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Chlorination and Nitration of Soy Isoflavones[1]

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Diets enriched in soy foods containing a high concentration of isoflavonoids are associated with a decrease in the incidence of several chronic inflammatory diseases. Studies with experimental models of diseases, such as atherosclerosis, suggest that these effects can be ascribed to the biological properties of the isoflavones. Since the isoflavones and tyrosine have structural similarities and modifications to tyrosine by inflammatory oxidants such as hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) have been recently recognized, we hypothesized that the isoflavones also react with HOCl and $ONOO^-$. Using an *in vitro* approach, we demonstrate in the present study that the isoflavones genistein, daidzein, and biochanin-A can be chlorinated and nitrated by these oxidants. These reactions were investigated using high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance. In the reaction with HOCl, both mono- and dichlorinated derivatives of genistein and biochanin-A are formed, whereas with daidzein only a monochlorinated derivative was detected. The reaction between genistein or daidzein and $ONOO^-$ yielded a mononitrated product. However, no nitrated product was detected with biochanin-A. Furthermore, the reaction between genistein and sodium nitrite and HOCl yielded a chloronitrogenistein derivative, as well as a dichloronitrogenistein derivative. These results indicate that the ability of the isoflavones to react with these oxidant species depends on their structure and suggest that they could be formed under conditions where these reactive species are generated under pathological conditions. © 1999 Academic Press

Key Words: **soy isoflavones; chlorination; hypochlorous acid; nitration; peroxynitrite; mass spectrometry.**

Genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone) (Fig. 1) are the principal isoflavones in the soybean (*Glycine max*) (1, 2). Foods derived from soy containing these isoflavones are associated with the reduction of risk of several chronic diseases, including atherosclerosis, cancer, inflammatory bowel disease, and osteoporosis (3–10). Many of these beneficial effects of soy have been attributed to the isoflavones.

Several mechanisms of action have been proposed for the isoflavones (11–21). Based on the presence of the hydroxylated aromatic A-ring and a second oxygen in the phenolic B-ring, the isoflavones are frequently described as phytoestrogens (plant estrogens). Although the affinity of the classical estrogen receptor (ERα) for isoflavones is weak (22), the recently discovered estrogen receptor beta (ERβ) has a strong affinity for genistein, comparable to that of the physiological estrogen agonist 17β-estradiol (23, 24). In the absence of physiological estrogens and at nanomolar concentrations, genistein stimulates the growth of ER-positive human breast cancer cell lines (25, 26). However, at micromolar concentrations, genistein is an inhibitor of estrogen-stimulated cell growth, whether or not estrogen receptors are present in the cells (13), suggesting that other, non-ER-dependent mechanisms are important.

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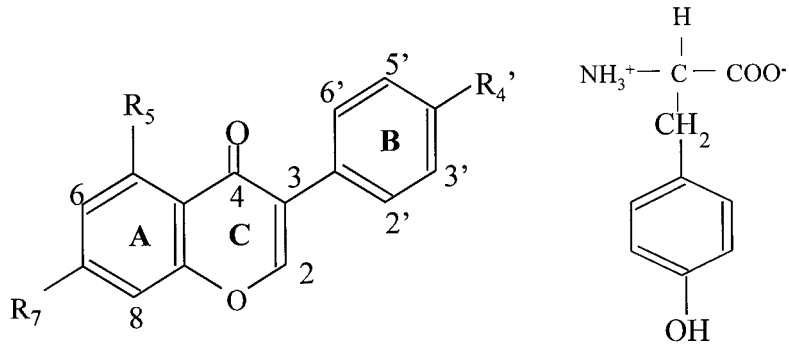

Isoflavone	R_4'	R_5	R_7
Genistein	OH	OH	OH
Biochanin-A	OCH_3	OH	OH
Daidzein	OH	H	OH

FIG. 1. Structures of the amino acid tyrosine and the isoflavones genistein, daidzein, and biochanin-A.

The B-rings of genistein and daidzein are structurally similar to those of the amino acid tyrosine. It is not surprising, therefore, that genistein is a potent *in vitro* inhibitor of protein tyrosine kinases (PTKs)[3] (27). Many investigators have associated changes in protein tyrosine phosphorylation in cells exposed in culture to genistein to *in vivo* inhibition of PTK. However, when used in short time course experiments (0–5 min) with human breast and prostate cancer cells, genistein has no effect on the autophosphorylation of the epidermal growth factor (EGF) receptor (11–13). Indeed, a recent study demonstrated that the decrease in tyrosine phosphorylation of the EGF receptor is a result of reduced expression of the EGF receptor, not inhibition of phosphorylation (28).

Other mechanisms of action of isoflavones have been proposed. Of these, their roles as DNA topoisomerase inhibitors (14, 16–18) and antioxidants (29–32) provide a link to the involvement of tyrosine in these processes. In the former case, DNA topoisomerase forms a covalent link via a tyrosine residue to one strand of the nicked DNA. In the latter case, isoflavones may have a role as scavengers of oxidant species, thereby preventing oxidation of proteins and lipids. However, although they can prevent LDL oxidation *in vitro* (33–35), it is unlikely that the circulating nanomolar concentrations of the isoflavones in the blood of soy consumers (36) are adequate to prevent oxidation *in vivo*. As an alternative, we are exploring the hypothesis that instead of being effective scavengers, the isoflavones may undergo metabolic activation during oxidative stress to generate molecules that could be potent regulators of cellular dysfunction. An important rationale for our hypothesis is the recently discovered presence of chlorinated and nitrated derivatives of tyrosine in patients with chronic inflammatory diseases (37–41). Chlorination occurs as a result of the production of hypochlorous acid (HOCl), whose formation is catalyzed by myeloperoxidase (MPO) from hydrogen peroxide and chloride during an immune response (42). Nitration occurs following the formation of peroxynitrite ($ONOO^-$) from the free radicals nitric oxide ($NO^\bullet$), a second messenger released from the vascular wall, and superoxide ($O_2^{\bullet-}$), the one-electron reduction product of oxygen (43). Furthermore, MPO, in the presence of nitrite, is also capable of nitrating polyphenols, as does HOCl when reacted with nitrite (44). Given the structural similarity between tyrosine and the isoflavones, we propose that the isoflavones form chlorinated and nitrated conjugates upon exposure to HOCl and reactive nitrogen species.

