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Mass spectrometric methods for the analysis of chlorinated and nitrated isoflavonoids: a novel class of biological metabolites

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Electrospray ionization combined with tandem mass spectrometry was applied to a study of some representative chlorinated and nitrated isoflavones — potential metabolites of isoflavones in inflammatory cells. Upon collision-induced dissociation of deprotonated $[M - H]^-$ ions of these compounds, a number of structurally characteristic product ions were produced. The product ion analysis of 3′- and 8-chlorodaidzein in the tandom mass spectra led to ready differentiation of these isomers. 3-Nitro derivatives of both genistein and daidzein have product ions due to the losses of HNO_2 and two OH groups. Chlorinated derivatives of isoflavones were detected in cell-based experiments and their structures were proposed by comparing the tandem mass spectra of their product ions with those of standards. This work provides a suitable analytical basis to aid the characterization of chlorinated and nitrated metabolites in studies *in vivo* and *in vitro*. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: electrospray ionization; tandem mass spectrometry; chlorinated isoflavones; nitration; identification

INTRODUCTION

Halogenation and nitration of biomolecules by reactive oxygen species (ROS) and reactive nitrogen species (RNS) are key mechanisms used by host defense systems in the body. However, since ROS and RNS also have potential to damage normal host tissues, they have been implicated in various chronic diseases, ranging from atherosclerosis to neurodegeneration.[1,2] Indeed, chlorinated and nitrated derivatives of protein tyrosine residues have been described in patients with chronic inflammatory diseases.[3–7] Chlorination of tyrosine is the result of its reaction with hypochlorous acid (HOCl), the production of which from hydrogen peroxide and chloride is catalyzed by myeloperoxidase (MPO) in activated neutrophils.[8] Nitration involves the reaction with peroxynitrite ($ONOO^-$), generated from the free radicals nitric oxide ($NO^\bullet$) and superoxide ($O_2^{-\bullet}$), to form 3-nitrotyrosine.[9] HOCl can also react with nitrite, a metabolite of $ONOO^-$, to form nitryl chloride, another potent chlorinating and nitrating agent.[10]

Isoflavonoids are polyphenolics found in soy foods[11] and some other dietary supplements.[12] The isoflavones genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone) are the major source of isoflavones in the diet.[13] Biochanin A (4′-methoxygenistein) is a major isoflavone found in red clover-based dietary supplements.[12] Isoflavones are undergoing intense research because of the apparent association of soy intake and a reduction in risk of atherosclerosis,[14] cancer,[15] neurodegeneration[16] and osteoporosis.[17] Several mechanisms of biological action have been proposed for isoflavones, such as estrogen receptor interaction,[18] as antioxidants[19,20] and as sinks for reaction with ROS and RNS.[21] The last two mechanisms relate to the structural analogies of isoflavones with the amino acid tyrosine.[22]

We have recently proposed that dietary polyphenolics may react with ROS and RNS produced at sites of inflammation.[21–23] This is potentially significant since pre-clinical studies in cell culture and animal experiments have revealed that diets enriched with polyphenolics may provide protection against cancer[24,25] and cardiovascular diseases.[26] We are currently investigating the hypothesis[21] that chlorinated and nitrated polyphenolics are not only end products of metabolism, but

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are also new pharmacophores, with their own biological activities.

In previous work, using *in vitro* approaches, we demonstrated that the isoflavones genistein, daidzein and biochanin A can be chlorinated and nitrated by ROS and RNS.[21] These reactions were investigated using liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS).[21,27] However, systematic studies using tandem mass spectrometry (MS/MS) of the molecular ions of these metabolites have not been reported previously. An initial purpose of the present study, therefore, was to elucidate the fragmentation patterns of synthetic chlorinated and nitrated isoflavones, in addition to genistin, the β-glucoside of genistein, a model of the circulating metabolite of genistein, genistein-7β-glucuronide.

Using the insights gained from the elucidation of the fragmentation patterns, the structures of isoflavonoid metabolites produced from HL-60 cells or *in vitro* reactions with chlorinating and nitrating reagents were deduced. Our results show that 3′- and 8-chlorodaidzein can be distinguished based on their product ions in MS/MS analysis and that this approach may be extended to other members of the polyphenol family.

