NEW METABOLIC PATHWAYS FOR FLAVANONES CATALYZED BY RAT LIVER MICROSOMES

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ABSTRACT:

Flavonoids represent a diverse group of natural pigments widely distributed in the plant kingdom and are an important component of human diet due to their high content in fruits and vegetables. Since many flavonoids have been shown to be potent inhibitors, substrates, and even inducers of various cytochrome P450 isoforms, there is considerable interest in studying interactions of this class of molecules with the cytochrome P450 enzyme system. In this study, the metabolism of several simple flavanones by rat liver microsomes was investigated and compared. In addition to the expected aromatic hydroxylation products, several novel metabolic pathways were observed including C-ring desaturation to form the corresponding flavones, oxidation of the B-ring to generate an unusual quinol oxidation product, B-ring cleavage with the formation of chromone derivatives, and reduction of carbonyl group to form flavan-4-ol derivatives. The metabolites were characterized and identified primarily by using liquid chromatography-tandem mass spectrometry with comparison to authentic standards. Formation of flavones from dietary flavanones might have biological significance since flavones often exhibit pharmacological activities that are different from those of flavanones. However, little is known about the pharmacological activities of the other types of flavonone metabolites.

Flavonoids are a diverse group of natural products encompassing more than 5000 compounds. Chemically, flavonoids contain a common phenylchromanone structure (C₆-C₃-C₃) with at least one hydroxyl group substituent or a hydroxyl derivative such as a methoxy group. Flavonoids are classified based on the level of oxidation and substitution pattern of the C-ring, whereas members within a class differ in the pattern of substitution on the A- and B-rings. Plants often synthesize O-linked or C-linked glycosides of these compounds.

Present in significant quantities in fruits and vegetables, flavonoids are an important component of the human diet. Estimates of daily intake range from 28 mg/day in the Netherlands (Hertog et al., 1993) to 1 g/day in the United States (Kuhnau, 1976). Many beneficial pharmacological properties have been attributed to flavonoids including antioxidant, anti-inflammatory, anticarcinogenic, chemopreventive, and cytochrome P450-inhibitory activities.

Although less abundant in plants than some other classes of flavonoids, many flavanones also exhibit important biological activities. Major dietary sources of flavanones include citrus fruits and juices, propolis, and honey. Naringenin, one of the most abundant flavanones in citrus fruits, has been shown to possess antioxidant (Wang and Goodman, 1999), antiproliferative (Manthey and Guthrie, 2002), and weakly estrogenic activities in vitro (Miksicek, 1993). Erlund et al. (2002) estimated that a high vegetable diet can provide up to 132 mg of hesperetin and 29 mg of naringenin per day, and that the plasma level of naringenin can reach 113 nM. The prenylated analog of naringenin, 8-prenylnaringenin, is a much more potent phytoestrogen (Milligan et al., 1999). Propolis and honey contain pinocembrin and pinobanksin, as well as several other flavanones. The methylated derivative of pinocembrin is called pinostrombin and has recently been shown to be a potent inducer of quinone reductase (Fahey and Stephenson, 2002). For comprehensive recent reviews of other pharmacological activities of flavonoids see Ross and Kasum (2002) and Havsteen (2002).

Recently, there has been considerable interest in studying the interactions of flavonoids with cytochrome P450 isozymes, since this process has the potential to interfere with the metabolism of various drugs (see Hodek et al., 2002). Although early studies indicated that many flavonoids could act as cytochrome P450 inhibitors, much of this activity may be attributed to the competitive metabolism of flavonoids. Some flavonoids such as diosmin and flavanone have also been shown to be inducers of cytochrome P450 enzymes (Canivenc-Lavier et al., 1996; Ciolino et al., 1998). Another important aspect of flavonoid metabolism is that their structural similarities can result in metabolic interconversion. Since metabolism might transform one class of flavonoid into another, new pharmacological activity might result. Indeed, a comprehensive study by Nielsen et al. (1998) showed that many methoxylated flavonoids can be demethylated to their oxidized analogs. For example, hesperetin is O-demethylated to form naringenin, or kaempferide is metabolized to kaempferol (Otake and Walle, 2002). Nielsen et al. (1998) showed that ring oxidation is
another metabolic pathway for conversion of one flavanoid into another, such as, for example, metabolism of apigenin to form luteolin.

In the present study, metabolic transformations of several flavanones catalyzed by rat liver microsomes were investigated (see structures in Fig. 1). Flavanones contain a saturated C₂-C₃ bond as well as aromatic rings that can undergo metabolic transformation. We discovered that flavanones are metabolized via several previously unreported routes such as desaturation, quinol formation, B-ring cleavage, and reduction of the keto group. These new metabolites were detected and identified primarily by using liquid chromatography-tandem mass spectrometry (LC/MS-MS) utilizing high sensitivity and high resolution quadrupole/time-of-flight mass spectrometry. Since human liver microsomes usually show low drug-metabolizing activities, dexamethasone-induced rat liver microsomes were used to enhance the levels of minor mammalian metabolites for characterization using LC/MS-MS.

Materials and Methods

Chemicals. Flavanones used in the study (see Fig. 1) were purchased from Indofine Chemical Company (Hillsborough, NJ) and were used without further purification. The purity of the material exceeded 98% according to the manufacturer’s specifications. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). HPLC (Optima) grade solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Preparation of 7,4'-Dihydroxyflavanone. 7,4'-Dihydroxyflavanone (liquiritigenin) was prepared from the corresponding chalcone by cyclization in mild alkaline medium. Briefly, the chalcone was dissolved in methanol/water (1:1; v/v) containing 0.1 M KOH and left at room temperature for 5 h.

