The metabolism of diallyl disulfide (DADS), a garlic sulfur compound, was investigated in human liver microsomes. Diallyl thiosulfinate (allicin) was the only metabolite observed and its formation followed Michaelis-Menten kinetics with a $K_m = 0.61 \pm 0.2 \text{mM}$ and a $V_{max} = 18.5 \pm 4.2 \text{nmol/min/mg protein}$, respectively (mean $\pm$ S.E.M., $n = 4$). Both flavin-containing monooxygenase and the cytochrome P-450 monooxygenases (CYP) were involved in DADS oxidation, but the contribution of CYP was predominant. The cytochrome P-450 isoforms involved in this metabolism were investigated using selective chemical inhibitors, microsomes from cells expressing recombinant CYP isoenzymes, and studying the correlation of the rate of DADS oxidation with specific monooxygenase activities of human liver microsomes. Diethylthiocarbamate and tranylcypromine inhibited allicin formation, whereas other specific inhibitors had low or no effect. Most of the different human microsomes from cells expressing CYP were able to catalyze this reaction, but CYP2E1 showed the highest affinity with a substantial activity. Furthermore, allicin formation by human liver microsomes was correlated with $p$-nitrophenol hydroxylase activity, a marker of CYP2E1, and tolbutamide hydroxylase activity, a marker of CYP2C9. Among these approaches only CYP2E1 was identified in each case, which suggested that DADS is preferentially metabolized to allicin by CYP2E1 in human liver. However the minor participation of other CYP forms and flavin-containing monooxygenases is likely.

The chemistry of the sulfur-containing compounds of garlic (Allium sativum) is complex. Fresh garlic contains allicin ($S$-allylcysteine sulfoxide) from which many molecules are formed when a garlic clove is crushed. Among the transformation products, ajoene, sulfides, and allicin (diallyl thiosulfinate or DADSO; Fig. 1) were noticed for their medicinal properties such as antimicrobial, antifungal, antitumoral, antithrombotic, hypotensive, hypoglycemic, and hypolipemic properties (see Lin, 1989, for review).

Diallyl disulfide (DADS) is one of the major volatile degradative compounds of garlic formed from DADSO (Block, 1992; Augusti, 1996). It was found in the breath of human subjects who ate dry or fresh garlic (Cai et al., 1995). Many studies on animals showed its protective effects against chemically induced toxicity and against carcinogenesis (Ip et al., 1992; Reddy et al., 1993). The modulation of the metabolism of carcinogens by DADS was considered one of the possible mechanisms of its protective effect against the occurrence of cancer (Reddy et al., 1993). Previous studies in our laboratory demonstrated that the administration of DADS to rats modified the quantity and the activity of several hepatic drug metabolism enzymes (Haber et al., 1994; 1995). DADS produced an enhancement of the microsomal level of cytochrome P-450 monooxygenase (CYP2B1/2 and of the activities of UDP glucuronyltransferases and of glutathione $S$-transferases. It decreased the nitrosodimethylamine demethylase activity and the level of CYP2E1. In addition, there are many reports about the chemical analysis and biological activities of sulfur-containing compounds of garlic. However, few studies have dealt with the metabolism and pharmacokinetics of garlic constituents. One study performed in mice with radioactive DADS indicated that the uptake of radioactivity was highest in the liver at 90 min after i.p. injection (Pushpendran et al., 1980). In metabolic studies using perfused rat liver, DADS appeared to be converted to allyl mercaptan (Egen et al., 1992). This information and previous results concerning the ability of DADS to modulate drug-metabolizing enzymes led us to hypothesize that DADS could be metabolized by cytochrome P-450 enzymes in the liver.

The flavin-containing monooxygenases (FMOs) play a role in xenobiotic and drug metabolism. These enzymes have been characterized in several mammalian species, including human. Among the identified isoenzymes, FMO3 is the predominant form in adult human liver. The FMOs are able to metabolize a wide variety of xenobiotics including thiols, sulfides, and disulfides but the involvement of such enzymes in the metabolism of garlic compounds has not been reported.

