Identification of new flavone-8-acetic acid metabolites using mouse microsomes and comparison with human microsomes

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d) **Abbreviations used:** FAA, flavone-8-acetic acid; 2'-OH-FAA, 2'-hydroxy-FAA; 3'-OH-FAA, 3'-hydroxy-FAA; 4'-OH-FAA, 4'-hydroxy-FAA; 3-OH-FAA, 3-hydroxy-FAA; 5-OH-FAA, 5-hydroxy-FAA; 6-OH-FAA, 6-hydroxy-FAA; 7-OH-FAA, 7-hydroxy-FAA; 3',4'-epoxy-FAA, 3',4'-dihydro-3',4'-epoxy-FAA; 3',4'-dihydrodiol-FAA, 3',4'-dihydro-3',4'-dihydroxy-FAA; 5,6-epoxy-FAA, 5,6-dihydro-5,6-epoxy-FAA; 5,6-dihydrodiol-FAA, 5,6-dihydro-5,6-dihydroxy-FAA; TFA, trifluoroacetic acid; CYP, cytochrome P450; NADPH, β-nicotinamide adenine dinucleotide 2'-phosphate reduced; RP-HPLC, reversed-phase high-performance liquid chromatography; MS, mass spectrometry; MS/MS, mass fragmentation pattern of molecular ion; Rt, retention time; UV, ultraviolet; amu, atomic mass unit(s).
Abstract

Flavone-8-acetic acid (FAA) is a potent anticancer agent in mouse but has not shown activity in humans. Because FAA metabolism could play a role in this interspecies difference, our aim was to identify the metabolites formed in vitro using mouse microsomes and compare to human microsomes. Mouse microsomes produced 6 metabolites as detected by reversed-phase high-performance liquid chromatography-mass spectrometry. Three metabolites were identified as the 3'-, 4'-, or 6-hydroxy-FAA, by comparison with retention times, UV and MS spectra of standards. Two metabolites presented a molecular weight of 296 (FAA=280) indicating the presence of one oxygen, but did not correspond to any monohydroxylated FAA derivative. These two metabolites were identified as epoxides because they were sensitive to epoxide hydrolase. The position of the oxygen was determined by the formation of the corresponding phenols under soft acidic conditions: one epoxide yielded the 3'- and 4'-hydroxy-FAA, thus corresponding to the 3',4'-epoxy-FAA, whereas the other epoxide yielded the 5- and 6-hydroxy-FAA, thus identifying the 5,6-epoxy-FAA. The last metabolite was assigned to the 3',4'-dihydrodiol-FAA because of its molecular weight (314) and sulfuric acid dehydration that indicated that the 3' and 4' positions were involved. Compared to mouse microsomes, human microsomes (2 pools and 15 individual microsomes) were unable to metabolize FAA to a significant extent. In conclusion, we have identified 6 new FAA metabolites formed by mouse microsomes, whereas human microsomes could not metabolize this flavonoid to a significant extent. The biological importance of the new metabolites identified herein remains to be evaluated.
Flavonoids represent one of the most important classes of plant metabolites that are widely distributed throughout the plant kingdom. The high interest toward this class of compounds is due to their rather large pharmacological activities that include anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, and antiviral properties (Middleton, Jr. et al., 2000). Flavonoids have also been reported to exert several anticancer activities that are linked to either cancer prevention and/or therapy (Lopez-Lazaro, 2002).

Among the flavonoids that have been tested in cancer therapy, flavone-8-acetic acid (FAA; 2-phenyl-8-(carboxymethyl)benzopyran-4-one; NSC-347512; LM975) (Figure 1) emerged as an interesting lead because it has demonstrated a potent \textit{in vivo} antitumor activity in mouse solid tumors (Bibby et al., 1988; Corbett et al., 1986; Plowman et al., 1986; Pratesi et al., 1988), and also in human tumors transplanted in nude mice (Giavazzi et al., 1988). However, contrary to its potent antitumor activity reported in mice, FAA has not shown anticancer activity in humans (de Forni et al., 1995; Kerr et al., 1989; Weiss et al., 1988). This interspecies difference in FAA anticancer activity has been hypothesized to be, at least partially, due to a possible metabolic activation of this flavonoid \textit{in vivo} in mice (Capolongo et al., 1987; Chabot et al., 1989).

The FAA mechanism of anticancer activity in mice is presently poorly understood. Because FAA is weakly cytotoxic \textit{in vitro} in murine and human cancer cell lines (Capolongo et al., 1987; Drewinko and Yang, 1986; Schroyens et al., 1987) which are otherwise responsive to FAA when these tumor cells are implanted in mice, several indirect mechanisms of anticancer action have therefore been suggested: augmentation of natural killer activity and induction of interferon (Hornung et al., 1988); antiangiogenic properties (Lindsay et al., 1996); tumor blood flow shut down and induction of extensive hemorrhagic necrosis (Bibby et al., 1989; Corbett et al., 1986; Evelhoeh et al., 1988; Hill et al., 1989; Zwi et al., 1989).
the molecular level, FAA has been reported to induce DNA damage in Glasgow osteogenic sarcoma after *in vivo* administration, which could indicate the *in vivo* formation of reactive metabolites in mice (Bissery *et al*., 1988).

In view of the poor cytotoxic activity of FAA *in vitro* and its potent anticancer activity *in vivo* in mice, the requirement for FAA metabolic activation has therefore been hypothesized (Capolongo *et al*., 1987; Chabot *et al*., 1989). The aims of the present study were therefore to a) identify the principal FAA phase 1 metabolites formed by mouse microsomes, and b) to compare the metabolic activity of mouse and human microsomes. We report here the identification of six new FAA metabolites formed by mouse microsomes. In addition we show that human microsomes do not metabolize FAA as efficiently as mouse microsomes. These data indicate a marked interspecies difference in FAA metabolism that could be involved in the differential activity of this molecule observed between the two species. The importance of the newly identified metabolites for the *in vivo* anticancer activity of FAA remains to be determined.
Materials and Methods

Chemicals. Flavone-8-acetic acid (FAA) and its 3-, 5-, 7-, 2’-, 3’-, or 4’-monohydroxylated derivatives (Figure 1) were kindly provided by Dr Jean-Jacques Berthelon (Merck-Lipha Santé, Lyon, France), and their synthesis has previously been reported (Briet et al., 1989). The abbreviations used for the compounds throughout this report and their code names are as follows: flavone-8-acetic acid (FAA, LM975, NSC347512); 2’-hydroxy-FAA (2’-OH-FAA, EMD107818, LM2795); 3’-hydroxyl-FAA (3’-OH-FAA, EMD107510, LM2728); 4’-hydroxy-FAA (4’-OH-FAA, EMD107433, LM2711; 3-hydroxy-FAA (3-OH-FAA, EMD106896, LM2594); 5-hydroxy-FAA (5-OH-FAA, LM2798); and, 7-hydroxy-FAA (7-OH-FAA, LM2771). Solvents and common chemicals were obtained from commercial sources and were of the highest purity available.

