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In Vitro Flavon-3-ol Oxidation Mediated by a B Ring Hydroxylation Pattern

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Flavonols are potent naturally occurring antioxidants. Chemical oxidation reactions in combination with modern spectroscopic techniques have been employed to identify oxidized flavonoid products. Although many oxidized derivatives have been generated from commercially available starting materials, few studies have developed a sequence of flavonoid substrates with a particular hydroxylation pattern to probe the mechanism of flavonoid oxidation. Here, we use AIBN (2,2′-azobisisobutyronitrile) in combination with a series of hydroxylated flavonols to probe the mechanism of flavonoid dimer formation and the role of singly or doubly oxidized species in generating and promoting oxidized flavonoid products. 3-Methoxyquercetin (3-hydroxyl group blocked) and luteolin (lack of 3-hydroxyl) were reacted with AIBN alone or with a second flavonol to serve as a C-3 hydroxyl donor to examine the mechanism of dimer formation. 3-Hydroxyflavones with increasing hydroxyl substitutions in the B ring were also reacted with AIBN in the presence or absence of an external nucleophile to examine the role of various hydroxyls in the formation of a carbocation intermediate via a doubly oxidized species. The presence of a free C-3 hydroxyl, coupled with a B ring ortho hydroxy unit, appears essential for dimer formation. An increase in the number of hydroxyls in the B ring facilitates products generated from a doubly oxidized species.

Introduction

Flavonoids are ubiquitous throughout the plant kingdom and, as a result, are an integral part of the human diet (*1*). The consumption of foods rich in flavonoids is inversely correlated with the risk of heart disease, and chemopreventive properties associated with these polyphenolic compounds have been ascribed to their capacity to scavenge free radicals and/or chelate metal ions (*2*). Quercetin has been shown to serve as a chain-breaking antioxidant that traps peroxyl radicals and thus suppresses radical chain propagation (*3*, *4*). Oxidation mechanisms vary with the oxidizing agent, and the mechanism described for a specific oxidizing agent may not directly apply to biological antioxidant systems. For example, the oxidation of quercetin with a superoxide anion radical is thought to generate a C-2-centered anion followed by a cyclic peroxide rearrangement to form the depside product (*5*, *6*), while AIBN[1]-generated azo radicals have been proposed to proceed at least in part through a carbocation intermediate at the C-2 of quercetin with subsequent attack by water or peroxide to form 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)benzofuranone or the depside, respectively (*7*). However, whatever the oxidation mechanism, the quenching of radical species and the termination of the radical chain reaction usually proceed by the coupling of two radical intermediates, either within a single molecule or between two separate chemical species and subsequent inter- and/or intramolecular reactions to generate nonradical product(s).

Flavonoid oxidation by either hydrogen atom abstraction or electron donation results in the formation of carbon- or oxygen-centered radical species. Such radical intermediates have been invoked to explain the array of oxidized products observed (*8*−*11*). The subsequent second oxidation of the initially formed radical species either by hydrogen atom abstraction or by disproportionation is known to produce quinone or quinone methide intermediates (*12*−*14*).

Earlier studies on the oxidation of quercetin carried out in our laboratory (*7*) and elsewhere (*15*) indicated the formation of novel dimers that were linked through a dioxane linkage following the formation of two σ-bonds. Although the dimers were characterized unequivocally, the mechanistic pathway involved and the structural requirements for their formation remained unclear. We envisaged that the appropriate manipulation of the functional groups on the flavonoid skeleton and the use of suitable donors with a free C-3 hydroxyl could lead to the identification of the intermediate species involved, resulting in a greater understanding of the mechanism of dimer formation in these systems. The present study is aimed at investigating the formation of such doubly linked dimers by examining the role of C-3 hydroxyl substitution. In addition, this study examines the role of hydroxyl groups in B and C rings in generating doubly oxidized, i.e., *o*-quinone or *p*-quinone methide species, vs singly oxidized or a C-2 radical species in the process of

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[1] Abbreviations: AIBN, 2,2′-azobisisobutyronitrile; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); DPPH, diphenylpicrylhydrazyl; CAN, ceric ammonium nitrate; ESI-MS, electrospray ionization mass spectrometry; APCI-MS, atmospheric pressure chemical ionization mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; MS-MS, tandem mass spectrometry; ROESY, rotating frame Overhauser effect spectroscopy; HF, hydroxyflavone.

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quenching peroxyl radical species generated from in vitro AIBN-initiated oxidation reaction.

Materials and Methods

Chemicals. 4-Hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic-acid, phloroglucinol, methoxyacetonitrile, and AIBN were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 4-Hydroxybenzoic acid used as an internal standard was obtained from Acros Organics (Fischer Scientific, Houston, TX), and 3-hydroxyflavone (3-HF, **4**) was purchased from Indofine Chemical Co. (Somerville, NJ). Acetonitrile and methanol were HPLC grade, and other chemicals were reagent grade. 3-Methoxyquercetin (**1**), 3,4′-dihydroxyflavone (**5**), 3,3′,4′-trihydroxyflavone (**6**), and 3,3′-dihydroxy-4′-methoxyflavone (**7**) were synthesized in our laboratory. Diazomethane was generated from diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co.) following standard procedures.

Synthesis of 3-Methoxyquercetin (1). It was prepared following the procedure of Anand et al (*16*).

Synthesis of 3,4′-Dihydroxyflavone (5). This was prepared by the modified AFO procedure (*17*). 4-Hydroxybenzaldehyde (5.0 g) was refluxed with benzyl bromide (12 mL) and K_2CO_3 (11 g) in acetone (200 mL) overnight to yield 4-benzyloxybenzaldehyde (8.4 g, 97%) after the usual workup. 4-Benzyloxybenzaldehyde (1.56 g) was then condensed with *o*-hydroxyacetophenone (0.88 mL) using NaOH (1 g/1.5 mL) in ethanol (20 mL). The chalcone formed was oxidized subsequently by adding a combination of H_2O_2 (3 mL, 30%) and NaOH (2.5 mL, 20%). The reaction mixture was then acidified using 10% H_2SO_4 to yield crude 3-hydroxy-4′-benzyloxyflavone (1.8 g). The crude 3-hydroxy-4′-benzyloxyflavone (500 mg) was hydrogenated for 8 h at atmospheric pressure in EtOAc using 10% Pd/C as a catalyst. The EtOAc layer was filtered and concentrated to yield crude **5**. Pure **5** (200 mg), with an overall yield of 54% from 4-benzyloxybenzaldehyde, was obtained by crystallization from a minimum quantity of methanol.

Synthesis of 3,3′,4′-Trihydroxyflavone (6). This was prepared following a procedure similar to that of Ozawa et al. (*18*). Dibenzyloxybenzaldehyde (5.0 g) was condensed with *o*-hydroxyacetophenone (2 mL) to obtain the corresponding chalcone (6 g, 87%). The finely powdered chalcone (2 g) was oxidized using a combination of H_2O_2 (3 mL, 30%) and NaOH (25 mL, 16%) to yield the crude 3-hydroxy-3′,4′-dibenzyloxyflavone (2 g). Subsequent debenzylation by hydrogenation at atmospheric pressure using 10% Pd/C as a catalyst yielded crude **6** (1.2 g). Crystallization from a minimum amount of methanol resulted in pure **6** (700 mg), in an overall yield of 54% from the chalcone.

