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B-Ring-modified and/or 5-demethylated nobiletin congeners: Inhibitory activity against pro-MMP-9 production

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ABSTRACT

Three metabolites and 12 analogues of nobiletin (**1**) were synthesized. Whereas nobiletin derivatives **2–4** inhibited pro-MMP-9 production similarly in both PMA- and TNF-α-stimulated human lens epithelial cells, the 2′-hydroxylated analogue **5a** exerted marked inhibitory effects (IC_{50}: 0.4 μM) on PMA-treated cells, which were 170-fold more potent than those on TNF-α-treated cells. This activity may be closely related to PKC-mediated transcriptional regulation of pro-MMP-9.

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1. Introduction

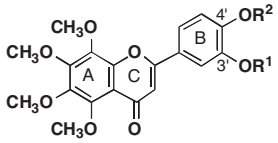
Over the past two decades, increasing attention has been given to polymethoxyflavones (PMFs).[1] Among them, nobiletin (**1**), one of the most abundant PMFs in citrus peel, has been of particular interest and its diverse properties beneficial to health including anticancer,[2] antimetastatic,[3] antiinflammatory,[4] antidiabetic,[5] and neurotrophic activities[6] have been reported by many research groups. However, systematic studies on the structure–activity relationships of PMFs have not been widely reported, with the notable exceptions of studies by Manthey et al.[7] and Li et al.[8,9]

Recently, we have reported a divergent synthesis of nobiletin metabolites **2–4** (Fig. 1) and their potent inhibitory activity against matrix metalloproteinase (MMP)-9.[10] MMP-9 is one of the most highly expressed enzymes in and around a wide variety of tumors and inflamed tissues.[11] Overactivation of MMP-9 leads to excessive degradation of the tissue, which causes diverse pathological states including arthritic diseases,[12] tumor metastasis,[13] cataract,[14] etc. In the recent communication,[10] we demonstrated that metabolite **3** inhibits the production of pro-MMP-9 much more potently than **1**, whereas metabolites **2** and **4** possess activity comparable to that of **1** in the phorbol 12-myristate 13-acetate (PMA)- or tumor necrosis factor (TNF)-α-treated human lens epithelial cell line SRA01/04.[15] These results suggested that the position of the hydroxyl group on the B ring of nobiletin may be closely linked with its inhibitory activity against pro-MMP-9 production. Here we discuss our structure–activity relationship study on B-ring-modified analogues as well as the effects on demethylation of the methoxy group at the C-5 position on the A ring (Fig. 2).

2. Chemistry

2.1. Synthesis of B-ring-modified analogues

Following the previously reported protocol,[10] eight nobiletin analogues **5a–d** and **6a–d** (Fig. 3) modified on the B ring were synthesized from the key A-ring synthon **7**. B-Ring synthons **8a–d** were prepared from the corresponding carboxylic acids. Acylation of **7** with **8a–d** gave rise to aryl esters **9a–d**, which underwent the Baker–Venkataraman rearrangement[16] to afford 1,3-diketones **10a–d** in good yields. Dehydrative closure of the flavone C ring proceeded smoothly, and subsequent hydrogenolysis of the benzyl ethers **11a–d** in the presence of Pearlman's catalyst gave analogues **5a–d** bearing a hydroxyl group(s) on the B ring in the range of 60–65% overall yield. Subsequent methylation of the hydroxyl group(s) on



1: $R^1 = R^2 = CH_3$ (nobiletin)
2: $R^1 = H$, $R^2 = CH_3$
3: $R^1 = CH_3$, $R^2 = H$
4: $R^1 = R^2 = H$

Figure 1. Structures of nobiletin and its metabolites.

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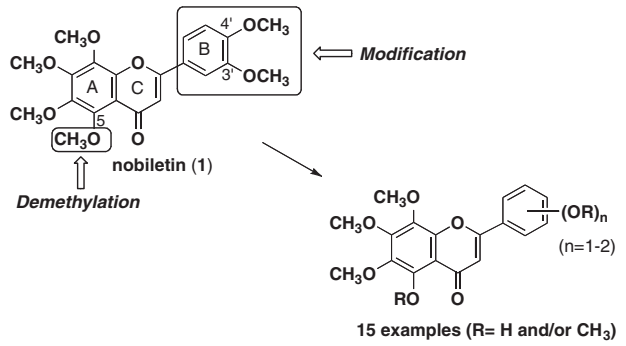


Figure 2. Structural modification of nobiletin (**1**).

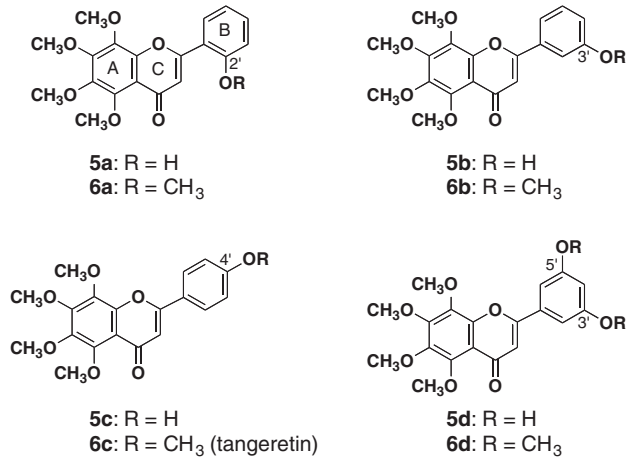


Figure 3. Structures of eight nobiletin congeners **5a–d** and **6a–d**.

the B ring gave rise to the corresponding PMFs **6a–d** in good yields, as shown in Scheme 1. The spectroscopic data for compounds **5a**,[17] **5c**,[18,19] and **6c**[18,20] thus obtained were identical to those reported previously.

2.2. Synthesis of 5-demethylated analogues

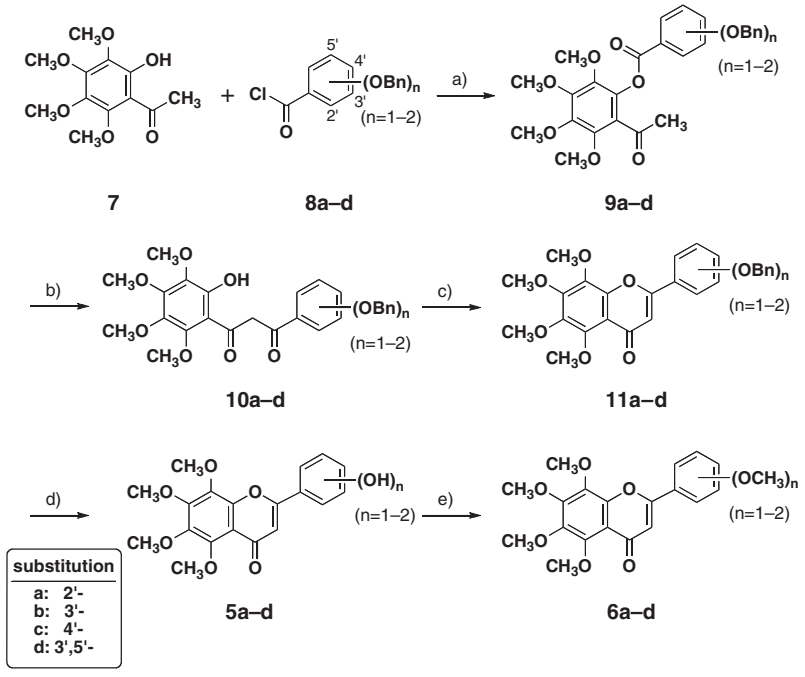
It is well known that after treatment with Lewis acid such as boron trichloride, nobiletin (**1**) undergoes regioselective demethylation of the C5-methoxy group to give 5-demethylnobiletin (**12**)[21] due to the neighboring-group participation of the C3 carbonyl oxygen.[8] According to the modified protocol of Li et al., **12** was obtained in 88% yield, whereas exposure of the 4′-benzyloxyflavone **13**,[10] a synthetic intermediate of nobiletin derivative **3**, to boron trichloride (1.2 mol equiv) yielded the 5-hydroxylated flavone **14** (43% yield) and 4′,5-dihydroxylated flavone **15** (36% yield). When two molar equivalents of boron trichloride were used, 3′-benzyloxyflavone **16**[10] and the tangeretin intermediate **11c** afforded the corresponding dihydroxylated flavones **17** and **18** in good yields, respectively (Scheme 2).