In the present study we have used a combination of ultraviolet (UV) absorption spectrophotometry, high-performance liquid chromatography (HPLC), HPLC-heated nebulizer–atmospheric pressure chemical ionization–mass spectrometry (LC-HN-APCI-MS), and nuclear magnetic resonance (NMR) to investigate the reactions of HOCl and $ONOO^-$ with isoflavonoids and to identify their chlorinated and nitrated products. In order to determine the effects of isoflavone structure on

[3] Abbreviations used: HOCl, hypochlorous acid; $ONOO^-$, peroxynitrite; GS, genistein; HPA, hydroxyphenylacetic acid; MPO, myeloperoxidase; DTPA, diethylenetriamine pentaacetic acid; TNM, tetranitromethane; ER, estrogen receptor; PTK, protein tyrosine kinase; EGF, epidermal growth factor; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline.

the sites of chlorination and nitration, a methylated analog of genistein, biochanin-A (5,7-dihydroxy-4′-methoxyisoflavone), was included in these investigations.

MATERIALS AND METHODS

Materials. Genistein was extracted and purified as described previously (13). Daidzein was obtained from LC Labs (Worburn, MA). Biochanin-A and DMSO-d_6 were obtained from Aldrich Chemicals (Milwaukee, WI). Glutathione, tetranitromethane (TNM), and hydroxyphenylacetic acid (HPA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dithionite ($Na_2S_2O_4$) and diethylenetriamine pentaacetic acid (DTPA) were obtained from Acros Organics (Fairlawn, NJ). $ONOO^-$ was synthesized as reported previously (45) and quantified spectrophotometrically at 302 nm (pH 12, $\epsilon = 1700$ M^{-1} cm^{-1}) in 1 M NaOH. HOCl concentrations were determined spectrophotometrically at 290 nm (pH 12, $\epsilon = 350$ M^{-1} cm^{-1}).

Reactions of isoflavones with HOCl. Buffered solutions of phosphate-buffered saline with 1 mM DTPA with 50 μM of each isoflavone were reacted with HOCl (0 to 500 μM). Aliquots of a stock solution of 10 mM HOCl were added to the reaction mixture with continuous mixing to achieve the nominal concentration. Decomposed HOCl was made by reacting the acid with an equimolar solution of glutathione for 20 min (46). This mixture was added, while vortexing, to each isoflavone reaction mixture. The reactions were analyzed using UV–visible spectrophotometry and reversed-phase HPLC. Samples analyzed by HPLC were extracted prior to injection as follows: 200 μl of the reaction mixture was added to 800 μl of water followed by 2 ml of diethyl ether. The samples were vortexed and then centrifuged at 3000 rpm, whereupon the ethereal, top layer was removed and the ether evaporated under N_2. Prior to injection, 150 μl 80% methanol was added to redissolve the dried residues.

Reactions of isoflavones with $ONOO^-$ and TNM. Buffered solutions of 200 mM sodium phosphate (pH 7.4) with 1 mM DTPA and 50 μM of each isoflavone were reacted with $ONOO^-$ (0 to 500 μM). Aliquots of a 10 mM $ONOO^-$ stock solution were added to the reaction mixture with continuous mixing to obtain the final concentration. Decomposed $ONOO^-$ was prepared by adding the $ONOO^-$ to the buffer solution first and allowing this to react for 10 min (pH 7.4). The isoflavones were then added to the decomposed reaction mixture with continuous mixing. Genistein was also nitrated by adding 500 μM TNM to 50 μM genistein in a buffer solution composed of 50% sodium bicarbonate (50 mM, pH 9)/50% ethanol with 1 mM DTPA. To carry out reduction of nitrated groups, $Na_2S_2O_4$ ($\sim$60 μM) was added to the 500 μM sample of the isoflavones and $ONOO^-$. Following each reaction, samples were extracted as previously described and subjected to HPLC and MS. Changes in the UV absorption of the isoflavones were monitored at 262 nm.

Reactions of genistein and HPA with $NaHCO_3$ and $ONOO^-$. Reaction mixtures containing 50 μM genistein or 1 mM HPA, in 100 mM sodium phosphate buffer (pH 7.4) with 1 mM DTPA which was purged with nitrogen gas for 30 min, were reacted with 25 mM sodium bicarbonate followed by 200 μM $ONOO^-$, with continuous mixing in an anaerobic environment. The reaction mixtures were extracted as previously described and then aliquots were subjected to LC–MS.

Reactions of genistein with $NaNO_2$ and HOCl. Reaction mixtures containing 50 μM genistein in 200 mM sodium phosphate buffer (pH 7.4) with 1 mM DTPA were reacted with 0.5 mM sodium nitrite followed by 100 μM HOCl, or vice versa, with continuous mixing. The reaction mixtures were extracted as previously described and then aliquots were subjected to LC–MS.

HPLC analysis of reaction products. All analyses were performed using a Beckman HPLC 125 solvent module, diode array Model 168 detector, and Beckman DU 7500 diode array spectrophotometer (Beckman Instruments, Fullerton, CA). Aliquots (40 μl) of the reaction mixtures were analyzed by reversed-phase HPLC, using an Aquapore octyl RP-300, C-8, 22 cm $\times$ 4.6-mm-i.d., 7-μm column preequilibrated with 10% aqueous acetonitrile in 0.1% trifluoroacetic acid (TFA). The column was eluted at a flow rate of 1.5 ml/min with the following mobile phase composition: 0–10 min, linear gradient (10–50%) of acetonitrile in 0.1% TFA; 10–12 min, linear gradient (50–90%) of acetonitrile in 0.1% TFA; and 12–15 min, isocratically with 90% aqueous acetonitrile in 0.1% TFA. The eluent was moni-

