STRUCTURAL ELUCIDATION OF HYDROXYLATED METABOLITES OF THE ISOFLAVAN EQUOL BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT:

Equol has, as have other isoflavonoids, recently gained considerable interest due to its possible health effects. However, detailed studies on the metabolism of equol are scarce. Therefore, we investigated the phase I metabolism of equol using liver microsomes from Aroclor-treated male Wistar rats as well as from a male human. The identification of the metabolites formed was elucidated using high performance liquid chromatography (HPLC) with diode array detection, HPLC/atmospheric pressure ionization electrospray mass spectrometry, and gas chromatography-mass spectrometry, as well as reference compounds. (+)-Equol was converted to 11 metabolites by the liver microsomes from Aroclor-pretreated rats comprising three aromatic monohydroxylated and four aliphatic monohydroxylated as well as four dihydroxylated products. The main metabolite was identified as 3′-hydroxy-equol. Using human liver microsomes, equol was converted to six metabolites with 3′-hydroxy- and 6-hydroxy-equol as main products. Furthermore, the aliphatic hydroxylated metabolite 4-hydroxy-equol, which was recently detected in human urine after soy consumption, was formed. On the basis of these findings, it is suggested that phase I metabolism of equol is part of a complex biotransformation of the soy isoflavone daidzein in humans in vivo.

Equol (7,4′-dihydroxy-isoflavan) is a nonsteroidal estrogen of the isoflavone family. In 1932, Marrian et al. were the first researchers to isolate equol from the urine of pregnant mares and elucidated its chemical structure. The equine origin gave the basis for its name (Marrian et al., 1932). Four decades later, in 1982, it was identified in human urine (Axelson et al., 1982). Equol was established as a bacterial metabolite of daidzein in 1968 (Shutt and Braden, 1968). It is one of the end products of the intestinal bacterial biotransformation of the isoflavone daidzein, which is one of the two predominant phytoestrogens found in soy and soy-derived products (Fig. 1). Therefore, equol is not a phytoestrogen, itself, since it is not a natural constituent of plants. Equol is unique in having a chiral center at C-3 due to the lack of a double bond in the heterocyclic C-ring. In vivo, the absolute configuration of equol was determined to be S-(−)-equol (Setchell et al., 2005).

Only 30 to 50% of the adult population excretes equol in urine after having consumed soy foods (Rowland et al., 2000), an observation that is still not understood. Even when pure daidzein is administered, which removes the influence of the food matrix, a high percentage of humans do not convert daidzein to equol. This phenomenon has led to the terminology of being an “equol-producer” or a “nonequol-producer” to describe the two distinct populations. Recently, the question arose whether “equol-producers” have particular benefit in the treatment and the prevention of hormone-dependent diseases by consuming soy products, since the biological properties of equol are interesting. Equol possesses high estrogenic activity: the binding affinities to both estrogen receptors, α and β, are similar to those of genistein, with a preference for estrogen receptor β. However, equol induces binding of the activated receptor to the estrogen response element as well as a transcription more strongly, especially with estrogen receptor α (Morito et al., 2001; Kostelac et al., 2003; Muthyala et al., 2004). Recently, the binding affinities of R- and S-equol as well as the racemate to both estrogen receptors have been separately elucidated. Interestingly, S-equol possesses high binding affinity for estrogen receptor β, whereas R-equol binds less strongly and with a preference for estrogen receptor α (Muthyala et al., 2004). Equol can act as an anti-androgen by binding 5α-dihydrotestosterone with high affinity, which prevents 5α-dihydrotestosterone from binding the androgen receptor (Lund et al., 2004). Furthermore, equol has superior antioxidant properties compared with all other isoflavones (Arora et al., 1998; Turner et al., 2004).

