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Separation of Quercetin's Biological Activity from Its Oxidative Property through Bioisosteric Replacement of the Catecholic Hydroxyl Groups with Fluorine Atoms

Suh Young Cho,[†,∥] Mi Kyoung Kim,[†,∥] Hyejung Mok,[†] Hyunah Choo,[§] and Youhoon Chong*[,†]

[†]Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Korea

[§]Neuro-Medicine Center, Korea Institute of Science and Technology, 39-1 Hawolgok-dong, Seoungbuk-gu, Seoul 136-791, Korea

ABSTRACT: An oxidative property has endowed quercetin with numerous biological benefits, and some of quercetin's biological activities may be related, at least partly, to its antioxidant activity. On the other hand, the oxidative property and associated susceptibility to oxidative decomposition has hampered in-depth investigation of the biological targets as well as underlying mechanisms for quercetin's biological activity. This study was undertaken to separate quercetin's biological activities from its antioxidant properties through bioisosteric replacement of the phenolic hydroxyl groups. The novel quercetin derivative 3′,4′-difluoroquercetin (**2**), thus prepared, showed nonoxidizable property with no attenuation of biological activity. Rather, **2** showed a subtle but significant increase in biological activity compared with quercetin, which might be attributed to its lack of oxidative property. The nonoxidizable nature along with the potent biological activity of the quercetin mimic **2** suggests possible oxidation-independent mechanisms for the biological activities of the quercetin that do not require oxidative formation of the highly electrophilic metabolites.

KEYWORDS: *quercetin, oxidative property, bioisostere, antiproliferative effect, antibacterial effect*

INTRODUCTION

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) (**1**, Figure 1) is one of the most important flavonoids in the diet due to its abundance in foods (apples, onions, grapes, red wine, and green tea) as well as its various physiological effects (antioxidant,[1] antiviral,[2,3] antibacterial,[4] and anticancer[5] activities).

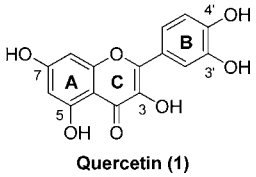

Figure 1. Structure of quercetin (**1**) with numbering system.

Among the various biological activities associated with quercetin, the antioxidative effect has long been investigated because oxidative damage is involved in several malignant pathologies such as cancers and cardiovascular and neurodegenerative diseases. However, despite many studies on quercetin's antioxidant properties, much less is known about how the oxidation of quercetin is related with its therapeutic effects. Rather, facile oxidative degradation of quercetin, particularly under in vitro conditions such as high pH and high concentration of transition metal ions and oxygen molecules, has hampered in-depth investigation of the biological targets as well as the underlying mechanisms of quercetin's biological activities.[6−11] As a result, a nonoxidizable quercetin mimic was anticipated to serve as a stable surrogate for quercetin to help investigation of its mechanisms of bioactivity. In this study, with the aim to separate quercetin's biological activities from its antioxidant properties, we sought to undertake a structural modification of the quercetin scaffold.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen. Nuclear magnetic resonance spectra were recorded on a Bruker 400 AMX spectrometer (Karlsruhe, Germany) at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR with tetramethylsilane as an internal standard. Chemical shifts are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Coupling constants are reported in hertz. The chemical shifts are reported as parts per million (δ) relative to the solvent peak. TLC was performed on silica gel-60 F254 purchased from Merck. Column chromatography was performed using silica gel 60 (220−440 mesh) for flash chromatography. Mass spectrometric data (MS) were obtained by electron spray ionization (ESI).

Chemistry. *3,5,7-Tris(benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-4H-chromen-4-one (**4**).* To a stirred solution of quercetin (**1**) (10 g, 33.1 mmol) in DMF (150 mL) were added K_2CO_3 (45.7 g, 331 mmol) and BnBr (39.3 mL, 331 mmol). The reaction mixture was stirred for 3 days at 80 °C. Upon completion of reaction, precipitate and K_2CO_3 were removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was crystallized from CH_2Cl_2/EtOAc to give **4** (20.2 g, 26.8 mmol, 81% yield) as a pale yellow powder, which was used for the next step without further purification.

*2-(Benzyloxy)-1-(2,4-bis(benzyloxy)-6-hydroxyphenyl)ethanone (**5**).* To a stirred solution of **4** (10 g, 13.3 mmol) in a mixture of

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pyridine (40 mL) and diethylene glycol (20 mL) was added 18 N KOH (20 mL). The reaction mixture was stirred for 12 h at 120 °C. After cooling to room temperature, the reaction mixture was neutralized with 2 N HCl and then extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, concentrated under reduced pressure, and purified by column chromatography (16:1:1 = Hex/CH_2Cl_2/EtOAc) on silica gel to give **5** (2.0 g, 4.4 mmol, 33% yield) as yellow powder: ^{1}H NMR (500 MHz, $CDCl_3$) δ 13.74 (s, 1H), 7.39−7.21 (m, 15H), 6.18 (s, 1H), 6.06 (s, 1H), 5.05 (s, 2H), 4.98 (s, 2H), 4.56 (s, 2H), 4.52 (s, 2H).