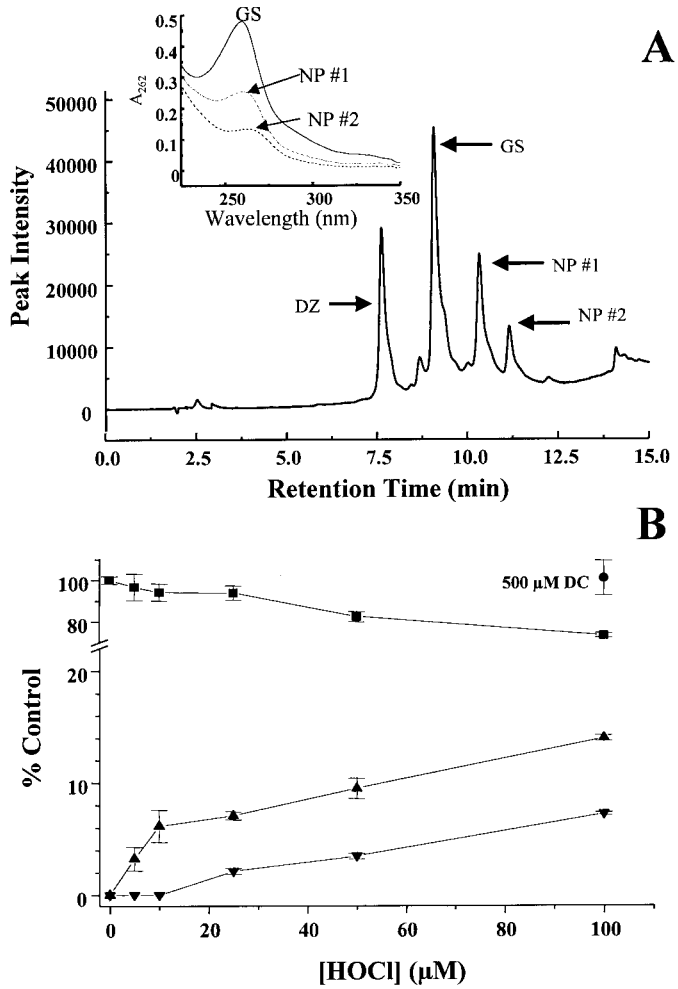

FIG. 2. Analysis of reaction products formed between HOCl and genistein. (A) 60 μM genistein (GS) was reacted with 100 μM HOCl in PBS + 1 mM DTPA and extracted with ether. Aliquots (40 μl) of the reaction mixture were analyzed by reversed-phase HPLC. Daidzein (DZ) (20 μM) was added after the reaction as an internal standard. The eluent was monitored at 262 nm and the following peaks were found: daidzein (7.56 min), genistein (9.01 min), new product (NP) 1 (10.25 min), and new product 2 (11.08 min). The inset shows the UV-absorption spectra at 200–400 nm for each peak: genistein, NP 1, and NP 2. (B) Substrate–product concentration curve of 60 μM genistein with HOCl (0–100 μM and 500 μM decomposed) which illustrates the loss of genistein (■) and the increase of the two novel products (▲, ▼, respectively) as the concentration of HOCl increases. (The ● represents the genistein concentration in the decomposed (DC) sample.) These values represent the nominal concentrations assuming the extinction coefficient is the same for the isoflavone and the products.

TABLE I

Oxidant	Isoflavone	Retention time HPLC (min)	Retention time HPLC–MS (min)	λ_{max} (nm)	% Isoflavone reacted
+HOCl					
	Genistein	9.0	10.0	257	56
	New product 1	10.2	9.2	260	—
	New product 2	11.1	8.2	263	—
	Biochanin-A	11.5	12.0	260	89
	New product 1	12.5	10.3	263	—
	New product 2	13.2	9.0	265	—
	Daidzein	7.6	8.7	245	100
	New product 1	9.2	7.7	248	—
$+ONOO^-$					
	Genistein	9.0	10.0	257	33
	New product	10.8	10.2	255	—
	Biochanin-A	11.5	12.0	260	0
	New product	Not detected	Not detected	N/A	—
	Daidzein	7.6	8.7	242	N/A
	New product	9.2	9.0	235	—

Note. Isoflavones (50 μM) were reacted with HOCl (100 μM) or $ONOO^-$ (200 μM). Reaction mixtures were then analyzed by reversed-phase HPLC using a C-8 column and a mobile phase with acetonitrile with 0.1% TFA. The mixtures were then further analyzed by reversed-phase LC–MS with a C-8 column and a mobile phase of 10 mM ammonium acetate (refer to Materials and Methods). Thus, different retention times were observed for the two methods. λ_{max} was obtained from the diode array on the HPLC.

tored at 262 nm by the diode array detector and was collected in 0.5-min fractions. Fractions collected 30 s prior to and following the peak of interest were pooled and dried under N_2. The dried residues were then resuspended with 300 μl of 80% methanol and a 100-μl aliquot was reinjected into HPLC to verify that the intended peak had been collected.

Mass spectrometry analysis of reaction products. Reaction mixtures were separated by HPLC using a 10 cm × 4.6-mm-i.d., C-8 Aquapore reversed-phase column preequilibrated with 10 mM ammonium acetate (NH_4OAc). The mobile phase composition was 0–10 min, linear gradient (0–50%) of acetonitrile in 10 mM NH_4OAc; 10–12 min, isocratically with 50% aqueous acetonitrile in 10 mM NH_4OAc; 12–15 min, linear gradient (50–90%) of acetonitrile in 10 mM NH_4OAc; and 15–17 min, isocratically with 90% aqueous acetonitrile in 10 mM NH_4OAc. The column eluent was passed into the HN–APCI interface of a PE-Sciex (Concord, Ontario, Canada) API III triple-quadrupole mass spectrometer. The voltage on the corona discharge needle was −8000 V and the orifice potential was set at −60 V. Negative-ion spectra were recorded over a *m/z* range of 200–400. Selected $[M\text{-}H]^-$ molecular ions were analyzed by collision-induced dissociation with 90% argon–10% nitrogen, and the daughter ion mass spectra were recorded over a range from *m/z* 20 to the *m/z* of the selected parent ion. Data were analyzed using software provided by the manufacturer on Macintosh Quadra 950 and PowerPC 9500 computers (Apple Computers, Cupertino, CA).

NMR analysis. Specific reaction products between phytoestrogens and proinflammatory oxidants were isolated by preparative HPLC. Concentrated aliquots (1 ml) of 1.5 mM genistein reacted with 5 mM HOCl or 200 μM genistein with 500 μM $ONOO^-$ were extracted and isolated on an Aquapore octyl RP-300, C-8, 25 cm × 10-mm-i.d., 20-μm column preequilibrated with 10% aqueous acetonitrile in 0.1% TFA. The column was eluted at a flow rate of 7 ml/min with the same mobile-phase composition as described for the analytical separation. The eluent was monitored at 262 nm by the diode array detector and was collected in 1-min fractions. Fractions collected 30 s prior to and following the peak of interest were pooled together, evaporated under N_2, and dried *in vacuo* over phosphorus pentoxide. They were reconstituted in 0.5 ml anhydrous DMSO-d_6 immediately prior to analysis. ^1H NMR spectra were obtained at 400.1 MHz with a 8013-Hz sweep width, 2.65-s repetition rate, and 300° sweep angle on a Bruker DRX 300 spectrometer. Spectra were internally referenced to the DMSO proton resonance at 2.49 ppm. Approximately 15,000 transients were collected for each sample.

RESULTS

Reaction of Isoflavones with HOCl

In the first series of experiments, biochanin-A, daidzein, and genistein were reacted with a range of HOCl concentrations, and the reaction mixtures were subjected to analysis using reversed-phase HPLC (Fig. 2A). The HPLC chromatograms revealed additional peaks for each of the isoflavones after reaction with HOCl, indicative of the formation of novel products. A typical chromatogram for the reaction of genistein with HOCl is shown in Fig. 2A and the data for biochanin-A and daidzein are reported in Table I. Genistein and biochanin-A reaction mixtures had similar patterns, with two additional peaks with longer retention times than the unreacted isoflavone. In contrast, the daidzein reaction mixture had only one new peak that had a longer retention time than daidzein. The inset in Fig. 2A shows the UV spectra of the new products resulting from the addition of HOCl to genistein. A small shift of approximately 3–6 nm in the absorption maximum of the modified isoflavone supports the idea that a modification of the aromatic structure of the isoflavones has occurred. Similar results were obtained with biochanin-A and daidzein (Table I).