The resulting flavanone was purified by using semipreparative reversed phase chromatography on a YMC (Milford, MA) AQ column, 10 × 250 mm, using acetonitrile/0.5% acetic acid (30:70; v/v) as the mobile phase and was >97% pure as determined by LC/MS analysis.

Preparation of Flavan-4-ols. Flavan-4-ols were prepared from the respective flavanones by reduction with sodium borohydride. Samples dissolved in tetrahydrofuran were stirred with sodium borohydride for several hours at room temperature. Reactions were stopped by adding excess water drop-wise, and the resulting products were extracted using ethyl acetate.

Rat Liver Microsomes. Female Sprague-Dawley rats (Sasco Inc., Wilmington, MA) were administered 100 mg/kg body weight dexamethasone in corn oil i.p. for 3 days and sacrificed on day 4. Liver microsomes were prepared, and protein and cytochrome P450 concentrations were determined as described previously (Thompson et al., 1987).

Microsomal Incubations. A typical incubation mixture (0.4 ml) contained 1 mg/ml rat liver microsomal protein, 50 μM substrate, and 1 mM NADPH in 50 mM phosphate buffer, pH 7.4. After 2-min preincubation of the substrate and the microsomal protein, the reaction was initiated by the addition of NADPH. Incubations were carried out for 60 min at 37°C and terminated by chilling the mixture on ice followed by addition of 1.6 ml of ice-cold acetonitrile/ethanol (1:1, v/v) to precipitate proteins. Samples were centrifuged for 20 min at 20,000g, and the supernatant was evaporated to dryness under nitrogen. The residue was reconstituted in the mobile phase before LC/MS-MS analysis. Control incubations were carried out either without microsomal protein or without NADPH and showed no evidence of any metabolites.

LC/MS-MS. Reversed-phase HPLC separations were carried out using a Supelco (Belleville, PA) Discovery C₁₈ column, 2.1 × 100 mm (5-μm particle size) connected to a Waters 2690 solvent delivery system. The flow rate was 0.2 ml/min. To maximize the signal intensity during positive and negative ion electrospray mass spectrometry, different HPLC solvents were used for each ionization mode. In addition, the gradients were optimized for each group of metabolites as follows.

Method A. During negative ion electrospray LC/MS-MS analysis of metabolites of naringenin, pinocembrin, and apigenin, the HPLC solvent system consisted of a 30-min linear gradient from 25 to 60% methanol in water (containing 0.05% acetic acid), 60 to 100% methanol over 5 min, and then 100% isocratic methanol for 5 min.

Method B. For separations of flavanone and monohydroxylated flavanone metabolites using negative ion electrospray LC/MS-MS, the solvent system was identical to that in method A except that the gradient consisted of 35 to 70% methanol over 30 min, 70 to 100% methanol over 5 min, and 100% isocratic methanol for 5 min.

Method C. Metabolites of 7,4'-dihydroxyflavanone were analyzed using negative ion electrospray LC/MS-MS and the solvent system of methods A and B except that the gradient consisted of 20 to 50% methanol over 30 min, 50 to 80% methanol over 5 min, and isocratic 100% methanol for 10 min.

Method D. For positive ion electrospray LC/MS-MS, the solvent system consisted of a gradient from 0.5% acetic acid in water to methanol. First, a 30-min gradient was used from 20 to 70% methanol followed by 70 to 100% methanol over 5 min, and finally isocratic 100% methanol for 5 min.

Mass spectrometric measurements were carried out using a Micromass (Manchester, UK) Q-TOF-2 hybrid quadrupole/time-of-flight mass spectrometer equipped with a Z-spray electrospray ionization source. The resolving power was 6000 FWHM at m/z 500. For elemental composition determinations using exact mass measurements, raffinose ([M + H]⁺ at m/z 503.1612) or reserpine ([M + H]⁺ at m/z 609.2812) was introduced postcolumn as a lock mass. The electrospray ion source parameters were as follows: capillary 2.3 kV (negative ion) or 3.5 kV (positive ion mode), cone voltage 30 V, source block temperature 120°C, and drying gas temperature 320°C. Tandem mass spectra were acquired using collision-induced dissociation (CID) at slightly less than unit resolution on the quadrupole, argon as the collision gas at a collision energy of 20 or 25 eV, and a collision gas pressure of 2.2 × 10⁻³ mBar.

Results

Metabolism of Flavanone. To identify the sites of metabolic transformation in flavanone, it was helpful to first obtain and interpret the electrospray product ion tandem mass spectrum of flavanone itself.
Due to the lack of acidic hydroxyl groups, flavanone ionized more efficiently in positive than in negative mode, and therefore only positive ion electrospray tandem mass spectra were obtained for the unmetabolized molecule. The fragment ion nomenclature described by Ma et al. (1997) and Fabre et al. (2001) was used in the interpretation of the tandem mass spectra of flavanone and its metabolites. Briefly, the superscript numbers indicate the bonds broken during fragmentation, and capital letters represent the aromatic ring that retains the charge (see labeled bonds and rings in the flavanone structure in Fig. 2).

During CID, the protonated molecule of flavanone produced an abundant fragment ion of \( m/z \) 121 (\( ^{1,3}\text{A}^+ \)), which was the product of a retro Diels-Alder reaction that is class-characteristic of flavonoids (Ma et al., 1997). In addition, a \( ^{1,4}\text{B}^+ \) ion was detected at \( m/z \) 131 corresponding to the other ring of flavanone. When analyzing metabolites or other derivatives of flavanone, changes in the masses of these ions would be indicative of the site of metabolic transformation. An interesting and unexpected fragment ion was observed at \( m/z \) 210 and represented loss of a methyl radical from the protonated molecule of flavanone. Although the formation of odd-electron ions from even-electron precursors has been reported during CID (Zhou et al., 2000), this is an unusual fragmentation pathway for a flavonoid carbon skeleton during low energy CID. The methyl group was probably eliminated from position 3 of the C-ring along with a hydrogen from the B-ring.

