Metabolism of Genistein by Rat and Human Cytochrome P450s

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The metabolism of genistein (4′,5,7-trihydroxyisoflavone), a phytoestrogen derived from soy products, was investigated using rat and human liver microsomes and recombinant human cytochrome P450 enzymes. Metabolism of genistein by microsomes obtained from rats treated with pyridine, phenobarbital, β-naphthoflavone, isosafrole, pregnenolone-16α-carbonitrile, or 3-methylcholanthrene resulted in very different product profiles consisting of five different NADPH- and time-dependent metabolites as observed by HPLC reverse-phase analysis at 260 nm. The metabolism of genistein was also investigated with recombinant human cytochrome P450 1A1, 1A2, 1B1, 2B6, 2C8, 2E1, or 3A4. P450s 1A1, 1A2, 1B1, and 2E1 metabolized genistein to form predominantly one product (peak 3) with smaller amounts of peaks 1 and 2. P450 3A4 produced two different products (peaks 4 and 5). Product peaks 1−3 eluted off the HPLC column prior to the parent compound genistein, and the UV/vis spectra, GC/MS, and ESI/MS/MS analyses support the conclusion that these products result from hydroxylation of genistein. The product peak 3 has been identified by tandem mass spectrometry as 3′,4′,5,7-tetrahydroxyisoflavone, also known as orobol, and peaks 1 and 2 appear to be hydroxylated at position 6 or 8.

Introduction

Dietary intake of soy is associated with lower incidence rates of hormonally dependent and independent cancers (*1*). Soy contains large amounts of the isoflavones, genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone) (*2*). Long-term systemic exposure to isoflavonoids, including genistein, may prevent the development of breast cancer and delay progression of latent prostatic carcinoma (*3*−*5*). Genistein is also known to suppress the growth of solid tumors (*6*, *7*) and various forms of leukemia (*8*−*10*). While a specific mechanism of action has not been identified, genistein is known to inhibit tyrosine specific protein kinases (*9*, *10*), DNA topisomerase II (*9*, *11*), epidermal growth factor-induced phosphatidylinositol turnover (*12*), S6 kinase activation (*13*), and angiogenesis (*14*). In addition, genistein also may exert its effect during the administration of a carcinogen to the animal by decreasing the activity of the enzymes that activate the carcinogen (*15*).

While the metabolism of isoflavones is poorly understood in humans, the metabolism of several isoflavones has been characterized in sheep (*16*, *17*). In plants, genistein exists as the aglycone, the 7-*O*-glucoside, the 6″-*O*-acetylglucoside, and the 6″-*O*-malonylglucoside as reviewed by Reinli and Block (*18*). The hydrolysis of the glycosides occurs in the gastrointestinal tract. The absorbed phytoestrogen metabolites undergo enterohepatic circulation and may be excreted in the bile, deconjugated by intestinal flora, reabsorbed, reconjugated by the liver, and excreted in the urine (*19*, *20*). Isoflavones have been found in human fluids, including urine, plasma, and breast milk (*21*, *22*). Concentrations of the different phytoestrogen metabolites in the body vary widely and depend on intestinal flora, antibiotic use, bowel disease, gender, and genetic factors as reviewed by Murkies et al. (*19*) and Aldercreutz and Mazur (*23*).

The cytochrome P450 superfamily of genes encodes a variety of proteins found in nearly every tissue, and these enzymes catalyze the metabolism of both endogenous compounds and xenobiotics (*24*). P450s also play a significant role in the metabolism of some procarcinogens to carcinogens (*24*). P450s are known to hydroxylate estradiol and also play a role in the biosynthesis of isoflavones in cell cultures of *Pueraria lobata* (*25*) and the metabolism of isoflavones in rats (*26*−*28*). Since P450s are abundant in the liver and small intestine (*29*) where much of the metabolism of isoflavones is reported to occur (*19*, *20*), P450s may play a role in the metabolism of isoflavones, including genistein. In this study, we investigate the metabolism of genistein in liver microsomes from rats treated with various P450-inducing agents, including pyridine, phenobarbital, β-naphthoflavone, isosafrole, pregnenolone-16α-carbonitrile, or 3-me-

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thylcholanthrene. We also investigate the metabolism of genistein in several human liver microsomal samples and with recombinant human P450s. The metabolites were characterized by HPLC, UV/vis spectroscopy, GC/MS, and ESI/MS/MS.

