Oxidative in Vitro Metabolism of the Soy Phytoestrogens Daidzein and Genistein

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The oxidative metabolism of the major soy isoflavones daidzein and genistein was investigated using liver microsomes from Aroclor-treated male Wistar rats. Both daidzein and genistein were extensively metabolized and are therefore excellent substrates for cytochrome P450 enzymes. The identity of the metabolites was elucidated using high-performance liquid chromatography (HPLC) with diode array detection, gas chromatography−mass spectrometry (GC/MS), and HPLC/atmospheric pressure ionization electrospray mass spectrometry (API-ES MS) as well as reference substances. Daidzein was converted to nine metabolites, comprising four monohydroxylated, four dihydroxylated, and one trihydroxylated metabolite. Genistein was metabolized to four monohydroxylated and two dihydroxylated products. With both isoflavones the additional hydroxy groups are exclusively introduced into the ortho positions of existing phenolic hydroxy groups. The major metabolites of daidzein were identified as 6,7,4′-trihydroxyisoflavone, 6,7,3′,4′-tetrahydroxyisoflavone, 7,8,4′-trihydroxyisoflavone, and 5,6,7,4′-tetrahydroxyisoflavone. The main microsomal metabolites of genistein were 5,6,7,4′-tetrahydroxyisoflavone and 5,7,8,4′-tetrahydroxyisoflavone. Furthermore, the GC/MS and HPLC/API-ES MS analysis support the conclusion that one monohydroxylated metabolite of daidzein and genistein is hydroxylated at the aliphatic position C-2 of the C-ring. The UV−vis, GC/MS, and HPLC/MS data of all detected metabolites as well as the derived chemical structure of the metabolites are presented. Most metabolites are reported in this paper for the first time. On the basis of these findings it is suggested that hydroxylation reactions may also play an important role in the in vivo metabolism of the soy isoflavones daidzein and genistein.

Keywords: *Phytoestrogens; isoflavones; daidzein; genistein; microsomal metabolism; soy*

INTRODUCTION

The phytoestrogens daidzein and genistein are naturally occurring isoflavones that are found in numerous edible plants, especially soybeans. There is a growing scientific interest in phytoestrogens and particularly daidzein and genistein due to their potential beneficial properties for human health. Several epidemiological studies in humans have suggested that isoflavonoid intake was inversely associated with the incidence of hormone-dependent diseases, especially breast and prostate cancer as well as osteroporosis (Adlercreutz, 1990; Adlercreutz et al., 1995). These findings are supported by experimental data from in vivo and in vitro studies. Various mechanisms for the potential effects of daidzein and genistein have been described and discussed. These include hormone-mediated as well as non-hormone-mediated effects and are summarized by Bingham et al. (1998).

Despite the increasing interest in the issue, data available on the absorption of conjugated and unconjugated isoflavones and their metabolism in humans or experimental animals are still limited. In soybeans as well as in nonfermented soy products, the isoflavones occur in an esterified form as glycosides, malonylglycosides, and acetylglycosides, whereas in fermented soy products (for example, miso) the agylycons predominate (Coward et al., 1993). Following ingestion, it is generally believed that the glycosides daidzin and genistin are hydrolyzed in the intestine colon by the action of bacteria to release the aglycons daidzein and genistein, prior to absorption. However, in analogy to the structurally related flavonoids, for example, quercitin (Graefe et al., 1999), it can also be discussed that unhydrolyzed isoflavone glycosides may be taken up by the enterocyte via a glucose transporter and are subsequently hydrolyzed there by a broad-specific cytosolic β-glucosidase before entering the portal vein (Day et al., 1998). After absorption in the small and probably large intestine, the isoflavone aglycons are reconjugated with glucuronic acid and to a smaller extent with sulfate by the phase II enzymes UDP-glucuronosyltransferases and sulfotransferases in the liver. Like endogenous estrogens, these conjugates are excreted through both urine and bile and undergo enterohepatic circulation. In addition to the hydrolysis of glycosides the flora of the large bowel plays another important role in the metabolism of the isoflavone. It has been described that daidzein can be converted to dihydrodaidzein, which is further metabolized to both equol and *O*-desmethylangolensin, whereas genistein is transformed to dihydrogenistein and is further metabolized to 6′-hydroxy-*O*-desmethy-

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10.1021/jf000524i CCC: $19.00
Published on Web 09/06/2000

langolensin. Genistein can also be degraded by the action of bacteria to ethylphenol (Kurzer and Xu, 1997).

However, only 7−30% of the ingested amount of daidzein and genistein may be recovered in urine and <10% in the feces (Kelly et al., 1993; Cassidy et al., 1994; Xu et al., 1994, 1995; Bingham et al., 1998). One possible explanation for this low recovery is the formation of hitherto unknown metabolites. We have therefore studied the oxidative metabolism of daidzein and genistein with rat liver microsomes to clarify whether these isoflavone are also substrates for the hepatic cytochrome P450 enzymes.

MATERIALS AND METHODS

Chemicals. Daidzein, genistein, 6,7,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone (purity > 99% according to GC/MS analysis), $NADP^+$, NADH, isocitrate, isocitrate dehydrogenase, resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, and tyrosinase were obtained from Sigma-Aldrich Chemical Co. (Deisenhofen, Germany). HPLC grade acetonitrile was from Merck (Darmstadt, Germany). Bicinchoninic acid protein assay reagent was from Pierce Chemical Co. (Rockford, IL). All other chemicals were of the highest grade available.

Preparation and Characterization of Liver Microsomes. Microsomal preparations were prepared from the livers of Aroclor-treated male Wistar rats according to standard procedures as described by Lake (1987). Treatment with Aroclor 1254 (one intraperitonal injection on day 1 of a dose of 500 mg/kg of body weight dissolved in sesame oil at 100 mg/mL, sacrifice on day 6) was carried out to induce hepatic cytochrome P450 enzymes. Protein concentrations were estimated by using Pierce bicinchoninic reagent. Cytochrome P450 concentrations were measured as described by Omura and Sato (1964). The microsomes were characterized by the specific activities of major cytochrome P450 enzymes as determined according to the method of Burke et al. (1994).

Incubation with Microsomes and Extraction. Standard incubation mixtures contained 2 mg of microsomal protein, 50 nmol of isoflavone dissolved in 40 μL of DMSO, and a NADPH-generating system (3 mM $MgCl_2$, 1 mM $NADP^+$, 8 mM D,L-isocitrate, and 0.5 U of isocitrate dehydrogenase) in a final volume of 2 mL of 0.05 M potassium phosphate buffer, pH 7.4. After 2 min of preincubation at 37 °C in a shaking water bath, the reaction was started by adding the NADPH-generating system and stopped after 60 min by extraction with 4 × 2 mL of ice-cold ethyl acetate. The organic solvent was evaporated under reduced pressure at room temperature and the residue dissolved in 0.2 mL of methanol and subjected to HPLC analysis. Controls were carried out by omitting the NADPH-generating system or by using heat-inactivated microsomes.