Synthesis of 6-hydroxyflavone-8-acetic acid (6-OH-FAA). The so far unknown 6-OH-FAA (Figure 1) was prepared according to our previously described methodology using appropriate starting materials (Dauzonne and Demerseman, 1990). The multistep synthesis of this compound is detailed in the Supplemental Data Section (Bauvois et al., 2003; Dauzonne et al., 1997).

Synthesis of FAA methyl ester. Esterification of FAA was accomplished as previously described (Chabot and Gouyette, 1991). Briefly, 400 mg of FAA dissolved in 95% ethanol was added to a solution of potassium hydroxide in water (5 g/8 ml). Diazomethane generated from p-toluenesulfonylmethyl nitrosamide (Diazald®, Aldrich Chemicals) was added as an ethereal solution (21.4 g/130 ml diethylether). FAA methyl ester molecular weight was 294 as determined by FAB mass spectrometry (glycerol matrix). The product was analytically pure based on HPLC.

Synthesis of methyl ester derivatives of FAA monohydroxylated standards. Three µl of concentrated sulfuric acid (96%) was added to 100 µl of a methanolic solution of either
FAA, 2'-OH-FAA, 3'-OH-FAA, or 4'-OH-FAA at 1.5 µg/ml. The methanolic solution was refluxed for 2 h, cooled, added with 200 µl of water, saturated with NaCl, and extracted with 500 µl of ethyl acetate under agitation for 20 min. The ethyl acetate phase was harvested, washed with 100 µl 5% NaHCO₃, decanted, dried over anhydrous MgSO₄, and evaporated under a nitrogen stream. The dry residue was reconstituted with 100 µl of mobile phase (28% methanol, 15% acetonitrile, and 57% aqueous 0.1% TFA, v:v:v), and 50 µl were injected onto the HPLC-UV-MS system.

**Human microsomes.** Two pools of human microsomes were used in this study, pool A from twenty-nine donors was purchased from BD Gentest (Le Pont-de-Claix, France, catalog No. 452161, Lot 24), and pool B composed of twenty donors was from Biopredic International (Rennes, France, batch No. MIC 259005). Individual human microsomes were derived from fifteen different donors and were purchased from Biopredic International (Rennes, France).

**Preparation of aroclor 1254-induced microsomes.** Aroclor-1254 induced microsomes were prepared as previously described (Breinholt *et al.*, 2002). Aroclor 1254 was used because this inducer is a potent inducer of several drug metabolizing enzymes including the major CYP families (Easterbrook *et al.*, 2001). In brief, 26 female C57Bl/6 mice, eight weeks of age were purchased from Janvier (Le Genest-St-Isle, France) and acclimated for a week in our animal facility and kept on a 12 h light/dark cycle with free access to food. Mice were injected intraperitoneally with aroclor-1254 (500 mg/kg body weight, dissolved in corn oil) on day 0. Five days later the mice were sacrificed by cervical dislocation after a 24 h fasting period. All animal experiments complied with the French regulations concerning the protection of animals used for experimental and other scientific purposes (D2001-486), and with the European Commission regulations (OJ of ECL358 12/18/1986). The hepatic microsomes were prepared as follows: the livers were excised and rinsed in ice-cold KCl.
1.15% solution and cut into small pieces with scissors; four volumes of 0.1 M phosphate buffer (pH 7.4) were added and the livers were homogenized using a Teflon potter at a speed of 50 rpm with 10 strokes; the liver homogenate was centrifuged at 15,000×g for 20 min, and after aspiration of the upper lipidic layer, the supernatant was ultracentrifuged at 100,000×g during 60 min, at 4°C; the pellets containing the microsomes were collected, covered with a phosphate buffer containing 20% glycerol, snap frozen on dry ice, and stored at -80°C until use. Total microsomal protein was determined using the Pierce BCA Protein Assay Reagent Kit (Rockford, IL, U.S.A.).

**Optimization of incubation conditions.** To optimize the incubation conditions for FAA metabolism, we first used a previously described protocol (Breinholt et al., 2002). However, the various incubation parameters had to be optimized with regard to: FAA and microsomal protein concentrations, NADPH alone or a NADPH regenerating system (BD Biosciences), incubation time, buffer type, shaking speed, opened or closed vials, and presence or absence of bovine serum albumin (BSA), vitamin C or MgCl₂. After a 1 h incubation time at 37°C the mixture (300 µl) was extracted twice with 800 µl ethyl acetate containing 2% acetic acid (v/v). The organic phase was dried under a gentle nitrogen steam, the dry residue was reconstituted with 300 µl of the HPLC mobile phase (described below), and 100 µl were injected onto the HPLC-UV system described thereafter. It was found that the optimal conditions yielding the maximum formation of metabolites were as follows: FAA concentration 8 µg/ml, microsomal protein 0.5 mg/ml, NADPH generating system corresponding to 1.3 mM, in 300 µl TRIS-HCl 0.1 M buffer, pH 7.4, containing 1.6 mg/ml BSA, and 5 mM MgCl₂. The incubation was carried out in opened 2 ml polypropylene tubes in a shaking water bath (150 oscillations/min) at 37°C. The reaction mixture was prewarmed at 37°C for 3 min before the NADPH generating system was added, and the incubation was extended for an additional 60 min.
**Epoxide hydrolase incubation.** The presence of possible FAA epoxides was assessed by using recombinant epoxide hydrolase from *Aspergillus niger* (Sigma Aldrich) at 0.5 mg protein/ml (0.85 U) in the optimal incubation conditions described above with aroclor-induced microsomes and FAA at 8 µg/ml. After the 1 h incubation time, the mixture was extracted with ethyl acetate, dried under nitrogen, reconstituted with 300 µl of the HPLC mobile phase (26% methanol, 19% acetonitrile, 55% aqueous acetic acid 2%, v:v:v) and 100 µl were injected onto the Shimadzu HPLC-UV system described below.

