Reactions of Genistein with Alkylperoxyl Radicals

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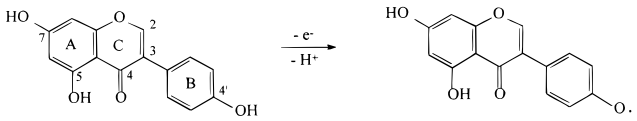
Antioxidant actions of the soy isoflavone genistein are believed to contribute to its overall chemopreventive activity. However, the mechanisms of its antioxidant reactions remain unknown. The objective of this study was to characterize the reaction products of genistein (5,7,4′-trihydroxyisoflavone) with peroxyl radicals generated by thermolysis of 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). Genistein oxidations with AMVN-derived peroxyl radicals yielded orobol (5,7,3′,4′-tetrahydroxyisoflavone), a hydroxylated derivative of genistein, and several stable adducts of 4′-oxogenistein with AMVN-derived radicals. Some of these adducts include novel structures resulting from secondary oxidations of the AMVN-derived moiety. For all the observed oxidation products, the modifications occurred on the B-ring of the molecule. Genistein oxidation product structures provide potentially useful markers of genistein antioxidant chemistry.

The potential role of soy products in cancer prevention has received much attention in recent years. Epidemiological data indicate that consumption of soybean-containing diets is associated with a lower incidence of certain human cancers in Asian than in Caucasian populations (*1*, *2*). Genistein is one of the two principal isoflavones found in soy (*3*) and, as such, has been the primary focus in studying the role of soy isoflavones in cancer prevention. Numerous biological activities have been reported for genistein, of which the protective effects against cancer are the most notable (*4*−*7*). Genistein is thought to act as an anticancer agent in large part through its ability to scavenge oxidants involved in carcinogenesis. It has been reported to inhibit tumor promoter-induced formation of hydrogen peroxide in vivo and in vitro in mouse skin (*8*). Other studies have demonstrated the antioxidant activity of genistein in a coupled linoleic acid/*β*-carotene system (*9*), in a liposomal system against UV or peroxyl radical-induced peroxidation (*10*), and against microsomal lipid peroxidation induced by the Fe(II)/ADP/NADPH system (*11*).

Genistein (Scheme 1) fulfills many of the structural requirements considered essential for effective radical scavenging by flavonoids and isoflavonoids. It has a C-2,3 double bond in conjugation with a 4-oxo function in the C-ring, which together can participate in electron delocalization from the B-ring (*12*). Additionally, the positions of its phenolic hydroxyl groups favor a high antioxidant activity. Previous structure−activity studies suggest that hydroxyl groups at positions C-5 and C-7 on the A-ring (*13*) and C-4′ of the B-ring (*14*, *15*) contribute to inhibition of lipid peroxidation.

Scheme 1. Formation of the Genistein Phenoxyl Radical by One-Electron Oxidation



Although published data describe structure−activity relationships for genistein antioxidant activity, the oxidative fate of genistein in mechanistic terms remains poorly understood. Like other phenolic antioxidants, genistein would primarily act as a chain-breaking antioxidant by scavenging peroxyl radicals, thereby suppressing radical chain autoxidations. Genistein has been proposed to react with peroxyl radicals by a single electron transfer followed by deprotonation (Scheme 1); however, the specific products formed during these antioxidant reactions have not been characterized.

The objective of this investigation was to identify reaction products formed by oxidation of genistein by peroxyl radicals. In the study presented here, we have succeeded in isolating and characterizing reaction products of genistein with alkylperoxyl radicals from AMVN[1] in a homogeneous acetonitrile system. The use of a selective generator of peroxyl radicals permitted selective study of the major radical-scavenging reaction of phenolic antioxidants. The oxidation products formed are orobol, a hydroxylated derivative of genistein, and a group of adducts of 4′-oxogenistein with AMVN-derived radicals.

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[1] Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); APCI-MS, atmospheric-pressure chemical ionization mass spectrometry; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography/mass spectrometry; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum coherence; LC/MS, liquid chromatography/mass spectrometry; MPLC, medium-pressure liquid chromatography; MS-MS, tandem mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; TMCS, trimethylchlorosilane; TMS, trimethylsilyl.

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These product structures indicate reactivity of the genistein B-ring in reactions with peroxyl radicals.

Experimental Procedures

Chemicals. *N*,*O*-(Bistrimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Pierce Chemical Co. (Rockford, IL). $H_2{}^{18}O$ was from Isotec, Inc. (Miamisburg, OH). Acetone-d_6 and CD_3CN were obtained from Cambridge Isotopes (Woburn, MA). All other reagents were of the highest purity available.

Genistein (**1**) was synthesized as yellowish needle-like crystals as described previously (*16*): UV (CH_3CN/H_2O) λ_{max} 200, 260 nm; GC/MS $[M - 15]^+$ *m*/*z* 471; positive APCI-MS $[M + H]^+$ *m*/*z* 271; negative ESI-MS $[M - H]^-$ *m*/*z* 269; positive high-resolution FAB-MS for $C_{15}H_{11}O_5$ $[M + H]^+$ calcd 271.0607, found 271.0608.

