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POLYMYRIFLINE A, A NEW POLYMETHOXYFLAVONE FROM *CITRUS AURANTIUM* VAR. *MYRTIFOLIA*

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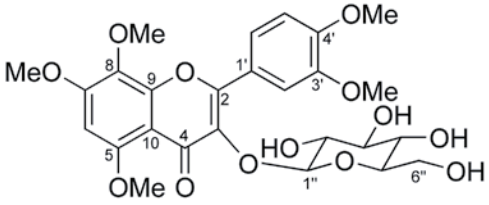
Abstract – A new polymethoxyflavone, polymyrifline A (**1**) was isolated together with known polymethoxyflavones from the peels of *Citrus aurantium* var. *myrtifolia*. Its structure was elucidated by spectroscopic methods and chemical means. Polymyrifline A (**1**) showed a moderate inhibition of the NO production in LPS-stimulated J774.1.

The genus *Citrus* is known to be a rich source of flavonoids,[1] limonoids,[2] coumarins,[3] acridone alkaloids,[4] cyclic peptides[5], and so on. Recently, some of highly methoxylated flavonoids have been shown to be a potent inhibitory activities against the proliferation, differentiation, and induce apoptosis of cancer cell lines.[6] In our search for structurally and biologically interesting flavonoids, a new polymethoxyflavone, polymyrifline A (**1**) was isolated from *Citrus aurantium* var. *myrtifolia*. In this paper, we describe the isolation and structure elucidation of **1**.



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†Dedicated to Prof. Dr. Albert Eschenmoser on the occasion of his 85th birthday.

The peels of *C. aurantium* var. *myrtifolia* were extracted with MeOH, and the MeOH extract was in turn partitioned with $CHCl_3$ and H_2O. The $CHCl_3$ layer was subjected to a silica gel column chromatography, in which a fraction eluted with $CHCl_3$/MeOH (1:1) was purified by an ODS HPLC column (MeCN/H_2O, 0.1% TFA) to afford polymyrifline A (**1**, 0.0003%) and known polymethoxy flavones, nobiletin[7] and tangeritin.[8]

Polymyrifline A (**1**), yellow amorphous solid, $[\alpha]_D^{23}$ -48° (*c* 1.0, MeOH), showed molecular formula, $C_{26}H_{30}O_{13}$, which was determined by HRESITOFMS [*m/z* 551.1774 $(M+H)^+$, Δ +0.9 mmu], indicating 12 degrees of unsaturation in the molecule. The IR absorption bands were characteristic of hydroxyl (3416 cm^{-1}) and conjugated ketone (1630 cm^{-1}) groups. The ^1H NMR spectrum of **1** showed the presence of five methoxyls, seven protons corresponding to sugar moiety, and four aromatic protons. Among them, three of four aromatic protons (δ_H 7.08, δ_H 7.83, and δ_H 7.94) indicated the presence of 1,2,4-trisubstituted benzene ring by its typical coupling patterns.

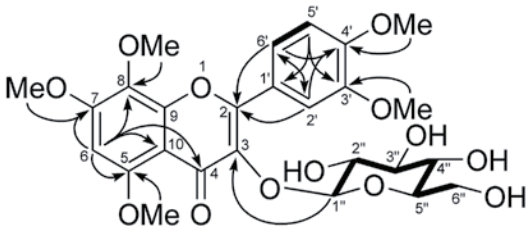

Figure 1. Selected 2D NMR correlations of polymyrifline A in pyridine-d_5

Remaining one aromatic proton (δ_H 6.60) was assigned as H-6 at flavone skeleton by HMBC correlations with C-4 (δ_C 175.3), C-5 (δ_C 157.1), C-7 (δ_C 156.7), C-8 (δ_C 130.8), and C-10 (δ_C 115.5). The location of sugar moiety was determined from the HMBC spectrum. The anomeric proton (δ_H 6.20) of the sugar moiety showed a correlation with C-3 (δ_C 137.6). Connections between each methoxyl and flavone skeleton were assigned by the HMBC cross-peaks for five methoxyls to C-5, C-7, C-8, C-3', and C-4' respectively. Thus, the gross structure of polymyrifline A was assigned as shown. The relative stereochemistry of the hexopyranose moiety was elucidated on the basis of the NOESY data and ^1H-^1H couplings. The NOESY correlations for H-1''/H-3'', H-1''/H-5'', and H-3''/H-5'' suggested that the hexopyranose took a boat form with axial orientations for H-1'', H-3'', and H-5''. Anti orientations for H-1''/H-2'' and H-3''/H-4'' were deduced from the $^3J_{(H\text{-}1''/H\text{-}2'')}$ and $^3J_{(H\text{-}3''/H\text{-}4'')}$ values (6.3 and 9.0 Hz, respectively). Thus, the hexopyranose unit was assigned as a β-glucopyranoside. The absolute configuration of the glucose moiety was determined as the D-form on the basis of HPLC analysis with chiral detector of the acid hydrolysate of **1**.

