Bioorganic & Medicinal Chemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Stereospecific inhibition of nitric oxide production in macrophage cells by flavanonols: Synthesis and the structure-activity relationship. Part 2

Wen-Jun Jiang [a], Susumu Kitanaka, Tomoko Takamiya, Hiroshi Iijima *

School of Pharmacy, Nihon University, 7-7-1 Narashino-dai, Funabashi-shi, Chiba 274-8555, Japan

ARTICLE INFO

Article history:
Received 6 May 2017
Revised 25 May 2017
Accepted 30 May 2017
Available online xxxx

Keywords:
Flavonoid
Nitric oxide
Macrophage
Structure-activity relationship
Stereoisomer

ABSTRACT

To explore the structure-activity relationships of flavanonols on the inhibition of nitric oxide (NO) production in RAW 264.7 cells, we have prepared a series of synthetic flavanonols. In our previous study, the 2′,3′-dihydroxyphenyl substructure was found to be the most potent B ring substructure among the flavanonols having 3,5,7-trihydroxychroman-4-one as the A/C ring. In this study, we examined the effect of diverse substitutions on the A ring of the 2-(2,3-dihydroxyphenyl)-3-hydroxychroman-4-one scaffold, *i.e.*, by fixing the B ring to the 2′,3′-dihydroxyphenyl substructure. Eighteen stereoisomers and 4 racemic mixtures were prepared, and their inhibitory potency on NO production in RAW 264.7 cells was tested. We observed higher inhibitory activity in the (2*R*,3*R*) stereoisomers than in the (2*S*,3*S*) stereoisomers. The presence of a hydroxy or a methoxy group at the 7-postiion enhanced the inhibitory potency, and the additional substitutions at the 6- or 8-position in the A ring increased potency and stereospecificity. A representative compound, (2*R*,3*R*)-2′,3′,7,8-tetrahydroxyflavanonol **5e**, had an IC_{50} value of 17 μM, whereas its (2*S*,3*S*) stereoisomer did not inhibit NO production at all at a concentration of 100 μM. In this study, it was necessary to determine the absolute configuration of the stereoisomers of the synthesized flavanonols that carry methoxy substitutions in the A ring. The procedure to determine their absolute configuration by the CD excitation chirality method is also discussed.

1. Introduction

Flavonoids are well known to have many biological activities, including anti-inflammatory,[1–4] anti-oxidative,[5–10] anti-allergic,[1,11] anti-microbial,[12–14] anti-viral,[15–17] anti-diarrheal,[5] anti-cancer,[2,18–21] and anti-diabetic[22] effects. We have been interested in the anti-inflammatory effects of flavonoids, especially in their effects on chronic inflammation leading to metabolic disease. Flavonoids, widely distributed in plants such as vegetables, fruits, teas, and some herbs, are considered an important dietary material that contributes to the control of chronic inflammation. The production of nitric oxide (NO) is a primary indicator of macrophage activation. NO is produced once the NF-κB complex has been activated by extracellular stimuli. Thus, chemicals that suppress NO production in macrophage cells can be expected to be useful suppressors of inflammation. We have examined the inhibitory effect of herbal materials from many sources on NO production in RAW 264.7 cells, which are stimulated by lipopolysaccharide (LPS).[23]

Indeed, many flavonoids showed inhibitory potency on NO production in RAW 264.7 cells. However, the structure-activity relationship of flavonoids on inhibitory potency against NO production remained a qualitative one. In 2011, Daikonya et al. isolated flavonoids that carry symmetrically substituted B rings from Tibetans herbal plants and determined their IC_{50} values for suppression of NO production.[24,25] These structure-activity data successfully led us to establish a quantitative structure-activity relationship (QSAR) model by comparative molecular field analysis (CoMFA).[26] This CoMFA model suggested the importance of the electrostatic feature of the B ring and prompted us to synthesize a series of 5,7-dihydroxyflavavonol derivatives that diversify in their B ring substitutions.[27] In the QSAR study, we found that (i) flavanonols that carry a 2′,3′-dihydroxyphenyl ring as the B ring show strong activity, (ii) the inhibition is stereospecific, *i.e.*, the (2*R*,3*R*) stereochemistry is relevant for the activity in macrophage RAW 264.7 cells, and (iii) the presence of a 4′-hydroxy group on the B ring of flavanonols is unfavorable for such activity. This disadvantage of the presence of the 4′-hydroxy group on the B ring is rather unexpected because in natural flavonoids, the 4′-position is

* Corresponding author.
E-mail address: iijima.hiroshi@nihon-u.ac.jp (H. Iijima).
[a] Present address: Jiangsu Yew Pharmaceutical Co., Ltd, QinXin Village, City WuXi, Province JiangSu 214199, China.

http://dx.doi.org/10.1016/j.bmc.2017.05.060
0968-0896/

often substituted by a hydroxy group due to biosynthetic destiny, and this might be one reason why it is rare to encounter a flavanonol that strongly inhibits NO production in macrophage cells.

In this study, to expand the structure-activity relationship of flavanonols on the inhibition of NO production, we synthesized flavanonols that carry a 2′,3′-dihydroxyphenyl substructure as the B ring, but which have diversity in the A ring.

2. Material and methods

2.1. General experimental procedures

Optical rotations were measured using a JASCO P-1020 polarimeter. The ^{1}H 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 a JASCO FT/IR-4200 spectrometer, UV spectra were recorded on JASCO V-730 spectrometer, and CD spectra were recorded on JASCO J-600 spectrometer. Melting points were determined by using AS-ONE ATM-02.

2.2. Separation of stereoisomers by chiral column chromatography

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

2.3. NO assay

The amount of released nitrite (NO) was quantified by the Griess method.[28] 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 cells/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 solution (Sigma Aldrich, L-2880) was then added to each well. The final concentration of LPS was 100 ng/mL. After 16 h of incubation in the CO_2 incubator, 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 1% sulfanilamide solution (in 5 v/v% aqueous phosphoric acid) 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 the mixture was 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 the ultraviolet absorption at λ_{max}.

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, followed by incubation at 37 °C for 4 h. Absorbance was measured at 570 nm (reference wavelength: 600 nm).

2.5. Synthesis of MOMO protection acetophenones **1**

NaH (1.5 equiv; depending on the number of hydroxyl groups) in dry THF was slowly added while stirring at 0–5 °C (in an ice-water bath) to a solution of hydroxyacetophenone (1 equiv) in dry DMF. When the solution was cooled to 0–5 °C, chloromethyl methyl ether (1.5 equiv; depending on the number of hydroxyl groups) was slowly added over a period of 15 min so that the temperature was maintained below 5 °C. The reaction mixture was then 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).

2.6. Synthesis of bis(2-metholmethyl)benzaldehyde **2**

K_2CO_3 (10 equiv) was added while stirring at 0-5 °C (ice-water bath) to a solution of 2,3-dihydroxylbenzaldehyde (1 equiv) in dry acetone. When the solution was cooled to 0–5 °C, chloromethyl methyl ether (3.0 equiv) was slowly added over a period of 15 min to keep the temperature below 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 oil (90% yield).