Incubation with Tyrosinase. The isoflavone (500 nmol in 50 μL of DMSO) was incubated with tyrosinase (500 U) and NADH (5 mg) in 4 mL of 0.1 M Tris-HCl buffer, pH 7.4, for 30 min at 37 °C. The medium was extracted three times with ethyl acetate, and the combined organic phase was evaporated under reduced pressure to dryness. The residues were dissolved in 0.2 mL of methanol and subjected to HPLC analysis. Controls were carried out by omitting the enzyme.

HPLC Analysis of Metabolites. The HPLC system consisted of two Knauer model 64 liquid chromatograph pumps (Knauer, Germany) equipped with a Kortec K45 gradient controller (ICI Instruments), a 100 μL injection loop, and a photodiode array UV−vis detector model SPD-M6A (Shimadzu). HPLC separation of the ethyl acetate extract was carried out on an RP-18 column (Tox 1, 250 mm × 4.6 mm i.d., particle size 5 μm, Sepserv, Germany) using a linear gradient of 15−35% acetonitrile in water (pH 3) for 50 min, followed by a 10 min isocratic elution. The flow rate was 1 mL/min, and the eluent was recorded with a diode array detector.

GC/MS Analysis of Metabolites. GC/MS was carried out on a Finnigan GCQ system connected to an ion trap mass detector. The metabolites were isolated by HPLC and freeze-dried. For GC/MS analysis, the residues were derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 2 h at room temperature. The TMS derivatives were analyzed using a nonpolar capillary column (MDN-5S, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Supelco, Germany) and a linear temperature gradient (60 °C for 1 min, then 30 °C/min to 250 °C, hold for 10 min, then 1 °C/min to 275 °C, and hold for 5 min). The injector port temperature was 50 °C. Mass spectra were obtained by electron impact ionization at 70 eV and an ion source temperature of 200 °C. Full-scan spectra (mass range of 50−750 amu) were recorded at rate of 2 spectra/s. The selected ion mode was used for the detection of daidzein, genistein, 6,7,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone, and their metabolites.

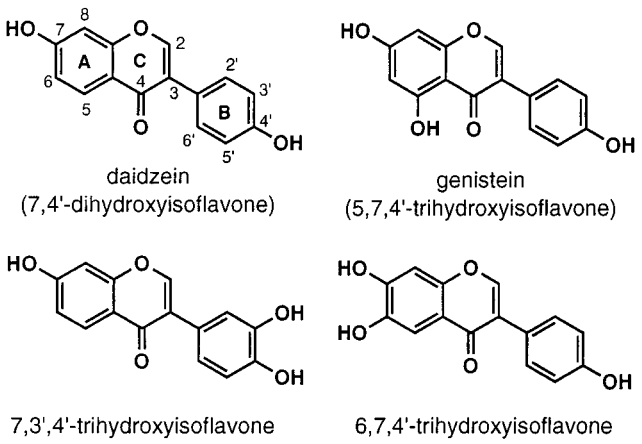

Figure 1. Chemical structures of daidzein, genistein, 6,7,4′-trihydroxyisoflavone, and 7,3′,4′-trihydroxyisoflavone.

Table 1. Characterization of Liver Microsomes from Aroclor 1254-Induced Male Wistar Rats[a]

protein content (mg of microsomal protein/g of liver)	23
CYP450 (total) (nmol/mg of protein)	1.63
ECOD activity (nmol/min/mg of protein)	23.5
EROD activity (nmol/min/mg of protein)	15.1
PROD activity (nmol/min/mg of protein)	0.22

[a] Each number represents the mean of three independent determinations. ECOD, 7-ethoxycoumarin-deethylase; EROD, 7-ethoxyresorufin-*O*-deethylase; PROD, 7-pentoxyresorufin-*O*-dealkylase.

HPLC/MS Analysis of Metabolites. HPLC/MS analysis was performed on an HP 1100 series HPLC equipped with an autoinjector, quaternary HPLC pump, column heater, UV detector, and HP Chem Station for data collection and handling. The HPLC was interfaced to an HP series 1100 mass selective detector equipped with an API-ES ionization chamber. Conditions for analysis in the positive mode were as follows: capillary voltage, 4 kV; fragmentor, 160 or 180 V; nebulizing pressure, 50 psi; drying gas temperature, 350 °C; drying gas flow, 10 L/min. Data were collected using both the scan mode and selected ion monitoring (SIM) mode. Spectra were scanned over a mass range of *m*/*z* 80−500 at 0.65 s/ per cycle.

RESULTS

Formation and Separation of Microsomal Metabolites of Daidzein and Genistein. Daidzein and genistein (Figure 1) were incubated with hepatic microsomes from male Wistar rats pretreated with Aroclor 1254. The microsomes were characterized as shown in Table 1. The complete organic extract of each incubation mixture was analyzed by reversed phase HPLC with diode array detection. Both daidzein and genistein were extensively metabolized. At least eight metabolites of daidzein (peaks 1, 2, 3, 4/5, 7, 8, 9, and 12) and six

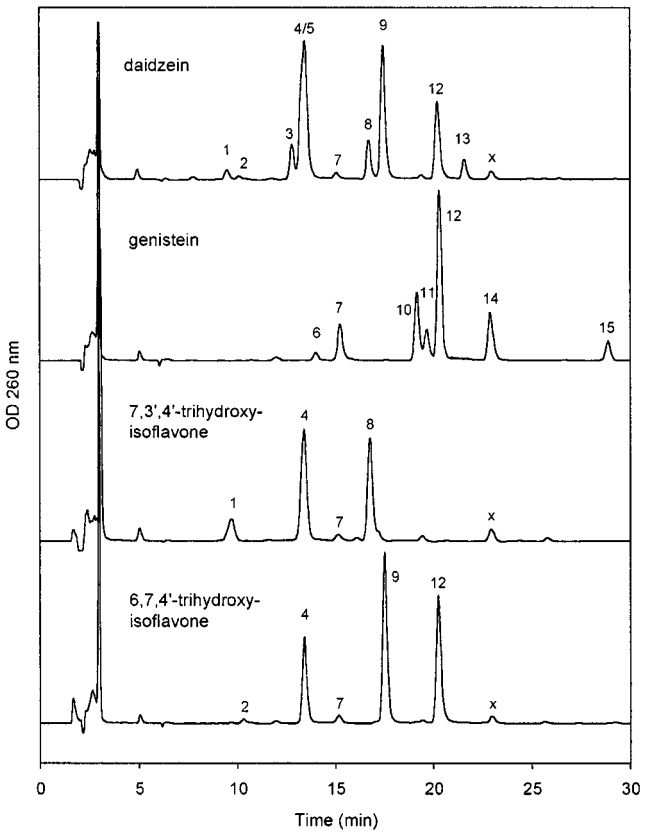

Figure 2. HPLC profiles of daidzein, genistein, 6,7,4′-trihydroxyisoflavone, and 7,3′,4′-trihydroxyisoflavone metabolites from Aroclor-induced rat liver microsomes. Peak numbers refer to Table 2. Peak x occurs also in the HPLC chromatograms of control incubations and is therefore not a metabolite.