In this study we investigated the in vitro metabolism of DADS in presence of human liver microsomes. We identified one metabolite formed and determined the kinetic parameters of the reaction. The contribution of both CYP and FMO were shown. Then we identified...
the CYP isoenzymes mainly involved in the reaction using several
approaches: 1) effect of selective chemical inhibitors on DADS ox-
idation, 2) determination of DADS oxidation by microsomes from
cells expressing recombinant CYP isoenzymes, and 3) study of the
correlation of DADS oxidation with marker activities of CYP isoen-
zymes in different human liver microsomes.

Experimental Procedures

Materials. DADS (purity of 80%) was obtained from Aldrich Chemical Co.
(Strasbourg, France). The other 20% were identified by HPLC as diallyl
sulfide and diallyl trisulfide. DADS was used without purification for the
study of individual human CYPs or baculovirus-infected insect cells ex-
pressing individual human FMOs. DADSO was donated by Professor Auger, Institut de
Recherche sur la Biologie de l’Insecte, University F. Rabelais of Tours
(France). It was synthesized from the corresponding disulfide according to
the method of Ferary (1996). 5,5-Dithiobis(2-nitrobenzoic acid), α-naphthofla-
vine, chlorozoxazone, coumarin, dextromethorphan, diethyldithiocarbamate,
NADPH, nifedipine, orphenadrine, quinidine, sulfaphenazole, tolbutamide,
tranylcypromine, and troleandomycin were purchased from Sigma-Aldrich
Chemie (Saint Quentin Fallavier, France). Dextrophan and 5-(-)-mepheny-
thon were purchased from Ultrafine Chemicals (Salford, England). 1-Amino-
benzotriazole was donated by Dr. Cabanne, Laboratoire de Phytopharmacologie,
Institut National de la Recherche Agronomique (Dijon, France). [1-14C]Lauric
acid and [14C] 5-(-)-mephenyton were purchased from Amersham Pharmacia
Biotech (Les Ulis, France). Microsomes from B-lymphoblastoid cell lines
expressing individual human CYPs or baculovirus-infected insect cells ex-
pressing individual human FMOs were purchased from Gentest (Woburn,
MA). All other chemicals and reagents used were of the highest commercial
quality available.

Human Liver Samples. Human liver samples were provided by Professor
Favre, Département de Chirurgie Digestive Thoracique et Cancérologique of
the General Hospital of Dijon, France. The protocol was approved by a local
ethics committee. The used tissues came from patients undergoing liver resec-
tion for various clinical reasons. The samples were frozen in liquid nitrogen
and stored at −80°C until use for microsome preparation as described below.
Biochemical characteristics of the microsomes are mentioned in Table 1.

Preparation of Microsomal Fractions and Determination of Protein
and Total CYP Contents. The microsomes were prepared as described previously
(Haber et al., 1994). Microsomal proteins were quantified by the method of
Bradford (1976) using bovine serum albumin fraction V as standard. Cyto-
chrome P-450 was assayed according to Omura and Sato (1964).

Microsomal Metabolism of DADS. The reaction medium described below
was the same for each experiment. This reaction medium consisted of human
liver microsomes corresponding to 300 pmol of CYP, 1 nM DADS, 1 mM

TABLE 1
Biochemical characteristics of human liver samples

<table>
<thead>
<tr>
<th>CYP</th>
<th>EROD&lt;sup&gt;a&lt;/sup&gt; &lt;br&gt;1A2</th>
<th>COH&lt;sup&gt;a&lt;/sup&gt; &lt;br&gt;2A6</th>
<th>TDB&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;2C9</th>
<th>Mph&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;2C19</th>
<th>DOD&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;2D6</th>
<th>PNPH&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;2E1</th>
<th>(α-1)&lt;/sup&gt;LAH&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;2E1</th>
<th>NO&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;3A4</th>
<th>(α-1)&lt;/sup&gt;LAH&lt;sup&gt;b&lt;/sup&gt; &lt;br&gt;4A</th>
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<td>39.3</td>
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<sup>a</sup> pmol/min/nmol CYP  
<sup>b</sup> nmol/min/nmol CYP  
nd, not determined
NADPH, 50 mM Tris-HCl pH 7 in a total volume of 500 μl. After 30 min at 37°C, the reaction was stopped by adding 320 μl of acetonitrile. After 15 min of protein precipitation, the mixture was centrifuged at 10,500 g for 10 min and 40 μl of the supernatant was analyzed by HPLC. The same protocol was applied with 25 pmol for each cDNA-expressed human CYP microsome. In case of cDNA-expressed human FMO3 microsomes, 100 μg of protein was added in a total volume of 125 μl. Many DADS concentrations were used to determine the kinetic parameters.