^{1}H NMR (600 MHz, $CDCl_3$), δ: 3.49 (3H, s, OCH_3-3), 3.56 (3H, s, OCH_3-2), 5.21 (2H, s, OCH_2-3), 5.23 (2H, s, OCH_2-2), 7.12 (1H, t, J = 7.8 Hz, H-5), 7.38 (1H, dd, J = 7.8, 1.2 Hz, H-4), 7.48 (1H, dd, J = 7.8, 1.2 Hz, H-6), 10.44 (1H, s, CHO). ^{13}C NMR (150 MHz, $CDCl_3$), δ: 56.5 (OCH_3-3), 58.1 (OCH_3-2), 95.4 (OCH_2-3), 99.9 (OCH_2-2), 121.1 (C-4), 122.4 (C-5), 124.7 (C-6), 130.8 (C-1), 150.1 (C-3), 150.3 (C-2), 190.4 (C=O).

2.7. 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).

2.8. 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 (95% yield).

Please cite this article in press as: Jiang W.-J., et al. Bioorg. Med. Chem. (2017), http://dx.doi.org/10.1016/j.bmc.2017.05.060

2.9. Synthesis of flavanonol **5**

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 a red-yellow oil that was purified by silica gel column chromatography, eluting with *n*-hexane and EtOAc to give **5a**–**5m** (30–55% yield).

2.9.1. 2′,3′-Dihydroxyflavavonol **5a**

Colorless crystal, mp 198–200 °C UV λ_{max} (MeOH) nm (logε): 252 (3.90), 286 (3.48), 320 (3.48). ^{1}H NMR (600 MHz, acetone-d_6) δ: 4.92 (1H, d, J = 12.0 Hz, H-3), 5.67 (1H, d, J = 12.0 Hz, H-2), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.91 (1H, dd, J = 7.8, 1.8 Hz, H-4′), 7.05 (1H, d, J = 8.4 Hz, H-8), 7.10 (1H, dd, J = 7.8, 1.8 Hz, H-6′), 7.13 (1H, t, J = 7.8 Hz, H-6), 7.61 (1H, td, J = 7.8, 1.8 Hz, H-7), 7.86 (1H, dd, J = 7.8, 1.8 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.8 (C-3), 79.5 (C-2), 116.2 (C-4′), 118.8 (C-8), 120.2 (C-6′), 120.4 (C-5′), 120.4 (C-10), 122.6 (C-6), 124.8 (C-1′), 127.8 (C-5), 137.2 (C-7), 145.2 (C-2′), 146.0 (C-3′), 162.7 (C-9), 195.0 (C-4). HR-EI-MS: m/z 272.0685 [M]$^+$ (Calcd 272.0685 for $C_{15}H_{12}O_5$).

Separation by chiral chromatography: EtOH-*n*-hexane (35:65 v/v) retention time (2*R*,3*R*)-isomer: 10.77 min, (2*S*,3*S*)-isomer: 7.25 min. (2*R*,3*R*)-isomer: $[\alpha]_D^{18}$ +55.8° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −4.77 (309.9),+2.88 (339.9) ($c = 6.0 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{18}$ −63.4° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +4.19 (310.8), −3.31 (339.9) ($c = 6.0 \times 10^{-5}$).

2.9.2. 2′,3′,5-Trihydroxyflavavonol **5b**

Colorless crystal, mp 224–226 °C. UV λ_{max} (MeOH) nm (logε): 276 (3.99), 351 (3.48) ^{1}H NMR (600 MHz, acetone-d_6) δ: 4.99 (1H, d, J = 11.4 Hz, H-3), 5.69 (1H, d, J = 11.4 Hz, H-2), 6.48 (1H, d, J = 7.8 Hz, H-6), 6.53 (1H, d, J = 7.8 Hz, H-8), 6.78 (1H, t, J = 7.8 Hz, H-5′), 6.90 (1H, dd, J = 7.8, 1.8 Hz, H-4′), 7.06 (1H, dd, J = 7.8, 1.8 Hz, H-6′), 7.48 (1H, t, J = 7.8 Hz, H-7). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.1 (C-3), 79.3 (C-2), 107.6 (C-10), 108.4 (C-6), 109.9 (C-8), 116.3 (C-4′), 120.3 (C-6′), 120.4 (C-5′), 124.5 (C-1′), 139.5 (C-7), 145.3 (C-2′), 146.0 (C-3′), 162.9 (C-5), 163.0 (C-9), 201.1 (C-4). HR-EI-MS: m/z 288.0635 [M]$^+$ (Calcd 288.0634 for $C_{15}H_{12}O_6$).

Separation by chiral chromatography: EtOH-*n*-hexane (35:65 v/v) retention time (2*R*,3*R*)-isomer: 6.83 min, (2*S*,3*S*)-isomer: 10.08 min. (2*R*,3*R*)-isomer: $[\alpha]_D^{18}$ +67.9° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −2.39 (316.4), +1.47 (346.7) ($c = 6.0 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{18}$ −66.6° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +1.40 (316.0), −1.48 (346.7) ($c = 6.0 \times 10^{-5}$).

2.9.3. 2′,3′,6-Trihydroxyflavanonol **5c**

Colorless crystal, mp 179–181 °C. UV λ_{max} (MeOH) nm (logε): 255 (sh), 284 (sh), 360 (3.33). ^{1}H NMR (600 MHz, acetone-d_6) δ: 4.84 (1H, d, J = 12.0 Hz, H-3), 5.58 (1H, d, J = 12.0 Hz, H-2), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.89 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 6.92 (1H, d, J = 9.0 Hz, H-8), 7.09 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.13 (1H, dd, J = 9.0, 3.0 Hz, H-7), 7.26 (1H, d, J = 3.0 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 74.3 (C-3), 79.5 (C-2), 111.4 (C-5), 116.1 (C-4′), 119.9 (C-8), 120.2 (C-6′), 120.4 (C-5′), 120.5 (C-10), 125.2 (C-1′), 125.6 (C-7), 145.1 (C-2′), 146.1 (C-3′), 152.9 (C-6), 156.4 (C-9), 195.1 (C-4). HR-ES-MS: m/z 311.0532 [M]$^+$ (Calcd 311.0532 for $C_{15}H_{12}O_6Na$).

Separation by chiral chromatography: EtOH-*n*-hexane (55:45 v/v) retention time (2*R*,3*R*)-isomer: 6.08 min, (2*S*,3*S*)-isomer: 3.92 min. (2*R*,3*R*)-isomer: $[\alpha]_D^{18}$ +87.2° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −4.59 (318.3), +3.94 (363.2) ($c = 6.0 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{18}$ −87.4° (c = 1.0, MeOH), CD(MeOH): $\Delta\varepsilon$ (nm) +3.37 (320.3), −3.86 (362.3) ($c = 6.0 \times 10^{-5}$).

2.9.4. 2′,3′,7-Trihydroxyflavanonol **5d**

Colorless crystal, mp 231–233 °C. UV λ_{max} (MeOH) nm (logε): 277 (4.22), 309 (sh). ^{1}H NMR (600 MHz, acetone-d_6) δ: 4.77 (1H, d, J = 12.0 Hz, H-3), 5.59 (1H, d, J = 12.0 Hz, H-2), 6.42 (1H, d, J = 1.8 Hz, H-8), 6.63 (1H, dd, J = 8.4, 1.8 Hz, H-6), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.90 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.08 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.75 (1H, d, J = 8.4 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.5 (C-3), 79.5 (C-2), 103.7 (C-8), 111.8 (C-6), 113.3 (C-10), 116.1 (C-4′), 120.2 (C-6′), 120.5 (C-5′), 125.0 (C-1′), 129.9 (C-5), 145.1 (C-2′), 146.1 (C-3′), 164.8 (C-9), 165.8 (C-7), 193.3 (C-4). HR-EI-MS: m/z 288.0631 [M]$^+$ (Calcd 288.0634 for $C_{15}H_{12}O_6$).

