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Stereospecific inhibition of nitric oxide production in macrophage cells by flavanonols: Synthesis and the structure–activity relationship

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ABSTRACT

To explore the structure–activity relationships on the inhibitory activity of flavanonols against nitric oxide (NO) production in inflammatory cells, we synthesized 19 flavanonols which shared a common 3,5,7-trihydroxychroman scaffold. A range of substitutions was included in the B ring in order to investigate the structure–activity relationship. We also succeeded in isolating stereoisomers from 16 of the flavanonols using chiral column chromatography. The inhibitory effects of these compounds on NO production were examined in RAW 264.7 cells (a murine macrophage-like cell line), which were activated by lipopolysaccharide (LPS). We only observed inhibitory activity against NO production in (2*R*,3*R*) stereoisomers, while the inhibitory activities of (2*S*,3*S*) stereoisomers were significantly weaker. We also evaluated the free radical scavenging potential of the flavanonols using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Each stereoisomer indicated the equivalent DPPH scavenging potential as expected. The radical scavenging activity was not correlated with the inhibitory activity against NO. The inhibition of NO production by flavanonols is stereospecific and cannot simply be explained by their radical scavenging activity. We propose the possible existence of a 'target' molecule for flavanonols which is involved in the production and/or regulation of NO in RAW 264.7 cells.

1. Introduction

Flavonoids are widely distributed in plants. Humans ingest them from fruits, vegetables, teas, dietary supplements and other sources. Some flavonoids are known to show anti-allergic,[1] anti-inflammatory,[1–4] anti-microbial,[5–7] anti-viral,[8–10] anti-cancer,[2,11] anti-diarrheal,[12] antitumor,[13–15] anti-diabetic,[16] and antioxidant[12,17–20] activities. The anti-inflammatory and antioxidant functions of flavonoids from many plants have often reported. These two activities are the most important and versatile functions of flavonoids.

In the inflammation processes, the expression of cytokines or mediators such as tumor necrosis factor-α, interleukin-1β and interleukin-6, as well as nitric oxide synthase are induced in immune cells by lipopolysaccharide (LPS) and interferon-γ. They play a critical causative role in rheumatoid arthritis, asthma, and atherosclerosis. Nitric oxide (NO) plays an important physiological role as a defense molecule in the immune system, while the excess production of NO by macrophages contributes to numerous pathological processes. In particular, when NO reacts with superoxide anion radicals, it produces peroxynitrite anions, which are strongly reactive. Aside from this direct action, the production of NO is one of the primary indicators of macrophage activation. Therefore, compounds that suppress NO production might have therapeutic value.

Oxidative stress arises from disturbances in the balance between the production of reactive oxygen species (ROS) and the antioxidant defenses. ROS can be beneficial as a means of attacking pathogens;[21] however, excessive ROS activity, which may occur when the balance of the ROS and antioxidant potential is disturbed, has a self-destructive effect. Animals ingest antioxidants through many foods, including fruits and vegetables. Recent research[2] shows that flavonoids are the predominant source of antioxidants in the animal body.

Numerous structure–activity relationship studies have investigated the NO production inhibitory activity[22,23] and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity[24,25] of flavonoids. In the present study, we aimed to synthesize flavanonols with an identical chroman scaffold in their A and C

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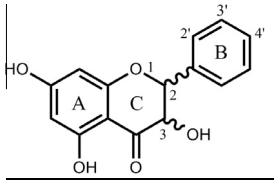
Table 1
The structure and activity of the flavanonols



Compound	Substitution in the B ring	NO-production inhibitory % at 100 μM		DPPH radical scavenging EC_{50} (μM)	
		(2*R*,3*R*)	(2*S*,3*S*)	(2*R*,3*R*)	(2*S*,3*S*)
AG		72			
Gallic acid				10–12	
5	None	<10	<10	>100	>100
5a	2′-OH	40	<15	>100	>100
5b	3′-OH	35	<15	>100	>100
5c	4′-OH	<10	<10	>100	>100
5d	2′,6′-OH	<10	<10	>100	>100
5e	2′,3′-OH	70	45	12.5	13.5
5f	2′,4′-OH	<10	<10	>100	>100
5g	2′,5′-OH	30	<15	24.5	25.4
5h	3′,4′-OH	<10	<10	13.9	12.6
5i	3′,5′-OH	<10	<10	>100	>100
5j	2′-OCH_3	Racemic mixture <10		Racemic mixture >100	
5k	3′-OCH_3	<10	<10	>100	>100
5l	4′-OCH_3	<10	<10	>100	>100
5m	2′,6′-OCH_3	Racemic mixture <10		Racemic mixture >100	
5n	2′,3′-OCH_3	<10	<10	>100	>100
5o	2′,4′-OCH_3	<10	<10	>100	>100
5p	2′,5′-OCH_3	<10	<10	>100	>100
5q	3′,4′-OCH_3	Racemic mixture <10		Racemic mixture >100	
5r	3′,5′-OCH_3	<10	<10	>100	>100

rings but with alterations to the structure of their B ring and in their stereochemistry (Table 1). The synthesized flavanonols were used to evaluate the effects of the structure–activity relationship on the inhibition of NO production in RAW 264.7 cells and on DPPH radical scavenging ability.

2. Material and methods

2.1. General experimental procedures

Optical rotations were measured using a JASCO P-1020 polarimeter. The ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a JEOL JNM-ECX600 spectrometer. Mass spectra were obtained on a JEOL GCmate mass spectrometer. IR spectra were recorded on JASCO FT/IR-4200 spectrometer. UV spectra were recorded on JASCO V-730 spectrometer. CD spectra were recorded on JASCO J-600 spectrometer. Melting points were determined by using AS-ONE ATM-02.

2.2. The separation of enantiomers by a chiral column

All of the synthesized flavanonols were further submitted to purification by a chiral column (DAICEL, CHIRALPAK, IA, 5 μm, 10 ϕ mm × 250 mm) to isolate the enantiomers using a high performance liquid chromatography system (JASCO PU-1580, UV-1575). The elution solvent was ethanol–hexane and the flow rate was 5 mL/min. Detection wavelength was 254 nm.

2.3. NO assay

The amount of released nitrite (NO) was quantified by the Griess method[26]. RAW 264.7 cells were cultured in F-12 Ham medium (Sigma Aldrich, N4888) containing 200 mM L-glutamate (Sigma Aldrich, G7513), penicillin (100 U/mL)—streptomycin (0.1 mg/mL) (Sigma Aldrich, P4333) and immobilized fetal bovine serum (10 v/v %) (Biowest, S1780). One hundred fifty microliters of cell suspension (1.6×10^6 cell/mL culture medium) was dispensed in a well of a 96-well plate (Sumitomo Bakelite, #8096R) and 40 μL of test compound solution was added. The test compound solution was prepared by diluting the DMSO solution of the flavanonols by a ratio of 1:100 with culture medium. The cells were incubated for 2 h at 37 °C in a CO_2 incubator. Cells adhered to the culture well during this process. Ten microliters of LPS (Sigma Aldrich, #L-2880) solution was then added to each well. The final concentration of LPS was 100 ng/mL. After 16 h of incubation, 100 μL of supernatant medium was transferred to another plate. The remaining cells were submitted to a cell viability test as described in Section 2.4. Fifty microliters of sulfanilamide solution (50 mg of sulfanilamide dissolved in a mixture of 250 μL of phosphoric acid and 5 mL of water) was added to each well. A few minutes later, 50 μL of 0.1% *N*-1-naphthylethylnediamine (Wako Pure Chemical Inc., 147-04141) solution was added and incubated at room temperature in the dark for 10 min. Absorbance at 540 nm (reference wavelength: 655 nm) was then measured using a microplate reader (BioRad Model 3550). Aminoguanidine hydrochloride (Wako Pure Chemical Inc., 328-26432) was used as a positive control. The concentrations of the test compounds were precisely determined from ultraviolet absorption at λ_{max} using a UV spectrometer (JASCO V-730).