**Oxidation products.** Computer-reconstructed mass chromatograms of oxidation products formed during incubation of flavanone with rat liver microsomes are shown in Fig. 3. The most abundant oxidation product (M1) was a mono-oxygenated metabolite with a retention time of 25.5 min. This and other mono-oxygenated flavanones including M2, M3, M4, and M5, eluting at 15.4, 21.6, 23.1, and 24.6 min, formed protonated molecules of \( m/z \) 241 as shown in Fig. 3A.

The most abundant product ions in the tandem mass spectrum of the flavanone metabolite M1 were detected at \( m/z \) 131 and 137 (data not shown). Since the fragment ion of \( m/z \) 131 indicated that the B-ring of flavanone was unchanged, oxidation must have occurred on the A-ring. However, the exact position of the additional oxygen on the A-ring could not be located based on this mass spectrum alone. By comparison of HPLC retention times and product ion tandem mass spectra with an authentic standard, M1 was identified as 6-hydroxyflavanone.

The minor mono-oxygenated metabolite M4, eluting at 23.1 min in Fig. 3A, was identified as 4′-hydroxyflavanone by comparison of the retention time and product ion mass spectrum with an authentic standard. The positive ion electrospray product ion tandem mass spectrum of M2, eluting at 15.4 min, was almost identical to that of M4. The base peak in each of the tandem mass spectra of M2 and M4 was the \( ^{1,3}\text{A}^+ \) ion of \( m/z \) 121, indicating that the A-ring was unchanged (see Table 1). During negative ion electrospray product ion tandem mass spectrometry of the deprotonated molecule of \( m/z \) 239 of M2 and M4 (data not shown), the base peak in each spectrum was the \( ^{1,3}\text{B}^- \) ion of \( m/z \) 119, which is characteristic of flavanones that are monohydroxylated on the B-ring (Fabre et al., 2001). Among the remaining hydroxylation sites that might lead to M2, substitution at the 3′-C was excluded by comparison of HPLC retention times with the authentic standard. Therefore, M2 was identified as 2′-hydroxyflavanone.

The positive ion electrospray product ion tandem mass spectrum of the mono-oxygenated metabolite M5 (protonated molecule detected at \( m/z \) 241) was dominated by the base peak of \( m/z \) 167. The \( ^{1,3}\text{A}^+ \) ion was detected at \( m/z \) 121, indicating that the A-ring was unchanged. Unlike flavanone, no loss of a methyl radical was observed during positive ion electrospray tandem mass spectrometry of the protonated molecule of M5, suggesting that this product was probably a 3-C hydroxylation product.

Finally, another mono-oxygenated metabolite of flavanone, M3, was detected in low abundance eluting at 21.3 min in Fig. 3A. The protonated molecule of M3 fragmented to produce the \( ^{1,3}\text{A}^+ \) ion of \( m/z \) 137, suggesting that it was an A-ring hydroxylation product. No additional structural information was obtained for M3.

**Formation of flavone.** Flavanone metabolite M6 eluted at 28.7 min in the computer-reconstructed positive ion electrospray mass chro-
matogram shown in Fig. 3B. The protonated molecule of \( m/z \) 223 suggested the net loss of two hydrogen atoms from the precursor molecule flavanone. M6 was identified as flavone by comparing HPLC retention time and tandem mass spectrometric fragmentation patterns with the authentic standard.

**Reduction of the C4 carbonyl group.** Two isomeric metabolites of flavanone, M7 and M8, eluted at 26.9 and 28.2 min, respectively, in Fig. 3C. The ions of \( m/z \) 209 and the molecular formula of \( C_{14}H_{10}O_3 \) (+6.0 ppm) suggest that M7 and M8 were formed by addition of two hydrogens to flavanone and then elimination of a molecule of water. Under very gentle positive ion electrospray source conditions, an ion of \( m/z \) 227 was just detectable, indicating that the ions of \( m/z \) 209 were probably dehydration products formed in the ion source. Therefore, M7 and M8 were probably formed by addition of two hydrogens to flavanone. One pathway for their formation might be reduction of the prochiral keto group to generate two flavanols. In the electrospray ion source, these flavanols might easily dehydrate to form a stable flavene. This pathway is supported by the observation that the CID product ion mass spectra of \( m/z \) 209 for the peaks at 26.9 and 28.2 min were identical (Table 1). This suggests that the ions of \( m/z \) 209 were structurally identical. Additional confirmation was obtained by comparing the retention times and mass spectra of the products of the reduction of flavanone by sodium borohydride. The flavanol reduction products of flavanone showed HPLC retention times and electrospray mass spectra that were identical to M7 and M8. Therefore, flavanone metabolites M7 and M8 were identified as flavan-4-ols.

**Metabolism of 4'-Hydroxy- and 3'-Hydroxyflavanone.** Following the investigation of the metabolism of flavanone, the hepatic microsomal incubation products of 4'-hydroxyflavanone and 3'-hydroxyflavanone were investigated to explore the influence on metabolism of hydroxyl groups at different positions on the B-ring. It should be noted that unlike flavanone, the presence of the phenolic group in these compounds facilitated the formation of abundant deprotonated molecules for use during tandem mass spectrometry with CID and product ion scanning. The negative ion electrospray tandem mass spectra of 4'-hydroxyflavone and 3'-hydroxyflavone contained the abundant ion of \( m/z \) 119 (Table 1), which permitted localization of the hydroxyl group to the B-ring. In particular, this \( 1^{3}\text{B} \) ion of \( m/z \) 119 was formed by retro Diels-Alder cleavage with charge retention on the B-ring.