**Acidic treatment of potential epoxides.** To determine the exact position of the oxygen on the putative arene oxide, the metabolite was treated under soft acidic conditions, which are known to yield the corresponding monohydroxylated derivative(s) after aromatization, as previously described (Coombs *et al.*, 1981). In brief, the suspected epoxide metabolite was incubated with TFA (final concentration 0.5 M) at 37°C for 30 min, and the resulting products were analyzed by HPLC-UV-MS and compared to available monohydroxylated standards of FAA.

**Dehydration of potential dihydrodiol.** To determine the presence of a dihydrodiol, the suspected metabolite fraction was dehydrated as described (Coombs *et al.*, 1981). In brief, the dry residue corresponding to 25 microsomal incubations was dissolved in 100 µl of methanol, added with 100 µl of 2.5 M sulfuric acid and heated at 80°C for 2 h. After cooling, 200 µl of water were added, the mixture was saturated with NaCl, and extracted with 500 µl of ethyl acetate under continuous agitation for 20 min. The organic phase was washed with 100 µl of 5% NaHCO₃, decanted, and evaporated to dryness under a nitrogen stream. The dry residue was reconstituted in 70 µl of mobile phase (28% methanol, 15% acetonitrile, and 57% aqueous 0.1% TFA, v:v:v), and 50 µl was injected onto the HPLC-UV-MS system described below. In these dehydration conditions, two monohydroxylated compounds were afforded as methyl ester derivatives on the carboxylic acid function. For identification purposes, FAA and
its monohydroxylated derivatives on the 2'-, 3'-, or 4'-positions were also submitted to identical strong acidic conditions to yield their corresponding methyl ester derivatives.

**HPLC-UV and HPLC-UV-MS conditions.** Two HPLC systems were used during this study. The first HPLC-UV system was a Shimadzu CLASS-VP® (version 5.3), equipped with a C\textsubscript{18} column (Beckman Ultrasphere ODS, 5 µm; 4.6 × 25 mm) thermostated at 20°C, and a UV detector set at 300 nm. The mobile phase consisted of a solution of 26% methanol, 19% acetonitrile, 55% aqueous acetic acid (2%), v:v:v, at a flow rate of 1 ml/min. This HPLC system was used to collect the metabolic fractions. In order to compare the quantitative yield of metabolites from different microsomal preparations, we first determined the area under the curve of each new metabolite peak expressed in µV×sec. In our conditions, the limit of detection corresponding to 3 times the baseline noise, was calculated as a peak area ≥ 360 µV×sec.

To further characterize the collected metabolic fractions, a second HPLC system was used. This system was composed of a Surveyor® HPLC coupled with a UV diode array detector and a mass spectrometer (LCQ-Advantage®, ThermoElectron). The mass spectrometer consisted of an electrospray ionization system used in positive ion polarity mode and an ion trap mass analyzer. The operating parameters where as follows: the spray needle voltage was set at 4.0 kV and the spray was stabilized with a nitrogen sheath gas. Electrospray capillary voltage was 4.0 V, nitrogen was used as auxilliary gas, and the capillary temperature was 250°C. In order to obtain ion masses and to propose a plausible scheme of fragmentation for each compound studied, two scan modes were used. The MS full scan mode was used to obtain the parent ion masses, and the MS/MS scan mode in full scan was used to fragment the parent ions into their product ions. The fragmentation was accomplished by collision-induced dissociation with a collision energy between 30 and 40% of maximum energy, and helium was used as the buffer gas. A C\textsubscript{18} reversed-phase column (EC. 250/2 Nucleodur Sphinx RP, 5
µm, 250 mm × 2.1 mm, Macherey-Nagel) thermostated at 30°C was used, and the mobile phase was composed of 28% methanol, 15% acetonitrile, and 57% aqueous 0.1% TFA, v:v:v, at a flow rate of 0.2 ml/min. The initial mobile phase composition was maintained for 14 min, then changed to 35% methanol, 19% acetonitrile and 46% aqueous 0.1% TFA, during the next 2 min, and maintained in these conditions for an additional 5 min. The Xcalibur® software was used for data acquisition and analysis.

**Data presentation and Statistics.** Results are presented as the means ± SEM. The total metabolite areas (µV×sec) are arbitrary values, because each metabolite has a different UV extinction coefficient. To compare the metabolic activity of the various microsomal preparations, the sum of the metabolites areas was used and compared to the sum of metabolites of the uninduced mouse microsomes using the Dunnett's t test. Comparison between groups was considered significant if the p value was <0.05.
Results

A- FAA metabolism by mouse microsomes and identification of metabolites.

Control microsomal incubation with FAA in absence of a NADPH generating system did not yield any metabolite peak as shown in Figure 2-A, whereas several metabolites were formed in presence of NADPH with aroclor-induced mouse microsomes (Figure 2-B). Uninduced mouse microsomes yielded the same metabolite profile as the aroclor-induced ones, but with a lower yield of each metabolite as shown in Table 1. In Figure 2-B, the metabolite peaks were labeled according to their retention times (Rt), as follows: M1 (Rt 4.1 min), M2 (Rt 7.6 min), M3A (Rt 9.0 min), M3B (Rt 10.0 min), M3C (Rt 11.2 min), and M4 (Rt 12.3 min). The parent compound FAA eluted with a Rt of 17.1 min. All the metabolites eluted before FAA in this RP-HPLC system, thus indicating that they were more hydrophilic than the parent compound. To obtain a sufficient quantity of these metabolites for identification purposes, extractions corresponding to 25 incubations were pooled and each metabolite peak was collected using a first RP-HPLC system, before submission to a second RP-HPLC-UV-MS in order to determine the UV and MS spectra.