2.4. Cell viability test

Cell viability was measured using AlamarBlue® reagent (Bio-Rad AbD Serotec Ltd.). Ten microliters of AlamarBlue® solution was added to the RAW 264.7 cells left in each well of the 96-well plate from the above-mentioned NO assay, which was then incubated at 37 °C for 4 h. Absorbance was measured at 570 nm (reference wavelength: 655 nm).

2.5. DPPH radical scavenging assay[27]

One hundred twenty microliters of ethanol-buffer solution (ethanol-0.5 M sodium acetate buffer pH 5.6, 105:15) was added to each well of a 96-well plate (Sumitomo Bakelite, #8096R). Forty microliters of compound solution (in ethanol) was added. Next, 40 μL of DPPH solution (1,1-diphenyl-2-picrylhydrazyl, Tokyo Chemical Industry. D4313, 0.5 mM ethanol solution) was added. The plate was shaken on a shaker for 1 min and kept in the dark at room temperature for 30 min. Absorbance was measured at 517 nm (reference wavelength: 655 nm). Gallic acid monohydrate (Wako Pure Chemical Inc., 077-06092) was used as the standard compound (EC_{50} = 10–12 μM).

2.6. Synthesis of 2,4,6-trimomoacetophenone 1

NaH (4.5 equiv) in dry DMF was slowly added while stirring at 0–5 °C (in an ice-water bath) to a solution of 2,4,6-trihydroxyacetophenone (1 equiv) in dry DMF. When the solution was cooled to 0–5 °C, chloromethyl methyl ether (4.5 equiv) was slowly added over a period of 15 min so that the temperature was maintained at less than 5 °C. The reaction mixture was stirred at room temperature for another 30 min, quenched by the addition of cold distilled water and extracted with EtOAc. The combined organic layer was washed with distilled water and brine and then dried over Na_2SO_4. The filtered organic layer was concentrated under a vacuum and the residue was purified by silica gel column chromatography eluting with *n*-hexane and EtOAc to give compound **1**: a colorless oil (80–95% yield).

^{1}H NMR (600 MHz, $CDCl_3$), δ: 2.47 (3H, s, C(=O)CH_3), 3.44 (6H, s, OCH_3-2, OCH_3-6), 3.45 (3H, s, OCH_3-4), 5.12 (6H, d, J = 2.4 Hz, OCH_2-2, OCH_2-4, OCH_2-6), 6.49 (2H, s, H-3, H-5). ^{13}C NMR (150 MHz, $CDCl_3$), δ: 32.5 (C(=O)CH_3), 56.1 (OCH_3-4), 56.3 (OCH_3-2, OCH_3-6), 94.4 (OCH_2-4), 94.7 (OCH_2-2, OCH_2-6), 97.0 (C-3, C-5), 116.8 (C-1), 155.1 (C-2, C-6), 159.4 (C-4), 201.4 (C=O). HR-EI-MS: m/z 300.1209 $[M]^+$ (Calcd 300.1209 for $C_{14}H_{20}O_7$).

2.7. Synthesis of MOMO protection benzaldehyde 2

K_2CO_3 (10 equiv) was added with stirring at 0–5 °C (ice-water bath) to a solution of hydroxylbenzaldehyde (1 equiv) in dry acetone, When the solution was cooled to 0–5 °C chloromethyl methyl ether (1.5 equiv; dependent on the number of the hydroxyl groups) was slowly added over a period of 15 min to keep the temperature under 5 °C. The reaction mixture was stirred at room temperature for another 30 min, quenched by the addition of cold distilled water and extracted with EtOAc. The combined organic layer was washed with distilled water and brine and then dried over Na_2SO_4. The filtered organic layer was concentrated under a vacuum and the residue was purified by silica gel column chromatography eluting with *n*-hexane and EtOAc to give compound **2**: a colorless or light yellow oil (80–95% yield).

2.8. Synthesis of chalcone 3

KOH (3 equiv) ethanol solution was added to a solution of **1** (1 equiv) in EtOH. Then **2** (1 equiv) was added to the reaction mixture solution and stirred at room temperature for 3 h. Distilled water was added and extracted with EtOAc and the combined organic layer was washed with distilled water and brine and dried over Na_2SO_4. The filtered organic layer was concentrated under a vacuum and the residue was purified by silica gel column chromatography eluting with *n*-hexane and EtOAc to give **3**: a light yellow oil (60–90% yield).

2.9. Synthesis of epoxide 4

H_2O_2 (30%) and aqueous 2 M NaOH were added to a methanol solution of chalcone **3**, and the mixture was stirred for 3 h at room temperature. Methanol was removed under a vacuum. Distilled water was added to the resultant aqueous suspension and extracted with EtOAc. The combined organic layer was dried over Na_2SO_4. The organic layer was concentrated under a vacuum to give compound **4**: a colorless oil (85–95% yield; in the cases of **4m** and **4o**, the yield was 50–60%).

2.10. Synthesis of flavanonols

Epoxide **4** was dissolved in HCl-MeOH (1 M), and stirred at 55 °C for 25 min. MeOH was removed under a vacuum. Distilled water was added to the residue and the mixture was extracted with EtOAc. The organic layer was combined and dried over Na_2SO_4. The filtered EtOAc was evaporated to give red-yellow oil which was purified by silica gel column chromatography eluting with *n*-hexane and EtOAc to give **5**, and **5a**–**5r** (30–55% yield).

2.10.1. 3,5,7-Trihydroxy flavanonol (5)

^{1}H NMR (600 MHz, acetone-d_6), δ: 4.68 (1H, d, J = 12.0 Hz, H-3), 5.19 (1H, d, J = 12.0 Hz, H-2), 5.98 (1H, d, J = 2.4 Hz, H-6), 6.01 (1H, d, J = 2.4 Hz, H-8), 7.44 (1H, m, H-4′), 7.44 (2H, m, H-3′, H-5′), 7.63 (2H, m, H-2′, H-6′). ^{13}C NMR (150 MHz, acetone-d_6), δ: 73.3 (C-3), 84.5 (C-2), 96.2 (C-6), 97.3 (C-8), 101.6 (C-10), 128.9 (C-2′, C-6′), 129.2 (C-3′, C-5′), 129.7 (C-4′), 138.4 (C-1′), 164.1 (C-5), 165.1 (C-9), 168.0 (C-7), 198.1 (C-4). HR-EI-MS: m/z 272.0684 $[M]^+$ (Calcd 272.0685 for $C_{15}H_{12}O_5$).