Thermal inactivation of microsomes was performed by a preincubation of microsomes in Tris-HCl buffer for 10 min at 37°C in the absence of NADPH or in the presence of NADPH for control microsomes. Then DADS and NAPDH were added subsequently to start the incubation for 30 min.

Inhibition of DADS Metabolism. Inhibitors were added to the incubation mixtures before initiation of the reaction. Only with the mechanism-based inhibitors such as diethylthiocarbamate or aminobenzotriazole, microsomes were preincubated for 10 min at 37°C before the addition of DADS. Nonhydrolyzable inhibitors were dissolved in 100% ethanol and the following volumes of ethanol were added to the incubation medium (total volume, 500 μl): 0.2 μl for sulphanilamide and orphenadrine, 0.3 μl for coumarin, 0.4 μl for diethylthiocarbamate, 0.5 μl for aminobenzotriazole and quinidine, 0.8 μl for α-naphthoflavone, and 2.5 μl for nifedipine. The human samples KS1, K12, K25, KS28, K33, K40, KS41, and KS63 were used for the inhibitory experiments.

HPLC Analysis. HPLC analysis was carried out using a Waters (Saint Quentin-en-Yvelines, France) system equipped with a model 600 pump, a model 717 auto sampler, a model 996 photodiode array UV detector, and a GL Sciences Inc. (Tokyo, Japan) Intersil ODS-3 column (4.6 × 150 mm). The flow rate was 0.6 ml/min and the solvent-isocratic program was 30:70 (v/v, acetonitrile/water) for 20 min. The spectrum from 190 to 300 nm was used to detect DADS and its metabolites. The quantification was made at 254 nm. Data were processed by Waters Millennium software.

Determination of Kinetic Constants. \( K_m \) and \( V_{max} \) were determined with a range of substrate concentrations of 0 to 7.5 mM with human liver microsomes and FMO cDNA-expressed microsomes and 0 to 5 mM with CYP cDNA-expressed microsomes. The values were estimated by fitting the Michaelis-Menten equation using a nonlinear regression program of SAS Software (Cary, NC). The human samples KS1, KS30, K33, and K40 were used in this experiment. The apparent \( V_{max} \) of FMO3 cDNA-expressed microsomes was determined considering that 100 μg of protein corresponded to approximately 100 pmol of enzyme. This consideration was based on the specific activity of recent lots of FMO3 from Gentest.

Enzyme Assays. Detailed information for these assays are given Table 2.

<table>
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<tr>
<th>Buffer</th>
<th>COH</th>
<th>DOD</th>
<th>ECOD</th>
<th>EROD</th>
<th>LAH</th>
<th>Mph</th>
<th>MMO</th>
<th>NIO</th>
<th>PNP</th>
<th>TDH</th>
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</thead>
<tbody>
<tr>
<td>NADPH (mM)</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>B</td>
<td>F</td>
<td>G</td>
<td>H</td>
<td>B</td>
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<tr>
<td>Microsomal proteins (mg/ml)</td>
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<td>1</td>
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<td>450</td>
<td>586</td>
<td>450</td>
<td>312</td>
</tr>
</tbody>
</table>