Experimental Procedures

Materials. Genistein and NADPH were from Sigma Chemical Co. (St. Louis, MO). Supersomes, microsomes obtained from baculovirus insect cells containing cDNA-expressed reductase, and P450s 1A1, 1A2, 1B1, 2B6, 2C8, 2E1 (with cytochrome b_5), and 3A4 were from Gentest Corp. (Woburn, MA). Human liver microsomes were a gift from F. P. Guengerich (Vanderbilt University, Nashville, TN). Rat liver microsomes were prepared according to the method of Saito and Strobel (*30*) from the livers of male, 10-week-old Fisher 344 rats (Charles River Laboratories, Portage, MI) given 100 mg of pyridine/kg (ip for 3 days), 80 mg of β-naphthoflavone/kg (ip for 3 days), 0.1% phenobarbital in the drinking water for 12 days, 50 mg of pregnenolone-16α-carbonitrile/kg (ip for 4 days), 150 mg of isosafrole/kg (ip for 4 days), or 25 mg of 3-methylcholanthrene/kg (ip for 3 days).

Metabolism of Genistein. Genistein was incubated with microsomes (0.5 mg of rat or human liver microsomal protein or 0.05−0.1 nmol of baculovirus-expressed P450) for 0−30 min at 37 °C with 1.0 mM NADPH in 50 mM potassium phosphate buffer (pH 7.4) in a final volume of 1.0 mL. The incubations containing P450 2E1 or 3A4 also contained 3 mM $MgCl_2$. Genistein and its metabolites were isolated from incubations that had been quenched with 50 μL of 30% phosphoric acid and then extracted with ethyl acetate. The organic layer was dried under N_2, and the residue dissolved in HPLC buffer. These fractions were analyzed on a Gilson HPLC system at 260 nm on a C_{18} reverse-phase column with solvent A (0.1% trifluoroacetic acid) and solvent B (95% CH_3CN/5% H_2O/0.1% trifluoroacetic acid). A linear gradient of 5 to 60% B over the course of 30 min separated the components. Quantitation of metabolites was based on a standard curve using known amounts of genistein since the proposed metabolites are not commercially available. This is a good approximation of the amount of products formed since (1) the peak maxima of the products are within 7 nm of the peak maximum of genistein (as determined by HPLC analysis with diode array detection), (2) they are hydroxylated metabolites of genistein (as determined by mass spectrometry), and (3) several isoflavones closely related to genistein, including daidzein, formononetin, and biochanin A, have extinction coefficients that differ at the most by a factor of 1.5 (*31*).

Mass Spectrometry. GC/MS and ESI/MS/MS were performed at the Medical Mass Spectrometry Facility (Washington University). Genistein and its metabolites were separated by HPLC and extracted into ethyl acetate. For GC/MS analysis, isoflavones in the dried extracts were converted to their trimethylsilyl (TMS)[1] ether derivatives by heating for 15 min at 65 °C with BSTFA. The gas chromatograph was a Hewlett-Packard model 5890, and the column that was used was a DB1 (12 m, 0.2 mm i.d., 0.33 mm methyl silicone film coating; P. J. Cobert, St. Louis, MO). The injector and detector temperatures were set to 250 °C. The isothermal column temperature was 260 °C. EI/MS spectra were acquired on a Hewlett-Packard 5988A mass spectrometer (Palo Alto, CA). The emission current was set to 300 mA. The electron energy was 70 eV, and the source temperature was 200 °C. The source pressure was maintained at about 0.5 Torr with methane as the reagent gas.

ESI/MS/MS anaylses were performed on a Finnigan quadrupole ion trap (LCQ) mass spectrometer equipped with a Finnigan ESI source operated in the positive ion mode with the automatic gain control active. The dried samples were dissolved in 200 μL of MeOH/10 mM NH_4OAC (50:50) and were sprayed into the ion source by continuous infusion at a rate of 5 μL/min. The temperature of the heated capillary was 150 °C, and the sheath gas (N_2) flow rate was 50 mL/min. The spray voltage was 4.5 kV. Product ion spectra were obtained using a collision energy setting of 27% of the resonance RF energy. Helium was introduced to an estimated pressure of 1 mTorr for improving trapping efficiency.

[1] Abbreviations: TMS, trimethylsilyl; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane; CAD, collision-activated dissociation; ESI, electrospray ionization.