AMVN was obtained from Polysciences, Inc. (Warrington, PA): ^{1}H NMR (CD_3CN, 500 MHz) δ 1.95 (m, $C\underline{H}_2$), 1.83 (m, $C\underline{H}_2$), 1.65 (m, $C\underline{H}$), 1.50 (s, $C\underline{H}_3$), 0.87 (d, $J = 6.5$ Hz, $C\underline{H}_3$), 0.77 (d, $J = 6.5$ Hz, $C\underline{H}_3$); ^{13}C NMR (CD_3CN, 125 MHz) δ 120.12 (C≡N), 72.84 (C), 47.04 (CH_2), 25.79 (CH), 25.19 (CH_3), 23.80 (CH_3), 23.62 (CH_3).

Liquid Chromatography. HPLC analyses were conducted using a Hewlett-Packard model 1040M diode-array detector coupled to a Hewlett-Packard 1050 four-channel gradient pump (Hewlett-Packard, Palo Alto, CA) and an Allsphere ODS-2 HPLC column (5 μm, 4.6 mm × 250 mm, Alltech Associates, Deerfield, IL). System control and data analyses were performed using Hewlett-Packard Chemstation Software. The medium-pressure liquid chromatography (MPLC) system that was used was equipped with a Büchi 688 pump, a D-Star Instruments DFV-20 fixed-wavelength detector, a Linseis L200E recorder, and a Büchi borosilicate 3.3 column (15 mm × 460 mm) filled with Polygoprep 100-30 C18 (25−40 μm, Machery & Nagel, Düren, Germany).

Mass Spectrometry. LC/MS was performed on a Finnigan TSQ-7000 triple-quadrupole mass spectrometer using either an atmospheric-pressure chemical ionization (APCI) or electrospray ionization (ESI) source (Finnigan MAT, San Jose, CA). Spectra obtained with high-resolution fast atom bombardment mass spectrometry (FAB-MS) were recorded with a JEOL HX 110 instrument equipped with a FAB (Xe) ionization gun. GC/MS spectra were recorded on a Fisons-VG MD-800 analyzer coupled to a Carlo Erba 8000 gas chromatograph fitted with a Fisons A200S autosampler and an on-column injector (Fisons Instruments, Beverly, MA). Samples for GC/MS were derivatized with 200 μL of a BSTFA/TMCS mixture (10:1, v/v) at 60 °C for 60 min. TMS-derivatized samples were introduced into the GC/MS apparatus by on-column injection at 140 °C and separated on a 30 m × 0.25 mm DB-5MS column (J & W Scientific, Folsom, CA). One minute after injection, the column temperature was increased at a rate of 20 °C/min to 320 °C and held at that temperature for 20 min. Compound detection was conducted in the electron ionization mode at 70 eV. Fisons-VG Lab-Base system software was used for system control and data processing.

NMR Spectroscopy. ^{1}H, ^{13}C, and ^{1}H−^{13}C heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and nuclear Overhauser effect (NOESY) spectra were acquired using either a Bruker AMX-500 or a Bruker DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) instrument at 25 °C. Samples were dissolved in 0.6 mL of deuterated acetone or acetonitrile. All spectra were referenced to residual undeuterated acetone or acetonitrile.

Oxidation of Genistein with AMVN. Genistein was oxidized with a 10-fold molar excess of AMVN in acetonitrile. Typical oxidation mixtures contained 1 μmol of genistein and 10 μmol of AMVN in 7 mL of acetonitrile. Large-scale oxidations were carried out using 150 μmol of genistein and 1.5 mmol of AMVN in 250 mL of acetonitrile. The reaction mixture was incubated at 50 °C for 3 h. The oxidation was terminated at the end of 3 h by chilling the reaction mixture on ice. Excess AMVN was removed from the reaction mixture by extractions with hexane. The residue was evaporated in vacuo and stored at −80 °C until further analyses could be carried out.

Isolation of Reaction Products. The reaction mixture was first subjected to reverse-phase MPLC using a 0.1% aqueous CF_3COOH/CH_3CN mixture (60:40, v/v) as the mobile phase at a rate of 15 mL/min. This was changed to a 0.1% aqueous CF_3COOH/CH_3CN mixture (30:70, v/v) at 18 min and the run terminated at 30 min. MPLC runs yielded three crude fractions that were further separated using a HPLC system. Analytical-scale HPLC was conducted using gradient elution at a rate of 1 mL/min and detection at 260 nm. Initially, the mobile phase consisted of 100% solvent A ($CH_3CN/H_2O/CF_3COOH$, 28:72:0.1, v/v/v). At 15 min, solvent B (0.1% CF_3COOH in CH_3CN) was introduced in a linear gradient to 40% by 25 min. From 40 to 45 min, the system was returned to 100% solvent A in a linear gradient.

Genistein Oxidations in Acetonitrile and $H_2{}^{18}O$. Genistein (1 μmol) and AMVN (10 μmol) were dissolved in 5 mL of an acetonitrile/^{18}O-labeled water mixture (95:5, v/v) and heated for 3 h in a screw-cap test tube at 50 °C. The reaction mixture was extracted with hexane, and the acetonitrile fractions were evaporated in vacuo and then redissolved in the HPLC mobile phase. Products were purified by reversed-phase HPLC as described above. Orobol samples were collected and analyzed by positive APCI-MS. Control oxidations were conducted in acetonitrile and unlabeled water (95:5, v/v).

