3-*O*-Methylquercetin Glucosides from *Ophioglossum pedunculosum* and Inhibition of Lipopolysaccharide-Induced Nitric Oxide Production in RAW 264.7 Macrophages

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The three new 3-*O*-methylquercetin glucosides **1**–**3**, together with three known congeners and 3-*O*-methylquercetin, were isolated from the fern *Ophioglossum pedunculosum* (quercetin = 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one). The new compounds were identified on the basis of spectroscopic analysis as 5′-isoprenyl-3-*O*-methylquercetin 4′,7-di-*β*-D-glucopyranoside (**1**), 3-*O*-methylquercetin 4′-*β*-D-glucopyranoside 7-[*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranoside] (**2**), and 3-*O*-methylquercetin 7-[*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranoside] (**3**). The effect of the isolated compounds on lipopolysaccharide (LPS)-induced NO production was evaluated. The inhibitory activity of 3-*O*-methylquercetin derivatives decreased markedly with the increasing number of glucosyl groups in the structures.

Introduction. – *Ophioglossum* is a genus of 25–30 species in the family of the Ophioglossaceae, which is distributed in tropical and subtropical zones. Seven species of this genus occur in China. *Ophioglossum pedunculosum* DESV. is a small fern used as a detoxifying herb in traditional Chinese medicine to treat diseases caused by snake venoms [1]. Our previous phytochemical research on this plant afforded seven homoflavonoid glucosides with two showing modest anti-HBV activity [2]. The study of the related species *O. petiolatum* afforded six homoflavonoids, together with five known flavonoids, among which ophioglonin and 3-*O*-methylquercetin showed slight anti-HBV surface antigen activity [3] (quercetin = 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one). In addition, a triglycoside, 3-*O*-methylquercetin 7-(glucosylglucoside) 4′-glucoside whose sugar–sugar linkage in the disaccharide portion was undetermined was isolated from *O. vulgatum* [4]. Although 3-*O*-methylquercetin is a well known compound with anti-inflammatory activity, its glucosides have not been investigated for this activity [5–7]. In our present research, three new 3-*O*-methylquercetin glucosides, **1**–**3**[1]), together with three known congeners 3-*O*-methylquercetin 4′,7-di-*β*-D-glucopyranoside (**4**) [4], 3-*O*-methylquercetin 7-*β*-D-glucopyranoside (**5**) [8], 3-*O*-methylquercetin 4′-*β*-D-glucopyranoside (**6**) [8], and 3-*O*-methylquercetin (**7**) [8][9] were isolated from *O. pedunculosum* (*Fig. 1*).

[1]) Trivial atom numbering; for systematic names, see *Exper. Part.*

Herein we describe the structure identification of the new compounds and the inhibitory activity of the main isolates on NO production in macrophage RAW 264.7 cells.