3. Results and discussion

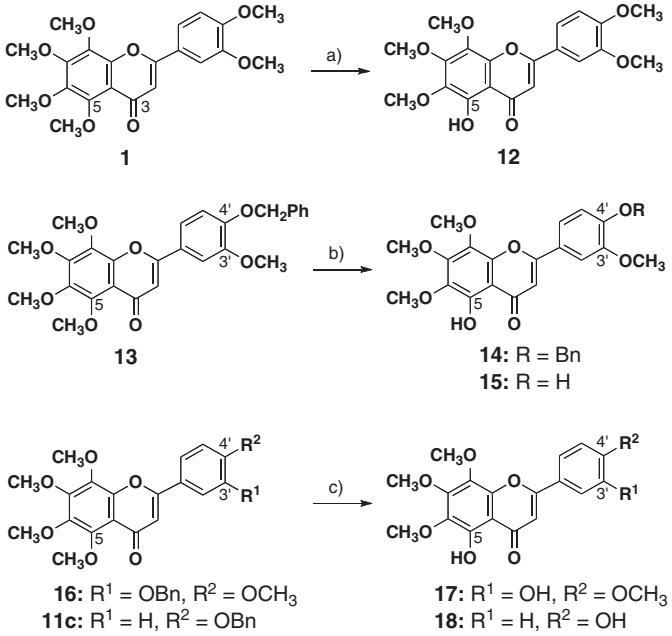
Since MMP-9 plays a pivotal role in cataract formation,[14] our interest has been focused on the effects of PMFs in human lens epithelial cells. Thus we evaluated the inhibitory activities of the synthesized PMFs against pro-MMP-9 production in the human lens epithelial cell line SRA01/04.[15]

3.1. Cytotoxicity of synthesized PMFs

First we examined the cytotoxicities of the synthesized PMFs in SRA01/04 cells using the lactate dehydrogenase (LDH) assay. As depicted in Figure 4, most of the tested PMFs, with a few exceptions such as benzyl ethers **11b** and **11c**, exerted little cytotoxic effect at 64 μM. Therefore, nobiletin metabolites **2–4**, B-ring-modified



Scheme 1. Synthesis of nobiletin analogues **5a–d** and **6a–d**. Reagents and conditions: (a) Et_3N (1.2 equiv), DMAP (0.1 equiv), CH_2Cl_2; **9a** 94%; **9b** 96%; **9c** 96%; **9d** 93%; (b) *t*-BuOK (1.1 equiv), THF, reflux, 1–1.5 h; (c) *p*-TsOH·H_2O (0.25 equiv), benzene, reflux (Dean-Stark), 5–10 h; **11a** 75%; **11b** 78%; **11c** 69%; **11d** 79% in two steps; (d) H_2 (1 atm), 20% $Pd(OH)_2$/C (cat.), ethyl acetate/ethanol (1:1), 0.5–1 h; **5a** 86%; **5b** 84%; **5c** 97%; **5d** 89%; e) CH_3I (5 equiv), K_2CO_3 (3 equiv), DMF; **6a** 80%; **6b** 90%; **6c** 84%; **6d** 76%.



Scheme 2. Synthesis of 5-demethylated congeners **12**, **14**, **15**, **17**, and **18**. Reagents and conditions: (a) BCl_3 (1.1 mol equiv), CH_2Cl_2, −78 °C to rt, 88%; (b) BCl_3 (1.2 mol equiv), CH_2Cl_2, −78 °C to rt, **14** 43%, **15** 36%; (c) BCl_3 (2.1 mol equiv), CH_2Cl_2, −78 °C to rt, **17** 92%; **18** 87%.

analogues **5a–d** and **6a–d**, and 5-hydroxylated flavones **12**, **15**, **17**, and **18**, all of which had almost no cytotoxic effects on SRA01/04 cells at 64 μM, were next subjected to gelatin zymography at various concentrations (0.25, 1, 4, 16, and 64 μM), and the observed results were shown not to be due to any cytotoxic effect of these PMFs.

3.2. Inhibitory activity against pro-MMP-9 production

MMP-9 belongs to a gelatinase subgroup, of which the main substrates are type IV collagens and denatured collagens (gelatins). Accordingly, the gelatin zymography method[22–24] was employed to evaluate the expression of pro-MMP-9 in SRA01/04 cells treated with PMA[25] or TNF-α[26] as an inducer agent for pro-MMP-9 production in the presence of the previously mentioned flavones. Briefly, gelatinolytic activities in the conditioned media were detected by electrophoresis (SDS–PAGE) on a gelatin containing 10% (w/v) polyacrylamide gel. The relative intensities of gelatin-digested bands were quantified using the image-processing program ImageJ[27] after the gel was stained. The IC_{50} values shown in Table 1 were estimated from the relative amounts of pro-MMP-9[28] at various concentrations (0.25, 1, 4, 16, or 64 μM) of the test samples.

Table 1
IC_{50} values (μM) of nobiletin (**1**) and synthesized congeners for pro-MMP-9 production[a]

Compound	PMA-treated cells	TNF-α-treated cells
1	20.9 ± 6.5	17.0 ± 1.6
2	16.9 ± 2.0	16.4 ± 6.6
3	3.7 ± 0.3	5.5 ± 0.8
4	12.3 ± 1.2	10.8 ± 1.9
5a	0.4 ± 0.1	68.0 ± 17.2
5b[b]	2.5 ± 3.4	23.5 ± 3.4
5c	2.6 ± 1.4	14.3 ± 8.1
5d	8.1 ± 4.4	24.0 ± 7.8
6a	13.7±5.8	24.0 ± 7.7
6b	5.5 ± 4.0	15.5 ± 6.7
6c	6.8 ± 3.9	33.2 ± 5.9
6d	12.0±6.6	25.8 ± 0.4
12[b]	3.0 ± 2.7	42.4 ± 11.6
15	0.7 ± 0.9	10.6 ± 5.4
17[b]	1.5 ± 1.7	29.6 ± 13.0
18	3.5 ± 3.1	22.3 ± 6.2

[a] Means of triplicate measurements.
[b] Rebound effects were observed (see Fig. 5).