Synthesis of 3,3′-Dihyroxy-4′-methoxyflavone (7). This was prepared following a similar procedure as that of **6**, by condensing 3′-benzyloxy-4′-methoxybenzaldehyde with *o*-hydroxyacetophenone, with an overall yield of 10%.

Oxidation with AIBN (Condition 1). AIBN (20 equiv) was added to a solution of the flavonoid [**1**, luteolin (**2**), **4**, **5**, **6**, or **7**] in CH_3CN (250 mL). The temperature of the reaction mixture was maintained at 60 °C for 3 h. Acetonitrile was then removed under reduced pressure, and the resulting solid was washed with hexane to remove the unreacted AIBN.

Oxidation with AIBN in the Presence of External Nucleophiles and under Modified pH. Compounds **4**−**7** (50 mg) were dissolved in a total volume of 250 mL of solvent with a varied ratio of acetonitrile/methanol/ethanol/concentrated HCl. To this solution, AIBN (20 equiv) was added and the reaction mixture was stirred at 60 °C for 3 h. Differences in the reaction mixtures are noted as follows: condition 2 (low pH), 100 μL of concentrated HCl was added to a solution of the flavonoid in acetonitrile (250 mL); condition 3 (external nucleophile), 10 mL of MeOH was added to the solution of the flavonoid in acetonitrile (240 mL); condition 4 (low pH with methanol as the external nucleophile), 100 μL of concentrated HCl was added to a solution of the flavonoid in acetonitrile (240 mL) and methanol (10 mL); and condition 5 (low pH with ethanol as the external nucleophile), 100 μL of concentrated HCl was added to a solution of the flavonoid in acetonitrile (240 mL) and ethanol (10 mL).

Oxidation with AIBN in the Presence of a Second HF. AIBN (20 equiv) was added to a solution of **1** or **2** (100 mg) in CH_3CN (250 mL). The reaction mixture was maintained at 60 °C for 30 min. Then, a CH_3CN solution of **4** (1.2 equiv) or **5** was added to the mixture and the reaction was continued for a further 2.5 h at 60 °C. At the end of the reaction, CH_3CN was removed under reduced pressure and the resulting solid was washed with hexane to remove unreacted AIBN.

Oxidation of **1** in the presence of **4** was repeated four times, and the pooled products were passed through a silica gel column (70−230 mesh) eluting first with $CHCl_3$ and then with $CHCl_3$ with an increasing proportion of MeOH. Fractions with common TLC profiles were pooled and subsequently run through sephadex LH-20 columns eluted with MeOH to yield the pure dimer **8** (75 mg, 11%). The reaction of **1** and **5** was performed four times. The pooled reaction mixture was run through sephadex LH-20 columns eluting with MeOH to yield the pure dimer **9** (100 mg, 14%).

Purification of Oxidized Products from the 3,3′,4′-Trihydroxyflavone−AIBN Reaction. The 3′,3′,4′-trihydroxyflavone oxidation reaction was run 10 times, and the pooled products were passed through a silica gel (70−230 mesh) column (55 cm × 2 cm) eluting first with $CHCl_3$ and then with $CHCl_3$ with an increasing proportion of MeOH. Fractions containing the dimer (**21**) were pooled and methylated using diazomethane. The crude methylated mixture was passed through silica gel columns eluting with EtoAc−hexane. Further purification by preparative TLC yielded the pure methylated dimer (**22**, 6 mg). Fractions containing the depside (**18**) were pooled and methylated. Silica gel purification using hexanes−EtOAc as the solvent system yielded the methylated depside (**20**, 21 mg).

Methylation of Heterodimer 8. To a suspension of **8** (45 mg, 82 μmol) and silica gel (catalytic/100−200 mesh) in MeOH, an excess of diazomethane (100 equiv) in ether was added, and the reaction mixture was allowed to stir overnight. Purification over sephadex LH-20 eluting with methanol and preparative TLC over silica gel using EtoAc−hexane as the solvent system yielded the methylated derivative **10** (8 mg, 17%).

Methylation of Heterodimer 9. To a suspension of **9** (100 mg, 0.18 mmol) and silica gel (catalytic/100−200 mesh) in MeOH, an excess of diazomethane (100 equiv) in ether was added and the reaction was allowed to stir overnight. Purification over sephadex LH-20 eluting with MeOH yielded the methylated derivative **11** (55 mg, 50%).

Product Quantification and Data Analysis. The oxidized products were quantified by a Hewlett-Packard 1100 series HPLC system that was equipped with a variable wavelength UV detector. Two milligrams of 4-hydroxybenzoic acid as an internal standard was added to the reaction mixtures at the end of the reaction. The components of the reaction mixture (10 μL) were separated on an Econosphere C18 column (250 mm × 2.1 mm, 5 μm, Alltech) using a water containing 0.1% CF_3COOH (v/v)/methanol gradient and detected at 254 nm. The mobile phase gradient program was started with 40% methanol, maintained at 40% for 10 min, linearly increased to 80% within 25 min, and kept at 80% for another 15 min. The flow rate was set to 0.2 mL/min. ANOVA was performed using SAS statistical software. Means were separated using Tukeys mean separation method (*19*) at a P value less than 0.05.

LC-MS. Analyses were performed on a Thermo Separation Products HPLC system coupled with a photodiode array (PDA) detector and a Thermo Finnigan (San Jose, CA) LCQ deca mass spectrometer in sequence. An Alltech Altima C18 column (250 mm × 2.1 mm, 5 μm) was used for separation using the same mobile phase gradient program described in the product quantification section. Formic acid (1%) instead of trifluoracetic acid was used as a mobile phase modifier to facilitate ionization. A

flow was set at 0.2 mL/min and directed to a PDA detector and then a quadrupole ion trap mass spectrometer via an ESI or APCI interface. The mass spectrometer was operated either in a negative or in a positive mode or both depending on the nature of the compound. MS-MS was performed as needed.

Electron Impact (EI) MS. Samples were introduced inside a GCQ plus quadrupole ion trap mass spectrometer using a direct insertion probe operated in an EI mode at 50 eV.

^{1}H and ^{13}C NMR Spectroscopy. ^{1}H, ^{13}C, and two-dimensional ^{1}H−^{13}C correlated NMR spectra were obtained in DMSO-d_6/CD_3CN/CD_3OD using a Bruker drx 500 MHz instrument.