Separation by chiral chromatography: EtOH–*n*-hexane (20:80 v/v) Retention time (2*R*,3*R*)-isomer: 9.08 min, (2*S*,3*S*)-isomer: 7.75 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{23}$ +12.8° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −5.66 (287.6),+2.03 (327.2) ($c = 7.35 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{23}$ −12.2° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm)+5.21 (289.0), −1.94 (326.0) ($c = 7.35 \times 10^{-5}$).

2.10.2. 2′,3,5,7-Tetrahydroxy flavanonol (5a)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.89 (1H, d, J = 12.0 Hz, H-3), 5.62 (1H, d, J = 12.0 Hz, H-2), 5.96 (1H, d, J = 2.4 Hz, H-6), 6.00 (1H, d, J = 2.4 Hz, H-8), 6.45 (1H, dd, J = 8.4, 8.4 Hz, H-5′), 6.93 (1H, d, J = 8.4 Hz, H-3′), 7.24 (1H, ddd, J = 8.4, 8.4, 2.4 Hz, H-4′), 7.53 (1H, dd, J = 8.4, 2.4 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 72.4 (C-3), 79.2 (C-2), 96.1 (C-6), 97.1 (C-8), 101.7 (C-10), 116.9 (C-5′), 120.6 (C-3′), 124.3 (C-1′), 129.7 (C-6′), 130.8 (4′-C), 156.8 (C-2′), 164.5 (C-5), 165.2 (C-9), 167.9 (C-7), 198.4 (C-4). HR-EI-MS: m/z 288.0632 $[M]^+$ (Calcd 288.0634 for $C_{15}H_{12}O_6$).

Separation by chiral chromatography: EtOH–*n*-hexane (20:80 v/v) Retention time (2*R*,3*R*)-isomer: 9.67 min, (2*S*,3*S*)-isomer: 7.88 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +80.1° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −2.98 (284.9), +1.67 (325.8) ($c = 6.94 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −72.2° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +3.02 (284.1), −1.70 (324.1) ($c = 6.94 \times 10^{-5}$).

2.10.3. 3,3′,5,7-Tetrahydroxy flavanonol (5b)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.63 (1H, d, J = 11.4 Hz, H-3), 5.12 (1H, d, J = 11.4 Hz, H-2), 5.98 (1H, d, J = 1.8 Hz, H-6), 6.00 (1H, d, J = 1.8 Hz, H-8), 6.89 (1H, dd, J = 8.4, 3.0 Hz, H-4′), 7.06 (1H, d, J = 8.4 Hz, H-6′), 7.07 (1H, d, J = 3.0 Hz, H-2′), 7.25 (1H, dd, J = 8.4, 8.4 Hz, H-5′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.3 (C-3), 84.4 (C-2), 96.2 (C-6), 97.3 (C-8), 101.6 (C-10), 115.8 (C-2′), 116.6 (C-4′), 120.0 (C-6′), 130.3 (C-5′), 139.8 (C-1′), 158.3 (C-3′), 164.1 (C-5), 165.1 (C-9), 167.9 (C-7), 198.0 (C-4). HR-EI-MS: m/z 288.0632 $[M]^+$ (Calcd 288.0634 for $C_{15}H_{12}O_6$).

Separation by chiral chromatography: EtOH–*n*-hexane (50:50 v/v) Retention time (2*R*,3*R*)-isomer: 5.30 min, (2*S*,3*S*)-isomer: 3.88 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +9.4° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −5.19 (289.5), +1.57 (327.4) ($c = 6.94 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −7.3° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +5.36 (291.1), −1.80 (326.7) ($c = 6.94 \times 10^{-5}$).

2.10.4. 3,4′,5,7-Tetrahydroxy flavanonol (5c)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.66 (1H, d, J = 11.4 Hz, H-3), 5.09 (1H, d, J = 11.4 Hz, H-2), 5.95 (1H, d, J = 1.8 Hz, H-6), 6.00 (1H, d, J = 1.8 Hz, H-8), 6.90 (2H, d, J = 8.4 Hz, H-3′, H-5′), 7.42 (d, 2H, J = 8.4 Hz, H-2′, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.2 (C-3), 84.4 (C-2), 96.1 (C-6), 97.2 (C-8), 101.6 (C-10), 116.0 (C-3′, C-5′), 129.2 (C-1′), 130.4 (C-2′, C-6′), 158.9 (C-4′), 164.3 (C-5), 165.1 (C-9), 167.9 (C-7), 198.4 (C-4). HR-EI-MS: m/z 288.0627 $[M]^+$ (Calcd 288.0634 for $C_{15}H_{12}O_6$).

Separation by chiral chromatography: EtOH–*n*-hexane (30:70 v/v) Retention time (2*R*,3*R*)-isomer: 7.55 min, (2*S*,3*S*)-isomer: 6.10 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +23.3° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −6.48 (289.4), +1.77 (327.4) ($c = 6.94 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −20.5° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +6.75 (289.7), −1.94 (326.7) ($c = 6.94 \times 10^{-5}$).

2.10.5. 2′,3,5,6′,7-Pentahydroxy flavanonol (5d)

^{1}H NMR (600 MHz, acetone-d_6) δ: 5.52 (1H, d, J = 12.0 Hz, H-3), 5.81 (1H, d, J = 12.0 Hz, H-2), 5.92 (1H, d, J = 2.4 Hz, H-6), 5.96 (1H, d, J = 2.4 Hz, H-8), 6.46 (2H, d, J = 8.4 Hz, H-3′, H-5′), 7.03 (1H, t, J = 8.4 Hz, H-4′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 70.4 (C-3),

76.9 (C-2), 95.9 (C-8), 96.8 (C-6), 101.7 (C-10), 108.3 (C-3′, C-5′), 110.7 (C-1′), 131.2 (C-4′), 158.9 (C-2′, C-6′), 165.2 (C-9), 165.3 (C-5), 167.6 (C-7), 199.8 (C-4). HR-EI-MS: *m*/*z* 304.0582 $[M]^+$ (Calcd 304.0583 for $C_{15}H_{12}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (20:80 v/v) Retention time (2*R*,3*R*)-isomer: 9.80 min, (2*S*,3*S*)-isomer: 10.18 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +102.2° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −0.50 (298.6), +1.69 (327.4) (*c* = 6.58 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −97.3° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +0.45 (297.8), −1.63 (324.2) (*c* = 6.58 × 10^{-5}).

2.10.6. 2′,3,3′,5,7-Pentahydroxy flavanonol (5e)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.86 (1H, d, *J* = 11.4 Hz, H-3), 5.61 (1H, d, *J* = 11.4 Hz, H-2), 5.96 (1H, d, *J* = 1.8 Hz, H-6), 6.00 (1H, d, *J* = 1.8 Hz, H-8), 6.78 (1H, dd, *J* = 7.8, 7.8 Hz, H-5′), 6.89 (1H, dd, *J* = 7.8, 1.8 Hz, H-4′), 7.03 (1H, dd, *J* = 7.8, 1.8 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 72.5 (C-3), 79.2 (C-2), 96.1 (C-6), 97.1 (C-8), 101.7 (C-10), 116.2 (C-4′), 120.2 (C-6′), 120.4 (C-5′), 124.6 (C-1′), 145.2 (C-2′), 146.0 (C-3′), 164.5 (C-5), 165.2 (C-9), 167.9 (C-7), 198.3 (C-4). HR-EI-MS: *m*/*z* 304.0583 $[M]^+$ (Calcd 304.0583 for $C_{15}H_{12}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (40:60 v/v) Retention time (2*R*,3*R*)-isomer: 5.83 min, (2*S*,3*S*)-isomer: 5.03 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +71.5° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −3.12 (283.0), +1.48 (325.0) (*c* = 6.58 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −64.6° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +2.88 (284.5), −1.44 (324.2) (*c* = 6.58 × 10^{-5}).