Results

AMVN-Initiated Peroxyl Radical Oxidations. Previous work from our laboratory and others has employed the azo initiator AMVN as a source of peroxyl radicals for studies of antioxidant chemistry. AMVN decomposes thermally to yield alkyl radicals ($R^\bullet$) which, in turn, react with molecular oxygen to generate peroxyl radicals ($ROO^\bullet$) (eqs 1 and 2) (*17*):

$$RN{=}NR \rightarrow 2R^\bullet + N_2 \quad (1)$$

$$R^\bullet + O_2 \rightarrow ROO^\bullet \quad (2)$$

Advantages of using AMVN for these studies include the selectivity with which AMVN yields peroxyl radicals, the ease of controlling oxidation rates (via the AMVN concentration and temperature), and the lack of transition metals used in other oxidant generating systems, which often degrade primary oxidation products.

HPLC Analysis of Oxidation Products of Genistein. Genistein was oxidized with peroxyl radicals generated by thermolysis of AMVN in oxygenated acetonitrile. These oxidations were conducted using a 10:1 molar ratio of AMVN to genistein. This ratio was selected because it resulted in a low rate of genistein oxidation. A low rate of oxidation ensured that genistein was the major component present in the reaction mixture at all times, thereby suppressing the generation of secondary oxidation products. Although this molar ratio appears high, thermolysis at 50 °C consumes AMVN with nearly zero-order kinetics and allows the oxidation of genistein to be reproducibly controlled. Analysis of the reaction mixture at 3 h by reverse-phase HPLC with diode-array UV detection (Figure 1) indicated the presence of residual genistein **1** and formation of five oxidation products **2**−**6**. Compound **2** was more polar than genistein, whereas the other oxidation products were less polar as suggested by RP-HPLC retention times.

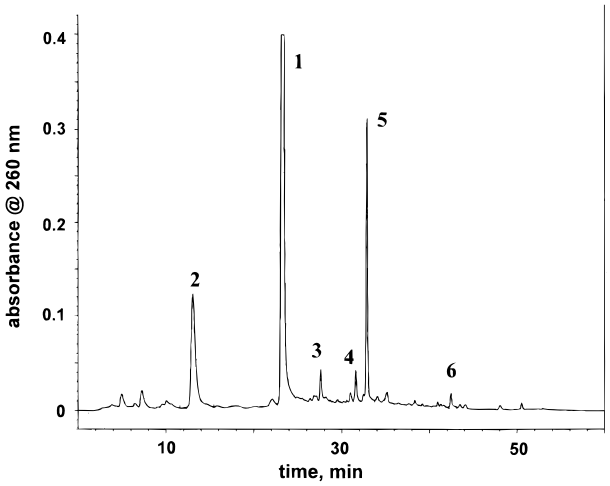

Figure 1. Reverse-phase HPLC analysis of oxidation products formed during the reaction of genistein and AMVN-derived peroxyl radicals in oxygenated acetonitrile for 3 h at 50 °C.

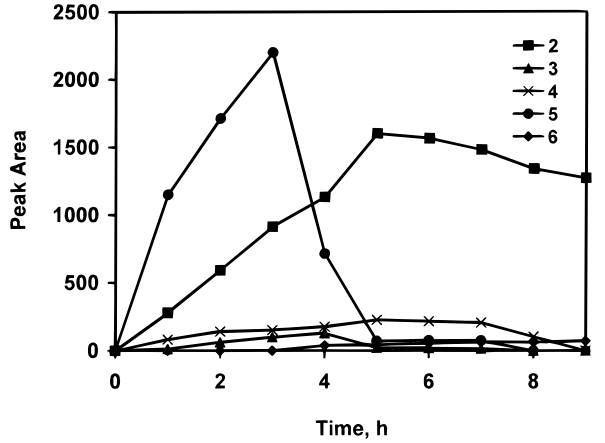

Figure 2. Time course of the appearance of oxidation products in oxygenated acetonitrile containing 1 μmol of genistein and 10 μmol of AMVN incubated at 50 °C.

Time Course Studies of Product Formation. The time course of formation of the oxidation products was investigated using HPLC analysis. The reaction was followed over 9 h, and the results are shown in Figure 2. Relative product yields were estimated from integrated peak areas. This is based on the similar UV/vis spectra of genistein and its oxidation products (vide infra) and the presumption of similar molar absorptivities. Time course studies indicated that oxidation products **2** (λ_{max} = 202 and 260 nm) and **5** (λ_{max} = 210 and 254 nm) were formed in much greater yields than products **3** (λ_{max} = 208 and 254 nm), **4** (λ_{max} = 208 and 254 nm), and **6** (λ_{max} = 208 and 254 nm). The distribution of products changed over time. Compound **5** was the principal product formed until 3 h and then rapidly disappeared from the reaction mixture. Compound **2**, the other major oxidation product, was formed more gradually and was still present in the reaction mixture at the end of the 9 h time course study. Oxidation products **3** and **4** were formed in very low yields and had essentially disappeared by the end of the 9 h oxidation. The other minor oxidation product, compound **6**, began to form in very low yields after 3 h of oxidation and was still present in the reaction mixture at the end of the 9 h study.

Isolation and Characterization of Oxidation Products. The oxidation products were isolated by MPLC and HPLC and characterized on the basis of their spectroscopic data. GC/MS analysis of TMS-derivatized genistein yielded a small molecular ion at *m/z* 486 and a strong $[M - 15]^+$ base peak at *m/z* 471. In contrast, the TMS derivative of **2** exhibited a molecular ion at *m/z* 574 and a strong $[M - 15]^+$ ion at *m/z* 559. This mass corresponded to the molecular weight of fully derivatized genistein plus an additional OTMS group, suggesting that **2** was a hydroxylated derivative of genistein. Positive APCI-MS yielded a $[M + H]^+$ ion at *m/z* 287 which corresponds to the molecular weight of hydroxylated genistein. The presence of an additional oxygen in **2** was confirmed by high-resolution positive FAB-MS data which gave a $[M + H]^+$ at *m/z* 287.0549 for **2** (calcd 287.0556), corresponding to a molecular formula of $C_{15}H_{11}O_6$.