The metabolism of soy isoflavones is well documented. Daidzein is reduced to dihydrodaidzein by the gut microflora, which is further metabolized to equol and O-desmethylandolensin (Fig. 1) (Chang and...
Daidzein and equol was identified by Wessely and Prillinger (1939) and Lamberton et al. (1978). The purity of 6-hydroxy-daidzein, and 3'-hydroxy-daidzein, respectively) as described by (7,4'-dihydroxyisoflavan), 6-hydroxy-equol (6,7,4'-trihydroxyisoflavan), and 3'-hydroxy-equol (7,3',4'-trihydroxyisoflavan) were synthesized in a microscale approach by reduction of the corresponding isoflavones (daidzein, 6-hydroxy-daidzein, and 3'-hydroxy-daidzein, respectively) as described by Wessely and Prillinger (1939) and Lambertson et al. (1978). The purity of daidzein and equol was >99% according to GC/MS analysis. NADP⁺ (β-nicotinamide adenine dinucleotide phosphate), N,O-bis-(trimethylsilyl)trifluorooracetamide (BSTFA), deuterated N,O-bis-(trimethylsilyl)acetamide (d9-BSA), and dimethyl sulfoxide were obtained from Sigma-Aldrich (Taufkirchen, Germany). All other chemicals were of the highest grade available.

**Preparation and Characterization of Rat Liver Microsomes and HLMs.** Microsomes were prepared and pooled from the livers of 10 Aroclor-treated male Wistar rats according to standard procedures as described by Lake (1987). Treatment with Aroclor 1254 (one intraperitoneal injection on day 1 of a dose of 500 mg/kg body weight dissolved in sesame oil at 100 mg/ml, killed on day 6) was carried out to induce hepatic P450 enzymes. HLMs were a friendly gift of Prof. M. Metzler, University of Karlsruhe (Karlsruhe, Germany). They were prepared from a normal liver tissue sample of a middle-aged white man in the same way as described above. The liver sample was obtained with appropriate human use approvals. Protein concentrations were determined using the Pierce Chemical (Rockford, IL) bicinchoninic acid reagent. P450 concentrations were measured as described by Omura and Sato (1964). The rat liver microsomes were further characterized by the specific activities of major P450 enzymes as determined by the method of Burke et al. (1994). Hepatic microsomes from Aroclor-treated rats were found to contain 1.69 nmol of P450 per mg of protein. 7-Ethoxyccumarin O-deethylase, 7-ethoxyresorufin O-deethylase, and 7-pentoxysresorufin O-dealkylase activity was found to be 23.5, 15.1, and 0.22 nmol/mg protein/min, respectively. HLMs contained 0.32 nmol of P450 per mg of protein.

**Incubation with Microsomes and Extraction.** Standard incubation mixtures with rat liver microsomes contained 2 mg of microsomal protein, 100 nmol of equol (dissolved in 40 μl of DMSO) in a final volume of 2 ml of 50 mM 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 (3 mM MgCl₂, 1 mM NADP⁺, 8 mM dL-isocitrate, and 0.5 unit of isocitrate dehydrogenase) and stopped after 60 min by extraction four times with 2 ml of ice-cold ethyl acetate. Controls were carried out by omitting the NADPH-generating system or by using heat-inactivated microsomes. The organic solvent was dried under nitrogen at room temperature. The residue was dissolved in 50 μl of methanol and 3950 μl of water and applied to a reversed-phase C-18 cartridge (Waters, Eschborn, Germany). The column was first rinsed with 5 ml of 20% aqueous methanol and equol, and the metabolites were then eluted with 5 ml of 70% aqueous methanol. The eluate was dried under nitrogen and the residues were dissolved in 200 μl of aqueous 50% methanol. The samples were analyzed immediately by HPLC. All glass tubes used in this study were presilanized with 5% (v/v) dimethylchlorosilane in toluene to avoid loss of hydroxylated equol metabolites due to adsorption to the glass surface. Incubations with HLMs were carried out in the same way with the exception that the incubation mixtures contained 4 mg of microsomal protein and 50 nmol of equol.