2.10.7. 2′,3,4′,5,7-Pentahydroxy flavanonol (5f)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.87 (1H, d, *J* = 11.4 Hz, H-3), 5.49 (1H, d, *J* = 11.4 Hz, H-2), 5.93 (1H, d, *J* = 1.8 Hz, H-6), 5.98 (1H, d, *J* = 1.8 Hz, H-8), 6.43 (1H, dd, *J* = 8.4, 3.0 Hz, H-5′), 6.47 (1H, d, *J* = 3.0 Hz, H-3′), 7.32 (1H, dd, *J* = 8.4, 3.0 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 72.2 (C-3), 79.3 (C-2), 96.0 (C-6), 97.0 (C-8), 101.7 (C-10), 103.7 (C-3′), 107.9 (C-5′), 115.5 (C-1′), 130.8 (C-6′), 158.1 (C-2′), 159.9 (C-4′), 164.7 (C-5), 165.1 (C-9), 167.8 (C-7), 198.8 (C-4). HR-EI-MS: *m*/*z* 304.0584 $[M]^+$ (Calcd 304.0583 for $C_{15}H_{12}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (25:75 v/v) Retention time (2*R*,3*R*)-isomer: 9.03 min, (2*S*,3*S*)-isomer: 7.25 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +84.3° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −4.05 (292.2), +1.70 (328.3) (*c* = 6.58 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −80.4° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +4.11 (293.2), −1.63 (326.7) (*c* = 6.58 × 10^{-5}).

2.10.8. 2′,3,5,5′,7-Pentahydroxy flavanonol (5g)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.79 (1H, d, *J* = 11.4 Hz, H-3), 5.57 (1H, d, *J* = 11.4 Hz, H-2), 5.96 (1H, d, *J* = 1.8 Hz, H-6), 5.99 (1H, d, *J* = 1.8 Hz, H-8), 6.72 (1H, dd, *J* = 8.4, 3.0 Hz, H-4′), 6.79 (1H, d, *J* = 8.4 Hz, H-3′), 6.99 (1H, d, *J* = 3.0 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 72.6 (C-3), 79.1 (C-2), 96.1 (C-6), 97.1 (C-8), 101.6 (C-10), 115.6 (C-6′), 117.4 (C-4′), 117.7 (C-3′), 124.9 (C-1′), 149.5 (C-2′), 151.4 (C-5′), 164.4 (C-5), 165.2 (C-9), 167.9 (C-7), 198.1 (C-4). HR-EI-MS: *m*/*z* 304.0584 $[M]^+$ (Calcd 304.0583 for $C_{15}H_{12}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (30:70 v/v) Retention time (2*R*,3*R*)-isomer: 7.37 min, (2*S*,3*S*)-isomer: 5.62 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +48.2° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −3.88 (287.3), +1.8 (317.2) (*c* = 6.58 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −45.6° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +3.99 (287.3), −1.87 (318.2) (*c* = 6.58 × 10^{-5}).

2.10.9. 3,3′,4′,5,7-Pentahydroxy flavanonol (5h)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.61 (1H, d, *J* = 11.4 Hz, H-3), 5.02 (1H, d, *J* = 11.4 Hz, H-2), 5.95 (1H, d, *J* = 1.8 Hz, H-6), 5.99 (1H, d, *J* = 1.8 Hz, H-8), 6.86 (1H, d, *J* = 8.4 Hz, H-5′), 6.92 (1H, dd, *J* = 8.4, 1.8 Hz, H-6′), 7.07 (1H, d, *J* = 1.8 Hz, H-2′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.2 (C-3), 84.6 (C-2), 96.1 (C-6), 97.1 (C-8), 101.6 (C-10), 115.8 (C-5′), 116.0 (C-2′), 120.9 (C-6′), 129.9 (C-1′), 145.8 (C-3′), 146.7 (C-4′), 164.2 (C-5), 165.1 (C-9), 167.9 (C-7), 198.3 (C-4). HR-EI-MS: *m*/*z* 304.0584 $[M]^+$ (Calcd 304.0583 for $C_{15}H_{12}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (50:50 v/v) Retention time (2*R*,3*R*)-isomer: 5.1 min, (2*S*,3*S*)-isomer: 4.25 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +17.8° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −6.95 (294.2), +1.93 (327.2) (*c* = 6.58 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −15.7° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +7.15 (294.1), −1.95 (329.9) (*c* = 6.58 × 10^{-5}).

2.10.10. 3,3′,5,5′,7-Pentahydroxy flavanonol (5i)

^{1}H NMR (600 MHz, acetone-d_6) δ: 4.57 (1H, d, *J* = 11.4 Hz, H-3), 5.02 (1H, d, *J* = 11.4 Hz, H-2), 5.97 (1H, d, *J* = 1.8 Hz, H-6), 5.99 (1H, d, *J* = 1.8 Hz, H-8), 6.38 (1H, t, *J* = 1.8 Hz, H-4′), 6.56 (2H, d, *J* = 1.8 Hz, H-2′, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.3 (C-3), 84.6 (C-2), 96.1 (C-6), 97.2 (C-8), 101.6 (C-10), 103.8 (C-4′), 107.4 (C-2′, C-6′), 140.4 (C-1′), 159.4 (C-3′, C-5′), 164.1 (C-5), 165.1 (C-9), 168.0 (C-7), 198.0 (C-4). HR-EI-MS: *m*/*z* 304.0580 $[M]^+$ (Calcd 304.0583 for $C_{15}H_{12}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (40:60 v/v) Retention time (2*R*,3*R*)-isomer: 6.50 min, (2*S*,3*S*)-isomer: 4.50 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +10.9° (*c* = 0.5, MeOH), CD (MeOH): Δε (nm) −4.32 (289.0), +1.68 (327.8) (*c* = 6.58 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −9.0° (*c* = 0.5, MeOH), CD (MeOH): Δε (nm) +4.53 (289.8), −1.86 (326.9) (*c* = 6.58 × 10^{-5}).