Further information about the position of the hydroxyl group on **2** was obtained by positive APCI-MS-MS analysis of the $[M + H]^+$ ion at *m/z* 287. Fragmentation of the *m/z* 287 ion yielded product ions at *m/z* 271, 259, 241, 231, 161, and 153. The ion at *m/z* 271 resulted from the loss of a hydroxyl group. The ions $[MH - CO]^+$, $[MH - H_2O - CO]^+$, and $[MH - COCO]^+$ at *m/z* 259, 241, and 231, respectively, represented typical losses for hydroxyisoflavones (*18*). The base peak observed at *m/z* 153 was attributed to a dihydroxylated A-ring fragment following retro-Diels−Alder rearrangement characteristic of all flavonoids. The presence of this fragment demonstrated that ring A in **2** had remained unchanged when compared to the parent genistein. The ion at *m/z* 161 had only been observed earlier with isoflavones as a rearrangement product for a dihydroxylated B-ring fragment (*18*). Taken together, the MS-MS data suggested that hydroxylation had occurred on ring B for product **2**.

In the ^{1}H NMR (CD_3CN) spectrum of **2** (Table 1), the signals of H-2 (δ_H 7.99) in ring C and H-6 (δ_H 6.25) and H-8 (δ_H 6.38) in ring A were similar to those observed earlier for genistein (*16*). The signals observed for ring B in **2** were a doublet at δ_H 6.86 (J = 8 Hz), a double doublet at δ_H 6.90 (J = 8 and 2 Hz), and a doublet at δ_H 7.04 (J = 2 Hz), indicating that the additional OH group is located at C-3′ in **2**. On the basis of the spectroscopic data and comparison to literature ^{1}H NMR data in acetone-d_6 (*19*, *20*), compound **2** was assigned as orobol (5,7,3′,4′-tetrahydroxyisoflavone) (Figure 3).

The other major oxidation product that formed, compound **5,** was very labile and decomposed readily. Compound **5** could not be analyzed by GC/MS as it decomposed in part to genistein during either sample workup or analysis. Positive APCI-MS analysis indicated a strong signal at *m/z* 271, which was equivalent to genistein. A small ion at *m/z* 444 observed in the positive APCI-MS spectrum and the base peak at *m/z* 442 in the negative ESI-MS spectrum suggested a product with a molecular weight of 443. This molecular weight was confirmed by positive high-resolution FAB-MS which gave a $[M + H]^+$ ion at 444.1288 (calcd 444.1295), corresponding to a molecular formula of $C_{22}H_{22}O_9N$ for **5**. The presence of a nitrogen atom in **5**, as deduced from the high-resolution MS data, together with the ^{1}H and ^{13}C NMR data suggested the incorporation of an AMVN-derived moiety on the genistein molecule.

The ^{1}H NMR spectrum of **5** (Table 1) showed the H-2 of ring C at δ_H 8.15 and the H-6 and H-8 of ring A at δ_H 6.24 and 6.34, respectively. The ^{13}C NMR data (Table 2) and the HSQC and HMBC correlations of the protons mentioned above indicated that rings A and C were

Table 1. ^{1}H NMR Chemical Shifts of Compounds 2−5 in CD_3CN

	δ_H			
position	**2**	**3**	**4**	**5**
2	7.99 (s)	8.14 (s)	8.13 (s)	8.15 (s)
3				
4				
4a				
5				
5-OH	12.96 (s)	12.56 (s)	12.50 (s)	12.52 (s)
6	6.25 (d) (J = 2 Hz)	6.26 (d) (J = 2 Hz)	6.25 (d) (J = 2.5 Hz)	6.24 (d) (J = 2 Hz)
7				
8	6.38 (d) (J = 2 Hz)	6.38 (d) (J = 2 Hz)	6.37 (d) (J = 2 Hz)	6.34 (d) (J = 3 Hz)
8a				
1′				
2′	7.04 (d) (J = 2 Hz)	7.20−7.16 (m)	7.19−7.15 (m)	7.17−7.13 (m)
3′		6.32 (br d) (J = 10.5 Hz)	6.31 (br d) (J = 10.5 Hz)	6.30 (br d) (J = 9.5 Hz)
4′				
5′	6.86 (d) (J = 8 Hz)	6.32 (br d) (J = 10.5 Hz)	6.31 (br d) (J = 10.5 Hz)	6.30 (br d) (J = 9.5 Hz)
6′	6.90 (dd) (J = 2 and 8 Hz)	7.20−7.16 (m)	7.19−7.15 (m)	7.17−7.13 (m)
1″				
2″a		2.14 (d) (J = 15 Hz)	2.13 (d) (J = 15.5 Hz)	2.26 (d) (J = 15 Hz)
2″b		2.07 (d) (J = 15 Hz)	2.06 (d) (J = 15 Hz)	2.16 (d) (J = 15 Hz)
3″				
4″		1.40[a] (s)	1.39 (s)	1.36 (s)
5″		1.39[a] (s)	1.38 (s)	1.33 (s)
6″				
7″		1.57 (s)	1.56 (s)	1.62 (s)

[a] Assignments interchangeable.