Flavone-8-acetic (FAA) UV and MS spectra. The parent compound FAA (Rt 17.1 min, Figure 2-B) presented a UV spectrum with a band I at 300 nm, a band II at 255 nm, and a shoulder at 235 nm (Figure 3-A). The MS spectrum presented in Figure 3-B showed a molecular ion $m/z$ 281 [M+H]$^+$ (i.e., MW=280), and a MS fragmentation pattern of this molecular ion showed several product ions. The $m/z$ 235 corresponded to a H$_2$CO$_2$ loss [M+H-H$_2$CO$_2$]$^+$. As presented below, the $m/z$ values of the following three ions were of particular importance for the further identification of FAA metabolites: the $m/z$ 179 $^{13}$A$^+$ was due to a retro Diels-Alder fragmentation pattern of ring C; the $m/z$ 161 and $m/z$ 133 were due to the loss of a water molecule [$^{13}$A$^+\text{-H}_2\text{O}$] or a formic acid molecule [$^{13}$A$^+\text{-H}_2\text{CO}_2$], respectively (Wu et al., 2004, and references therein). The main FAA fragmentation patterns
are presented in Figure 3-C. The MS analysis of all possible monohydroxylated FAA derivatives indicated that the three ions at \( m/z \) 179, 161 and 133 were indicative of monohydroxylation on the B or the C ring only (i.e., 2', 3', 4', and 3-OH-FAA). For the FAA derivatives hydroxylated on the A ring (i.e., 5-, 6-, and 7-OH-FAA), the ions at \( m/z \) 195, 177 and 149, (i.e., + 16 amu) were observed (data not shown).

**Identification of metabolite 4 (M4).** The metabolite M4 (Rt 12.3 min) was more hydrophilic than FAA (Figure 2-B), and co-eluted with our synthesized standard 6-OH-FAA. The UV spectrum of M4 was superimposable to the one of standard 6-OH-FAA, and both spectra presented a band I at 310 nm and a band II at 270 nm (Figures 4-A and 4-B). It is noteworthy that a bathochromic shift from 250 nm for FAA to 270 nm for 6-OH-FAA is suggestive of a substitution on the A ring (Mabry et al., 1970). M4 presented a molecular ion \( m/z \) 297 \([M+H]^+\) (Figure 4-C) indicating the presence of an oxygen atom (+16 amu) on the FAA molecule (FAA \( m/z \) 281 \([M+H]^+\)). The MS fragmentation pattern of the M4 molecular ion \( m/z \) 297 presented two product ions at \( m/z \) 251 and 149 (Figure 4-C). The standard 6-OH-FAA also presented a molecular ion at \( m/z \) 297 with a MS spectrum exhibiting product ions \( m/z \) 251 and 149 corresponding to \([M+H-H_2CO_2]^+\) and \([1,3A^+-H_2CO_2]\), respectively (Figure 4-D). As stated above, the ion \( m/z \) 149 is specifically observed with FAA derivatives hydroxylated on the A ring. Because of Rt, UV and MS spectra similar to those of standard 6-OH-FAA, metabolite M4 was therefore assigned to the structure of the 6-OH-FAA (Figure 4).

**Identification of metabolite 3A (M3A).** Metabolite M3A (Figure 2-B) was identified as the 4'-OH-FAA by comparison with an authentic standard. M3A and 4'-OH-FAA exhibited identical Rt (data not shown), similar UV spectrum (Figures 5-A and 5-B), and matching MS fragmentation pattern (Figures 5-C and 5-D).
Identification of metabolite 3B (M3B). The M3B peak was also identified by comparison with the authentic standard 3'-OH-FAA by comparing Rt (data not shown), UV spectra (Figures 6-A and 6-B), and MS fragmentation pattern (Figures 6-C and 6-D).

Identification of metabolite 3C (M3C). Metabolite M3C eluted with a Rt of 11.2 min (Figure 2-B). This metabolite had a UV spectrum with two maxima at 315 nm (band I) and 250 nm (band II) (Figure 7-A). The position of bands I and II was similar to the ones observed above for FAA derivatives hydroxylated on the B-ring which could suggest a modification on the same ring. The MS analysis of M3C yielded a molecular ion m/z 297 [M+H]+ indicating the presence of an oxygen atom on the FAA molecule (i.e., +16 amu). In addition, the MS fragmentation pattern of the M3C molecular ion yielded three product ions at m/z 179, 161 and 133, indicating that the oxygen atom was not on the A ring (Figure 7-B).

Because this mono-oxygenated metabolite did not co-elute with any of the monohydroxylated standards of FAA, an epoxidation of FAA was therefore hypothesized, probably on the B ring, as suggested by the UV spectrum and the MS fragmentation pattern. To verify the presence of an epoxide, this metabolite was then submitted to recombinant epoxide hydrolase from *Aspergillus niger*. As shown in Figure 8, this enzyme caused the almost complete disappearance of the M3C peak (Figure 8). As a matter of fact, the M3C peak area was reduced by 96% in presence of the enzyme within a 1 h incubation period (compare the M3C peak on Figures 8-A and 8-B). In order to determine the exact position of the epoxidic oxygen, we next submitted the metabolite M3C to soft acidic conditions (TFA), because arene oxide exposure to such conditions are known to yield the corresponding phenolic compounds by protonation (Coombs *et al.*, 1981). In those conditions, M3C was readily transformed into two hydroxylated derivatives of FAA that were identified as the 3'-OH-FAA (23%) and the 4'-OH-FAA (77%), as determined by coelution with authentic standards, and by comparing their UV and MS spectra (data not shown). Based on M3C
metabolite UV and MS data, its aromatization to 3'-OH-FAA and 4'-OH-FAA under soft acidic conditions, and its sensitivity to epoxide hydrolase, this metabolite was therefore assigned to the structure of the 3',4'-epoxy-FAA (Figure 7-C).