EXPERIMENTAL

Materials

Genistein and genistin were extracted and purified as described previously.[24] Daidzein and biochanin A were obtained from Indofine Chemicals (Somerville, NJ, USA). Acetonitrile and ammonium acetate were of HPLC grade and obtained from Fisher Chemical (Norcross, GA, USA). Hypochlorous acid (HOCl) was purchased from Aldrich Chemicals (Milwaukee, WI, USA). Catalase, β-glucosidase and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals were the best grades available.

Synthesis of chlorinated and nitrated isoflavonoids

Chlorinated and nitrated derivatives of isoflavones were obtained by reaction with HOCl and $ONOO^-$ or tetranitromethane (TNM), respectively.[21] The 3′-chlorinated derivatives were synthesized from commercially available 3-chloro-4-hydroxyphenylacetic acid. 8-Chlorodaidzein was synthesized from 2-chlororesorcinol, which was itself prepared via chlorination of 1,3-cyclohexanedione and subsequent aromatisation. Hydrolysis of chlorinated genistin was carried out with β-glucosidase in ammonium acetate buffer (pH 5.0). The synthetic isoflavones were fully characterized by ^1H and ^{13}C NMR spectroscopy and their purity was established by reversed-phase high-performance liquid chromatography (HPLC), using acetonitrile–water as eluent. Nitration of prunetin (5 mM, in 1 ml of methanol) was carried out in 10 ml of a buffer solution composed of 50% sodium hydrogen-carbonate (50 mM, pH 9)–50% ethanol with 1 mM diethylenetriaminepentaacetic acid (DTPA) and TNM (125 mM, in 658 µl of buffer). The reaction was allowed to proceed for 30 min with constant shaking and the products were extracted with ethyl acetate for MS analysis.

Cell culture and detection of chlorinated isoflavones

HL-60 cells obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin–streptomycin and 2 mM L-glutamine. Cells were incubated at 37 °C in an atmosphere of 95% O_2–5% CO_2. DMSO (1.3%, v/v) was added to the cells for 7 days to induce differentiation to mature granulocytes. On day 7, differentiated HL-60 cells were washed twice with KRPG. Viable cells were counted on a hemocytometer using trypan blue exclusion.

Differentiated HL-60 cells were suspended in Krebs–Ringer phosphate buffer with 5 mM glucose (KRPG) (0.9% NaCl, 1.15% KCl, 2.11% $CaCl_2$, KH_2PO_4, 3.8% $MgSO_4$, 0.1 M sodium phosphate, pH 7.4) at 1×10^6 cells ml^{-1}. Genistein (final concentration 0–100 µM) was added to 1 ml of cell suspension in 5 µl of DMSO. Cells were activated with 10 µM PMA and incubated for 0–60 min. Each sample was treated with catalase (5 U ml^{-1}) and placed on ice for 10 min. The cells were sedimented by centrifugation for 5 min at 800 *g* at 4 °C. The supernatant was aspirated and extracted as follows: the cell supernatant (~950 µl) was added to water (4 ml) and diethyl ether (2 ml). The samples were vortex mixed and centrifuged at $2000 \times g$ for 10 min, then the ethereal, top layer was removed. Additional aliquots (2 ml) of diethyl ether were added and the same steps were repeated until a total volume of 10 ml of diethyl ether has been added. The ether layers were combined and evaporated to dryness under air. A similar procedure was followed for genistin, except that enzymatic hydrolysis of chlorinated genistin was carried out prior to diethyl ether extraction.

Instrumentation

Reaction mixtures were separated by HPLC using a 10 cm × 4.6 mm i.d. C-8, 300 Å Aquapore reversed-phase column, pre-equilibrated with 10 mM ammonium acetate (NH_4OAc). Elution was carried out at a flow-rate of 1 ml min^{-1}. The mobile phase composition was as follows: 0–10 min, linear gradient (0–50%) of acetonitrile in 10 mM NH_4OAc; 10–12 min, isocratic 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, isocratic with 90% aqueous acetonitrile in 10 mM NH_4OAc. A portion of the column eluate (25 µl min^{-1}) was passed into the Ionspray ionization interface attached to a PE-Sciex (Concord, Ontario, Canada) API III triple-quadrupole mass spectrometer. The voltage on the Ionspray interface was −4900 V and the orifice potential was set at −50 V. Negative ion mass spectra were recorded over an m/z range of 200–500. Selected $[M - H]^-$ (molecular) ions were analyzed by collision-induced dissociation with 90% argon-10% nitrogen, and product ion spectra were recorded.