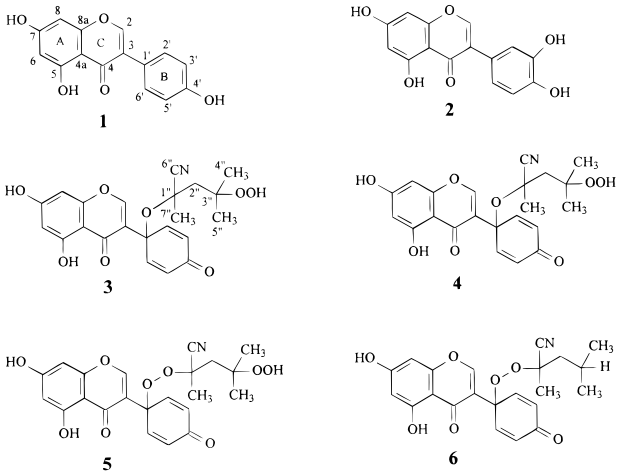

Figure 3. Structures of **1** (genistein), and oxidation products **2** (orobol) and **3**−**6**.

unchanged in **5** when compared to those of genistein. This left ring B as the only possible location where the modification could have occurred.

The NOESY spectrum revealed a small cross-peak between the H-2 of ring C and the H-2′ and H-6′ of ring B. Both B-ring protons (H2′ and H6′) showed HSQC correlations to two overlapping carbons at δ_C 147.1. The broad doublet at δ_H 6.30 (2H, J = 9.5 Hz), which correlated to two overlapping carbons at δ_C 131.2, was assigned to the H-3′ and H-5′ of the B-ring. The HMBC experiment revealed correlations between H-2′ and/or H-6′ and a carbonyl at δ_C 186.2, indicating that the 4′-hydroxyl in genistein was oxidized to a keto group in **5**. HMBC correlations between H-3′ and/or H-5′ and a quaternary carbon at δ_C 77.6 (C-1′) were also observed, indicating that the C-1′ location was the only possible connection between the B-ring of the isoflavone moiety and the AMVN-derived moiety in **5**.

For the AMVN-derived moiety, the ^{1}H NMR spectrum of **5** revealed three methyl groups at δ_H 1.33, 1.36, and 1.62 and two doublet signals of a methylene group at δ_H 2.26 (J = 15 Hz) and δ_H 2.16 (J = 15 Hz). The isopropyl proton which appears at δ_H 1.65 in AMVN was not seen in the spectrum of **5**, indicating a substituent at that position. Additionally, the isopropyl methyl groups in **5**, when compared to those in AMVN, exhibited HMBC correlations to a downfield-shifted quaternary carbon at δ_C 82.7 (C-3″) and to the methylene carbon at δ_C 45.2 (C-2″) (Figure 4). The methyl signal at δ_H 1.62 (C-7″) exhibited HMBC cross-peaks to the nitrile carbon at δ_C 121.1 (C-6″), to the methylene carbon C-2″, and to a quaternary carbon at δ_C 78.9 that was assigned as C-1″. When compared to those in AMVN, C-1″ and C-3″ in **5** experienced downfield shifts of 6.1 and 56.9 ppm, respec-

Table 2. ^{13}C NMR and HMBC Data of Compounds 3−5 in CD_3CN

	3		4	5	
position	δ_C[a]	HMBC	δ_C	δ_C	HMBC
2		3, 8a	157.4	157.1	3, 4, 8a
3	118.8		119.9	119.7	
4			180.9	180.6	
4a	105.9		106.7	106.5	
5	163.0		163.9	163.7	
5-OH		4a, 5, 6			4a, 5, 6
6	99.7		100.8	100.4	4a, 5, 7, 8
7			165.5	165.0	
8		4a, 6	95.3	95.0	4a, 6, 7, 8a
8a	158.0		159.1	158.8	
1′	76.7		78.0	77.6	
2′			147.1	147.1	4′, 6′
3′		1′, 5′	131.6	131.2	1′, 5′
4′			186.4	186.2	
5′		1′, 3′	131.6	131.2	1′, 3′
6′	146.4		147.1	147.0	2′, 4′
1″	66.1		67.2	78.9	
2″a	48.1	1″, 3″, 6″	49.4	45.2	1″, 3″−7″
2″b		1″, 3″, 6″			1″, 3″−7″
3″	83.0		84.0	82.7	
4″	25.2	2″, 3″, 5″	26.3	26.5	2″, 3″, 5″
5″	25.2	2″−4″	26.3	25.4	2″−4″
6″	123.1		124.3	121.1	
7″	29.9	1″, 2″, 6″	30.9	25.2	1″, 2″, 6″

[a] Based on HMBC data.

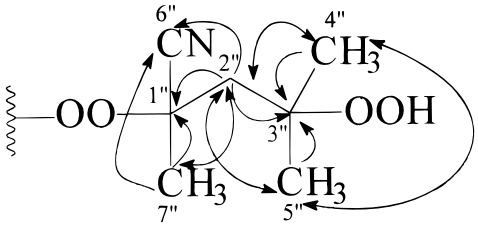

Figure 4. HMBC correlations (C → H) of the AMVN moiety of **5**.

tively, indicating oxygen substituents on both ends of the AMVN-derived moiety in **5**. On the basis of all spectroscopic data, the structure of **5** was assigned as 1′-(alkyldioxyhydroperoxide)-4′-oxogenistein. This is equivalent to 4′-oxogenistein with an OO-X-OOH substitution at the 1′-position of the molecule, where X corresponds to $C(CN)(CH_3)CH_2C(CH_3)_2$ derived from AMVN.