11a-Hydroxy-9-(3-methoxy-5,7-dihydroxy-4*H*-1-benzopyran-4-on-2-yl)-5a-phenyl-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (8). ^{1}H NMR (CD_3OD, 500 MHz): 3.77 [s, 3H, C-3 OMe (C*)], 6.15 [d, J = 2 Hz, 1H, H-6 (A*)], 6.34 [d, J = 2 Hz, 1H, H-8 (A*)], 7.05 [dd, J = 8 Hz and 2 Hz, 1H, H-8 (A)], 7.09 [t, J = 8 Hz, 1H, H-6 (A)], 7.18 [d, J = 8.5 Hz, 1H, H-5′ (B*)], 7.28−7.34 [m, 3H, H-3′ (B), H-4′ (B), H-5′ (B)], 7.58 [t, J = 8 Hz, 1H, H-7 (A)], 7.70−7.84 [m, 5H, H-2′ (B), H-6′ (B); H-2′ (B*), H-6′ (B*), H-5 (A)]. ^{13}C NMR (CD_3OD, 125 MHz): 60.70 [O*Me* (C*)], 92.19 [C-3 (C)], 94.85 [C-8 (A*)], 99.87 [C-6 (A*)], 102.61 [C-2 (C)], 105.96 [C-4a (A*)], 118.61 [C-5′ (B*), C-2′ (B)], 118.92 [C-6′ (B)], 119.15 [C-8 (A)], 120.09 [C-4a (A)], 123.89 [C-6 (A)], 124.34 [C-2′ or C-6′ (B*)], 126.43 [C-1′ (B*)], 128.39 [C-5 (A)], 129.09 [C-3′ or C-5′ (B)], 131.09 [C-4′ (B)], 135.22 [C-1′ (B)], 138.86 [C-7 (A)], 140.22 [C-3 (C*)], 142.33 [C-3′ (B*)], 144.30 [C-4′ (B*)], 156.25 [C-2 (C*)], 158.32 [C-7 (A*)], 159.32 [C-8a (A)], 163.03 [C-5 (A*)], 166.04 [C-8a (A*)], 179.92 [C-4 (C*)], 187.84 [C-4 (C)]. EI-MS: 551.9 $[M]^+$, 317.1 [3-Q-OMe + H]$^+$, 105, 91.

11a-Hydroxy-9-(3-methoxy-5,7-dihydroxy-4*H*-1-benzopyran-4-on-2-yl)-5a-(4-hydroxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (9). ^{1}H NMR (CD_3OD, 500 MHz): 3.76 [s, 3H, C-3-OMe (C*)], 6.16 (d, 1H, J = 2.5 Hz, H-6 (A*)], 6.35 [d, 1H, J = 2 Hz, H-8 (A*)], 6.69 [d, 2H, J = 8.5 Hz, H-3′ (B), H-5′ (B)], 7.04 [d, 1H, J = 8 Hz, H-8 (A)], 7.09 [t, 1H, J = 8 Hz, H-6 (A)], 7.19 [d, 1H, J = 8.5 Hz, H-5′ (B*)], 7.55−7.57 [m, 3H, H-2′ (B), H-6′ (B), H-7 (A)], 7.69 [d, 1H, J = 2.5 Hz, H-2′ (B*)], 7.74 [dd, 1H, J = 8.5 Hz, 2.5 Hz, H-6′ (B*)], 7.80 [dd, 1H, J = 8 Hz, 1.5 Hz, H-5 (A)]. ESI-MS-MS: 569 $[M + H]^+$, 550.8 $[(M + H) - H_2O]^+$, 315 [(3-methoxyquercetin subunit) + H]$^+$, 255.1 [(3,4′-dihydroxyflavone subunit) + H]$^+$.

11a-Methoxy-9-(3,5,7-trimethoxy-4*H*-1-benzopyran-4-on-2-yl)-5a-phenyl-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (10). ^{1}H NMR ($CDCl_3$, 500 MHz): 3.75 [s, 3H, C-3 OMe (C)], 3.89 [s, 3H, C-7 OMe (A*)], 3.92 [s, 3H, C-3 OMe (C*)], 3.96 [s, 3H, C-5 OMe (A*)], 6.34 [d, 1H, J = 2 Hz, H-6 (A*)], 6.50 [d, 1H, J = 2.5 Hz, H-8 (A*)], 7.08 [d, 1H, J = 8.5 Hz, H-8 (A)], 7.12 [t, 1H, J = 7.5 Hz, H-6 (A)], 7.17 [d, 1H, J = 9 Hz, H-5′ (B*)], 7.34−7.39 [m, 3H, H-3′ (B), H-4′ (B), H-5′ (B)], 7.58 [dt, 1H, J = 8 Hz, 1 Hz, H-7 (A)], 7.73−7.76 [m, 2H, H-2′ (B), H-6′ (B)], 7.83−7.89 [m, 3H, H-5 (A), H-6′ (B*), H-2′ (B*)]. ESI-MS-MS: 617.1 $[M + Na]^+$, 595.1 $[M + H]^+$, 343 [5,7-dimethoxy-3-methoxyquercetin subunit]$^+$, 315, 253 [3-methoxyflavone subunit]$^+$.

11a-Methoxy-9-(3,5,7-trimethoxy-4*H*-1-benzopyran-4-on-2-yl)-5a-(4-methoxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (11). ^{1}H NMR: 3.76 [bs, 3H, C-3 OMe (C)], 3.78 [s, 3H, C-4′ OMe (B)], 3.89 [s, 3H, C-7 OMe (A*)], 3.92 [s, 3H, C-3 OMe (C*)], 3.95 [s, C-5 OMe (A*)], 6.34 [d, 1H, J = 2.5 Hz, H-6 (A*)], 6.49 [d, 1H, J = 2.5 Hz, H-8 (A*)], 6.86 [d, 2H, J = 9 Hz, H-3′ (B), H-5′ (B)], 7.06 [d, 1H, J = 8.5 Hz, H-8 (A)], 7.10 [t, 1H, J = 7.5 Hz, H-6 (A)], 7.16 [d, 1H, J = 9 Hz, H-5′ (B*)], 7.56 [t, 1H, J = 7 Hz, H-7 (A)], 7.68 [d, 1H, J = 9 Hz, H-2′ (B), H-6′ (B)], 7.83−7.88 [m, 3H, H-2′ (B*), H-5 (A), H-6′ (B*)]. ESI-MS: 647 $[M + Na]^+$, 625 $[M + H]^+$, 343 [5,7-dimethoxy 3-methoxyquercetin subunit]$^+$, 315, 283.

2-(3,4-Dimethoxybenzoyloxy)methylbenzoate (20). ^{1}H NMR ($CDCl_3$, 500 MHz): 3.75 (s, 3H, $COOCH_3$), 3.97 (s, 3H, $ArOCH_3$), 3.98 (s, 3H, $ArOCH_3$), 6.97 (d, J = 8.5 Hz, 1H, H-5′), 7.24 (dd, J = 8 Hz and 1 Hz, 1H, H-8), 7.36 (dt, J = 8 Hz and 2 Hz, 1H, H-6), 7.61 (dt, J = 8 and 2 Hz, 1H, H-7), 7.70 (d, J = 2 Hz, 1H, H-2′), 7.89 (dd, J = 8.5 and 2 Hz, 1H, H-6′), 8.07 (dd, J = 8 Hz and 2 Hz, 1H, H-5). ^{13}C NMR ($CDCl_3$, 125 MHz): 52.17 ($COOCH_3$), 56.04 ($ArOCH_3$), 56.07 ($ArOCH_3$), 110.42 (C-5′), 112.42 (C-2′), 121.87 (C-1′), 123.55 (C-4a), 124.01 (C-8), 124.54 (C-6′), 125.94 (C-6), 131.83 (C-5), 133.77 (C-7), 148.75 (C-3′), 150.87 (C-8a), 153.51 (C-3′), 165.06 (C-2/$COOCH_3$), 165.08 ($COOCH_3$/C-2). EI-MS: 316 $[M]^+$, 285 $[M - 31]^+$, 165 $[C_9H_9O_3]^+$, 137 $[165 - CO]^+$, 122 $[137 - CH_3]^+$, 77 $[C_6H_5]^+$.