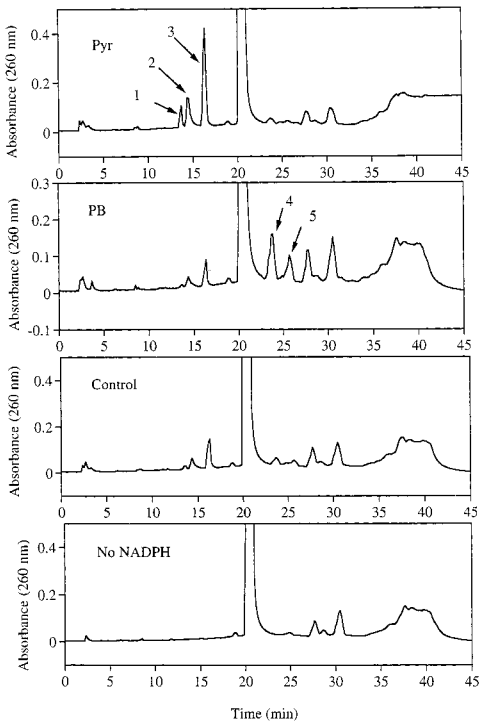


Figure 1. Representative reverse-phase HPLC profiles of ethyl acetate extracts of incubations containing rat liver microsomes and genistein. Each reaction mixture contained 150 μM genistein and 0.5 mg of microsomal protein from untreated rats (control) or rats treated with pyridine (Pyr) or phenobarbital (PB). Incubations and HPLC analyses were as described in Experimental Procedures. Genistein eluted at 21 min, and product peaks 1−5 are shown.

Results

When genistein was incubated with rat liver microsomes in the presence of NADPH, several product peaks were observed by HPLC analysis at 260 nm (Figure 1). Five different products were observed when genistein was metabolized by microsomes from pyridine- or phenobarbital-induced rats. These products were not observed when NADPH was omitted from the incubation mixtures. The formation of these products with microsomes from pyridine or phenobarbital-treated rats was time-dependent (data not shown). The formation was somewhat linear early in the incubation but appeared to deviate significantly from linearity at later incubation times (15−30 min). If microsomes from rats treated with other P450-inducing agents were used to metabolize genistein, the results in Figure 2 were obtained. Induction with pyridine, 3-methylcholanthrene, or β-naphthoflavone favored the production of products 1−3, while induction with phenobarbital or pregnenolone-16α-carbonitrile favored the production of peaks 4 and 5. Thus, the metabolism of genistein is NADPH- and time-dependent and results in the production of five different metabolites. The relative amount of each metabolite is dependent upon the inducing agent that was used.