RESULTS AND DISCUSSION

Product ion analysis of standards

To understand the mass spectrometric behavior of chlorinated and nitrated isoflavones, LC/MS analysis of authentic

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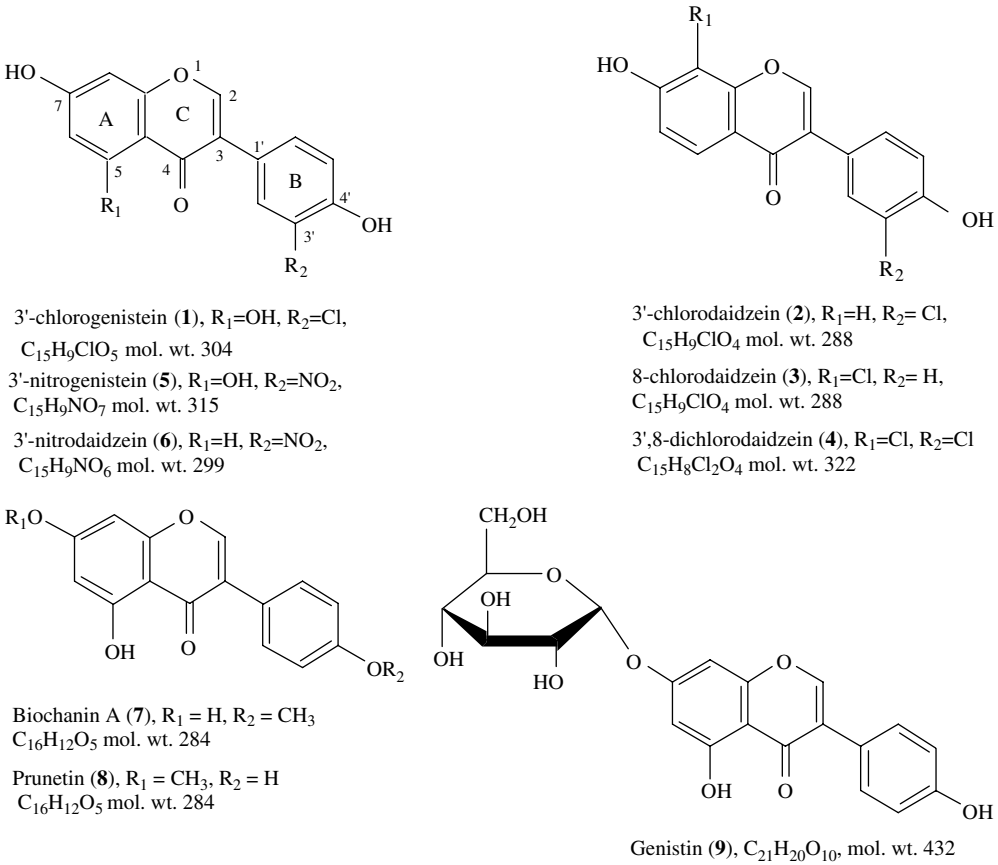


Figure 1. Structures of isoflavones **1**–**9**.

3′-chlorogenistein (**1**), 3′-chlorodaidzein (**2**), 8-chlorodaidzein (**3**), 3′,8-dichlorodaidzein (**4**), 3′-nitrogenistein (**5**) and 3′-nitrodaidzein (**6**) was performed using ESI in the negative ion mode. We observed molecular ions of monochlorinated compounds including the prominent $[M-H]^-$ ion together with an $[M-H+2]^-$ ion, approximately one-third the intensity of the former ion, indicating the presence of the ^{37}Cl isotope. The molecular masses and structural and elemental formulae of these standards are shown in Fig. 1. Product ion spectra of deprotonated molecules of **1** and **2** are displayed in Fig. 2(a) and (b), respectively. In the cases of **1** and **2**, prominent product ions at m/z 267 and 251 were observed, respectively, due to the neutral loss of HCl. Together with these, a series of product ions were also observed differing by 28 Da owing to the loss of CO, representing a chlorinated polyphenolic ring system. Comparison of the product ion spectra of $[M-H]^-$ and $[M-H+2]^-$ of both **1** and **2** showed no product ions containing chlorine, indicating that loss of HCl from the deprotonated molecular ion of 3′-chloroisoflavonoids was a particularly facile process. In contrast, the product ion spectrum of **3** showed ions containing chlorine at m/z 125, 169 and 259. Another interesting feature is the presence of product ions at m/z 183 (base peak), 169 and 117, produced via a retro-Diels–Alder reaction in the product ion spectrum of **3**. Analogous ions are absent in the cases of **1** and **2**. The possible fragmentation pathways of **1** and **3** are shown in Schemes 1 and 2, respectively. A limited number of structural isomers of product ions are shown in the schemes since the loss of CO could occur from several different sites.