11a-Methoxy-9-(3-methoxy-4*H*-1-benzopyran-4-on-2-yl)-5a-(3,4-dimethoxyphenyl)-5,6,11-hexahydro-5,6,11-trioxanaphthacene-12-one (22). ^{1}H NMR ($CDCl_3$, 500 MHz): 3.79 [3H, s, C-3 OMe (C)], 3.84 [3H, s, C-3′ OMe (B)], 3.85 [3H, s, C-4′ OMe (B)], 3.94 [3H, s, C-3 OMe (C*)], 6.79 [1H, d, J = 8.5 Hz, H-5′ (B)], 7.07 [1H, d, J = 8 Hz, H-8 (A)], 7.11 [1H, t, J = 8 Hz, H-6 (A)], 7.19 [1H, d, J = 9 Hz, H-5′ (B*)], 7.29 [1H, d, J = 7 Hz, H-6′ (B)], 7.14 [1H, d, J = 2.5 Hz, H-2′ (B)], 7.39 [1H, t, J = 8 Hz, H-6 (A*)], 7.52 [1H, d, J = 8.5 Hz, H-8 (A*), 7.57 [1H, t, J = 8 Hz, H-7 (A)], 7.68 [1H, dt, J = 8 Hz, 2 Hz, H-7 (A*)], 7.84 [1H, d, J = 7.5 Hz, H-5 (A)], 7.92−7.93 [2H, m, H-2′ (B*), H-6′(B*)], 8.23 [1H, dd, J = 8 Hz, 2 Hz, H-5 (A*)]. LC-MS-MS: 595.1 $[M + H]^+$, 563 $[(M + H) - CH_3OH]^+$, 313 $[(C_{15}H_7O_2(OCH_3)_3) + H]^+$.

3′,4′-Dihydroxy-2-ethoxy-3,4-flavandione-3-hydrate (24). LC-MS-MS: 313.0 $[M - H - H_2O]$, 267 $[M - 2H - OEt]$, 193, 164.9.

Results and Discussion

To examine the importance of the C-3 hydroxyl in dimer formation, two substrates were reacted separately with the radical generator AIBN: compound **1** with a blocked C-3 hydroxyl and compound **2** lacking a C-3 hydroxyl altogether. Although AMVN has been used widely as a mild radical generator, AIBN was preferred for the current studies as it resulted in a higher yield of the oxidized products and is in continuation with our preceding work on quercetin (*7*). Both of the substrates (**1** and **2**), when reacted with AIBN, did not result in dimers, and the starting material was recovered as such. To investigate the presence of any reactive species during the AIBN oxidation of **1** and **2**, **4** and **5** with a free C-3 hydroxyl were used as trapping agents for any radical species generated. The NMR and MS data of the purified products from the above reaction confirmed the generation of dimers. The major product resulting from the oxidative coupling of **1** and **4** was purified and subjected to spectral analyses. LC-MS-MS analyses using both negative APCI and positive ESI revealed two chromatographic peaks with identical nominal mass (552) and collision-induced dissociation mass spectra, suggesting the presence of stereo- and/or regioisomers. Characteristic ^{1}H NMR signals from the A ring protons of both **1** (H-6 at δ 6.15, H-8 at δ 6.34) and **4** (H-5 at δ 7.7−7.84) confirmed that the compound was a dimer consisting of one unit of **1** and one unit of **4**. The integration on the C-3 methoxyl signal of the 3-methoxyquercetin unit revealed the presence of an isomeric mixture of the dimer in the ratio of 3:2. The ^{13}C NMR showed the disappearance of the olefinic carbon signals (C-2 and C-3) from the 3-HF unit, whereas all of the carbon signals from the 3-methoxyquercetin unit were intact. Furthermore, the appearance of two quaternary carbon signals at δ 102.61 and δ 92.19, corresponding to C-2 and C-3 of the 3-HF unit, respectively, clearly indicated a dioxane linkage between the C2−C3 of the 3-HF unit and the B ring hydroxyls of the 3-methoxyquercetin unit as shown in structure **8** of Figure 2. All of the carbon signals were assigned based on HMQC, HMBC, and ^{1}H−^{1}H COSY experiments.

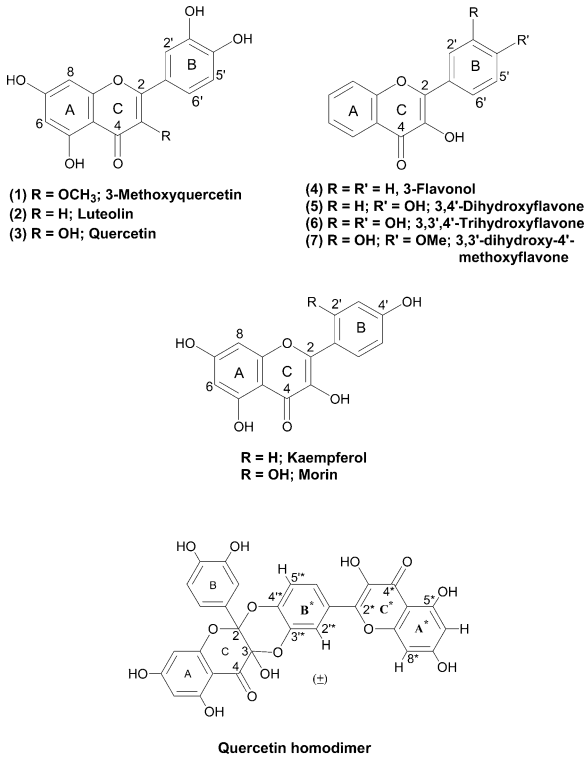


Figure 1. Chemical structures of various flavon-3-ols and the quercetin homodimer.

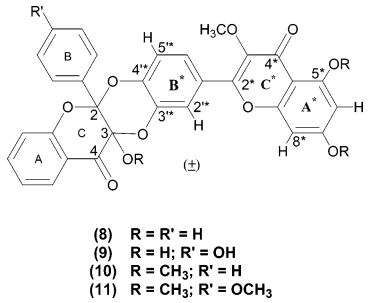


Figure 2. Chemical structures of various heterodimers.

Heterodimer **9** (Figure 2) was the predominant product (MW 568) obtained from the oxidative coupling of **1** with **5**. The ESI-MS and the ^{1}H NMR data were consistent with the structure of this mixed dimer (**9**). ^{1}H NMR integration of the methoxy signal at δ 3.76 and δ 3.79 and the A ring signals at δ 6.16, δ 6.17 and δ 6.35, δ 6.38 indicated a mixture of isomers in the ratio of 1:4.

It was of particular interest to determine the regio- and stereochemistry of the heterodimers to provide insight into the mechanism of their formation. The stereo- and regiochemistry of the dioxane linkage were ascertained by a combination of ROESY and geometry optimization studies (AM1) carried out on the syn and the anti isomers of the methylated dimer (**10**). The ROESY spectrum of the methylated dimer (**10**) indicated several informative correlations. The tertiary methoxy at C-3 of **4** showed a correlation with H-2′ of the 3-methoxyquercetin unit (Figures 2 and 3) and with H2′−H6′ of the same unit but none with the H-5′ of the 3-methoxyquercetin unit. The correlation with H-2′ and not with H-5′ indicates that the major isomer exists in a regioisomeric geometry of C2−C4′, C3−C3′ while the correlation with H2′−H6′ points to a syn geometry at the dioxane linkage. Prominent ROESY correlations for the methylated heterodimers **10** and **11** are shown in Figure 3. All of the correlations

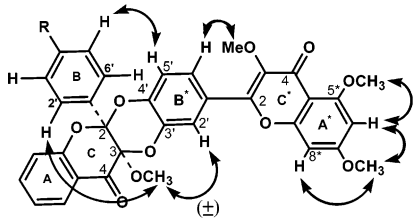


Figure 3. Structure of the methylated heterodimer showing prominent interactions observed in its ROESY spectrum.