Separation by chiral chromatography: EtOH-*n*-hexane (35:65 v/v) retention time (2*R*,3*R*)-isomer: 5.08 min, (2*S*,3*S*)-isomer: 3.92 min. (2*R*,3*R*)-isomer: $[\alpha]_D^{18}$ +61.5° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −4.66 (304.0), +2.64 (329.2) ($c = 6.0 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{18}$ −64.4° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +3.82 (304.9), −3.13 (329.2) ($c = 6.0 \times 10^{-5}$).

2.9.5. 2′,3′,7,8-Tetrahydroxyflavanonol **5e**

Colorless crystal, mp 241–243 °C. UV λ_{max} (MeOH) nm (logε): 292 (4.21). ^{1}H NMR (600 MHz, acetone-d_6) δ: 4.78 (1H, d, J = 12.0 Hz, H-3), 5.56 (1H, d, J = 12.0 Hz, H-2), 6.66 (1H, d, J = 8.4 Hz, H-6), 6.80 (1H, t, J = 8.4 Hz, H-5′), 6.90 (1H, dd, J = 8.4, 1.2 Hz, H-4′), 7.08 (1H, dd, J = 0.4, 1.2 Hz, H-6′), 7.33 (1H, d, J = 8.4 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 73.7 (C-3), 80.0 (C-2), 111.3 (C-6), 113.8 (C-10), 116.1 (C-4′), 119.2 (C-5), 120.5 (C-6′), 120.5 (C-5′), 125.0 (C-1′), 133.6 (C-8), 145.1 (C-2′), 146.1 (C-3′), 151.8 (C-7), 153.3 (C-9), 193.6 (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 (35:65 v/v) retention time (2*R*,3*R*)-isomer: 10.75 min, (2*S*,3*S*)-isomer: 12.08 min. (2*R*,3*R*)-isomer: $[\alpha]_D^{18}$ +86.1° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −2.89 (305.9), +1.89 (336.8) ($c = 6.0 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{18}$ −83.1° (c = 1.0, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +1.84 (306.2), −2.95 (337.2) ($c = 6.0 \times 10^{-5}$).

2.9.6. 2′,3′-Dihydroxy-6-methoxyflavanonol **5f**

Colorless crystal, mp 222-224 °C. UV λ_{max} (MeOH) nm (logε): 252 (sh), 283 (sh), 351 (3.55). ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.84 (3H, s, OCH_3-6), 4.87 (1H, d, J = 12.0 Hz, H-3), 5.61 (1H, d, J = 12.0 Hz, H-2), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.89 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.00 (1H, d, J = 9.0 Hz, H-8), 7.09 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.25 (1H, dd, J = 9.0, 2.4 Hz, H-7), 7.29 (1H, d, J = 2.4 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.2 (OCH_3-6), 74.1 (C-3), 79.6 (C-2), 108.5 (C-5), 116.1 (C-4′), 120.1 (C-8), 120.2 (C-6′), 120.3 (C-10), 120.4 (C-5′), 125.0 (C-1′), 125.7 (C-7), 145.1 (C-2′), 146.0 (C-3′), 155.4 (C-6), 157.3 (C-9), 194.9 (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 (40:60 v/v) retention time (2*R*,3*R*)-isomer: 9.50 min, (2*S*,3*S*)-isomer: 5.87 min. (2*R*,3*R*)-isomer: $[\alpha]_D^{25}$ −186.0° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +13.27 (230.0), −5.84 (295.3) ($c = 6.62 \times 10^{-5}$). (2*S*,3*S*)-isomer: $[\alpha]_D^{25}$ +183.5° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −15.69 (229.6), +45.80 (295.7) ($c = 6.62 \times 10^{-5}$).

2.9.7. 2′,3′-Dihydroxy-7-methoxyflavanonol **5g**

Colorless crystal, mp 211–213 °C. UV λ_{max} (MeOH) nm (logε): 274 (4.17), 310 (sh). ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.89 (3H, s, OCH_3-7), 4.81 (1H, d, J = 12.0 Hz, H-3), 5.62 (1H, d, J = 12.0 Hz, H-2), 6.54 (1H, d, J = 2.4 Hz, H-8), 6.70 (1H, dd, J = 9.0, 2.4 Hz,

H-6), 6.80 (1H, t, J = 7.8 Hz, H-5′), 6.90 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.09 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.78 (1H, d, J = 9.0 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.4 (OCH_3-7), 73.4 (C-3), 79.7 (C-2), 101.7 (C-8), 111.3 (C-6), 113.8 (C-10), 116.2 (C-4′), 120.2 (C-6′), 120.4 (C-5′), 124.9 (C-1′), 129.5 (C-5), 145.1 (C-2′), 146.0 (C-3′), 164.8 (C-9), 167.5 (C-7), 193.3 (C-4). HR-EI-MS: m/z 302.0791 $[M]^+$ (Calcd 302.0790 for $C_{16}H_{14}O_6$).

Separation by chiral chromatography: EtOH-n-hexane (50:50 v/v) retention time (2R,3R)-isomer: 7.42 min, (2S,3S)-isomer: 12.83 min. (2R,3R)-isomer: $[\alpha]_D^{25}$ −86.4° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +6.49 (304.8), −4.80 (329.8) ($c = 6.62 \times 10^{-5}$). (2S,3S)-isomer: $[\alpha]_D^{25}$ +85.8° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −6.76 (304.8), +4.35 (330.8) ($c = 6.62 \times 10^{-5}$).

2.9.8. 2′,3′-Dihydroxy-6,7-dimethoxyflavanonol **5h**

Colorless crystal, mp 245-247 °C. UV λ_{max} (MeOH) nm (logε): 237 (4.35), 276 (4.11), 340 (3.84). ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.84 (3H, s, OCH_3-7), 3.91 (3H, s, OCH_3-6), 4.77 (1H, d, J = 12.0 Hz, H-3), 5.58 (1H, d, J = 12.0 Hz, H-2), 6.59 (1H, s, H-8), 6.80 (1H, t, J = 7.8 Hz, H-5′), 6.89 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.10 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.23 (1H, s, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.5 (OCH_3-7), 56.6 (OCH_3-6), 73.5 (C-3), 79.9 (C-2), 101.4 (C-8), 107.7 (C-5), 111.9 (C-10), 116.1 (C-4′), 120.2 (C-6′), 120.4 (C-5′), 125.1 (C-1′), 145.1 (C-2′), 146.1 (C-3′), 146.2 (C-7), 157.9 (C-6), 159.3 (C-9), 193.3 (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 (70:30 v/v) retention time (2R,3R)-isomer: 8.37 min, (2S,3S)-isomer: 10.75 min. (2R,3R)-isomer: $[\alpha]_D^{25}$ −87.6° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +5.16 (309.0), −5.25 (343.1) ($c = 6.02 \times 10^{-5}$). (2S,3S)-isomer: $[\alpha]_D^{25}$ +100.6° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −5.96 (308.6), +5.54 (343.9) ($c = 6.02 \times 10^{-5}$).