The first group in Table 1 shows the effects of nobiletin (**1**) and its metabolites **2–4** on the inhibition of the production of pro-MMP-9 in PMA- or TNF-α-stimulated SRA01/04 cells. Regardless of whether PMA or TNF-α was used as an inducing agent, similar results were obtained.

In sharp contrast to metabolites **2**–**4**, hydroxylated analogues **5a**–**d** and PMFs **6a**–**d** preferentially inhibited PMA-induced pro-MMP-9 production, as demonstrated in the second group in Table 1.[29] It is of particular interest that the 2′-hydroxylated flavone **5a** showed markedly potent inhibitory activity against pro-MMP-9 production in PMA-stimulated SRA01/04 cells (IC_{50}: 0.4 μM) with extremely high selectivity (Cf. IC_{50} against TNF-α-stimulated cells:

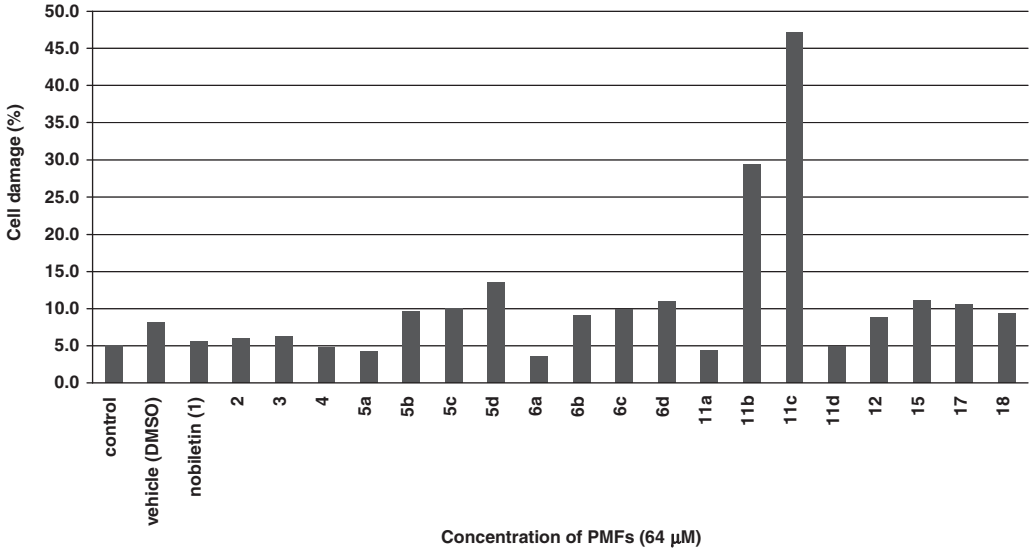
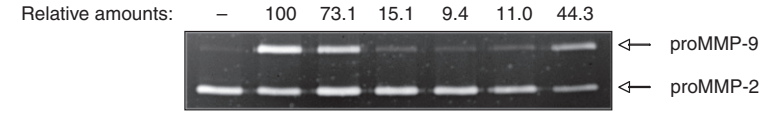


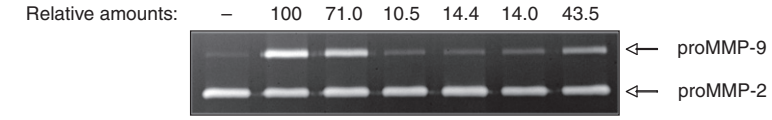
Figure 4. Cytotoxicity of nobiletin (**1**) and synthesized PMFs.

(a) Results of gelatin zymography of compound **5b**



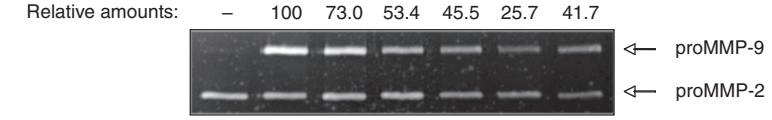
PMA (10 nM)	–	+	+	+	+	+	+
5b (μM)	0	0	0.25	1	4	16	64

(b) Results of gelatin zymography of compound **12**



PMA (10 nM)	–	+	+	+	+	+	+
12 (μM)	0	0	0.25	1	4	16	64

(c) Results of gelatin zymography of compound **17**



PMA (10 nM)	–	+	+	+	+	+	+
17 (μM)	0	0	0.25	1	4	16	64

Figure 5. Rebound effects observed with compounds **5b**, **12**, and **17**.

68.0 μM). As PMA is one of the most well-known activators of protein kinase C (PKC), this observation suggests that the inhibitory activity of **5a** is closely related to the PKC-mediated transcriptional regulation of pro-MMP-9. Meanwhile, hydroxylated analogues **5b–d** exhibited almost similar properties to **6b–d**, respectively.

We next examined the effects on demethylation at the C-5 methoxy group. As depicted in the third group in Table 1, 5-demethylated PMFs **12**, **15**, **17**, and **18** exerted 6- to 15-fold more potent inhibitory effects on PMA-treated cells than on TNF-α-treated cells. To our surprise, rebound effects were observed in a few cases. Usually, the higher the concentration of PMFs, the stronger the inhibitory activity observed. However, a higher dosage (64 μM) of compounds **5b**, **12**, and **17** diminished the inhibitory effects on PMA-induced pro-MMP-9 production, as shown in Figure 5.[30,31] To the best of our knowledge, such observations on the inhibition of pro-MMP-9 production have not been reported previously. It should be emphasized that these rebound effects were observed not in TNF-α-treated cells but in PMA-treated cells. Although the detailed mechanism remains to be elucidated, it could be assumed that a higher concentration of such PMFs negates in part the inhibitory effects on PKC-mediated pro-MMP-9 production.

4. Conclusion

In summary, we synthesized 15 congeners of nobiletin (**1**). Three nobiletin metabolites **2–4** inhibited pro-MMP-9 production similarly in both PMA- and TNF-α-stimulated SRA01/04 cells much more potently than nobiletin (**1**). On the other hand, hydroxylated analogues **5a–d**, PMFs **6a–d**, and 5-demethylated PMFs **12**, **15**, **17**, and **18** inhibited pro-MMP-9 production more selectively in PMA-treated SRA01/04 cells than in TNF-α-treated cells. Compound **5a** showed the most potent activity (IC_{50}: 0.4 μM) in PMA-stimulated cells, which was 170-fold greater than the activity in TNF-α-treated cells. Therefore, this compound could be a clue to unravel the detailed mechanism of the inhibition of pro-MMP-9 production. Moreover, at higher concentrations of flavones **5b**, **12**, and **17**, the inhibitory effects of PMFs on pro-MMP-9 production were reduced in PMA-treated cells. Ongoing studies to elucidate the mechanism of action, as well as the synthesis and evaluation of other nobiletin congeners, are currently underway in our laboratories.