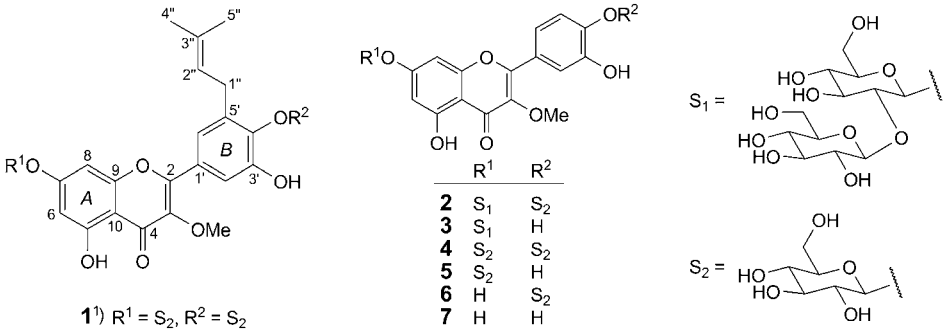

Fig. 1. *Compounds* **1**–**7**, *isolated from* Ophioglossum pedunculosum

Results and Discussion. – Compound **1** was obtained as an optically active, yellow amorphous powder with a molecular formula $C_{33}H_{40}O_{17}$ as deduced from its HR-ESI-MS (*m*/*z* 707.2176). The IR spectrum showed absorptions for OH (3395 cm^{-1}), conjugated C=O (1654 cm^{-1}), and aromatic moieties (1603 and 1491 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **1** (*Table*) revealed the presence of two glucose residues by two anomeric-H-atom signals at δ(H) 5.08 (*d*, *J* = 7.4 Hz) and 4.66 (*d*, *J* = 7.8 Hz) and the related anomeric C-atom signals at δ(C) 99.9 and 105.2. In the ^{1}H-NMR spectrum, one MeO group (δ(H) 3.82 (*s*), one isoprenyl (= 3-methylbut-2-en-1-yl) group (δ(H) 1.73 (*s*, 3 H), 1.71 (*s*, 3 H), 5.29–5.37 (*m*, 1 H), 3.46–3.50 and 3.53–3.61 (*m*, 2 H)) [10], four *meta*-coupled aromatic H-atoms (δ(H) 6.46 and 6.77 (*d*, *J* = 2.0 Hz, 1 H each), and 7.40 and 7.37 (*d*, *J* = 2.0 Hz, 1 H each)), one phenolic OH group (δ(H) 9.46 (br. *s*), and one H-atom-bonded OH group (δ(H) 12.57 (br. *s*) were observed. The ^{13}C-NMR chemical shifts of their corresponding C-atoms were assigned by an HSQC experiment (*Table*). The HMBC spectrum of **1** showed a cross-peak δ(H) 3.82/δ(C) 138.8 (C(3)) suggesting the location of the MeO moiety at C(3) (*Fig. 2*); the cross-peaks from the phenolic OH at δ(H) 9.46 to C(2′), C(3′), and C(4′) and from the H-atom-bonded OH at δ(H) 12.57 to C(4), C(5), and C(6) indicated that the two OH groups were located at position C(3′) and C(5), respectively; the cross-peaks from CH_2(1″) to C(4′), C(5′), and C(6′) and from H–C(6′) to C(1″) suggested that the isoprenyl group was attached at C(5′) of ring *B*. Furthermore, the HMBC cross-peaks between the anomeric H-atom signals of H–C(1‴) (δ(H) 5.08) and C(7) (δ(C) 163.1), and of H–C(1⁗) (δ(H) 4.66) and C(4′) (δ(C) 145.4) indicated that one glucosyloxy residue was linked to C(4′) at ring *B*, and the other one was connected with C(7) at ring *A*, which was also supported by the ROESY correlations (*Fig. 2*) H–C(6) (δ(H) 6.46) and H–C(8) (δ(H) 6.77)/H–C(1‴) (δ(H) 5.08), as well as OH–C(3′) (δ(H) 9.46) and H–C(1″) (δ(H) 3.53–3.61 and 3.46–3.50)/H–C(1⁗) (δ(H) 4.66). The ^3J coupling constants (7.4 and 7.8 Hz) of the anomeric H-atom signal suggested β-configuration for the glucosyl moieties. The absolute configuration of the glucose residues was determined to be D by GC/MS

Table. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.; (D_6)DMSO)[a]) *of* **1**–**3**[1])