2.9.9. 2′,3′,8-Trihydroxy-7-methoxyflavanonol **5i**

Colorless crystal, mp 220–222 °C. UV λ_{max} (MeOH) nm (logε): 290 (4.21), 320 (sh). ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.94 (3H, s, OCH_3-7), 4.80 (1H, d, J = 11.4 Hz, H-3), 5.59 (1H, d, J = 11.4 Hz, H-2), 6.80 (1H, t, J = 7.8 Hz, H-5′), 6.85 (1H, d, J = 9.0 Hz, H-6), 6.89 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.10 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.40 (1H, d, J = 9.0 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.8 (OCH_3-7), 73.9 (C-3), 79.9 (C-2), 107.2 (C-6), 115.0 (C-10), 116.1 (C-4′), 118.6 (C-5), 120.4 (C-6′), 120.5 (C-5′), 125.0 (C-1′), 135.7 (C-8), 145.1 (C-2′), 146.1 (C-3′), 151.1 (C-9), 154.6 (C-7), 194.1 (C-4). HR-EI-MS: m/z 318.0736 $[M]^+$ (Calcd 318.0740 for $C_{16}H_{14}O_7$).

Separation by chiral chromatography: EtOH-n-hexane (40:60 v/v) retention time (2R,3R)-isomer: 13.0 min, (2S,3S)-isomer: 11.7 min. (2R,3R)-isomer: $[\alpha]_D^{25}$ −74.5° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) +2.49 (304.0), −2.27 (338.5) ($c = 6.28 \times 10^{-5}$). (2S,3S)-isomer: $[\alpha]_D^{25}$ +64.1° (c = 0.1, MeOH), CD (MeOH): $\Delta\varepsilon$ (nm) −2.70 (305.2), +2.09 (338.9) ($c = 6.28 \times 10^{-5}$).

2.9.10. 2′,3′-Dihydroxy-5,7-dimethoxyflavanonol **5j**

Colorless crystal, mp 224–226 °C. UV λ_{max} (MeOH) nm (logε): 285 (4.28), 318 (sh). ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.87 (3H, s, OCH_3-5), 3.87 (3H, s, OCH_3-7), 4.61 (1H, d, J = 12.0 Hz, H-3), 5.50 (1H, d, J = 12.0 Hz, H-2), 6.15 (1H, d, J = 1.8 Hz, H-8), 6.23 (1H, d, J = 3.0 Hz, H-6), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.89 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.10 (1H, dd, J = 7.8, 1.2 Hz, H-6′). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.3 (OCH_3-5), 56.4 (OCH_3-7), 73.1 (C-3), 78.6 (C-2), 93.8 (C-8), 94.5 (C-6), 104.1 (C-10), 116.1 (C-4′), 120.0 (C-6′), 120.5 (C-5′), 125.0 (C-1′), 145.0 (C-2′), 146.1 (C-3′), 163.2 (C-5), 166.0 (C-9), 167.6 (C-7), 191.0 (C-4). HR-EI-MS: m/z 332.0897 $[M]^+$ (Calcd 332.0896 for $C_{17}H_{16}O_7$). The stereoisomers of **5j** could not be separated by chiral chromatography.

2.9.11. 2′,3′-Dihydroxy-6-methylflavanonol **5k**

Colorless crystal, mp 206–208 °C. UV λ_{max} (MeOH) nm (logε): 255 (3.91), 285 (sh), 332 (3.49). ^{1}H NMR (600 MHz, acetone-d_6) δ: 2.33 (3H, s, CH_3-6), 4.87 (1H, d, J = 12.0 Hz, H-3), 5.62 (1H, d, J = 12.0 Hz, H-2), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.90 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 6.94 (1H, d, J = 9.0 Hz, H-8), 7.09 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.42 (1H, dd, J = 9.0, 2.4 Hz, H-7), 7.64 (1H, d, J = 2.4 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 20.4 (CH_3-6), 74.0 (C-3), 79.4 (C-2), 116.1 (C-4′), 118.6 (C-8), 120.0 (C-6), 120.2 (C-6′), 120.4 (C-5′), 124.9 (C-1′), 127.3 (C-5), 132.0 (C-10), 138.2 (C-7), 145.1 (C-2′), 146.0 (C-3′), 160.8 (C-9), 195.0 (C-4). HR-EI-MS: m/z 286.0841 $[M]^+$ (Calcd 286.0841 for $C_{16}H_{14}O_5$). The stereoisomers of **5k** could not be separated by chiral chromatography.

2.9.12. 2′,3′-Dihydroxy-7-methylflavanonol **5l**

Colorless crystal, mp 189–191 °C. UV λ_{max} (MeOH) nm (logε): 255 (3.91), 285 (sh), 332 (3.49). ^{1}H NMR (600 MHz, acetone-d_6) δ: 2.37 (3H, s, CH_3-7), 4.85 (1H, d, J = 12.6 Hz, H-3), 5.63 (1H, d, J = 12.6 Hz, H-2), 6.79 (1H, t, J = 7.8 Hz, H-5′), 6.86 (1H, s, H-8), 6.90 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 6.96 (1H, d, J = 7.8 Hz, H-6), 7.08 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.74 (1H, d, J = 7.8 Hz, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 21.9 (CH_3-7), 73.8 (C-3), 79.4 (C-2), 116.1 (C-4′), 118.0 (C-10), 118.8 (C-8), 120.2 (C-6′), 120.4 (C-5′), 123.9 (C-6), 124.9 (C-1′), 127.7 (C-5), 145.1 (C-2′), 146.1 (C-3′), 148.8 (C-7), 162.8 (C-9), 194.5 (C-4). HR-EI-MS: m/z 286.0841 $[M]^+$ (Calcd 286.0841 for $C_{16}H_{14}O_5$). The stereoisomers of **5l** could not be separated by chiral chromatography.

2.9.13. 2′,3′-Dihydroxy-6,7-dimethylflavanonol **5m**

Colorless crystal, mp 178–180 °C. UV λ_{max} (MeOH) nm (logε): 237 (4.35), 276 (4.11), 340 (3.84). ^{1}H NMR (600 MHz, acetone-d_6) δ: 2.26 (3H, s, CH_3-7), 2.30 (3H, s, CH_3-6), 4.80 (1H, d, J = 12.0 Hz, H-3), 5.59 (1H, d, J = 12.0 Hz, H-2), 6.80 (1H, t, J = 7.8 Hz, H-5′), 6.88 (1H, s, H-8), 6.89 (1H, dd, J = 7.8, 1.2 Hz, H-4′), 7.08 (1H, dd, J = 7.8, 1.2 Hz, H-6′), 7.58 (1H, s, H-5). ^{13}C NMR (150 MHz, acetone-d_6) δ: 18.9 (CH_3-7), 20.5 (CH_3-6), 73.9 (C-3), 79.4 (C-2), 116.1 (C-4′), 118.0 (C-10), 119.3 (C-8), 120.1 (C-6′), 120.4 (C-5′), 125.1 (C-1′), 127.6 (C-5), 131.3 (C-6), 145.0 (C-2′), 146.1 (C-3′), 147.7 (C-7), 161.1 (C-9), 194.6 (C-4). HR-EI-MS: m/z 300.0998 $[M]^+$ (Calcd 300.0998 for $C_{17}H_{16}O_5$). The stereoisomers of **5m** could not be separated by chiral chromatography.

2.10. Synthesis of 2-(2,3-dimethoxyphenyl)-6,7-dimethoxy-4-oxochroman-3-yl 4-chlorobenzoate **7h**

Compound **5h** (2R,3R) or (2S,3S) (8 mg) was dissolved in 1 mL of EtOH and stirred in an ice bath until the temperature of the solution dropped below 4 °C. Then 3 mL of CH_2N_2-Et_2O was added, and the mixture was kept at 4 °C for 16 h. The organic solution was removed under a vacuum, and then 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, and the residue was purified by silica gel column chromatography, eluting with n-hexane and EtOAc to give white powder **6h** (75–80% yield). Compound **6h** was dissolved in 1 mL of pyridine, and one drop of 4-chlorobenzoyl chloride was added, followed by stirring of the mixture solution at room temperature for 18 h. Distilled water was added to the mixture, and then it was extracted with EtOAc three times, followed by collection and evaporation of the EtOAc. The residue was purified by preparative HPLC to give compound **7h** (70–80% yield).