**HPLC Analysis.** HPLC separation of the oxidative metabolites was carried out on a Prontosil (250 mm × 4.6 mm i.d., particle size 3 μm) reversed-phase
column (Bischoff, Leonberg, Germany). The solvent system consisted of 0.1% formic acid in water (pH 3) (A) and acetonitrile (B) with the following linear gradient: from 15% to 35% B in 50 min, from 35% to 50% in 10 min, and from 50% to 60% in 30 min. The flow rate was 0.9 mL/min and the eluate was recorded with a diode array detector at 280 nm. Observed peaks were scanned between 190 and 400 nm.

**GC/MS Analysis of Hydroxylated Metabolites.** GC/MS was carried out on a Finnigan MAT system (gas chromatograph model GCQ connected to an ion trap mass detector) (Thermo Electron Corporation, Bremen, Germany). The metabolites were isolated by HPLC and freeze-dried. For GC/MS analysis, the dry residues were derivatized with BSTFA or d9-BSA for at least 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, Taufkirchen, 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 250°C. Mass spectra were obtained by electron impact (EI) ionization at 70 eV and an ion source temperature of 150°C. Full-scan spectra (mass range 50–750 atomic mass units) were recorded at a rate of 2 spectra per second. Furthermore, the selected ion mode was used for the detection of equol and its metabolites.

**HPLC/MS Analysis.** HPLC/MS analysis was performed on an HP 1100 series HPLC apparatus (Agilent Technologies, Waldbronn, Germany) equipped with an autoinjector, quaternary HPLC pump, column heater, UV detector, and HP ChemStation for data collection and handling. The HPLC apparatus was interfaced to an HP series 1100 mass selective detector equipped with an API-ES chamber. For the analysis of oxidative metabolites, conditions in the positive mode were as follows: capillary voltage, 4 kV; fragmentor voltage, 80 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 mode. Spectra were scanned over a mass range of m/z 100 to 500 at 1.03 s/cycle.

**Results**

**Formation and Separation of Rat Microsomal Metabolites of Equol.** Equol (Fig. 1) was incubated with hepatic microsomes of Aroclor-treated male Wistar rats. Aroclor 1254 induces P450 enzymes, especially of the 1A but also of the 2B subfamily (Burke et al., 1985; Correia, 1995). Therefore, the use of Aroclor-induced microsomes increases both the variety of the metabolites formed and their yield, thus facilitating the elucidation of the structures.

The complete organic extract of each incubation was analyzed by reversed-phase HPLC with diode array detection. Equol was extensively metabolized. Eleven metabolites were clearly detectable by HPLC analysis (Fig. 2). These products were not observed when NADPH was omitted or heat-inactivated microsomes were used.

Furthermore, the extracted metabolites were analyzed by HPLC/MS with positive API-ES and by GC/EI-MS after trimethylsilylation. The mass spectra indicated the formation of seven mono- and four dihydroxylated metabolites. For the correlation of HPLC and GC peaks, each HPLC peak was isolated and analyzed by GC/MS after derivatization. The chromatographic and MS data and the UV maxima, as well as the assigned chemical structures and the percentage of total metabolite formed, are summarized in Table 1.

**Approach for the Elucidation of Metabolite Structures.** Use of Reference Compounds. 6-Hydroxy-equol and 3'-hydroxy-equol were synthesized in a microscale approach (see Materials and Methods) and used as reference compounds for cochromatography and comparison with mass spectra. These standards proved to be helpful tools for the identification of equol metabolites; e.g., equol metabolites, which are also generated in the microsomal metabolism of 3'-hydroxy-equol, must contain an additional hydroxyl group in position C-3' of the B-ring.