*3,5-Bis(benzyloxy)-2-(2-(benzyloxy)acetyl)phenyl 3,4-Difluorobenzoate (**6**).* To a solution of 3,4-difluorobenzoic acid (270 mg, 1.7 mmol) in CH_2Cl_2 (7 mL) cooled to 0 °C were added oxalyl chloride (0.3 mL, 3.4 mmol) and DMF (0.05 mL, catalytic amount). The reaction mixture was stirred for 2 h at 45 °C and concentrated under reduced pressure to give the corresponding acid chloride as white powder, which was used for the next step without further purification. To a stirred solution of **5** (500 mg, 1.1 mmol) in CH_2Cl_2 (7 mL) was added the 3,4-difluorobenzoyl chloride obtained above (300 mg, 1.7 mmol), and the mixture was cooled to 0 °C. TEA (0.24 mL, 1.7 mmol) was added to this solution in dropwise fashion. The reaction mixture was stirred for 4 h at room temperature. Upon completion of the reaction, the mixture was concentrated under reduced pressure, and the residue was purified by column chromatography on silica (30:30:1 = Hex/CH_2Cl_2/EtOAc) to give **6** (450 mg, 0.76 mmol, 70% yield) as a colorless oil: ^{1}H NMR (400 MHz, acetone-d_6) δ 8.02−7.97 (m, 2H), 7.56−7.49 (m, 5H), 7.43−7.36 (m, 6H), 7.29−7.20 (m, 5H), 6.84 (d, J = 2.1 Hz, 1H), 6.71 (d, J = 2.1 Hz, 1H), 5.24 (s, 2H), 5.20 (s, 2H), 4.51 (s, 2H), 4.40 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.8, 164.0, 162.9, 159.9, 153.0 (ddd, J = 12.9 Hz, 251.3 Hz, 396.8 Hz, 1C), 151.4, 138.5, 136.3, 129.6, 129.5, 129.4, 129.3, 129.2, 128.8, 128.7, 128.6, 128.5, 127.0, 119.5 (dd, J = 18.3 Hz, 204.6 Hz, 1C), 115.2, 102.8, 99.4, 76.9, 72.1, 71.4; LC-MS (ESI) m/z found 595.20 $[M + H]^+$, calcd for $C_{36}H_{28}F_2O_6$ 594.60.

*3,7-Bis(benzyloxy)-2-(3,4-difluorophenyl)-5-hydroxy-4H-chromen-4-one (**7**).* To a solution of **5** (450 mg, 0.76 mmol) in toluene (5 mL) were added K_2CO_3 (420 mg, 3.04 mmol) and tetra-*n*-butylammonium bromide (367 mg, 1.14 mmol). The reaction mixture was stirred for 2 h at 90 °C. Upon completion of reaction, precipitate and K_2CO_3 were removed by filtration. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (50:50:1 = Hex/CH_2Cl_2/EtOAc) to give **7** (250 mg, 0.51 mmol, 67% yield) as a yellow oil: ^{1}H NMR (400 MHz, acetone-d_6) δ 12.94 (s, 1H), 7.97−7.91 (m, 2H), 7.52−7.29 (m, 11H), 6.79 (d, J = 2.00 Hz, 1H), 6.46 (d, J = 2.4 Hz, 1H), 5.27 (s, 2H), 5.20 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.8, 165.8, 163.1, 157.7, 153.1 (ddd, J = 12.0 Hz, 99.0 Hz, 277.8 Hz, 1C), 139.1, 137.0, 136.7, 130.1, 129.8, 129.6, 129.5, 128.5, 126.6, 118.7 (dd, J = 18.5 Hz, 75 Hz, 1C), 107.2, 99.9, 94.1, 75.7, 71.6; LC-MS (ESI) m/z found 487.11 $[M + H]^+$, calcd for $C_{29}H_{20}F_2O_5$ 486.46.

*2-(3,4-Difluorophenyl)-3,5,7-trihydroxy-4H-chromen-4-one (**2**).* The degassed suspension of **7** (250 mg, 0.51 mmol) obtained above in a mixture of MeOH (2 mL), THF (5 mL), and Pd/C (10% w/w, 25 mg), under an atmosphere of hydrogen gas (balloon), was vigorously stirred for 12 h at room temperature. The reaction mixture was filtered through a short Celite pad, concentrated under reduced pressure, and purified by column chromatography on silica gel (8:1 = CH_2Cl_2/acetone) to give the desired product, 3′,4′-difluoroquercetin, **2** (46.8 mg, 0.15 mmol, 30% yield) as a yellow powder: ^{1}H NMR (400 MHz, acetone-d_6) δ 12.00 (s, 1H), 8.22 (ddd, J = 1.8, 7.9, 12.4 Hz, 1H), 8.13−8.15 (m, 1H), 7.54 (dd, J = 8.6, 18.9, 1H), 6.60 (s, 1H), 6.30 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.8, 166.0, 162.3, 159.4, 157.8, 151.1 (ddd, J = 63.0 Hz, 270.4 Hz, 990.4 Hz), 144.7, 139.1, 130.0, 126.4, 118.8 (dd, J = 73.4 Hz, 525.2 Hz), 114.0, 104.8, 100.0, 95.3; LC-MS (ESI) m/z found 307.00 $[M + H]^+$, calcd for $C_{15}H_8F_2O_5$ 306.22.

*7-(Benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-3,5-dihydroxy-4H-chromen-4-one (**9**).* To a solution of rutin (**8**) (10 g, 16.4 mmol) in 30 mL of DMF were added K_2CO_3 (7.6 g, 54.9 mmol) and benzyl bromide (6.5 mL, 54.9 mmol) at room temperature. The reaction mixture was stirred for 5 h at 60 °C, acidified with 2 N HCl in EtOH (150 mL) at 0 °C, and then stirred for 2 h at 70 °C. The precipitate was filtered through a filter paper to afford **9** (5.6 g, 9.84 mmol, 60% yield) as a yellow powder: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.91 (d, J = 1.9 Hz, 1H), 7.85 (dd, J = 2.0, 8.6 Hz, 1H), 7.33−7.48 (m, 5H), 7.44−7.51 (m, 10H), 6.86 (d, J = 2.1 Hz, 1H), 6.45 (d, J = 2.1 Hz, 1H), 5.24 (s, 4H), 5.21 (s, 2H).