metabolites of genistein (peaks 6, 7, 10, 11, 12, and 14) were clearly detectable by HPLC analysis (Figure 2). These products were not observed when NADPH was omitted or when heat-inactivated microsomes were used in the incubation mixture (data not shown). Furthermore, the extracted metabolites were analyzed by HPLC/MS with atmospheric pressure ionization electrospray (API-ES) and by GC/MS after trimethylsilylation with BSTFA. The GC profiles are shown in Figure 3. In the case of daidzein, the mass spectra indicated the formation of nine hydroxylated metabolites (four monohydroxylated, four dihydroxylated, and one trihydroxylated metabolite). In the case of genistein, the mass spectra demonstrated the formation of six hydroxylated metabolites (four monohydroxylated and two dihydroxylated metabolites). For the correlation of HPLC and GC peaks, each HPLC peak was isolated and analyzed by GC/MS after trimethylsilylation. The chromatographic data, the UV maxima, and the MS data as well as the assigned chemical structures (see below) are summarized in Table 2.

Approach for the Elucidation of Metabolite Structures. *Use of Reference Compounds.* 6,7,4′-Trihydroxyisoflavone and 7,3′,4′-trihydroxyisoflavone (Figure 1) were commercially available and employed as reference compounds for cochromatography and comparison of mass spectra. Furthermore, the microsomal metabolism of these two isoflavones was studied (Figure 2). The generated metabolites proved to be helpful tools for the identification of daidzein and genistein metabolites with unknown structures, as are outlined below. For example, daidzein metabolites that are also generated in the metabolism of 6,7,4′-trihydroxyisoflavone must contain an additional hydroxy group in position C-6 of the A-ring, whereas daidzein metabolites that are also generated in the metabolism of 7,3′,4′-trihydroxyisoflavone must contain an additional hydroxy group in position C-3′ of the B-ring (Figure 1).

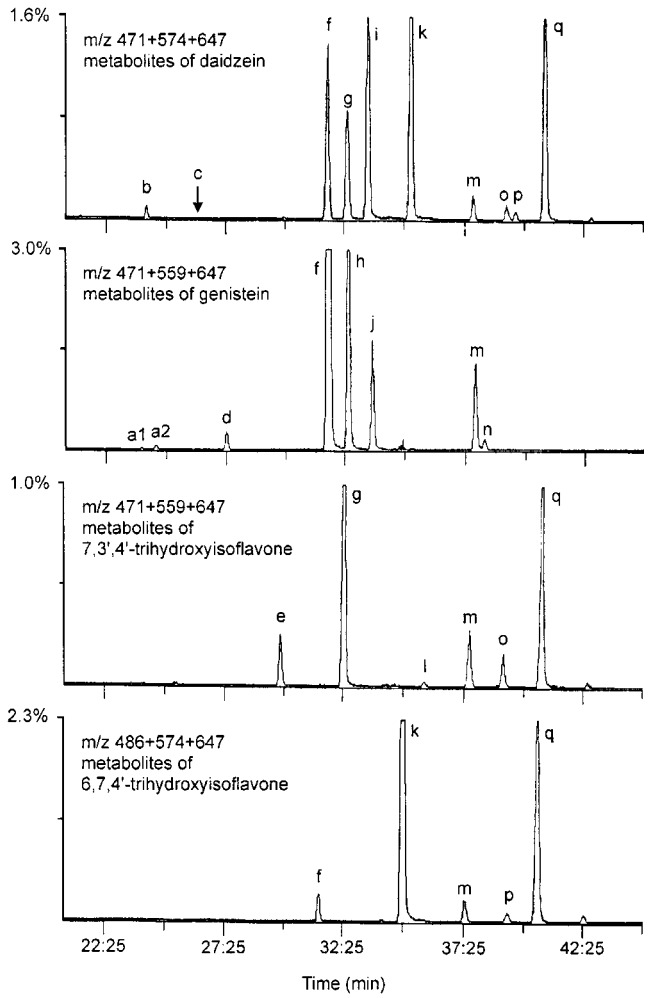

Figure 3. GC selected ion chromatograms of hydroxylated metabolites of daidzein, genistein, 6,7,4′-trihydroxyisoflavone, and 7,3′,4′-trihydroxyisoflavone from Aroclor-induced liver microsomes. Peak labels refer to Table 2.

HPLC/MS and GC/MS Analysis. Mass spectrometry can be used to determine the molecular weight of the metabolites and to establish the distribution of hydroxy groups between the A- and B-rings of the isoflavone molecule. GC/MS with electron impact (EI) ionization of TMS derivatives of the metabolites gave mass spectra with an intense molecular ion or $[M^+ - 15]$ ion (loss of CH_3 radical), as is demonstrated for several of the metabolites in Figure 4. EI mass spectra can thus be used to determine the molecular weight of the metabolites. The other fragment ions are of rather low intensity and were not used to obtain information about the chemical structure of the metabolites; they are formed through loss of a TMSO group and breakdown of the A- and B-ring.

Valuable information about the location of the hydroxy groups can be obtained from HPLC/MS with positive API-ES, if a relatively high fragmentor voltage is used (see Materials and Methods). Representative API-ES mass spectra of isoflavone with one, two, and three hydroxy groups in the A-ring are shown in Figure 5. They display a $[M + 1]^+$ base peak ion as well as a

Table 2. Chromatographic and Spectroscopic Data of the Oxidative Metabolites of Daidzein and Genistein

HPLC peak[a]	RT in HPLC (min)	UV max (nm)	$[M + 1]^+$ HPLC/MS	GC peak[b]	RT in GC (min)	M^+, GC/MS TMS derivative	isoflavone substitution pattern
1	10.5	260	287	o	38.5	574	7,8,3′,4′-tetrahydroxyisoflavone
2	11.0	264/322	287	p	39.1	574	6,7,8,4′-tetrahydroxyisoflavone
3	13.6	248/300	271	b	23.4	486	2,7,4′-trihydroxyisoflavone
4	14.4	258/288/325	287	q	40.2	574	6,7,3′,4′-tetrahydroxyisoflavone
5	14.4	nm[c]	271	i	33.0	486	7,8,4′-trihydroxyisoflavone
6	14.9	nm[d]	303	n	37.5	662	5,7,8,3′,4′-pentahydroxyisoflavone
7	16.0	271	303	m	37.3	662	5,6,7,3′,4′-pentahydroxyisoflavone
8	17.7	249/292	271	g	32.1	486	7,3′,4′-trihydroxyisoflavone
9	18.4	256/324	271	k	34.5	486	6,7,4′-trihydroxyisoflavone
10	20.1	268/351	287	h	32.2	574	5,7,8,4′-tetrahydroxyisoflavone
11	20.6	258	287	a1	23.4	502	2,5,7,4′-tetrahydroxyisoflavone
				a2	23.9	574	
12	21.3	270	287	f	31.3	574	5,6,7,4′-tetrahydroxyisoflavone
13	22.5	249/303	255	c	26.0	398	7,4′-dihydroxyisoflavone (daidzein)
14	23.9	262/(287)	287	j	33.2	574	5,7,3′,4′-tetrahydroxyisoflavone
15	29.5	262	271	d	27.0	486	5,7,4′-trihydroxyisoflavone (genistein)
nd[e]				e	29.3	574	not identified
nd[e]				l	35.2	662	not identified

[a] According to Figure 2. [b] According to Figure 3. [c] nm, not measurable, coelution with HPLC peak 4. [d] nm, not measurable (intensity too low for UV spectrum). [e] nd, not detected.

