Relationship between Structure and Antiproliferative Activity of Polymethoxyflavones towards HL60 Cells

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Abstract. *As part of our continuing investigation of polymethoxyflavone (PMF) derivatives as potential anticancer substances, a series of PMF derivatives was synthesized. The synthesized compounds were evaluated for cytotoxicity against the promyelocytic leukemic HL60 cell line, and structure–activity relationship correlations were investigated along with previously isolated PMFs from the peel of king orange (Citrus nobilis). 7,3'-Dimethoxyflavone demonstrated the most potent activity among the synthetic PMFs. Consideration of correlation between the methoxylation pattern and antiproliferative activity revealed the importance of the 3'-methoxyl group and the higher degree of methoxylation on the A-ring moiety of PMFs.*

Flavonoids are diverse plant-derived chemicals that are produced by various higher plants (1), which can therefore be found in numerous food sources such as fruits, vegetables, legumes, and whole grains (2). Citrus plants are rich sources of flavonoid and they contain a wide range of flavonoid constituents. Polymethoxyflavones (PMFs) are found almost exclusively in the *Citrus* genus, particularly in the peel of king orange (*Citrus nobilis*), sweet orange (*Citrus sinensis*), and mandarin orange (*Citrus reticulata*). They are of particular interest due to their broad spectrum of biological activity, including anti-inflammatory, anticarcinogenic, antiatherogenic, neurotropic and memory-enhancing properties (3-10). PMFs have been shown to inhibit the growth of the human leukemia HL60 cell line *in vitro* and to suppress proliferation, while promoting apoptosis (11).

Their biological properties have led not only to horticultural breeding attempts to produce new citrus cultivars containing high amounts of PMFs (12), but also to development of a rapid, large-scale method for the preparation of PMF-rich fraction from peel of *Citrus sunki* (13).

In previous studies, we found potent differentiation-inducing activity towards HL-60 leukemia cells from the juice of *C. nobilis*. The active principles were isolated and identified as four PMFs, namely tangeretin (**8**), nobiletin (**9**), heptamethoxyflavone (**10**) and natsudaidain (**11**) (Figure 2) and these PMFs inhibited the growth of several cancer cell lines (14). Among these PMFs, **11** demonstrated the most potent activity (15, 16).

In the course of our investigation on the structure–activity relationship of flavonoids, the correlation between the number/position of methoxyl groups and the antiproliferative activity of PMFs drew our attention. In order to study the effect of the methoxylation pattern of PMFs on their bioactivity, we synthesized 15 PMFs with variations of methoxylation of the A-ring moiety, since most natural PMFs have a highly methoxylated A-ring moiety.

Materials and Methods

General procedures. Chemicals and solvents from commercial sources were used without further purification unless specified. Reactions were carried out under argon and monitored by thin-layer chromatography on silica gel (mesh size 60, F_{254}) with visualization under UV light. Standard and flash column chromatography procedures were not optimized. Nuclear magnetic resonance (NMR) spectra were recorded on a 400-MHz JEOL ECP-400 spectrometer (JOEL, Tokyo, Japan), and chemical shifts values are expressed in ppm (δ) relative to the residual ^{1}H signal of the solvents. Unless otherwise specified, compounds were dissolved in $^2HCCl_3$. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectrometry were performed on Thermo Exactive (Thermo Fisher Scientific K.K, Yokohama, Japan) and Hitachi M8000 instruments (Hitachi, Tokyo, Japan), respectively.

Synthesis of 7,3'-dimethoxyflavone (Figure 1). General procedure for synthesis of PMFs (Figure 2, **6b-6o**). To a suspension of dicyclohexylcarbodiimide (1.00 g, 4.85 mmol) and *N,N*-dimethyl-4-aminopyridine (107 mg, 0.876 mmol) in dry dichloromethane (12 ml), 2'-hydroxy-4'-methoxyacetophenone (718 mg, 4.32 mmol) and then 3-methoxybenzoic acid (977 mg, 6.42 mmol) were added. The reaction