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
C(2)		155.5		155.5		155.8
C(3)		138.8		138.4		137.8
C(4)		178.3		178.2		178.1
C(5)		160.9		160.8		160.8
H–C(6)	6.46 (*d*, *J* = 2.0)	99.3	6.50 (*d*, *J* = 2.0)	99.4	6.49 (*d*, *J* = 2.0)	99.3
C(7)		163.1		162.8		162.7
H–C(8)	6.77 (*d*, *J* = 2.0)	94.6	6.84 (*d*, *J* = 2.0)	94.7	6.81 (*d*, *J* = 2.0)	94.7
C(9)		156.0		155.9		156.2
C(10)		106.0		105.9		105.8
C(1′)		126.4		123.8		120.6
H–C(2′)	7.40 (*d*, *J* = 2.0)	114.2	7.59 (*d*, *J* = 2.0)	115.7	7.58 (*d*, *J* = 2.0)	115.6
C(3′)		149.5		146.5		145.2
C(4′)		145.4		147.8		148.8
H–C(5′)		136.2	7.28 (*d*, *J* = 8.5)	115.8	6.92 (*d*, *J* = 8.5)	115.7
H–C(6′)	7.37 (*d*, *J* = 2.0)	120.2	7.53 (*dd*, *J* = 8.5, 2.0)	120.2	7.46 (*dd*, *J* = 8.5, 2.0)	120.6
CH_2(1″)	3.53 – 3.61 (*m*), 3.46 – 3.50 (*m*)	28.1				
H–C(2″)	5.29 – 5.37 (*m*)	122.5				
C(3″)		132.1				
Me(4″)	1.73 (*s*)	25.5				
Me(5″)	1.71 (*s*)	17.7				
7-Glc						
H–C(1‴)	5.08 (*d*, *J* = 7.4)	99.9	5.22 (*d*, *J* = 7.2)	98.2	5.22 (*d*, *J* = 7.0)	98.3
H–C(2‴)	3.26 (*t*, *J* = 9.0)	73.1	3.51 (*dd*, *J* = 7.2, 9.0)	82.6	3.51 (*dd*, *J* = 7.0, 9.0)	82.6
H–C(3‴)	3.29 (*t*, *J* = 9.0)	76.4	3.52 (*t*, *J* = 9.0)	75.6	3.52 (*t*, *J* = 9.0)	75.6
H–C(4‴)	3.18 (*t*, *J* = 9.0)	69.5	3.25 (*t*, *J* = 9.0)	69.1	3.24 (*t*, *J* = 9.0)	69.1
H–C(5‴)	3.41 – 3.47 (*m*)	77.1	3.47 – 3.53 (*m*)	76.9	3.46 – 3.52 (*m*)	76.9
CH_2(6‴)	3.70 (br. *d*, *J* = 12.0), 3.47 (*dd*, *J* = 5.7, 12.0)	60.5	3.70 (br. *d*, *J* = 12.5), 3.48 (*dd*, *J* = 5.7, 12.5)	60.7	3.71 (br. *d*, *J* = 10.0), 3.48 (*dd*, *J* = 5.7, 10.0)	60.4
2‴-Glc						
H–C(1⁗)			4.47 (*d*, *J* = 7.8)	104.8	4.47 (*d*, *J* = 7.8)	104.6
H–C(2⁗)			2.99 (*dd*, *J* = 7.8, 8.0)	74.6	2.99 (*dd*, *J* = 7.8, 9.0)	74.6
H–C(3⁗)			3.20 (*t*, *J* = 9.0)	76.1	3.20 (*t*, *J* = 9.0)	76.2
H–C(4⁗)			3.14 (*t*, *J* = 9.0)	69.6	3.13 (*t*, *J* = 9.0)	69.6
H–C(5⁗)			3.11 – 3.17 (*m*)	76.8	3.11 – 3.17 (*m*)	76.8
CH_2(6⁗)			3.70 (br. *d*, *J* = 12.5), 3.48 (*dd*, *J* = 5.7, 12.5)	60.5	3.71 (br. *d*, *J* = 10.0), 3.48 (*dd*, *J* = 5.7, 10.0)	60.6
4′-Glc						
H–C(1⁗)	4.66 (*d*, *J* = 7.8)	105.2	4.89 (*d*, *J* = 7.3)	101.5		
H–C(2⁗)	3.36 (*t*, *J* = 9.0)	73.9	3.34 (*dd*, *J* = 7.3, 9.0)	73.2		
H–C(3⁗)	3.29 (*t*, *J* = 9.0)	76.2	3.31 (*t*, *J* = 9.0)	75.9		
H–C(4⁗)	3.21 (*t*, *J* = 9.0)	69.7	3.19 (*t*, *J* = 9.0)	69.8		
H–C(5⁗)	3.19 – 3.25 (*m*)	77.4	3.36 – 3.42 (*m*)	77.2		
CH_2(6⁗)	3.70 (br. *d*, *J* = 12.0), 3.49 (*dd*, *J* = 5.7, 12.0)	60.8	3.70 (br. *d*, *J* = 12.5), 3.48 (*dd*, *J* = 5.7, 12.5)	60.4		
HO–C(5)	12.57 (br. *s*)		12.60 (br. *s*)		12.70 (br. *s*)	
HO–C(3′)	9.46 (br. *s*)		9.06 (br. *s*)		9.40 (br. *s*)	
HO–C(4′)					9.84 (br. *s*)	
MeO–C(3)	3.82 (*s*)	59.8	3.82 (*s*)	59.8	3.80 (*s*)	59.8