**Identification of metabolite 2 (M2).** The M2 metabolite (Rt 7.6 min) did not co-elute with any possible FAA monohydroxylated derivatives (Figure 2-B). The UV spectrum of this metabolite presented two poorly resolved bands at 295 nm (band I) and 270 nm (band II, shoulder at 235 nm) (Figure 9-A). The band II was identical to the one observed above with the A ring substituted 6-OH-FAA (cf. Figure 4-B), and also with authentic standard 5-OH-FAA (235, 270-II and 340-I nm, not shown), thus suggesting that M2 was probably resulting from an A ring modification of FAA. The MS analysis of M2 yielded a molecular ion \( m/z \) 297 \([\text{M+H}]^+\), indicating the addition of an oxygen atom (+16 amu) on the FAA molecule (Figure 9-B). The MS spectrum of the \( m/z \) 297 ion presented 3 product ions, of which \( m/z \) 149 and 177 are typical of the fragmentation pattern of A ring-substituted compounds, as observed with 6-OH-FAA (cf. Figure 4-D). Because M2 was an oxygenated derivative of FAA and since it did not co-elute with any A ring monohydroxylated standard of FAA (i.e., 5-, 6-, or 7-OH-FAA), these results were therefore suggestive of an epoxidation on the A ring of FAA.

We next submitted M2 to hydrolysis by *Aspergillus niger* recombinant epoxide hydrolase, and it was found that the M2 peak area was decreased by 55% within a 1 h incubation time (Figures 8-A and 8-B), thus indicating that it was an FAA epoxide. To further determine the exact position of the epoxidic oxygen on M2, we submitted this metabolite to soft acidic conditions (TFA) in order to protonate the epoxide and to induce aromatization into the corresponding phenolic compounds (Coombs *et al.*, 1981). Under such acidic conditions, metabolite M2 yielded two compounds which presented identical Rt to the standards 5-OH-FAA (16%) and 6-OH-FAA (84%) (data not shown). In addition, the UV and MS spectra of the M2 products after acidification were superimposable to the ones obtained
with standards of 5-OH-FAA (data not shown) and 6-OH-FAA (cf. Figures 4-B and 4-D).
Based on its UV and MS spectra, its acidic aromatization to 5-OH-FAA and 6-OH-FAA, and
its sensitivity to epoxide hydrolase, M2 was therefore assigned to the structure of the 5,6-
epoxy-FAA (Figure 9-C).

Identification of metabolite 1 (M1). This metabolite was the most hydrophilic and
eluted with a short Rt of 4.1 min (Figure 2-B). The UV spectrum of M1 exhibited a band I at
315 nm and a more intense band II at 245 nm suggesting a B-ring modification of FAA
(Figure 10-A). M1 did not match any B-ring monohydroxylated standards of FAA (i.e., 2’-, 3’-
, or 4’-OH-FAA) in terms of Rt, UV and MS spectra. The MS analysis of M1 presented a
molecular ion \( m/z \) 315 \([\text{M+H}]^+\), i.e., a gain of 34 amu over the parent compound FAA which
was suggestive of the addition of two hydrogen atoms and two oxygen atoms. The
fragmentation pattern of the molecular ion \( m/z \) 315 yielded six product ions (Figure 10-B).
The first three ions at \( m/z \) 297, 279, and 251, corresponded to the loss of one water molecule
\([\text{M+H-H}_2\text{O}]^+\), two water molecules \([\text{M+H-2H}_2\text{O}]^+\), and the loss of a water molecule plus a
formic acid molecule \([\text{M+H-H}_2\text{O-H}_2\text{CO}_2]^+\), respectively. Loss of two water molecules (-36
amu) substantiated the hypothesis that M1 was a dihydrodiol. For the ions smaller than \( m/z \)
251, i.e., \( m/z \) 179, 161, and 133, we found the typical fragmentation pattern indicating that the
A ring was not modified, as suspected above by the UV spectrum bands. Therefore, because
of its UV and MS spectra, metabolite M1 was most likely a dihydrodiol of FAA on the B ring.

In order to determine the exact position of the two hydroxyl groups on the B ring, we
next submitted M1 to sulfuric acid dehydration in methanolic solution (Coombs et al., 1981).
Because it was found that these dehydration conditions also allowed the formation of the
methyl ester of the carboxylic function of FAA in the presence of methanol, we therefore
compared the results of M1 dehydration with the methyl esters of the B ring
monohydroxylated FAA derivatives (i.e., methyl esters of 2’-, 3’-, and 4’-OH-FAA). In these
conditions, it was observed that the dehydration of M1 yielded 2 compounds with Rt, UV and MS spectra identical to the methyl ester derivatives of 3'- and 4'-OH-FAA. Based on these results, metabolite M1 was therefore assigned to the structure of the 3',4'-dihydrodiol-FAA (Figure 10-C).

B- FAA metabolism by human microsomes.

After the identification of the principal FAA metabolites formed with mouse microsomes, we were interested to investigate the metabolism of FAA with human microsomes. To do so, we tested two different pools of human microsomes and also 15 microsomal preparations from individual donors. For comparison purposes, the average metabolite peak areas formed by uninduced and aroclor-induced mouse microsomes are also presented in Table 1. Concerning the human microsomes, one pool composed of 29 donors (pool A) could not metabolize FAA (Table 1), whereas another pool of 20 donors (pool B) yielded only a small metabolite peak corresponding to the Rt of the 5,6-epoxy-FAA (Table 1). For comparison purposes, it was noteworthy that uninduced mouse microsomes formed more metabolites than either pools of human microsomes with a total metabolites area of 22698 µV×sec, whereas pool A did not yield any detectable metabolite, and pool B yielded total areas of only 1154, which were 20-fold lower than uninduced mouse microsomes (Table 1).

Because only one metabolite of FAA was barely detected using the above human microsomal pools, we were next interested to test human microsomes from individual donors to assess inter-individual variation. As presented in Table 1, six out of 15 individual preparations did not yield any detectable metabolite peak (samples 1-6). Seven microsomal preparations (samples 7-13) could produce a small peak corresponding to the Rt of the 5,6-epoxy-FAA. Only one human microsome could yield a small peak corresponding to the Rt of the 4'-OH-FAA (sample 14). Finally, only one microsomal preparation (sample 15) could
clearly produce 3 metabolites corresponding to the Rt of the 3',4'-dihydrodiol-FAA, the 4'-OH-FAA, and the 3',4'-epoxy-FAA.