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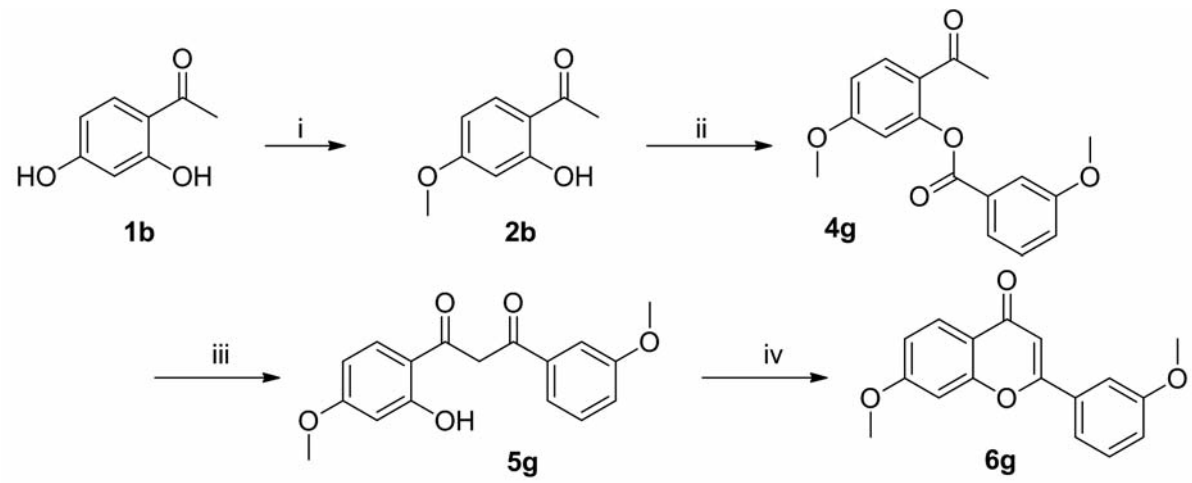


Figure 1. *Synthesis of 7,3'-dimethoxyflavone. i) MeI, K_2CO_3, acetone, rt, 6 h; ii) dicyclohexylcarbodiimde, N,N-dimethyl-4-aminopyridine, 3-methoxybenzoic acid; iii) KOH, pyridine, 100˚C, 10 min; iv) 20% H_2SO_4/acetic acid, 100˚C, 10 min.*

mixture was stirred at room temperature for 20 hours. The mixture was filtered to remove dicyclohexylurea as white precipitate. The solvent of the filtrate was removed under reduced pressure, and the residue was chromatographed over silica gel [hexane/ dichloromethane (DCM); 2:8] to afford 4'-methoxy-2'-(3-methoxybenzoyloxy)acetophenone (**4g**) as a white solid. To a suspension of KOH (212 mg, 3.79 mmol) in dry pyridine (3 ml) **4g** (573 mg, 2.12 mmol) was added and the mixture was stirred at 100˚C for 10 min. After being cooled to room temperature, the reaction mixture was neutralized with acetic acid (approximately 3.0 ml). The mixture was added to ethanol (3.0 ml) and deionized water (3.0 ml). The resulting precipitate was filtered and washed with cold ethanol to give 1-(3-methoxyphenyl)-3-(2'-hydroxy-4'-methoxyphenyl) propan-1,3-dione (**5g**, 350 mg, yield from **4g**: 61%). To a solution of **5g** (431 mg, 1.30 mmol) dissolved in acetic acid (6 ml) kept at 100˚C, 20% H_2SO_4/acetic acid (1 ml) was added. The mixture was stirred at 100˚C for 10 min. After cooling to room temperature, deionized water was added to the mixture. The resulting precipitate was filtered and washed with water, and was then chromatographed over silica gel (hexane/DCM; 2:8) to afford 7,3'-dimethoxyflavone (**6g**, 379 mg, yield from **5g**: 93%).

For **4g**: ^{1}H-NMR ($CDCl_3$) δ 2.49 (s, 3H), 3.87 (m, 6H), 6.71 (d, 1H, *J*=2.2 Hz), 6.87 (dd, 1H, *J*=8.8, 2.2 Hz), 7.19 (dt, 1H, *J*=7.7, 1.5 Hz), 7.42 (t, 1H, *J*=7.7 Hz), 7.71 (t, 1H, *J*=1.5 Hz), 7.81 (dt, 1H, *J*=7.7, 1.5 Hz), 7.90 (d, 1H, *J*=8.8 Hz). HRMS (M + H)$^+$: calcd for $C_{17}H_{16}O_5$, 300.0994; found 300.0997.