**HPLC/MS and GC/MS Analysis.** Mass spectrometry can be used to determine the molecular weight of the metabolites and to clarify the distribution of hydroxyl groups between the A- and the B-ring of the isoflavone molecule. GC/MS with EI ionization (70 eV) of trimethylsilylated (TMS) derivatives of the metabolites gave, in most of the cases, mass spectra with an intense molecular ion or [M+15] ion (loss of a CH₃ radical) as demonstrated in Fig. 3. EI mass spectra can thus be used to determine the molecular weight of the metabolites. Furthermore, in contrast to genistein and daidzein, equol showed a distinct fragmentation pattern using EI ionization. To minimize the uncertainty in the interpretation of the mass spectra, we used BSTFA as well as d9-BSA for the derivatization. With BSTFA, every proton in the hydroxyl groups is substituted by a trimethylsilyl group (TMS; m/z = 73), whereas per-deuterated TMS groups (d9-TMS; m/z = 82) are formed when using d9-BSA. By comparison of the mass spectra of the derivatives, information about the number of hydroxyl groups in the molecule and about the structure of the formed fragments is obtained. Figure 3 shows the EI mass spectra of equol, 3'-hydroxy-equol, and 6-hydroxy-equol after derivatization with BSTFA and d9-BSA. In Fig. 4, the proposed fragmentation pathway of the TMS derivative of equol is depicted. Important fragments are formed via retro Diels-Alder (rDA) reaction in the C-ring. The molecule is fragmented into a dien- (a) and an en- (b) component. It depends on the molecule which component is the main product; e.g. for equol and 3'-hydroxy-equol, the en-component is formed more exclusively (m/z 192 and 280, respectively), whereas in the case of 6-hydroxy-equol, the en- and the dien-component are formed to the same extent (m/z 192 and 282) (Fig. 3). Furthermore, it must be taken into account that the dien-component a might react to c by migration of the TMS group to the keto group. The rDA-fragments provide important information about the degree of the substitution at each phenolic ring.

Besides the rDA reaction, further fragmentation is observed in the C-ring. The fragment at m/z 206 in the mass spectrum of equol is assigned to be the alkene ionic radical d, which is stabilized by the +1-effect of the methyl and phenyl groups (Fig. 4). Mass spectra of

![Fig. 2. Representative HPLC chromatogram of equol metabolites formed by the incubation of equol with liver microsomes of Aroclor-induced male rats. Peak numbers refer to Table 1.](Image)
A-ring-substituted equol derivatives show a fragment ion at \( m/z \) 295, which is proposed to occur via the fragmentation pathway shown in Fig. 5. Valuable information about the location of the hydroxyl groups can also be obtained by HPLC/MS with positive API-ES if a relatively low fragmentor voltage of 80 V is used (see Materials and Methods). A higher fragmentor voltage leads to the complete breakdown of the molecule. In Fig. 6, the fragmentation pathway of equol and aromatic hydroxylated equol derivatives is depicted. The fragmentation pattern

<table>
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<tr>
<th>HPLC Peak</th>
<th>RT in HPLC</th>
<th>UV Maxima</th>
<th>Percentage of Total Metabolite Formed</th>
<th>([M + H]^+) HPLC/MS</th>
<th>RT in GC</th>
<th>M(^+), GC/MS TMS Derivative</th>
<th>Proposed Chemical Structure</th>
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<td>1</td>
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<td>233, 200, 304</td>
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<td>386, 192</td>
<td>Equol</td>
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*Each peak was separated into two peaks during GC/MS analysis.

Fig. 3. GC/EI-MS spectra of the TMS and per-deuterated d9-TMS derivatives of equol, 3'-, 4'-, and 6-hydroxy-equol obtained after derivatization with BSTFA and d9-BSA. The mass shift caused by the deuterium labeling is a helpful tool for a detailed interpretation of the fragmentation pattern. Further information is given under Results (HPLC/MS and GC/MS Analysis). A detailed explanation of the EI-MS spectra of the TMS and the d9-TMS derivative of equol is shown in Fig. 4.

Fig. 4. Proposed fragmentation pathways of equol after trimethylsilylation with BSTFA (a) and d9-BSA (b). The percentage of the fragment ion formed is given in parentheses.