Colorless crystal, UV λ_{max} (MeOH) nm (log ε): 205 (4.77), 241 (4.62), 276 (4.25), 338 (3.90). ^{1}H NMR (600 MHz, acetone-d_6) δ: 3.84 (3H, s, OCH_3-3′), 3.85 (3H, s, OCH_3-7), 3.87 (3H, s, OCH_3-2′), 3.93 (3H, s, OCH_3-6), 6.06 (1H, d, J = 12.0 Hz, H-3), 6.21 (1H, d, J = 12.0 Hz, H-2), 6.57 (1H, s, H-8), 7.06 (1H, d, J = 8.4 Hz, H-4′), 7.14 (1H, t, J = 8.4 Hz, H-5′), 7.23 (1H, s, H-5), 7.29 (1H, d, J = 8.4 Hz, H-6′), 7.53 (2H, d, J = 8.4 Hz, H-3″, H-5″), 7.95 (2H, d, J = 8.4 Hz, H-2″, H-6″). ^{13}C NMR (150 MHz, acetone-d_6) δ: 56.2 (OCH_3-3′), 56.4 (OCH_3-7), 56.7 (OCH_3-6), 61.6 (OCH_3-2′), 74.6 (C-2), 77.5 (C-3), 101.4 (C-8), 107.6 (C-5), 112.4 (C-10), 114.6 (C-4′), 120.7 (C-6′), 125.1 (C-5′), 129.1 (C-1′), 129.8 (C-3″, C-5″), 130.3 (C-1″), 132.2 (C-2″, C-6″), 140.2 (C-4″), 146.4 (C-7), 149.1 (C-2′), 153.8 (C-3′), 158.1 (C-6), 158.8 (C-9), 164.2 (C-12), 187.2 (C-4). HR-EI-MS: m/z 498.1080 $[M]^+$ (Calcd 498.1081 for $C_{26}H_{23}O_8Cl$). (2R,3R)-isomer: CD (MeOH): $\Delta\varepsilon$ (nm) −22.51 (207.0), −13.82 (233.0), +3.26 (248.0), +4.23 (307.0), −6.89 (344.0) ($c = 4.02 \times 10^{-5}$). (2S,3S)-isomer: CD (MeOH): $\Delta\varepsilon$ (nm) +25.53 (207.0), +13.88 (233.0), −4.75 (248.0), −5.97 (307.0), +6.67 (344.0) ($c = 4.02 \times 10^{-5}$).

3. Results

3.1. Synthesis of 2′,3′-dihydroxyflavanonols

The preparation of the 2′,3′-dihydroxyflavanonols was carried out as illustrated in Scheme 1. Hydroxyl acetophenones were protected with chloromethyl methyl ether (MOMCl) to give compounds **1** (yield 80–95%). Benzaldehydes were also protected with MOMCl to give compounds **2** (yield 90%). Compounds **1** and **2** were treated with KOH in EtOH to give the intermediates **3** (yield 60–90%), which were then oxidized with H_2O_2 under alkaline condition to give epoxides **4** (yield 95%). Flavanonols **5a**–**5m** (yield 30–55%) were then obtained by hydrolysis of the epoxides.[9,29]

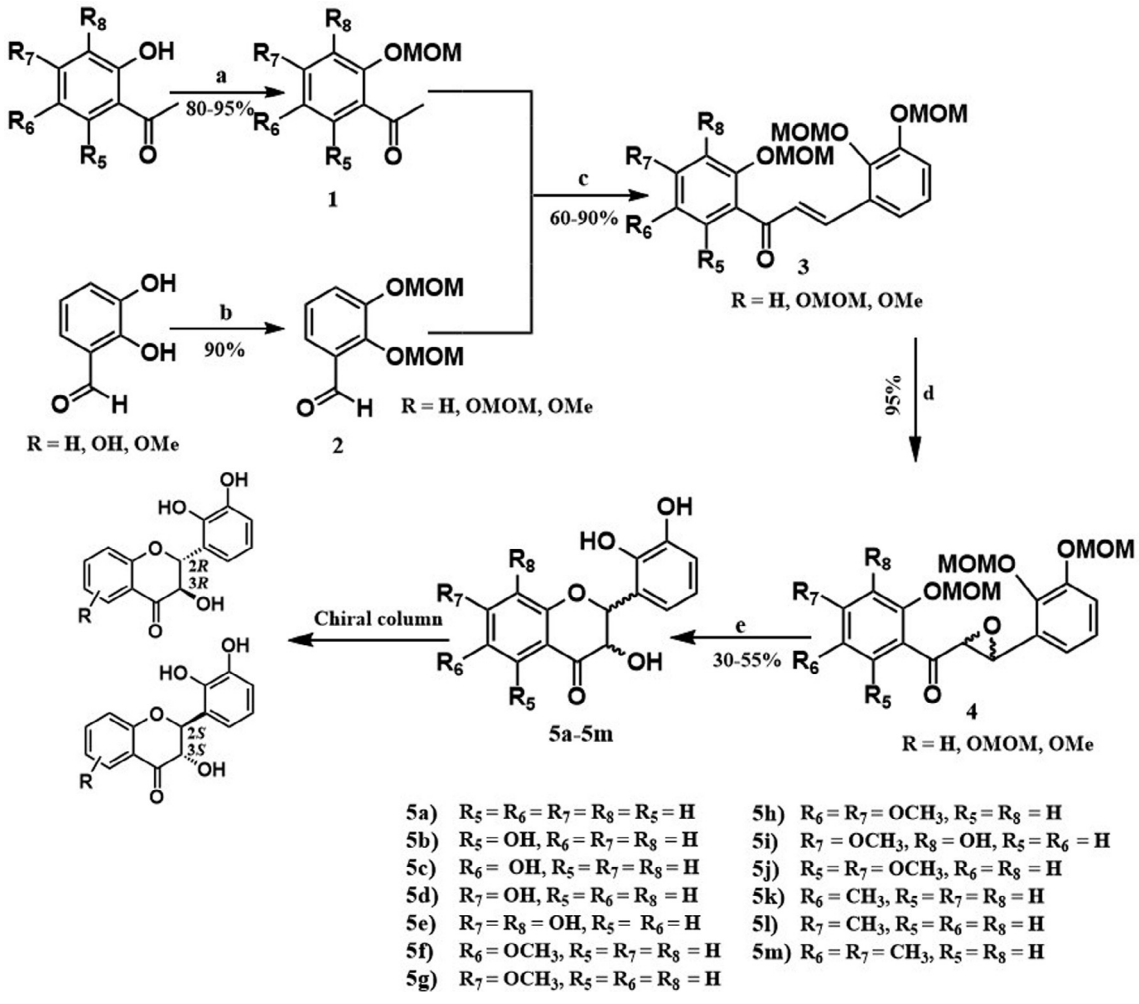
3.2. Separation of stereoisomers using a chiral column

Nine pairs of stereoisomers (enantiomers) were successfully isolated from the racemic mixtures by using a chiral column. However, 4 racemates (**5j**, **5k**, **5l**, and **5m**) were inseparable. Thus **5j**, **5k**, **5l**, and **5m** were used as racemic mixtures to measure the NO assay.