2.10.11. 3,5,7-Trihydroxy-2′-methoxy flavanonol (5j)

Colorless powder, mp 218–220 °C; IR (KBr) ν_{max}: 3444, 1643 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 290 (4.16), 320 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.86 (3H, s, OCH_3-2′), 4.86 (1H, d, *J* = 10.8 Hz, H-3), 5.62 (1H, d, *J* = 10.8 Hz, H-2), 5.94 (1H, d, *J* = 1.8 Hz, H-6), 6.00 (1H, d, *J* = 1.8 Hz, H-8), 7.04 (1H, ddd, *J* = 8.4, 8.4, 1.2 Hz, H-5′), 7.08 (1H, d, *J* = 8.4 Hz, H-3′), 7.39 (1H, ddd, *J* = 8.4, 8.4, 1.2 Hz, H-4′), 7.58 (1H, dd, *J* = 8.4, 1.2 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.2 (OCH_3-2′), 72.3 (C-3), 78.7 (C-2), 96.0 (C-6), 97.2 (C-8), 101.8 (C-10), 112.3 (C-3′), 121.4 (C-5′), 125.9 (C-1′), 129.7 (C-6′), 131.1 (C-4′), 159.2 (C-2′), 164.5 (C-5), 165.2 (C-9), 167.9 (C-7), 198.5 (C-4). HR-EI-MS: *m*/*z* 302.0789 $[M]^+$ (Calcd 302.0790 for $C_{16}H_{14}O_6$). The enantiomers of **5j** could not be separated using chiral chromatography.

2.10.12. 3,5,7-Trihydroxy-3′-methoxy flavanonol (5k)

Colorless powder, mp 203–205 °C; IR (KBr) ν_{max}: 3435, 1637 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 291 (4.15), 318 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.83 (3H, s, OCH_3-3′), 4.68 (1H, d, *J* = 12.0 Hz, H-3), 5.16 (1H, d, *J* = 12.0 Hz, H-2), 5.98 (1H, d, *J* = 1.8 Hz, H-6), 6.01 (1H, d, *J* = 1.8 Hz, H-8), 6.97 (1H, dd, *J* = 7.8, 1.2 Hz, H-4′), 7.16 (1H, d, *J* = 7.8 Hz, H-6′), 7.18 (1H, d, *J* = 1.8 Hz, H-2′), 7.35 (1H, dd, *J* = 7.8, 7.8 Hz, H-5′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 55.7 (OCH_3-3′), 73.2 (C-6), 84.4 (C-2), 96.2 (C-6), 97.3 (C-8), 101.6 (C-10), 114.6 (C-2′), 115.1 (C-4′), 121.1 (C-6′), 130.3 (C-5′), 139.9 (C-1′), 160.7 (C-3′), 164.1 (C-5), 165.1 (C-9), 168.0 (C-7), 198.0 (C-4). HR-EI-MS: *m*/*z* 302.0790 $[M]^+$ (Calcd 302.0790 for $C_{16}H_{14}O_6$).

Separation by chiral chromatography: EtOH–*n*-hexane (70:30 v/v) Retention time (2*R*,3*R*)-isomer: 5.17 min, (2*S*,3*S*)-isomer: 3.95 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +4.7° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −6.52 (288.7), +2.08 (327.1) (*c* = 6.62 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −5.5° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +6.54 (289.6), −2.01 (324.2) (*c* = 6.62 × 10^{-5}).

2.10.13. 3,5,7-Trihydroxy-4′-methoxy flavanonol (5l)

^{1}H NMR (600 MHz, acetone-d_6) δ: 3.83 (3H, s, OCH_3-4′), δ 4.67 (1H, d, *J* = 11.2 Hz, H-3), 5.13 (1H, d, *J* = 11.2 Hz, H-2), 5.96 (1H, d, *J* = 1.8 Hz, H-6), 6.00 (1H, d, *J* = 1.8 Hz, H-8), 7.00 (2H, d, *J* = 9.0 Hz, H-3′, H-5′), 7.52 (2H, d, *J* = 9.0 Hz, H-2′, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 54.8 (OCH_3-4′), 72.3 (C-3), 83.3 (C-2), 95.2 (C-6), 96.3 (C-8), 100.7 (C-10), 113.7 (C-3′, C-5′), 129.4 (C-2′, C-6′), 129.5 (C-1′), 160.3 (C-4′), 163.3 (C-5), 164.2 (C-9), 167.0 (C-7), 197.4 (C-4). HR-EI-MS: *m*/*z* 302.0788 $[M]^+$ (Calcd 302.0790 for $C_{16}H_{14}O_6$).

Separation by chiral chromatography: EtOH–*n*-hexane (25:75 v/v) Retention time (2*R*,3*R*)-isomer: 10.22 min, (2*S*,3*S*)-isomer: 8.58 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +15.6° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −7.68 (288.4), +2.26 (327.4) (*c* = 6.62 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −21.0° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +8.02 (288.8), −2.23 (326.7) (*c* = 6.62 × 10^{-5}).

2.10.14. 3,5,7-Trihydroxy-2′,6′-dimethoxy flavanonol (5m)

Colorless powder, mp 268–270 °C; IR (KBr) ν_{max}: 3478, 1637 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 288 (4.22), 320 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.83 (6H, s, OCH_3-2′, OCH_3-6′), 5.40 (1H, d, *J* = 12.6 Hz, H-3), 5.81 (1H, d, *J* = 12.6 Hz, H-2), 5.90 (1H, d, *J* = 2.4 Hz, H-6), 5.97 (1H, d, *J* = 2.4 Hz, H-8), 6.72 (1H, d, *J* = 8.4 Hz, H-3′, H-5′), 7.36 (1H, t, *J* = 8.4 Hz, H-4′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.4 (OCH_3-2′, OCH_3-6′), 70.4 (C-3), 76.3 (C-2), 95.9 (C-6), 96.8 (C-8), 101.6 (C-10), 105.5 (C-3′, C-5′), 113.2 (C-1′), 131.9 (C-4′), 161.0 (C-2′, C-6′), 165.2 (C-5), 165.3 (C-9), 167.5 (C-7), 199.6 (C-4). HR-EI-MS: *m*/*z* 332.0895 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$). The Enantiomers of **5m** could not be separated using chiral chromatography.

2.10.15. 3,5,7-Trihydroxy-2′,3′-dimethoxy flavanonol (5n)

Colorless powder, mp 233–235 °C; IR (KBr) ν_{max}: 3432, 1632 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 290 (4.33), 320 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.83 (3H, s, OCH_3-3′), 3.89 (3H, s, OCH_3-2′), 4.81 (1H, d, *J* = 12.0 Hz, H-3), 5.58 (1H, d, *J* = 12.0 Hz, H-2), 5.95 (1H, d, *J* = 1.8 Hz, H-6), 6.01 (1H, d, *J* = 1.8 Hz, H-8), 7.09 (1H, dd, *J* = 7.8, 1.8 Hz, H-6′), 7.16 (1H, dd, *J* = 7.8, 7.8 Hz, H-5′), 7.22 (1H, dd, *J* = 7.8, 1.8 Hz, H-4′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.3 (OCH_3-2′), 61.4 (OCH_3-3′), 72.4 (C-3), 78.9 (C-2), 96.1 (C-6), 97.2 (C-8), 101.6 (C-10), 114.1 (C-6′), 120.9 (C-5′), 124.9 (C-4′), 131.6 (C-1′), 149.3 (C-2′), 154.0 (C-3′), 164.3 (C-5), 165.2 (C-9), 167.9 (C-7), 198.4 (C-4). HR-EI-MS: *m*/*z* 332.0897 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (25:75 v/v) Retention time (2*R*,3*R*)-isomer: 7.13 min, (2*S*,3*S*)-isomer: 6.05 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +61.6° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −2.76 (290.7), +1.35 (323.2) (*c* = 6.06 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −73° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +2.75 (290.9), −1.41 (324.6) (*c* = 6.06 × 10^{-5}).