To examine the yield of products formed between HOCl and isoflavones, the appearance of new products and the loss of the starting molecule were monitored by

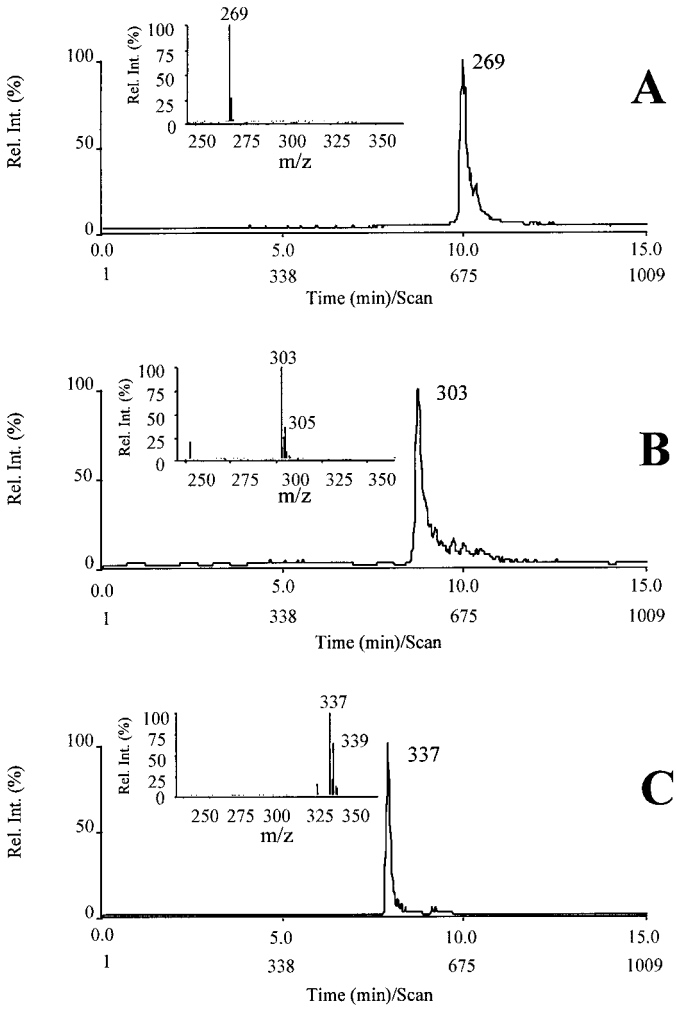

FIG. 3. Mass spectra of new products formed between HOCl and genistein. Selected ion chromatograms of (A) *m/z* 269 (genistein), (B) *m/z* 303 (chlorogenistein), and (C) *m/z* 337 (dichlorogenistein) by reversed-phase LC–MS. The inset shows the negative-ion mass spectra for each of the selected ions formed by electrospray ionization–MS.

HPLC as a function of increasing HOCl. The results with genistein are shown in Fig. 2B and indicate that the novel product with the shortest retention time was formed before accumulation of the second product. Since it is known that HOCl is capable of incorporating a chlorine group into an aromatic structure, these results would be consistent with a monochlorination followed by dichlorination.

The exact yield of the reaction products formed from the isoflavones with HOCl has not been determined, but, assuming the same extinction coefficient as the unreacted isoflavone, an estimate can be made. The loss of genistein parallels the formation of the new products with a yield of approximately 20%, with respect to HOCl (Fig. 2B). When decomposed HOCl was reacted with each of the isoflavones, no new products were detected in these samples (Fig. 2B). Summation of the nominal concentrations of the products accounts for the amount of genistein reacted, suggesting that the excess HOCl is lost through nonspecific decomposition reactions. Similar results were obtained with biochanin-A and daidzein and the formation of their products (Table I).

In order to identify the novel products, each peak from the reaction mixture of 50 μM isoflavone with 100 μM HOCl was collected and subjected to HPLC–MS. The conditions for chromatography were modified to be compatible with mass spectral analysis, which resulted in a slight change in the relative retention times for the unreacted isoflavones and the novel products (Table I). Figure 3A shows the typical elution profile of genistein, with a retention time 10 min, as a $[M\text{-}H]^-$ molecular ion of mass to charge ratio (*m/z*) 269 (47). The first new product, retention time 9.2 min, was determined to have a *m/z* value of 303, corresponding to addition of *m/z* 34 units to the genistein $[M\text{-}H]^-$ molecular ion (Fig. 3B, Table II). This increase in mass corresponds to chlorine being added to the polyphenolic structure. Further evidence for chlorination is the presence of a chlorine isotope peak at *m/z* 305. The second product peak, at retention time 8.2 min, had a *m/z* 337 (and an isotope peak at *m/z* 339), confirming dichlorination with the addition of *m/z* 34 to the monochlorinated derivative or *m/z* 68 to genistein (Fig. 3C). The two novel products detected in the biochanin-A samples were also mono- and dichlorinated derivatives (Table II). In the case of daidzein, only a monochlorinated derivative was detected, coinciding with the single novel product peak seen in this reaction (Table II).

The positions of chlorine substitution in genistein were investigated by ^{1}H NMR (Table III). The tyrosine-like B-ring of genistein has two pairs of equivalent protons ($H_{2'}/H_{6'}$ and $H_{3'}/H_{5'}$) giving rise to two sets of doublets (δ7.41 and δ6.88). Their chemical shifts were unchanged in the ^{1}H NMR spectra of both the mono- and dichlorinated products. However, their integrated intensities were lower than those in genistein, suggesting that some chlorination of the B-ring occurred. The intensities of resonances of the two protons in the A-ring (H_6 and H_8) were either decreased (for monochlorogenistein) or totally absent (dichlorogenistein). Overall, these data are consistent with chlorination occurring in both the A- and B-rings.