**Oxidation products.** The computer-reconstructed negative ion electrospray LC/MS chromatograms for the mono-oxygenated metabolites of 4'-hydroxyflavanone and 3'-hydroxyflavanone are shown in Figs. 4A and 5A, respectively. Both compounds formed the same abundant mono-oxygenated metabolite M9 with a deprotonated molecule of \( m/z \) 255 and a retention time of 11.9 min. M9 was identified

<table>
<thead>
<tr>
<th>Substrate/Metabolite</th>
<th>Precursor Ion, ( m/z )</th>
<th>Major Fragment Ions (Relative Abundance)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavanone</td>
<td>[M + H]^+ 241.0885</td>
<td>241.09 (11); 226.06 (12); 223.07 (9); 147.04 (42)</td>
<td>2'-Hydroxyflavanone</td>
</tr>
<tr>
<td>M3</td>
<td>[M + H]^+ 241.080</td>
<td>137.01 (100) (1^{3}\text{A})</td>
<td>A-Ring hydroxylation</td>
</tr>
<tr>
<td>M4</td>
<td>[M + H]^+ 241.084</td>
<td>241.09 (15); 226.06 (14); 147.04 (22) (1^{3}\text{B}^+); 121.03 (100) (1^{3}\text{A}^+)</td>
<td>4'-Hydroxyflavanone</td>
</tr>
<tr>
<td>M5</td>
<td>[M + H]^+ 241.083</td>
<td>241.08 (3); 195.08 (20); 167.09 (100); 121.03 (52)</td>
<td>3'-Hydroxyflavanone</td>
</tr>
<tr>
<td>M7</td>
<td>[MH - H2O]^+ 209.0979</td>
<td>209.09 (42); 194.07 (32); 181.10 (25); 166.07 (30); 131.05 (83); 115.05 (100); 91.06 (35)</td>
<td>Flavan-4-ol</td>
</tr>
<tr>
<td>M8</td>
<td>[MH - H2O]^+ 209.0980</td>
<td>209.09 (42); 194.07 (24); 181.10 (25); 166.07 (30); 165.07 (11) 131.05 (86); 115.05 (100); 91.06 (36)</td>
<td>Flavan-4-ol</td>
</tr>
</tbody>
</table>

**TABLE 1**

Electrospray tandem mass spectra with CID of flavanones and selected metabolites
as 3',4'-dihydroxyflavanone based on both the positive and negative ion electrospray tandem mass spectra that contained a 1,3-A$^+$ ion of m/z 121 and a 1,3-B$^-$ ion of m/z 135 (Table 1).

In addition to M9, 3'-hydroxyflavanone formed another, but less abundant, mono-oxygenated metabolite, M12, that eluted at 9.9 min. The 1,3-B$^-$ ion of m/z 135 was the base peak in the negative ion product ion tandem mass spectrum of M12 (Table 1). In contrast to the negative ion tandem mass spectrum of M9 (3',4'-dihydroxyflavanone), several other fragment ions were detected in addition to the 1,3-B$^-$ ion. During positive ion electrospray tandem mass spectrometry, the 1,3-A$^+$ of m/z 121 was the base peak confirming that oxidation did not take place on the A-ring. Combined with the presence of an abundant 1,3-B$^-$ ion of m/z 135, these data suggest that M12 contains a dihydroxylated B-ring. However, the exact substitution pattern could not be determined based on the available data. Incubation of 4'-hydroxyflavanone with rat liver microsomes formed a second but less abundant mono-oxygenated metabolite, M10, which eluted at 8.9 min. During negative ion and positive ion tandem mass spectrometry, M10 formed a 1,3-B$^-$ ion of m/z 119 (Table 1) and a 1,3-A$^+$ ion of m/z 137 (data not shown), respectively. These data are consistent with oxidation on the A-ring at C-6, although the exact position of oxidation could not be determined based on these data.

**Formation of flavones.** As shown in the negative ion LC/MS chromatograms in Figs. 4B and 5B, 4'-hydroxyflavanone but not 3'-hydroxyflavanone formed a flavone metabolite of m/z 237. This flavone metabolite, M11, eluted at 17.9 min (Fig. 4B). M11 was identified as 4'-hydroxyflavone, based on the molecular formula of $C_{19}H_{12}O_5$ (+5.5 ppm) and the fragmentation pattern in the negative ion tandem mass spectrum (Table 1), which contained an abundant 1,3-B$^-$ fragment ion of m/z 117, which is 2 units less than the corresponding ion of m/z 119 in the 4'-hydroxyflavanone tandem mass spectrum (Table 1).

