported the inhibitory effects of hydroxyflavones and aminoflavones on protein-tyrosine kinases.[19,20] It is not obvious, however, if the amino substituents offer any overall advantage over hydroxyl group, but both are approximately the same size and can function as hydrogen donors as well a hydrogen bond acceptors. We have focused on the 6-hydroxyl group of **1** and been interested in the α-glucosidase inhibitory activity of aminoflavones.

In the present study, we studied the influence of the C-ring of **1** on the α-glucosidase inhibitory activity in order to clarify further structural requirements of **1** for inhibition of this enzyme. And furthermore, we prepared a series of 2,3,4-trihydroxybenzoyl-containing flavonoid analogs of **1** as well as 6-aminoflavones, and evaluated their α-glucosidase inhibitory effect.

2. Results and discussion

2.1. Synthetic approach

Compound **1** was prepared as previously described.[21] The synthesis of compound **2** is shown in Scheme 1. Reaction of 3,4,5-trimethoxyaniline with benzoyl chloride in the presence of triethylamine gave *N*-(3,4,5-trimethoxyphenyl)benzamide **2a**.[22] Friedel-Crafts acylation with acetyl chloride in the presence of $SnCl_4$ gave *N*-(2-acetyl-3,4,5-trimethoxyphenyl)benzamide **2b**. Cyclization of **2b** in the presence of *t*-BuOK in THF gave a quinolone **2c**, which was demethoxylated with excess BBr_3 afforded 5,6,7-trihydroxy-2-phenyl-4-quinolone **2**.[22] Methylation of **1** gave 5,6,7-trimethoxyflavone, [23] which was treated with excess Lawesson's reagent in dry toluene to convert into the corresponding 5,6,7-trimethoxy-4-thioflavone.[24] As the method described for **2**, demethylation of 5,6,7-trimethoxy-4-thioflavone gave 5,6,7-trihydroxy-4-thioflavone **3**. Compounds **4** and **11** were obtained by the hydrogenation of **1** in the presence of palladium black.[25] A 3-hydroxy substituent was introduced to 5,6,7-trimethoxyflavone by quenching the corresponding 3-lithioflavone with trimethyl borate followed by oxidation with hydrogen peroxide in acetic acid to give 3-hydroxy-5,6,7-trimethoxyflavone, [26] which was demethylated to give

3,5,6,7-tetrahydroxyflavone **7**. The synthesis of compounds **8** and **9** were shown in Scheme 2. Treatment of 3,4,5-trimethoxyphenol with phenylacetyl chloride in the presence of BF_3-Et_2O complex gave 6-hydroxy-2,3,4-trimethoxyphenyl benzyl ketone **8a**.[27] This was treated with *N*,*N*-carbonyldiimodazole/formic acid in THF to give 5,6,7-trimethoxyisoflavone **8b**.[28] Demethylation of **8b** with BBr_3 provided the corresponding 5,6,7-trihydroxyisoflavone **8**. 4',5,6,7-

Tetrahydroxyisoflavone **9** was obtained by the same procedure described for **8** using *p*-methoxyphenylacetyl chloride as the starting material. Reaction of 3,4,5-trimethoxyphenol with cinnamoyl chloride in the presence of BF_3-Et_2O complex gave 6'-hydroxy-2',3',4'-trimethoxychalcone **10a**,[27] which was cyclized to the corresponding 5,6,7-trimethoxyflavanone **10b** with KF in methanol under reflux.[29] By the procedure described for **2**, **10b** was demethylated to give 5,6,7-trihydroxyflavanone, which was spontaneously converted to 2',3',4',6'-terahydroxychalcone **10** in the reaction condition. 1,2,3-Trihydroxyxanthone **14** was obtained as previously described.[30] Condensation of 3,4,5-trimethoxyphenol and 2,5-dihydroxy-4-methoxybenzoic acid by anhydrous zinc chloride in phosphorus oxychloride gave 7-hydroxy-1,2,3,6-tetramethoxyxanthone **15a**, which was demethylated with hydriodic acid to give 1,2,3,6,7-pentahydroxyxanthone **15** (Scheme 3).[30,31]

The synthesis of compounds **16**, **17** and **21** was shown in Scheme 4. Nitration of 5,7-dihydroxyflavone with 70% HNO_3 in acetic acid gave 8-nitro-5,7-dihydroxyflavone **16a**, which was hydrogenated to give 8-amino-5,7-dihydroxyflavone **17**.[32,33] Methylation of **16a** with MeI followed by hydrogenation in the presence of 10% Pd-C afforded 8-amino-5,7-dimethoxyflavone **16c**, which was rearranged to 6-amino-5,7-dihydroxyflavone **16** by demethylation in the Wessely-Moser reaction condition.[32] Treatment of 5,7-dihydroxyflavone with excess 70% HNO_3 in acetic acid gave 5,7-dihydroxy-6,8-dinitroflavone **21a**, which was reduced with Zn-acetic acid system to give **21**.[34] Treatment of 2,6-dihydroxy-3-nitroacetophenone with sodium benzoate and benzoic anhydride gave 5-hydroxy-6-nitroflavone.[35] Reduction of 5-hydroxy-6-nitroflavone with Zn-acetic acid system gave 6-amino-5-hydroxyflavone **18**.[34] The same procedure as described above can smoothly produce 6-amino-7-hydroxyflavone **19** from 4,6-dihydroxy-3-nitroacetophenone as a starting-material. Structures of the compounds **1**-**4**, **7**-**11** and **14**-**21** were confirmed by spectroscopic data (NMR, Mass) and satisfactory analytical values were obtained for all the

compounds.

2.2. Biological activity tests

As shown in Fig. 1, a replacement of the O-1 for **1** by a nitrogen atom did not affect the activity [**2** (IC_{50}=45 μM) and **1** (IC_{50}=52 μM)], whereas a sulfur-substitution of the C-4 carbonyl oxygen (**3**) led to a dramatic drop in the activity. Reduction of the double bond at C_2/C_3 of **1** led to a flavanone **4** (IC_{50}=79 μM), which was slightly less active than **1** or **2**. These results suggested that a γ-pyrone structure of the C-ring of **1** was not essential but advantageous to the activity. This rationale is supported by the activity of compound **5** (IC_{50}=170 μM) and **6** (IC_{50}=201 μM), which have neither a B- nor C-ring, as described in the previous study.[18] From these results, it is fair to conclude that the 2,3,4-trihydroxybenzoyl structure of **1** is essential in eliciting α-glucosidase inhibitory activity.

Having identified the importance of 2,3,4-trihydroxybenzoyl moiety of **1**, we decided to evaluate the activity of a series of 2,3,4-trihydroxybenzoyl-containing flavonol (**7**), isoflavones (**8**, **9**) and chalcones (**10**, **11**), together with anthraqinone (**12**) and xanthones (**13-15**). Unfortunately, all of these compounds tended to decrease the potency compared to **1** (Fig. 1). As for a flavonol **7** (IC_{50}=153 μM), an addition of a hydroxyl group to position 3 of **1** partially reduced the activity. Furthermore, isoflavone **8** (IC_{50}=640 μM) or **9** (IC_{50}=624 μM), which has B-ring (phenyl group) at position 3, was 12-fold less active than **1**. Rossi et al. [36] have described a relationship between molecular structure and activity toward enzymes of baicalein (**1**), and revealed that **1** could act as a template for attractive hydrogen-bonding interactions with the amino acids so as to reduce or eliminate enzyme activity. Based on their study, the results might be explained by the interaction between **1** with α-glucosidase. This 2,3,4-trihydroxybenzoyl moiety of **1** can be considered to

bind to the enzyme by hydrogen-bonding interactions. As predicated previously, the phenyl group (B-ring) at position 2 for **1** seems not to greatly affect the activity. However, a bulky substitution at position 3 may hinder the approach of a 2,3,4-trihydroxybenzoyl moiety to the binding site in this enzyme. On the other hand, compounds **9**-**15**, which do not have flavone skeleton although possessing the 2,3,4-trihydroxybenzoyl moiety, showed almost inactive. Hence, it was suggested that the structure near the C-ring of **1** was important for the potency and the structure of flavone itself was taken part in the α-glucosidase inhibitory activity.