Reaction of Isoflavones with $ONOO^-$ and TNM

The isoflavones were reacted with a range of $ONOO^-$ concentrations, and changes in the UV-absorption spectra for genistein and daidzein were detected, as were product peaks upon analysis of the reaction mixture by HPLC (Fig. 4A). The reaction of genistein with

TABLE II

m/z Values for Molecular Ions of Isoflavones and Novel Chlorinated and Nitrated Derivatives

Oxidant	Isoflavone	Free	Mono-Cl	Di-Cl
+HOCl	Genistein	269	303	337
	Biochanin-A	283	317	351
	Daidzein	253	287	Not detected
		Free	Nitrated	
+ONOO−	Genistein	269	314	
	Biochanin-A	283	Not detected	
	Daidzein	253	298	
		Mono-Cl-nitro	Di-Cl-nitro	
+HOCl and $NaNO_2$	Genistein	348	382	

Note. Isoflavones (50 μM) were reacted with HOCl (100 μM) or $ONOO^-$ (200 μM) or HOCl (100 μM) and $NaNO_2$ (0.5 mM). Reaction mixtures were then analyzed by reversed-phase LC–MS with a C-8 column and a mobile phase of 10 mM ammonium acetate (refer to Materials and Methods). Values indicate *m/z* values for molecular ions of isoflavones and novel chlorinated and nitrated derivatives.

$ONOO^-$ resulted in the formation of a single novel peak with a longer retention time than that of the unreacted isoflavone. A similar result was obtained for the reaction of daidzein with $ONOO^-$, whereas analysis of the biochanin-A reaction products showed no decrease in biochanin-A and no reaction products were detected (Table I). The inset to Fig. 4A shows the UV spectra of the new product created from the reaction of genistein and $ONOO^-$. Under the acidic conditions of the HPLC solvent, the modified isoflavone resulted in a slight shift of approximately 2 nm in the absorption maximum. As with the HOCl experiments, this result supports the idea that a modification of the aromatic structure of the isoflavones has occurred. Visible-absorption spectra of the $ONOO^-$–isoflavone reaction mixture at basic pH revealed a novel absorption band at 416 nm similar to that found with nitrotyrosine (48). Figure 4B illustrates an inverse correlation between the amount of novel product detected and the amount of unreacted genistein as the concentration of $ONOO^-$ increased. Since it is known that $ONOO^-$ is capable of nitrating tyrosine, these results would be consistent with the nitration of the isoflavone. However, when the nominal concentrations of the product are added together, they do not account for the amount of genistein reacted. This suggests that some of the genistein or nitrated product is also oxidized, as might be expected given the complexity of the oxidation reactions that

TABLE III

NMR Chemical Shifts and Integral Values for Genistein and Chlorinated Metabolites

		Genistein		GS-Cl		$GS\text{-}Cl_2$	
Ring	Proton	δ	Integral	δ	Integral	δ	Integral
A-ring	H_6	6.226	0.99	6.220	0.570	N/D	N/D
	H_8	6.392	1.03	6.380	1.11	N/D	N/D
	C_5OH	12.93	1.00	12.916	1.00	13.72	1.00
	C_7OH	10.86	1.00	N/D	N/D	N/D	N/D
B-ring	H_2	8.323	0.96	8.416	0.99	8.360	1.65
C-ring	$H_{3'}$–$H_{5'}$	6.504	2.14	6.803	1.68	6.806	1.91
		6.512		6.827		6.827	
	$H_{2'}$–$H_{6'}$	7.363	2.13	7.370	1.48	7.369	1.30
		7.384		7.380		7.389	
	$C_{4'}OH$	9.569	1.06	9.641	1.48	9.60	1.32
				9.654			

Note. Genistein (1.5 mM) was reacted with HOCl (5 mM) and reaction mixtures were separated and collected by reversed-phase LC–MS with a semiprep C-8 column (as described under Materials and Methods). Values indicate chemical shift (δ) and integral values of genistein and the novel metabolites chlorogenistein (GS-Cl) and dichlorogenistein ($GS\text{-}Cl_2$) as determined by ^{1}H-NMR.