A: 50 mM sodium phosphate buffer (pH 7.4)
B: 100 mM potassium phosphate buffer (pH 7.4)
C: 100 mM Tris-HCl buffer (pH 7.7), 1 mM EDTA
D: 100 mM D-MOPS buffer (pH 7.7), 25 mM MgCl₂
E: 66 mM Tris-HCl buffer (pH 7.4)
F: 0.1 M tricine (pH 8.4), 1 mM EDTA, 0.06 mM 5,5’-dithiobis(2-nitrobenzoate), 6 mM potassium phosphate (pH 8.4), 0.02 mM dithiothreitol
G: 50 mM potassium phosphate buffer (pH 7.4), 3 mM MgCl₂
H: 100 mM sodium phosphate buffer (pH 6.8)
tion did not modify the ECOD activity; this reaction is often used as a standard marker for CYP-mediated reactions as several forms of CYP are involved in this activity), and 3) microsomes prepared from baculovirus-infected insect cell lines expressing the human FMO3 (in this medium, the same product DADSO was obtained as with human liver microsomes; the kinetic parameters of the reaction were calculated: $K_{m} = 10.15$ mM and $V_{max} = 41.9$ pmol/min/pmol FMO3).

**Effect of Human CYP Inhibitors on DADS Oxidation.** To further assess which CYP isoenzymes are involved in DADS oxidation, the effects of the following chemical inhibitors were determined: $\alpha$-naphthoflavone (specific of CYP1A), chlorzoxazone (CYP2E1; Met et al., 1995), coumarin (CYP2A6; Harris et al., 1994), diethyl-dithiocarbamate (CYP2E1; Guengerich et al., 1991), mephenytoin (CYP2C19; Harris et al., 1994), nifedipine (CYP3A4; Harris et al., 1994), orphenadrine (CYP2B6; Chang et al., 1993), quinidine (CYP2D6; Harris et al., 1994), sulfaphenazole (CYP2C9; Baldwin et al., 1995), tolbutamide (CYP2C9; Harris et al., 1994), tranylcypromine (CYP2C19; Postlind et al., 1998), and troleandomycin (CYP3A4; Rodrigues, 1994).

The results are presented in Fig. 4. The strongest inhibitions were observed in the presence of diethyl-dithiocarbamate, followed by tranylcypromine. A slighter inhibition rate appeared with coumarin, chlorzoxazone, mephenytoin, nifedipine, and sulfaphenazole. Orphenadrine, tolbutamide, and troleandomycin had almost no effect on the DADSO formation, and $\alpha$-naphthoflavone and quinidine had no effect at all. To verify the selectivity of some of the inhibitors used, we measured PNPH activity, a marker of CYP2E1, in the presence of 30 $\mu$M tranylcypromine, or of 22 or 200 $\mu$M coumarin. The result is shown in Table 4. Tranylcypromine and coumarin, described as selective inhibitors of CYP2C19 and CYP2A6 respectively, were also inhibitors of PNPH activity. These results suggest that tranylcypromine and coumarin can inhibit other CYPs, at least CYP2E1.

**Metabolism by Microsomes Containing cDNA-Expressed CYPs.** The abilities of different human CYP enzymes to catalyze the $S$-oxidation of DADS to DADSO were investigated in many microsomes derived from cells expressing CYP DNA. CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 catalyzed the $S$-oxidation of DADS. CYP2D6, CYP2C19, and CYP2E1 were the most active enzymes. We can observe in Fig. 5, that every CYP isoform followed a Michaelis-Menten kinetic except CYP2E1. For this latter enzyme, the DADS oxidase activity decreased quickly as soon as a certain amount of DADSO was produced. This could be explained by the CYP2E1 mechanism-based inhibitor property of DADSO. No detectable amounts of DADSO were found when using microsomes expressing CYP1A2, CYP3A4, or CYP4A11, or when using microsomes from B-lymphoblastoid cells transfected with an empty vector. The kinetic parameters were determined for CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (Table 5). CYP2E1 exhibited the smallest $K_{m}$ whereas CYP2D6, CYP2C19, and CYP2E1 had close $V_{max}$ values. The intrinsic clearance ($V_{max}/K_{m}$ ratio) was 2010 for CYP2E1 whereas it reached the maximum value of 108 for the other CYPs (Table 5).