A replacement of the hydroxyl group (-OH) at C-6 in **1** with an amino group ($-NH_2$) afforded **16**. Interestingly, **16** (IC_{50}=2.4 μM) showed a dramatic improvement in potency, whereas 8-amino-5,7-dihydroxyflavone **17** was inactive. And also, **19** (IC_{50}=135 μM) was found to show a moderate activity even though 6,7-dihydroxyflavone[17] was inactive. Hence, it was suggested that the amino group at position 6 of the flavones gave an additional favorable effect on α-glucosidase inhibition. Having established that the potency could be improved through this modification, we sought to prepare a series of 6-aminoflavones and tested their activity (Fig. 1). Compound **18**, which lacks a hydroxyl group at position 7, was found to be inactive. The introduction of an amino group to position 8 in **19** afforded **20**, which showed a dramatic loss of potency. To our surprise, **21** (IC_{50}=82 μM), which possessed an additional amino group substitution on position 8 of **16**, decreased the activity 34-fold compared to **16** in spite of the presence of 6-amino-5,7-dihydroxyflavone structure. These results suggested that the 6-amino group and 7-hydroxyl group of **16** were crucial for the activity; 5-hydroxyl substitution was favorable to the activity, whereas the substitution of 8-amino group was unfavorable.

Having identified a potent series of rat intestinal α-glucosidase inhibitors, we evaluated the selectivity of **1**, **2**, **4** and **16** for several selected sugar hydrolases inhibition (Table 1). As indicated

above, each of these compounds showed the high activity against rat intestinal sucrase. Compound **1** (IC_{50}= 500 μM) was a mild rat intestinal maltase inhibitor, while **16** (IC_{50}=4.4 μM) strongly inhibited this enzyme. However, **2** or **4** showed no inhibitory activity against maltase. It is interesting to note that all of the compounds **1**, **2**, **4** and **16** did not inhibit the other glycosidases, ex. isomaltase, β-glucosidase, α-mannosidase, α-galactosidase, β-galactosidase and porcine pancreatic α-amylase. Therefore, compounds **1**, **2**, **4** and **16** would belong to specific rat intestinal sucrase inhibitors. In addition, the result indicated that **16** was also a potent, specific rat intestinal maltase inhibitor.

Among the tested compounds **1**-**21**, **16** showed the strongest inhibitory activity against rat intestinal α-glucosidase; thus, we examined the type of inhibition of this enzyme by **16**. Lineweaver-Burk plot of α-glucosidase kinetics is shown in Fig. 2. The kinetic result demonstrated that the mechanism of α-glucosidase inhibition of **16** was un-competitive on sucrose- and maltose-hydrolyzing activity with K_i value of 1.4 μM and 2.2 μM, respectively. Next, we compared the α-glucosidase inhibitory activity and the inhibition-type against the enzyme between **16** and acarbose, a commercial medicine as an α-glucosidase inhibitor (Table 2). The inhibition-type of **16** (uncompetitive inhibition) against sucrase or maltase was different from that of acarbose (competitive inhibition), while the sucrase or maltase inhibitory activity of **16** (sucrase: IC_{50}=2.4 μM, K_i=1.4 μM; maltase: IC_{50}=4.4 μM, K_i=2.2 μM) was comparable to that of acarbose (sucrase: IC_{50}=1.8 μM, K_i=0.8 μM; maltase: IC_{50}=2.1 μM, K_i=0.7 μM).

In summary, we have reported rat intestinal α-glucosidase inhibitory activity of 2,3,4-trihydroxybenzoyl-containing flavonoid analogs of **1** and 6-aminoflavones. These results suggested that C-ring of **1** was not necessary for the activity, and validated the importance of 2,3,4-trihydroxybenzoyl structure of **1**. By SAR-studies, **16** was found to be a more potent and

specific rat intestinal α-glucosidase inhibitor than **1**. In addition, **16** showed the comparable α-glucosidase inhibitory activity to ararbose. Therefore, it is concluded that **16** would be a lead compound suitable for designing new potent α-glucosidase inhibitors of this kind.

3. Experimental

3.1. Chemistry

NMR spectra were recorded with a Bruker AMX500 instrument (^{1}H, 500MHz; ^{13}C, 125MHz). 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). 2',3',4'-Trihydroxyacetophenone (**5**), 2,3,4-trihydroxybenzaldehyde (**6**) and mangeferin

(**13**) were supplied by Sigma Aldrich Japan Co. (Tokyo, Japan). 1,2,3-Trihydroxyanthraquinone (**12**) was supplied by Tokyokasei Co. (Tokyo, Japan). Acetone and tetrahydrofuran were dried by storing over 3A molecular sieves. All solvents were distilled before use.

3.1.1. *N*-(3,4,5-Trimethoxyphenyl)benzamide (2a) [22]

To a solution of 3,4,5-trimethoxyaniline (3.6 g, 19.6 mmol) in dry THF (50 mL) was added Et_3N (4.1 mL, 29.4 mmol). The solution was stirred at 0 °C for 10 min and treated dropwise by benzoyl chloride (3.4 mL, 29.4 mmol). The reaction mixture was stirred at room temperature for 1 h, then quenched by adding water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography using hexane-ethyl acetate (8:2) as the eluent to yield **2a** as a gray solid (4.78 g, 85%): mp 141 °C; EIHRMS *m/z* 287.1165 (calcd. for $C_{16}H_{17}O_4N$, 287.1158); ^{1}H-NMR δ (chloroform-*d*) ppm: 4.14 (9H, s, OMe-3, 4 and 5), 7.24, 7.26 (each 1H, s, H-2 and 6), 7.81-7.85 (3H, m, H-3', 4' and 5'), 8.16-8.20 (2H, m, H-2' and 6').

3.1.2. *N*-(2-Acetyl-3,4,5-trimethoxyphenyl)benzamide (2b) [22]

To an ice-cooled solution of **2a** (5.25 g, 18.3 mmol) in dry 1,2-dichloroethane (60 mL) under argon gas was added dropwise $SnCl_4$ (4.3 mL, 36.6 mmol) and acetyl chloride (1.41 mL, 20 mmol). After stirring at room temperature for 1.5 h, the solution was poured into ice-cooled water, extracted with ethyl acetate, washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography using hexane-ethyl acetate (1:1) as the eluent to yield **2b** as an oil (4.98g, 83%): EIHRMS *m/z* 329.1255 (calcd. for $C_{18}H_{19}O_5N$, 329.1264); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 2.66 (3H, s, H-8), 3.85, 3.99, 4.00 (each 3H,

s, OMe-3, 4 and 5), 7.51-7.55 (3H, m, H-3', 4' and 5'), 8.02 (2H, dd, *J*=6.8, 1.3, H-2' and 6').

3.1.3. 5,6,7-Trimethoxy-2-phenyl-4-quinolone (2c) [22]

To a stirred solution of **2b** (2.5 g, 7.6 mmol) in dry THF (50 mL) under argon gas was added *t*-BuOK (4.21 g, 38 mmol). The reaction mixture was then refluxed for 20 h. After cooling to room temperature, the resultant mixture was added to a saturated aqueous solution of NH_4Cl and extracted with ethyl acetate. The extract was washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography using hexane-ethyl acetate (4:6) as the eluent to yield **2c** as a white solid (1.51 g, 64%): mp 230 °C; EIHRMS *m/z* 311.1140 (calcd. for $C_{18}H_{17}O_4N$, 311.1158); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.93, 3.97, 4.14 (each 3H, s, OMe-5, 6 and 7), 7.00 (1H, s, H-3), 7.39 (1H, s, H-8), 7.43 (2H, d, *J*=6.6, H-2' and 6'), 7.37-7.40 (3H, m, H-3', 4' and 5').

3.1.4. 5,6,7-Trihydroxy-2-phenyl-4-quinolone (2) [22]

To a stirred solution of **2c** (0.35 g, 1.13 mmol) in dry CH_2Cl_2 (10 mL) at –80 °C was added BBr_3 (1 M CH_2Cl_2 solution, 10 mL, 10 mmol). The reaction mixture was then stirred for 1 h at –80 °C and 12 h at room temperature. The reaction was stopped by adding ice-cooled water (100 mL). To this mixture was added 1-butanol (200 mL). The organic phase was separated, washed with brine, dried over anhydrous Na_2SO_4 and evaporated. The residue was crystallized from hexane-ethyl acetate to give **2** as a yellow solid (0.16 g, 52%): mp 292 °C; FABHRMS (negative) *m/z* 268.0617 ($[M-H]^-$, calcd. for $C_{15}H_{10}O_4N$, 268.0610); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.16 (1H, s, H-3), 6.62 (1H, s, H-8), 7.55-7.56 (3H, m, H-3', 4' and 5'), 7.77 (2H, d, *J*=3.1, H-2' and 6'), 9.98, 11.71, 14.58 (each 1H, s, OH-5, 6 and 7).