With regard to the quantitative analysis of these results, it can be noted that the individual human microsomes that did produce some metabolite(s) were doing so in very small yield (Table 1) compared with uninduced mouse microsomes. In the same experimental conditions, uninduced mouse microsomes yielded a total area of metabolite production that was 12- to 32-fold higher than human microsomes, with the exception of one microsomal preparation (sample 15) that could produce a meaningful metabolite yield that was however 3-fold lower than the uninduced mouse microsomes.
Discussion

Although FAA metabolism has been suggested to possibly play a role in its antitumor activity in mice (Capolongo et al., 1987; Chabot et al., 1989), the FAA metabolic pathways in this species have not yet been elucidated. The only mouse metabolite identified so far is the acyl glucuronide of FAA (Chabot and Gouyette, 1991; Cummings et al., 1989), a metabolite that has also been identified in humans, but has not shown cytotoxic activity in vitro (Cummings et al., 1989). In the present study, we were interested to elucidate the principal phase 1 metabolic pathways of FAA using mouse microsomes. Because the metabolic profile obtained with uninduced mouse microsomes was identical to the one obtained with aroclor-induced microsomes, we therefore used the latter preparation to obtain a sufficient quantity of metabolites to allow their identification. Aroclor 1254 is a known broad inducer of several different CYPs including the subfamilies CYP1A, CYP2B, and CYP3A, and can increase their metabolic capacity toward planar compounds like the polycyclic aromatic hydrocarbons and flavones (Easterbrook et al., 2001; Nielsen et al., 1998). In this study, we have shown that FAA can indeed be metabolized in vitro using aroclor-induced mouse microsomes into 6 unpreviously described metabolites. In addition, we have shown that FAA metabolism is qualitatively and quantitatively more important in murine microsomes compared to human microsomes.

The FAA proposed phase 1 metabolic pathways in mouse microsomes is depicted in Figure 11. Of the 6 FAA metabolites identified, 4 metabolites involved the B ring, and 2 metabolites involved the A ring. The B ring metabolites included the 4'-OH-FAA (M3A), the 3'-OH-FAA (M3B), the 3',4'-epoxy-FAA (M3C), and the 3',4'-dihydrodiol-FAA (M1) (Figure 11). The B ring mono-hydroxylation at the 3' or 4' position has also been observed with other flavones (Nielsen et al., 1998). It is however interesting that beside the 3',4'-dihydrodiol-FAA possessing a saturated 3',4' bond, we did not observe the formation of a 3',4'-dihydroxylation
on the intact B ring (catechol), as has been observed with some B-ring unsubstituted flavones (Nielsen et al., 1998; Nikolic and Van Breemen, 2004).

The detection and identification of two epoxides, one on the B ring (3',4'-epoxy-FAA, M3C) and another one on the A ring of FAA (5,6-epoxy-FAA, M2) was rather surprising, given the fact that such metabolites are often unstable and readily undergo chemical degradation during the incubation period and/or in the process of extraction. In fact, these epoxides were relatively stable because they could not only sustain a 1 h incubation time at physiological conditions (pH 7.4, 37°C), but they could also resist to the extraction procedures at room temperature, both conditions not considered favorable for epoxide stability. To our knowledge, such stable epoxides have rarely been described for other flavones, perhaps with the exception of the 5,6-epoxy-α-naphthoflavone which is also chemically stable, apparently due to the adjacent C-4 carbonyl group (Vyas et al., 1983).

Although the sensitivity of the two epoxides to epoxide hydrolase can be considered as a rather convincing proof of the epoxidic character of these metabolites (Guengerich, 2003), a significant difference in their respective sensitivity to this enzyme was noted, as the 3',4'-epoxy-FAA was a better substrate compared to the 5,6-epoxy-FAA. It is noteworthy that another flavone epoxide at the same 5,6-position, i.e., the 5,6-epoxy-α-napthoflavone, has also been found to be quite resistant to epoxide hydrolase, probably due to the proximate carbonyl group at the C-4 position of the molecule which could cause steric hindrance of the epoxidic function to the enzyme catalytic site (Vyas et al., 1983).

Another intriguing observation about the two FAA epoxides is that we did not observe the formation of their corresponding dihydrodiols after treatment with Aspergillus niger epoxide hydrolase, under conditions where the epoxides were obviously hydrolyzed. This could be due to the fact that epoxide hydrolase converts their substrates via the intermediate formation of a covalent enzyme-substrate complex, and it has been shown that the formation
of the intermediate can proceed much faster than the subsequent hydrolysis, i.e., the formation of the terminal dihydrodiol products. Thus, the enzyme would act like a molecular sponge by binding and inactivating the epoxide very fast, whereas the subsequent release of the dihydrodiol product is considerably slower (Oesch et al., 2001). However, it should be noted that the observed formation of the 3’,4’-dihydrodiol-FAA (M1) during the incubation with mouse microsomes indicates that this preparation contained mouse epoxide hydrolase that could release the 3’,4’-dihydrodiol-FAA. In contrast, we did not detect 5,6-dihydrodiol-FAA probably because the mouse epoxide hydrolase could not hydrolyze 5,6-epoxy-FAA, or could not release the product of the reaction.

Although metabolism frequently results in the formation of more hydrophilic metabolites usually possessing a lower biological activity compared to the parent compound, there are several examples where metabolism can lead to the formation of more active compounds (Breinholt et al., 2002). The FAA phase 1 metabolites identified in this study were all more hydrophilic than the parent compound and are therefore expected to be more easily excreted. However, concerning the potential biological activity of the identified FAA metabolites, it is difficult to predict which one(s) could be involved (if any) in the in vivo activity of this compound. In the flavonoid family, it is often observed that minor differences in structure can profoundly influence their biological activity (Nikolic and Van Breemen, 2004). It is also of interest that several flavonoid metabolites have been reported to exert antiproliferative activity in vitro (Meng et al., 2006), thereby underlining the importance of the identification of these metabolites. Concerning the two relatively stable epoxides identified in this study, it would be possible that these compounds could be involved in the antitumor activity, considering that DNA strand breaks were observed in tumors after administration of FAA in mice (Bissery et al., 1988). Indeed, it has been reported that
epoxidic compounds can cause DNA damages through covalent binding (Xue and Warshawsky, 2005, and references therein).