**Correlation Study between DADS Oxidation and Specific CYP Activities.** The correlation between DADS oxidation and CYP marker activities was analyzed with a panel of at least 24 individual samples of human liver microsomes. The characteristic transformations by individual CYPs were EROD for CYP1A2, COH for CYP2A6, TDH for CYP2C9, MpH for CYP2C19, DOD for CYP2D6, (o-$\omega$)-LAH and PNPH for CYP2E1, NfO for CYP3A4, and (o-$\omega$)LAH for CYP4A. These results are shown in Table 6. The best correlations (r) were found with PNPH, TDH, and (o-$\omega$)-LAH activities, whereas the correlations with EROD, COH, DOD, NfO, MpH, and (o-$\omega$)LAH were very low.

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**Table 3**

<table>
<thead>
<tr>
<th>CYP</th>
<th>DADS Oxidase</th>
<th>MMO</th>
<th>ECOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min 37°C</td>
<td>20.0 ± 6.3</td>
<td>40.2 ± 9.6</td>
<td>0.25 ± 0.0</td>
</tr>
<tr>
<td>Control</td>
<td>23.0 ± 7.4</td>
<td>194 ± 50.1</td>
<td>0.25 ± 0.0</td>
</tr>
</tbody>
</table>

Values are means of four samples ± S.E.M. and are expressed relative to control (without inhibitor).

**Fig. 2.** HPLC profile of an incubation of DADS with microsomes and NADPH, obtained with UV detection at 254 nm.

If DADSO, has been formed, its elution would be between the peak of NADPH (AU = 0.03) and the peak of DADSO.

**Fig. 3.** Effects of 1-aminobenzotriazole on DADS oxidase activity.

Values are mean of four samples ± S.E.M. and are expressed relative to control (without inhibitor).

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**Fig. 4.** Effects of thermal treatment of microsomes on few activities.

Values are the means of five determinations ± S.E.M.
We have studied the metabolism of DADS in the presence of human liver microsomes and we have observed the appearance of DADSO formed by the oxidation of one sulfur atom. Both DADS and DADSO are natural compounds found in crushed garlic but the latter has been considered as the most important biologically active compound of garlic (Reuter, 1995; Augusti, 1996). Among several sulfides from *Allium* studied, DADS exhibited in vivo the strongest anticarcinogenic properties (Ip et al., 1992; Haber-Mignard et al., 1996) and protected against carcinogen-induced DNA strand breaks (Le Bon et al., 1997). These effects were attributed to in vivo modulation of hepatic drug-metabolizing enzymes by DADS (Haber et al., 1994). Knowing the in vitro DADS metabolism, we suppose that the in vivo effects of DADS are in fact due to DADSO.

In this study, we have observed the oxidation of DADS to sulfoxide. The in vivo metabolisms of diallyl sulfide (DAS; Brady et al., 1991, Chen et al., 1994) and of dipropyl sulfide (Nickson and Mitchell, 1994), two sulfur compounds from *Allium*, implicate a first step of oxidation to form a sulfoxide and a second one to form a sulfone. With DADS two steps of oxidation were not excluded, even if formation of diallyl thiosulfonate (DADSO₂) was not observed in the incubation medium. In fact we have characterized DADSO as a mechanism-based inactivator of CYP2E1 (results not published). This should mean that DADSO could be oxidized to a reactive species (possibly...
DADS oxidation that should interact with the enzyme and should not be released from the active site of the enzyme.

Egen-Schwind et al. (1992) have studied the metabolism of DADS in a perfused rat liver. They observed that while passing through the liver, DADS was metabolized to DADS. The discrepancy could be explained by the models that were used in both studies. They did not add NADPH during the liver perfusion. Due to the sensitivity of DADS to temperature, a dismutation of DADS in DADS could be possible. We observed the formation of DADS when DADS was incubated with microsomes at 37°C without NADPH. In addition, Jin and Baille (1997) studied the metabolism of DAS in rat. They proposed that the reduction of allyl sulfide to DAS was impossible with respect to the glutathione conjugates observed with rat fed with DAS or allyl sulfoxide.