3.1.5. 5,6,7-Trimethoxyflavone (3a) [23]

A mixture of **1** (2.7 g, 10 mmol), 10 mL of methyl iodide and anhydrous potassium carbonate (6.9 g, 50 mmol) in dry acetone (100 mL) was well stirred under reflux for 8 h. The reaction mixture was cooled and filtered. The filtrate was evaporated and the residue was crystallized from acetic acid-water to give **3a** (2.56 g, 82%) as white plates: mp 145-147 °C; EIHRMS *m/z* 312.1029 (calcd. for $C_{18}H_{16}O_5$, 312.0999); ^{1}H-NMR δ (chloroform-*d*) ppm: 3.99 (9H, s, OMe-5, 6 and 7), 6.67 (1H, s, H-3), 6.82 (1H, s, H-8), 7.51-7.52 (3H, m, H-3', 4' and 5'), 7.87-7.89 (2H, m, H-2' and 6').

3.1.6. 5,6,7-Trimethoxy-4-thioflavone (3b) [24]

A mixture of **3a** (1.56 g, 5 mmol), Lawesson's reagent (1.23 g, 3 mmol) and anhydrous toluene (40 mL) was refluxed for 3 h. The solvent was evaporated and the residue was purified by silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to give **3b** as a purple solid (1.18 g, 72%): mp 116-117 °C; EIHRMS *m/z* 328.0756 (calcd. for $C_{18}H_{16}O_4S$, 328.0770); ^{1}H-NMR δ (chloroform-*d*) ppm: 3.93, 3.95, 4.01 (each 3H, s, OMe-5, 6 and 7), 6.83 (1H, s, H-3), 7.50-7.52 (3H, m, H-3', 4' and 5'), 7.59 (1H, s, H-8), 7.92-7.93 (2H, m, H-2' and 6').

3.1.7. 5,6,7-Trihydroxy-4-thioflavone (3)

Using the procedure described for **2**, **3b** (164 mg, 0.5 mmol) was demethylated to **3**. The solid was further purified by preparative HPLC (mobile phase, water-methanol-formic acid (20:80:0.1); flow rate, 5 mL/min; t_R 25.2 min; detection, UV 254 nm) to give **3** (108 mg, 76%) as green

crystals: mp 198 °C; EI-HR-MS *m/z* 286.0291 (calcd. for $C_{15}H_{10}O_4S$, 286.0300); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.69 (1H, s, H-3), 7.55 (1H, s, H-8), 7.56-7.60 (3H, m, H-3', 4' and 5'), 8.12 (2H, d, *J*=8.1, H-2' and 6').

3.1.8. 5,6,7-Trihydroxyflavanone (4) and 2',3',4',6'-tetrahydroxydihydrochalcone (11) [25]

A solution of **1** (108 mg, 0.4 mmol) in water-methanol-acetic acid (1:10:1, 24 mL) was hydrogenated for 36 h in the presence of palladium black (10 mg) at room temperature. The catalyst was removed by filtration and the filtrate was concentrated. The residue was dissolved in a small amount of methanol and subjected to preparative HPLC (mobile phase, water-methanol-formic acid (30:70:0.1); flow rate, 4.5 mL/min; detection, UV 254 nm) to give 24 mg of **4** (t_R 17.2 min) as yellow powders: mp 228-230 °C; EIHRMS *m/z* 272.0703 (calcd. for $C_{15}H_{12}O_5$, 272.0685); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 2.75 (1H, dd, *J*=17.2, 3.2, H-3_{eq}), 3.06 (1H, dd, *J*=17.2, 12.8, H-3_{ax}), 5.41 (1H, dd, *J*=12.8, 2.9, H-2), 6.00 (1H, s, H-8), 7.33 (1H, d, *J*=4.6, H-4'), 7.35-7.40 (2H, m, H-3' and 5'), 7.48 (1H, d, *J*=7.4, H-2' and 6'); and 32 mg of **11** (t_R 19.7 min) as a yellow solid: mp 140 °C; EIHRMS *m/z* 274.0870 (calcd. for $C_{15}H_{14}O_5$, 274.0841); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 2.86-2.91 (2H, m, H-α), 3.27-3.34 (2H, m, H-β), 5.88 (1H, s, H-5'), 7.18 (2H, d, *J*=1.5, H-2 and 6), 7.24-7.28 (3H, m, H-3, 4 and 5).

3.1.9. 3-Hydroxy-5,6,7-methoxyflavone (7a) [26]

n-Butyllithium (1.6 M solution in hexane, 5 mL, 8 mmol) was added to diisopropylamine (1.2 mL, 8 mmol) in dry THF (40 mL) at –80 °C under argon gas. The LDA solution was warmed to 0 °C for 30 min and re-cooled to –80 °C. **3a** (2.5 g, 8 mmol) in dry THF (80 mL) was added dropwise to the LDA solution and stirred for 1 h. After an addition of trimethyl borate (0.92 mL, 8 mmol)

in THF (20 mL), the resulting solution was then stirred at –80 °C for 30 min. The reaction mixture was acidified with 0.8 g of acetic acid, stirred for 15 min and then oxidized with 30% hydrogen peroxide (1.2 mL, 8 mmol). The solution was allowed to warm slowly to room temperature and was then shaken with aqueous saturated sodium bicarbonate solution (50 mL). The aqueous mixture was extracted with ethyl acetate (100 mL×3). The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography using chloroform-methanol (10:0.2) as the eluent to give **7a** (0.84 g, 32%) as yellow powders: mp 240-243 °C; EIHRMS *m/z* 328.0938 (calcd. for $C_{18}H_{16}O_6$, 328.0947); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.91, 3.97, 4.02 (each 3H, s, OMe-5, 6 and 7), 6.79 (1H, s, H-8), 7.43 (1H, t, *J*=7.4, H-4'), 7.50(2H, t, *J*=7.4, H-3' and 5'), 8.18 (2H, d, *J*=7.4, H-2' and 6').

3.1.10. 3,5,6,7-Tetrahydroxyflavone (7)

Using the procedure described for **2**, **7a** (130 mg, 0.4 mmol) was demethylated to **4**. Crystallization from hexane and ethyl acetate gave **7** (74 mg, 65%) as yellow powders: mp 246 °C; EIHRMS *m/z* 286.0472 (calcd. for $C_{15}H_{10}O_6$, 286.0300); ^{1}H-NMR δ (methanol-d_4) ppm: 6.53 (1H, s, H-8), 7.43-7.49 (3H, m, H-3', 4' and 5'), 8.18 (2H, d, *J* =7.1, H-2' and 6').

3.1.11. 6-Hydroxy-2,3,4-trimethoxyphenyl benzyl ketone (8a) [27]

A mixture of 3,4,5-trimethoxyphenol (3.7 g, 20 mmol), phenylacetyl chloride (3.4 g, 22 mmol) and BF_3-Et_2O complex solution (20 mL) was heated under reflux for 15 min and quenched with excess of water. The resulting mixture was extracted with ethyl acetate (200 mL×3) and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and

concentrated. The residue was purified by silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to give **8a** (5.98 g, 90%) as yellow pale oil: EIHRMS *m/z* 302.1171 (calcd. for $C_{17}H_{18}O_5$, 302.1154); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.78, 3.82, 3.88 (each 3H, s, OMe-2, 3 and 4), 4.37 (2H, s, H-8), 6.24 (1H, s, H-5), 7.23-7.26 (3H, m, H-3', 4' and 5'), 7.27-7.35 (2H, m, H-2' and 6').

3.1.12. 5,6,7-Trihydroxyisoflavone (8b) [28]

Formic acid (9.2 g, 0.1 mol) was gradually added to a solution of *N*,*N*'-carbonyldiimidazole (16.2 g, 0.1 mol) in THF (100 mL) at 0 °C. The resulting solution was stirred for 1 h. **8a** (4.53 g, 15 mmol) in dry THF (50 mL) was then added to it and stirred for 12 h at 0 °C. The solution was then heated at 100 °C for 30 min. The mixture was concentrated. The residue was treated with ice and the solid obtained was purified by silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to give **8b** as yellow powders (3.46 g, 74%): mp 156-158 °C; EIHRMS *m/z* 312.0984 (calcd. for $C_{18}H_{16}O_5$, 312.0848); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.92, 3.96, 3.97 (each 3H, s, OMe-5, 6 and 7), 6.70 (1H, s, H-8), 7.38 (1H, d, *J*=7.2, H-4'), 7.41-7.44 (2H, m, H-3' and 5'), 7.54 (2H, d, *J*=6.9, H-2' and 6'), 7.84 (1H, s, H-2).