2.10.16. 3,5,7-Trihydroxy-2′,4′-dimethoxy flavanonol (5o)

Colorless powder, mp 203–205 °C; IR (KBr) ν_{max}: 3478, 1639 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 289 (4.19), 318 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.83 (3H, s, OCH_3-2′), 3.83 (3H, s, OCH_3-4′), 4.85 (1H, d, *J* = 12.0 Hz, H-3), 5.51 (1H, d, *J* = 12.0 Hz, H-2), 5.91 (1H, d, *J* = 2.4 Hz, H-6), 5.98 (1H, d, *J* = 2.4 Hz, H-8), 6.59 (1H, d, *J* = 2.4 Hz, H-3′), 6.61 (1H, dd, *J* = 8.4, 2.4 Hz, H-5′), 7.47 (1H, d, *J* = 8.4 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 55.8 (OCH_3-4′), 56.2 (OCH_3-2′), 72.1 (C-3), 78.8 (C-2), 96.0 (C-6), 97.0 (C-8), 99.3 (C-5′), 101.6 (C-10), 105.9 (C-3′), 118.1 (C-1′), 130.7 (C-6′), 160.4 (C-4′), 162.6 (C-2′), 164.6 (C-5), 165.1 (C-9), 167.9 (C-7), 198.7 (C-4). HR-EI-MS: *m*/*z* 332.0897 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (20:80 v/v) Retention time (2*R*,3*R*)-isomer: 11.50 min, (2*S*,3*S*)-isomer: 10.50 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +77.7° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −4.84 (292.3), +1.70 (326.7) (*c* = 6.06 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −75.0° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +4.28 (291.5), −1.70 (324.7) (*c* = 6.06 × 10^{-5}).

2.10.17. 3,5,7-Trihydroxy-2′,5′-dimethoxy flavanonol (5p)

Colorless powder, mp 223–225 °C; IR (KBr) ν_{max}: 3428, 1638 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 293 (4.13), 320 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.78 (3H, s, OCH_3-2′), 3.80 (3H, s, OCH_3-5′), 4.86 (1H, d, *J* = 12.0 Hz, H-3), 5.60 (1H, d, *J* = 12.0 Hz, H-2), 5.95 (1H, d, *J* = 1.8 Hz, H-6), 6.00 (1H, d, *J* = 1.8 Hz, H-8), 6.95 (1H, dd, *J* = 9.0, 2.4 Hz, H-4′), 7.02 (1H, d, *J* = 9.0 Hz, H-3′), 7.19 (1H, d, *J* = 2.4 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.0 (OCH_3-2′), 57.0 (OCH_3-5′), 72.4 (C-3), 78.6 (C-2), 96.1 (C-6), 97.2 (C-8), 101.7 (C-10), 113.8 (C-3′), 115.5 (C-6′), 115.6 (C-4′), 127.0 (C-1′), 153.3 (C-2′), 154.8 (C-5′), 164.4 (C-5), 165.2 (C-9), 167.9 (C-7), 198.4 (C-4). HR-EI-MS: *m*/*z* 332.0901 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (50:50 v/v) Retention time (2*R*,3*R*)-isomer: 5.00 min, (2*S*,3*S*)-isomer: 4.30 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +46.7° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −4.64 (286.6), +1.86 (325.2) (*c* = 6.06 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −47.4° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +4.43 (288.8), −1.84 (325.9) (*c* = 6.06 × 10^{-5}).

2.10.18. 3,5,7-Trihydroxy-3′,4′-dimethoxy flavanonol (5q)

^{1}H NMR (600 MHz, acetone-d_6) δ: 3.84 (3H, s, OCH_3-4′), 3.85 (3H, s, OCH_3-3′), 4.71 (1H, d, *J* = 12.0 Hz, H-3), 5.11 (1H, d, *J* = 12.0 Hz, H-2), 5.96 (1H, d, *J* = 1.8 Hz, H-6), 6.00 (1H, d, *J* = 1.8 Hz, H-8), 6.99 (1H, d, *J* = 1.8 Hz, H-5′), 7.11 (1H, dd, *J* = 8.4, 1.8 Hz, H-6′), 7.22 (1H, d, *J* = 1.8 Hz, H-2′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.2 (OCH_3-4′), 56.3 (OCH_3-3′), 73.2 (C-3), 84.6 (C-2), 96.1 (C-6), 97.2 (C-8), 101.6 (C-10), 112.4 (C-5′), 112.6 (C-2′), 121.8 (C-6′), 130.7 (C-1′), 150.3 (C-3′), 151.0 (C-4′), 164.2 (C-5), 165.1 (C-9), 167.9 (C-7), 198.3 (C-4). HR-EI-MS: *m*/*z* 332.0896 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$). The enantiomers of **5q** could not be separated using chiral chromatography.

2.10.19. 3,5,7-Trihydroxy 3′,5′-dimethoxy flavanonol (5r)

Colorless powder, mp 191–193 °C; IR (KBr) ν_{max}: 3474, 1644 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 289 (4.14), 318 (sh); ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.81 (6H, s, OCH_3-3′, OCH_3-5′), 4.67 (1H, d, *J* = 12.0 Hz, H-3), 5.11 (1H, d, *J* = 12.0 Hz, H-2), 5.98 (1H, d, *J* = 2.4 Hz, H-6), 6.01 (1H, d, *J* = 2.4 Hz, H-8), 6.52 (1H, dd, *J* = 2.4, 2.4 Hz, H-4′), 6.77 (2H, d, *J* = 2.4 Hz, H-2′, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 55.8 (OCH_3-3′, OCH_3-5′), 73.2 (C-3), 84.5 (C-2), 96.2 (C-6), 97.3 (C-8), 101.2 (C-4′), 101.6 (C-10), 107.0 (C-2′, C-6′), 140.5 (C-1′), 161.8 (C-3′, C-5′), 164.0 (C-5), 165.1 (C-9), 167.9 (C-7), 198.0 (C-4). HR-EI-MS: *m*/*z* 332.0901 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$).

Separation by chiral chromatography: EtOH–*n*-hexane (80:20 v/v) Retention time (2*R*,3*R*)-isomer: 6.83 min, (2*S*,3*S*)-isomer: 4.17 min.

(2*R*,3*R*)-isomer: $[\alpha]_D^{12}$ +0.8° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) −5.84 (292.0), +1.98 (328.3) (*c* = 6.06 × 10^{-5}). (2*S*,3*S*)-isomer: $[\alpha]_D^{12}$ −0.8° (*c* = 1.0, MeOH), CD (MeOH): Δε (nm) +5.46 (290.1), −1.84 (324.7) (*c* = 6.06 × 10^{-5}).