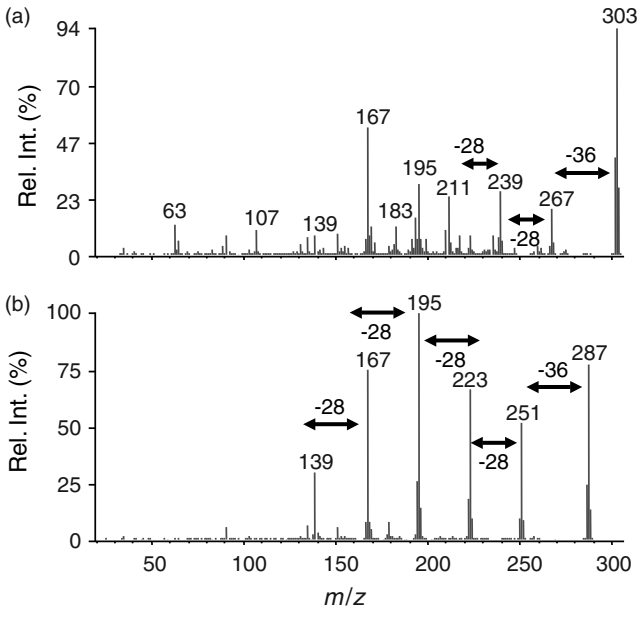
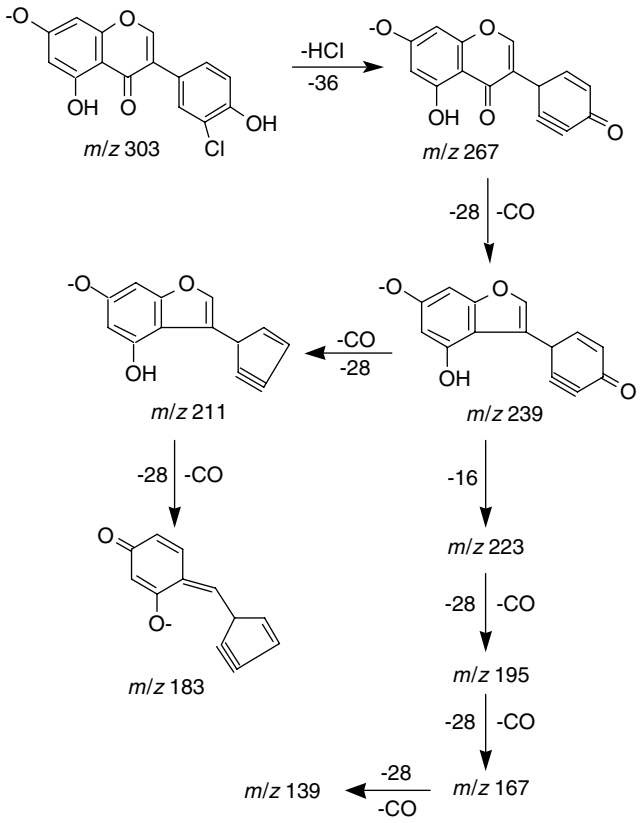


Figure 2. Comparison of the products ions obtained in ESI-MS/MS experiments on deprotonated (a) **1** and (b) **2**. The loss of HCl from the deprotonated parent ions appeared to be the initial event of fragmentation in both the cases.

The product ion spectrum of **4** shared the fragmentation pathways those of **2** and **3**. The product ions appearing at m/z 285, 257, 229 and 201 corresponded to those of **2**, whereas the ions at m/z 89 and 125 are common in both



Scheme 1. Major product ions and proposed structures observed in the product ion spectrum of deprotonated molecular ion of **1**. A limited number of possible structural isomers are shown.