**Reduction of the C-4 carbonyl group.** Another difference in the routes of metabolism of 4'-hydroxy- and 3'-hydroxyflavanone was the exclusive formation of reduction products M13 and M14 from 3'-hydroxyflavanone. M13 and M14 eluted at 13.4 and 14.0 min, respectively, in the negative ion LC/MS chromatogram of the metabolites of 3'-hydroxyflavanone shown in Fig. 5C. There were no corresponding metabolites for 4'-hydroxyflavanone in Fig. 4C. The deprotonated molecules of these metabolites of m/z 241 correspond to the addition of two hydrogens to 3'-hydroxyflavanone. Since the negative ion product ion tandem mass spectra of m/z 241 showed an abundant 1,3-B$^-$ fragment ion of m/z 119, which was also an abundant fragment ion of 3'-hydroxyflavanone (Table 1), this indicates that both the B-ring and C-ring carbons were unaffected. Therefore, the carbonyl group must have been reduced during the formation of M13 and M14. Furthermore, the observation of two isomeric metabolites of m/z 241 indicates that two diastereoisomers were formed as described above for the reduction of flavanone. The identification of M13 and M14 as diastereomers of 3'-hydroxyflavan-4-ol was confirmed by comparison with the products of chemical reduction of 3'-hydroxyflavanone by sodium borohydride.

**B-ring cleavage and formation of chromone (γ-pyrone).** Another difference between the hepatic microsomal metabolism of 3'-hydroxy- and 4'-hydroxyflavanone was the formation of M15, which was detected as a protonated molecule of m/z 147 (Fig. 6). M15 was detected only in hepatic microsomal incubations with 4'-hydroxyflavanone. During LC/MS, M15 eluted at a retention time of 10.0 min. The exact mass measurement of M15 was consistent with an elemental composition of $C_{19}H_{14}O_5$ (−7.5 ppm), and the positive ion electrospray product ion spectrum (see inset in Fig. 6) suggested that the structure was a simple aromatic molecule. These data indicate that M15 was formed from 4'-hydroxyflavanone by elimination of the entire B-ring to produce chromone. The identification of M15 as
chromone was confirmed by comparing the retention times and fragmentation patterns of M15 and a chromone standard.

**Metabolism of Pinocembrin and Naringenin.** Pinocembrin and its 4'-hydroxylated analog naringenin are abundant flavonoids in citrus fruits and honey. Some aspects of naringenin metabolism have been reported by Nielsen et al. (1998), but we are aware of no studies addressing the metabolism of pinocembrin. The negative ion product ion mass spectra of naringenin and pinocembrin (Table 1) both contained a common retro Diels-Alder fragment ion (1,3 \( \text{A}^- \)) of m/z 151. In addition, naringenin formed a 1,3 \( \text{B}^- \) ion of m/z 119 (see Table 1).

**Oxidation products.** The negative ion electrospray LC/MS analysis of the hepatic microsomal metabolites of pinocembrin are shown in Fig. 7. In particular, Fig. 7A shows the computer-reconstructed mass chromatogram for the mono-oxidation products of pinocembrin. One of the most abundant metabolites eluting at 18.2 min was naringenin (M16), which was formed by hydroxylation at the 4'-C of the B-ring on pinocembrin. Eluting at 17.8 min and only partially resolved from naringenin was the mono-oxygenated metabolite M17. By comparison of the HPLC retention time and tandem mass spectrometric fragmentation patterns with an authentic standard, M17 was identified as 3,5,7-trihydroxyflavanone (pinobanksin). It is interesting to note that the product ion spectrum of M17 (pinobanksin) contained an ion of m/z 125, which represents a 1,3 \( \text{A}^- \) fragment ion (data not shown). This type of fragment ion has been reported for 4'-hydroxylated catechins and catechin esters by Miketova et al. (2000), who noted that it is useful for characterizing this substitution pattern on the B-ring. Since flavanones have an extra carbonyl group, this type of fragmentation is possible even if the B-ring is unsubstituted. However, we found that the 1,3 \( \text{A}^- \) ion was much more abundant for 4'-hydroxylated flavanones than for unsubstituted analogs. Finally, there was a trace of another peak at 20.8 min (Fig. 7C) corresponding to a minor mono-oxygenated metabolite of pinocembrin that could not be identified.

The most abundant metabolite of naringenin was a mono-oxygenated metabolite M20 of m/z 287 with a retention time of 14.7 min (see the negative ion electrospray LC/MS chromatogram in Fig. 8A). M20 was identified as the 3'-hydroxylated analog, eriodictyol, based on previous studies (Nielsen et al., 1998). A novel but less abundant mono-oxygenated metabolite of naringenin, M21, eluted at a retention time of 9.7 min. This metabolite was not detected in the incubations with pinocembrin. The negative ion electrospray product ion tandem mass spectrum of M21 is shown in Fig. 9. The usual negative ion fragmentation that is characteristic of most flavanones is absent. For example, instead of the loss of 120 U that would be characteristic of flavanones containing a monohydroxylated B-ring, a loss to 110 U was observed to form the base peak of m/z 177. Also not observed were abundant fragment ions of m/z 119 or 135, which would be characteristic of compounds containing a mono- or dihydroxylated aromatic B-ring. Instead, two abundant radical anions of m/z 150 and 108 were detected (Fig. 9), which were not observed for other flavanone derivatives. Exact mass measurements of these fragment ions provided some insights into the structure of M21. Loss of 110 units represented loss of C\(_6\)H\(_4\)O\(_2\), and the elemental composition of m/z 108 was C\(_\text{a}\)H\(_{11}\)O\(_2\). This indicates either that the aromatic B-ring might be eliminated as a neutral or that it carries a negative charge in the form of radical anion. The most plausible structure consistent with this fragmentation pattern is a quinol (hydroxy quinone). A quinol metabolite might have been formed by ipso hydroxylation of the aromatic B-ring (see structure in Fig. 9). Loss of 110 units would correspond to loss of the hydroquinone moiety, and the ion of m/z 108 would be a radical anion as shown in Fig. 9. The ion of m/z 150 had an elemental composition of C\(_6\)H\(_{11}\)O\(_2\) and was probably formed by elimination of the B-ring as a semiquinone radical in addition to loss
of CO from the C-ring. This type of metabolic transformation is known, since quinols have been reported for steroidal estrogens as a C10 oxidation product (Ohe et al., 2000). In addition, this type of structure is frequently evoked as an intermediate in O-dearylation reactions (see below).