For **5g**: ^{1}H-NMR ($CDCl_3$) δ 3.86 (m, 8H), 6.47 (m, 2H), 6.69 (s, 1H), 7.07 (dt, 1H), 7.38 (t, 1H, *J*=8.8 Hz), 7.44 (st, 1H, *J*=2.2 Hz), 7.48 (dt, 1H), 7.68 (d, 1H, *J*=8.8 Hz). HRMS (M + H)$^+$: calcd for $C_{17}H_{16}O_5$, 300.0994; found 300.0994.

By combination of 3 2'-hydroxyacetophenone derivatives, namely 2'-hydroxy- (**1a**), 2'-hydroxy-4'-methoxy- (**1b**), and 2'-hydroxy-4',6'-acetophenone (**1c**), and benzoic acid (**2a**) and its derivatives, namely 3-methoxy- (**2b**), 4-methoxy- (**2c**), 3,4-dimethoxy- (**2d**), and 3,4,5-tirmethoxybenzoic acids (**2e**), 15 PMFs were synthesized. Spectral data for 6g are shown in Table I along with data for other synthetic PMFs.

Flavone (**6a**) and sinensetin (**7**) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and Funakoshi (Tokyo, Japan). Tangeretin (**8**), nobiletin (**9**), heptamethoxyflavone (**10**) and natsudaidain (**11**) were isolated from king orange juice. Fruits were juiced by hand, and 200 ml of juice was absorbed on 250 g of polystyrene resin (Diaion HP-20; Mitsubishi Chemical, Tokyo, Japan) and eluted with ethanol. The combined eluents were concentrated and subjected to silica-gel column chromatography (Wako Gel C-200; Wako Pure Chemicals, Tokyo, Japan) eluted with 10% ethanol in hexane, chloroform, and then 50% chloroform in methanol. The chloroform eluates were further purified by a reversed-phase high performance liquid chromatography (HPLC), giving **8** (yield; 11.8 mg), **9** (yield; 12.7 mg), **10** (yield; 30.4 mg), **11** and (yield; 12.0 mg).

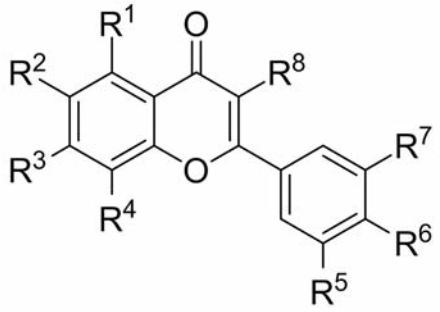
Cell proliferation assay. HL60 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. The level of cellular proliferation for HL60 cells grown in a 96-well microplate was measured by using alamar blue (Life Technologies Ltd., Tokyo, Japan). To each well, 100 μl of HL60 cell suspension (1.0×10^4 cells/100 μl) was inoculated and 100 μl of medium containing serial dilutions of the samples to be assayed was aded. After three days of incubation, 20 μl of alamar blue was aseptically added to each well, and cells were further incubated for approximately 20 hours. Cellular proliferation (as a percentage that of the untreated control) was calculated with the following equation: Proliferation (%)=

$$= \frac{[(A_{570}\text{-}A_{595}) \text{ of test agent dilution}]-[(A_{570}\text{-}A_{595}) \text{ of blank}]}{[(A_{570}\text{-}A_{595}) \text{ of positive growth control}]-[(A_{570}\text{-}A_{595}) \text{ of blank}]}$$

where A_{570} and A_{595} are the absorbance at 570 nm and 595 nm, respectively.