Polymyrifline A (**1**) was found to inhibit the NO production (15% inhibition at 50 μg/mL) in

LPS-stimulated J774.1 with scarcely affecting the cell viability.[9]

Table 1. ^{1}H and ^{13}C NMR Data of Polymyrifline A (**1**) in Pyridine-d_5 at 27 °C

	δ_H	δ_C	HMBC (^{1}H)
2		153.9	2', 6'
3		137.5	1''
4		174.1	6
5		156.7	6, 5-OMe
6	6.60 (1H, s)	93.6	
7		157.1	6, 7-OMe
8		130.8	8-OMe
9		151.1	
10		109.3	6
1'		123.7	5'
2'	8.45 (1H, s)	113.4	6'
3'		149.2	5', 3'-OMe
4'		151.8	2', 6', 4'-OMe
5'	7.00 (1H, d, 8.5)	111.6	
6'	8.12 (1H, d, 8.5)	122.4	2'
1''	6.20 (1H, d, 6.3)	104.2	
2''	4.36 (1H, m)	76.2	
3''	4.34 (1H, m)	78.6	
4''	4.27 (1H, dd, 9.0, 9.0)	71.3	
5''	3.93 (1H, m)	79.0	
6''a	4.25 (1H, m)	62.2	
6''b	4.34 (1H, m)		
5-OMe	3.94 (3H, s)	56.5	
7-OMe	3.91 (3H, s)	56.4	
8-OMe	4.00 (3H, s)	61.3	
3'-OMe	4.00 (3H, s)	56.0	
4'-OMe	3.77 (3H, s)	55.7	

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV spectra were recorded on a Shimadzu UVmini-1240 spectrophotometer and IR spectra on a JASCO FT/IR-4100 spectrophotometer. Mass spectra were obtained using a Waters ZQ-2000 and a LTQ Orbitrap XL (Thermo Scientific) spectrometers. 1D and 2D NMR spectra were recorded on Bruker AV 400 spectrometers, and chemical shifts were referenced to the residual solvent peaks (δ_H 7.21 and δ_C 135.5 for pyridine-d_5). Standard pulse sequences were employed for the 2D NMR experiments. HPLC was performed on a CAPCELL PAK C_{18} MG-II, 5 μm (ϕ 10 x 250 mm).

Plant Material. Peels of *C. aurantium* var. *myrtifolia* was collected in Shizuoka, Japan in 2008. The botanical identification was made by Dr. Kazunori Ogawa, National Institute of Fruit Tree Science,

Faculty of Science of Japan.

Extraction and Isolation. Peels (1 kg) of *C. aurantium* var. *myrtifolia* were extracted with MeOH, and the MeOH extract (48.3 g) was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction (4.2 g) was subjected to silica gel column chromatography (elution, $CHCl_3$/MeOH 1:0 to 0:1) to yield 9 fractions. The 7 th fraction (410 mg) which eluted by $CHCl_3$/MeOH (1:1) was purified by C_{18} HPLC (33~45% aqueous MeOH with 0.1% TFA) to afford polymyrifline A (**1**, 3.0 mg, 0.0003%) together with nobiletin (171.6 mg, 0.02%) and tangeritin (10.7 mg, 0.001%).

Polymyrifline A (1): yellow amorphous solid; $[\alpha]_D^{23}$ –48° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.25), 253 (4.04), 269 (3.96), and 351 (4.01) nm; IR (KBr) ν_{max} 3416, 2922, 1630, and 1595 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); ESIMS *m/z* 551 $(M + H)^+$; HRESITOFMS *m/z* 551.1774 (M + H; calcd for $C_{26}H_{31}O_{13}$, 551.1765).

Absolute Stereochemistry of β-D-glucose of 1. Polymyrifline A (**1**, 0.2 mg) was treated with 1N aqueous HCl (50 μL) at 100 ºC for 2 h. After neutralization with 1 N aqueous NaOH, the mixture was extracted with $CHCl_3$. The aqueous layer was submitted to HPLC analysis (GL science NH_2 column ϕ 4.6 x 250 mm, eluent: 70% aqueous MeCN, flow rate 1.0 mL/min, JASCO OR-1590 chiral detector). Retention times of authentic L- and D-glucose were as follows: L (9.1 min with negative intensity) and D (9.1 min with positive intensity). The retention time of glucose in the aqueous layer of hydrolysate of **1** was 9.1 min with positive intensity.

NO Production Assay by J774.1 Cell Lines. J774.1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin/streptmycin. J774.1 cells were seeded onto 96-well microtiter plate at 1×10^5 cells in 100 μL per well, and were preincubated for 12 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were cultured in the medium containing LPS (5 μg/mL) with or without the test sample of different concentrations for 24 h. The NO production was then determined by Griess assay. 100 μL of the supernatant of the cultured medium were transferred to 96-well microtiter plate, and then, added 100 μL of Griess reagent (1% sulfanilamide, 0.1% *N*-l-naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4). After incubation at room temperature for 15 min, the absorbance at 540 nm and 620 nm was measured with a microplate reader (Benchmark Plus microplate sqectrometer, Bio-Rad). L-N^G-monomethyl arginine (L-NMMA) (98%) was used as a positive control (IC_{50} = 22 μg/mL).

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