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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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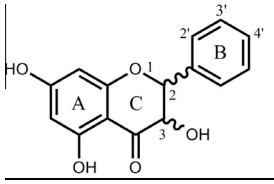
Table 1
The structure and activity of the flavanonols



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).