3.2.1. Determination of absolute configuration of flavanonols

The absolute configurations of the stereoisomers were determined by ^{1}H NMR and circular dichroism (CD). All of the synthesized compounds showed a coupling constant between H-2 and H-3 ($J_{2,3}$) of approximately 12 Hz. Thus, H-2 and H-3 are *trans* and diaxial, and therefore, the absolute configuration has to be either (2R,3R) or (2S,3S).

In the case of naturally occurring flavanonols, the configuration of the C-2 carbon atom can be reduced by applying a rule presented by Gaffield:[30] a positive Cotton effect at high wavelength (300–340 nm) indicates the 2R configuration, and a negative Cot-



Scheme 1. Synthesis of the 2′,3′-dihydroxyflavanonols. Reagents and conditions: (a) NaH, MOMCl, THF, 0 °C; (b) K_2CO_3, MOMCl, Me_2CO, 0 °C; (c) KOH, EtOH, rt; (d) 30% H_2O_2, NaOHaq, MeOH, rt; (e) MeOH-HCl, 55 °C.

ton effect indicates the 2*S* configuration. However, the absolute configuration of the synthetic flavanonols that have methoxy groups in the A ring (**5f**, **5g**, **5h**, and **5i**) could not be determined by this rule because the UV absorption spectra of these compounds differ from those of natural-type flavanonols (*O*-functional groups at the 5- and 7-positions in the A ring); the synthetic compounds show three absorption maxima at around 240 nm, 280 nm, and 340 nm (Fig. 1-(A)), whereas the natural-type flavanonols show a maxima at around 290 nm. An example of UV and CD spectra of a natural-type flavanonol, 2′,3′,5,7-tetrahydroxyflavanonol, is available as supplemental data. To determine the absolute configuration of the flavanonols substituted at the 6- and 7-positions by

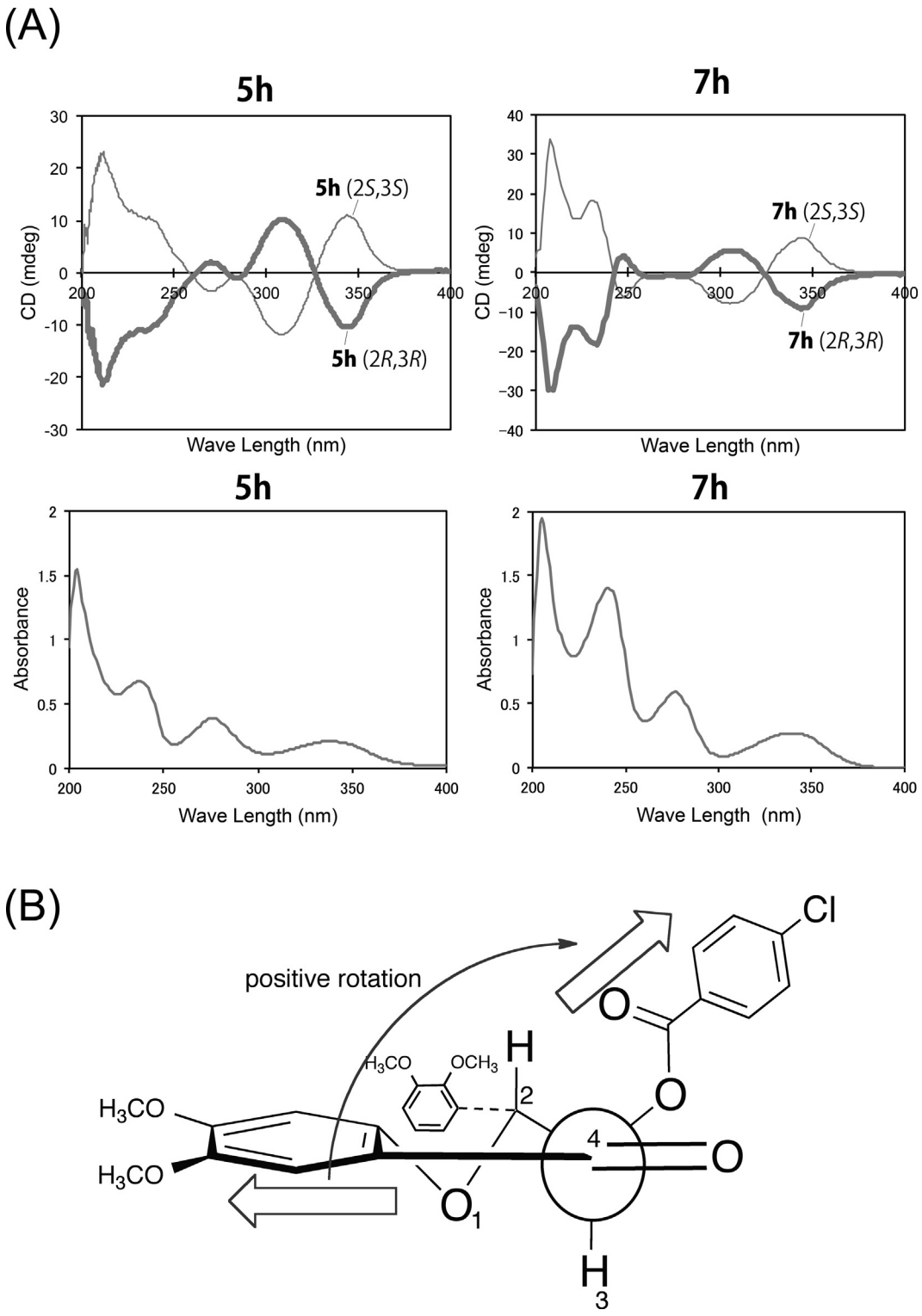


Fig. 1. (A) CD and UV spectra of **5h** and **7h**. The thick line corresponds to the (2*R*,3*R*)-isomer, and the thin line corresponds to the (2*S*,3*S*)-isomer. (B) (2*R*,3*R*) stereoisomer of **7h**: Newman projection about the C3–C4 bond is shown. Arrows represent two electronic transition moments, the benzoyl substructure of the A ring and the introduced 4-chlorobenzoyloxy substructure.

O-functional groups, we prepared the 4-chlorobenzoyl ester **7h** from **5h** (Scheme 2) and determined its absolute configuration based on the CD exciton chirality method.[31]

*3.2.2. Synthesis of 2-(2,3-dimethoxyphenyl)-6,7-dimethoxy-4-oxochroman-3-yl 4-chlorobenzoate **7h***

The preparation of each stereoisomer of 2-(2,3-dimethoxyphenyl)-6,7-dimethoxy-4-oxochroman-3-yl 4-chlorobenzoate **7h** was carried out as illustrated in Scheme 2. Each stereoisomer of **5h** was separated by chiral chromatography. Then, the 2′,3′-dihydroxy groups of **5h** were methoxylated by CH_2N_2. The product **6h** was reacted with 4-chlorobenzoyl chloride in pyridine to give compound **7h**, which corresponds to the original stereoisomer of **5h** (total yield 50–60%).

3.2.3. Determination of the absolute configuration of flavanonols

Fig. 1-(A) shows the CD and UV spectra of each stereoisomer of **5h** and **7h**. Introduction of the 4-chlorobenzoyl moiety of **5h** added an inflection point at 240 nm in the CD spectrum. In the (2*R*,3*R*) stereoisomer of **7h** as shown in Fig. 1-(B), two electronic transition moments, the 4-chlorobenzoyloxy chromophore and the benzoyl moiety of the A/C ring (the A ring and C4 carbonyl) indicated with arrows, make a clockwise (positive) rotation. Thus, the positive Cotton effect at 248 nm and the negative Cotton effect at 233 nm in the CD spectrum of **7h** assign the *R*-configuration to C-3.