*3-(Benzylamino)-7-(benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-5-hydroxy-4H-chromen-4-one (**10**).* To a solution of **9** (300 mg, 0.52 mmol) in pyridine (5 mL) was added MsCl (0.06 mL, 0.79 mmol) at 0 °C. The reaction mixture was stirred for 4 h at room temperature, concentrated under reduced pressure, and purified by column chromatography on silica gel (3:1 = Hex/EtOAc) to afford the 7-(benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-5-hydroxy-4-oxo-4H-chromen-3-ylmethanesulfonate (318 mg, 0.48 mmol, 94% yield) as a yellow powder: ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.1 (s, 1H), 7.71 (s, 1H), 7.62 (d, J = 9.1 Hz, 1H), 7.29−7.50 (m, 15H), 7.03 (d, J = 8.6 Hz, 1H), 6.50 (s, 1H), 6.46 (s, 1H), 5.24 (s, 2H), 5.22 (s, 2H), 5.13 (s, 2H), 3.54 (s, 3H). To a solution of the 3-*O*-mesylate obtained above (120 mg, 0.18 mmol) in THF (5 mL) was added benzylamine (0.2 mL, 1.8 mmol) at 0 °C. The reaction mixture was stirred for 5 days at room temperature and then washed with H_2O. The organic layer was dried over $MgSO_4$ and then concentrated under reduced pressure. The residue was triturated with Et_2O to afford **10** (97 mg, 0.15 mmol, 82% yield) as a yellow powder: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (s, 1H), 7.61 (d, J = 8.6 Hz, 1H), 7.49−7.54 (m, 4H), 7.28−7.45 (m, 16H), 7.02 (d, J = 8.6 Hz, 1H), 6.53 (s, 1H), 6.45 (s, 1H), 5.26 (s, 2H), 5.25 (s, 2H), 5.22 (s, 2H), 5.07 (s, 2H), 2.04 (s, 1H).

*3-Amino-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one (**3**).* The degassed suspension of **10** (97 mg, 0.15 mmol) obtained above in a mixture of MeOH (2 mL), THF (2 mL), and $Pd(OH)_2$ (10% w/w, 10 mg), under an atmosphere of hydrogen gas (balloon), was vigorously stirred for 12 h at room temperature. The reaction mixture was filtered through a short Celite pad and concentrated under reduced pressure. The residue was trituated with Et_2O to give the desired product, 3-aminoquercetin, **3** (22 mg, 0.07 mmol, 50% yield) as a yellow powder: ^{1}H NMR (400 MHz, MeOD) δ 7.35 (s, 1H), 7.19 (d, J = 8.3 Hz, 1H), 6.89 (d, J = 8.3, 1H), 6.11 (s, 1H), 5.99 (s, 1H); LC-MS (ESI) m/z found 302.20 $[M + H]^+$, calcd for $C_{15}H_8F_2O_5$ 301.25.

Oxidative Properties. *Radical Scavenging Activity.* The free radical-scavenging activities of quercetin (**1**) and the two quercetin derivatives (**2** and **3**) were measured using the DPPH radical.[12] Ascorbic acid was used as a reference compound. In a typical procedure, in each well of a 96-well plate was mixed a freshly prepared methanol solution of DPPH (190 μL, 0.1 mM) with 10 μL of a methanol (1% DMSO) solution of **1**, **2**, **3**, and ascorbic acid at different concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM). The absorption spectra at 517 nm were recorded every 2 min. For each sample concentration tested, the percentage of DPPH remaining reached the steady state after 30 min. Therefore, the percentage of residual DPPH after 30 min was plotted against the concentration of samples. The EC_{50} values, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, were determined from this plot.

Stability Against Oxidative Degradation. Quercetin (**1**) and the two quercetin derivatives synthesized (**2** and **3**) were dissolved in various pH buffer solutions [pH 2, PBS (pH 7.4), and pH 10] to reach final concentrations of 50 μM−10 mM. The solutions were incubated at 37 °C and, at different time points (0, 0.5, 1, 2, 3,4, 5, 6, 12, and 24 h), an aliquot (100 μL) of the reaction mixture was taken out, filtered, and injected into the HPLC equipped with a C-18 reverse phase column. Amounts of the remaining quercetin (**1**) and its derivatives (**2** and **3**) were evaluated by HPLC, which was performed according to the following conditions: flow rate, 1 mL/min; detection, UV 340 nm; mobile phase, 0−8 min (20% aqueous acetonitrile and 0.1% formic acid), 8−18 min (25% aqueous acetonitrile and 0.1% formic acid), 18−40 min (35% aqueous acetonitrile and 0.1% formic acid), 40−45 min (80% aqueous acetonitrile and 0.1% formic acid), 45−54 min

(100% aqueous acetonitrile and 0.1% formic acid), and 54−60 min (20% aqueous acetonitrile and 0.1% formic acid).

Quercetin (**1**) and its derivatives (**2** and **3**) were also dissolved in cDMEM [Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin] to reach final concentrations of 50 μM−10 mM. The solutions were incubated at 37 °C and 5% CO_2 in 24-well plates. The media were vortexed and analyzed with HPLC under the same analysis conditions as described above.