Results and Discussion

Chemistry. In this study, 15 PMFs were synthesized. The synthetic route to 7,3'-dimethoxyflavone (**6g**) is illustrated in Figure 1. The starting 2'-hydroxyacetophenone derivatives were first treated with methyl iodide and K_2CO_3 in dry



Compd	Substituent								IC_{50} (μM)	Growth at 100 μM (%)*	Log *P*
	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8			
6a	H	H	H	H	H	H	H	H	257	49	3.07
6b	H	H	H	H	OMe	H	H	H	215	46	2.95
6c	H	H	H	H	H	OMe	H	H	>400	84	2.95
6d	H	H	H	H	OMe	OMe	H	H	>400	79	2.82
6e	H	H	H	H	OMe	OMe	OMe	H	>400	55	2.69
6f	H	H	OMe	H	H	H	H	H	>400	83	2.95
6g	H	H	OMe	H	OMe	H	H	H	8	1.0	2.82
6h	H	H	OMe	H	H	OMe	H	H	>400	72	2.82
6i	H	H	OMe	H	OMe	OMe	H	H	>400	94	2.69
6j	H	H	OMe	H	OMe	OMe	OMe	H	>400	98	2.57
6k	OMe	H	OMe	H	H	H	H	H	31	2.0	2.82
6l	OMe	H	OMe	H	OMe	H	H	H	24	2.0	2.69
6m	OMe	H	OMe	H	H	OMe	H	H	23	2.0	2.69
6n	OMe	H	OMe	H	OMe	OMe	H	H	>400	72	2.57
6o	OMe	H	OMe	H	OMe	OMe	OMe	H	>400	82	2.44
7	OMe	OMe	OMe	H	OMe	OMe	H	H	>400	96	2.44
8	OMe	OMe	OMe	OMe	H	OMe	H	H	32	36	2.44
9	OMe	OMe	OMe	OMe	OMe	OMe	H	H	52	39	2.31
10	OMe	OMe	OMe	OMe	OMe	OMe	H	OMe	63	42	1.51
11	OMe	OMe	OMe	OMe	OMe	OMe	H	OH	5	2.0	1.15

Figure 2. *Structures, half maximal inhibitory concentration (IC_{50}), growth rate at 100 μM, and log P of polymethoxyflavones. Calculation of log P was carried out by ChemBioDraw Ultra 12.0 (Cambridge Soft). *Relative to the untreated control.*

acetone to afford their corresponding methoxylated derivatives, namely 2'-hydroxy- (**2a**), 2'-hydroxy-4'-methoxy- (**2b**), 2'-hydroxy-4',6'-dimethoxyactophenones (**2c**). Methylation selectively occurred at the 4'-position of 2',4'-dihydroxyacetophenone (**1b**) and at the 4'- and 6'-positions of 2',4',6'-trihydroxyacetophenone (**1c**). Fortunately, 2'-hydroxyl groups did not react with the methylation reagent, presumably because of the weakened nucleophilicity of the 2'-hydroxyl

Table I. *Analytical data of synthesized compounds*.