3 and **4**. The product ions at *m/z* 125, 201, 217, 229, 257 and 285 are chlorinated. As in the case of **1** and **2**, the product ion at *m/z* 285 is due to the loss of HCl from the deprotonated molecular ion. These observations support the assumption that chlorinated product ions may be generated in the MS/MS experiments only from ring A chlorination. Elimination of HCl from the deprotonated molecule ion is the first step in the principal fragmentation route of deprotonated **1** and **2**, whereas loss of CO seems to be the initial fragmentation step in the case of 8-chloro derivatives. Thus the loss of HCl from the deprotonated molecular ion is an indicative of B-ring chlorination in genistein and daidzein.

The loss of HCl from the 3′-position of **1, 2** and **4** could lead to the production of a stable keto group in the ring B with extended conjugation. This may justify the easy removal of HCl from the 3′-position compared with the 8-position of the ring A. The apparent difference in the fragmentation pathways of these isomers (**2** and **3**) can be utilized in the isomer differentiation of chlorinated isoflavones.

We next analyzed the nitrated isoflavones (**5** and **6**) by LC/MS/MS in order to obtain their diagnostic product ion(s). The product ion spectra of deprotonated ions of **5** and **6** showed characteristic elimination of one HNO_2, two OH groups and a series of ions originating from the losses of CO groups, albeit that the relative abundance of each varied with collision energy. Figure 3(a) and (b) show the MS/MS profiles of deprotonated **5** at collision energies 35 and 28 eV, respectively. The most abundant ion (*m/z* 280) at lower collision energy (28 eV) was due to the loss of two OH groups from the deprotonated parent ion. The relatively high abundance of this ion indicated that the loss of OH

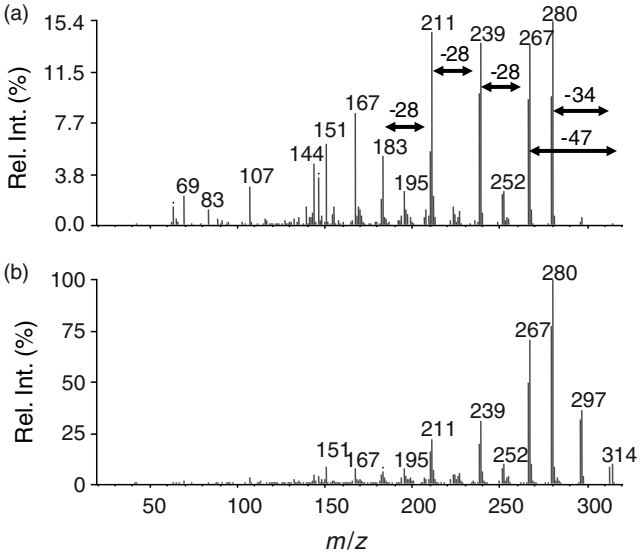
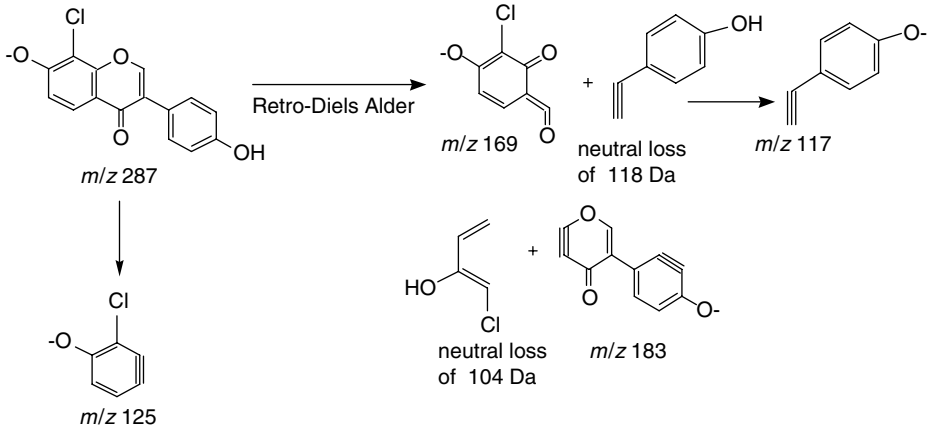


Figure 3. Product ion spectra for the ion *m/z* 314 of **5** at collision energy (a) 35 and (b) 28 eV.



Scheme 2. Major product ions and proposed structures observed in the product ion spectrum of deprotonated molecular ion of **3**.