A-ring-substituted equol derivatives show a fragment ion at \( m/z \) 295, which is proposed to occur via the fragmentation pathway shown in Fig. 5. Valuable information about the location of the hydroxyl groups can also be obtained by HPLC/MS with positive API-ES if a relatively low fragmentor voltage of 80 V is used (see Materials and Methods). A higher fragmentor voltage leads to the complete breakdown of the molecule. In Fig. 6, the fragmentation pathway of equol and aromatic hydroxylated equol derivatives is depicted. The fragmentation pattern
can be used to determine the number of hydroxyl groups in the A- or the B-ring. The loss of a hydroxystyrene derivative results in the ionic dien component [H9251], which gives information about the hydroxylation degree in the A-ring. The loss of a hydroxyphenol derivative leads to the fragment [H9252], which gives information about the number of hydroxyl groups in the B-ring.

Theoretical Considerations about the Stereochemistry of Aliphatic Hydroxylated Metabolites. Possible positions for the aromatic hydroxylation of equol by P450 enzymes are the positions C-8 and C-6 in the A-ring as well as C-3' (equivalent to C-5') in the B-ring. Possible positions for the aliphatic hydroxylation are C-2, C-3, and C-4 in the C-ring.

In contrast to genistein and daidzein, equol possesses a chiral center at C-3 in the C-ring. The synthesis of equol results in the mixture of the two enantiomers, R-(+)- and S-(-)-equol (Lamberton et al., 1978). With respect to the hydroxylation at the aromatic positions C-6 and C-8, and C-3', as well as at the aliphatic position C-3, the corresponding R- and S-enantiomers are formed. Hydroxylation at the aliphatic positions C-2 and C-4 leads to the formation of a second asymmetric center in the C-ring, and four stereoisomers can theoretically be formed. Figure 7 depicts the four possible stereoisomers of 4-hydroxy-equol. In analogy, the corresponding products can be postulated after introduction of a hydroxyl group at C-2.

Identification of Microsomal Metabolites of Equol after Incubation with Aroclor-Induced Rat Liver Microsomes. A representative HPLC profile of the microsomal metabolites of equol is depicted in Fig. 2.

The GC/MS analysis of the TMS derivative of the major microsomal metabolite (peak 11, Fig. 2) shows a molecular ion at m/z 474 and a fragment ion m/z 280 indicating a monohydroxylated equol structure with two hydroxy groups in the B-ring. The positive HPLC/API-ES mass spectrum displays a quasimolecular ion [M + 1]⁺ at m/z 256, indicating a monohydroxylation, and a diagnostic fragment ion at m/z 123, implying an unchanged A-ring. Furthermore, metabolite 11 coelutes with the synthesized reference compound 3'-hydroxy-equol. Taking these findings together, metabolite 11 can be identified as 3'-hydroxy-equol. It is the main product of the microsomal metabolism of equol and accounts for up to 60%, calculated on the basis of the peak area at λ = 280 nm.

Peak 10 was identified by cochromatography in GC/MS with the synthesized reference compound 6-hydroxy-equol. The positive API-ES mass spectrum displays a quasimolecular ion [M + 1]⁺ at m/z 256—which confirms the monohydroxylation—and a diagnostic fragment ion at m/z 139, implying a hydroxylated A-ring (Fig. 6).

Metabolite 9 represents an A-ring monohydroxylated isoflavan according to its mass spectral data (Table 1). The EI and API-ES mass spectra are found to have the same fragment ions as 6-hydroxy-equol (metabolite 10), which has already been identified. The spectra of
these two metabolites only differ in the intensities of the fragment ions. The remaining possible positions for the introduction of an additional hydroxyl group in the A-ring are C-5 and C-8. Hydroxylation at C-5 would mean a hydroxylation in the meta position relative to the existing hydroxy group at C-7, which is very unusual and is, to our knowledge, so far not described. Therefore, we assign peak 9 to be 8-hydroxy-equol.