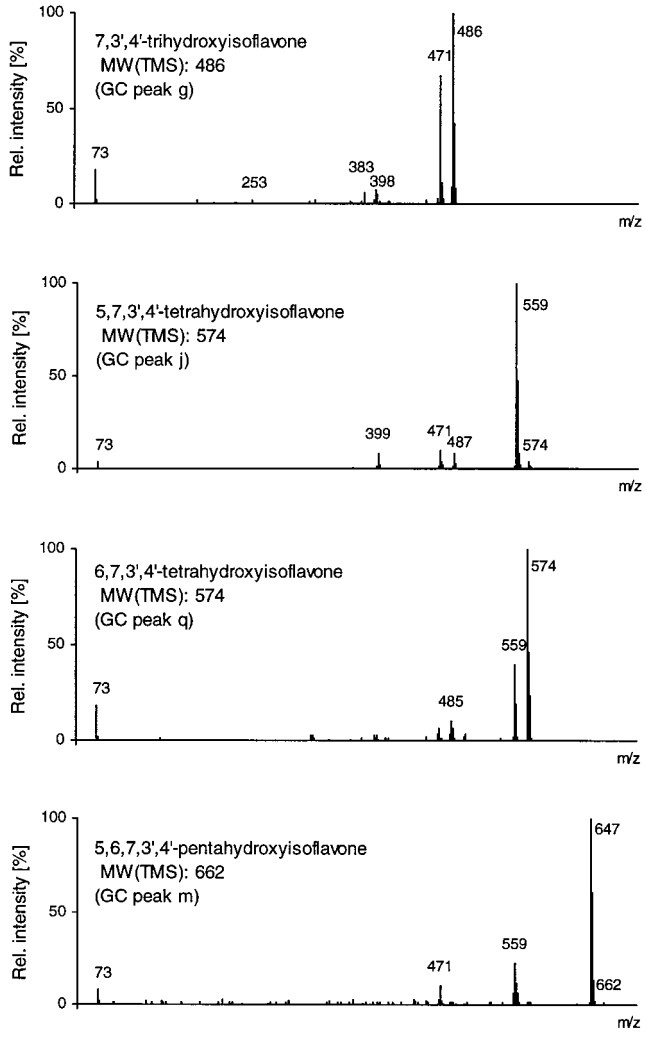

Figure 4. Representative electron impact mass spectra of TMS derivatives of daidzein and genistein metabolites. GC peaks refer to Figure 3.

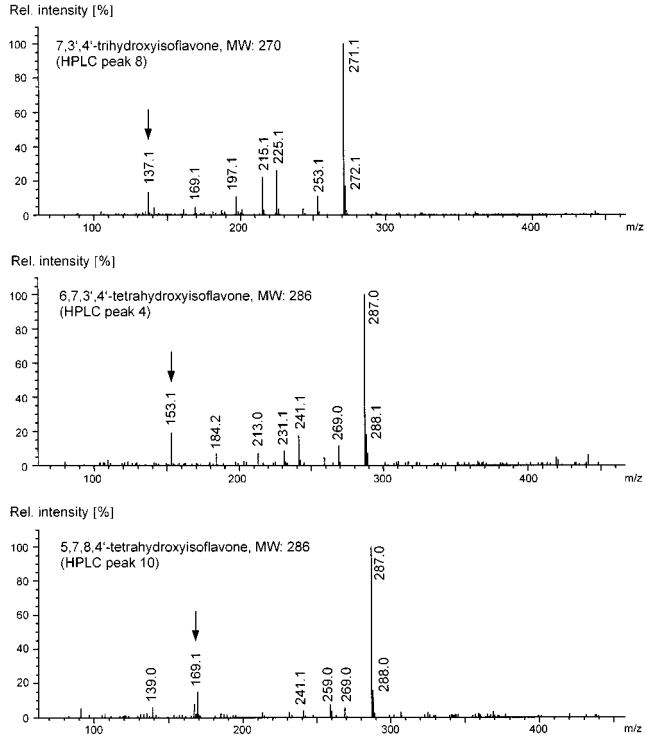

Figure 5. HPLC/API-ES mass spectra of metabolites with one, two, or three hydroxy groups in the A-ring.

fragment ion derived from the molecular ion by a retro-Diels−Alder reaction (Figure 6); this fragment ion can be used to determine the number of hydroxy groups in the A-ring of the molecule. Other fragment ions are generated by the loss of H_2O and CO (Figure 6).

Use of Tyrosinase. The enzyme tyrosinase was used to generate catechol metabolites of several isoflavones (summarized in Table 3). Interestingly, the reaction catalyzed by tyrosinase was dependent on the number and positions of existing hydroxy groups in the isoflavone molecule. Isoflavones with only one hydroxy group in the B-ring located at C-4′ were found to be hydroxylated by the enzyme to the corresponding catechol (3′,4′-dihydroxylated) structure. In contrast, no A-ring catechols were generated by tyrosinase. For example, daidzein (7,4′-dihydroxyisoflavone) was converted only to 7,3′,4′-trihydroxyisoflavone but not to 6,7,4′-trihydroxyisoflavone. Furthermore, the reaction with 7,3′,4′-trihydroxyisoflavone does not result in a defined product, whereas 6,7,4′-trihydroxyisoflavone was converted to 6,7,3′,4′-tetrahydroxyisoflavone.