Biological Activity. *Antibacterial Activity.* A modified agar diffusion Kirby−Baur method was used to determine antibacterial activity. Stock solutions of quercetin (**1**), its derivatives (**2** and **3**), and the positive control (ampicillin) were prepared at 1.25, 2.5, and 5 mM. Sterile paper disks (8 mm) were loaded with 50 μL of solutions and subsequently dried to remove acetone at room temperature. Each bacterium (*Staphylococcus epidermidis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Escherichia coli*) was inoculated in 2 mL of Mueller−Hinton medium. The culture grew until absorbance at 600 nm reached 1.2. The Mueller−Hinton agar was poured into Petri dishes to give a solid plate and spread with 30 μL of each bacterial culture broth. The disks were deposited on the surface of inoculated agar plates, which were then incubated at 37 °C for 24 h. Inhibition zone diameters around each of the disks were measured.

Anticancer Effect: Cell Viability. MCF-7 and Huh-7 cells (5×10^3 each) were seeded in tissue-cultured COSTAR clear-bottom 96-well plates in complete DMEM and incubated for 12 h (37 °C, 5% CO_2). Quercetin (**1**) and its derivatives (**2** and **3**) dissolved in DMSO were serially diluted to six concentrations (0.1, 0.5, 1, 5, 10, and 50 mM), and the resulting solutions were added to media. After 12 h, cell viability was estimated by MTT assay.

Anticancer Effect: Apoptosis Assay. MCF-7 cells were seeded in tissue-cultured COSTAR clear-bottom 6-well plates (10^6 cell/well) in cDMEM and incubated for 12 h (37 °C, 5% CO_2). After treatment with quercetin (**1**) (50 μM), 3′,4′-difluoroquercetin (**2**) (50 μM), and control (1% DMSO) for 12 h (37 °C, 5% CO_2), cells were trypsinized, collected, and washed twice with cold PBS. After loading 1× binding buffer (100 μL), cells were resuspended, and the solution (100 μL, 1×10^6 cells) was transferred to a 5 mL culture tube. The solution was stained with 5 μL of propidium iodide (PI) and 5 μL of FITC Annexin V and incubated for 15 min at room temperature in the dark. Then, 400 μL of 1× binding buffer was added to each tube. Apoptosis detection was analyzed using FACSCalibur (Becton Dickinson) within 1 h.

RESULTS AND DISCUSSION

According to Dangles et al.,[13] one-electron oxidation into aryloxy radicals ($\mathbf{QH_3}$, Figure 2) and subsequent fast disproportionation into quinone ($\mathbf{QH_4}$) constitute the key steps for quercetin oxidation. As a single-electron transfer specifically from 3-OH or 3′-OH to form the corresponding aryloxy radical initiates the entire degradation process, it was anticipated that replacement of the two hydroxyl groups with a biologically equivalent and non-radical-producing substituent would block the oxidation of quercetin.

In bioorganic chemistry, it is a common practice to replace a specific functional group of the active principle with a more relevant chemical entity that is structurally distinct but recognized similarly by biological systems. The term "bioisostere" coined by Hans Erlenmeyer represents this practice, and the bioisosteres are characterized by similar biological properties rather than exact structural mimetics. Due to close polarity and isosteric relationship with the hydroxyl group, both amino and fluorine functionalities are well-known classical monovalent bioisosteres of the hydroxyl group.[14−20] In addition, the high bond energies of N−H and C−F bonds are expected to render the corresponding derivatives resistance to oxidative degradation.[21]

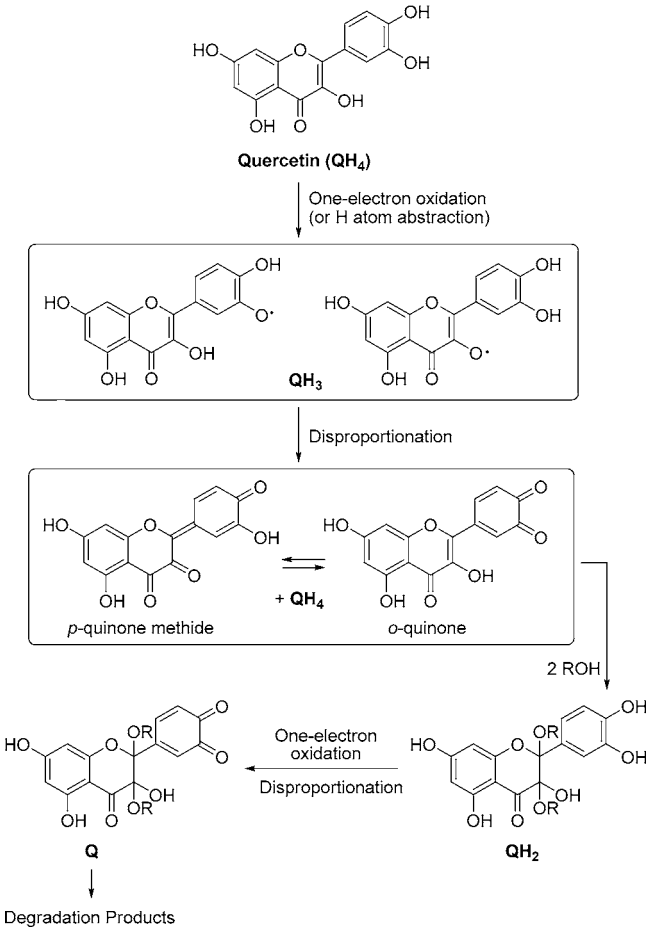

Figure 2. Mechanism for oxidative degradation of quercetin (**1**).