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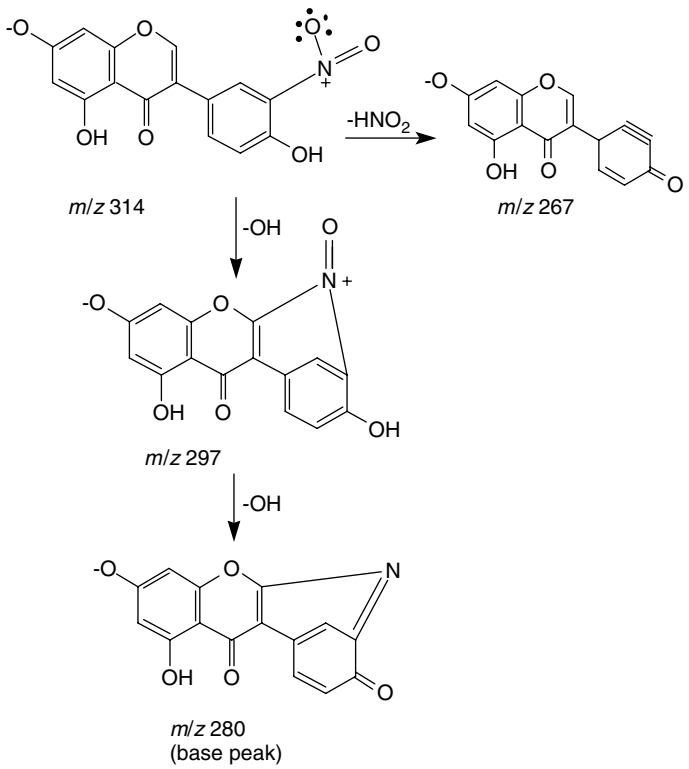

Scheme 3. Possible mechanism for the elimination of HNO_2 and OH groups from the ion of *m/z* 314 of **5**. A limited number of possible structural isomers are shown.

was a particularly facile process. At higher collision energy (35 eV), however, the abundances of other ions increased. Whereas losses of O, OH, NO and NO_2 are common in nitro aromatic compounds, losses of two OH and HNO_2 are not typical fragments.[28,29] We hypothesize that these losses are triggered by the interaction of the nitro group attached to the ring B with the ring C of the isoflavones. This hypothesis is supported by the fact that no losses of OH and HNO_2 were observed in the product ion spectrum of 2-nitrophenol.

The characteristic loss of two OH in **5** and **6** may occur via the formation of a six-membered ring as shown in Scheme 3. Validation of this hypothesis would require further analyses, including the use of isotope labeling. The loss of HNO_2 may be justified by the formation of a keto group in the ring B. The elimination of two OH groups from the deprotonated molecular ion of **5** may be possible as depicted in Scheme 3.

Identification of reaction products of biochanin A (7) with HOCl

LC/MS analysis of products formed by the reaction of biochanin A (**7**) with HOCl in phosphate buffer showed ions at *m/z* 317 (retention time t_R 10.36 min) and 351 (t_R 9.14 min), corresponding to mono- and dichloro products of **7**, respectively. Product ion spectra of the deprotonated ion of monochlorobiochanin A $[M-H]^-$ showed chlorine-containing ions at *m/z* 169, 245, 258 and 273, but did not generate a significant ion due to the loss of HCl (36 Da) from the ion at *m/z* 317, indicating that chlorine was in ring A. Another striking feature in favor of the ring A chlorination is the appearance of an ion at *m/z* 132, which is a diagnostic product ion of methoxylated isoflavonoids with one OH or OCH_3 group in ring B (J. Prasain and S. Barnes, unpublished data). This ion was invariably present in the deprotonated product ion spectra of biochanin and mono- and dichlorobiochanin A. Furthermore, the presence of an ion at *m/z* 169, which is one of the diagnostic product ions of **3**, supports the ring A chlorination. The possible sites of chlorination could be the 6- or 8-position. In the present studies, we could not elucidate the position of chlorination in ring A conclusively.