Determination of the stereochemistry of **7h** deduced the CD spectra of each of the stereoisomers of **5h**. For flavanonols such as **5f**–**5i**, which have methoxy-substituted A-rings, the (2*R*,3*R*) stereoisomers showed a negative Cotton effect at a longer wavelength (340 nm) and a positive Cotton effect at a shorter wavelength (310 nm).

3.3. Cell viability

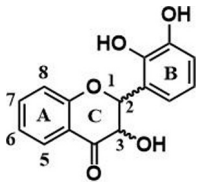
None of the compounds showed cell toxicity at 100 μM.

3.4. Inhibitory potential against NO production in RAW 264.7 cells

The inhibitory activity of the stereoisomers and racemic mixtures tested in the RAW 264.7 cells is summarized in Table 1. (2*R*,3*R*)-2′,3′,7,8-Tetrahydroxyflavanonol **5e** showed the highest inhibitory potential (95% inhibition at 100 μM, IC_{50} = 17.0 ± 1.1 - μM). Its inhibitory potential was comparable with the positive control, kaempferol (IC_{50} = 13.4 ± 0.5 μM). To our knowledge, compound **5e** is the most potent flavanonol ever reported. (2*R*,3*R*)-2′,3′,7-Trihydroxyflavanonol **5d** also showed comparative potency (70% inhibition at 100 μM, IC_{50} = 70.7 ± 7.4 μM). It should be noted that the (2*R*,3*R*) stereoisomer of **5d** and **5e** exhibited much higher inhibitory potency than the corresponding (2*S*,3*S*) stereoisomer. The (2*R*,3*R*) stereoisomers of 2′,3′-dihydroxy-6,7-dimethoxyflavanonol **5h** (93% inhibition at 100 μM, IC_{50} = 50.3 ± 1.4 μM) and 2′,3′,8-trihydroxy-7-methoxyflavanonol **5i** (93% inhibition at 100 μM, IC_{50} = 49.7 ± 2.3 μM) also showed higher inhibitory potency than the (2*S*,3*S*) isomers. The analogues with no substitution in the A ring, **5a**, with 6-hydroxy substitution, **5c**, and with 6-methoxy substitution, **5f**, are less potent. The magnitude of the difference in the potency between the stereoisomers roughly correlated with the stereo specificity of inhibition (Fig. 2).

The racemic mixtures of compounds **5j**, **5k**, **5l**, and **5m** also showed strong inhibitory potential against NO production (IC_{50} = 57.6 ± 2.9 μM, 45.3 ± 3.1 μM, 31.8 ± 0.6 μM, and 20.7 ± 2.4 μM, respectively). Regrettably, separation of their stereoisomers could not be done. The stereospecificity of their inhibitory potency remains to be determined.

Table 1
Structures and activity of the 2′,3′-dihydroxyflavanonols (IC_{50}).

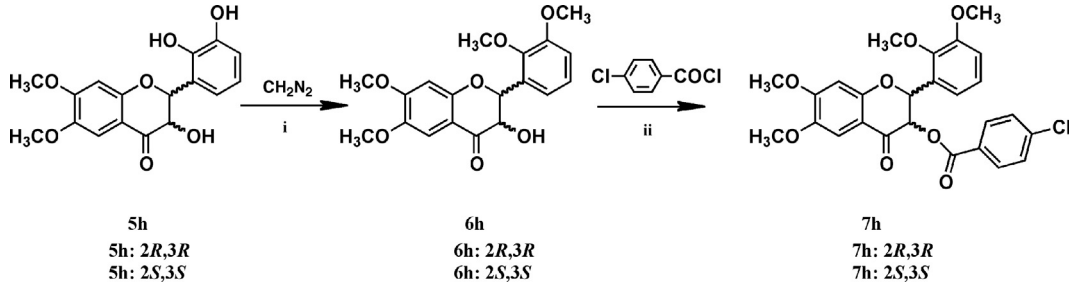


Compound	Substitution in the A ring	NO production inhibitory IC_{50} (μM)	
		(2*R*,3*R*)	(2*S*,3*S*)
5a	None	>100	>100
5b	5-OH	77.7 ± 0.9	>100
5c	6-OH	>100	>100
5d	7-OH	70.7 ± 7.4	>100
5e	7,8-OH	17.0 ± 1.1	>100
5f	6-OCH_3	>100	>100
5g	7-OCH_3	24.6 ± 2.0	30.3 ± 0.8
5h	6,7-OCH_3	50.3 ± 1.4	>100
5i	7-OCH_3, 8-OH	49.7 ± 2.3	88.5 ± 4.9
5j	5,7-OCH_3	Racemic mixture 57.6 ± 2.9	
5k	6-CH_3	Racemic mixture 45.3 ± 3.1	
5l	7-CH_3	Racemic mixture 31.8 ± 0.6	
5m	6,7-CH_3	Racemic mixture 20.7 ± 2.4	

Reference compound: Kaempferol, IC_{50} = 13.4 ± 0.5 μM.
Cell viability ≥ 100% in all of the tested compounds.
Data are expressed as the mean ± S.D. (n = 3).

4. Discussion

The inhibition rate (%) of the stereoisomers at the concentration of 100 μM are shown in Fig. 2. Compounds **5a** (no substitution in A ring), **5b** (5-OH), **5c** (6-OH), and **5g** (7-OCH_3) showed little



5h
5h: 2*R*,3*R*
5h: 2*S*,3*S*

6h
6h: 2*R*,3*R*
6h: 2*S*,3*S*

7h
7h: 2*R*,3*R*
7h: 2*S*,3*S*

Scheme 2. Synthesis of 2-(2,3-dimethoxyphenyl)-6,7-dimethoxy-4-oxochroman-3-yl 4-chlorobenzoate **7** h. Reagents and conditions: (i) EtOH, CH_2N_2-Et_2O solution, 4 °C; (ii) 4-chlorobenzoyl chloride, pyridine, room temperature.

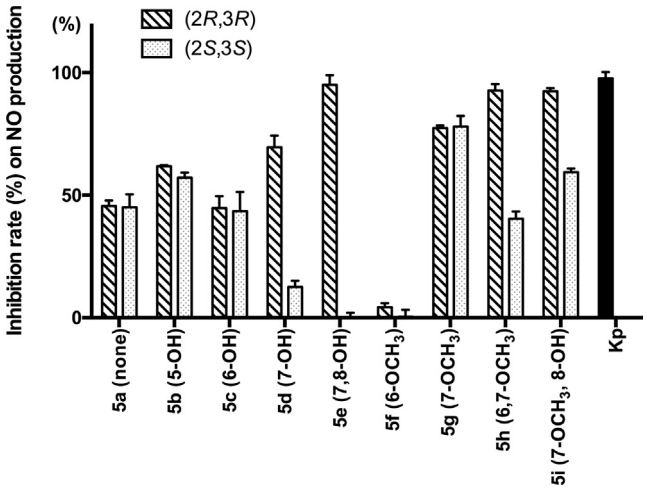


Fig. 2. Effects of the 2′,3′-dihydroxyflavanonols on NO production. The vertical axis shows the inhibition rate of the 2′,3′-dihydroxyflavanonols on NO production. The concentration of each specimen was 100 μM. All data are expressed as the mean ± S. D. (n = 3). Kp: Kaempferol (black bar: reference compound at 100 μM). The striped bars represent the inhibitory rate of the (2*R*,3*R*)-isomers, and the meshed bars represent that of the (2*S*,3*S*)-isomers.