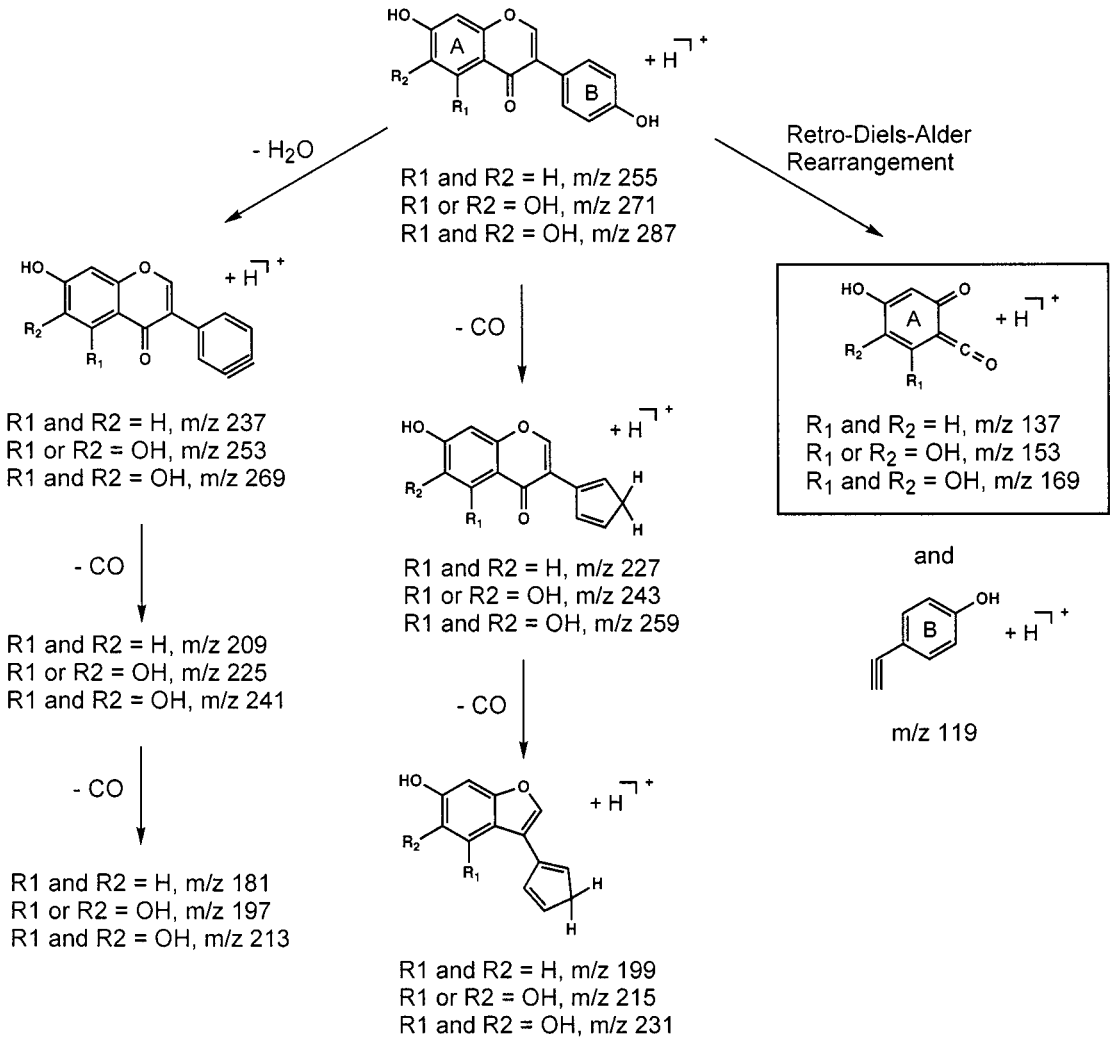

Figure 6. Proposed fragmentation pathways of isoflavone molecules according to Barbuch et al. (1989).

Table 3. Reaction of Various Isoflavones with Tyrosinase to Their Catechols

substrate	catechol metabolite (main product, >90%)
daidzein	7,3′,4′-trihydroxyisoflavone
genistein	5,7,3′,4′-tetrahydroxyisoflavone
6,7,4′-trihydroxyisoflavone	6,7,3′,4′-tetrahydroxyisoflavone
5,6,7,4′-tetrahydroxyisoflavone	5,6,7,3′,4′-pentahydroxyisoflavone
5,7,8,4′-tetrahydroxyisoflavone	5,7,8,3′,4′-pentahydroxyisoflavone
7,3′,4′-trihydroxyisoflavone	no defined products, polymers

Identification of the Microsomal Metabolites of Daidzein. A representative HPLC profile of the microsomal metabolites of daidzein is depicted in Figure 2. The chemical structures of all of the numbered peaks were elucidated and are listed together with the analytical data in Table 2.

The HPLC peaks 8 (GC peak g, Figure 3) and 9 (GC peak k, Figure 3) represent two major metabolites and were identified by cochromatography in GC/MS with reference substances as 7,3′,4′-trihydroxyisoflavone and 6,7,4′-trihydroxyisoflavone, respectively. The mass spectra of 7,3′,4′-trihydroxyisoflavone are depicted in Figures 4 and 5.

Another prominent daidzein metabolite, HPLC peak 12 (GC peak f, Figure 3), was identified by GC/MS and HPLC/MS as a dihydroxylated daidzein. This compound also appeared in the in vitro metabolism of genistein and 6,7,4′-trihydroxyisoflavone (Figures 2 and 3). The only dihydroxylated daidzein metabolite that can also be formed from genistein and 6,7,4′-trihydroxyisoflavone is 5,6,7,4′-tetrahydroxyisoflavone.

HPLC peak 3 in the metabolite profile of daidzein was not generated in the metabolism of 6,7,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone, and genistein (Figure 2). The positive API-ES MS gave a quasimolecular ion $[M + 1]^+$ at *m/z* 271, indicating a monohydroxylated daidzein, and a diagnostic fragment ion at *m/z* 137, indicating an unchanged A-ring. The only remaining positions for hydroxylation of the daidzein molecule are C-2′ in the B-ring and C-2 in the C-ring. C-2′ would mean a hydroxylation in meta position to the existing hydroxy group at C-4′, which would be a very unusual metabolic reaction. The GC/MS analysis of the isolated HPLC peak 3 after trimethylsilylation resulted in three peaks, two clear GC peaks with molecular ions of *m/z* 486 and *m/z* 414 and one peak also with a molecular ion at *m/z* 486 that only occurred in trace amounts. If the hydroxylation takes place in the aliphatic C-2 position of the C-ring, keto−enol tautomers can be formed and could explain these three GC peaks and the corresponding mass spectra (Figure 7). It is therefore proposed that HPLC peak 3 is 2,7,4′-trihydroxyisoflavone.

HPLC peak 4/5 was composed of two distinct compounds (peaks i and q, Figure 3) when analyzed by GC/MS. The mass spectra of the TMS derivatives exhibited