3.1.13. 6-Hydroxy-2,3,4-trimethoxyphenyl *p*-methoxybenzyl ketone (9a)

By the same method as **8a**, **9a** was obtained from 3,4,5-trimethoxyphenol (3.7 g, 20 mmol) and *p*-methoxyphenylacetyl chloride (4.1 g, 22 mmol). Chromatography on silica gel using hexane-ethyl acetate (3:7) as the eluent gave **9a** (4.78 g, 72%) as a yellow pale solid:
mp 95-96 °C; EIHRMS *m/z* 332.1262 (calcd. for $C_{18}H_{20}O_6$, 332.1160); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.77, 3.78, 3.86, 3.96 (each 3H, s, OMe-2, 3, 4' and 4), 4.29 (2H, s, H-8), 6.22 (1H,

s, H-5), 6.87 (2H, d, *J*=8.4, H-3’ and 5’), 7.13 (2H, d, *J*=8.4, H-2’ and 6’).

3.1.14. 4’,5,6,7-Tetramethoxyisoflavone (9b)

By the same method as **8b**, **9b** was obtained from **9a** (3.32 g, 10 mmol). The crude solid was purified with silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to **9b** (2.46 g, 72%) as a yellow solid: mp 175 °C; EIHRMS *m/z* 342.1086 (calcd. for $C_{19}H_{18}O_6$, 342.1103); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 3.81, 3.89, 3.94, 3.94 (each 3H, s, OMe-4’, 5, 6 and 7), 6.67 (1H, s, H-8), 6.94 (2H, d, *J*=8.6, H-3’ and 5’), 7.45 (2H, d, *J*=8.6, H-2’ and 6’), 7.79 (1H, s, H-2).

3.1.15. 5,6,7-Trihydroxyisoflavone (8) and 4’,5,6,7-tetrahydroxyisoflavone (9)

Using the procedure described for **2**, **8b** and **9b** was demethylated to give **8** (81%) and **9** (79%), respectively. **8**: yellow powders; mp 284 °C; EIHRMS *m/z* 270.0569 (calcd. for $C_{15}H_{10}O_5$, 270.0528); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.51 (1H, d, *J*=1.7, H-8), 7.41-7.44 (3H, m, H-3’, 4’ and 5’), 7.56 (2H, d, *J*=8.1, H-2’ and 6’), 8.39 (1H, d, *J*=0.7, H-2); ^{13}C-NMR δ (DMSO-d_6) ppm: 94.5 (C-8), 105.6 (C-10), 122.4 (C-1’), 128.7 (C-6), 129.0 (C-2’ and 6’), 129.9 (C-3’ and 5’), 130.2 (C-4’), 132.0 (C-3), 148.3 (C-5), 150.9 (C-9), 154.5 (C-7), 155.7 (C-2), 180.9 (C-4); **9**: yellow powders; mp 260 °C; EI-HR-MS *m/z* 286.0493 (calcd. for $C_{15}H_{10}O_6$, 286.0477); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.47 (1H, d, *J*=1.7, H-8), 6.81 (2H, d, *J*=1.7, H-3’ and 5’), 7.36 (2H, dd, *J*=8.4, 1.7, H-2’ and 6’), 8.38 (1H, d, *J*=1.0, H-2); ^{13}C-NMR δ (DMSO-d_6) ppm: 93.5 (C-8), 104.8 (C-10), 115.0 (C-3’ and 5’), 121.5 (C-3), 121.5 (C-1’), 129.2 (C-6), 130.2 (C-2’ and 6’), 147.4(C-5), 150.0 (C-9), 153.6 (C-7), 153.9 (C-2), 157.3 (C-4’), 180.4 (C-4).

3.1.16. 2’,3’,4’,6’-Tetrahydroxychalcone (10)

A mixture of 3,4,5-trimethoxyphenol (3.7 g, 20 mmol) and cinnamoyl chloride (3.7 g, 22 mmol) and BF_3-Et_2O complex (20 mL) was refluxed for 15 min, cooled and quenched with excess of water. The resulting mixture was extracted with ethyl acetate (200 mL×3) and the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to provide crude 6’-hydroxy-2’,3’,4’-trimethoxychalcone [27] (5.15 g, crude yield 82%) which was used without further purification.

Crude 6’-hydroxy-2’,3’,4’-trimethoxychalcone (3.77 g) was added to a stirred solution of KF (1 g) in methanol (300 mL) and the reaction mixture was refluxed for 24 h. The reaction mixture was diluted with water and extracted with chloroform (300 mL×3). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to give 1.06 g of 5,6,7-trimethoxyflavanone [29] (yield from 3,4,5-trimethoxyphenol, 23%) as pale yellow powders: mp 157-158 °C; EIHRMS *m/z* 314.1176 (calcd. for $C_{18}H_{18}O_5$, 314.1154); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 2.78 (1H, dd, *J*=16.7, 2.7, H-3_{eq}), 3.00 (1H, dd, *J*=16.7, 13.3, H-3_{ax}), 3.81, 3.86, 3.93 (each 3H, OMe-5, 6 and 7), 5.39 (1H, dd, *J*=13.3, 2.7, H-2), 6.34 (1H, s, H-8), 7.36-7.38 (2H, m, H-2’ and 6’), 7.40-7.44 (3H, m, H-3’, 4’ and 5’).

Using the procedure described for **2**, 5,6,7-trimethoxyflavanone (314 mg, 1 mmol) was demethylated to give 5,6,7-trihydroxyflavanone, which was spontaneously converted to 2’,3’,4’,6’-tetrahydroxychalcone by ring-opening under the reaction condition. The product 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 75 mg of **10** (t_R 19.4 min, 28%) as yellow powders: mp 175 °C; EIHRMS *m/z* 272.0643 (calcd. for $C_{15}H_{12}O_5$, 272.0685); ^{1}H-NMR δ (methanol-d_4) ppm (*J* in Hz): 5.92 (1H, s, H-5'), 7.38-7.40 (3H, m, H-3, 4 and 5), 7.63 (2H, d, *J*=1.7, H-2 and 6), 7.73 (1H, d, *J*=15.5, H-α), 8.25 (1H, d, *J*=15.5, H-β).

3.1.17. 1,2,3-Trihydroxyxanthone (14) [30]

A mixture of salicylic acid (2.07 g, 15 mmol), 2',4',6'-trihydroxyacetophenone (2.52 g, 15 mmol), anhydrous $ZnCl_2$ (6 g) and $POCl_3$ (20 mL) were heated at 70-80 °C for 2 h. The reaction mixture was cooled, poured into ice-cooled water and extracted with ethyl acetate (100 mL×3). The extracts were combined, washed with water, dried over Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography using hexane-ethyl acetate (7:3) as the eluent to give 1,3-dihydroxy-2-acetylxanthone (1.74 g, 43%) as pale yellow powders: mp 252-254 °C; EIHRMS *m/z* 270.0529 (calcd. for $C_{15}H_{10}O_5$, 270.0528); ^{1}H-NMR δ (chloroform-*d*) ppm: 2.79 (3H, s, H-15), 6.36 (1H, s, H-4), 7.40-7.41(1H, m, H-5), 7.42-7.44 (1H, m, H-7), 7.73 (1H, m, H-6), 8.22 (1H, d, *J*=7.6, H-8), 14.33 (1H, s, OH-3), 15.27 (1H, s, OH-1).

1,3-Dihydroxy-2-acetylxanthone (0.755 g, 2.79 mmol) was dissolved in a mixture of 6 mL of 4% NaOH and 6 mL of pyridine and the mixture was cooled in an ice bath. Hydrogen peroxide (6 mL, 20%) was added dropwise with stirring at 5 min. The reaction mixture was left for 1 h. Acidification with 10 mL of 1 M HCl (10 mmol) yielded **14** as a yellow solid, which was crystallized from ethanol to give **14** (442 mg, 65%) as yellow powders: mp 268-270 °C; EIHRMS *m/z* 244.0345 (calcd. for $C_{15}H_{10}O_5$, 244.0371); ^{1}H-NMR δ (acetone-d_6) ppm (*J* in Hz): 6.54 (1H, s, H-4), 7.44 (1H, t, *J*=7.9, H-7), 7.53 (1H, d, *J*=7.9, H-5), 7.83 (1H, t, *J*=7.9, H-6), 8.20 (1H, dd, *J*=7.9, 1.7, H-8), 12.75 (1H, s, OH-1).