5. Experimental section

5.1. Chemistry

5.1.1. General procedure

All air- or moisture-sensitive reactions were carried out under an argon atmosphere. Anhydrous solvents were purified following the standard methods. Other commercially available reagents were used as received unless otherwise noted. Melting points were measured with a Yanaco micromelting point apparatus and were uncorrected. Flash column chromatography was performed using Cica Silica Gel 60 N (spherical, neutral; 40–50 μm). IR spectra were

recorded on a JASCO FT/IR-4200 model using NaCl plates or KBr pellets. NMR spectra were recorded on a JEOL AL-400 (^{1}H NMR at 400 MHz, ^{13}C NMR at 100 MHz) or a JEOL EPC-600 (^{1}H NMR at 600 MHz, ^{13}C NMR at 150 MHz) model. Chemical shifts are given in δ units relative to internal tetramethylsilane (0 ppm for ^{1}H and ^{13}C NMR) or $CDCl_3$ (77.0 ppm for ^{13}C NMR), and the coupling constants (J) are given in Hertz (Hz). Splitting patterns are abbreviated as follows: broad (br), singlet (s), doublet (d), triplet (t), and multiplet (m). HR–MS spectra were obtained on a Shimadzu LCMS-IT-TOF model.

5.1.1.1. 2-(2-Benzyloxybenzoyloxy)-3,4,5,6-tetramethoxyacetophenone (9a). To a solution of **7** (205 mg, 0.800 mmol) and DMAP (10 mg, 0.82 mmol) in dichloromethane (3 mL) were successively added triethylamine (0.13 mL, 0.93 mmol) and a solution of **8a** (236 mg, 0.96 mmol) in dichloromethane (3 mL) at ice-water bath temp. The reaction mixture was stirred at rt for 1 h and partitioned between ethyl acetate (20 mL) and 0.5 M HCl (20 mL). The organic layer separated was washed with aq. sat. $NaHCO_3$ (20 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue (370 mg) was purified on a silica gel column (hexane/ethyl acetate = 4/1) to give **9a** (351 mg, 0.752 mmol, 94% yield) as yellow oil: IR (neat) 2941, 1751, 1704, 1600 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.99 (1H, dd, J = 1.6, 7.6 Hz), 7.51–7.47 (3H, m), 7.36–7.27 (3H, m), 7.04 (2H, m), 5.21 (2H s), 3.97 (3H, s), 3.93 (3H, s), 3.89 (3H, s), 3.79 (3H, s), 2.45 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 199.3, 163.7, 158.7, 148.9, 146.3, 144.8, 142.4, 136.6, 136.2, 134.2, 132.3, 128.5 (2C), 127.7, 126.9 (2C), 125.4, 120.6, 119.1, 113.7, 70.5, 62.1, 61.3 (2C), 61.2, 32.0; HRMS calcd. for $[M+Na]^+$ of $C_{26}H_{26}O_8$: 489.1520, found: 489.1522.

5.1.1.2. 2-(3-Benzyloxybenzoyloxy)-3,4,5,6-tetramethoxyacetophenone (9b). According to a similar procedure as described for the preparation of **9a**, **9b** was obtained in 96% yield as yellow oil: IR (neat) 2941, 1743, 1702, 1586 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.79–7.76 (2H, m), 7.48–7.32 (6H, m), 7.25 (1H, m), 5.13 (2H, s), 3.98 (3H, s), 3.94 (3H, s), 3.90 (3H, s), 3.81 (3H, s), 2.48 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 199.0, 164.5, 158.8, 149.1, 146.5, 145.0, 142.3, 136.4, 136.2, 130.0, 129.7, 128.6 (2C), 128.1, 127.5 (2C), 125.1, 123.0, 121.1, 115.7, 70.2, 62.1, 61.30 (2C), 61.28, 32.0; HRMS calcd. for $[M+Na]^+$ of $C_{26}H_{26}O_8$: 489.1520, found: 489.1528.

5.1.1.3. 2-(4-Benzyloxybenzoyloxy)-3,4,5,6-tetramethoxyacetophenone (9c). According to a similar procedure as described for the preparation of **9a**, **9c** was obtained in 96% yield as white crystals: mp 131 °C; IR (KBr) 2940, 1745, 1704, 1605 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 8.10 (2H, br d, J = 9.0 Hz), 7.46–7.34 (5H, m), 7.04 (2H, br d, J = 9.0 Hz), 5.16 (2H s), 3.98 (3H, s), 3.94 (3H, s), 3.89 (3H, s), 3.81 (3H, s), 2.47 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 199.2, 164.3, 163.2, 149.0, 146.4, 144.9, 142.4, 136.3, 136.1, 132.5 (2C), 128.7 (2C), 128.2, 127.4 (2C), 125.3, 121.2, 114.8 (2C), 70.2, 62.1, 61.29 (2C), 61.27, 32.0; HRMS calcd. for $[M+Na]^+$ of $C_{26}H_{26}O_8$: 489.1520, found: 489.1521.

5.1.1.4. 2-(3,5-Dibenzyloxybenzoyloxy)-3,4,5,6-tetramethoxyacetophenone (9d). According to a similar procedure as described for the preparation of **9a**, **9d** was obtained in 93% yield as white crystals: mp 95–96 °C; IR (KBr) 2937, 1737, 1707, 1598 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.45–7.33 (12H, m), 6.87 (1H, dd, J = 2.0, 2.4 Hz), 5.09 (4H s), 3.98 (3H, s), 3.94 (3H, s), 3.90 (3H, s), 3.79 (3H, s), 2.46 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 199.0, 164.4, 159.9 (2C), 149.1, 146.5, 145.0, 142.3, 136.3 (2C), 136.2, 130.5, 128.6 (4C), 128.1 (2C), 127.6 (4C), 125.1, 109.1 (2C), 108.1, 70.4 (2C), 62.1, 61.33, 61.30 (2C), 32.0; HRMS calcd. for $[M+H]^+$ of $C_{33}H_{32}O_9$: 573.2119, found: 573.2117.

5.1.1.5. 1-(2-Benzyloxyphenyl)-3-(2-hydroxy-3,4,5,6-tetramethoxyphenyl)propane-1,3-dione (10a). A mixture of **9a** (326 mg, 0.699 mmol) and potassium *t*-butoxide (86 mg, 0.77 mmol) in THF (6 mL) was heated at reflux for 1 h. After being cooled to rt, the reaction mixture was partitioned between ethyl acetate (20 mL) and 0.5 M HCl (20 mL). The organic layer was washed with brine (2 × 20 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue (310 mg) was purified on a silica gel column (hexane/ethyl acetate = 4/1) to give **10a** (262 mg, 0.562 mmol, 80% yield) as a mixture of tautomeric isomers (enol form: keto form = Ca. 3:2): IR (neat) 2936, 1672, 1602, 1562 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 12.97 (1H of the keto form, s, OH), 12.39 (1H of the enol form, s), 7.97 (1H of the keto form, dd, J = 2.0, 8.0 Hz), 7.91 (1H of the enol form, dd, J = 2.0, 8.0 Hz), 7.67 (1H of the enol form, s, OH, and 1H of the keto form, m) 7.53–7.25 (6H of the enol form and 5H of the keto form, m), 7.09–6.99 (2H of the enol form and 2H of the keto form, m), 5.26 (2H of the enol form, s, $PhCH_2O$), 5.09 (2H of the keto form, s, $PhCH_2O$), 4.60 (2H of the keto form, ArCO-CH_2-COAr), 4.07 (3H of the keto form, s, OCH_3), 4.06 (3H of the enol form, s, OCH_3), 3.88 (3H of the enol form, s, OCH_3), 3.85 (3H of the keto form, s, OCH_3), 3.76 (3H of the enol form, s, OCH_3), 3.71 (3H of the keto form, s, OCH_3), 3.68 (3H of the enol form, s, OCH_3), 3.64 (3H of the keto form, s, OCH_3); HRMS calcd. for $[M+Na]^+$ of $C_{26}H_{26}O_8$: 489.1520, found: 489.1521.