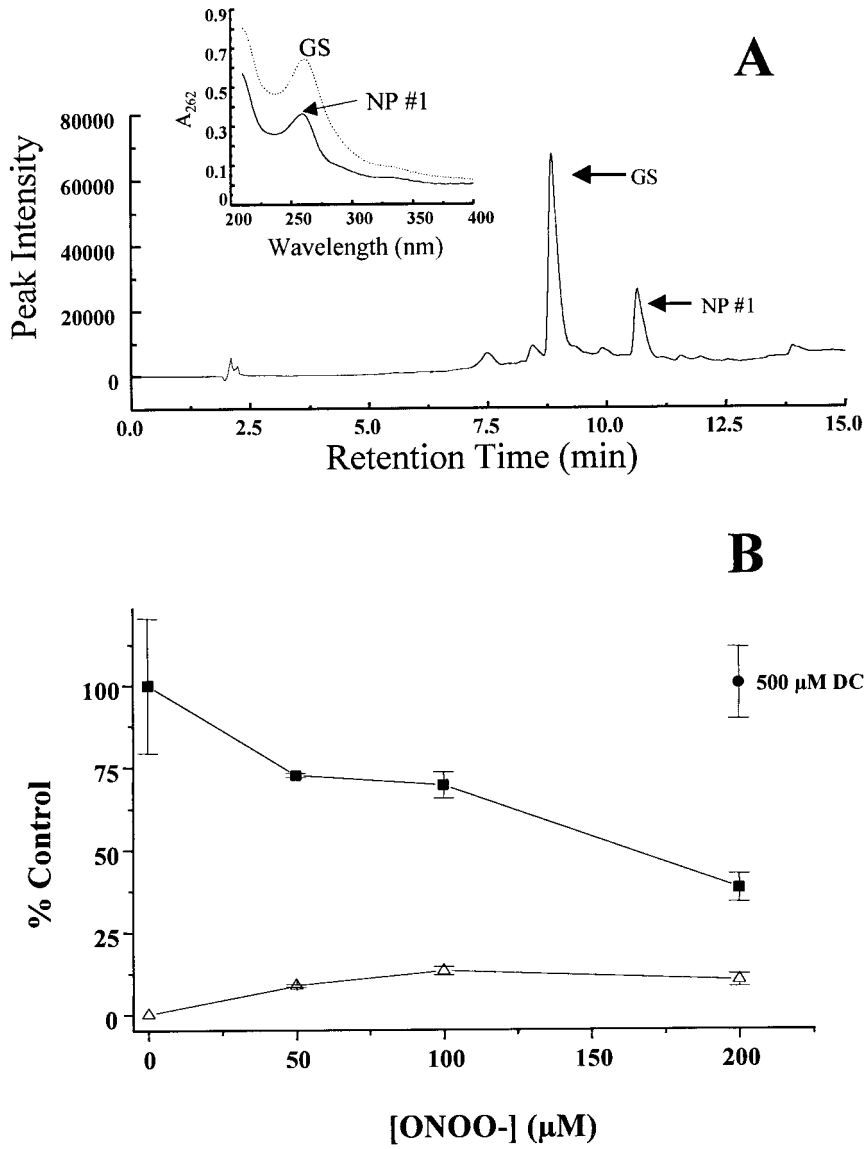

FIG. 4. New product formed between genistein and $ONOO^-$. (A) Genistein (100 μM) was reacted with 200 μM $ONOO^-$ in 200 mM sodium phosphate (pH 7.4) with 1 mM DTPA and extracted with ether. Aliquots (40 μl) of the reaction mixture were analyzed by reversed-phase HPLC. The eluent was monitored at 262 nm and the following peaks were found: genistein (8.93 min) and NP 1 (10.80 min). The inset shows the UV-absorption spectra (200–400 nm) at each retention time: genistein and NP 1. (B) Substrate–product concentration curve of genistein (100 μM) with $ONOO^-$ (0–200 μM and 500 μM decomposed), which illustrates the loss of genistein (■) and the increase of the novel product (▲) as the concentration of $ONOO^-$ increases. (The ● represents the genistein concentration in the decomposed (DC) sample.) These values represent the nominal concentrations assuming the extinction coefficient is the same for the isoflavone and the product.

$ONOO^-$ can mediate. Since such modifications are likely to result in extensive modification of the molecules leading to loss of the chromophore, it is probable that the products could no longer be detected. Similar results were obtained with daidzein and the formation of its nitrated product (Table I). When decomposed $ONOO^-$ was reacted with each of the isoflavones, no nitrated products were detected in those samples (Fig. 4B).

HPLC–MS was used to identify each novel product resulting from the reaction mixture of 50 μM isoflavone and 200 μM $ONOO^-$. Figure 5 shows the elution of the product of the reaction of genistein with $ONOO^-$. A single molecular ion (*m/z* 314) was detected, representing an increase in mass of *m/z* 45 from the parent molecule, and was consistent with the incorporation of a nitro group in the isoflavone. A similar result was obtained with daidzein and the formation of a com-

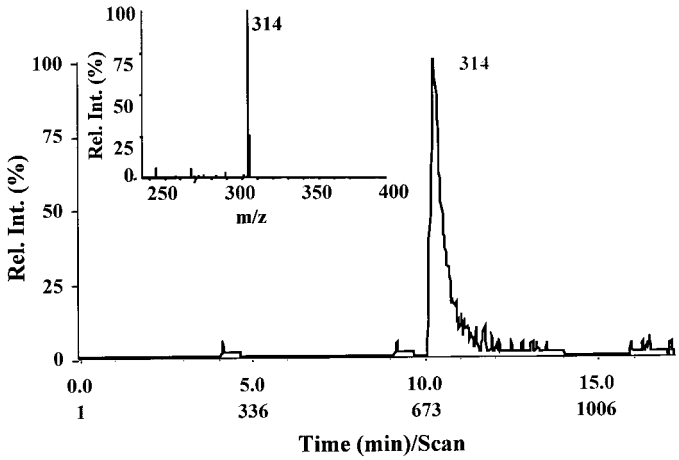

FIG. 5. Mass spectra of new products formed between $ONOO^-$ and genistein. Selected ion chromatogram of *m/z* 314 (nitrogenistein) by reversed-phase LC–MS. The inset shows the negative-ion mass spectra for the selected ion run on negative chemical ionization–MS.

pound with a *m/z* value of 298 (i.e., *m/z* 254 + 45, Table II). However, no similar products were detected with biochanin-A (data not shown).

In order to verify that the *m/z* 45 increase was due to a nitro group, the reaction mixtures of $ONOO^-$ and genistein or daidzein, which contained the putative nitrated products, were reacted with sodium dithionite ($Na_2S_2O_4$), which reduces nitro groups to amines. The HPLC analyses of these reaction mixtures showed the loss of the nitrated product peaks from the chromatograms for both genistein and daidzein and the appearance of novel peaks with a faster retention time, while the native isoflavone peaks were unchanged (Fig. 6A). HPLC–MS analysis showed the anticipated conversion of the nitro group (*m/z* 314) of genistein to the corresponding amine with a *m/z* value of 284 (i.e., *m/z* 269 + 15). Similarly, nitrodaidzein (*m/z* 298) was also reduced to the amine (*m/z* 270) (data not shown). As a further test of the assignment of the new product to nitrogenistein, genistein was reacted with a classical nitrating agent TNM (500 μM). A novel peak with the same retention time as the nitrogenistein formed by reaction with $ONOO^-$ was observed (Fig. 6B). Confirmation of its identity was further carried out by HPLC–MS analysis (data not shown).

Investigation of the site of nitration of genistein by NMR revealed that the chemical shifts of the H_6 and H_8 proton resonances were unchanged. In contrast, the doublets that correspond to proton resonances in the B-ring ($H_{2'}/H_{6'}$ and $H_{3'}/H_{5'}$) integrated to a single proton. In addition, the 4′-hydroxyl group proton resonance underwent a substantial upfield shift. These data are consistent with nitration of genistein in the B-ring (Table IV).

Reactions of Genistein and HPA with $NaHCO_3$ and ONOO

In the presence of CO_2, $ONOO^-$-dependent nitration of aromatic compounds has been shown to be twofold higher (49). A similar effect is reported using HPA (1 mM) as a positive control with 25 mM $NaHCO_3$ and 200 μM $ONOO^-$ (Fig. 7). On the other hand, when genistein (50 μM) is exposed to similar conditions, there is a decrease in the amount of nitrated product that is produced compared to the $ONOO^-$-generated product.

Reaction of Isoflavones with HOCl and Nitrite

An alternative mechanism for nitration of tyrosine, of possible biological relevance, is the reaction of HOCl