On the basis of this information, we attempted bioisosteric replacement of 3-OH and 3′-OH groups with either amino or fluorine functionalities. Taking ease of synthesis into consideration, two quercetin derivatives, 3′,4′-difluoroquercetin (**2**, Figure 3) and 3-aminoquercetin (**3**), were designed as potential quercetin mimics that would be resistant to oxidative degradation while maintaining biological activity.

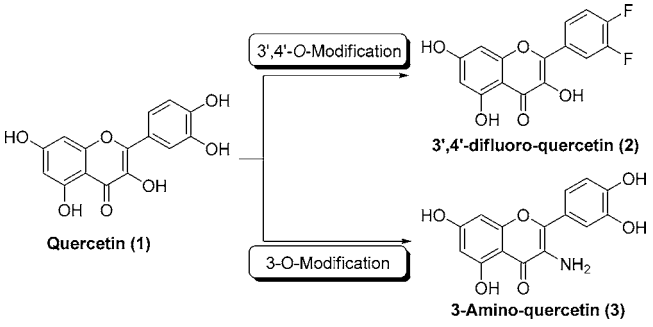

Figure 3. Bioisosteric modification of quercetin (**1**).

Chemistry. Syntheses of 3′,4′-difluoroquercetin (**2**) and 3-aminoquercetin (**3**) are summarized in Schemes 1 and 2, respectively.

For preparation of **2**, starting from quercetin (**1**), a well-known synthetic routine composed of fragmentation followed by acylation and ring closure was employed (Scheme 1).[22] Thus, the perbenzylated quercetin (**4**) was exposed to a strong

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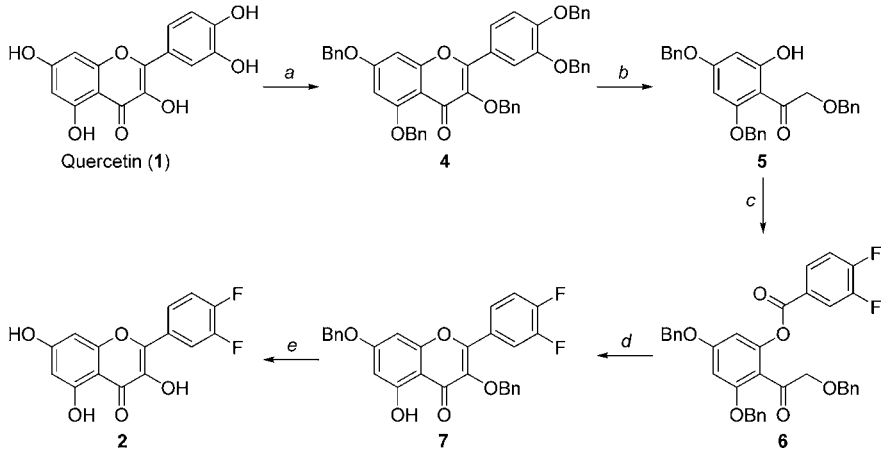
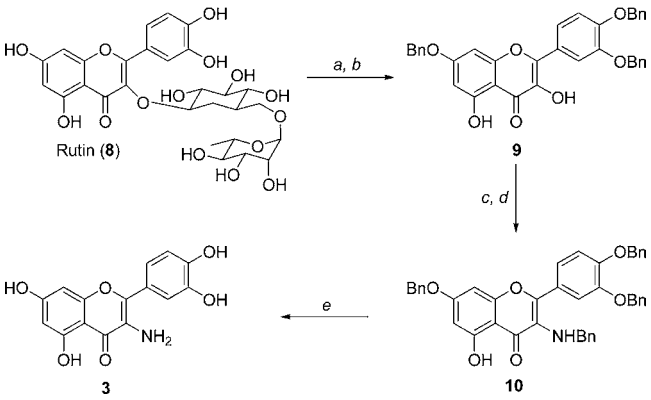
Scheme 1. Preparation of 3′,4′-Difluoroquercetin (2) from Quercetin (1)[a]



[a]Reagents and conditions: (a) BnBr, K_2CO_3, DMF, 80 °C; (b) 18 N KOH, diethylene glycol, 120 °C; (c) 3,4-difluorobenzoyl chloride, TEA, CH_2Cl_2; (d) K_2CO_3, TBAB, toluene, 90 °C; (e) H_2, Pd/C, MeOH−CH_2Cl_2.

Scheme 2. Preparation of 3-Aminoquercetin (3) from Rutin (8)[23] [a]



[a]Reagents and conditions: (a) BnBr, DMF, 60 °C; (b) aq HCl, EtOH, reflux; (c) MsCl, pyr, rt; (d) $BnNH_2$, THF, rt; (e) H_2, $Pd(OH)_2$, THF/EtOH.

base (18 N KOH) at elevated temperature to give the fragmented benzylquercetin (**5**) in 40% yield. The catechol-mimic, difluorophenyl B-ring, was introduced via condensation of **5** with 3,4-difluorobenzoyl chloride, prepared from the commercially available 3,4-difluorobenzoic acid, in 84% yield. Treatment of the resulting ester **6** with K_2CO_3 in the presence of a phase transfer catalyst (tetra-*n*-butylammonium bromide, TBAB) afforded the cyclized product **7**, which was smoothly converted to the desired quercetin derivative **2** upon global deprotection of the benzyl protecting groups in 41% yield.