Compound	3'-Methoxyflavone (**6b**)
weight (yield)	370 mg (68%)
HRMS $(M + H)^+$	calcd for $C_{16}H_{12}O_3$, 252.0786; found 252.0783
^{1}H-NMR	3.90 (s, 3H), 6.85 (s, 1H), 7.08 (dt, 1H, *J*=8.4, 2.9 Hz), 7.44 (3H, m), 7.51 (1H, dd, *J*=7.0, 1.1 Hz), 7.57 (1H, dd, *J*=7.0, 1.1 Hz), 7.71 (1H, dt, *J*=7.6, 1.8 Hz), 8.23 (dd, 1H, *J*=7.6, 1.8 Hz).
Compound	4'-Methoxyflavone (**6c**)
weight (yield)	151 mg (74%)
HRMS $(M + H)^+$	calcd for $C_{16}H_{12}O_3$, 252.0786; found 252.0788
^{1}H-NMR	3.88 (s, 3H), 6.74 (s, 1H), 7.02 (dd, 2H, *J*=9.2, 2.9 Hz), 7.39 (t, 1H, *J*=7 Hz), 7.53 (d, 1H, *J*=8 Hz), 7.67 (m, 1H), 7.88 (dd, 2H, *J*=9.2, 2.9 Hz), 8.21 (dd, 1H, *J*=7.7, 1.1 Hz)
Compound	3',4'-Dimethoxyflavone (**6d**)
weight (yield)	628 mg (87%)
HRMS $(M + H)^+$	calcd for $C_{17}H_{14}O_4$, 282.0892; found 282.0893
^{1}H-NMR	3.96 (s, 3H), 3.98 (s, 3H), 6.85 (s, 1H), 6.99 (d, 1H, *J*=8.8 Hz), 7.42 (m, 2H), 7.59 (m, 2H), 7.70 (t, 1H, *J*=7, 1 Hz), 8.23 (dd, 1H, *J*=8.0, 1.5 Hz)
Compound	3',4',5'-Trimethoxyflavone (**6e**)
weight (yield)	963 mg (89%)
HRMS $(M + H)^+$	calcd for $C_{18}H_{16}O_5$, 312.0998; found 312.1002
^{1}H-NMR	3.93 (m, 6H), 3.94 (s, 3H), 6.77 (s, 1H), 7.13 (s, 2H), 7.43 (t, 1H, *J*=7.5 Hz), 7.58 (d, 1H, *J*=8.5 Hz), 7.70 (dt, 1H, *J*=7, 1.5 Hz), 8.23 (dd, 1H, *J*=8.0, 1.1 Hz)
Compound	7-Methoxyflavone (**6f**)
weight (yield)	230 mg (71%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.94 (s, 3H), 6.85 (s, 1H), 7.00 (m, 2H), 7.52 (m, 3H), 7.92 (m, 2H), 8.14 (d, 1H, *J*=8.8 Hz)
Compound	7,3'-Dimethoxyflavone (**6g**)
weight (yield)	379 mg (93%)
HRMS $(M + H)^+$	calcd for $C_{17}H_{14}O_4$, 282.0892; found 282.0895
^{1}H-NMR	3.89 (s, 3H), 3.94 (s, 3H), 6.85 (s, 1H), 7.00 (m, 2H), 7.08 (d, 1H, *J*=7 Hz), 7.42 (m, 2H), 7.50 (dt, 1H, *J*=7.7, 1.1 Hz), 8.14 (d, 1H, *J*=8.8 Hz)
Compound	7,4'-Dimethoxyflavone (**6h**)
weight (yield)	275 mg (97%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.89 (s, 3H), 3.93 (s, 3H), 6.69 (s, 1H), 6.95 (m, 2H), 7.01 (d, 2H, *J*=9 Hz), 7.86 (dd, 2H, *J*=9 Hz), 8.12 (d, 1H, *J*=8.8 Hz)
Compound	7,3',4'-Trimethoxyflavone (**6i**)
weight (yield)	274 mg (97%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.93 (s, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 6.70 (s, 1H), 6.97 (m, 3H), 7.36 (d, 1H, *J*=1.8 Hz), 7.54 (dd, 1H, *J*=8.4, 1.6 Hz), 8.12 (d, 1H, *J*=8.8 Hz)
Compound	7,3',4',5'-Tetramethoxyflavone (**6j**)
weight (yield)	195 mg (57%)
HRMS $(M + H)^+$	calcd for $C_{19}H_{18}O_6$, 342.1103; found 342.1101
^{1}H-NMR	3.92 (s, 3H), 3.94 (s, 3H), 3.96 (s, 6H),6.71 (s, 1H), 6.98 (m, 2H), 7.11 (s, 2H), 8.13 (d, 1H, *J*=8.8 Hz)
Compound	5,7-Dimethoxyflavone (**6k**)
weight (yield)	206 mg (55%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.91 (s, 3H), 3.95 (s, 3H), 6.38 (d, 1H, *J*=2.2 Hz), 6.58 (d, 1H, *J*=2.2 Hz), 6.76 (s, 1H), 7.49 (m, 3H), 7.88 (m, 2H)
Compound	5,7,3'-Trimethoxyflavone (**6l**)
weight (yield)	257 mg (57%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.88 (s, 3H), 3.91 (s, 3H), 3.95 (s, 3H), 6.37 (d, 1H, *J*=2.6 Hz), 6.56 (d, 1H, *J*=2.6 Hz), 6.67 (s, 1H), 7.04 (dt, 1H, *J*=8.0, 1.1 Hz), 7.42 (m, 3H)
Compound	5,7,4'-Trimethoxyflavone (**6m**)
weight (yield)	202 mg (79%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.87 (s, 3H), 3.90 (s, 3H), 3.95 (s, 3H), 6.36 (d, 1H, *J*=2.2 Hz), 6.55 (d, 1H, *J*=2.2 Hz), 6.59 (s, 1H), 6.99 (d, 2H, *J*=6.8 Hz), 7.81 (d, 2H, *J*=6.8 Hz)
Compound	5,7,3',4'-Tetramethoxyflavone (**6n**)
weight (yield)	149 mg (63%)
HRMS $(M + H)^+$	nd
^{1}H-NMR	3.91 (s, 3H), 3.95 (s, 6H), 3.97 (s, 3H), 6.37 (d, 1H, *J*=2.2 Hz), 6.56 (d, 1H, *J*=2.2 Hz), 6.62 (s, 1H), 6.95 (d, 1H, *J*=8.4 Hz), 7.31 (d, 1H, *J*=2.2 Hz), 7.50 (dd, 1H, *J*=8.4, 2.2 Hz)
Compound	5,7,3',4',5'-Pentamethoxyflavone (**6o**)
weight (yield)	2.14 g (88%)
HRMS $(M + H)^+$	calcd for $C_{20}H_{20}O_7$, 372.1209; found 372.1211
^{1}H-NMR	3.90 (s, 6H), 3.92 (s, 6H), 3.94 (s, 3H), 6.38 (d, 1H, J=2.2 Hz), 6.56 (d, 1H, J=2.2 Hz), 6.63 (s, 1H), 7.07 (s, 2H)