Compounds **3** and **4** could not be analyzed by GC/MS either as they apparently decomposed in part to genistein during sample workup or analysis. For both compounds, positive APCI-MS yielded a small peak at *m/z* 428 and negative ESI-MS showed a base peak at *m/z* 426. High-resolution positive FAB-MS gave a $[M + H]^+$ peak at 428.1357 (calcd 428.1346) for **3** and 428.1341 (calcd 428.1346) for **4**, corresponding to a molecular formula of $C_{22}H_{22}O_8N$ for both compounds. The MS data indicated that compounds **3** and **4** differed from **5** only by possessing one fewer oxygen. The ^{1}H NMR data for **3** and **4** were very similar to those of **5**, suggesting that all three compounds were structurally related. Since **3** and **4** were formed in very low yields and decomposed over periods of extended storage, it was very difficult to generate sufficient quantities of these compounds to conduct ^{13}C NMR and HMBC experiments.

We were able to acquire HMBC data for **3** and used the HMBC data to assign most of the ^{13}C NMR resonances for that compound. The ^{1}H, ^{13}C, and two-dimensional NMR data for the isoflavone moiety of **3** were very similar to those in **5**, suggesting that this part of the molecule remained unchanged in **3** when compared to **5**. The only changes observed in the NMR spectra of **3** belonged to the AMVN-derived moiety, indicating the loss of one oxygen in **3** when compared to **5**. As in **5**, the methyl group (δ_H 1.57, H-7″) geminal to the nitrile group showed HMBC correlations to the nitrile carbon C-6″, to the methylene carbon C-2″, and to the quaternary C-1″ that had experienced an upfield shift of 12.8 ppm in **3**, when compared to that in **5**. As in **5**, the methyl protons H-4″ (δ_H 1.40) and H-5″ (δ_H 1.39) showed HMBC correlations to the quaternary C-3″ (δ_C 83.0) that had experienced an upfield shift of only 0.3 ppm in **3**. On the basis of the NMR data for **3**, we concluded that the only location possible for the loss of one oxygen atom, as indicated by MS data, could be the connection between the isoflavone- and the AMVN-derived moieties between C-1′ and C-1″ in **3**, when compared to **5**. Negative ESI-MS-MS experiments confirmed the site of the oxygen loss in **3**. Fragmentation of the $[M - H]^-$ ion at *m/z* 442 yielded strong product ions at *m/z* 268 and 285. These fragments corresponded to masses of the 4′-oxogenistein moiety and 4′-oxogenistein moiety with one oxygen, respectively. In contrast to the negative ESI-MS-MS data of **5**, we did not observe a fragment at *m/z* 301, which would have corresponded to the 4′-oxogenistein fragment with two oxygens attached. On the basis of our MS and NMR data, **3** was determined to be 1′-(alkyloxyhydroperoxide)-4′-oxogenistein. This is equivalent to 4′-oxogenistein with an O-X-OOH substituent at the 1′-position, where X corresponds to $C(CN)(CH_3)CH_2C(CH_3)_2$ derived from AMVN.

We were able to generate sufficient quantities of compound **4** to acquire ^{13}C NMR data. The ^{13}C NMR data for **4** were very similar to the carbon data of **3** derived from HMBC experiments. Both compounds exhibited the same chemical shifts when compared to **5**. In the case of **4**, C-1″ was at δ_C 67.2 and C-3″ was at δ_C 84.0, again suggesting that the AMVN radical substitution in **4** was O-X-OOH instead of the OO-X-OOH observed for **5** [where X is $C(CN)(CH_3)CH_2C(CH_3)_2$ derived from AMVN]. The negative ESI-MS-MS data were also similar for compounds **3** and **4**. On the basis of their similar MS and NMR spectra, we infer that **3** and **4** may be stereoisomers of each other, perhaps differing in configuration at C-1″.

The extremely low yield of compound **6** coupled with very fast degradation precluded us from acquiring high-resolution MS or NMR data for the compound. Positive APCI-MS indicated a $[M + H]^+$ ion at *m/z* 412 for **6**. This corresponded to a molecular mass of 411 for **6**, which would be equivalent to the mass of compound **5** minus two oxygen atoms. Under our RP-HPLC conditions, **5** eluted at 33 min whereas **6** eluted at 42 min. This indicated that **6** was less polar than **5**. On the basis of the MS information for **6**, we hypothesized that it was 1′-(alkyldioxy)-4′-oxogenistein. This is equivalent to 4′-oxogenistein with an OOX [where X is $C(CN)(CH_3)CH_2$-$CH(CH_3)_2$] substitution at the 1′ position instead of the OO-X-OOH [where X is $C(CN)(CH_3)CH_2C(CH_3)_2$] substitution observed for **5**.

Genistein Oxidations in Acetonitrile and $H_2{}^{18}O$. To ascertain whether the hydroxyl group at C-3′ in orobol originated from water or atmospheric oxygen, we conducted experiments with ^{18}O-labeled water. These experiments were conducted by carrying out the genistein and AMVN oxidations in an acetonitrile and ^{18}O-labeled water mixture (95:5, v/v) rather than in acetonitrile alone. Corresponding oxidations also were carried out using unlabeled water. Positive APCI-MS analysis of orobol purified from the ^{18}O-labeled and unlabeled water oxidations indicated that the molecular ion for orobol remained unchanged at $[M + H]^+$ *m/z* 287 for both samples (data not shown). If water was the source of oxygen necessary for genistein hydroxylation to orobol, we would have expected the ^{18}O label to be incorporated into orobol and for its mass to be shifted by 2 mass units. The ^{18}O-labeled water experiment demonstrated that the hydroxylation of genistein during peroxyl radical oxidation does not occur through hydrolysis.