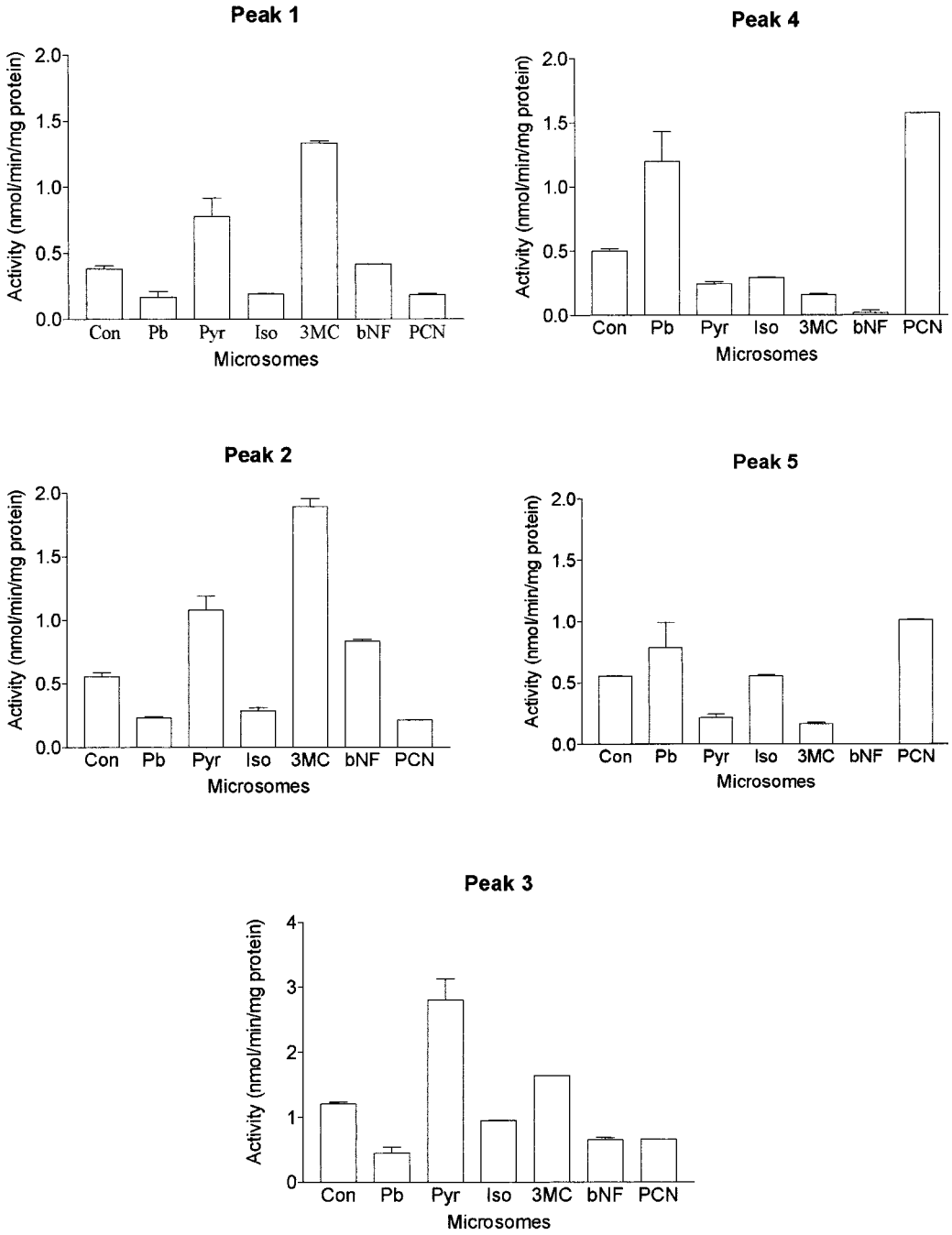


Figure 2. Metabolism of genistein by liver microsomes from rats treated with various inducing agents. Each reaction mixture contained 0.5 mg of protein, 80 μM genistein, and NADPH, and the incubation time was 10 min. Incubations and HPLC analysis were as described in Experimental Procedures. Quantitation is based on a standard curve using known amounts of genistein. Rats were untreated (Con) or treated with phenobarbital (Pb), pyridine (Pyr), isosafrole (Iso), 3-methylcholanthrene (3MC), β-naphthoflavone (bNF), or pregnenolone-16α-carbonitrile (PCN). Mean values ± standard deviations from three independent experiments are plotted.

The kinetic constants for the formation of peaks 1−3 from genistein by microsomes from pyridine-treated rats are given in Table 1. From Eadie−Hofstee plots of the data, high and low K_m values were determined for the metabolism of genistein to peak 3. This could be explained if microsomes from pyridine-treated rats contained a high- and low-K_m P450 responsible for the metabolism of genistein to peak 3.

The results from the metabolism of genistein by three different human liver microsomal samples are shown in Figure 3. Each microsomal sample exhibits a different product profile. Each of the human liver microsomal samples has been analyzed for various P450 marker activities; the sample designated as 100 has high 2E1-

Table 1. Kinetic Constants for the Formation of Peaks 1−3 from Genistein by Microsomes from Pyridine-Treated Rats[a]

peak	K_m (μM)	V_{max}[b] (nmol min^{-1} mg of protein^{-1})
1	30	0.89
2	35	1.2
3	16 (low K_m)	1.4
3	104 (high K_m)	4.4

[a] Incubation mixtures contained 2−200 μM genistein, and the incubation time was 10 min. Other incubation conditions were as described in Experimental Procedures. Kinetic constants were obtained from Eadie−Hofstee plots of the data. [b] The values are approximate and based on quantitation with a standard curve using known amounts of genistein as described in Experimental Procedures.

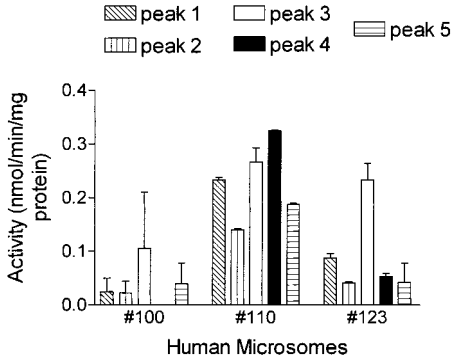


Figure 3. Genistein metabolism by human liver microsomes. Experimental conditions were as described in the legend of Figure 2 and in Experimental Procedures.

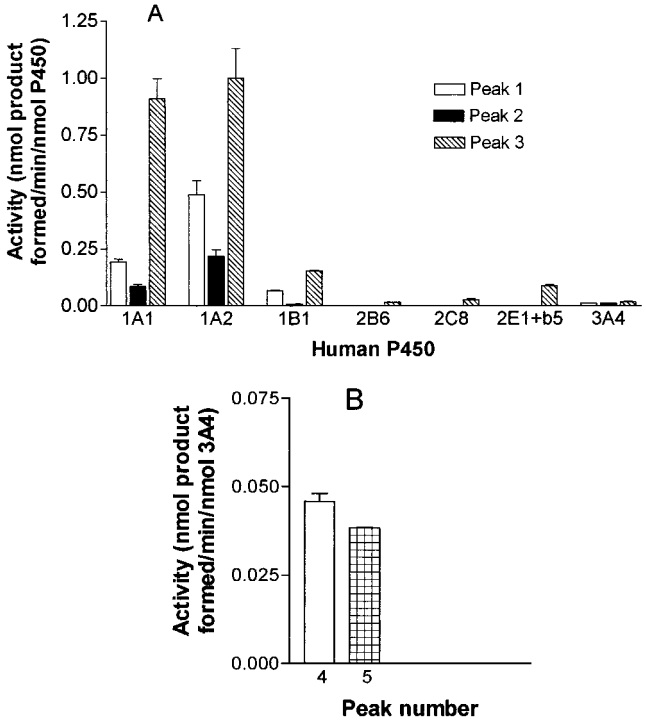


Figure 4. Genistein metabolism by recombinant human P450s. The incubation and HPLC conditions were as described in Experimental Procedures. (A) Production of product peaks 1−3. (B) Production of product peaks 4 and 5 by P450 3A4.