On the other hand, 3-aminoquercetin (**3**) was prepared by following the synthetic protocol published by Takecchi et al. (Scheme 2).[23] After protection of phenolic hydroxyl groups of the commercially available rutin (**8**) with benzyl moieties, the resulting perbenzylated rutin was treated with aqueous HCl to give the corresponding aglycon **9** in 60% yield. Reaction of **9** with methanesulfonyl chloride proceeded preferably at the 3-OH position. Upon treatment of the resulting quercetin-3-*O*-mesylate with benzylamine, the 3-benzylaminoquercetin derivative **10** was formed in 82% yield.[23] Global deprotection provided the desired 3-aminoquercetin (**3**) in 50% yield.

Radical-Scavenging Activity. As an electron transfer from 3-OH and/or 3′-OH to form the corresponding radical is known to initiate the oxidation of quercetin (Figure 2),[12] bioisosteric replacement of 3-OH or 3′-OH was expected to give a quercetin derivative without oxidative property. To determine the capacity of the quercetin derivatives to interact with electrons, their radical-scavenging activity was evaluated first. Thus, after quercetin (**1**) and the two quercetin derivatives (**2** and **3**) had been mixed with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in methanol, reduction of light absorption at 517 nm caused by the DPPH radical was monitored.[24,25] The monitoring was repeated at various concentrations of the test compound, and the remaining amount of DPPH radical was plotted against sample concentration (Figure 4).

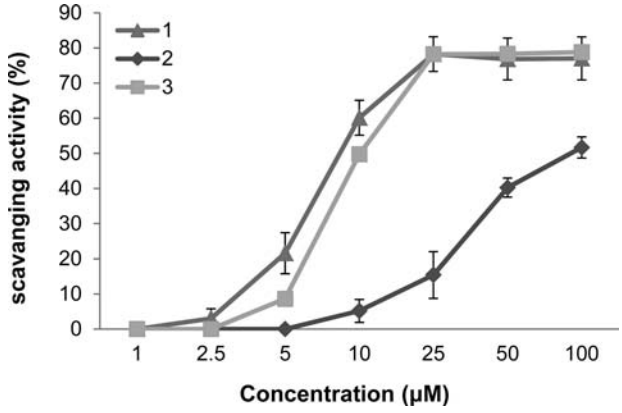


Figure 4. Radical-scavenging activity of quercetin (**1**) and its derivatives (**2** and **3**). Measurements were repeated three times. Error bars indicate SD ($P < 0.05$, *t* test).

It is worth noting that quercetin (**1**) and 3-aminoquercetin (**3**) showed similar potent radical-scavenging capacity but that 3′,4′-difluoroquercetin (**2**) acted as a weak radical scavenger. This result indicates that the catechol functionality is the key factor contributing to the radical-scavenging activity of the quercetin derivatives, whereas the 3-OH is replaceable.

Stability against Oxidative Degradation. As oxidation of quercetin is known to be accompanied by its degradation,[12] the oxidative property of quercetin can also be estimated by its

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stability. The stabilities of quercetin (**1**) and its newly synthesized derivatives (**2** and **3**) were determined by their half-lives, $t_{1/2}$ (h), in various buffer solutions [phosphate-buffered saline (PBS), pH 7.4; pH 2 and pH 11] as well as a cell culture medium (cDMEM) (Table 1).

Table 1. Stability of Quercetin (1), 3′,4′-Difluoroquercetin (2), and 3-Aminoquercetin (3) in Buffer Solutions and Cell Culture Medium

		half-life, $t_{1/2}$ (h)		
		quercetin	3′,4′-difluoroquercetin (**2**)	3-aminoquercetin (**3**)
buffer	pH 2.0	7	>24	>24
	PBS	10	>24	>24
	pH 10.0	<1	>24	>24
cDMEM		<0.5	>24	>24

In line with the literature,[6−11] quercetin (**1**) underwent facile decomposition ($t_{1/2}$ < 1 h) at high pH and cell culture medium, whereas it showed a moderate stability profile under acidic and neutral conditions with half-lives ($t_{1/2}$) of 7 and 10 h, respectively (Table 1). As anticipated by the lack of radical-scavenging activity, the replacement of a catechol with a 3,4-difluorophenyl functionality resulted in a stable quercetin derivative, **2**, without oxidative decomposition in all of the tested media even after 24 h of incubation (Table 1). Interestingly, however, despite the potent radical-scavenging activity, 3-aminoquercetin (**3**) also did not undergo oxidative decomposition. The mechanism for stability of the radical-scavenging 3-aminoquercetin (**3**) is not clear yet, but it could be attributed to the stability of the corresponding radical formed by an electron transfer from 3′-OH. It should be noted that quercetin quinones and quinone methides formed by radical disproportionation (Figure 2) have well-documented oxidizing and electrophilic properties to play critical roles in the oxidative decomposition of quercetin. In this context, the nonoxidizable nature of the 3-aminoquercetin radical might be attributed to its stability not to form the corresponding quinone and/or quinone methide.

Biological Activity. The purpose of this study was to separate the oxidative property of quercetin from its biological activities to provide a stable quercetin derivative having sustained biological activity. With the nonoxidizable stable quercetin derivatives **2** and **3** in hand, we then evaluated their biological activity.