3. Results

3.1. The synthesis of flavanonols

The preparation of flavanonols was carried out as illustrated in Scheme 1. 2,4,6-Trihydroxyacetophenone was protected with an excessive amount of chloromethyl methyl ether (MOMCl) in the presence of an excessive amount of NaH suspended in DMF, while cooling in an ice-water bath, to give compound **1**. The hydroxylbenzaldehydes were protected with an excessive amount of MOMCl in the presence of an excessive amount of K_2CO_3 in acetone, while cooling in an ice-water bath, to give compound **2**. Compounds **1** and **2** were treated with KOH in ethanol at room temperature for 3 h to give compound **3**. Compound **3** was epoxidized with H_2O_2 under alkaline conditions (NaOH aqueous) in ethanol to give compound **4**. Finally compound **5** was obtained by treating compound **4** with HCl in methanol at 55 °C. All of the compounds were characterized by ^{1}H NMR, ^{13}C NMR, and mass spectroscopy.

We prepared 19 flavanonols with a common 3,5,7-trihydroxychroman scaffold. The compounds differed in the substitution of the B ring. Stereoisomers were separated from 16 of the compounds. Thus 32 stereochemically pure compounds and 3 racemic mixtures were tested by examining their inhibitory potential against NO production in RAW 264.7 cells, and evaluating their DPPH radical scavenging potential.

3.2. The separation of enantiomers using a chiral column and determination of absolute configuration of flavanonols

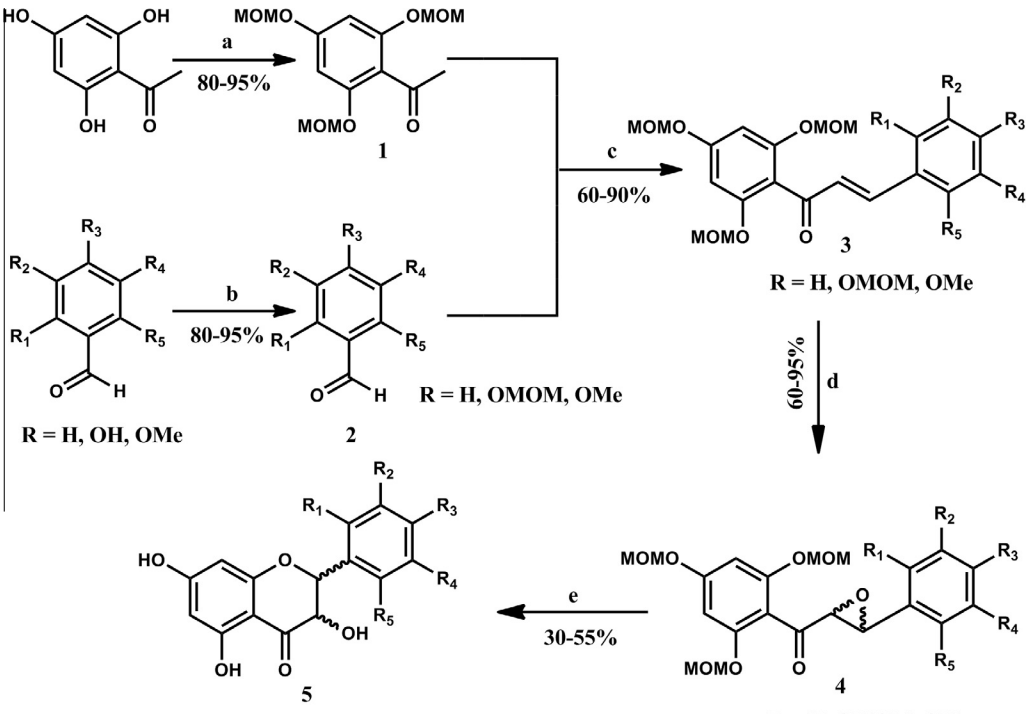
We separated 32 enantiomers (Table 1) from the racemic mixtures using chiral column chromatography. The separation of compounds **5j**, **5m** and **5q** was not satisfactory. Thus **5j**, **5m** and **5q** were used as racemic mixtures.

The absolute configurations of the stereoisomers were determined by circular dichroism (CD) and the coupling constant (*J*) between H-2 and H-3 measured by ^{1}H NMR. Positive Cotton effect around 320 nm and negative Cotton effect around 290 nm indicates that the C-2 is in *R* configuration[28,29]. The coupling constant of 10–12 Hz indicates that H-2 and H-3 are in *trans* configuration.

As far as the flavanonols that were synthesized in this study are concerned, the optical rotation ($[\alpha]_D$) of (2*R*,3*R*)-isomer was positive.

3.3. The inhibitory potential against NO production in RAW 264.7 cells

The (2*R*,3*R*)-isomer of **5e**, with a 2′,3′-dihydroxyl substitution in the B ring, indicated the highest inhibitory potential of the 35 specimens (70% inhibition at 100 μM). Its inhibitory potential was comparable with the positive control, aminoguanidine HCl (AG). The (2*R*,3*R*)-isomers of **5a** and **5b**, with 2′- and 3′-hydroxyl substitutions in the B ring, respectively, showed weaker but significant



5) $R_1 = R_2 = R_3 = R_4 = R_5 = H$

5a) $R_1 = OH, R_2 = R_3 = R_4 = R_5 = H$ 5j) $R_1 = OMe, R_2 = R_3 = R_4 = R_5 = H$

5b) $R_2 = OH, R_1 = R_3 = R_4 = R_5 = H$ 5k) $R_2 = OMe, R_1 = R_3 = R_4 = R_5 = H$

5c) $R_3 = OH, R_1 = R_2 = R_4 = R_5 = H$ 5l) $R_3 = OMe, R_1 = R_2 = R_4 = R_5 = H$

5d) $R_1 = R_5 = OH, R_2 = R_3 = R_4 = H$ 5m) $R_1 = R_5 = OMe, R_2 = R_3 = R_4 = H$

5e) $R_1 = R_2 = OH, R_3 = R_4 = R_5 = H$ 5n) $R_1 = R_2 = OMe, R_3 = R_4 = R_5 = H$

5f) $R_1 = R_3 = OH, R_2 = R_4 = R_5 = H$ 5o) $R_1 = R_3 = OMe, R_2 = R_4 = R_5 = H$

5g) $R_1 = R_4 = OH, R_2 = R_3 = R_5 = H$ 5p) $R_1 = R_4 = OMe, R_2 = R_3 = R_5 = H$

5h) $R_2 = R_3 = OH, R_1 = R_4 = R_5 = H$ 5q) $R_2 = R_3 = OMe, R_1 = R_4 = R_5 = H$

5i) $R_2 = R_4 = OH, R_1 = R_3 = R_5 = H$ 5r) $R_2 = R_4 = OMe, R_1 = R_3 = R_5 = H$

Scheme 1. Synthesis of flavanonols. Reagents and conditions: (a) NaH, MOMCl, DMF, 0 °C; (b) K_2CO_3, MOMCl, Me_2CO, rt; (c) KOH, EtOH, rt; (d) 30% H_2O_2, NaOH aq, MeOH, rt; (e) MeOH-HCl, 55 °C.