Another minor mono-oxygenated metabolite (M22) of naringenin was detected at 11.2 min during negative ion electrospray LC/MS (Fig. 8A). The product ion tandem mass spectrum of M22 consisted exclusively of the 1,4-\(\text{A}^-\) fragment ion of m/z 125. M22 was identified as 3-hydroxynaringenin (aromadendrin) by analogy with M17 (pino-

banksin), which is the corresponding metabolite of pinocembrin. This assignment was confirmed by comparison with the tandem mass spectra of other 3-hydroxylated derivatives such as taxifolin, which also produced an abundant 1,4-\(\text{A}^-\) ion of m/z 125.

**B-ring cleavage and formation of 5,7-dihydroxychromone.** During negative ion LC/MS analysis of metabolites of naringenin (Fig. 8C), a peak designated M24 was detected at 7.8 min with a deprotonated molecule of m/z 177. M24 was not detected as a metabolite of pinocembrin. Exact mass measurement indicated that the molecular formula was \(\text{C}_{10}\text{H}_{10}\text{O}_4\) (6.7 ppm). This formula is consistent with the structure 5,7-dihydroxychromone, which is a B-ring cleavage product analogous to that described above for 4'-OH flavanone. The negative ion product ion tandem mass spectrum of the deprotonated molecule of M24 at m/z 177 (Table 1) showed loss of 42 U at m/z 135 corresponding to loss of ketene, \([\text{M} - \text{H} - \text{CH}_2 = \text{C} = \text{O}]^-\), as well as an additional loss of CO to form the ion of m/z 91.

**Formation of flavones.** Both naringenin and pinocembrin formed flavone metabolites during incubation with rat liver microsomes. For example, pinocembrin formed M18, chrysin (see Fig. 7B), and naringenin was metabolized to M23, apigenin (Fig. 8B). Both of these flavone metabolites were identified by comparison of HPLC retention times and product ion tandem mass spectra with the authentic standards. Apigenin was a much more abundant metabolite than was chrysin, which is due to the influence of the 4'-hydroxyl group on the formation of this metabolite.

**Reduction of the C-4 carbonyl group.** Metabolite M19 of pinocembrin was detected at a retention time of 8.1 min during LC/MS (Fig. 7D). The deprotonated molecule of M19 at m/z 289 was determined to correspond to a molecular formula of \(\text{C}_{10}\text{H}_{10}\text{O}_5\) during exact mass measurement. Therefore, M19 was formed by double oxygenation of pinocembrin combined with the addition of two hydrogen atoms. During negative ion electrospray tandem mass spectrometry, structurally significant fragment ions were detected at m/z 135 and m/z 125, corresponding to 1,3-\(\text{B}^-\) and 1,4-\(\text{A}^-\) ions, respectively (Table 1). The observation of the 1,4-\(\text{A}^-\) ion suggested 3-hydroxylation as described above. Based on these data, M19 was tentatively identified as 3,4,5,7,4'-pentahydroxyflavan. It is interesting to note that no metabolite was detected corresponding to a carbonyl reduction product alone.

**Metabolism of 7,4’-dihydroxyflavanone.** To learn more about the metabolism and tandem mass spectrometric behavior of B-ring hydroxylated flavanones, 7,4'-dihydroxyflavanone was synthesized from the corresponding chalcone. This flavanone, also known as liquiritigenin, is found in many species of *Leguminosae* and occurs in licorice as a glycoside. The presence of the hydroxyl groups on the A- and B-rings facilitated the formation of a negative charge on either of these rings during negative ion electrospray. As a result, both 1,3-\(\text{A}^-\) and 1,3-\(\text{B}^-\) ions were detected during negative ion tandem mass spectrometry of the deprotonated molecule of m/z 255 (see Table 1). This is similar to the fragmentation pattern of naringenin and is typical of flavanones in general.

**Oxidation products.** The computer-reconstructed LC/MS chromatogram of the mono-oxygenation products of 7,4'-dihydroxyflavanone (liquiritigenin) is shown in Fig. 10A. The most abundant mono-oxygenated metabolite, M25, eluted at 16.8 min and formed a deprotonated molecule of m/z 271. M25 was identified as 7,3’,4’-trihydroxyflavanone based on the negative ion product ion tandem mass spectrum that gave a characteristic fragment ion of m/z 135 (see Table 1). It should be noted that the tandem mass spectrum of the deprotonated molecule of M25 contained two partially resolved product ions of nominal mass m/z 135, one at m/z 135.00 for 1,3-\(\text{A}^-\) and another of 135.04 for the 1,3-\(\text{B}^-\) ion. The resolving power of the
The most abundant metabolite, M26, which was hydroxylated at the 3’ position of the B-ring, was tentatively identified as a hydroxy quinone product similar to that described for naringenin.

The quadrupole/time-of-flight mass spectrometer used to obtain this measurement was sufficient to partially resolve and identify these two fragment ions.

The two mono-oxygenated metabolites M27 and M28 eluting at 14.5 and 16.1 min both fragmented to produce a $^{1,3}$-A$^-$ ion of $m/z$ 119 (Table 1) and a $^{1,2}$-A$^+$ ion of $m/z$ 137. These data suggested that M27 and M28 were isomeric A-ring oxidation products. Since no authentic standards were available, no definitive assignments could be made about the positions of oxidation. However, since two products were formed, at least one of them should represent an A-ring catechol formed by oxidation at one of the two ortho positions.