specific chloroxazone hydroxylase activity, while sample 110 has high 3A4-specific nifedipine oxidation activity (*32*). Thus, as seen previously with rat liver microsomes from animals treated with different P450-inducing agents, the genistein product profile is dependent on the P450 profile of the microsomes. Genistein was also metabolized to various products when incubated with insect cell microsomes containing recombinantly expressed human P450s and reductase (Figure 4). P450s 1A1, 1A2, and, to a lesser extent, 1B1 and 2E1 metabolized genistein to peaks 1−3. Only P450 3A4 was found to metabolize genistein also to give peaks 4 and 5.

The UV spectra of the product peaks were obtained by HPLC with diode array detection (data not shown). The UV spectrum appeared to be similar to that of genistein for each of the five compounds with peak maxima at 267 nm for peak 1, 270 nm for peak 2, 262 nm for peak 3, 265 nm for peak 4, and 264 nm for peak 5. The GC/MS (electron impact) analysis of the TMS derivative of peak 3 (Figure 5) demonstrated that peak

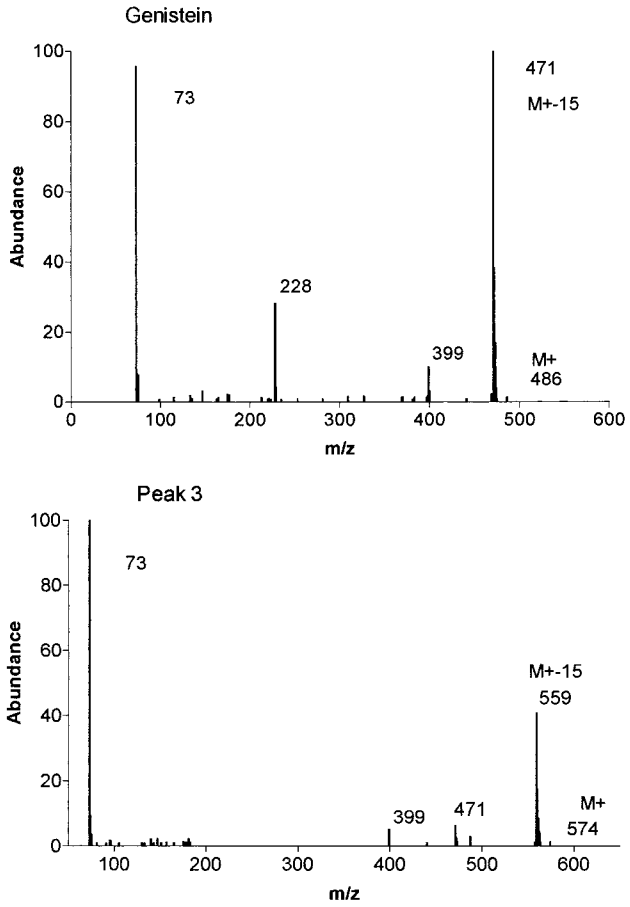


Figure 5. GC/MS analysis of the TMS derivative of genistein and peak 3. Peak 3 was isolated from an incubation containing microsomes from pyridine-induced rats.

3 is a hydroxylated metabolite of genistein. The TMS derivative of genistein has a molecular weight of 486, and while the M^+ ion is present at an intensity of <1%, the $M^+ - 15$ ion is strong and can be used to determine the molecular weight. The loss of an OTMS group results in the ion at *m/z* 399. Peak 3 has a strong ion at *m/z* 559 which corresponds to the addition of an OTMS group. The ions at *m/z* 471 and 399 result from losses of additional OTMS and CH_3 groups. More information about the position of the hydroxyl group was obtained by the use of electrospray ionization mass spectrometry (ESI). The ESI/MS spectrum of peak 3 displayed in Figure 6 shows the $[M + H]^+$ ion (panel A). Upon CAD, this ion is further fragmented as shown in the spectrum of panel B. Tandem mass spectrometry of isoflavones will fragment to give ions derived from consecutive losses of H_2O and CO as well as ions indicative of substituent positioning (*33*). The retro Diels−Alder decomposition product, a predominate ion in the CAD spectra of all flavonoids (Scheme 1), can be used to determine the number of substituents on the A ring. The mass at *m/z* 153 is a retro Diels−Alder fragment which demonstrates that there are two hydroxyl groups on the A ring. This ring has not been altered from the original parent genistein. An additional ion fragment arises from bond cleavage to give a fragment containing the B ring. The fragment, *m/z* 161, was only seen previously when there were 3′,4′-hydroxyl groups on the B ring (*33*). The possible structure is a quinone as shown in Scheme 1. The losses at $MH^+ - 18$ (269), $MH^+ - 28$ (259), $MH^+ - 18 - 28$ (241), and $MH^+ - 28 - 28$ (231) are common to hydroxy isoflavones,