In addition to substantial difference in FAA phase 1 metabolism between mice and humans, shown in this study, differences in phase 2 metabolism is also possible. FAA metabolism has already been looked at in humans and in mice, and the only phase 2 metabolite identified so far in both species is the acyl glucuronide of FAA (Cummings et al., 1989; Chabot and Gouyette, 1991). The six new FAA phase 1 metabolites identified in this study will undoubtedly facilitate the identification of other phase 2 metabolites potentially formed in mice. The identification of these metabolites is of importance, because they could be involved in the remarkable anticancer activity observed in mice and perhaps explain the lack of activity in humans.

In conclusion, we have identified 6 new FAA metabolites using mouse microsomes, the species in which this compound was shown to be a potent anticancer agent. We also have shown that human microsomes could not metabolize FAA as efficiently as mouse microsomes. This marked interspecies difference in metabolism could be involved, as least in part, in the differential anticancer activity observed between the two species. The implication of the these new metabolites, either unconjugated or conjugated (Williamson et al., 2005), in the FAA anticancer activity remains to be investigated.
References


Schroyens WA, Dodion PF, Sanders C, Loos M, Dethier NE, Delforge AR, Stryckmans PA, Kenis Y (1987) In vitro chemosensitivity testing of flavone acetic acid (LM975; NSC


Footnotes

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1 These authors are considered as first author because they collaborated equally to this work.
Legends for Figures

Figure 1. Structure of flavone-8-acetic acid (FAA; NSC-347512; LM975) and its monohydroxylated derivatives.

Figure 2. Representative RP-HPLC chromatogram of flavone-8-acetic metabolites (FAA) formed in vitro using mouse microsomes. The incubation mixture contained FAA at 8 µg/ml, aroclor-induced mouse microsomes (0.5 mg/ml), and was incubated for 1 h without NADPH (panel A), or with a NADPH generating system (panel B). Ethyl acetate extracts were dried under a nitrogen stream, reconstituted in the mobile phase and 100 µl were injected onto a RP-HPLC system with UV detection set at 300 nm, as detailed in the Materials and Methods section.

Figure 3. Flavone-8-acetic acid (FAA) UV, MS spectra and fragmentation pattern. A, FAA UV spectrum; B, MS/MS spectrum of parent ion m/z 281; C, main fragmentation pattern showing the retro Diels-Alder (rD-A) fragmentation of ring C. FAA was injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.

Figure 4. UV and MS spectra of metabolite M4 and standard 6-OH-FAA. A, UV spectrum of metabolite M4; B, UV spectrum of 6-OH-FAA; C, MS/MS spectrum of M4 parent ion m/z 297; and D, MS/MS spectrum of 6-OH-FAA parent ion m/z 297. The compounds were injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.

Figure 5. UV and MS spectra of metabolite M3A and standard 4’-OH-FAA. A, UV spectrum of M3A; B, UV spectrum of 4’-OH-FAA; C, MS/MS of M3A parent ion m/z 297; and D, MS/MS of 4’-OH-FAA parent ion m/z 297. The compounds were injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.
Figure 6. UV and MS spectra of metabolite M3B and standard 3’-OH-FAA. A, UV spectrum of M3B; B, UV spectrum of 3’-OH-FAA; C, MS/MS of M3B parent ion m/z 297; and D, MS/MS of 3’-OH-FAA parent ion m/z 297. The compounds were injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.

Figure 7. UV and MS spectra of metabolite M3C and proposed structure. A, UV spectrum of M3C; B, MS/MS of M3C parent ion m/z 297; C, proposed chemical structure of M3C assigned to the 3’,4’-epoxy-FAA. The compounds were injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.

Figure 8. Sensitivity of FAA metabolites to epoxide hydrolase. The incubation mixture contained FAA at 8 µg/ml, aroclor-induced mouse microsomes (0.5 mg/ml), a NADPH generating system, and was incubated for 1 h without (panel A), or with recombinant epoxide hydrolase from Aspergillus niger (panel B). Ethyl acetate extracts were dried under a nitrogen stream, reconstituted in the mobile phase and 100 µl were injected onto a RP-HPLC system with UV detection set at 300 nm.

Figure 9. UV and MS spectra of metabolite M2 and proposed structure. A, UV spectrum of M2; B, MS/MS of M2 parent ion m/z 297; C, proposed chemical structure of metabolite M2 assigned to the 5,6-epoxy-FAA. The compounds were injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.

Figure 10. UV and MS spectra of metabolite M1 and proposed structure. A, UV spectrum of M1; B, MS/MS of M1 parent ion m/z 315; C, proposed chemical structure of M1 assigned to the 3’,4’-dihydrodiol-FAA. The compounds were injected onto a RP-HPLC-UV-MS system, as detailed in the Materials and Methods section.

Figure 11. Flavone-8-acetic acid (FAA) proposed metabolic pathways obtained with aroclor-induced mouse microsomes. The abbreviations used are: Cyp, mouse cytochrome P450; EH, epoxide hydrolase; FAA, flavone-8-acetic acid; 4’-OH-FAA, 4’-
hydroxy-FAA; 3’-OH-FAA, 3’-hydroxy-FAA; 3’,4’-epoxy-FAA, 3’,4’-dihydro-3’,4’-epoxy-FAA; 3’,4’-dihydrodiol-FAA, 3’,4’-dihydro-3’,4’-dihydroxy-FAA; 6-OH-FAA, 6-hydroxy-FAA; 5,6-epoxy-FAA, 5,6-dihydro-5,6-epoxy-FAA; 5,6-dihydrodiol-FAA, 5,6-dihydro-5,6-dihydroxy-FAA. The 5,6-dihydrodiol-FAA is shown in brackets because it was not detected in our experimental conditions. The solid arrows indicate a direct production of a given metabolite, whereas the dashed arrows indicate a putative pathway.
Table 1. Comparison of in vitro FAA metabolism by different mouse or human microsomal preparations.