5.1.1.6. 1-(3-Benzyloxyphenyl)-3-(2-hydroxy-3,4,5,6-tetramethoxyphenyl)propane-1,3-dione (10b). According to a similar procedure as described for the preparation of **10a**, **10b** was obtained in 82% yield as a mixture of tautomeric isomers (enol form: keto form = ca. 5:1): IR (neat) 2936, 1595, 1573, 1547 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 13.01 (1H of the keto form, s, OH), 12.45 (1H of the enol form, s, OH), 7.60–7.12 (10H of the enol form and 9H of the keto form, m), 5.14 (2H of the enol form and 2H of the keto form, s, $PhCH_2O$), 4.60 (2H of the keto form, ArCO-CH_2-COAr), 4.09 (3H of the enol form and 3H of the keto form, s, OCH_3), 3.89 (3H of the enol form, s, OCH_3), 3.88 (3H of the enol form, s, OCH_3), 3.87 (3H of the keto form, s, OCH_3), 3.86 (3H of the enol form, s, OCH_3), 3.73 (3H of the keto form, s, OCH_3), 3.69 (3H of the keto form, s, OCH_3); HRMS calcd. for $[M+Na]^+$ of $C_{26}H_{26}O_8$: 489.1520, found: 489.1519.

5.1.1.7. 1-(4-Benzyloxyphenyl)-3-(2-hydroxy-3,4,5,6-tetramethoxyphenyl)propane-1,3-dione (10c). According to a similar procedure as described for the preparation of **10a**, **10c** was obtained in 99% yield as a mixture of tautomeric isomers (enol form:keto form = ca. 5:1): IR (neat) 2936, 1600, 1567, 1508 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 13.04 (1H of the keto form, s, OH), 12.49 (1H of the enol form, s, OH), 7.97–7.88 (2H of the enol form and 2H of the keto form, m), 7.47–7.30 (6H of the enol form and 5H of the keto form, m), 7.07–7.02 (2H of the enol form and 2H of the keto form, m), 5.15 (2H of the enol form and 2H of the keto form, s, $PhCH_2O$), 4.60 (2H of the keto form, ArCO-CH_2-COAr), 4.08 (3H of the enol form and 3H of the keto form, s, OCH_3), 3.892 (3H of the enol form, s, OCH_3), 3.885 (3H of the enol form, s, OCH_3), 3.867 (3H of the keto form, s, OCH_3), 3.862 (3H of the enol form, s, OCH_3), 3.74 (3H of the keto form, s, OCH_3), 3.73 (3H of the keto form, s, OCH_3); HRMS calcd. for $[M+Na]^+$ of $C_{26}H_{26}O_8$: 489.1520, found: 489.1520.

5.1.1.8. 1-(3,5-Dibenzyloxyphenyl)-3-(2-hydroxy-3,4,5,6-tetramethoxyphenyl)propane-1,3-dione (10d). According to a similar procedure as described for the preparation of **10a**, **10d** was obtained in 88% yield as a mixture of tautomeric isomers (enol form:keto form = ca. 3:1): IR (neat) 2932, 1579, 1548 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 12.97 (1H of the keto form, s, OH), 12.38 (1H of the enol form, s, OH), 7.44–7.30 (11H of the enol form and 10H of the keto form, m), 7.17–7.13 (2H of the enol form and 2H of the keto form,

m), 6.84 (1H of the keto form, m), 6.77 (1H of the enol form, m), 5.08 (4H of the enol form and 4H of the keto form, br s, $PhCH_2O$), 4.56 (2H of the keto form, $ArCO\text{-}CH_2\text{-}COAr$), 4.10 (3H of the enol form and 3H of the keto form, s, OCH_3), 3.90 (3H of the enol form, s, OCH_3), 3.880 (3H of the keto form, s, OCH_3), 3.877 (3H of the enol form, s, OCH_3), 3.86 (3H of the enol form, s, OCH_3), 3.74 (3H of the keto form, s, OCH_3), 3.69 (3H of the keto form, s, OCH_3); HRMS calcd. for $[M-H]^-$ of $C_{33}H_{32}O_9$: 571.1974, found: 571.1967.

5.1.1.9. 2′-Benzyloxy-5,6,7,8-tetramethoxyflavone (11a). A mixture of **10a** (248 mg, 0.532 mmol) and *p*-TsOH (monohydrate, 25 mg, 0.13 mmol) in benzene (5 mL) was heated at reflux for 7 h with azeotropic removal of water using Dean-Stark trap. After being cooled to rt, the mixture was diluted with ethyl acetate (30 mL) and washed with sat. aq. $NaHCO_3$ (2 × 20 mL). The organic layer was dried ($MgSO_4$) and concentrated. The residue was chromatographed (silica gel, toluene/ethyl acetate = 1/0–5/1–2/1) to afford **11a** (224 mg, 0.499 mmol, 94% yield) as yellow crystals: mp 99–101 °C; IR (KBr) 2941, 1640, 1447, 1359 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.90 (1H, dd, J = 2.0, 8.0 Hz), 7.43–7.30 (6H, m), 7.13–7.04 (2H, m), 7.01 (1H s) 5.25 (2H s), 4.09 (3H, s), 3.96 (3H, s), 3.95 (3H, s), 3.94 (3H, s); ^{13}C NMR ($CDCl_3$) δ:177.5, 158.9, 156.8, 151.2, 148.1, 147.9, 143.8, 138.0, 136.1, 132.0, 129.3, 128.6 (2C), 128.0, 126.9 (2C), 121.2, 121.0, 114.8, 113.3 (2C), 70.6, 62.2, 62.0, 61.8, 61.6; HRMS calcd. for $[M+H]^+$ of $C_{26}H_{24}O_7$: 449.1595, found: 449.1596.

5.1.1.10. 3′-Benzyloxy-5,6,7,8-tetramethoxyflavone (11b).

According to a similar procedure as described for the preparation of **11a**, **11b** was obtained in 95% yield as yellow crystals: mp 102–104 °C; IR (KBr) 2937, 1634, 1590, 1464 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.54–7.51 (2H, m), 7.48–7.35 (6H, m), 7.14 (1H, m), 6.67 (1H, s), 5.16 (2H s), 4.11 (3H, s), 4.00 (3H, s), 3.95 (6H, s); ^{13}C NMR ($CDCl_3$) δ: 177.3, 160.9, 159.2, 151.5, 148.3, 147.8, 144.2, 138.1, 136.4, 132.9, 130.2, 128.7 (2C), 128.2, 127.4 (2C), 118.7, 118.1, 114.9, 112.3, 108.2, 70.2, 62.23, 62.22, 62.0, 61.8; HRMS calcd. for $[M+H]^+$ of $C_{26}H_{24}O_7$: 449.1595, found: 449.1592.

5.1.1.11. 4′-Benzyloxy-5,6,7,8-tetramethoxyflavone (11c).