3.1.18. 7-Hydroxy-1,2,3,6-tetramethoxyxanthone (15a) [30]

A mixture of 2,5-dihydroxy-4-methoxybenzoic acid (1.98 g, 10 mmol), 3,4,5-trimethoxyphenol (3.13 g, 17 mmol), anhydrous $ZnCl_2$ (15 g) and $POCl_3$ (26 mL) were heated at 70-80 °C for 2 h. Treatment following the usual procedure gave **15a** (0.83 g, 25%) as yellow powders: mp 186-190 °C; EIHRMS *m/z* 332.0941 (calcd. for $C_{17}H_{16}O_7$, 332.0954); ^{1}H-NMR δ (chloroform-*d*) ppm: 3.93, 3.94, 3.96, 4.00 (each 3H, OMe-1, 2, 3 and 6), 6.38 (1H, s, H-4), 6.97 (1H, s, H-5), 7.53 (1H, s, H-8).

3.1.19. 1,2,3,6,7-Pentahydroxyxanthone (15) [31]

A mixture of **15a** (166 mg, 0.5 mmol), acetic anhydride (5 mL) and hydroiodic acid (*d*=1.6, 2 mL) was heated under reflux at 150 °C for 2 h. The cooled mixture was poured into a saturated solution of sodium hydrogen sulphite. The mixture was extracted with ethyl acetate (50 mL×3). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was dissolved in a small amount of methanol and subjected to preparative HPLC (mobile phase, water-methanol-formic acid (30:70:0.1); flow rate, 4.5 mL/min; detection, UV 254 nm) to give 36 mg of **15** (t_R 11.4 min, 26%) as yellow powders: mp 284 °C; EIHRMS *m/z* 276.0267 (calcd. for $C_{13}H_8O_7$, 276.0269); ^{1}H-NMR δ (methanol-d_4) ppm: 6.33 (1H, s, H-4), 6.92 (1H, s, H-5), 7.44 (1H, s, H-8).

3.1.20. 5,7-Dihydroxy-8-nitroflavone (16a) [32]

Nitric acid (*d*=1.42, 1 mL) in acetic acid (10 mL) was slowly added to a solution of 5,7-dihydroxyflavone (2.54 g, 10 mmol) in acetic acid (20 mL). The mixture was stirred for 1 h at 65 °C and the mixture was then poured into ice-cooled water (500 mL). The precipitated solid were filtered, wash with water and crystallized from ethanol to give **16a** (2.1 g, 70%) as yellow powders: mp 224 °C; EIHRMS *m/z* 299.0449 (calcd. for $C_{15}H_9O_6N$, 299.0430); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 6.38 (1H, s, H-6), 7.21 (1H, s, H-3), 7.61-7.64 (3H, m, H-3', 4' and 5'), 7.99 (2H, dd, *J*=8.3, 1.4, H-2' and 6').

3.1.21. 5,7-Dimethoxy-8-nitroflavone (16b)

By the method described for **3a**, **16a** was methylated to give **16b** (yield, 85%) as pale yellow powders: mp 207-208 °C; EIHRMS *m/z* 327.0751 (calcd. for $C_{17}H_{13}O_6N$, 327.0743); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 4.01, 4.08 (each 3H, s, OMe-5 and 7), 6.81 (1H, s, H-3), 6.92 (1H, s, H-6), 7.56-7.59 (3H, m, H-3', 4' and 5'), 7.87 (2H, dd, *J*=8.1, 1.7, H-2' and 6').

3.1.22. 8-Amino-5,7-dimethoxyflavone (16c) [33]

A solution of **16b** (200 mg) in THF (100 mL) was hydrogenated for 10 h in the presence of 10% palladium on charcoal (200 mg) at room temperature. The catalyst was removed by filtration and the filtrate was concentrated to give **16c** (yield 92%) as yellow powders: mp 215 °C; EIHRMS *m/z* 297.0968 (calcd. for $C_{17}H_{15}O_4N$, 297.1002); ^{1}H-NMR δ (chloroform-*d*) ppm : 3.96, 4.00 (each 3H, s, OMe-5 and 7), 6.65 (1H, s, H-6), 7.26 (1H, s, H-3), 7.50-7.52 (3H, m, H-3', 4' and 5'), 7.86-7.88 (2H, m, H-2' and 6').

3.1.23. 6-Amino-5,7-dihydroxyflavone (16) [32]

16c (100 mg) was heated with hydroiodic acid (*d*=1.6, 10 mL) in a sealed tube at 175-180 °C for 3 h, and the reaction mixture was poured into 10% sodium hydrogen sulfate. The solution was neutralized by an addition of 1M NaOH and the mixture was extracted with 1-butanol (100 mL × 3). The extracts were washed with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was crystallized from hexane-ethyl acetate to give **16** (51 mg, 55%) as yellow powders: mp 263 °C; EIHRMS *m/z* 269.0713 (calcd. for $C_{15}H_{11}O_4N$, 269.0688); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.62 (1H, s, H-8), 6.92 (1H, s, H-3), 7.54-7.59 (3H, m, H-3', 4' and 5'), 8.05 (2H, d, *J*=7.1, H-2' and 6'), 12.65 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 94.0 (C-8), 104.2 (C-10), 104.5 (C-3), 126.3 (C-2' and 6'), 129.1 (C-3' and 5'), 129.4 (C-6), 131.0 (C-1'), 131.8 (C-4'), 146.8 (C-5), 149.9 (C-9), 154.0 (C-7), 162.8 (C-2), 182.1 (C-4).

3.1.24. 8-Amino-5,7-dihydroxyflavone (17)

By the same method described for **16c**, **16a** was hydrogenated to give **17** (yield, 79%) as yellow powders: mp 244-246 °C; EIHRMS *m/z* 269.0663 (calcd. for $C_{15}H_{11}O_4N$, 269.0688); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.29 (1H, s, H-6), 6.90 (1H, s, H-3), 7.54-7.62 (3H, m, H-3', 4' and 5'), 8.21 (2H, d, *J*=7.6, H-2' and 6'), 12.07 (1H, s, OH-5); ^{13}C-NMR δ (DMSO-d_6) ppm: 98.4 (C-6), 103.7 (C-10), 104.5 (C-3), 116.5 (C-8), 126.7 (C-2' and 6'), 129.0 (C-3' and 5'), 130.9 (C-1'), 131.9 (C-4'), 143.3 (C-7), 151.3 (C-5), 151.4 (C-9), 162.9 (C-2), 182.4 (C-4).

3.1.25. 5,7-Dihydroxy-6,8-dinitroflavone (21a) [32]

Nitric acid (*d*=1.42, 5 mL) in acetic acid (10 mL) was slowly added to a solution of 5,7-dihydroxyflavone (2.54 g, 10 mmol) in acetic acid (20 mL). The mixture was stirred for 10 h at 65 °C and the mixture was then poured into ice-cooled water (500 mL). The precipitated solid

were filtered, washed with water and crystallized from ethanol to give **21a** (1.8 g, 52%) as pale yellow plates: mp 231 °C; EIHRMS *m/z* 344.0305 (calcd. for $C_{15}H_8O_8N_2$, 344.0280); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 6.91 (1H, s, H-3), 7.54-7.59 (3H, m, H-3', 4' and 5'), 7.88 (2H, d, *J*=7.4, H-2' and 6').

3.1.26. 6,8-Diamino-5,7-dihydroxyflavone (21) [34]

Zinc dust (100 mg) and acetic acid (20 ml) were added successively to a stirred solution of **21a** (200 mg) in CH_2Cl_2 (80 mL) at room temperature. After 1 h, the reaction mixture was filtered and 200 mL of CH_2Cl_2 was added to the filtrate. The solution was washed with water, dried over anhydrous Na_2SO_4, passed through a plug of silica gel and concentrated. The residue was crystallized from hexane-ethyl acetate to give **21** (117 mg, 71%) as pale purple powders: mp 182 °C; EIHRMS *m/z* 284.0820 (calcd. for $C_{15}H_{12}O_4N_2$, 284.0798); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.91 (1H, s, H-3), 7.54-7.61 (3H, m, H-3', 4' and 5'), 8.21 (2H, d, *J*=7.1, H-2' and 6').

3.1.27. 5-Hydroxy-6-nitroflavone (18a) [35]

A mixture of 2,6-dihydroxy-3-nitroacetophenone (3 g), sodium benzoate (4.25 g), benzoic anhydride (15 g) and pyridine (1.0 mL) was heated at 150-160 °C for 9 hr under argon gas. After cooled to room temperature, the reaction mixture was poured into water (100 mL) and extracted with chloroform (100 mL×3). The combined organic phase was washed with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was refluxed with alcoholic potassium hydroxide (5%, 100 mL) for 3 h under argon gas. After acidified with 1 M HCl, the mixture was extrated with chloroform (100 mL×3). The extract was washed with water, dried over anhydrous

Na_2SO_4 and concentrated. The residue was subjected to silica gel column chromatography using hexane-ethyl acetate (8:2) as the eluent to give **18a** (1.6 g, 40%) as pale yellow powders: mp 210 °C; EIHRMS *m/z* 283.0443 (calcd. for $C_{15}H_9O_5N$, 283.0481); ^{1}H-NMR δ (chlorofom-*d*) ppm (*J* in Hz): 6.86 (1H, s, H-3), 7.10 (1H, d, *J*=9.3, H-8), 7.57-7.60 (3H, m, H-3', 4' and 5'), 7.94 (2H, dd, *J*=6.9, 1.2, H-2' and 6'), 8.42 (1H, d, *J*=9.3, H-7).