nd; Not determined; HRMS; high-resolution mass spectrometry.

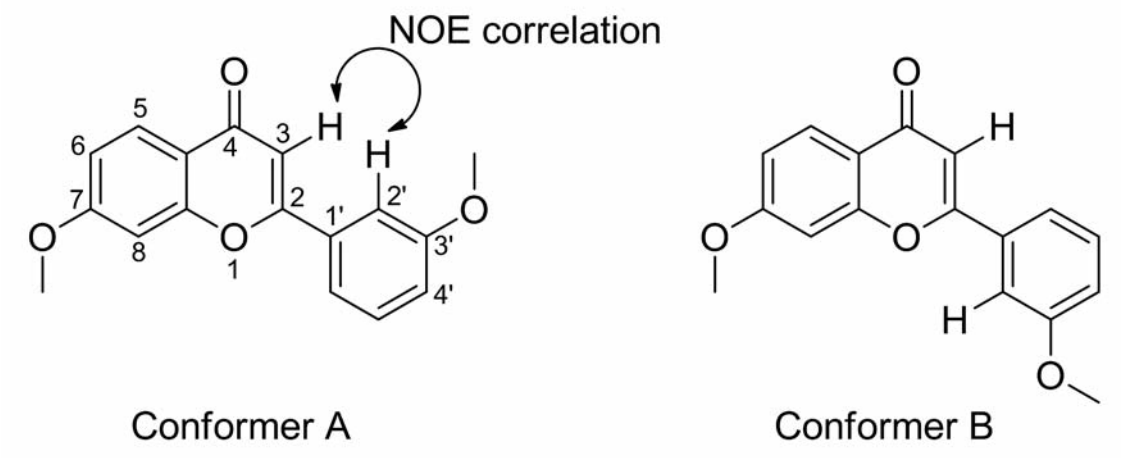


Figure 3. Nuclear Overhauser correlation found in conformer A of 7,3'-dimethoxyflavone **(6g).**

oxygen atom by its hydrogen bonding to the oxygen atom of the acetyl carbonyl group.

2'-Hydroxyacetophenone derivatives (**2a-2c**) were then reacted with a series of methoxylated derivatives of benzoic acids to give the corresponding esters. The intermediate esters were subjected to the Baker-Venkataraman rearrangement in dry pyridine in the presence of KOH, to afford the corresponding 1,3-diarylpropan-1,3-diones. Acid-catalyzed cyclization was carried out in acetic acid in the presence of H_2SO_4, which yielded the target products in yields of 25-35%.