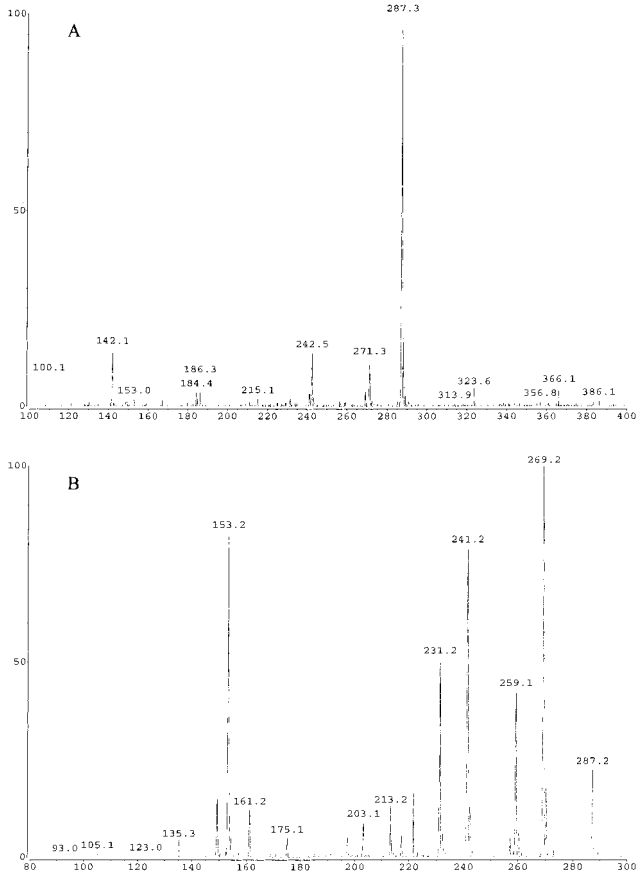


Figure 6. Tandem mass spectral analysis of peak 3. Experimental conditions were as described in Experimental Procedures. (A) Full scan spectrum of peak 3. (B) CAD tandem mass spectrum of $[M + H]^+$ (*m*/*z* 287.3).

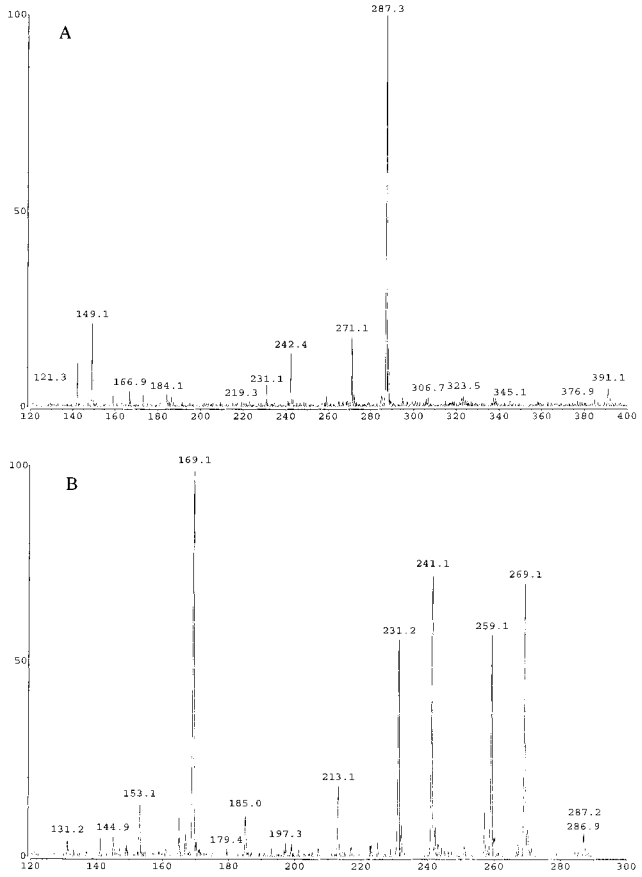
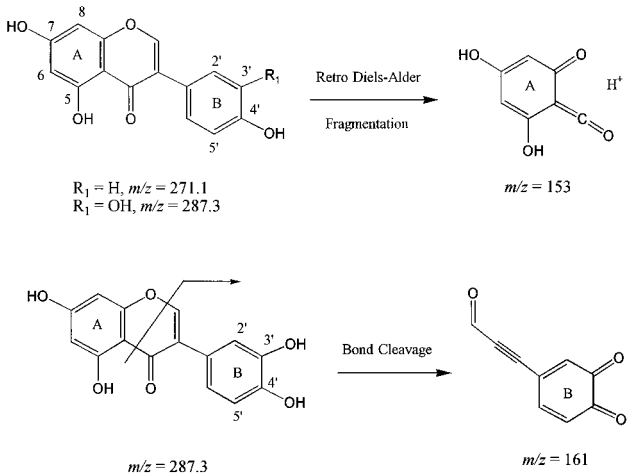