The product ion spectrum of the ion at *m/z* 351 displayed a fragmentation pattern similar to that of monochloro-biochanin A. The product ions at *m/z* 244, 279, 292 and 307 corresponded to chlorinated molecules. No product ion due to the loss of HCl from the deprotonated molecular ion of dichlorobiochanin A was observed, suggesting again no chlorination in ring B. Hence the dichlorination in ring A was confirmed, and the structure of the product is proposed as 6,8-dichlorobiochanin A. Dichlorination at the 6- and 8-positions has also been reported in the case of quercetin (3′,4′,5,7-tetrahydroxyflavon-3-ol).[30]

Identification of reaction products of genistin (9) with HOCl

We next observed a conversion of genistin (genistein-7-*O*-*β*-glucoside, **9**) to chlorinated products by the reaction of genistin with HOCl. The reaction product gave rise to ions at *m/z* 465 $[M-H]^-$ and 467 $[M-H+2]^-$, indicating the production of monochlorogenistin. Following hydrolysis with *β*-glucosidase and extraction with diethyl ether, LC/MS analysis of the hydrolyzed product revealed the production of two monochlorogenistein isomers. These monochloro products eluting at t_R 12.0 and 13.3 min produced similar but non-identical product ions on LC/MS/MS (Fig. 4), indicating

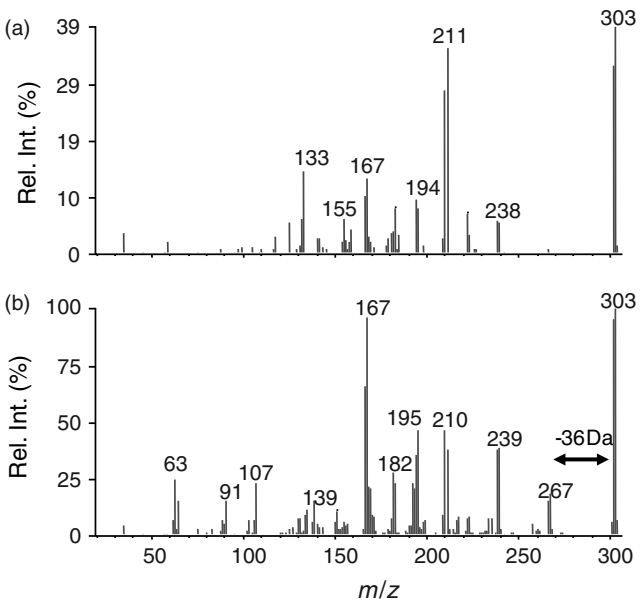

Figure 4. Product ion spectra of the hydrolyzed product of chlorogenistein at *m/z* 303 in LC/MS/MS experiments at t_R (a) 12.06 and (b) 13.37 min.

they could be isomeric products. The product ions of the chlorogenistein isomer eluting at t_R 13.3 min were found to be identical with those of 3′-chlorogenistein, whereas the product ion mass spectrum of the isomer eluting at 12.0 min did not show an ion due to the loss of HCl from the deprotonated molecular ion; instead, a chlorine-containing product ion at *m/z* 125 was observed, indicating that it could be 6- or 8-chlorogenistein. Hence these data demonstrated that genistin, the *β*-glucoside of genistein, is also chlorinated by HOCl and forms at least two isomers. By analogy, it should be expected that the main circulating form of genistein, its 7-*O*-*β*-glucuronide, would undergo a similar reaction.

Analysis of reaction product of prunetin (8) with TNM

Since prunetin is a methylated analogue of genistein and also an isomer of **7**, it is important to identify its nitrated metabolites by MS analysis. In order to obtain a nitrated prunetin, it was reacted with TNM and the reaction mixture was subjected to ESI-MS analysis. The ESI mass spectrum showed ions due to *m/z* 328 and 373, corresponding to deprotonated ions of mono- and dinitrated prunetin, respectively. The product ion spectrum of the ion at *m/z* 373 showed a prominent ion at *m/z* 358 due to the loss of a methyl radical (typical of methoxylated isoflavones) and it further gave rise to a weak ion at *m/z* 312 liberating an NO_2 group. An ion at *m/z* 281 may be generated by the losses of two NO_2 groups. In the present study, we could not conclusively describe the genesis and structure of other ions such as *m/z* 253, 225 and 200, owing to data limitations. Further analysis is under way to elucidate the structure of dinitroprunetin.