Antibacterial Activity. The antimicrobial activity of the polyphenolic compounds has long been considered to be related with its antioxidative activity and, on the basis of the analysis of a large number of spice and herb extracts, Shan et al. proposed a highly positive relationship ($0.70 < R^2 < 0.84$) between antimicrobial activity and antioxidant capacity of the phenolic compounds.[26,27] Thus, we reasoned that evaluation of antimicrobial activity of the quercetin derivatives with (**3**) or without (**2**) antioxidative activity would provide interesting insights into the possible interplay between antioxidant and antimicrobial properties of the polyphenolics. Antibacterial activities of **2** and **3** in comparison to quercetin (**1**) were determined by using a modified agar diffusion Kirby−Baur's method. Briefly, bacterial suspension (*Staphylococcus epidermidis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Escherichia coli*) was spread over the face of a sterile agar plate, and a sterile paper disk treated with quercetin (**1**), its derivatives (**2** and **3**), or ampicillin (a positive control) was placed at the center of the agar plate. After incubation, the size of the zone of inhibition was measured and summarized in Table 2.

Due to the potent radical-scavenging activity, 3-aminoquercetin (**3**) was expected to show better antibacterial activity than **2**. However, the efficient radical scavenger 3-aminoquercetin (**3**) failed to show any antibacterial activity against a series of bacterial strains (Table 2). In contrast, the nonoxidizable 3′,4′-difluoroquercetin (**2**) exhibited more potent antibacterial activity than quercetin against the Gram-positive strains (*S. epidermidis* and *M. luteus*). Clearly, this result shows that the antibacterial activity of quercetin might not be directly related with its oxidative property.

Cell Viability. Anticancer activity is another example in which quercetin's bioactivity is related with its ability to interact with electrons.[28] At high concentration, quercetin is known to promote oxidative breakage of double-stranded DNA, which results in increased apoptosis through activation of proteins, leading to the induction of many downstream pathways.[29,30] Also, quercetin increases oxidative stress and cytotoxicity in transformed cells by becoming a reactive oxygen species (ROS) itself. For example, highly electrophilic quinone methide, the key oxidation product of quercetin (Figure 2), is known to form adducts with other cellular macromolecules such as glutathione (GSH), and the resulting depletion of cellular GSH contributes to tumor cell apoptosis.[29,31−33] Thus, when quercetin exerts its anticancer effect, formation of the reactive quinone and quinone methide seems to be critical. In this context, it was of our great interest to evaluate the anticancer activity of the quercetin derivatives **2** and **3** of which stability against oxidative decomposition represents the lack of formation of the reactive quinones and quinone methides.

Breast cancer (MCF-7) and hepatoma (Huh-7) cell lines were treated with quercetin (**1**) and its two derivatives (**2** and **3**), and their viabilities were evaluated (Figure 5). Unexpectedly, in the MCF-7 cell line, 3′,4′-difluoroquercetin (**2**) showed an antiproliferative effect similar to that of quercetin (**1**),

Table 2. Antibacterial Activity of Quercetin Derivatives against Bacterial Strains in Different Dilutions (5, 2.5, and 1.25 mM)[a]

		quercetin (**1**)	3′,4′-difluoroquercetin (**2**)	3-aminoquercetin (**3**)	ampicillin
Gram-positive	*S. epidermidis*	11, 10, −	16, 16, 13	−	21, 19, 17
	M. luteus	−	11, 10, −	−	44, 40, 35
Gram-negative	*P. aeruginosa*	−	−	−	−
	E. cloacae	−	−	−	−
	E. coli	−	−	−	25, 25, 21

[a]Numerals are mean diameter of inhibition zones (in mm) at each concentration. The size of pores on agar is 4 mm. (−) resistant.

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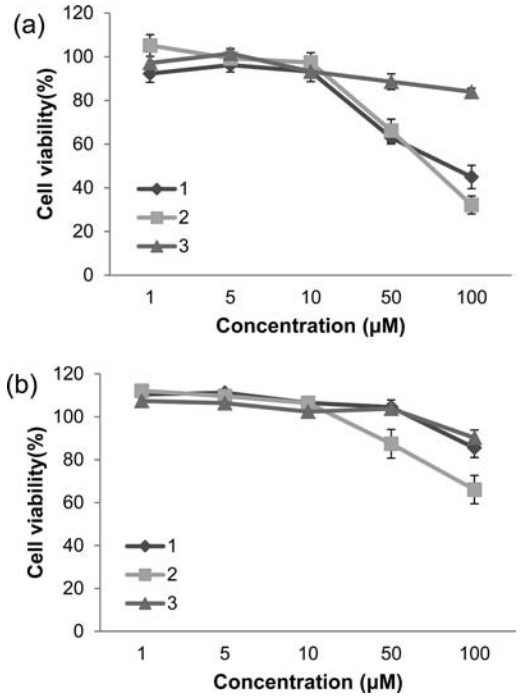

Figure 5. Effects of quercetin (**1**) and its analogues (**2** and **3**) on viability of two different cancer cell lines: (a) MCF-7; (b) Huh-7.

whereas 3-aminoquercetin (**3**) was not cytostatic up to 100 μM (Figure 5a). The quercetin derivatives were less cytostatic against Huh-7, and only 3′,4′-difluoroquercetin (**2**) showed a modest antiproliferative effect (Figure 5b). Along with the antibacterial activity, the unanticipated antiproliferative activity of **2** also indicates that the quercetin derivatives might have oxidation-independent mechanisms for their biological activities, which do not require oxidative formation of the highly electrophilic metabolites.