Antiproliferative activity. The antiproliferative activity of synthetic 15 PMFs along with several PMFs isolated from the peel of king orange was determined using promyelocytic leukemic HL60 cells based on the alamar blue assay. The results are summarized in Figure 2.

Among the compounds tested, 3-hydroxy-5,6,7,8,3',4'-hexamethoxyflavone (**11**, trival name: natsudaidain) demonstrated the most potent activity (IC_{50}=5.0 μM), followed by 7,3'-dimethoxyflavone (**6g**, IC_{50}=8.0 μM). Furthermore, the 5,7-dimethoxylated derivatives, namely 5,7,4'-trimethoxyflavone (**6m**, IC_{50}=23 μM), 5,7,3'-trimethoxyflavone (**6l**, IC_{50}=24 μM) and 5,7-dimethoxyflavone (**6k**, IC_{50}=31 μM) also demonstrated significant activity.

Structure and activity relationship. Since these results showed that there was not a simple correlation between the number of methoxyl groups on PMFs and their antiproliferative activity, the pattern of methoxylation, *i.e*. the number and the position of methoxylation, shoult be considered. Thus 20 PMFs tested in this study were classified based on their methoxylation pattern and were subjected to the consideration of the structure–activity relationship.

Increase of the number of methoxyl groups on the B-ring moiety tended to lower the activity among the PMFs with the same A-ring methoxylation pattern. When the number of A-ring methoxyl groups was 0-3, attachment of more than two methoxyl groups on the B-ring moiety completely diminished the activity (IC_{50} values of compounds **6d**, **6e**, **6i**, **6j**, **6n**, **6o**, and **7** were higher than 400 μM). Although each of the PMFs with four methoxyl groups on their A-ring moiety demonstrated significant activity, the tendency of activity reduction by the increase of B-ring methoxyl groups was maintained.

On the contrary, an increase in the number of A-ring methoxyl groups tended to enhance the activity. Typically, only compound **9**, which had the tetramethoxylated A-ring structure, demonstrated significant activity among a series of PMFs with 3',4'-dimethoxylated B-ring structure, namely compounds **6d**, **6i**, **6n**, **7**, and **9**. Similarly, compounds **6m** and **8** demonstrated potent activity among a series of PMFs with the 4'-methoxylated B-ring structure.

The position of methoxyl group on B-ring moiety had a significant effect on the activity. The activity was reduced when a methoxyl group was attached to the 4'-position; most of the 4'-methoxylated PMFs tested had no activity. Similar activity-weakening phenomena by 4'-substitution on the B-ring were reported for 1-azaflavanone (18). In contrast, many 3'-methoxylated PMFs demonstrated significant activity. For example, compounds **6b** and **6g** exhibited more potent activity than their corresponding 4'-methoxylated derivatives, namely compounds **6c** and **6h**, respectively. The importance of the 3'-methoxyl group was reduced when two methoxyl groups were attached to the A-ring moiety.

The free rotation of the C2-C1' single bond causes two typical conformers, namely conformers A and B as indicated in Figure 3. In order to determine which conformer contributes

to the activity, the nuclear Overhauser effect (NOE) spectroscopy was measured. The observed NOE correlation between H3 and H2' indicated the predominance of conformer A. While these results suggested the involvement of conformer A for activity, further studies are necessary.

In summary the data presented here demonstrated that an increase in the number of methoxyl groups on the A-ring enhanced the activity of PMFs, whereas the increase of B-ring methoxyl groups reduced the activity.

References

1 Dixon RA and Steele C: Flavonoids and isoflavonoids – gold mine for metabolic engineering. Trends Plant Sci *4*: 394-400, 1999.

2 Barnes J, Anderson LA and Phillipson JD: St John's wort (*Hypericum perforratum* L.): A review of its chemistry, pharmacology and clinical properties. J Pharm Pharmacol *53*: 583-600 2001.

3 Manthey JA, Grohmann K, Guthrie N: Biological properties of citrus flavonoids pertaining to cancer and inflammation. Curr Med Chem *8*: 135-153, 2001.