The last of the mono-oxygenated metabolites of 7,4’-dihydroxyflavanone eluted at 12.2 min (M26). The negative ion electrospay product ion tandem spectrum of M26 (Table 1) indicated a neutral loss of 110 U from the deprotonated molecule of $m/z$ 271 to form a product ion of $m/z$ 161, which is characteristic of naringenin quinols. In addition, the product ion of $m/z$ 135 was probably a $^{1,2}$-A$^+$ ion, indicating that the A-ring was unchanged. The low molecular weight region of the tandem mass spectrum of M26 contained an abundant ion of $m/z$ 109 and an ion of $m/z$ 108 corresponding to a hydroxy quinone. Although not identical to the fragmentation pattern of naringenin, these product ions suggested that M26 was probably a hydroxy quinone metabolite. Please note that these studies indicate that even minor differences in the positions of hydroxyl group substituents can produce profound changes in fragmentation patterns for flavanones. Therefore, the lack of a 5-hydroxy group in 7,4’-dihydroxyflavanone might have altered the relative abundances of the class-characteristic fragment ions of M26. Unfortunately, the signal for M26 was low in intensity, which prevented additional data from being acquired.

**B-ring cleavage and formation of 7-hydroxychromone.** Metabolite M30 eluted at 7.7 min in the LC/MS chromatogram shown in Fig. 10C, and the deprotonated molecule corresponding to this peak was detected at $m/z$ 161. The negative ion product ion tandem mass spectrum of the deprotonated molecule of M30 was very similar to that of the metabolite of $m/z$ 177 obtained from naringenin (M24), indicating that M30 is 7-hydroxychromone. This assignment was confirmed by comparison with a synthetic standard of 7-hydroxychromone. The detection of 7-hydroxychromone (M30) provides additional support for the assignment of M26 as a hydroxy quinone product.

**Formation of flavone.** The peak designated as M29 eluted at 26.4 min in the LC/MS chromatogram shown in Fig. 10B. The deprotonated molecule of M29 was detected at $m/z$ 253 corresponding to the loss of two hydrogens from 7,4’-dihydroxyflavanone during metabolism. This peak was identified as 7,4’-dihydroxyflavone based on the negative ion electrospay product ion tandem mass spectrum (Table 1) that gave a characteristic $^{1,3}$-B$^-$ fragment ion of $m/z$ 117.

**Discussion**

Since few studies have addressed the mammalian phase I metabolism of flavanones, we carried out this investigation of the metabolism of this important class of botanical flavonoids using rat liver microsomes. Previously, the most comprehensive study of flavanone metabolism was reported by Nielsen et al. (1998), who identified some abundant hydroxylation products of several flavanones. Since the work by Nielsen et al. (1998) and similar studies were carried out using synthetic standards representing only a few abundant metabolites, no comprehensive metabolic pathways of flavanones have been elucidated. In our study a highly sensitive HPLC-quadrupole/time-of-flight mass spectrometer was used that allowed us to obtain a more complete metabolic profile that included previously unknown metabolites (see below).

The major pathway of flavanone metabolism was aromatic hydroxylation (Fig. 11). In unsubstituted flavanones, hydroxylation occurred primarily at position 6 of the A-ring, which is para to the activating ether oxygen and meta to the deactivating carbonyl group. In flavanone, other hydroxylation sites included the 2’ and 4’ positions of the B-ring. Since 4’-hydroxylation was a dominant metabolic
pathway for pinocembrin, this flavanone may be considered a metabolic precursor of the related flavanone naringenin.

The presence of a single hydroxyl group on either the A- or B-ring directed oxidation to the ortho position to form the corresponding catechol (see Figs. 12 and 13). In the model compound 7,4'-dihydroxyflavanone, at least one of the ortho positions on the A-ring was oxidized as well as the 3' position on the B-ring (Fig. 13). Interestingly, the B-ring catechol derivative of 7,4'-dihydroxyflavanone was more abundant than the corresponding A-ring catechols. However, when the A-ring was unsubstituted or contained two hydroxyls in meta arrangement, a B-ring 3' or 4' hydroxyl group directed oxidation to the ortho position to produce a 3',4' catechol as the dominant oxidation product. This observation is consistent with the studies of Nielsen et al. (1998) and Breinholt et al. (2002), who found this type of oxidation to be a universal route for B-ring hydroxylated flavonoids regardless of the configuration of the C-ring. The formation of catechols can have important toxicological implications since oxidation produces electrophilic ortho quinones (Awad et al., 2002). In addition to aromatic hydroxylation of flavanones, aliphatic hydroxylation was observed on the C-ring such as at position 3 of pinocembrin to form pinobanksin. During MS-MS, the \(^{1,4}\)A\(^{-}\) fragment ion was useful for the confirmation of C-ring hydroxylation at position 3 as reported by Miketova et al. (2000) for catechol esters.

An unusual quinol oxidation product was observed that was formed by ipso oxidation of the flavanone B-ring. Quinol metabolites, also known as hydroxy quinones, have been described in the literature for other classes of compounds, such as oxidation of estradiol at carbon-10 (Ohe et al., 2000). A possible mechanism for this type of oxidation has been proposed by Guengerich (2001) and involves ipso attack of the reactive oxygen species from cytochrome P450 as shown in Fig. 14A. As observed in previous studies (Ohe et al., 1994, 2000; Solaja et al., 1996), a para hydroxyl group is a prerequisite for the formation of quinols. Furthermore, flavanones with a 3' hydroxyl group on the B-ring (meta position) or compounds lacking a hydroxyl group altogether did not form quinols. Finally, a saturated C-ring was also found to be important for the formation of quinols since apigenin (flavone) did not produce this type of metabolite (data not shown). We know of no previous reports of quinol metabolites of flavanoids. Furthermore, there have been no reports of the biological activities or toxicity of this type of metabolite. Quinols are quinoid-type molecules and as such have the potential to exhibit toxicity by reacting with biological nucleophiles.