Peaks 4, 5, 6, and 8 show molecular ions at m/z 474, implying the introduction of one additional hydroxyl group. The EI mass spectra of these metabolites exhibit [M − 90]⁺ ions at m/z 384, suggesting the introduction of one additional double bond into the equol molecule. The double bond is derived by the elimination of a trimethylsilylated aliphatic hydroxyl group during GC/MS analysis. Hydroxylation in the aliphatic position of equol can generally be recognized by the loss of a TMSOH group [M − 90]⁺. Thus, it is assumed that HPLC peaks 4, 5, 6, and 8 represent aliphatic monohydroxylated metabolites of equol. Possible positions of equol for the aliphatic hydroxylation are C-2, C-3, and C-4 at the C-ring.

The GC mass spectrum of the TMS derivatives of metabolite 4 lacks the ions m/z 192 and 177, respectively, or 179, which are minor fragments of all other metabolites and are derived by the formation of the en-component or a hydroxybenzylium ion (m/z 179). Furthermore, peak 4 additionally exhibits strong fragment ions at m/z 280 and m/z 268, which are formed by the introduction of a hydroxyl group at C-3. On the basis of these data, peak 4 is assigned to be 3-hydroxy-equol. Fig. 8 shows the proposed fragmentation pathway.

Examination of the isolated peaks 5 and 8 shows identical results in the GC/MS. Each peak was separated into two products, which differed in the retention times (16.20 and 17.12 min), but showed the same mass spectra with molecular ions at m/z 474 (Table 1). These metabolites can be tentatively identified as the stereoisomers of 4-hydroxy-equol. The EI mass spectra after derivatization with BSTFA and d9-BSA and the corresponding fragmentation pathways are depicted in Figs. 9 and 10. The EI mass spectra of these metabolites exhibit [M − 90]⁺ ions at m/z 384, suggesting a hydroxylation in an aliphatic position due to the loss of a TMSOH group. For the formation of the basic ion at m/z 267 and the fragment ions at m/z 282 and 355, we suggest the fragmentation pathway depicted in Fig. 10. Hydroxylation in the aliphatic positions C-4 in the C-ring of the (−)-equol molecule leads to the insertion of an additional asymmetric C-atom and, therefore, to the formation of four stereoisomers as shown in Fig. 7. The four possible stereoisomers were separated into two diastereomeric pairs (e.g., 3S,4R and 3S,4S separated from 3R,4S and 3R,4R) under the conditions used for liquid chromatography. These pairs then were separated into the corresponding diastereomers by GC/MS analysis. Since no chiral column material was used, the two corresponding enantiomers of peaks 5 and 8 show identical retention times in the GC/MS. The mass spectra were identical to...
those published by Joannou et al. (1995) and Heinonen et al. (1999), who identified the metabolites as the diastereomeric forms cis- and trans-4-hydroxy-equol.

For peak 6, the third aliphatic hydroxylated metabolite, we found a complex fragmentation pattern in the EI mass spectrum since this metabolite is rather unstable. The most intense ion in the EI mass

![Image of chemical structures and fragmentation pathways]

Fig. 12. Proposed fragmentation pathway of metabolite 6, identified as 2-hydroxy-equol according to the fragment ions derived by HPLC/API-ES MS analysis. The mass fragmentation spectrum is shown in Fig. 11.