Figure 7. Tandem mass spectral analysis of peaks 1 and 2. Experimental conditions were as described in Experimental Procedures. (A) Full scan spectrum of peaks 1 and 2. (B) CAD tandem mass spectrum of $[M + H]^+$ (*m*/*z* 287.3).

Scheme 1. Fragmentation Patterns for Isoflavones[a]



[a] The structure of genistein is shown when R_1 is H, and the structure of the hydroxylated metabolite, orobol, is shown when R_1 is OH.

resulting from the losses of H_2O and CO. Thus, peak number 3 corresponds to a hydroxylated metabolite of genistein that has been hydroxylated on the B ring at the 3′ position to form 3′,4′,5,7-tetrahydroxyisoflavone. This metabolite of genistein was identical with the aglycone of orobol-7-*O*-glucoside when analyzed by ESI/MS/MS (data not shown). A sample of orobol-7-*O*-glucoside was kindly provided by E. Wollenweber (Technische Universitaet Darmstadt, Darmstadt, Germany), and the identity of the material was confirmed by ^{1}H NMR (acetone-d_6, 600 MHz) with comparison to literature data (*34*)

Peaks 1 and 2 were collected together in one fraction and subjected to ESI/MS/MS analysis. Only one parent ion is shown in Figure 7 (in panel A with an ion at *m*/*z* 287.3). This also corresponds to a hydroxylated metabolite of genistein. Thus, peaks 1 and 2 could be hydroxylated metabolites (hydroxylation at different positions on the A ring) that have the same molecular weight. The fragmentation pattern of this ion is shown in panel B and is distinctly different from that seen with peak 3 (Figure 6B). The retro Diels−Alder fragment (*m*/*z* 169.1), which is one of the major ions in the CAD spectra of flavonoids, results from the A ring that has an additional hydroxyl group at either position 6 or 8. Thus, peak 1 or peak 2 is a tetrahydroxyisoflavone with hydroxylation at either position 6 or 8.

Discussion

We have found that genistein metabolism in rat liver microsomes is NADPH- and time-dependent. In addition, prototype inducers of P450 can substantially alter the profile of hepatic genistein metabolism in rats. From the different metabolite patterns, it is apparent that metabolism by individual P450s results in different product profiles. The Eadie−Hofstee plot of the results of genistein metabolism to peak 3 by microsomes from pyridine-treated rats indicates that there is a high-K_m and a low-K_m enzyme with similar turnover numbers. Thus, al-

though two different P450s may produce the same product, they appear to bind genistein with significantly different affinities. Some general conclusions can be drawn from the data obtained with the rat liver microsomes, human liver microsomes, and recombinant human P450s. The results from the rat liver microsomes indicated that peaks 1−3 were present in incubations containing microsomes from rats treated with 3-methylcholanthrene, isosafrole, and β-naphthoflavone (strong 1A inducers) (*35*) and pyridine (a strong 2E1 and moderate 2B and 1A inducer) (*35*, *36*). Peaks 4 and 5 were formed in incubations containing microsomes from pregnenolone-16α-carbonitrile (a strong 3A1 and 3A2 inducer) (*35*) and phenobarbital (a strong 2B and moderate 3A inducer) (*35*). The human liver microsomal sample 110, high in 3A4-dependent activity, produced all five peaks. Recombinant human 3A4 produced peaks 4 and 5, while 1A1 and 1A2 and, to a lesser extent, 1B1 and 2E1 produced peaks 1−3. More experiments are currently underway to definitely attribute a particular metabolite to a particular P450 by using inhibitory antibodies to specific P450s, a bank of 18 human liver microsomes typed for various P450-dependent activities, and specific inhibitors of P450 isozymes. From our results, it is apparent that (1) of the P450s tested 1A1 and 1A2 have the highest activities for genistein metabolism, (2) different P450s result in different product profiles when metabolizing genistein, and (3) P450s 1A1, 1A2, 1B1, and 2E1 produce peaks 1−3 while only 3A4 metabolizes genistein to peaks 4 and 5.