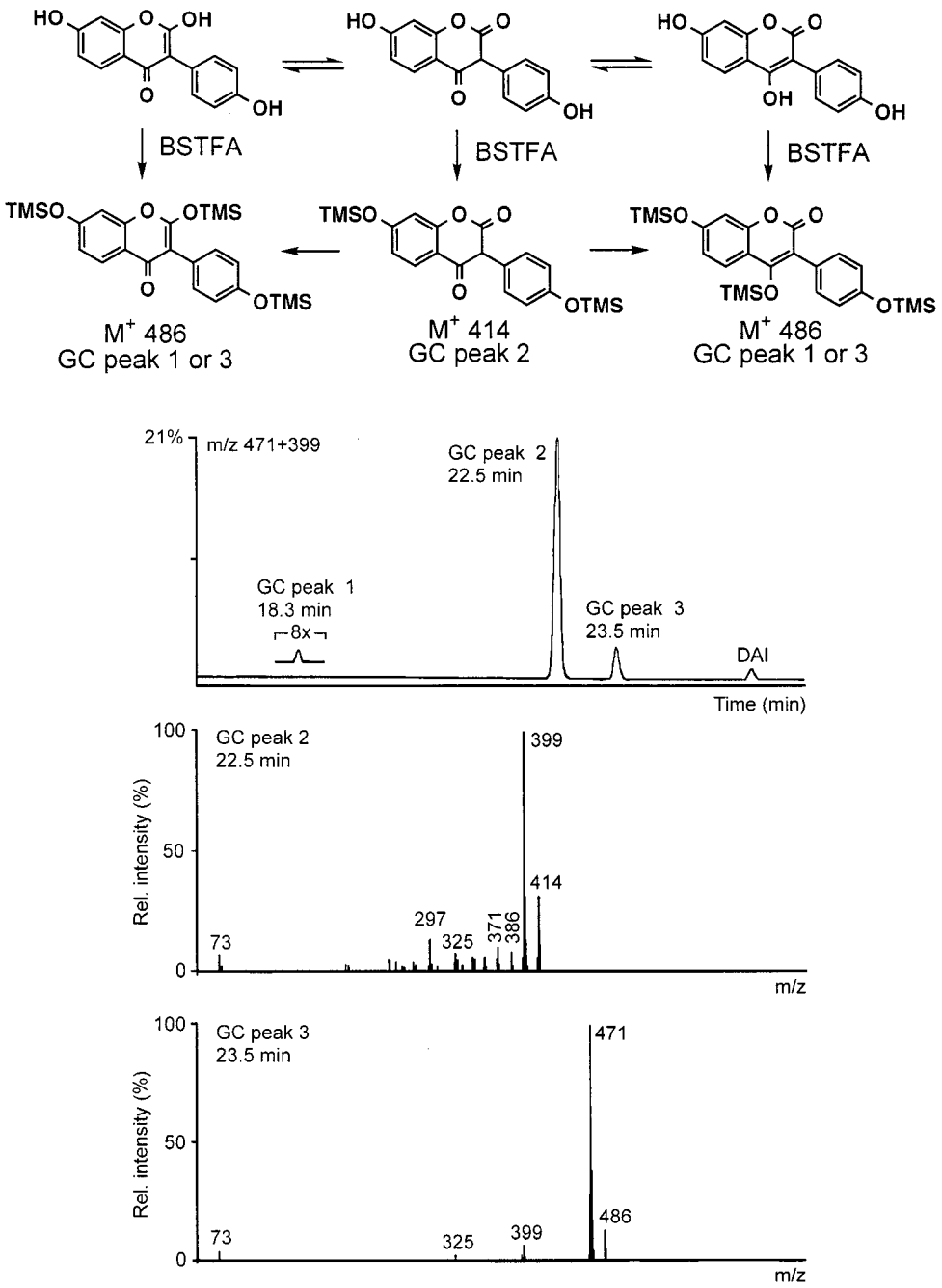

Figure 7. GC analysis of the TMS ether derivative of 2,7,4′-trihydroxyisoflavone: formation of keto−enol tautomers. GC peaks 1 and 3 show identical mass spectra.

molecular ions at *m/z* 486 and 574 (Figure 4), consistent with the addition of one and two hydroxy groups, respectively, to the daidzein molecule (Table 2). This result is in accordance with the HPLC/MS analysis, revealing one metabolite with $[M + 1]^+$ at *m/z* 271 (monohydroxylated daidzein) and one with $[M + 1]^+$ at *m/z* 287 (dihydroxylated daidzein, Figure 5). Both products exhibit the fragment ion *m/z* 153 but not *m/z* 137, indicating two hydroxy groups in the A-ring. The isolated HPLC peak 4 in the metabolism of 6,7,4′-trihydroxyisoflavone and 7,3′,4′-trihydroxyisoflavone (Figure 2) gave rise to only one product in GC/MS, which was identical with the dihydroxylated daidzein metabolite. Furthermore, this metabolite can also be generated by the reaction of 6,7,4′-trihydroxyisoflavone with tyrosinase (Table 3). Taken together, these data conclusively identify the dihydroxylated daidzein metabolite in HPLC peak 4 as 6,7,3′,4′-tetrahydroxyisoflavone. The monohydroxylated metabolite of daidzein in peak 4/5 carries the additional hydroxy group in ring A as discussed above. Possible positions are C-8, C-5, and C-6. Hydroxylation at C-5 would form genistein (5,7,4′-trihydroxyisoflavone), whereas hydroxylation at C-6 would generate 6,7,4′-trihydroxyisoflavone. As these two compounds have different retention times in HPLC and GC (Table 2), the second metabolite in HPLC peak 4/5 is most likely 7,8,4′-trihydroxyisoflavone.

Another prominent microsomal metabolite of daidzein, HPLC peak 12 (Figure 2), was a dihydroxylated daidzein according to GC/MS and HPLC/MS analysis. As this metabolite appeared also in the in vitro metabolism of genistein and 6,7,4′-trihydroxyisoflavone (Figure 2), the only possible structure is that of 5,6,7,4′-tetrahydroxyisoflavone.

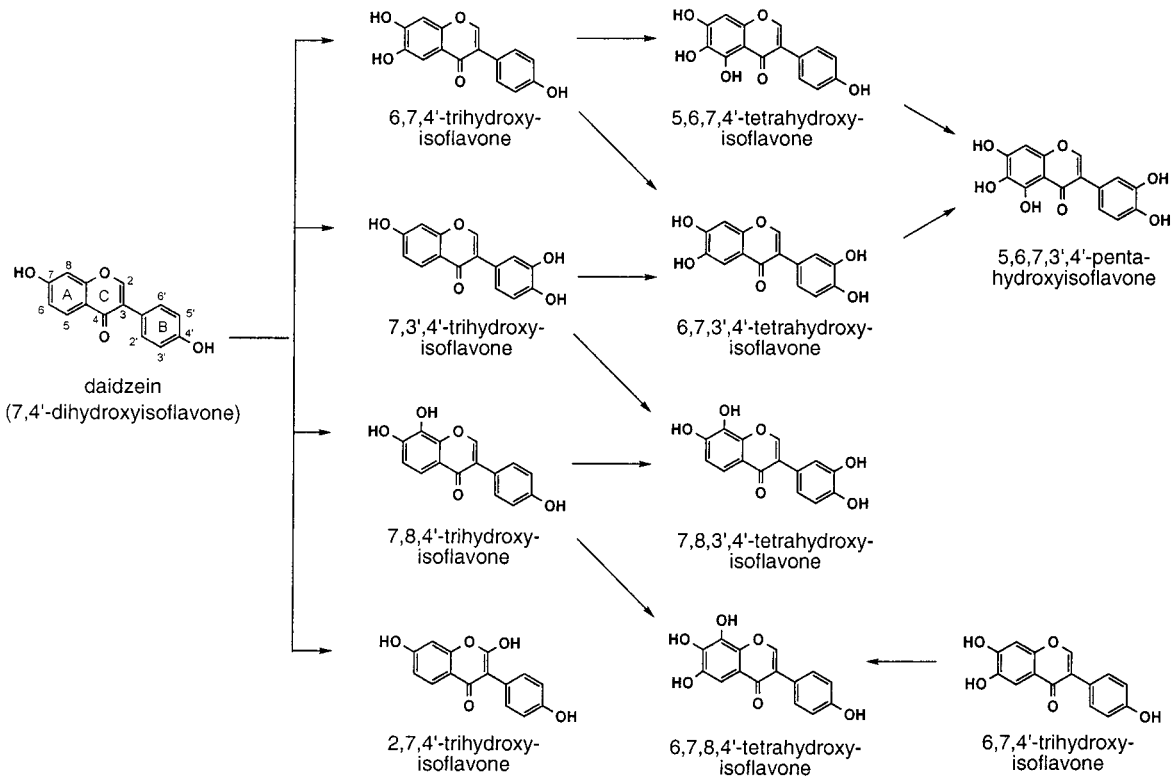

Figure 8. Microsomal metabolites of daidzein formed through aromatic and aliphatic hydroxylation.