A possible concern was that the ^{18}O label had been incorporated into orobol but was lost during the HPLC purification step due to an exchange with unlabeled water from the mobile phase. To check for exchange of the ^{18}O label, we incubated our orobol standard with 1 mL of acetonitrile and ^{18}O-labeled water (28:72, v/v) for 3 h at room temperature. The orobol standard was immediately evaporated in vacuo and analyzed by positive APCI-MS. The observed $[M + H]^+$ for orobol remained unchanged at *m/z* 287, confirming that no exchange of the label had occurred during the HPLC step (data not shown).

Discussion

The purpose of this investigation was to isolate and characterize the reaction products of genistein with alkylperoxyl radicals derived from AMVN in a homogeneous solution. The choice of AMVN as the model oxidant for these studies was dictated by the specificity of peroxyl radical generation from AMVN (*17*). In previous studies with α-tocopherol (vitamin E), antioxidant reactions with AMVN-derived peroxyl radicals in acetonitrile and acetonitrile/water mixtures (*21*, *22*) were similar to those subsequently detected in lipid bilayers (*23*, *24*), in subcellular fractions (*25*), and in whole organs (*26*) in vitro and in vivo (*27*). Thus, the model system used for these studies is reasonably predictive of antioxidant chemistry in more complex biological systems.

Here we report that genistein reacts with AMVN-derived peroxyl radicals in oxygenated acetonitrile to form orobol (**2**) and 1′-(alkyldioxyhydroperoxide)-4′-oxogenistein (**5**) as principal products, and 1′-(alkyloxyhydroperoxide)-4′-oxogenistein (**3** and **4**) and 1′-(alkyldioxy)-4′-oxogenistein (**6**) as minor oxidation products. Of these five oxidation products, orobol is the only compound that has been characterized previously. Ours, however, is the first study to report the formation of orobol as an oxidation product during the antioxidant reactions of genistein. A recent paper demonstrated that rat and human cytochrome P450 metabolism of genistein resulted in the formation of orobol (*18*). Orobol has also been isolated previously from the seeds of *Bolusanthus speciosus* (Bolus) Harms (*19*) and from the Podalyrieae and Lipariae papilionoid tribes of the Fabaceae (*20*), legume plants indigenous to South Africa. Orobol has biological activities that are similar to that of genistein. It has been shown to induce topoisomerase II-dependent DNA cleavage at a rate comparable to that of genistein (*28*), inhibit dihydroxyphenylalanine decarboxylase with an activity stronger than that of genistein (*29*), inhibit 15-lipoxygenase (*30*) and phosphatidylinositol turnover in cell culture with an IC_{50} similar to that of genistein (*31*), and increase cytoplasmic free calcium levels in isolated rat hepatocytes (*32*).

We were interested in understanding the mechanism for formation of orobol as a product of genistein antioxidant chemistry. Our experiments with ^{18}O-labeled water suggested that instead of water, atmospheric oxygen was the source of the oxygen needed for hydroxylation of genistein to orobol. On the basis of our findings, we proposed a mechanism for the formation of orobol during peroxyl radical oxidation of genistein (Figure 5).

The other family of oxidation products that we identified, the stable AMVN-derived radical adducts observed for genistein, appears to be unique in two ways. First, although previously observed with other phenolic antioxidants, peroxyl radical addition products have not been reported in flavonoid oxidations. Second, further oxidation of the AMVN-derived moiety in these adducts is unprecedented. Of these products, compound **5** was the major product that was formed. Compounds **3**, **4**, and **6** were generated in very low yields. For all four products, it appeared that AMVN-derived alkoxyl (HXO•) or peroxyl radicals (HXOO•) attached at the C-1′ position on the B-ring of the genistein phenoxyl radical. Further oxidation of the AMVN moiety led to formation of the corresponding O-X-OOH or OO-X-OOH hydroperoxide. A mechanism for

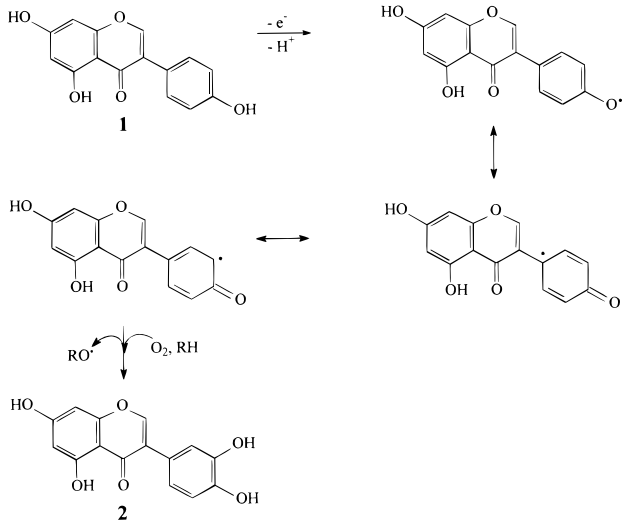

Figure 5. Proposed mechanism for the formation of **2** (orobol) during the peroxyl radical oxidation of **1** (genistein).