3.1.28. 6-Amino-5-hydroxyflavone (18)

By the same procedure as described for **21**, **18a** (566 mg, 2 mmol) was reduced by Zn-AcOH to give **18** (330 mg, 65%) as yellow powders: mp 183-185 °C; EIHRMS *m/z* 253.0743 (calcd. for $C_{15}H_{11}O_5N$, 253.0739); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.98(1H, s, H-3), 7.07 (1H, d, *J*=8.9, H-7), 7.15 (1H, d, *J*=8.9, H-8), 7.56-7.61 (3H, m, H-3', 4' and 5'), 8.09 (2H, d, *J*=6.6, H-2' and 6').

3.1.29. 6-Amino-7-hydroxyflavone (19)

According to the method using preparation of **18a**, 7-hydroxy-6-nitroflavone (**19a**) was obtained by the reaction of 4, 6-dihydroxy-3-nitroacetophenone with sodium benzoate and benzoic anhydride.to give **19a** (yield 35%) as pale yellow powders: mp 234 °C; EIHRMS *m/z* 283.0489 (calcd. for $C_{15}H_9O_5N$, 283.0481); ^{1}H-NMR δ (chlorofom-*d*) ppm (*J* in Hz): 6.79 (1H, s, H-3), 7.26, 7.27 (each 1H, s, H-5 and 8), 7.54-7.59 (3H, m, H-3', 4' and 5'), 7.90 (2H, d, *J*=6.9, H-2' and 6').

By the same procedure as described for **21**, **19a** was reduced by Zn-AcOH to give **19** (yield 71%) as yellow powders: mp 209 °C; EIHRMS *m/z* 253.0712 (calcd. for $C_{15}H_{11}O_5N$, 253.0739); ^{1}H-

NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.77 (1H, s, H-3), 6.92 (1H, s, H-5), 7.12 (1H, s, H-8), 7.54-7.59 (3H, m, H-3', 4' and 5'), 7.90-8.02 (2H, m, H-2' and 6').

3.1.30. 6,8-Diamino-7-hydroxyflavone (20)

Using the procedure for preparation of **21a**, 7-hydroxyflavone was nitrated to give 7-hydroxy-6,8-dinitroflavone **20a** (yield 72%) as yellow powders: mp 289-293 °C; EIHRMS *m/z* 328.0309 (calcd. for $C_{15}H_8O_7N_2$, 328.0331); ^{1}H-NMR δ (chloroform-*d*) ppm (*J* in Hz): 7.01 (1H, s, H-3), 7.56 (1H, s, H-5), 7.55-7.61 (3H, m, H-3', 4' and 5'), 7.96 (2H, d, *J*=7.9, H-2' and 6').

By the same procedure as described for **21**, **20a** was reduced using Zn-AcOH to give **20** (yield 68%) as yellow powders: 223 °C; EIHRMS *m/z* 268.0876 (calcd. for $C_{15}H_{12}O_3N_2$, 268.0849); ^{1}H-NMR δ (DMSO-d_6) ppm (*J* in Hz): 6.59 (1H, s, H-3), 6.76 (1H, s, H-5), 7.52-7.54 (3H, m, H-3', 4' and 5'), 8.15 (2H, d, *J*=8.6, H-2' and 6').

3.2. Biology

α-Glucosidase inhibitory activity determination

The α-glucosidase inhibitory activity was measured as described previously.[17,37] The crude enzyme solution prepared from rat intestinal acetone powder (Sigma Aldrich Japan Co., Tokyo, Japan), was used as the small intestinal α-glucosidases, sucrase, maltase and isomaltase. The reaction mixture consisted of crude enzyme solution (as surase, 0.2 mL; as maltase, 0.05 mL; as isomaltase, 0.2 mL), substrate (sucrose: 56 mM, 0.2 mL; maltose: 5 mM, 0.35 mL; isomaltose: 7 mM, 0.2 mL, respectively) 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 glucose measurement.[17] 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). For kinetic analyses of sucrase and maltase by 6-amino-5,7-dihydroxyflavone (**16**), the enzyme and **16** (10 and 20 μM for sucrase; 20 and 40 μM for isomaltase) were incubated with increasing concentrations of sucrose (7-56 mM) or maltose (1.25-15 mM), respectively. 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.

b-Glucosidase, a-mannosidase, a-glactosidase and b-galactosidase inhibitory activity determination

The inhibitory activity of β-glucosidase (Sigma, G0395; almonds), α-mannosidase (Sigma, M7257; Jack bean), α-galactosidase (Sigma, G8507; Green coffee bean), or β-galactosidase (Sigma, G1875; Bovine liver) was performed with a slight modification of the literature procedure.[38] The enzymatic hydrolysis of substrates was monitored by the amount of *o*- or *p*-nitrophenol released in the reaction mixture: β-glucosidase (6 μg/mL) and *p*-nitrophenyl β-D-glucopyranoside (4 mM) in 50 mM sodium citrate buffer (pH 5.0); α-mannosidase (10 μg/mL) and *p*-nitrophenyl α-D-mannopyranoside (4 mM) in 50 mM sodium citrate buffer (pH 4.5); α-galactosidase (3.8 μg/mL) and *o*-nitrophenyl α-D-galactopyranoside (4 mM) in 50 mM potassium phosphate buffer (pH 6.5); β-galactosidase (1.4 mg/mL) and *o*-nitrophenyl β-D-

galactopyranoside (40 mM) in 40 mM potassium phosphate buffer (pH 7.3). After the mixture of enzyme solution (0.2 mL) and substrate (0.2 mL) was preincubated for 5 min at 37 °C, 0.2 mL of test compounds in 50% DMSO was added and then incubated for 15 min at 37 °C. After stopping the reaction by addition of 1 M Na_2CO_3 (0.4 mL), the amount of *o*-, or *p*-nitrophenol in the mixture was measured spectrophotometrically at 405 nm.

Porcine pancreatic a **-amylase inhibitory activity determination**

The inhibitory effect of the compounds on α-amylase activity was assessed by the usual method.[39] Briefly, starch azure (Sigma; 2.0 mg) used as a substrate was suspended in 50 mM Tri-HCl buffer (pH 6.9) containing 10 mM $CaCl_2$, and boiled for 5 min at 100 °C. Then, the starch azure solution was pre-incubated for 10 min at 37 °C. The test samples in 50% DMSO, and 0.2 mL of porcine pancreatic α-amylase solution (Sigma, A-6255; 2.0 U/mL; 50 mM Tri-HCl buffer containing 10 mM $CaCl_2$, pH 6.9) were added into each assay. The reaction was carried out at 37 °C for 10 min and stopped by adding 0.5 mL of 50% acetic acid. The reaction mixture was then centrifuged at 2000 rpm for 5 min at 4 °C. The absorbance of the supernatant at 595 nm was measured.

Acknowledgements

We are grateful to Mr. Kenji Watanabe and Dr. Eri Fukushi, GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University, for their skillful mass spectral measurements.

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Legends of figures and schemes

Fig. **1**. Structures and IC_{50} values of compounds **1**-**21** for inhibition of rat intestinal α-glucosidase (sucrose, 56 mM; phosphate buffer,100 mM; PH 6.3). [a] no inhibition, less than 30% inhibition at the concentration of 1000 μM.

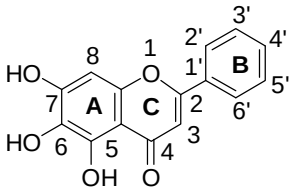
Fig. **2**. Lineweaver-Burk plot analysis of the inhibition kinetics of rat intestinal α-glucosidase inhibitory effects by 6-amino-5,7-dihydroxyflavone (**16**).

Scheme 1. Reagents and conditions: (1)Et_3N, THF, 85%; (2) CH_3COCl, $SnCl_4$, 1,2-dichloroethane, 83%; (3) *t*-BuOK, THF, 80 °C, 64%; (4) BBr_3/CH_2Cl_2, 52%.