The similar antiproliferative effects of quercetin (**1**) and 3′,4′-difluoroquercetin (**2**) allowed us to compare their apoptotic properties as well. Quercetin is known to induce apoptosis in various cancer cell lines when administered in high doses.[34] Also, interference of the pathways concerned with cell proliferation and maintenance as well as inhibition of protein chaperon are known mechanisms by which quercetin promotes apoptosis.[34] Evaluation of apoptosis was performed by a flow cytometric method that uses double labeling with Annexin V and PI.[35] After treatment with quercetin (**1**) and 3′,4′-difluoroquercetin (**2**), MCF-7 cells were incubated for 12 h and apoptosis was measured by flow cytometric counting. The percentages of cells in early (EA; Annexin$^+$, PI$^-$; lower right quadrant, Figure 6) and late apoptotic−necrotic stages (LA; Annexin$^+$, PI$^+$; upper right quadrant, Figure 6) are indicated in Figures 6 and 7.

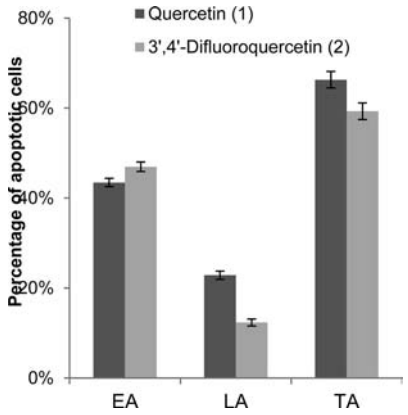

Figure 7. Different subsets of apoptotic cells as percentages of all cells measured by Annexin V/PI fluorescence staining (EA, early apoptotic; LA, late apoptotic-necrotic; TA, total apoptotic). Measurements were repeated three times. Error bars indicate SD ($P < 0.05$, t test).

As shown in Figures 6 and 7, both quercetin (**1**) and 3′,4′-difluoroquercetin (**2**) induced early apoptosis (Annexin$^+$, PI$^-$; EA) in MCF-7 cells in a similar fashion (44.1 and 47.7% for **1** and **2**, respectively). As compared to untreated cells (Figure 6a), apoptosis became apparent. In contrast, there was a significant difference in the number of cells with positive Annexin V and PI signal (Annexin$^+$, PI$^+$; LA), and quercetin (**1**) (23.5%) produced cells in the late-stage apoptotic−necrotic stage almost twice as much as 3′,4′-difluoroquercetin (**2**) (12.9%). Taken together, 3′,4′-difluoroquercetin (**2**) shows a profile of early apoptosis similar to that of quercetin (**1**) but is less effective than quercetin (**1**) in inducing cells in late apoptotic−necrotic stage, and this result indicates that the oxidative capacity of quercetin might be more closely related to its property to induce late apoptosis−necrosis rather than early apoptosis.

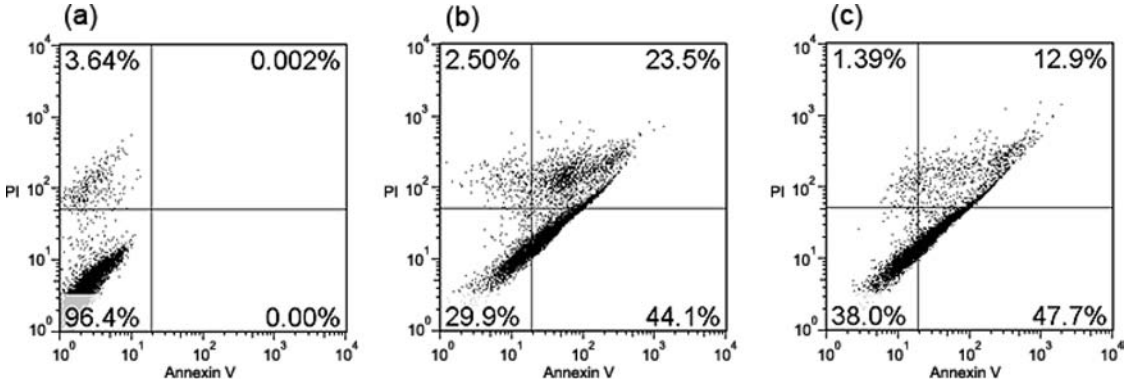

Figure 6. MCF-7 cells (a) without treatment or after treatment with 50 μM (b) quercetin (**1**) or (c) 3′,4′-difluoroquercetin (**2**) were incubated for 12 h. After treatment, cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry. The results are representative of two independent experiments.

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In conclusion, as quercetin's oxidative property and thereby susceptibility to oxidative degradation has hampered in-depth investigation of its biological targets as well as underlying biological mechanisms, we intended to separate quercetin's biological activities from its antioxidant properties through bioisosteric replacement of the phenolic hydroxyl groups. The bioisosteric replacement of the catecholic hydroxyl groups of quercetin with fluorine atoms provided a nonoxidizable stable quercetin derivative but without attenuation of its biological activities. Rather, 3′,4′-difluoroquercetin (**2**) showed a subtle but significant increase in biological activity compared with quercetin, which might be attributed to its lack of oxidative property. The nonoxidizable nature along with the potent biological activity of the quercetin mimic **2** suggests possible oxidation-independent mechanisms for quercetin's biological activities that do not require oxidative formation of the highly electrophilic metabolites.

AUTHOR INFORMATION

Corresponding Author

*Phone: +82-2-2049-6100. Fax: +82-2-454-8217. E-mail: chongy@konkuk.ac.kr.

Author Contributions

‖These two authors contributed equally to this work.

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Notes

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