In this study, peak 3 was identified by GC/MS and ESI/MS/MS analyses as a hydroxylated metabolite of genistein (hydroxylated on the B ring at position 3′). Peak 1 and/or peak 2 may be a metabolite hydroxylated at positions 6 or 8 on the A ring. Peak 3, the 3′,4′,5,7-tetrahydroxyisoflavone, is also known as orobol and has previously been isolated as a glucoside from a plant, *Orobus tuberosus* (*37*), and a fungal strain, *Tritirachium* sp. F3707 (*38*). *O. tuberosus* is a herb belonging to the family Leguminosae, and is now usually included in the genus *Lathyrus* (*39*). In Europe, the plants are sometimes cultivated for their tubers, which have an agreeable, pleasant taste (*40*). Orobol has been reported to induce topoisomerase II-dependent DNA cleavage to the same extent as genistein (*41*), inhibit dihydroxyphenylalanine decarboxylase with an IC_{50} similar to that of genistein (*42*), increase the level of cytoplasmic free calcium in isolated rat hepatocytes (*43*), inhibit 15-lipoxygenase (*38*), and inhibit phosphatidylinositol turnover and EGF receptor tyrosine specific kinase in cell cultures to the same extent as genistein (*12*).

In rats, P450s are reported to catalyze the transformation of the isoflavone galangin (3,5,7-trihydroxyisoflavone) to kaempferol (4′,3,5,7-tetrahydroxyisoflavone) and finally to quercetin (3′,4′,3,5,7-pentahydroxyisoflavone) (*26*, *27*). 1A1 is reported to hydroxylate kaempferol on the B ring at the 3′ position to yield quercetin (*27*), while an unidentified P450 is responsible for the first hydroxylation (*27*). Similarly, we have evidence that P450s 1A1, 1A2, and 1B1 hydroxylate genistein at the 3′ position on the B ring to yield orobol. The metabolism of genistein by cytochrome P450 may be another mechanism of cancer chemoprevention. The 1A proteins are responsible for the metabolic activation of more than 90% of the known procarcinogenic environmental chemicals, toxins, and toxic drugs (*44*). P450 1A1 is an extrahepatic enzyme highly induced in smokers. The protein is detected in the lungs of smokers, and gene expression is observed in pulmonary carcinoma cells and malignant breast cancer (*44*). P450 1B1 is also an extrahepatic enzyme found in many tissues, most notably in both normal breast tissue and breast tumors. P450 1B1 catalyzes the hydroxylation of 17β-estradiol at position 4, resulting in a catechol that can directly or indirectly damage macromolecules (*45*) and thus initiate cancer. Isoflavones may inhibit P450s 1A1, 1A2, and 1B1, resulting in the decreased level of formation of hydroxylated estradiol products known to damage macromolecules. Adlercreutz and Mazur (*23*) have recently reviewed the hypothesis that the isoflavones play a significant inhibitory role in cancer development in the promotional phase of the disease, and recent evidence points to a role in the initiation stage of carcinogenesis.

Genistein is thought to be metabolized in the gastrointestinal tract and liver (*19*, *20*) which are also sites of abundant levels of P450. In the small intestine where there are high levels of 3A4, extensive biotransformation of orally administered drugs occurs during absorption across the intestinal wall (*46*). Much of the metabolism of genistein is believed to be due to microbial metabolism, but there have been observations of individual variability in genistein metabolism that cannot be explained solely on the basis of microbial metabolism (*47*). Furthermore, there are some unidentified metabolite peaks when urine obtained from people on soy-based diets was analyzed by HPLC (*22*). More recently, genistein was found to be metabolized to a hydroxylated metabolite in a transformed human breast cancer MCF-7 cell line (*48*). Thus, the available experimental information, including the abundance of P450s in several tissues known for genistein metabolism, the ability of P450 to metabolize a vast number of xenobiotics and endogenous compounds, including estradiol and other isoflavones, the data presented herein, reports of unidentified isoflavone metabolites in human urine (*21*, *22*), and genetic individual differences in metabolism (*47*), suggests that human P450s may play important roles in genistein metabolism in vivo. Many times, P450 metabolism results in metabolites that are less biologically active and more easily excreted than the substrates, but in this case, P450 metabolism results in a compound, orobol, that is at least as active as genistein in several in vitro tests (*41*−*43*). P450s may have important roles in the biochemical action of flavonoids such as gensitein by producing active metabolites. In addition, the metabolism of isoflavonoids by P450 may affect the metabolism of other P450 substrates, including procarcinogens.

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