Fig. 13. GC-selected ion chromatogram of the incubation of equol with HLM. *, 4-hydroxy-equol is overlapped by an unknown compound having the same retention time.
spectrum is not the molecular ion (m/z 474 only 5%) but m/z 402, which can be derived by the loss of a TMS group. In contrast, the API-ES mass spectrum allows an unambiguous interpretation: It shows a quasimolecular ion [M + 1] at m/z 259 (Fig. 11), implying a monohydroxylated derivative. Further fragment ions are m/z 241 and 231, which are derived by the loss of H2O and CO, respectively. The most intense ion is m/z 137, which can be explained by the formation of the ionic 3-phenylethanal a. This product can further be degraded to b. In Fig. 12, the proposed fragmentation pathway is shown. On the basis of these findings, peak 6 is tentatively identified as 2-hydroxy-equol. Nonetheless, the final allocation of the structure requires further spectroscopic information or reference compounds. From a theoretical point of view, the formation of four stereoisomers, as shown for 4-hydroxy-equol, is possible. However, with the analytical methods used, we got no indication of further stereoisomeric 2-hydroxy-equol derivatives.

Peak 7 is formed in the microsomal metabolism of equol and 3'-hydroxy-equol. In the EI mass spectrum, a molecular ion at m/z 562 is observed as well as a quasimolecular ion [M + 1] at m/z 287 in the API-ES mass spectrum. It is concluded that metabolite 7 is a monohydroxylated 3'-hydroxy-equol derivative. Based on the EI and API-ES mass spectra, a dihydroxylated A-ring derivative is likely. Since the metabolite does not coelute with 6,3'-dihydroxy-equol (a metabolite that is formed during incubation of 3'-hydroxy-equol with...
the enzyme tyrosinase; data not shown), we preliminarily assign peak 7 to be 8,3'-dihydroxy-equol.

Metabolites 1, 2, and 3 are formed only in small yields. According to the API-ES mass spectra, they correspond to dihydroxylated equol derivatives. The isolated HPLC peaks degraded during GC/MS analysis. Therefore, the structures have not yet been identified. These three metabolites are also formed in the microsomal metabolism of 3'-hydroxy-equol, implying that peaks 1, 2, and 3 are monohydroxylated 3'-hydroxy-equol derivatives.

**Human Microsomal Metabolites of Equol.** HLMs have been used to gain information about the metabolites formed in vitro by humans in comparison to the rat. Equol is converted to six metabolites by HLMs. The main metabolites are the aromatic monohydroxylated products 3'- and 6-hydroxy-equol. Further metabolites are 8-hydroxy-equol and both diastereomeric pairs of 4-hydroxy-equol (Fig. 13). In trace amounts, two monohydroxylated 3'-hydroxy-equol derivatives are found.

**Discussion**

In the present study, we have investigated the in vitro phase I metabolism of equol using pooled Aroclor-induced rat liver microsomes. Eleven metabolites were formed, separated by HPLC and GC, and identified with the help of their HPLC/API-ES and GC/EI-MS spectra (Fig. 9) and by comparison with reference compounds. Our results clearly indicate that equol is a substrate for P450 enzymes. A scheme of the identified metabolites is shown in Fig. 11. Preliminary experiments with HLM from one male individual suggest that the oxidative metabolism of equol may also be relevant to humans, although HLMs caused, in our experiments, compared with the Aroclor-induced rat liver microsomes, a less complex metabolite profile. This is not very surprising since Aroclor 1254 induces a variety of different P450 enzymes including, also, P450 enzymes that are usually not expressed in the liver; e.g., CYP 1A1. Furthermore, the amount of P450 enzyme per nanomole of equol substrate was higher in incubation mixtures with rat liver microsomes. However, in both cases, the rat and the human microsomal metabolism, 3'-hydroxy-equol, as well as 6- and 8-hydroxy-equol, and the aliphatic hydroxylated metabolite 4-hydroxy-equol are the main products. Further studies with pooled HLMs or with a larger number of different HLMs as well as with human recombinant P450 enzymes are needed to get additional information on the interindividual variation of the metabolite pattern in humans and on the P450 enzymes involved in the formation of the different metabolites.