Detection of chlorinated products of genistein and genistin (9) in PMA-activated HL-60 cells

In previous studies, we have shown that genistein when incubated with PMA-activated HL-60 cells produces monochlorogenistein (37%), dichlorogenistein (5%) and genistein (42%) (B. J. Boersma, R. P. Patel, M. R. Benton, M. Kirk, L. S. Wilson, N. P. Botting, J. K. Prasain, S. Barnes and V. M. Darley-Usmar, unpublished data). These products were analyzed by LC/MS and LC/MS/MS using an isocratic solvent system (40% aqueous acetonitrile in 10 mM NH_4OAc) in order to identify their structures. Inspection of the LC/MS/MS data showed that two different monochlorinated products of genistein were produced in cells treated with genistein and PMA. One eluting at t_R 1.6 min had product ions containing chlorine and there was no ion due to the loss of HCl (36 Da) from the deprotonated molecular ion. These data indicated that the product was 6- or 8-chlorogenistein. The other yielded product ions at t_R 3.8 min consistent with those of 3′-chlorogenistein. Hence scavenging of HOCl by genistein in the PMA activated HL-60 cells results in at least two chlorinated isomers.

The fate of **9** when incubated with HL-60 cells during the respiratory burst was also investigated. After incubation for 60 min, the cell medium was subjected to enzymatic hydrolysis with *β*-glucosidase. The reaction products were extracted with diethyl ether and analyzed by LC/MS/MS with multiple reaction ion monitoring (MRM). The MS/MS/MRM scan (parent ion/fragment ion combination) is very sensitive technique that can detect genistein (*m/z* 269/133), chlorogenistein (*m/z* 303/221 or 303/167), dichlorogenistein (*m/z* 337/209 or 337/224), and nitrogenistein (*m/z* 314/280 or 314/267). These ions are selected because they are diagnostic for the particular compound. Because of the high selectivity of the MRM technique and the use of

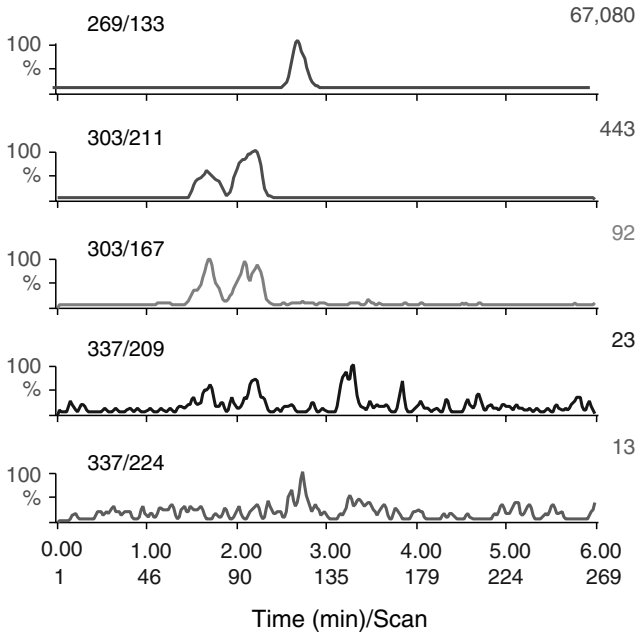

Figure 5. LC/MS/MS/MRM analysis for genistein (*m/z* 269/133), chlorogenistein (*m/z* 303/167, 303/211) and dichlorogenistein (*m/z* 337/209, 337/224) in the reaction mixture obtained from hydrolysis of genistin in PMA activated HL-60 cells. The numbers on the top right corner of each chromatogram represent the full-scale value of the ion abundance.

isocratic conditions, the throughput of sample analysis can be enhanced substantially.

Figure 5 shows LC/MS/MS/MRM ion chromatogram of the reaction product. Genistein, chlorogenistein, and dichlorogenistein were analyzed. The intensities of the peaks clearly indicated that genistein was the most abundant, followed by monochlorogenistein. Thus we have demonstrated by the application of ESI-MS/MS that the activation of the respiratory burst can generate HOCl, capable of chlorinating the soy isoflavones genistein and genistin. However, the extent of chlorination in the case of **9** is comparatively low compared with that of genistein.

CONCLUSION

LC/MS/MS analyses of authentic chlorinated and nitrated isoflavones were used to obtain information about the typical product ions that are structurally characteristic. Elimination of HCl occurs for 3′-chloro derivatives of genistein and daidzein from the deprotonated molecular ions, and distinguishes them from their isomers (6- or 8-chloro derivatives of genistein and daidzein). We developed an LC/MS/MS/MRM method for detection of the chlorinated genistein in biological samples. These data will be invaluable in the characterization and quantitation of chlorinated and nitrated metabolites in *in vivo* and *in vitro* future studies.

Acknowledgements

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