[a]) The assignments were based upon ^{1}H- and ^{13}C-NMR, HSQC, HMBC, and ROESY experiments.

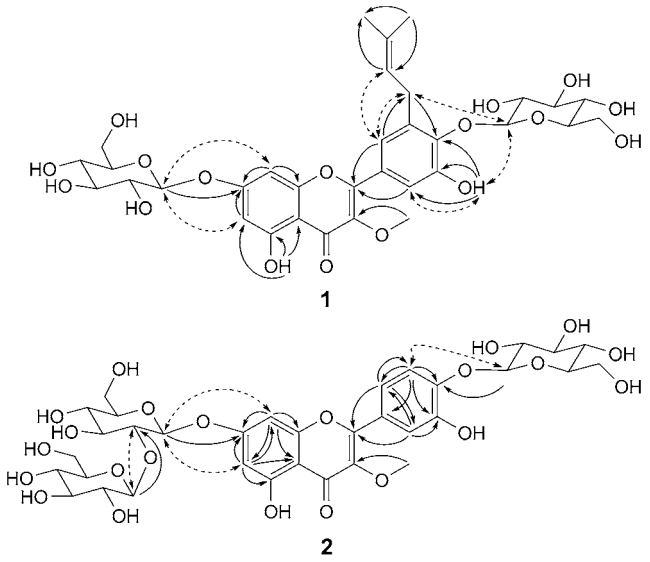

Fig. 2. *Key HMBC* (H → C) *and ROESY* (H ←--→ H) *correlations of compounds* **1** *and* **2**

analysis after acid hydrolysis (2.0M HCl at 100° for 4 h) and derivatization with L-cysteine methyl ester according to the method previously described [2]. Therefore, the structure of compound **1** was identified as 5′-isoprenyl-3-*O*-methyl quercetin 4′,7-di-β-D-glucopyranoside[1]).