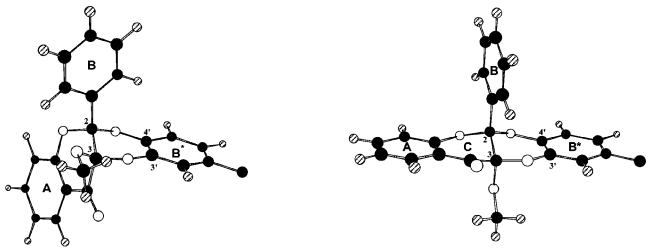
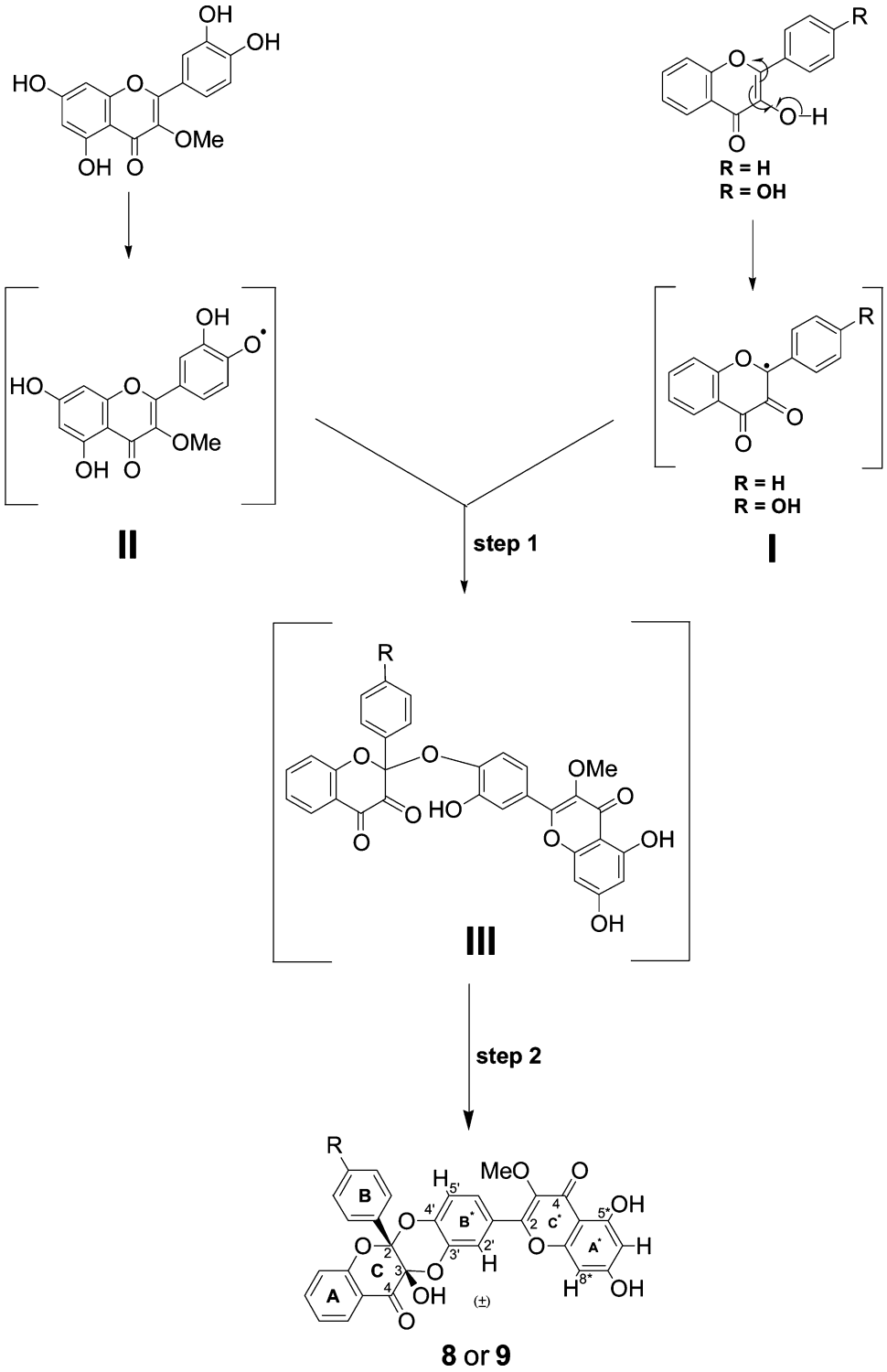


Figure 4. AM1-optimized partial structures of the syn and the anti isomer of the methylated heterodimer.

obtained from the ROESY spectrum were in agreement with the relative distances obtained through AM1 calculations (*20*). AM1 has been found to be a satisfactory method for predicting molecular geometry and energies for small molecules (*20*) as well as large molecular clusters (*21*). The geometries of the syn and the anti isomers of **10** were optimized using Gaussian 98 (*22*) (Figure 4). The spatial distances between the protons present in the 3-methoxyquercetin unit and the tertiary methoxy group at C-3 of the 3-HF unit were obtained from these optimized structures. For the syn isomer (**10A**), the distance between the tertiary methoxyl at C-3 and H-2′ (3-methoxyquercetin unit) was found to be 3.9 Å while the C3−H5′ (3-methoxyquercetin unit) distance was found to be 6.9 Å. All of the above data confirmed a regioselectivity of C2−C4′, C3−C3′ and a syn stereochemistry for the heterodimers. The calculated heats of formation for **10A** and **B** were found to be −187.0 and −183.4 kcal/mol, respectively (*23*), in agreement with the predicted structure.

The formation of flavonoid dimers through a dioxane linkage between the *o*-hydroxy groups in the B ring of one unit and the C2−C3 olefinic system in the second unit could proceed either via a radical intermediate (Scheme 1) forming the C2−C4′ linkage or via a Diels−Alder type cyclo addition through the intermediacy of *o*-quinone (Scheme 2). A regioisomeric geometry of C2−C4′, C3−C3′ points to a radical process in which radical species at C2 and the oxygen of C4′ would generate the first linkage. The second cyclization step to form the stable dioxane linkage being highly favored due to the proximity of the C-3′ hydroxyl of 3-methoxyquercetin unit and the C-3 carbonyl of the flavonol unit might proceed either through a radical or nucleophilic addition (Scheme 1). Initially, it would be expected that such a radical mechanism (Scheme 1) would also give rise to singly linked dimers. However, singly linked dimers such as **III** (Scheme 1) were not observed from the reaction of flavonols such as **5**, kaempferol, or morin (Figure 1) with AIBN (unpublished results). In such a reaction, if the entropy outweighs the enthalpy, singly linked dimers

Scheme 1. Proposed Mechanism for the Formation of the Heterodimer through the Radical Pathway