HPLC peak 1 is a metabolite that occurs in small amounts in the oxidative metabolism of both daidzein and 7,3′,4′-trihydroxyisoflavone (Figure 2). The GC/MS analysis (peak o, Figure 3) of its TMS derivative gave a molecular ion at *m/z* 574, indicating an isoflavone molecule with four hydroxy groups. Further characterization was achieved by means of its positive API-ES mass spectrum, exhibiting a quasi-molecular ion $[M + 1]^+$ at *m/z* 287 together with a fragment ion at *m/z* 153, indicating two hydroxy groups in the A-ring. Of the two possible structures, that is, 7,8,3′,4′-tetrahydroxyisoflavone and 6,7,3′,4′-tetrahydroxyisoflavone, the latter can be ruled out because it has been already allocated to HPLC peak 4. Thus, the structure of HPLC peak 1 must be that of 7,8,3′,4′-tetrahydroxyisoflavone.

HPLC peak 2 was detected in trace amounts among the metabolites of daidzein and 6,7,4′-trihydroxyisoflavone (Figure 2). The mass spectrum of its TMS derivative (peak p, Figure 3) showed a molecular ion at *m/z* 574 and the positive API-ES mass spectrum a $[M + 1]^+$ at *m/z* 287 and a characteristic fragment ion at *m/z* 169. On the basis of these MS data it can be concluded that metabolite 2 has the structure of 6,7,4′-trihydroxyisoflavone plus one additional hydroxy group in ring A. Possible positions for this hydroxy group are C-5 and C-8. As the structure of 5,6,7,4′-tetrahydroxyisoflavone has already been allocated to HPLC peak 12 (see above), it is concluded that metabolite 2 must be 6,7,8,4′-tetrahydroxyisoflavone.

HPLC peak 7 was formed in the metabolism of daidzein, genistein, 6,7,4′-trihydroxyisoflavone, and 7,3′,4′-trihydroxyisoflavone (Figure 2) and, according to its mass spectral data (Figure 4, GC peak m), represents an isoflavone with five hydroxy groups. The only conceivable metabolite structure containing the structures of all four parent compounds is that of 5,6,7,3′,4′-pentahydroxyisoflavone.

A complete scheme of the microsomal metabolism of daidzein is depicted in Figure 8.

Identification of the Microsomal Metabolites of Genistein. The HPLC profiles of the microsomal metabolites of genistein are given in Figure 2. The six numbered peaks were all structurally identified (Table 2). Two of the genistein metabolites, HPLC peaks 7 and 12, also occurred in the metabolism of daidzein (Figure 2) and were identified as 5,6,7,3′,4′-pentahydroxyisoflavone and 5,6,7,4′-tetrahydroxyisoflavone, respectively, as described above.

HPLC peaks 6, 10, 11, and 14 were generated neither from daidzein nor from 7,3′,4′- or 6,7,4′-trihydroxyisoflavone (Figure 2). GC/MS analysis of HPLC peak 10 (peak h, Figure 3) indicated a monohydroxylated genistein metabolite (molecular ion *m/z* 574). The positive API-ES mass spectrum (Figure 5) exhibited a $[M + 1]^+$ ion at *m/z* 287 and a fragment ion at *m/z* 169, indicating that the hydroxylation has taken place in the A-ring of genistein. As there are only two possible positions, that is, C-6 and C-8, and because the structure of 5,6,7,4′-tetrahydroxyisoflavone has already been allocated to metabolite 12**,** metabolite 10 must be 5,7,8,4′-tetrahydroxyisoflavone.

The positive API-ES mass spectrum of HPLC peak 11 displayed an $[M + 1]^+$ ion at *m/z* 287 and a fragment ion at *m/z* 153, indicating a monohydroxylated genistein with an unchanged A-ring. The GC/MS analysis of the TMS derivative resulted in two peaks with molecular ions at *m/z* 574 and 502 (peaks a1 and a2, Figure 3), which can be explained by the formation of keto−enol tautomers, as was discussed above for the daidzein metabolite 3 (Figure 7). In comparison to daidzein

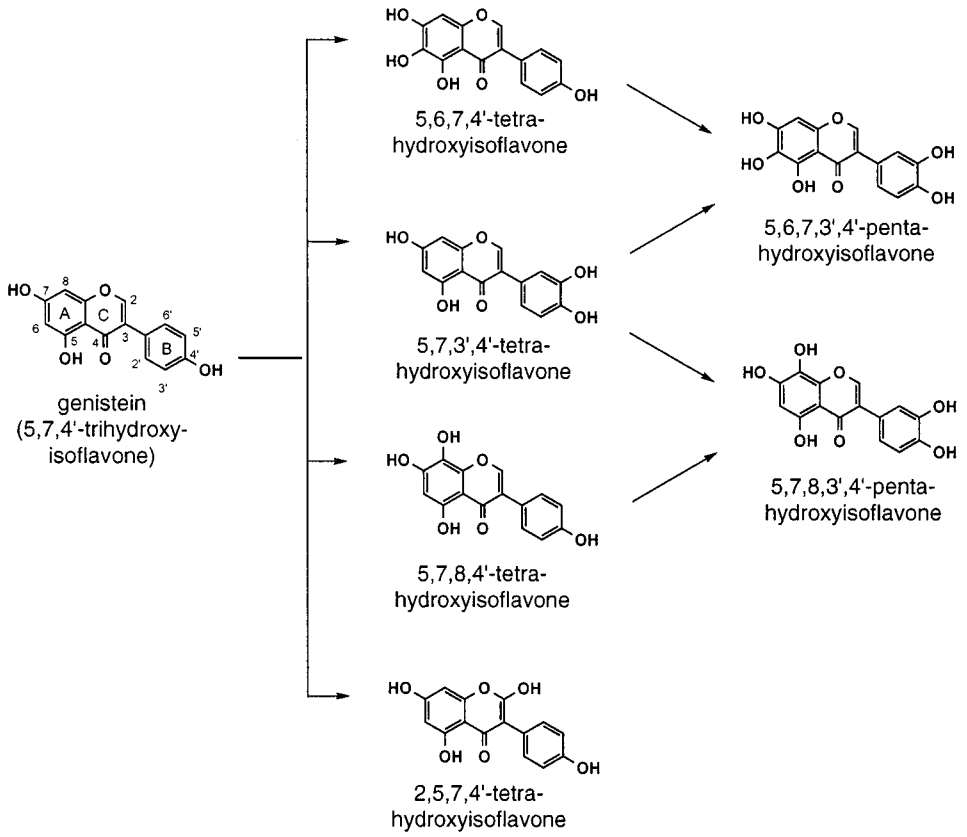

Figure 9. Microsomal metabolites of genistein formed through aromatic and aliphatic hydroxylation.

metabolite 3 the third expected peak with a molecular ion at *m/z* 574 (second enol tautomer) could not be detected. Nevertheless, it is concluded that the structure of HPLC peak 11 is 2,5,7,4′-tetrahydroxyisoflavone.