Compound **2** was also isolated as a yellow amorphous powder. Its molecular formula was $C_{34}H_{42}O_{22}$ as determined from its HR-ESI-MS (*m/z* 801.2061). Its IR spectrum showed absorption bands similar to those of **1** at 3424 cm^{-1} (OH), 1684 cm^{-1} (C=O), and 1626, 1597, and 1464 cm^{-1} (aromatic moiety). The ^{1}H-NMR spectrum of **2** (*Table*) displayed three anomeric-H-atom signals at δ(H) 5.22 (*d*, *J* = 7.2 Hz), 4.47 (*d*, *J* = 7.8 Hz), and 4.89 (*d*, *J* = 7.3 Hz) indicating the presence of three glucosyl residues. Analysis of the ^{1}H-NMR spectrum also revealed the presence of one MeO group at δ(H) 3.82 (*s*), one phenolic OH group at δ(H) 9.06 (br. *s*), one H-atom-bonded OH group at δ(H) 12.60 (br. *s*), two *meta*-coupled aromatic H-atoms at δ(H) 6.50 and 6.84 (each *d*, *J* = 2.0 Hz), and an aromatic *ABX* system (δ(H) 7.59 (*d*, *J* = 2.0 Hz, 1 H), 7.53 (*dd*, *J* = 8.5, 2.0 Hz, 1 H), and 7.28 (*d*, *J* = 8.5 Hz, 1 H)) indicating a 1,3,4-trisubstituted benzene moiety (ring *B*). The ^{13}C-NMR chemical shifts of their corresponding C-atoms were assigned by HSQC experiments (*Table*). The HMBC spectrum of **2** showed cross-peaks from δ(H) 3.82 to δ(C) 138.4 (C(3)) suggesting the location of the MeO group at C(3) (*Fig. 2*). The HMBCs of H–C(1‴) (δ(H) 5.22)/C(7) (δ(C) 162.8) and H–C(1⁗) (δ(H) 4.89)/C(4′) (δ(C) 147.8) revealed that one glucosyloxy residue was linked to C(4′) at ring *B* and the other one connected with C(7) at ring *A*. The remaining glucosyloxy residue was linked to the C(2‴) of glucosyloxy moiety at C(7) according to the significantly shifted signals of C(2‴) (δ(C) 82.6) and the HMBC H–C(1⁗) (δ(H) 4.47)/C(2‴) (δ(C) 82.6). In addition, the ROESY cross-peak H–C(2‴) (δ(H) 3.51)/H–C(1⁗) (δ(H) 4.47) unambiguously established the 1 → 2 interglycosidic linkage

between the carbohydrate residues [11]. Therefore, the structure of compound **2** was confirmed as 3-*O*-methylquercetin 4′-*β*-D-glucopyranoside 7-[*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranoside][1]).

Compound **3** was obtained as a yellow amorphous powder whose molecular formula was $C_{28}H_{32}O_{17}$ according to its HR-ESI-MS (*m*/*z* 639.1536). From the ^{1}H- and ^{13}C-NMR data of **3** and **2** (*Table*), it was apparent that compound **3** possessed the same aglycone and the same type of sugar units as those of **2**, while the difference between **2** and **3** was the number of the sugar units. The ^{1}H-NMR spectrum of **3** showed two anomeric H-atom signals at δ(H) 5.22 (*d*, *J* = 7.0 Hz) and 4.47 (*d*, *J* = 7.8 Hz) and the absence of signal at δ(H) 4.89 (*d*, *J* = 7.3 Hz) observed for **2**, indicating the absence of the glucosyloxy group at C(4′) of **3**. Therefore, the structure of **3** was proposed to be 3-*O*-methylquercetin 7-[*O*-*β*-D-glucopyranosyl-(1 → 2)-*β*-D-glucopyranoside][1]), which was further confirmed by 2D-NMR data.