stereospecificity of the inhibitory effects on NO production. In contrast, evident stereospecificity was found in compounds **5d** (7-OH), **5e** (7,8-OH), and **5i** (7-OCH_3, 8-OH). The (2*R*,3*R*)-isomer of **5e** showed the highest inhibitory potency, whereas the (2*S*,3*S*)-isomer showed a very weak inhibitory rate at 100 μM (0%). Comparing the structures of **5d** with **5e**, introduction of a hydroxyl group at the 8-position would increase both stereospecificity and inhibitory activity. Compounds **5c** (6-OH) and **5f** (6-methoxy) showed very weak inhibitory activity on NO production. However, compound **5k** (6-CH_3, racemic mixture [Table 1]) showed higher inhibitory potency. Compound **5h** (6,7-OCH_3) showed stronger inhibitory potential with enhanced stereospecificity than that of **5f**. The presence of a methoxy group at the 7-position of the A ring would enhance inhibitory potency and stereospecificity. Compound **5g** (7-OCH_3) showed strong inhibitory potency but little stereospecificity. Compound **5l** (7-methyl), which is a racemic mixture, also showed high potency (Table 1). The additional substitution of a hydroxyl group at the 8-position in **5g** (7-OCH_3) resulted in clear stereospecificity (**5i**: 7-OCH_3, 8-OH). The substituent at the 7-position of the A ring is essential for 2′,3′-dihydroxyflavanonols to exhibit inhibitory activity, and the stereospecificity of the inhibition will increase when an additional substituent is introduced at the 6- or 8-positions. It should be noted that compound **5m** (6,7-CH_3, racemic mixture [Table 1]) exhibited an IC_{50} value of 20 μM. Considering the high potency of **5m**, its (2*R*,3*R*) stereoisomer might be a very potent compound. The hydrophobicity of the substituents at the 7-position of the A ring might be important (Table 1: **5d** < **5l** < **5g**, **5h** < **5m**). Compound **5j** (5,7-OCH_3, racemic mixture [Table 1]) showed an inhibition rate of 58% on NO production. Considering that the (2*R*,3*R*) stereoisomer of 2′,3′,5,7-tetrahydroxyflavanonol, *i.e.*, (2*R*,3*R*)-desmethoxy-**5j**, showed 70% inhibition at 100 μM,[27] substituents at the 5- and 7-positions would function as hydrogen-bonding acceptors or molecular volumes.

We observed stronger inhibitory activity in the (2*R*,3*R*) stereoisomers than in the (2*S*,3*S*) stereoisomers. The results of this study indicate that the inhibitory potency of flavanonols relates to their three-dimensional features such as the existence of a substituent at the 7-position and an additional substituent in the neighborhood of the 7-position.

The existence of a structure-activity relationship strongly suggests the possibility of designing a novel compound for suppression of NO production. In fact, none of the compounds synthesized in this study have yet been isolated from natural sources or synthesized elsewhere.

5. Conclusion

We prepared a series of 2′,3′-dihydroxyflavanonols with diversity in the A ring structures. (2*R*,3*R*)-2′,3′,7,8-Tetrahydroxyflavanonol **5e** was found to be a potent inhibitor of NO production (IC_{50} = 17.1 μM). To our knowledge, this is the most potent flavanonol ever reported.

The (2*R*,3*R*) stereoisomers generally showed stronger inhibitory activity against NO production than the (2*S*,3*S*) stereoisomers. For the studied flavanonols, the stronger the inhibitory potency of an analogue was, the larger was the difference between the potency of the (2*R*,3*R*) stereoisomer and the (2*S*,3*S*) stereoisomer. The structure–activity relationship obtained in this study suggests the existence of a biological target that can interact with the flavanonols and modulate NO production in RAW 264.7 cells.

Acknowledgments

We thank the Watanuki International Scholarship Foundation and Otsuka Toshimi Scholarship Foundation for supporting the study and research of author W-J. J.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.05.060. These data include MOL files and InChiKeys of the most important compounds described in this article.

References

1. Yamamoto Y, Gaynor RB. *J Clin Invest*. 2001;107:135–142.
2. Cazarolli LH, Zanatta L, Alberton EH, et al. *Mini-Rev Med Chem*. 2008;8:1429–1440.
3. Osadebe PO, Okoye FB. *J Ethnopharmacol*. 2003;89:19–24.
4. Rotelli AE, Guardia T, Juárez AO, de la Rocha NE, Pelzer LE. *Pharmacol Res*. 2003;48:601–606.
5. Schuier M, Sies H, Illek B, Fischer H. *J Nutr*. 2005;135:2320–2325.
6. Havsteen BH. *Pharmacol Ther*. 2002;96:67–202.
7. Ng TB, Liu F, Wang ZT. *Life Sci*. 2000;66:709–723.
8. Weisburger JH. *Food Chem Toxicol*. 1999;37:943–948.
9. Pandurangan N, Bose C, Banerji A. *Bioorg Med Chem Lett*. 2011;21:5328–5330.
10. Pietta PG. *J Nat Prod*. 2000;63:1035–1042.
11. Mlcek J, Jurikova T, Skrovankova S, Sochor J. *Molecules*. 2016;21.
12. Rahman M, Gray AI. *Phytochemistry*. 2002;59:73–77.
13. Cushnie TP, Lamb AJ. *Int J Antimicrob Agents*. 2011;38:99–107.
14. Manner S, Skogman M, Goeres D, Vuorela P, Fallarero A. *Int J Mol Sci*. 2013;14:19434–19451.
15. Vlietinck AJ, De Bruyne T, Apers S, Pieters LA. *Planta Med*. 1998;64:97–109.
16. Kaul TN, Middleton Jr E, Ogra PL. *J Med Virol*. 1985;15:71–79.
17. Friedman M. *Mol Nutr Food Res*. 2007;51:116–134.
18. Way TD, Kao MC, Lin JK. *FEBS Lett*. 2005;579:145–152.
19. Yilmaz Y, Toledo RT. *Trends Food Sci Technol*. 2004;15:422–433.
20. Moridani MY, Galati G, O' Brien PJ. *Chem Biol Interact*. 2002;139:251–264.
21. Sousa RR, Queiroz KC, Souza AC, et al. *J Enzyme Inhib Med Chem*. 2007;22:439–444.
22. Asgari S, Naderi GH, Sarrafzadegan N, et al. *Pharm Acta Helv*. 1999;73:223–236.
23. Kitanaka S. *Yakugaku Zasshi*. 2016;136:1195–1216.
24. Daikonya A, Kitanaka S. *Chem Pharm Bull*. 2011;59:1567–1569.
25. Daikonya A, Kitanaka S. *Jpn J Food Chem Saf*. 2011;18:183–190.
26. Jiang W-J, Daikonya A, Ohkawara M, et al. *Bioorg Med Chem*. 2017;25:779–788.
27. Jiang W-J, Ishiuchi K, Furukawa M, Takamiya T, Kitanaka S, Iijima H. *Bioorg Med Chem*. 2015;23:6922–6929.
28. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. *Anal Biochem*. 1982;126:131–138.
29. Jr BR, Fink RC, McMichael MD, Li D, Alberte RS. *Phytochemistry*. 2009;70:1255–1261.
30. Gaffield W. *Tetrahedron*. 1970;26:4093–4108.
31. Harada N, Nakanishi K. *Circular Dichroic Spectroscopy Exciton Coupling in Organic Stereochemistry*. CA: University Science Books; 1983. Oxford: Oxford University Press.