Flavin or CYPs are the only enzymes present in microsomes that can catalyze NADPH- and oxygen-dependent oxidation of xenobiotics. The inhibition of CYP by 1-aminobenzotriazole, a suicide inhibitor, as well as the irreversible inactivation of FMO by heating induced a decrease of the rate of DADS oxidation. Moreover, the DADS oxidation was observed with microsomes prepared from cells expressing human FMO3. These results suggest a contribution of both CYP and FMO-containing monooxygenases. Some results allow the evaluation of the FMO implication in this oxidation: its K_m is much higher than the one obtained for FMOs associated with CYP in human microsomes (10.15 versus 0.61 mM). The similar comparison made with cDNA-expressed isoenzymes gave 10.15 mM for FMO3 and 0.03 mM for CYP2E1. When the incubation of microsomes and DADS was made in the presence of inhibited CYP, the DADS oxidase activity is very low, whereas when the FMOs were inactivated this activity was slightly decreased. Each of these results suggests that FMOs are less active than CYP in the metabolism of DADS. Nevertheless, to our knowledge, this is the first study that describes the implication of FMOs in the oxidation of sulfur compounds issued from garlic.

Three approaches have been developed to identify the CYP isoenzymes involved in DADS oxidation. They were based on: 1) the use of cDNA-expressed CYP isoenzymes, 2) the use of selective inhibitors, and 3) the study of the correlation of DADS oxidation activity with marker activities of CYP isoenzymes. Several pieces of evidence indicate that CYP2E1 is the major cytochrome P-450 responsible for the metabolic process: 1) the rate of formation of DADS was substantially inhibited by the CYP2E1 inhibitors diethylthiocarbamate and chlorozoxazone, 2) PNPH and (ω-1)-LAH activities (two marker activities of CYP2E1) in 25 individual human liver microsomes exhibited the best correlation with the formation of DADS, and 3) with a K_m of 0.03 mM and a V_max/K_m ratio of 2010
calculated with cDNA-expressed CYP2E1, this isoenzyme exhibited the highest affinity and the highest intrinsic clearance among the isoenzymes tested. Even if the quantity of CYP2E1 in human microsomes was evaluated to 7% of whole CYP versus 18% for CYP2C and 29% for CYP3A (Shimada et al., 1994), the relative involvement of CYP2E1 is predominant.

Nevertheless, our results suggest the involvement of other CYPs. Many isoenzymes, such as CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 were able to oxidize DADS to DASO. Their K_m values showed that in a competitive context like in human microsomes, only CYP2E1 would be involved. The apparent velocity of CYP2E1 was not the highest one. We demonstrated that DADS was a mechanism-based inhibitor of CYP2E1 (Martin, Teysseyer and Siess, publication in preparation). This means that the more DADS is produced, more CYP2E1 is inhibited. This observation may serve as an explanation for the low V_max of CYP2E1.

In the inhibitory experiments, tranylcypromine, an inhibitor described as being specific of CYP2C19 (Postlind et al., 1998), strongly inhibited DADS oxidase activity, suggesting the participation of this isoenzyme. However, the specificity of this inhibitor should be reconsidered because Draper et al. (1997) recently demonstrated that this molecule is among the strongest inhibitors of the coumarin hydroxylase activity (CYP2A6 activity marker), and we showed the PNPH activity inhibition by tranylcypromine. Therefore, CYP2C19 does not seem to be involved in DADS oxidation.

DADS has a chemical structure similar to DAS; they differ only by one sulfur atom. These two molecules are metabolized by CYP2E1 and produce a mechanism-based inhibitor of CYP2E1. The metabolism of DADS and its pathway is one more similarity between these two molecules issued of garlic.

In conclusion, the oxidation of DADS to DASO in human liver microsomes is mainly mediated by CYPs but also by FMOs. Among the CYP isoenzymes, CYP2E1 seems to be the most involved isoenzyme even if a few other isoenzymes are also able to catalyze this reaction.

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