According to a similar procedure as described for the preparation of **11a**, **11c** was obtained in 70% yield as yellow crystals: mp 142–144 °C (Lit.[18] 136–139 °C); IR (KBr) 2941, 1638, 1605, 1514 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.87 (2H, br d, J = 8.8 Hz), 7.46–7.34 (5H, m), 7.09 (2H, br d, J = 8.8 Hz), 6.61 (1H s), 5.15 (2H s), 4.10 (3H, s), 4.02 (3H, s), 3.95 (6H, s); ^{13}C NMR ($CDCl_3$) δ: 177.1, 161.3, 161.0, 151.2, 148.2, 147.6, 143.9, 137.9, 136.1, 128.6 (2C), 128.1, 127.6 (2C), 127.3 (2C), 123.9, 115.3 (2C), 114.8, 106.6, 70.1, 62.2, 62.0, 61.8, 61.6; HRMS calcd. for $[M+H]^+$ of $C_{26}H_{24}O_7$: 449.1595, found: 449.1593.

5.1.1.12. 3′,5′-Dibenzyloxy-5,6,7,8-tetramethoxyflavone (11d).

According to a similar procedure as described for the preparation of **11a**, **11d** was obtained in 90% yield as white crystals: mp 88–90 °C; IR (KBr) 2937, 1641, 1595 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.46–7.33 (10H, m), 7.14 (2H, br d, J = 2.0 Hz), 6.77 (1H, dd, J = 2.0, 2.0 Hz), 6.64 (1H s), 5.12 (4H s), 4.10 (3H, s), 3.97 (3H, s), 3.95 (6H, s); ^{13}C NMR ($CDCl_3$) δ: 177.3, 160.8, 160.3 (2C), 151.5, 148.3, 147.8, 144.2, 138.1, 136.3 (2C), 133.5, 128.7 (4C), 128.2 (2C), 127.5 (4C), 114.9, 108.4, 105.3 (3C), 70.3 (2C), 62.3, 62.0, 61.8, 61.7; HRMS calcd. for $[M+H]^+$ of $C_{33}H_{30}O_8$: 555.2013, found: 555.2013.

5.1.1.13. 2′-Hydroxy-5,6,7,8-tetramethoxyflavone (5a). A mixture of **11a** (32.4 mg, 72.2 μmol) and 20% $Pd(OH)_2$ on carbon (6 mg) in ethyl acetate (1.5 mL) and ethanol (1.5 mL) was stirred at rt for 2 h under a hydrogen atmosphere. The mixture was passed through a pad of Celite and the cake was washed with ethyl acetate. The combined organic layers were concentrated. The residual yellow solids were purified on a silica gel column (hexane/ethyl acetate = 2/1) to give **5a** (22.2 mg, 61.9 μmol, 86% yield) as white crystals: mp 223 °C; IR (KBr) 3442, 2935, 1624, 1574, 1450 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.99 (1H, dd, J = 1.6, 8.0 Hz), 7.86 (1H, br s), 7.41 (1H, ddd, J = 1.6, 8.0, 8.0 Hz), 7.30 (1H, br d, J = 8.0 Hz), 7.02 (1H, ddd, J = 1.6, 8.0, 8.0 Hz), 4.14 (3H, s), 4.05 (3H, s), 4.02 (3H, s), 3.99 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 179.9, 160.8, 157.6, 151.8, 148.2, 147.8, 144.3, 138.0, 132.9, 127.9, 119.8, 118.3, 116.8, 114.1, 110.3, 62.4, 62.1, 61.9, 61.7; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_7$: 359.1125, found: 359.1120.

5.1.1.14. 3′-Hydroxy-5,6,7,8-tetramethoxyflavone (5b).

According to a similar procedure as described for the preparation of **5a**, **5b** was obtained in 84% as white crystals. mp 177 °C; IR (KBr) 3314, 2941, 1644, 1605 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.66 (1H, br t, J = 1.8 Hz), 7.51 (1H, br d, J = 8.4 Hz), 7.38 (1H, dd, J = 8.4, 8.4 Hz), 7.10 (1H, dd, J = 1.8, 8.4 Hz), 6.84 (1H s), 4.12 (3H, s), 4.03 (3H, s), 3.99 (3H, s), 3.96 (3H, s); ^{13}C NMR ($CDCl_3$) δ:178.1, 162.1, 157.4, 151.9, 148.4, 147.9, 144.3, 138.1, 132.4, 130.3, 119.4, 117.8, 114.7, 113.2, 107.8, 62.25, 62.16, 61.8, 61.7; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_7$: 359.1125, found: 359.1123.

5.1.1.15. 4′-Hydroxy-5,6,7,8-tetramethoxyflavone (5c).

According to a similar procedure as described for the preparation of **5a**, **5c** was obtained in 97% yield as white crystals: mp 199–201 °C (Lit.[18,19] 197–199 °C); IR (KBr) 3480, 2941, 1622, 1583, 1558 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.82 (2H, br d, J = 8.8 Hz), 7.07 (2H, br d, J = 8.8 Hz), 6.63 (1H s), 4.12 (3H, s), 4.03 (3H, s), 3.97 (3H, s), 3.96 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 178.0, 162.2, 160.2, 151.6, 148.2, 147.7, 144.1, 138.0, 128.0 (2C), 122.6, 116.3 (2C), 114.4 105.9, 62.3, 62.1, 61.8, 61.7; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_7$: 359.1125, found: 359.1120.

5.1.1.16. 3′,5′-Dihydroxy-5,6,7,8-tetramethoxyflavone (5d).

According to a similar procedure as described for the preparation of **5a**, **5d** was obtained in 89% yield as white crystals: mp 246–248 °C; IR (KBr) 3326, 3188, 2941, 1636, 1595 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 9.72, (2H, s), 6.84 (2H, d, J = 1.8 Hz), 6.57 (1H, s), 6.42 (1H, dd, J = 1.8, 1.8 Hz), 4.01 (3H, s), 3.95 (3H, s), 3.83 (3H, s), 3.76 (3H, s); ^{13}C NMR (DMSO-d_6) δ: 175.8, 160.6, 159.0 (2C), 151.0, 147.5, 147.2, 143.6, 137.7, 132.6, 114.3, 107.2, 105.7, 104.0 (2C), 61.94, 61.88, 61.5, 61.4; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_8$: 375.1074, found: 375.1078.

5.1.1.17. 2′,5,6,7,8-Pentamethoxyflavone (6a). To a mixture of **5a** (28.4 mg, 79.3 μmol) and K_2CO_3 (33 mg, 0.24 mmol) in DMF (2 mL) was added iodomethane (0.03 mL, 0.4 mmol). The reaction mixture was stirred at rt for 5 h, and then partitioned between water (20 mL) and toluene (20 mL). The organic layer separated was washed with brine, and dried ($MgSO_4$). Filtration followed by concentration gave yellowish solids (29 mg), which were purified on a silica gel column (hexane/ethyl acetate = 2/1–1/1) to afford **6a** (23.5 mg, 63.1 mmol, 80% yield) as white crystals: mp 82–84 °C; IR (KBr) 2941, 1634, 1458, 1445 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.94 (1H, dd, J = 1.6, 7.6 Hz), 7.48 (1H, ddd, J = 1.6, 7.6, 7.6 Hz), 7.12 (1H, ddd, J = 1.6, 7.6, 7.6 Hz), 7.06–7.03 (2H, m), 4.10 (3H, s), 3.99 (3H, s), 3.96 (6H, s), 3.94 (3H, s); ^{13}C NMR ($CDCl_3$) δ:177.8, 158.7, 158.0, 151.3, 148.3, 148.0, 143.9, 138.1, 132.3, 129.1, 120.9, 120.6, 114.8, 113.2, 111.7, 62.3, 62.0, 61.9, 61.7, 55.6; HRMS calcd. for $[M+H]^+$ of $C_{20}H_{20}O_7$: 373.1282, found: 373.1293.