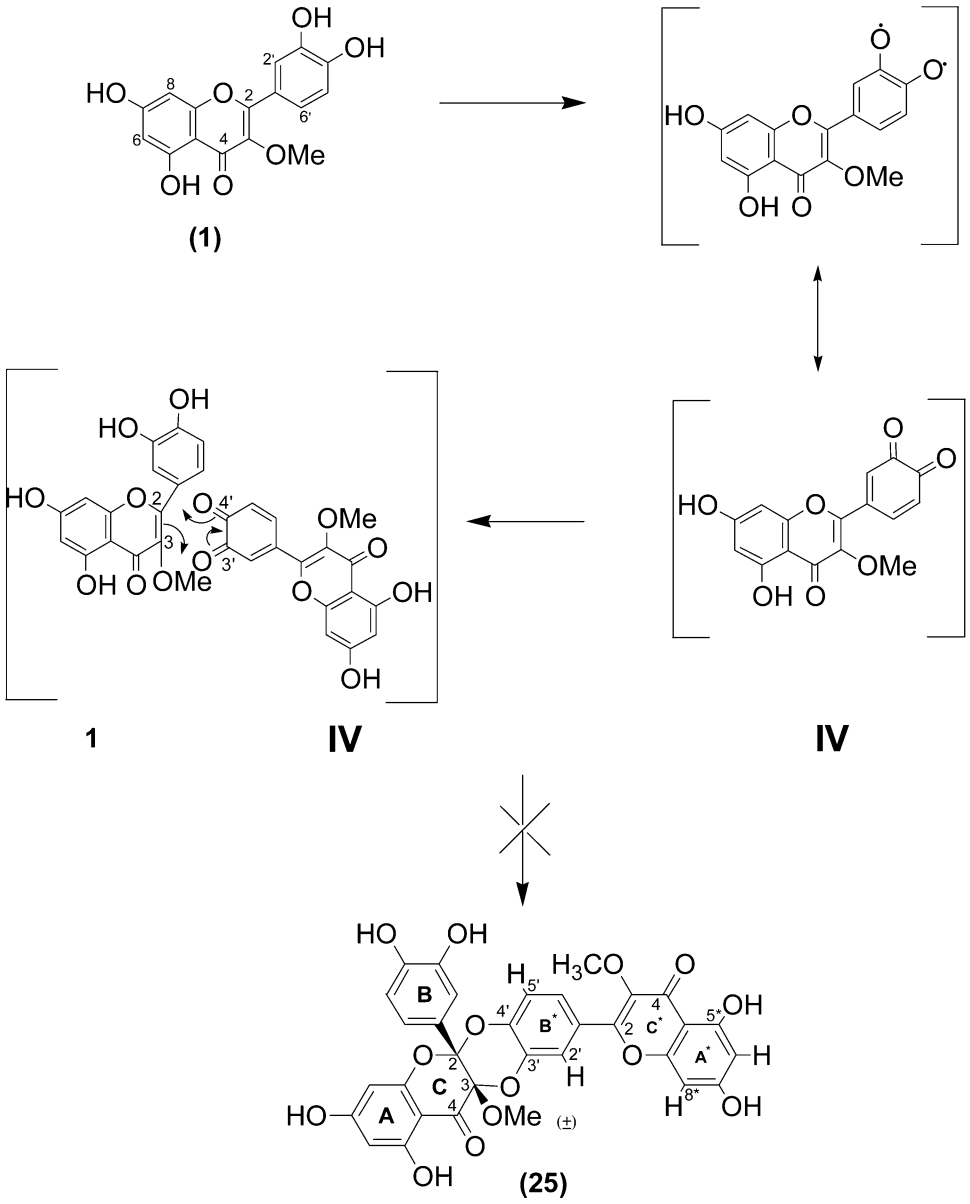


would not be observed. In contrast, the doubly linked dimers are stabilized by the six-membered dioxane linkages that preclude its dissociation. The absence of homodimer when the hydroxyl group at C3 is either blocked or absent confirms the importance of proton abstraction in initiating dimer formation and argues against the operation of a Diels−Alder type cycloaddition mechanism. In fact, the reaction of **1** with AIBN did not produce detectable levels of oxidized products and is in line with the earlier observations that blocking the 3-hydroxy group highly reduces the generation of observable oxidized products (*24*−*26*). The importance of a free C-3 hydroxyl group in dimer formation is further substantiated by the fact that AIBN oxidation of **1** in the presence of either **4** or **5** results in dimeric products.

LC-MS-MS analyses of the luteolin−flavonol reaction mixture also revealed the presence of a dimer with a nominal mass of 522, along with unreacted luteolin. As the mechanism of formation of this dimer is expected to be similar to that of **8**, the structure and stereochemistry of the dimer are also proposed to be analogous to the dimer **8**.

To examine the role of hydroxyl groups in B and C rings in generating doubly oxidized, i.e., *o*-quinone or

Scheme 2. Pathway Showing the Operation of a Possible Cyclic Mechanism



p-quinone methide species, vs singly oxidized or a C-2-centered radical species, 3-HFs with an increasing number of hydroxyl groups in the B ring, viz., **4**−**7**, were reacted with the radical generator AIBN. Compound **7** was chosen in order to understand the effect of an additional C-3′ hydroxyl on the secondary oxidation of the singly oxidized species. A blocked C-4′ hydroxyl would simplify the study by preventing the formation and thus the contribution of the *o*-quinone species toward a secondary oxidation process.

Compounds **4**, **5**, and **7** reacted with AIBN to give rise to corresponding depsides (**16** and **17**, Figure 5) as the predominant products along with trace amounts of substituted 3(*2H*)benzofuranones (**12** and **13**, Figure 5). These products were confirmed by LC-MS analysis of the reaction mixture.

The reaction of **6** with AIBN resulted in the formation of a depside (**18**), 2-(3,4-dihydroxybenzoyl)-2-hydroxy-3(*2H*)benzofuranone (**14**), and a homodimer (**21**) as major products as indicated by the LC-MS and LC-UV analyses of the crude reaction mixture. Although compounds with nominal masses of 274 and 286 corresponding to the depside and substituted 3(*2H*)benzofuranone were found in this mixture of oxidized flavonoids, difficulty was encountered in obtaining components in a pure form. However, methylation of depside-containing fractions by diazomethane yielded a stable, fully methylated depside derivative (**20**, Figure 5) that could be isolated pure for spectroscopic analyses. The ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, and MS data for the methylated depside were consistent with **20**.

Silica gel chromatography yielded fractions rich in the dimer component. Methylation of the dimer-enriched fractions by diazomethane yielded a more stable tetramethylated compound. The MS and ^{1}H NMR data were in agreement and indicated the presence of a fully methylated dimer.

It has been proposed that the depside is generated predominantly via a single oxidation reaction through the intermediacy of a C-2-centered radical species (*8*−*11*) and the benzofuranone via a double oxidation reaction through the intermediacy of a *p*-quinone methide species (*12*−*14*, *27*, *28*). To shed more light on the active intermediate species involved, we carried out the AIBN oxidation of **6**

Table 1. HPLC Peak Areas for the Oxidized Products of 6[a]

product	CH_3CN (condition 1)[b]	CH_3CN/H^+ (condition 2)[b]	CH_3CN/MeOH (condition 3)[b]	CH_3CN/MeOH/H^+ (condition 4)[b]
BF	0.65 ± 0.06[c]	1.09 ± 0.10	0.69 ± 0.03	0.27 ± 0.01
C-2-OMe	ND[d]	ND	ND	0.43 ± 0.02
Dep 1	0.83 ± 0.03	0.72 ± 0.03	0.69 ± 0.07	0.43 ± 0.01
dimer	1.42 ± 0.29	0.86 ± 0.11	0.94 ± 0.15	1.23 ± 0.16

[a] Values (× e^4) represent mean peak areas from four replications. [b] Condition 1, CH_3CN; condition 2, CH_3CN/H^+; condition 3, CH_3CN/MeOH; condition 4, CH_3CN/MeOH/H^+. [c] Indicates standard error. [d] Not detected.