Compounds **1**, **4**, **5**, and **7** which were obtained in sufficient amounts were investigated for their inhibitory effects on lipopolysaccharide (LPS)-induced NO production in macrophage RAW 264.7 cells. Compounds **5** and **7** with an IC_{50} of 23.7 ± 2.0 and 12.4 ± 2.1 μM showed stronger inhibition than **1** and **4** ($IC_{50} > 50$ μM). In this assay, the positive control *N*-nitro-L-arginine methyl ester (L-NAME) exhibited an IC_{50} of 25.6 ± 1.7 μM. The percentages of inhibition of **1**, **4**, **5**, and **7** were 46.5, 35.8, 66.8, and 93.1% at 50 μM, and 12.3, 7.5, 22.3, and 42.0% at 10 μM, respectively. Comparison of the inhibition of the 3-*O*-methylquercetin glucosides **1**, **4**, and **5** with their aglycone **7** revealed that the inhibitory activity decreased markedly with the increasing number of glucosyl groups in the structures. The compounds showed no sign of cytotoxicity up to 50 μM on RAW 264.7 cells.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; *Qingdao Haiyang Chemical Co., Ltd.*), *MCI* gel (*CHP20P*, 75 – 150 μm; *Mitsubishi Chemical Industries Ltd.*), and *Sephadex LH-20* (20 – 100 μm; *Pharmacia*). Prep. HPLC: *Agilent 1100* system coupled with a *Shim-pack RP-C$_{18}$* column (200 × 20 mm i.d., 10 μm; *Shimadzu*). Optical rotations: *Jasco P-1020* digital polarimeter (*Shimadzu*). UV Spectra: *UV-2450* UV/VIS spectrophotometer (*Shimadzu*) ; λ_{max} (log ε) in nm. IR Spectra (KBr discs): *Bruker-Tensor-27* spectrometer; $\tilde{\nu}$ in cm^{-1}. 1D- and 2D-NMR Spectra: *Bruker-AV-500* spectrometer; in (D_6)DMSO at 303 K; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. ESI- and HR-ESI-MS: *Agilent 1100 LC/MSD* trap mass spectrometer (ESI-MS) and *Agilent-G1969A* TOF-MS instrument; in *m*/*z*.

Plant Material. The whole plants of *O. pedunculosum* were purchased in August 2008 from the local traditional Chinese medicine (TCM) market in Bozhou, Anhui Province, China, and identified by Prof. *Mian Zhang*, Department of Pharmacognosy, China Pharmaceutical University. A voucher specimen (No. 200808101) was deposited with the Department of Natural Medicinal Chemistry, China Pharmaceutical University.

Extraction and Isolation. The air-dried materials of *O. pedunculosum* (4.0 kg) were powdered and successively extracted three times with 75% EtOH (3 × 30 l, each 3 h) at 80° under reflux. After evaporation, the EtOH extract (692 g) was subjected to CC (SiO_2, gradient 0, 10, 30, 50, 70, and 100% MeOH/CH_2Cl_2). Fractions eluted with 30 – 50% MeOH/CH_2Cl_2 (22.0 g) were combined and further

separated by CC (*MCI* gel, 30, 50, and 70% MeOH/H_2O): *Fractions I–III*. *Fr. I* (with 30% MeOH; 6.8 g) was further separated by CC (*Sephadex LH-20*, 50% MeOH/H_2O) and the compounds finally purified by prep. HPLC (10 → 27.5% MeOH/H_2O): **2** (2.0 mg), **3** (6.6 mg), and **4** (80.0 mg). *Fr. II* (with 50% MeOH; 9.6 g) was further separated by CC (*Sephadex LH-20*, MeOH) and prep. HPLC (30 → 40% MeOH/H_2O): **5** (40.0 mg) and **6** (9.0 mg). *Fr. III* (with 70% MeOH; 4.9 g) yielded **1** (22.4 mg) and **7** (90.0 mg) after CC (*Sehpadex LH-20*, MeOH) and prep. HPLC (50% MeOH/H_2O).

*5′-Isoprenyl-3-*O*-methylquercetin 4′,7-Di-β-*D*-glucopyranoside* (= *7-(β-*D*-Glucopyranosyloxy)-2-[4-(β-*D*-glucopyranosyloxy)-3-hydroxy-5-(3-methylbut-2-en-1-yl)phenyl]-5-hydroxy-3-methoxy-4*H*-1-benzopyran-4-one*; **1**). Yellow amorphous powder. $[\alpha]_D^{28} = -34.4$ ($c = 0.10$, MeOH/H_2O 1:1). UV: 337 (4.07), 262 (4.25), 202 (4.41). IR: 3395, 2929, 1654, 1603, 1491, 1443, 1368, 1302, 1206, 1178, 1073, 815. ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS: 707 ($[M-H]^-$). HR-ESI-MS: 707.2176 ($[M-H]^-$, $C_{33}H_{39}O_{17}^-$; calc. 707.2193).