5.1.1.18. 3′,5,6,7,8-Pentamethoxyflavone (6b). According to a similar procedure as described for the preparation of **6a**, **6b** was obtained in 90% as white crystals: mp 100 °C; IR (KBr) 2940, 1651, 1593, 1467 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 7.52 (1H, ddd, J = 0.9, 1.8,

7.6 Hz), 7.45 (1H, dd, J = 1.8, 2.4 Hz), 7.43 (1H, dd, J = 7.6, 8.4 Hz), 7.07 (1H, ddd, J = 0.9, 2.4, 8.4 Hz), 6.68 (1H, s), 4.11 (3H, s), 4.03 (3H, s), 3.96 (3H, s), 3.89 (3H, s); ^{13}C NMR ($CDCl_3$) δ:177.1, 160.8, 159.8, 151.4, 148.2, 147.6, 144.0, 138.0, 132.7, 130.0, 118.3, 117.1, 114.8, 111.2, 108.1, 62.2, 62.0, 61.8, 61.6, 55.4; HRMS calcd. for $[M+H]^+$ of $C_{20}H_{20}O_7$: 373.1282, found: 373.1288.

5.1.1.19. 4′,5,6,7,8-Pentamethoxyflavone (tangeretin, 6c).

According to a similar procedure as described for the preparation of **6a**, **6c** was obtained in 84% as white crystals: mp 150–152 °C (Lit.[18] 153–154 °C, Lit.[20] 150–151 °C,); IR (KBr) 2945, 1651, 1608, 1514 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.86 (2H, br d, J = 8.8 Hz), 7.01 (2H, br d, J = 8.8 Hz), 6.59 (1H s), 4.09 (3H, s), 4.01 (3H, s), 3.94 (6H, s), 3.87 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 177.1, 162.1, 161.0, 151.2, 148.2, 147.6, 143.9, 138.0, 127.6 (2C), 123.7, 114.8, 114.4 (2C), 106.6, 62.2, 62.0, 61.8, 61.6, 55.5; HRMS calcd. for $[M+H]^+$ of $C_{20}H_{20}O_7$: 373.1282, found: 373.1289.

5.1.1.20. 3′,5,5′,6,7,8-Hexamethoxyflavone (6d). According to a similar procedure as described for the preparation of **6a**, **6d** was obtained in 76% yield as white crystals: mp 124–125 °C; IR (KBr) 2937, 1636, 1593, 1463 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 7.06 (2H, d, J = 2.0 Hz), 6.67 (1H, s), 6.62 (1H, dd, J = 2.0, 2.0 Hz), 4.11 (3H, s), 4.03 (3H, s), 3.95 (6H, s), 3.87 (6H, s); ^{13}C NMR ($CDCl_3$) δ: 177.4, 161.2 (2C), 160.9, 151.6, 148.3, 147.8, 144.1, 138.1, 133.4, 108.4, 104.1 (3C), 103.5, 62.2, 62.0, 61.8, 61.7, 55.5 (2C); HRMS calcd. for $[M+H]^+$ of $C_{21}H_{22}O_8$: 403.1387, found: 404.1390.

5.1.1.21. 5-Hydroxy-3′,4′,6,7,8-pentamethoxyflavone (12).

To a solution of **1** (111 mg, 0.276 mmol) in dichloromethane (5 mL) was added a solution of BCl_3 in toluene (1.0 M, 0.30 mL, 0.30 mmol) at −78 °C. The reaction mixture was allowed to warm to rt over a period of 15 h, to which was added 1 M aq. NaOH (10 mL). The mixture was stirred at rt for additional 1 h, acidified with 2 M HCl (10 mL), and extracted with chloroform (2 × 15 mL). The combined organic layer was dried ($MgSO_4$) and concentrated. The residue (108 mg) was chromatographed (silica gel, toluene/ethyl acetate = 10/1–8/1–6/1) to afford **12** as yellow solids (95.0 mg, 0.244 mmol, 88% yield): mp 147–148 °C (Lit.[21] mp 144–145 °C); IR (KBr) 3443, 2936, 2837, 1651, 1596 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 12.55 (1H, s, OH), 7.59 (1H, dd, J = 2.0, 8.5 Hz), 7.43 (1H, d, J = 2.0 Hz), 7.01 (1H, d, J = 8.5 Hz), 6.62 (1H s), 4.12 (3H, s), 3.990 (3H, s), 3.985 (3H, s), 3.979 (3H, s), 3.96 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 182.9, 163.8, 152.9, 152.4, 149.5, 149.3, 145.7, 136.5, 132.9, 123.6, 120.1, 111.2, 108.6, 106.9, 103.9, 62.0, 61.7, 61.1, 56.1, 55.9; HRMS calcd. for $[M-H]^-$ of $C_{20}H_{20}O_8$: 387.1085, found: 387.1079.

5.1.1.22. 4′-Benzyloxy-5-hydroxy-3′,6,7,8-tetramethoxyflavone (14) and 4′,5-dihydroxy-3′,6,7,8-tetramethoxyflavone (15).

To a solution of 4′-benzyloxy-3′,5,6,7,8-pentamethoxyflavone (**13**) (114 mg, 0.238 mmol) in dichloromethane (3 mL) was added a soln of BCl_3 in toluene (1.0 M, 0.25 mL, 0.25 mmol) at −15 °C. The reaction mixture was allowed to warm to rt over a period of 12 h, to which was added 1 M aq. NaOH (5 mL). The mixture was stirred at rt for additional 1 h, acidified with 2 M HCl (5 mL), and extracted with chloroform (2 × 15 mL). The combined organic layer was dried ($MgSO_4$) and concentrated. The residue (108 mg) was chromatographed (silica gel, toluene/ethyl acetate = 5/1–3/1) to give **14** (47.0 mg, 0.101 mmol, 43% yield) and **15** (32 mg, 0.086 mmol, 36% yield). **14**: mp 135–136 °C; IR (KBr) 3422, 2945, 2360, 1655, 1611, 1587 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ:12.55 (1H, s, OH) 7.52–7.33 (7H, m) 7.00 (1H, d, J = 8.5 Hz), 6.59 (1H, s), 5.25 (2H, s), 4.11 (3H, s), 3.99 (3H, s), 3.97 (3H, s), 3.95 (3H, s); ^{13}C NMR ($CDCl_3$) δ:182.8, 163.7, 152.8, 151.4, 149.7, 149.4, 145.6, 136.4, 136.1, 132.8, 128.6 (2C), 128.1, 127.1 (2C), 123.8, 119.9, 113.4, 109.1, 106.9, 103.9, 70.9, 62.0, 61.7, 61.1, 56.1; HRMS calcd. for $[M+H]^+$ of $C_{26}H_{24}O_8$: 465.1544, found: 465.1544. **15**: mp 194–196 °C (Lit.[32] 192 °C); IR (KBr) 3316, 2935, 1651, 1608, 1582 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 12.55 (1H, s) 7.53 (1H, dd, J = 2.0, 8.4 Hz) 7.41 (1H, d, J = 2.0 Hz), 7.05 (1H, d, J = 8.4 Hz), 6.59 (1H, s), 6.22 (1H, s), 4.12 (3H, s), 4.00 (3H, s), 3.98 (3H, s), 3.96 (3H, s); ^{13}C NMR ($CDCl_3$) δ:182.8, 163.9, 152.9, 149.39, 149.36, 146.8, 145.6, 136.5, 132.8, 123.1, 120.7, 115.1, 108.2, 106.9, 103.7, 62.1, 61.7, 61.1, 56.0; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_8$: 375.1074, found: 375.1075.