The GC/MS analysis of HPLC peak 14 (peak j, Figures 3 and 4) revealed a molecular ion at *m/z* 574, indicating a monohydroxylation. As the positive API-ES mass spectrum gave a quasi-molecular ion at *m/z* 287 and a fragment ion at *m/z* 153, the hydroxylation has not occurred in the A-ring. HPLC peak 14 was also detected by HPLC and GC/MS after incubation of genistein with tyrosinase (Table 3). This indicates that metabolite 14 has a catechol structure and hydroxylation has taken place in the B-ring. Therefore, the structure of metabolite 14 is 5,7,3′,4′-tetrahydroxyisoflavone.

Genistein metabolite 6, which was formed only in trace amounts (Figure 2), had a molecular ion of *m/z* 662 in GC/MS (TMS derivative) and an $[M + 1]^+$ ion of *m/z* 303 in HPLC/MS, implying two additional hydroxy groups. Moreover, the positive API-ES mass spectrum contained a fragment ion at *m/z* 169, indicative for three hydroxy groups in the A-ring. The only possible positions for the third hydroxy group in the A-ring of genistein are C-6 and C-8. As metabolite 6 is also formed by incubation of metabolite 10, identified before as 5,7,8,4′-tetrahydroxyisoflavone, with tyrosinase (Table 3), the two hydroxy groups introduced into genistein must be located at C-8 of the A-ring and C-3′ of the B-ring, establishing the structure of 5,7,8,3′,4′-pentahydroxyisoflavone for metabolite 6. A complete scheme of the microsomal metabolism of genistein is depicted in Figure 9.

DISCUSSION

In the present study, we have investigated the in vitro metabolism of the dietary isoflavones daidzein and genistein with Aroclor-induced rat liver microsomes. Daidzein was converted into nine hydroxylated metabolites and genistein into six hydroxylated products in an NADPH-dependent reaction. The metabolites were separated by HPLC and GC and identified by their EI and API-ES mass spectra. Our results demonstrate that daidzein and genistein are excellent substrates for cytochrome P450 enzymes. The monohydroxylated daidzein and genistein metabolites are not the end products of metabolic oxidation but are themselves substrates for the cytochrome P450 monooxygenase system and are converted to di- or tri-hydroxylated metabolites. Our results are in accordance with the findings of Roberts-Kirchhoff et al. (1999), who reported that genistein is metabolized by human and rat liver microsomes to 5,7,3′,4′-tetrahydroxyisoflavone and four other unidentified metabolites.

Recent preliminary studies in our laboratory show that oxidative metabolites of daidzein and genistein also occur in humans in vivo. We have analyzed the urine of volunteers after consumption of a soy-rich diet for 2 days and found (besides genistein, daidzein, glycitein, and equol) three oxidative daidzein metabolites identified as 6,7,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone, and 6,7,3′,4′-tetrahydroxyisoflavone and two monohydroxylated genistein metabolites, tentatively identified as 5,7,8,4′-tetrahydroxyisoflavone and 5,6,7,4′-tetrahydroxyisoflavone by EI-mass spectra and retention time in GC (data not shown). Thus, cytochrome P450 enzymes appear to play an important role in the metabolism of the soy isoflavones daidzein and genistein. Most of the oxidative metabolites formed by rat liver microsomes or detected in human urine have a catechol or pyrogallol structure. It can be expected that these metabolites will occur in vivo as methoxy derivates generated by the reaction with catechol-*O*-methyltrans-

ferase and *S*-adenosyl-L-methionine as methyl-group donor. Preliminary analysis of human urine has provided clear evidence for at least two methylated derivatives of the above-mentioned catechol metabolites (data not shown). Therefore, our findings might have a substantial impact on the understanding of the biotransformation and bioavailability of isoflavones in vivo and could be one reason for the low recovery of genistein and daidzein in former biotransformation studies (Kelly et al., 1993; Cassidy et al., 1994; Xu et al., 1995).

Moreover, the hydroxylated metabolites might have other biological activities than their parent compounds. For example, they may differ in their estrogenic activities. The number of hydroxy groups strongly affects the biological activities of isoflavones. Thus, genistein is described as a topoisomerase and tyrosine kinases inhibitor (Yamashita et al., 1990; Akiyama et al., 1987), whereas daidzein, the chemical structure of which differs by only one hydroxy group (Figure 1), exhibits a completely different spectrum of biological properties. For example, we and others have recently reported that genistein and coumestrol are mutagenic and genotoxic in various mammalian cell culture systems (Kulling and Metzler, 1997; Morris et al., 1998; Kulling et al., 1999; Abe 1999), whereas daidzein proved to be inactive in these studies.

In this context it cannot be excluded that the aromatic hydroxylation of daidzein and genistein, which leads to metabolites with catechol and pyrogallol moieties, might represent a metabolic activation process because *o*-hydroquinones can be further oxidized to the semiquinone or quinone species. Generation of radicals (e.g., superoxide) can then occur as a result of redox cycling between the catechol and the quinone. Free radical damage of proteins and DNA as well as covalent binding of semiquinones and quinones to these macromolecules can therefore be envisioned. This may be of interest because an analogous process has been implicated in the mechanism of carcinogenesis induced by 17β-estradiol and diethylstilbestrol (Liehr and Roy, 1990; Pasagian-Macaulay et al., 1996; Liehr and Ricci, 1996). Therefore, the estrogenic and other possible biological activities, both benefical and adverse, of the major oxidative metabolites of soy isoflavones should be investigated.

ABBREVIATIONS USED

API-ES, atmospheric pressure ionization electrospray; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; DMSO, dimethyl sulfoxide; EI, electron impact; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; HPLC/MS, high-performance liquid chromatography/mass spectrometry; NADH, β-nicotinamide adenine dinucleotide, reduced form; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; SIM, single ion monitoring; RT, retention time; TIC, total ion current; TMS, trimethylsilyl; U, units.

ACKNOWLEDGMENT

We thank Dr. Renate Loske for her help with the GC/MS analyses.

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Received for review April 26, 2000. Revised manuscript received July 11, 2000. Accepted July 12, 2000. This study has been supported by the Deutsche Forschungsgemeinschaft (Grant Ku 1079/5-1 and Me 574/9-2).

JF000524I