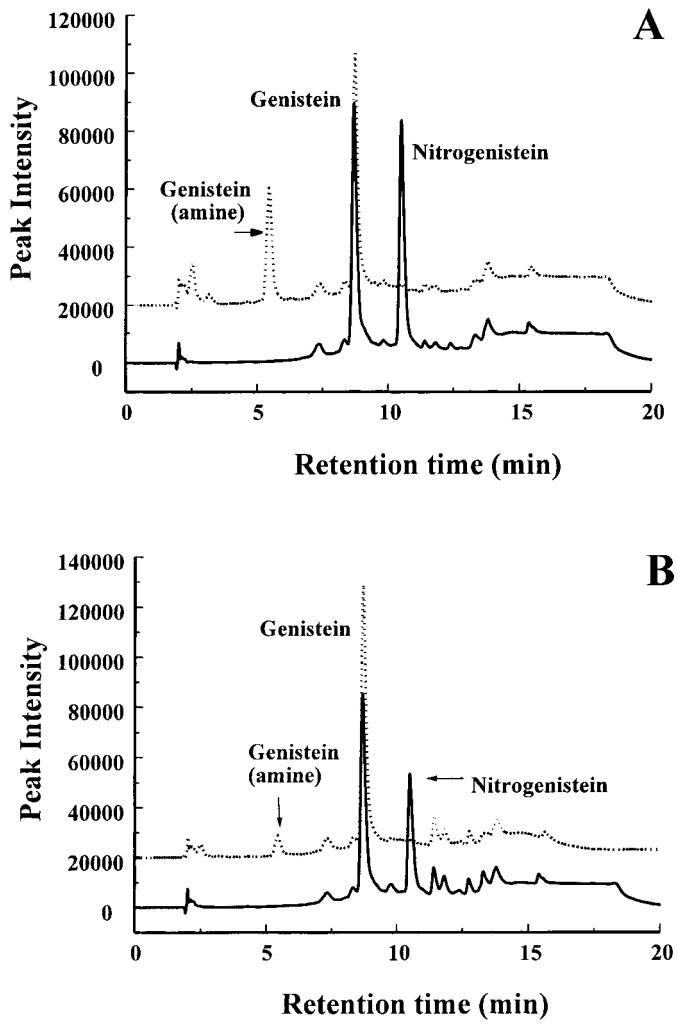

FIG. 6. Nitration of genistein by $ONOO^-$ and TNM. 50 μM genistein in 200 mM sodium phosphate (pH 7.4) with 1 mM DTPA was reacted with $ONOO^-$ (200 μM) (A) or TNM (500 μM) (B) and extracted with ether. Aliquots (40 μl) of the reaction mixture were analyzed by reversed-phase HPLC. The nitrated product peak was detected at a retention time of 10.5 min. Sodium dithionite (· · ·) was added to the reaction mixtures, which resulted in the disappearance of the nitrated product peak and the appearance of a new peak at a retention time of 5.2 min.

TABLE IV

NMR Results from Nitrogenistein

Ring	Proton	δ	Integral
A-ring	H_6	6.24	1.08
	H_8	6.41	1.10
	C_5OH	12.75	0.94
	C_7OH	N/D	N/D
B-ring	H_2	8.13	0.99
		8.48	1.00
C-ring	$H_{3'}$–$H_{5'}$	7.134	1.47
		7.154	
	$H_{2'}$–$H_{6'}$	7.697	1.14
		7.720	
	$C_{4'}OH$	8.13	0.99
		8.48	1.00

Note. Genistein (0.2 mM) was reacted with $ONOO^-$ (0.5 mM) and reaction mixtures were separated and collected by reversed-phase LC–MS with a semiprep C-8 column (as described under Materials and Methods). Values indicate chemical shift (δ) and integral values of the novel metabolite nitrogenistein as determined by ^1H NMR.

with nitrite (44). To determine whether these conditions could result in the nitration of isoflavones, genistein (50 μM) was reacted with $NaNO_2$ (0.5 mM) and HOCl (100 μM) at room temperature for 20 min. These conditions result in the nitration of tyrosine. The reaction mixture was analyzed by HPLC–MS and showed mono- and dichlorinated derivatives along with nitrogenistein in addition to the molecular ions indicating chloronitrogenistein (*m/z* 348) and dichloronitrogenistein (*m/z* 382) (Fig. 8, Table II).

DISCUSSION

In the present study we have demonstrated, using a combination of analytical techniques, that chlorinated or nitrated derivatives of the isoflavones are formed when isoflavones are reacted *in vitro* with HOCl or $ONOO^-$, respectively. Furthermore, both nitration and chlorination occur on the same isoflavone molecule when it is reacted with sodium nitrite and HOCl.

The significance of these findings is in their potential for providing insight into the mechanism of action of isoflavones. The isoflavones have received a significant amount of attention as prospective therapeutic agents for several types of cancers and heart disease (3–7). However, the mechanisms for their biological actions are not clear. Since these diseases are also associated with the formation of reactive oxygen and nitrogen species, it has been argued that isoflavonoids, and other dietary polyphenolic substances, are able to scavenge these species and act as antioxidants (29–32). This rationale is based on the structural similarities that exist between isoflavones and the amino acid tyrosine. However, the recent detection of tyrosine residues modified by chlorination and nitration (50) raised the possibility the isoflavones may also undergo similar reactions.

We have confirmed using LC–MS that HOCl reacts with isoflavones to form mono- and/or dichlorinated derivatives. HOCl is formed by the reaction of MPO with H_2O_2 and Cl^- in neutrophils. The involvement of HOCl in inflammation was derived from the detection of chlorotyrosine and MPO at sites of inflammation (37–39). Subtle structural differences among the isoflavones result in a rather striking difference in the reactivities toward HOCl. The lack of a hydroxyl group at the C_5 position on the A-ring of daidzein results in monochlorination by HOCl, rather than dichlorination detected with the other isoflavones. While chlorination could feasibly occur on any of the three rings, these structural differences led us to hypothesize that chlorination largely occurs at C_6 and C_8 sites of the A-ring of biochanin-A and genistein, while chlorination of daidzein occurs at one of these two sites or at the C_5 position. Proton NMR analysis of mono- and dichlorogenistein confirmed this hypothesis, although the possibility of B-ring chlorination could not be excluded. In an analogous reaction, it has been previously shown that iodination of the isoflavone biochanin-A (catalyzed by the reaction of thyroid peroxidase with H_2O_2 and I^-) occurs at three sites, C_6, C_8, and $C_{3'}$ (51). It is possible that selective chlorination occurs in an MPO-catalyzed reaction *in vivo* if the substrate binds to the enzyme as reported for iodination by thyroid peroxidase.

We also demonstrated that the isoflavones genistein and daidzein can be nitrated by the oxidant $ONOO^-$. Peroxynitrite is a nitrating agent that has been associated with the detection of nitrotyrosine at sites of inflammatory damage (40, 41). A single novel product

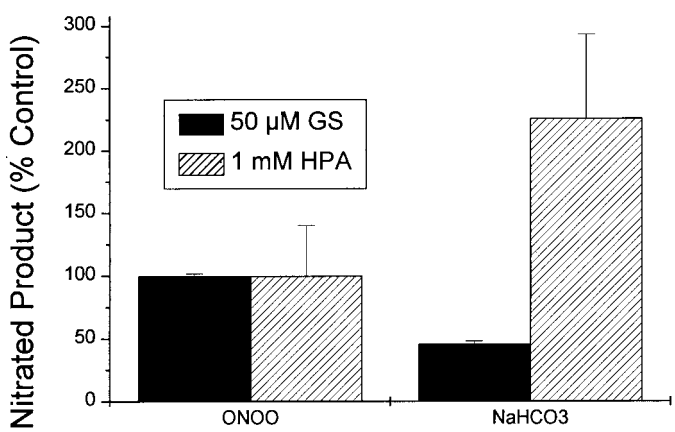

FIG. 7. Nitration of genistein and HPA by $NaHCO_3$ and $ONOO^-$. Genistein (50 μM) (solid) or HPA (1 mM) (striped) in 100 mM sodium phosphate (pH 7.4) with 1 mM DTPA purged with nitrogen gas were reacted with $NaHCO_3$ (25 mM) followed by $ONOO^-$ (200 μM) and extracted with ether. Aliquots (40 μl) of the reaction mixture were analyzed by reversed-phase HPLC. Samples were normalized according to nitrated products generated by $ONOO^-$.