5.1.1.23. 3′,5-Dihydroxy-4′,6,7,8-tetramethoxyflavone (17).

To a solution of **16** (100 mg, 0.209 mmol) in dichloromethane (5 mL) was added a soln of BCl_3 in toluene (1.0 M, 0.42 mL, 0.42 mmol) at −78 °C. The reaction mixture was allowed to warm to rt over a period of 12 h, to which was added 1 M aq. NaOH (10 mL). The mixture was stirred at rt for additional 30 min, acidified with 2 M HCl (10 mL), and extracted with ethyl acetate (2 × 15 mL). The combined organic layer was dried ($MgSO_4$) and concentrated. The residual orange-yellow solids (92 mg) were purified on a silica gel (toluene/ethyl acetate = 3/1) to give **17** (72.0 mg, 0.192 mmol, 92% yield): mp 198–199 °C; IR (KBr) 3379, 2937, 1653, 1605, 1586 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 12.57 (1H, s, OH) 7.49 (2H, m), 6.97 (1H, d, J = 8.8 Hz), 6.58 (1H, br s), 5.90 (1H, m, OH), 4.12 (3H, s), 3.98 (6H, s), 3.95 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 182.9, 163.8, 152.9, 149.7, 149.3, 146.0, 145.7, 136.4, 132.9, 124.3, 119.2, 112.3, 110.7, 106.9, 104.1, 62.2, 61.7, 61.1, 56.2; HRMS calcd. for $[M+H]^+$ of $C_{19}H_{18}O_8$: 375.1074, found: 375.1076.

5.1.1.24. 4′,5-Dihydroxy-6,7,8-trimethoxyflavone (18).

According to a similar procedure as described for the preparation of **17**, **18** was obtained in 87% yield as white crystals: mp 230–231 °C (Lit.[33] 225 °C); IR (KBr) 3386, 3287, 2937, 1659, 1605, 1562 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 12.56, (1H, br s, OH) 7.86 (2H, br d, J = 8.8 Hz), 6.99 (2H, br d, J = 8.8 Hz), 6.60 (1H, s), 5.87 (1H, br s), 4.12 (3H, s), 3.98 (3H, s), 3.96 (3H, s); ^{13}C NMR ($CDCl_3$) δ: 183.0, 164.0, 159.2, 153.0, 149.5, 145.8, 136.6, 133.0, 128.4 (2C), 123.7, 116.2 (2C), 107.0, 103.8, 62.2, 61.7, 61.1; HRMS calcd. for $[M-H]^-$ of $C_{18}H_{16}O_7$: 343.0823, found: 343.0805.

5.2. Cytotoxicity evaluation (LDH assay)

Cytotoxicity was quantified as the increase in LDH leakage using LDH Cytotoxic Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocol. Cultures were seeded on 96-well microtiter plates at a density of 5,000 cells per well. After 15–18 h, the medium was removed, and the cultures were washed with serum-free medium and exposed to the medium containing the stock solution of the test sample (100 mM) in DMSO (the final concentration of the test sample in the culture medium was 64 μmol/L). Maximum releasable LDH was assessed by incubating cells with 0.2% Tween-20 (positive control), while the background was evaluated in both cell- and serum-free medium (negative control). After 24-h incubation, LDH release in the supernatant was monitored. The percentage of specific release of LDH was calculated according to the following formula: % specific lytic activity = $(S - N)/(P - N) \times 100$, where S is the activity of LDH released (measured by absorbance at 570 nm) in the medium containing the test sample, N is the activity of LDH released from the negative control cells, and P is the activity of LDH released from positive control cells. The values shown in Figure 4 are the means of the triplicate measurements. Cell damage of 'control' in Figure 4 reflects the background release of LDH after incubation of cells in serum-free medium.

5.3. Cell culture and treatment

The human lens epithelial cell line SRA01/04 was a kind gift from Dr. Nobuhiro Ibaraki (Jichi Medical University, Tochigi, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 20% (v/v) heat-inactivated (56 °C for 30 min) fetal bovine serum (Biowest, Nuaille, France) including PSN antibiotic mixture (Invitrogen, penicillin/streptomycin/neomycin: 100 μg/mL each) at 37 °C in a humidified 5% CO_2 atmosphere. After reaching confluence, the cells were treated with the test sample in the presence of PMA (Sigma Aldrich, 10 nM) or TNF-α (Sigma Aldrich, 10 ng/mL) for 24 h. The harvested culture media were stored at 4 °C until immediately before use.

5.4. Gelatin zymography

Aliquots (20 μL) of the harvested culture media were subjected to SDS–PAGE with 10% acrylamide gel containing gelatin (0.6 mg/mL) (Difco Laboratories, Detroit, MI, USA). The gel was washed with 50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.1% Triton X-100 and then incubated in 50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$ at 37 °C. Then the gel was stained with 0.1% Coomassie Brilliant Blue R-250, and gelatinolytic activity[28] was detected as unstained bands on a blue background. The IC_{50} values (means of triplicate measurements) shown in Table 1 were estimated from the relative amount of pro-MMP-9 (quantified using ImageJ[27]) at various concentrations (0.25, 1, 4, 16, or 64 μM) of the test samples.

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27. ImageJ is public-domain software of the US National Institutes of Health. http://rsb.info.nih.gov/ij/.
28. Under the influence of SDS, pro-MMP-9, the precursor (proenzyme) of MMP-9, was denatured to acquire gelatinolytic activity without any proteolytic activation (cleavage).
29. In addition, inhibitory activities of noncytotoxic benzyl ethers **11a** and **11d** for pro-MMP-9 production were assessed. Although **11a** possessed considerable activity (IC_{50} value in PMA-treated cells, 7.9 ± 2.0 μM; IC_{50} value in TNF-α-treated cells, 14.0 ± 3.6 μM), **11d** showed no inhibitory activity at 64 μM in either PMA- or TNF-α-treated cells.
30. These results were reproducibly observed in triplicate experiments.
31. At a higher dosage (64 μM) of compounds **5b** and **17**, decrease of the gelatinolytic activity of MMP-2 was observed. These findings are interesting because the regulation of pro-MMP-2 production is known to be independent on PKC activation in most cell species. Elucidation of detailed mechanism is ongoing in our laboratories.
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