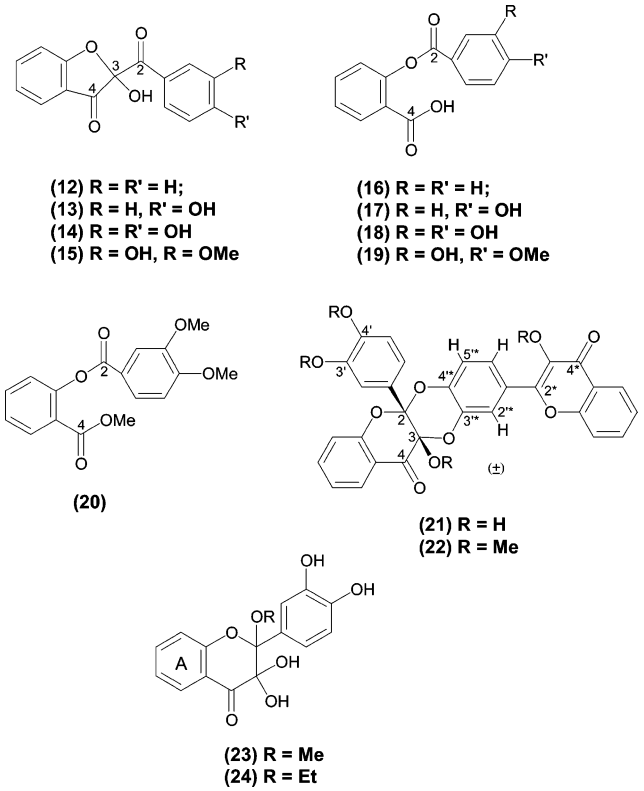
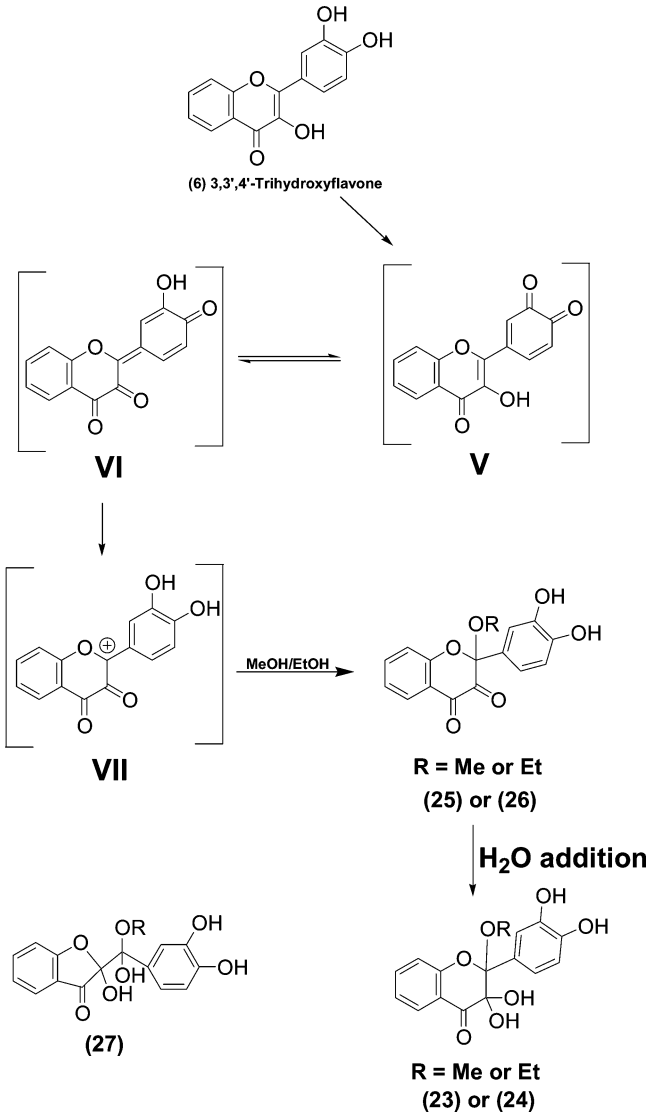


Figure 5. Structures of the oxidized products obtained from the AIBN oxidation of **6**.

in the presence or absence of external nucleophile under differing proton concentrations. The addition of external nucleophiles in flavonoid oxidation has been used to a great advantage to determine the nature of the reactive intermediates involved particularly in the carbocation or *p*-quinone methide species (*13*, *27*, *28*).