In addition to being primary oxidation products, quinols have been evoked as intermediates in \(O\)-dearylation reaction (Testa, 1995). Consistent with this hypothesis, we observed that all 4'-hydroxylated flavanones were metabolized to chromones (\(\gamma\)-pyrones) by cleavage of the B-ring. Thus, it appears that this reaction is common for 4'-hydroxylated flavanones. In addition, we found that other flavanones such as prenylated naringenin and isoxanthohumol can also form this cleavage product (Nikolic et al., 2004). Patzlaff and Barz (1978) observed a chromone product as a result of peroxidative

**Fig. 12.** Proposed metabolic pathway for rat liver microsomal metabolism of 3',4'-dihydroxyflavanone.

Note that 3',4'-dihydroxyflavanone was a common metabolite for both compounds.

**Fig. 13.** Proposed metabolic pathways for rat liver microsomal metabolism of pinocembrin (A), and liquiritigenin and naringenin (B).

Pinocembrin is converted into naringenin (M16) via 4'-hydroxylation pathway. Liquiritigenin and naringenin share similar metabolic pathways, and both formed an unusual hydroxy quinone (quinol) metabolite which was further cleaved to produce the corresponding chromone.

**Fig. 14.** Proposed pathways for formation of a hydroxy quinone metabolite of naringenin (A) (adapted from Guengerich, 2001 and proposed pathway for formation of chromone (B).

Hydroxy quinone is proposed to be an intermediate for the B-ring cleavage pathway. Note that B-ring cleavage was observed for all of the 4'-hydroxylated flavanones tested in this study.
degradation of naringenin by horseradish peroxidase and proposed that this transformation occurred via an unidentified intermediate. Our data suggest that this intermediate is the hydroxy quinone product. A potential mechanism for this transformation might involve base-assisted removal of one of the alpha protons from the C-ring with the expulsion of hydroquinone phenolate as a good leaving group as shown in Fig. 14B. Since a C-C bond is cleaved, this reaction may be described as C-dearylation.

Another metabolic transformation of potential biological significance was desaturation of the flavano C-ring to form the corresponding flavones (Fig. 15). Formation of flavones was observed for B-ring unsubstituted and 4′-OH flavanone. These observations suggest that the reaction mechanism might involve hydrogen abstraction to form a radical at position 2 of the C-ring. A hydroxyl group in the para position would stabilize this radical but, in the meta position, would destabilize it through an inductive effect. Next, a second hydrogen abstraction would form the unsubstituted C-ring as shown in Fig. 15. If hydrogen abstraction began at carbon-3 instead, this alternate mechanism could not explain why desaturation was not observed for 3′-hydroxylated analog.

Since flavones are aromatic and planar, they exhibit biological activities different from those of the bulkier flavone precursors. For example, apigenin, which is a flavone analog of naringenin, is weaker phytoestrogen than naringenin (Miksicek, 1993), 4′-hydroxyflavone is ~20-fold more potent than 4′-hydroxyflavone as an inhibitor of aromatase (Ibrahim and Abdul-Hajj, 1990), and chrysin, a flavone analog of pinocembrin, is ~5-fold more potent at inducing quinone reductase (Fahey and Stephenson, 2002). Furthermore, flavones are typically more potent antioxidants than flavonanes (Foti et al., 1996). Therefore, desaturation of the C-ring to form aromatic flavones can alter potency or produce new pharmacological activity.

Reduction of the carbonyl group on the C-ring to the corresponding alcohol is a metabolic pathway that was observed for flavanone, 3′-OH flavonane, and, tentatively, for pinocembrin. Although no quantitative method was developed for these two flavonols under authentic standards, it is interesting to compare the relative stereospecificity of the reduction using the nominal peak area from the LC/MS profile. In the case of flavanone and 3′-OH flavonane, the reaction proceeded with some stereospecificity (67% ee for flavanone and 43% ee for 3′-OH flavonane). In contrast, chemical reduction by sodium borohydride proceeded with higher stereospecificity ranging from 85% ee for flavanone to 100% ee for 3′-OH flavonane. Even more interesting was the fact that the preferred stereoisomer for enzymatic reduction was opposite that obtained by chemical reduction. The mechanism for these differences in stereoselectivity will be the subject of future studies.

Flavonoids represent a large and diverse group of natural products in which minor differences in structure often have profound effects on biological activity. Therefore, it is important to understand the metabolic pathways of classes of flavonoids such as flavonanes, since metabolic transformation of these compounds can introduce new pharmacological activity. The results of this study provide a framework for further elucidation of metabolic pathways of this class of flavonoids in humans and underscore the importance of careful and thorough evaluation of metabolic pathways of natural products.

Conclusions

We report several new metabolic pathways for flavanones that are catalyzed by rat liver microsomes. These include formation of flavones, formation of quinol-type oxidation products, B-ring cleavage, and reduction of the C-ring carbonyl group. The formation of flavones was a relatively prominent route of metabolism, particularly for 4′-hydroxylated flavanones, but the other metabolic transformations represented relatively minor pathways. The biological significance of these pathways and the pharmacological or toxicological effects of these metabolites remain to be established.

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References