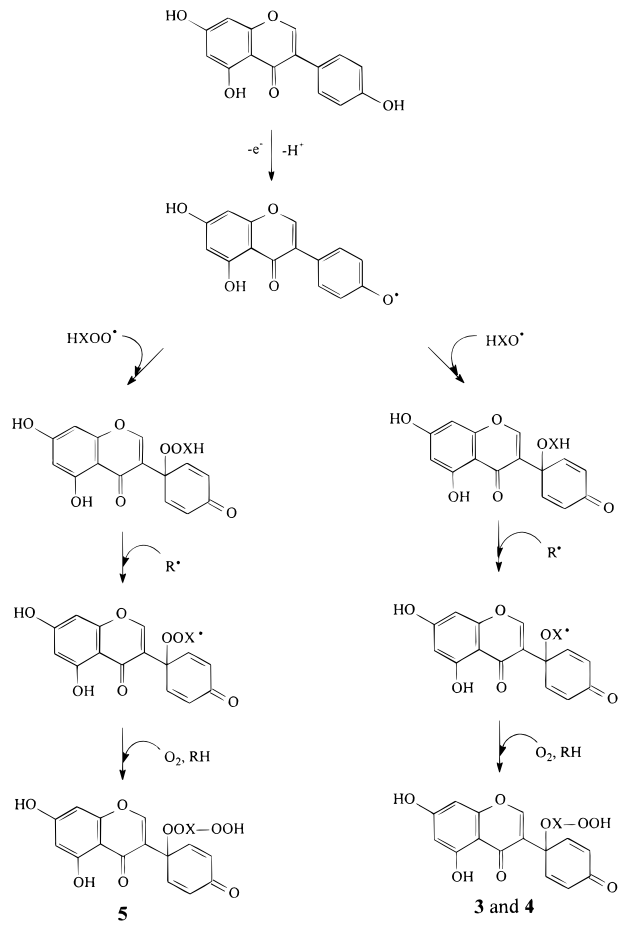

Figure 6. Proposed mechanism for the formation of compounds **3**−**6** during the peroxyl radical oxidation of **1** (genistein).

the formation of these compounds is proposed in Figure 6.

An initial one-electron oxidation of genistein by a peroxyl radical yields a genistein phenoxyl radical. Recombination with a peroxyl or alkoxyl radical would form either 1′-(alkyldioxy)-4′-oxogenistein or 1′-(alkyloxy)-4′-oxogenistein, respectively. Further abstraction of a proton from the alkyl side chain of the AMVN moiety, followed by addition of oxygen in the presence of a proton donor, would result in the formation of product **5** or

products **3** and **4**. These appeared to be stable end products of genistein reactions with AMVN.

Interestingly, 1′-(alkyldioxyhydroperoxide)-4′-oxogenistein (**5**) was the major product formed during these oxidations of genistein with alkylperoxyl radicals. Its precursor, 1′-(alkyldioxy)-4′-oxogenistein (**6**), was formed in almost negligible yield, suggesting that **6** was less stable than genistein in oxidations with AMVN. Products **3** and **4,** which were formed through a parallel oxidation pathway involving an AMVN alkoxyl radical, were also generated in low yields, indicating that this was a minor pathway for the oxidation of genistein by AMVN.

There have been many studies conducted to characterize the oxidation products of reactions of other phenolic antioxidants with AMVN-derived peroxyl radicals. With α-tocopherol and other phenolic antioxidants, adducts that are structurally analogous to compound **6** have been previously observed (*21*, *22*, *33*). However, further oxidation of the AMVN moiety to give products analogous to **3**, **4**, or **5** has not been observed. We cannot presently explain this surprising secondary oxidation of AMVN-derived alkyl chains in genistein−AMVN oxy radical adducts. It seems highly unlikely that these isopropyl moieties are intrinsically more reactive toward radicals than genistein itself. However, noncovalent association of genistein molecules and/or initial reaction products (such as **6**) may alter relative reactivities enough to favor formation of products **3**−**5**. Further work will be required to sort out the chemistry that is involved.

The site of antioxidant chemistry for formation of all these reaction products was the B-ring of genistein. This finding agrees with earlier studies that suggested that ring B was the principal site of antioxidant reactions for flavonoids (*12*, *34*). Our study provides the first conclusive proof of this hypothesis for genistein oxidations by peroxyl radicals. A previous paper from our laboratory had reported that the site of antioxidant reactions for the green tea catechin (−)-epigallocatechin-3-gallate when oxidized with peroxyl radicals also resides on the B-ring of the molecule (*35*). What is particularly interesting about the results of our product studies with flavonoids is the variety of antioxidant chemistries displayed by the B-ring phenols. Genistein produced products that are relatively typical of well-documented phenol antioxidant reactions: B-ring hydroxylation and formation of radical addition products. In contrast, (−)-epigallocatechin and (−)-epigallocatechin-3-gallate yield neither B-ring hydroxylation nor stable peroxyl radical addition products. Instead, one-electron oxidations appear to initiate radical polymerization reactions, and transient radical addition products decompose with B-ring expansion.[2] Thus, antioxidant chemistry of simpler phenols does not necessarily predict the course of flavonoid phenol antioxidant reactions.

The characterization of these product structures provides potential markers of genistein antioxidant reactions. Sensitive assays for specific genistein oxidation products may thus be useful markers for antioxidant reactions of the isoflavone in biological systems. Currently, we are investigating whether reactions of genistein and peroxyl radicals in lipid bilayers in vitro and in biological membranes in vitro and in vivo yield products similar to those observed in this study in a homogeneous solution system.

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