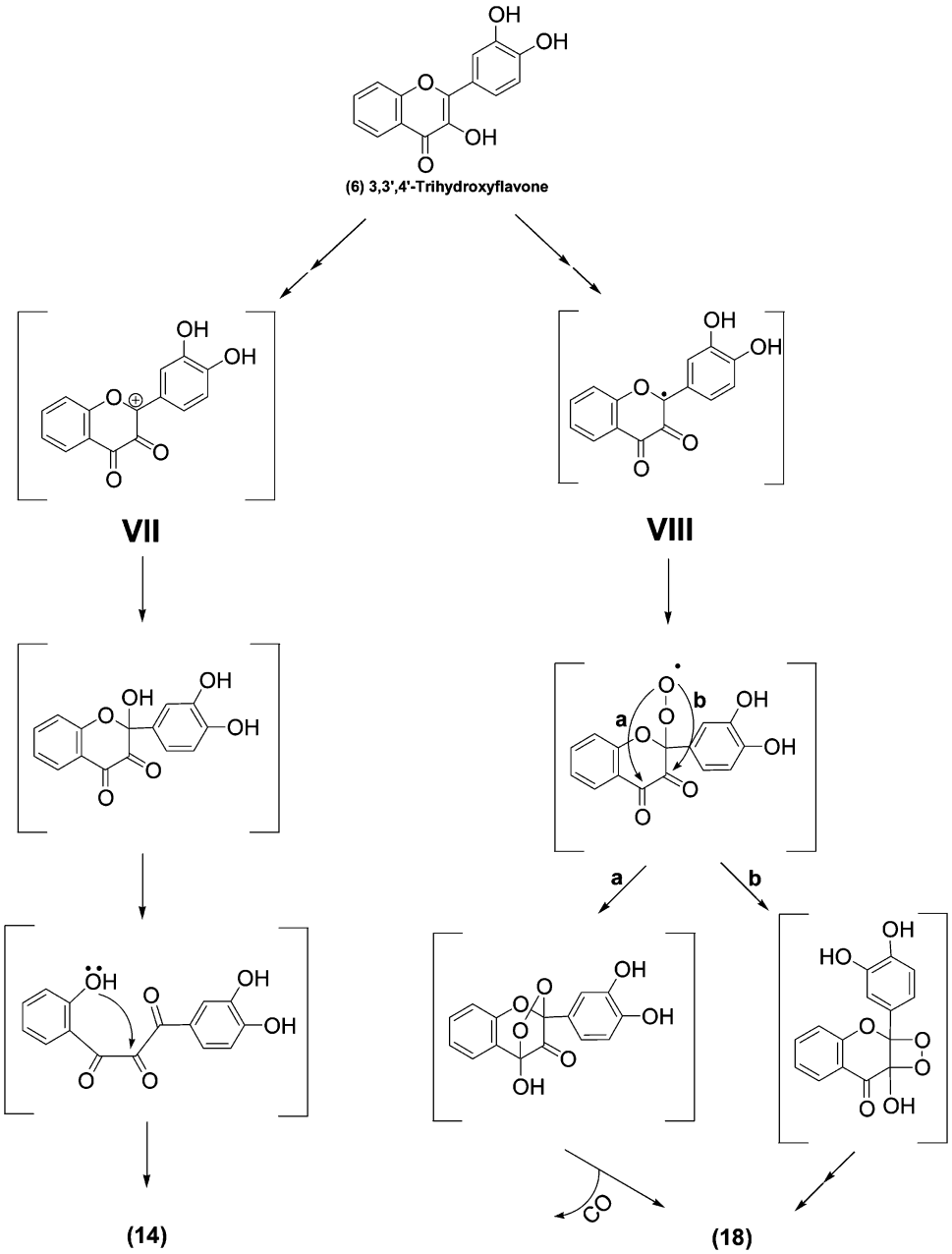
The abundance of the major oxidized products formed from **6**, i.e., the depside, the substituted 3(2*H*)benzofuranone, and the dimer, under different proton concentrations and external nucleophiles was measured (Table 1). The levels of substituted 3(2*H*)benzofuranone (**14**) and depside (**18**) decreased significantly in the presence of an external nucleophile at reduced pH (condition 4) as compared to that of acetonitrile alone ($p < 0.0001$). The decrease in the accumulation of products **14** and **18** was accompanied by the appearance of the methoxylated adduct **23** (Figure 5). When ethanol was used as the external nucleophile, formation of a 3,3′,4′-trihydroxyflavone−ethanol adduct (**24**) was observed. The formation of the methoxylated and ethoxylated adducts, confirmed by LC-MS analyses, suggests the predominance of doubly oxidized species, i.e., carbocation or *p*-quinone methide species. In an aqueous system, the attack of water on the carbocation/*p*-quinone methide species with subsequent attack on the C-2 carbonyl by an external nucleophile results in substituted 3(2*H*)benzofuranone (*27*).

Scheme 3. Proposed Mechanism for the Formation of the C-2 Adduct from 6



Although one-dimensional NMR and MS data cannot differentiate between a substituted 3(2*H*)benzofuranone (**27**) and **23/24** (Scheme 3), we have previously established through HMBC correlations that the C-2 adduct generated from quercetin under otherwise similar conditions yields a hydrated benzopyranone and not a 3(2*H*)-benzofuranone skeleton (*7*). Compound **6** and quercetin are identical in terms of B and C ring structures and thus can be expected to react similarly with AIBN. By analogy, methanol or ethanol is predicted to serve as the primary nucleophile (Scheme 3) resulting in **23** or **24** after water addition on **25** or **26**, respectively. The formation of the C-2 adduct in the case of **6** suggests the presence of a carbocation (**VII**, Scheme 3) or *p*-quinone methide species (**VI**, Scheme 3).

Scheme 4. Proposed Mechanism for the Formation of the Oxidized Products from 6 through Radical as Well as Carbocation Intermediates



Substituted **14** and **18** obtained from the reaction of **6** with AIBN can potentially be formed from two different intermediates (Scheme 4), i.e., the C-2-centered radical species (**VIII**, Scheme 4) and the *p*-quinone methide and/or the C-2-centered carbocation species (**VI** and **VII**, Scheme 3). The decrease in the levels of **14** and **18** in the presence of an external nucleophile at reduced pH as compared to that of acetonitrile alone and the corresponding increase in the amounts of the C-2-methoxylated adduct (**23**) most likely represent the level of carbocation/*p*-quinone methide species present in the reaction mixture. The remaining levels of **14** and **18** under condition 4 (CH_3CN/MeOH/H^+) (Table 1) then represent the contribution of the C-2 radical species (**VIII**) in the formation of **14** and **18**.

Oxidation of **4** and **5** with AIBN under varying conditions of external nucleophiles and proton concentrations revealed no formation of C-2 adducts as observed from the LC-MS analyses of the reaction mixtures. However, with **7**, trace amounts of the C-2 methoxy and C-2 ethoxy adducts were observed. In the case of **4** and **5**, this indicates a limited role for the doubly oxidized species and a greater role for the singly oxidized C-2-centered radical species and is in line with earlier studies on the oxidation of flavon-3-ols (*8*, *9*, *11*, *12*). In the case of **7**, an alternative oxidation pathway for the formation of the C-2-centered carbocation may exist via a second oxidation of the initially formed C-2-centered radical species. In other words, it appears that the additional C-3′ hydroxyl may lower the ionization potential of species such as **VIII**, significantly enough to allow secondary oxidation to form the carbocation such as **VII**.

Conclusions

Oxidation of 3-HFs has been studied under several oxidizing conditions including peroxidase oxidation (*28*), two electron copper ion-mediated oxidation (*29*−*31*), and

DPPH/CAN treatment (*13*). The advantages of using azo radical generators such as AIBN for probing oxidation mechanisms include the selectivity with which the radical generator yields peroxyl radicals, the ability to control oxidation rates by changing temperature and/or radical generator concentrations, and the stability of the oxidized product (*32*).

The presence of a free C-3 hydroxyl in combination with a B ring ortho hydroxy group present either within the same flavonoid unit or in a second flavonoid unit appears to be essential for the formation of doubly linked dimers under AIBN oxidation conditions. This can be understood from the fact that the formation of doubly linked dimers proceeds through a radical mechanism initiated by the formation of the C-2-centered radical, which then couples with the C-4′ oxygen radical species to finally yield the dimer after cyclization.

The presence of both a catechol unit in the B ring and a free C-3 hydroxyl as in **3** and **6** (Figure 1) appears to be a prerequisite for the formation of a carbocation or *p*-quinone methide species (**VII** and **VI**, Scheme 3). This can be understood by presuming that the formation of a *p*-quinone methide (**VI**, Scheme 3) proceeds predominantly through its tautomer the *o*-quinone (**V**, Scheme 3). Compounds **4** and **5** (Figure 1), which do not have a catechol unit in the B ring skeleton, cannot form an *o*-quinone species or generate its tautomeric form the *p*-quinone methide species. Compound **7** possesses an additional hydroxyl at C-3′ but is blocked at C-4′ and does not generate the *o*-quinone species or the C-2-centered carbocation to any significant amount.

To the best of our knowledge, this is the first report on the isolation and characterization of heterodimers from the reaction of 3-HFs with the radical generator AIBN. Furthermore, a comparative study of the products formed from the AIBN-mediated oxidation of 3-HFs having different substitution patterns in the B and C rings, in the presence and absence of an external nucleophile, provides insight as to the mechanism of formation of singly oxidized species, i.e., C-2-centered radical species and the doubly oxidized species, i.e., *p*-quinone methide or carbocation species with chemical oxidation.

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