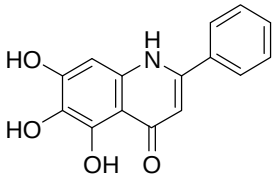
Scheme 2. Reagents and conditions: (1) $PhCH_2COCl$ (or (*p*-OMe)PhCOCl), BF_3-Et_2O; (2) *N*,*N*'-carbonyldiimidazole, formic acid, THF; (3) BB_3/CH_2Cl_2.

Scheme 3. Reagents and conditions: (1) $ZnCl_2$, $POCl_3$, 70-80 °C, 2 h, 25%; (2) HI (*d*=1.5), reflux, 2 h, 26%.

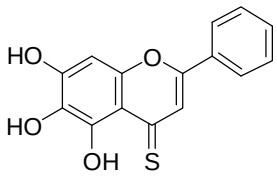
Scheme 4. Reagents and conditions: (1) 70% HNO_3, AcOH, 65 °C, 1h, 70%; (2) MeI, K_2CO_3, acetone, 8 h, 85%; (3) H_2, 10% Pd-C, THF, 10 h, 92%; (4) HI (*d*=1.6), reflux, 3h, 55%; (5) H_2, 10% Pd-C, THF, 10 h, 79%; (6) 70% HNO_3, AcOH, 65 °C, 10 h, 52%; (7) Zn, AcOH, 1 h, 71%.



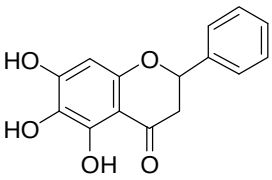
1 52 μM



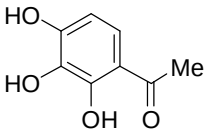
2 45 μM



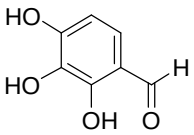
3 NI[a]



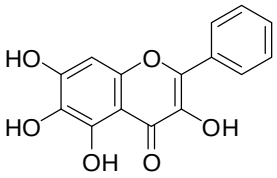
4 79 μM



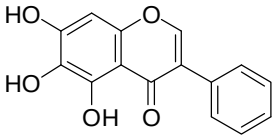
5 170 μM



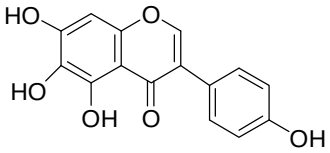
6 201 μM



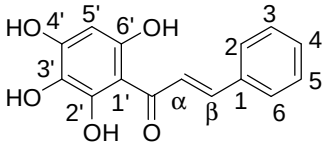
7 153 μM



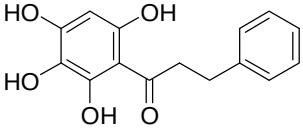
8 640 μM



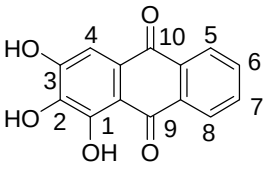
9 624 μM



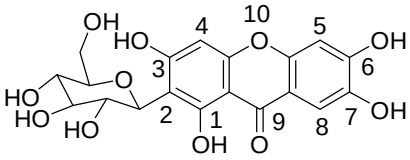
10 NI



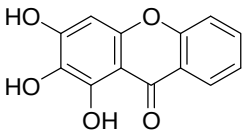
11 NI



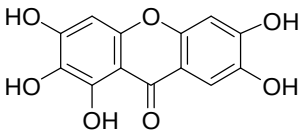
12 NI



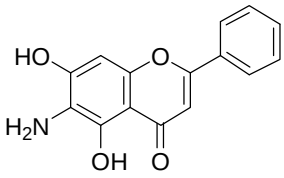
13 NI



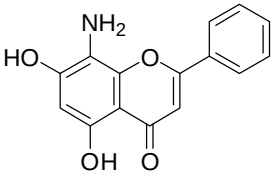
14 NI



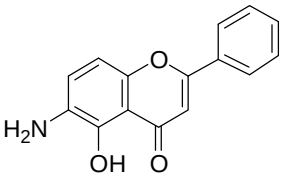
15 NI



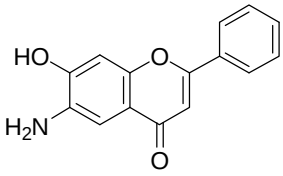
16 2.4 μM



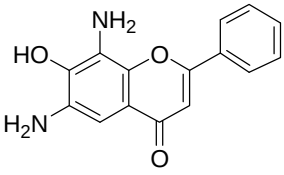
17 NI



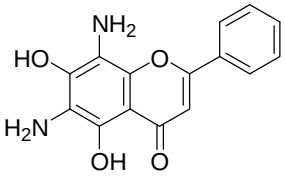
18 NI



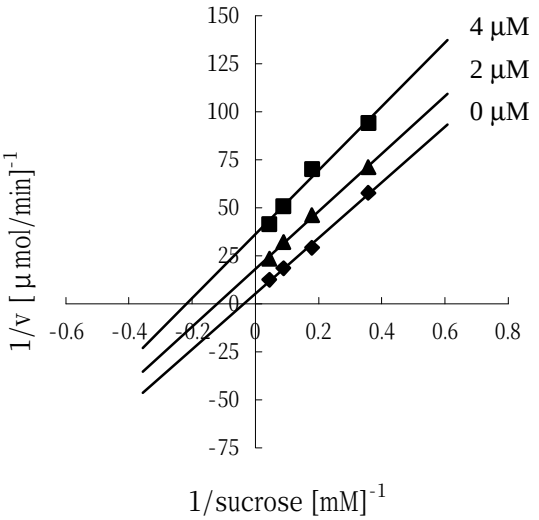
19 135 μM



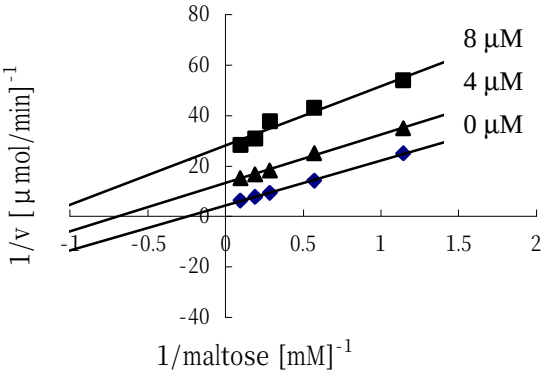
20 NI



21 82 μM

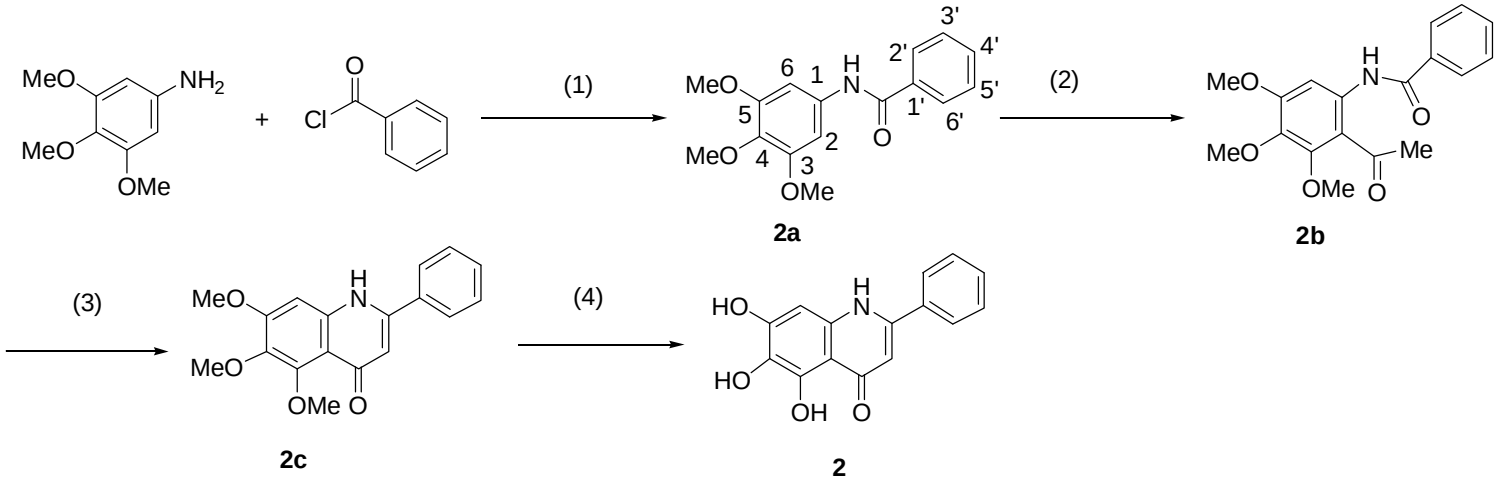

150
125
100
75
50
25
0
-25
-50
-75
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
4 μM
2 μM
0 μM
1/v [μ mol/min]-1
1/sucrose [mM]-1