4 Middleton E, Kandaswami C and Theoharides TC: The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev *52*: 673-751, 2000.

5 Lopez-Lazaro M: Flavonoids as anticancer agents: Structure–activity relationship study. Curr Med Chem Anticancer Agents *2*: 691-714, 2002.

6 Whitman SC, Kurowska EM, Manthey JA and Daugherty A: Nobiletin, a citrus flavonoid isolated from tangerines, selectively inhibits class A scavenger receptor-mediated metabolism of acetylated LDL by mouse macrophages. Atherosclerosis *178*: 25-32, 2005.

7 Yoshimizu N, Otani Y, Saikawa Y, Kubota T, Yoshida M, Furukawa T, Kumai K, Kameyama K, Fujii M, Yano M, Sato T, Ito T and Kitajima M: Anti-tumour effects of nobiletin, a citrus flavonoid, on gastric cancer include: antiproliferative effects, induction of apoptosis and cell cycle deregulation. Aliment Pharmacol Ther *20(Suppl 1)*: 95-101, 2004.

8 Lin N, Sato T, Takayama Y, Mimaki Y, Sashida Y, Yano M and Ito A: Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. Biochem Pharmacol *65*: 2065-2071, 2003.

9 Guo S, Qiu P, Xu G, Wu X, Dong P, Yang G, Zheng J, McClements DJ and Xiao H: Synergistic anti-inflammatory effects of nobiletin and sulforaphane in lipopolysaccharide-stimulated RAW 264.7 cells. J Agric Food Chem *60*: 2157-2164, 2012.

10 Al Rahim M, Nakajima A, Saigusa D, Tetsu N, Maruyama Y, Shibuya M, Yamakoshi H, Tomioka Y, Iwabuchi Y, Ohizumi Y and Yamakuni T: 4'-Demethylnobiletin, a bioactive metabolite of nobiletin enhancing PKA/ERK/CREB signaling, rescues learning impairment associated with NMDA receptor antagonism *via* stimulation of the ERK cascade. Biochemistry *48*: 7713-7721, 2009.

11 Li S, Pan MH, Lai CS, Lo CY, Dushenkov S and Ho CT: Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines. Bioorg Med Chem *15*: 3381-3389, 2007.

12 Kawaii S, Tomono Y, Katase E, Ogawa K, Nonomura-Nakano M, Nesumi H, Yoshida T, Sugiura M and Yano M: Quantitative study of fruit flavonoids in citrus hybrids of King (*C. nobilis*) and Mukaku Kishu (*C. kinokuni*). J Agric Food Chem *49*: 3982-3986, 2001.

13 Ko HC, Jang MG, Kang CH, Lee NH, Kang SI, Lee SR, Park DB and Kim SJ: Preparation of a polymethoxyflavone-rich fraction (PRF) of *Citrus sunki* Hort. ex Tanaka and its antiproliferative effects. Food Chem *123*: 484-488, 2010.

14 Kawaii S, Tomono Y, Katase E, Ogawa K and Yano M: Antiproliferative activity of flavonoids on several cancer cell lines. Biosci Biotechnol Biochem *63*: 896-899, 1999.

15 Kawaii S, Tomono Y, Katase E, Ogawa K and Yano M: HL-60 differentiating activity and flavonoid content of the readily extractable fraction prepared from citrus juices. J Agric Food Chem *47*: 128-135, 1999.

16 Kawaii S, Tomono Y, Katase E, Ogawa K and Yano M: Quantitation of flavonoid constituents in citrus fruits. J Agric Food Chem *47*: 3565-3571, 1999.

17 Ganguly AK, Kaur S, Mahata PK, Biswas D, Pramanik BN and Chan TM: Synthesis and properties of 3-acyl-γ-pyrones, A novel class of flavones and chromones. Tetrahedron Lett *46*: 4119-4121, 2005.

18 Kawaii S, Endo K, Tokiwano T and Yoshizawa Y: Relationship between structure and antiproliferative activity of 1-azaflavanones. Anticancer Res *32*: 2819-2825, 2012.

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