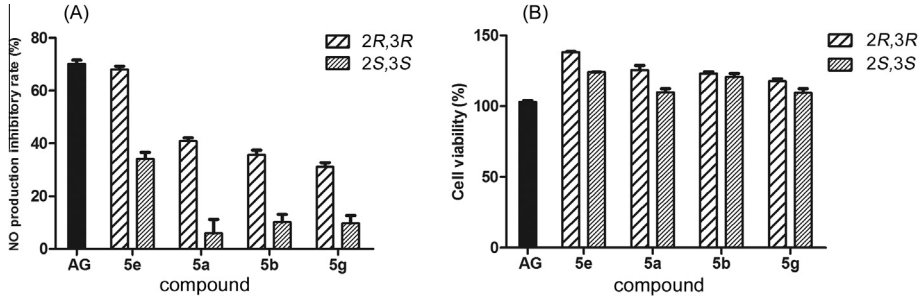


Figure 1. The effects of the flavanonols on the NO production (A) and the cell viability of RAW 264.7 cells (B). **5e**: 2′,3,3′,5,7-Pentahydroxy flavanonol, **5a**: 2′,3,5,7-tetrahydroxy flavanonol, **5b**: 3,3′,5,7-tetrahydroxy flavanonol, **5g**: 2′,3,5,5′,7-pentahydroxy flavanonol, AG: aminoguanidine hydrochloride. The final concentration of the tested specimen including AG was 100 μM. All data are expressed as the mean ± S.D (n = 3).

inhibitory activity (40% and 35% inhibition at 100 μM). The (2*R*,3*R*)-isomer of **5g**, with 2′,5′-dihydroxyl substitution, also showed weaker inhibitory potential (30% inhibition at 100 μM). Their enantiomers (i.e., the (2*S*,3*S*)-isomers) indicated lower or very weak potential (Fig. 1A and Table 1). The inhibitory activity of the other compounds was not detectable at 100 μM. The presence of the hydroxyl group at the 2′- and 3′-positions of the B ring is important for the inhibition of NO production. The methylation of the 2′-hydroxyl group (**5j**), 3′-hydroxyl group (**5k**) and both groups together (**5n**) resulted in a loss of activity, suggesting the importance of the 2′- and 3′- hydroxyl groups for the inhibitory activity. The substitution at the 4′-position lowered the inhibitory potential (compounds **5c**, **5f** and **5h**). The potent flavanonols were not found to be toxic to RAW 264.7 cells at 100 μM. No significant difference was found in the toxicity of the stereoisomers (Fig. 1B).

The configuration of all of the potent compounds was (2*R*,3*R*). The inhibition of NO production by the flavanonols was stereospecific. It should be noted that most flavanonols from natural sources have the (2*R*,3*R*) configuration[30].

3.4. Radical scavenging potential

Table 1 shows the results of a radical scavenging potential (EC_{50}) assay of the flavanonols. The most potent compounds were the (2*R*,3*R*)- and (2*S*,3*S*)-isomers of 2′,3′-dihydroxyl analogue **5e**, and 3′,4′-dihydroxyl analogue **5h**. The EC_{50} value of the (2*R*,3*R*)-isomer of 2′,3′-dihydroxy flavanonol **5e** was found to be 12.5 μM, while that of the (2*S*,3*S*)-isomer of **5e** was 13.2 μM. The (2*R*,3*R*)-isomer of **5h**, called dihydroquercetin, is often used as a standard specimen for DPPH assays. Dihydroquercetin and its enantiomer (2*S*,3*S*)-**5h** had EC_{50} values of 13.9 and 12.6 μM, respectively.

The activity of 2′,5′-dihydroxy analogue **5g** (25 μM) was half that of **5e** and **5h**. Again, both the (2*R*,3*R*)- and (2*S*,3*S*)-isomers of **5g** were found to have equivalent radical scavenging potential (EC_{50}: 24.5 μM and 25.4 μM, respectively).

Radical scavenging activity was not detected in any compounds other than **5e**, **5g** and **5h**. In short, the flavanonols with *ortho*- or *para*-hydroquinone moiety in the B ring showed radical scavenging activity. The stereochemistry was irrelevant to the radical scavenging potential.

4. Discussion

Many studies have been carried out about the anti-inflammatory activity of flavonoids using RAW 264.7 cells [e.g., 22–25]. Analyses of the complied results suggest that, in general, flavonoids having C2-C3 double bond show stronger NO production inhibitory activity as well as DPPH radical scavenging activity than C2-C3 is single bond. In our knowledge, no flavanonol has been recognized as a strong inhibitor for NO-production in RAW 264.7 cells. This may be due to the fact that number of flavanonols isolated from nature is significantly smaller than other flavonoids. Moreover there was no report that stereoisomers of flavonoids have different effects on NO production inhibitory activity. Therefore in order to explore wider chemical space to look for bioactive flavonoids, we synthesized flavonoids which structures are unnatural, rare and new.

The (2*R*,3*R*)-isomers of **5e**, **5a**, **5b** and **5g** were found to be potent flavanonols for the inhibition of NO production (Fig. 1A). Flavanonols (2*R*,3*R*)-**5e** and (2*R*,3*R*)-**5g** were also found to have radical scavenging potential (Table 1). However, (2*R*,3*R*)-**5a** and (2*R*,3*R*)-**5b** which exhibited inhibitory activity on NO production did not exhibit radical scavenging potential. On the other hand, (2*R*,3*R*)- and (2*S*,3*S*)-isomers of **5h** both of which demonstrated strong radical scavenging potential, did not inhibit the NO production.

As expected the radical scavenging potential was not stereospecific. Our results support the long-speculated hypothesis that the radical scavenging potential of flavanonols might simply be explained by the oxidation–reduction potential of the B ring moiety. Our study supports this hypothesis and confirms that it is also valid for unnatural flavonoids.

In contrast, the inhibitory potential of NO production in RAW 264.7 cells was stereospecific and irrelevant to the radical scavenging potential. This suggests that flavanonols are stereospecifically recognized by RAW 264.7 cells and that active flavanonols would bind to a biological target that plays a role in the production and/or regulation of NO.

5. Conclusion

We prepared 35 flavanonols with an identical chroman scaffold in the A and C rings but with differences in their B ring structure and stereochemistry. Nineteen of the synthesized flavanonols were new compounds that have not been isolated from nature. We carried out an NO production inhibitory assay and a DPPH radical scavenging assay. The NO production inhibitory activity was stereospecific and the (2*R*,3*R*)-configuration was required for this activity to occur. Existence of 2′- and 3′-hydroxy groups in the B ring was important for the inhibition of NO production. The flavanonols with *ortho*- or *para*-hydroquinone moiety in their B ring exhibited strong radical scavenging potential. No correlation was found between the inhibition of NO production and the radical scavenging activity. Taken together the inhibitory effect of flavanonols against NO production cannot solely be explained by anti-oxidative potential. An existence of a biological target could be proposed to explain the inhibitory effect.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.09.042. These data include MOL files and InChiKeys of the most important compounds described in this article.

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30. Structural search by SciFinder (American Chemical Society; https://scifinder.cas.org) indicated that 994 flavanonols having (2*R*,3*R*) configuration were known, while number of the flavanonols having (2*S*,3*S*) configuration was 67. In the reviews by Kazi, A. et al. (*World Journal of Pharmaceutical research* **2015**, *4*, 560) and by Sareedenchai, V. et al. (*Biochem. Syst. Ecol.* **2010**, *38*, 93), all flavanonols listed have (2*R*,3*R*) configuration. Takahashi, H. et al. (*Chem. Pharm. Bull.* **1984**, *32*, 4852) also stated the dominancy of (2*R*,3*R*) configuration among naturally occurring flavanonol.