*3-*O*-Methylquercetin 4′-β-*D*-Glucopyranoside 7-[*O*-β-*D*-Glucopyranosyl-(1 → 2)-β-*D*-glucopyranoside* (= *7-[(2-*O*-β-*D*-Glucopyranosyl-3-β-*D*-glucopyranosyl)oxy]-2-[4-(β-*D*-glucopyranosyloxy)-3-hydroxyphenyl]-5-hydroxy-3-methoxy-4*H*-1-benzopyran-4-one*; **2**): Yellow amorphous powder. $[\alpha]_D^{28} = -62.0$ ($c = 0.10$, MeOH/H_2O 1:1). UV: 348 (3.87), 257 (4.01), 205 (4.36). IR: 3424, 2931, 1684, 1626, 1597, 1464, 1385, 1357, 1208, 1137, 1077, 842, 804. ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS: 801 ($[M-H]^-$). HR-ESI-MS: 801.2061 ($[M-H]^-$, $C_{34}H_{41}O_{22}^-$; calc. 801.2095).

*3-*O*-Methylquercetin 7-[*O*-β-*D*-Glucopyranosyl-(1 → 2)-β-*D*-glucopyranoside]* (= *2-(3,4-Dihydroxyphenyl)-7-[(2-*O*-β-*D*-glucopyranosyl-β-*D*-glucopyranosyl)oxy]-5-hydroxy-3-methoxy-4*H*-1-benzopyran-4-one*; **3**): Yellow amorphous powder. $[\alpha]_D^{28} = -34.9$ ($c = 0.07$, MeOH/H_2O 1:1). UV: 359 (3.77), 256 (3.86), 204 (4.16). IR: 3451, 2921, 1656, 1601, 1496, 1460, 1375, 1123, 1071, 918, 804. ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS: 639 ($[M-H]^-$). HR-ESI-MS: 639.1536 ($[M-H]^-$, $C_{28}H_{31}O_{17}^-$; calc. 639.1567).

Cell Culture, Cell Viability, and Determination of NO Production. The macrophage cell line RAW 264.7 was obtained from the *CBCAS* (*Cell Bank of the Chinese Academic of Sciences*, Shanghai, P. R. China), and cultured in DMEM (*Dulbecco*'s modified *Eagle*'s medium) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% heat-inactivated FBS (fetal bovin serum at 37° with 5% CO_2.

Cell viability was determined colorimetrically by using the MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. After treatment with the test compounds (50, 40, 20, 10, 5, and 1 μM) for 24 h, 20 μl of 5 mg/ml MTT soln. was added to each well and incubated for the next 4 h. The supernatants were aspirated, the formazan crystals in each well were dissolved in 200 μl of DMSO for 30 min at 37.0°, and the optical density at 570 nm was read with a *Microplate Reader* (*Bio-Rad*, CA, USA). The mean absorbance for the triplicate of the triplicate culture of each compound was calculated, and the mean blank value was subtracted from these.

The level of NO production was determined by measuring the amount of nitrite from the cell-culture supernatants as described previously [12]. Briefly, cells were plated at a density of 10^5 cells per well in 96-well culture plates and were pretreated with the test compounds (50, 40, 20, 10, 5, and 1 μM) for 2 h. Subsequently the LPS (100 ng/ml) was added and continually stimulated for 18 h. Afterwards, 100 μl of supernatants were mixed with an equal volume of *Griess* reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at r.t. for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring the optical density at 540 nm. All experiments were performed in triplicate, and the *N*-nitro-L-arginine methyl ester (L-NAME, *Sigma–Aldrich*) was used as positive control.

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