(a)

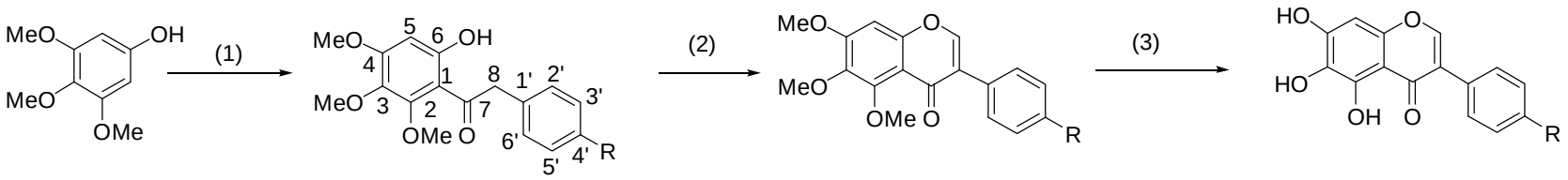

80
60
40
20
0
-20
-40
-1
-0.5
0
0.5
1
1.5
2
8 μM
4 μM
0 μM
1/v [μ mol/min]-1
1/maltose [mM]-1

(b)

Fig. 2.


MeO
MeO
OMe
NH2
+
Cl
O
(1)
MeO
MeO
OMe
H
N
O
2a
(2)
MeO
MeO
OMe
H
N
O
Me
O
2b
(3)
MeO
MeO
OMe
H
N
O
2c
(4)
HO
HO
OH
H
N
O
2

Scheme 1.

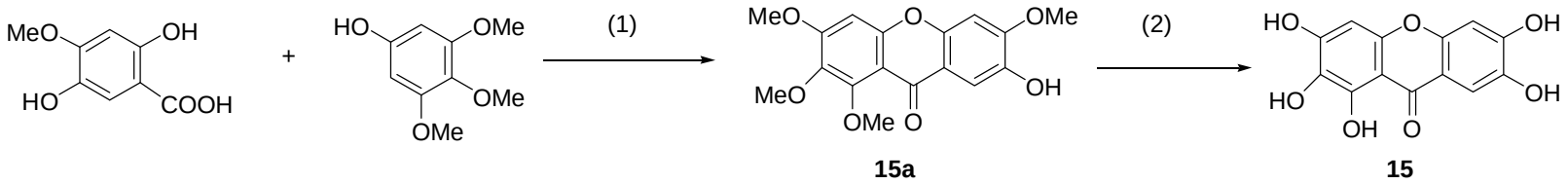


8a: R=H (yield, 90%)
9a: R=OMe (yield, 72%)

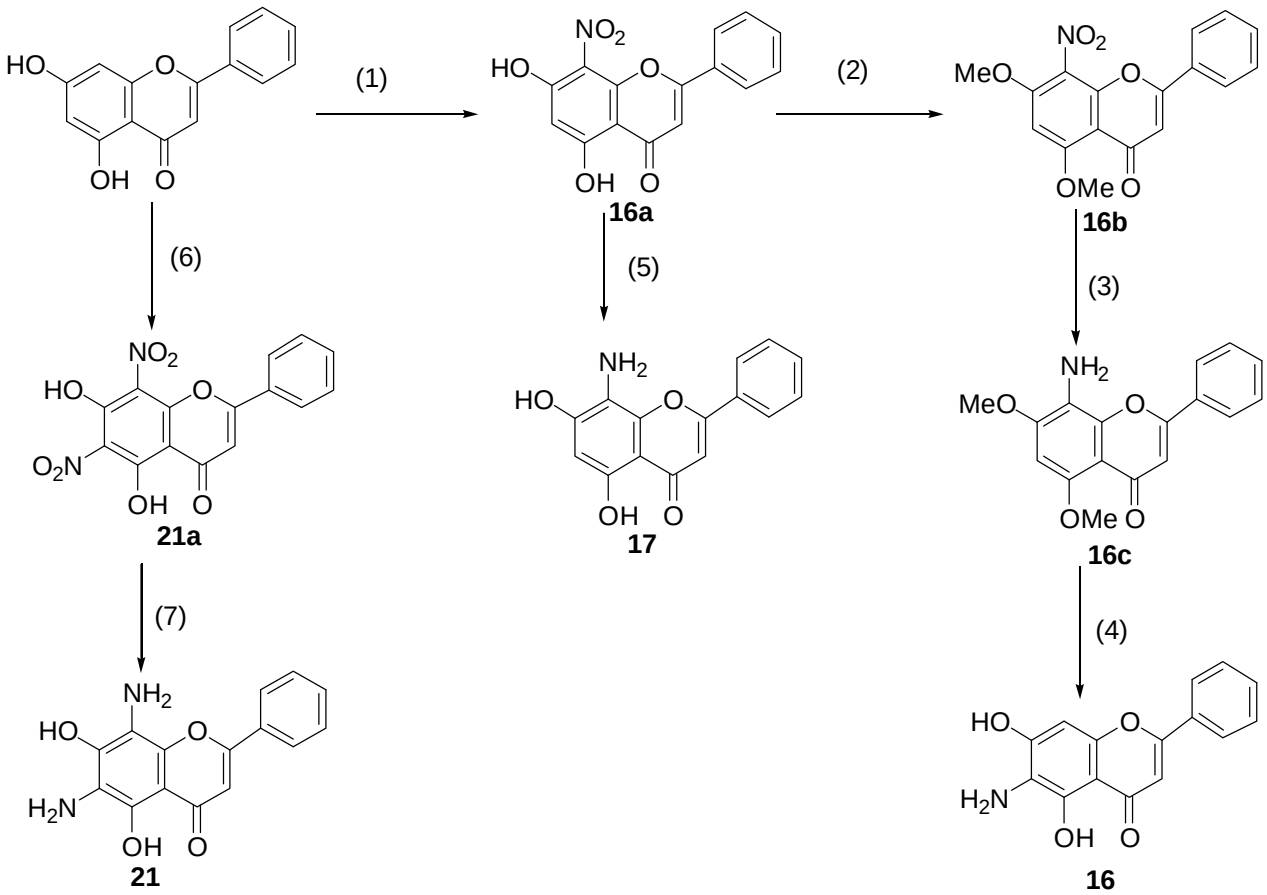
8b: R=H (yield, 74%)
9b: R=OMe (yield, 72%)

8: R=H (yield, 81%)
9: R=OH (yield, 79%)

Scheme 2.


MeO
OH
HO
COOH
+
HO
OMe
OMe
OMe
(1)
MeO
O
OMe
MeO
OH
OMe
O
15a
(2)
HO
O
OH
HO
OH
OH
O
15

Scheme 3.


HO
O
OH
O
(1)
NO2
HO
O
OH
O
16a
(2)
NO2
MeO
O
OMe
O
16b
(6)
(5)
(3)
NO2
HO
O2N
O
OH
O
21a
NH2
HO
O
OH
O
17
NH2
MeO
O
OMe
O
16c
(7)
(4)
NH2
HO
O
H2N
OH
O
21
HO
O
H2N
OH
O
16

Scheme 4.

Table 1. Selectivity of compounds **1**, **2**, **4** and **16** for the inhibitory effect on eight sugar hydrolases

	IC_{50} (μM)			
	1	**2**	**4**	**16**
Sucrase (rat intestine)	52	45	79	2.4
Maltase (rat intestine)	500	NI[a]	NI	4.4
Isomaltase (rat intestine)	NI	NI	NI	NI
β-Glucosidase (almonds)	NI	NI	NI	NI
α-Mannosidase (jack bean)	NI	NI	NI	NI
α-Galactosidase (green coffee been)	NI	NI	NI	NI
β-Galactosidase (bovine liver)	NI	NI	NI	NI
α-Amylase (porcine pancreas)	NI	NI	NI	NI

[a] no inhibition, less than 30% inhibition at the concentration of 1000 μM.

Table 2. IC_{50}, K_i values and inhibition-type of **16** and acarbose for rat small intestinal disaccharidase

Substrate	K_m (mM)	IC_{50} (μM)		K_i (μM)		inhibition type	
		16	acarbose	**16**	acarbose	**16**	acarbose
Sucrose	27	2.4	1.8	1.4	0.8	uncompetitive	competitive
Maltose	4	4.4	2.1	2.2	0.7	uncompetitive	competitive