4-Hydroxy-equol has already been identified by Kelly et al. (1993), Joannou et al. (1995), and Heinonen et al. (1999) in urine of human volunteers after the consumption of soy. The in vivo formation of 4-hydroxy-equol is proposed to occur via the reductive metabolism of daidzein to dihydrodaidzein and a further reduction step of the keto group in the C-ring of dihydrodaidzein to 4-hydroxy-equol (Fig. 1). Our in vitro experiments with rat liver microsomes and HLMs show that the formation of 4-hydroxy-equol can also occur via hydroxylation of equol by P450 enzymes. Perhaps both pathways are relevant for the in vivo situation. The same may hold for the aromatic hydroxylated equol derivatives formed by human and rat liver microsomes. Kulling et al. (2000, 2001) have been able to show that the soy isoflavones daidzein and genistein are good substrates for P450 enzymes in vitro. Furthermore, several mono- and dihydroxylated daidzein and genistein as well as monohydroxylated equol metabolites have been identified in vivo (Kulling et al., 2001; Heinonen et al., 2003). Therefore, the formation of the aromatic hydroxylated equol metabolites may occur via reduction of the corresponding hydroxylated isoflavones or via hydroxylation of equol by P450 enzymes as shown in this study. Further studies are needed to clarify the importance of these two metabolic pathways. It seems likely that both pathways are of relevance for the in vivo formation of the hydroxylated equol metabolites. Therefore, our findings might have a substantial impact on the understanding of the biotransformation of isoflavones.

Joannou et al. (1995) and Kelly et al. (1993) tentatively identified the detected derivatives of 4-hydroxy-equol in human urine as cis- and trans-form based on mass spectrometric data and microscale synthesis of possible reference compounds, the structures of which were characterized by GC/MS. However, Heinonen et al. (1999) detected only cis-4-hydroxy-equol. They used reference compounds fully characterized by two-dimensional $^1$H and $^{13}$C NMR as well as molecular mechanical calculations (Wahala et al., 1997; Heinonen et al., 1999). Therefore, it seems likely that, in vivo, only cis-4-hydroxy-equol is formed. Hydroxylation of racemic (±)-equol with human and rat liver microsomes leads to the formation of all four possible stereoisomers of 4-hydroxy-equol, the cis-isomers 3R,4R- and 3S,4S-4-hydroxy-equol as well as the trans-isomers 3S,4R- and 3R,4R-4-hydroxy-equol (Fig. 7). cis-4-Hydroxy-equol, the intermediate metabolite in the intestinal bacterial metabolism of daidzein to S-(−)-equol as identified by Heinonen et al. (1999), has to be 3S,4S-4-hydroxy-equol. Probably, 3S,4S-4-hydroxy-equol is formed by P450 enzymes in vivo (Fig. 15).

The hydroxylated metabolites exert interesting biological properties: the anticancer activities of both 4-hydroxy-equol isomers have been studied by Wahala et al. (1997). Both isomers inhibit the growth of prostate cells (LNCaP 90) much more effectively than the parent compound daidzein. The activities were comparable to that of genistein; however, cis- and trans-4-hydroxy-equol were less cytotoxic. Regarding the antioxidant activity, it has been shown that equol is more potent than genistein or daidzein and that the hydroxylated metabolites of genistein and daidzein exert an even higher antioxidant efficacy than equol (Arora et al., 1998; Rimbach et al., 2003; Turner et al., 2004). Therefore, studies on the antioxidant capacity of the hydroxylated equol metabolites seem to be interesting. Reference compounds are needed to investigate the biological effects in detail.

In conclusion, this is the first study, to our knowledge, to show that...
equol is a substrate for P450 enzymes and is therefore not inevitably the metabolic end product of daidzein. It is well established that the biological activities of isoflavones are dramatically altered with the chemical structure. On the basis of these findings, it can be assumed that the hydroxylated products have biological properties other than those of the parent compound. Recent studies show that the formation of equol through the action of intestinal bacteria might play a central role regarding the chemopreventive potential of isoflavones (Frankenfeld et al., 2004). Therefore, it seems necessary to completely understand the metabolism pathways of isoflavones and to identify all metabolites that were formed during this process, even if these metabolites might appear in small quantities.

References


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