<table>
<thead>
<tr>
<th>Microsomal preparations</th>
<th>3’,4’-dihydrodiol-FAA</th>
<th>5,6-epoxy-FAA</th>
<th>4’-OH-FAA</th>
<th>3’-OH-FAA</th>
<th>3’,4’-epoxy-FAA</th>
<th>6-OH-FAA</th>
<th>Total metabolite area b</th>
</tr>
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<tr>
<td><strong>Mouse microsomes</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Uninduced control</td>
<td>719 ± 201</td>
<td>4195 ± 336</td>
<td>12832 ± 2643</td>
<td>856 ± 36</td>
<td>1137 ± 242</td>
<td>2961 ± 761</td>
<td>22698 ± 2293</td>
</tr>
<tr>
<td></td>
<td>0.02 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>0.35 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.09 ± 0.03</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>Aroclor-induced</td>
<td>24571 ± 2268</td>
<td>58174 ± 3348</td>
<td>11356 ± 1458</td>
<td>2650 ± 718</td>
<td>37992 ± 4168</td>
<td>26256 ± 2411</td>
<td>160998 ± 9863*</td>
</tr>
<tr>
<td></td>
<td>0.96 ± 0.22</td>
<td>2.18 ± 0.35</td>
<td>0.43 ± 0.10</td>
<td>0.10 ± 0.02</td>
<td>1.34 ± 0.10</td>
<td>1.03 ± 0.24</td>
<td>6.04 ± 0.98</td>
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<td><strong>Pooled human microsomes</strong></td>
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</tr>
<tr>
<td>Pool A (Gentest)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pool B (Biopredic)</td>
<td>-</td>
<td>1154 ± 209</td>
<td>0.04 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1154 ± 209*</td>
</tr>
<tr>
<td><strong>Individual human microsomes</strong></td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>7 (003HLA) f</td>
<td>-</td>
<td>872 ± 275</td>
<td>0.02 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>872 ± 275*</td>
</tr>
<tr>
<td>8 (MIC 25007)</td>
<td>-</td>
<td>904 ± 44</td>
<td>0.03 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>904 ± 44*</td>
</tr>
<tr>
<td>9 (216 B)</td>
<td>-</td>
<td>953 ± 117</td>
<td>0.03 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>953 ± 17*</td>
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<tr>
<td>10 (MIC 259025)</td>
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<td>1136 ± 99</td>
<td>0.04 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1136 ± 99*</td>
</tr>
<tr>
<td>11 (010 HLA)</td>
<td>-</td>
<td>1471 ± 386</td>
<td>0.04 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1471 ± 386*</td>
</tr>
<tr>
<td>12 (MIC 259009)</td>
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<td>1895 ± 340</td>
<td>0.06 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1895 ± 340*</td>
</tr>
<tr>
<td>13 (MIC 259013)</td>
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<td>903 ± 201</td>
<td>0.03 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>903 ± 201*</td>
</tr>
<tr>
<td>14 (MIC 259006)</td>
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<td>710 ± 155</td>
<td>0.02 ± 0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>710 ± 155*</td>
</tr>
<tr>
<td>15 (MIC 259008)</td>
<td>2555 ± 164</td>
<td>2673 ± 10</td>
<td>0.08 ± 0.01</td>
<td>-</td>
<td>2282 ± 308</td>
<td>0.07 ± 0.01</td>
<td>7510 ± 448</td>
</tr>
</tbody>
</table>

Notes:
- Microsomal incubations were carried out as described in the Materials and Methods section.
- Total metabolite area (µV×sec) refers to the sum of the areas of new metabolite peaks, as determined by UV detection at 300 nm as described in Materials and Methods. These data are arbitrary values because each metabolite has a different UV extinction coefficient.
- Metabolite area (µV×sec), mean ± SEM of 3 to 6 determinations.
- Percent conversion of total peak area including all metabolites and FAA.
- Human microsomal preparations 1 to 6 refer to the following Gentest code numbers: MIC 259011; MIC 259017; MIC 259016; MIC 259014; MIC 259010; 012 HLA.
- The code number in parentheses refers to the Gentest preparation number. *p < 0.05, Dunnett’s t test vs uninduced mouse microsomes.
Flavone-8-acetic acid (FAA) and its hydroxylated derivatives

<table>
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<tr>
<th>Compound</th>
<th>R²</th>
<th>R³</th>
<th>R⁴'</th>
<th>R³'</th>
<th>R⁵</th>
<th>R⁶</th>
<th>R⁷</th>
</tr>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>2'-OH FAA</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
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<tr>
<td>3'-OH FFA</td>
<td>H</td>
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</tr>
<tr>
<td>4'-OH FAA</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
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<tr>
<td>3-OH FAA</td>
<td>H</td>
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<td>H</td>
<td>OH</td>
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<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>6-OH FAA</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>7-OH FAA</td>
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<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>

Figure 1
Figure 2

- NADPH

+ NADPH

UV Detector Response at 300 nm (mV)

Time (min)

A

B

Figure 2
Figure 3

(A) FAA

- 235 (I)
- 255 (II)
- 300

(B) FAA

- $[M+H-H_2CO_2]^+$
- $[M+H]^+$

(C) FAA

- $1,3A^+\cdot H_2O$
- $1,3A^+\cdot H_2CO_2$
- $1,3A^+$
- $1,3A^+\cdot H_2O$
- $1,3A^-\cdot H_2CO_2$

Figure 3
Figure 4
Figure 5

A) Spectra of M3A

B) Spectra of 4'-OH-FAA

C) Mass spectra of M3A

D) Mass spectra of 4'-OH-FAA

Legend:
- [M+H]⁺: Molecular ion
- [M+H-H₂CO₂]⁺: Molecular ion minus one CO₂
- [M+H-H₂O]⁺: Molecular ion minus one H₂O
- 1,3A⁺: Unlabeled

Wavelength (nm)

Relative intensity

m/z
Figure 6
Figure 7

(A) M3C

(B) M3C

Relative intensity

Wavelength (nm)

m/z

220 250 (II)

315 (I)

133

161

251

279

1,3A+-H2CO2

1,3A+-H2O

[M+H+H2CO2]+

[M+H-H2O]+

297

[M+H]+

0 10 20 30 40 50 60 70 80 90 100

0 10 20 30 40 50 60 70 80 90 100

M3C
Figure 8
Figure 9
Figure 10
Figure 11