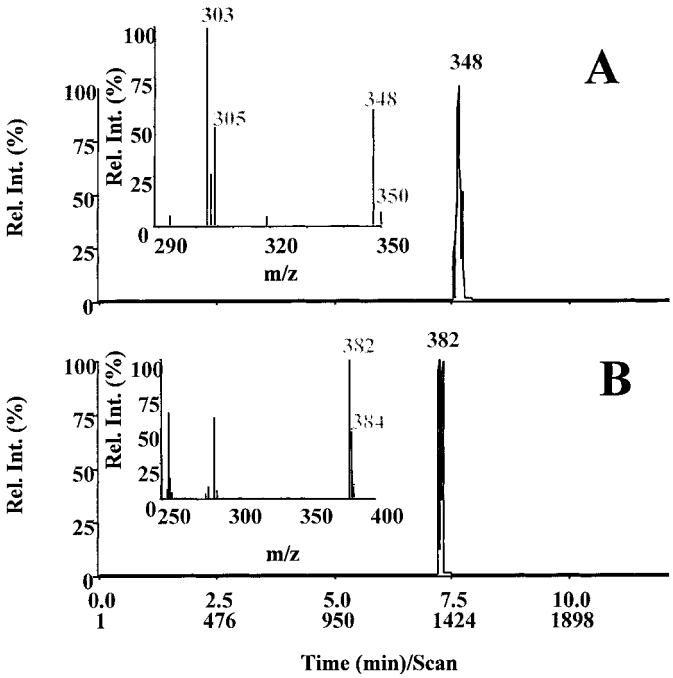

FIG. 8. Mass spectra of reaction between $NaNO_2$ and HOCl with genistein. Selected ion chromatogram of (A) *m/z* 348 (chloronitrogenistein) and (B) *m/z* 382 (dichloronitrogenistein) by reversed-phase LC–MS. The inset shows the negative-ion mass spectra for the selected ions run on negative chemical ionization–MS.

peak was present in the chromatograms of these isoflavones following reaction with $ONOO^-$. These novel products were identified as nitrogenistein and nitrodaidzein by mass spectrometry analysis. The addition of $Na_2S_2O_4$ resulted in the reduction of the nitrated products to their respective amines. Another nitrating agent, TNM, formed products identical to the $ONOO^-$-treated isoflavone. Nitration of the isoflavones was also tested by using HOCl reacted with nitrite. Under the conditions tested, both nitration and chlorination were found to occur on the same isoflavone molecule. While it has been reported that the anaerobic addition of sodium bicarbonate to $ONOO^-$ enhances nitration of aromatic molecules (49), when genistein is subjected to these conditions the yield of nitration product decreased by 50% of the control (Fig. 7). $ONOO^-$ is also known to hydroxylate aromatic structures; however, no hydroxylation was detected in the mass spectra of the products of the isoflavones and $ONOO^-$.

Analysis of the differences in the nitration reactions among the isoflavones makes it apparent that nitration occurs on the tyrosine-like B-ring. The methyl group of biochanin-A at the $C_{4'}$ position on the B-ring is the only structural difference between genistein and biochanin-A, but only genistein is nitrated. Therefore, this methyl group must block the nitration of biochanin-A by $ONOO^-$. It has been reported previously that the $C_{4'}$-hydroxyl group is responsible for the antioxidant activity of the isoflavones, as it is believed to be an effective H donor (29, 51, 52). Since tyrosine itself is nitrated at the C_3 position, we speculate that genistein and daidzein are nitrated at the $C_{3'}$ site of the B-ring. Proton NMR data for nitrogenistein were consistent with B-ring nitration.

Since genistein is both nitrated and mono- and/or dichlorinated when reacted with sodium nitrite and HOCl, this strongly suggests that nitration and chlorination occur at independent sites. Since nitration is believed to occur in the B-ring at the $C_{3'}$ position, it is therefore unlikely that chlorination also occurs in the B-ring, thereby suggesting that chlorination occurs in the A-ring (at C_6 and C_8).

A number of studies show that reactions between flavonoids and isoflavonoids with free radical species occur, although the products have not been identified (29–32, 52–54). Specifically, the flavonoids and isoflavonoids have been shown to react with peroxyl radicals, superoxide, hydroxyl radicals, and $ONOO^-$ (52–54). The antioxidant behavior of the flavonoids and isoflavonoids is related to the structure of the compound. That is, antioxidant properties increase as a function of the number of hydroxyl groups and donatable H^+ atoms (52). Other studies have shown that phenolic compounds may inhibit the nitration of tyrosine by either preferential nitration of the compound or acting as an electron donor (55).

A limiting feature of a hypothesis based on the reduction of free radical damage by the isoflavones by means of a scavenger mechanism is their low plasma concentrations. In a diet enriched in soy, the total plasma isoflavone concentration is less than 1 μM (36), with only nanomolar concentrations of unconjugated isoflavones. This effectively precludes them from acting as competitive scavengers of prooxidants. However, the affinity of estrogen receptor β for genistein is in the subnanomolar concentration range. Since both chlorination and nitration of isoflavones could change their binding affinities to the estrogen receptor β, modifications of their biological effects mediated via the estrogen receptors should be anticipated. Similar modifications may significantly alter cell-signaling mechanisms dependent on the interaction of isoflavones with other receptor systems.

In summary, we have demonstrated *in vitro* that the proinflammatory oxidants HOCl and $ONOO^-$ are capable of chlorinating and nitrating the isoflavones, respectively. These reactions produce novel, covalent modifications to the isoflavone structures. Since isoflavones are biologically active and have known biochemical targets such as the estrogen receptors, we hypothesize that the nitrated and chlorinated products will modify these inherent properties. Indeed, chlorinated derivatives of isoflavones have been detected in two different human cellular models (B. Boersma, M. Kirk,

R. Patel, V. Darley-Usmar, and S. Barnes, unpublished data). Further studies are currently being undertaken to isolate these species and determine their biological effects.

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