A NOVEL P450-CATALYZED TRANSFORMATION OF THE 2,2,6,6-TETRAMETHYL PIPERIDINE MOIETY TO A 2,2-DIMETHYL PYRROLIDINE IN HUMAN LIVER MICROSONES: CHARACTERIZATION BY HIGH RESOLUTION QUADRUPOLE-TIME-OF-FLIGHT MASS SPECTROMETRY AND 1H-NMR

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ABSTRACT:

We describe herein a novel metabolic fate of the 2,2,6,6-tetramethyl-piperidine (2,2,6,6-TMPi) moiety to a ring-contracted 2,2-dimethyl pyrrolidine (2,2-DMPy) in human liver microsomal incubations. The existence of this pathway was demonstrated for three compounds (I-III) of varied structures. GC-MS analysis of the three compounds with human liver microsomes was characterized by online high performance liquid chromatography and fragmentation data. High resolution hybrid quadrupole-time-of-flight mass spectrometry suggested elemental composition obtained from accurate mass measurements of the parent compound. Two-dimensional correlation NMR experiments on a metabolite purified from liver microsomal incubations, which showed only two geminal methyl groups, indicated that one of the original two gem-dimethyl groups was intact in the metabolite structure. Proof of a ring-contracted 2,2-DMPy structure was obtained using 1H-NMR experiments on a metabolite purified from liver microsomal incubations, which showed only two geminal methyl groups, instead of four in the parent compound. Two-dimensional correlation spectroscopy and decoupling experiments established aliphatic protons arranged in a pyrrolidine ring pattern. The fact that the formation of 2,2-DMPy metabolites in human liver microsomes was NADPH-dependent suggested that this novel metabolic reaction was catalyzed by the cytochrome P450 (P450) enzyme(s). Immunoinhibition studies in human liver microsomal incubations revealed at least 48 compounds with a 2,2,6,6-TMPi moiety that have entered preclinical and/or clinical testing for various disease indications. This allowed for overcoming limitations of the unsubstituted piperidine. This strategy has also been successful at obtaining improvements in pharmacokinetics and blocking iminium ion formation among our new drug candidates (unpublished observations). However, to our knowledge, the metabolic fate of the 2,2,6,6-TMPi moiety has not been examined. Here we describe a novel metabolic transformation of the 2,2,6,6-TMPi moiety in human liver microsomal incubations that results in the loss of three carbon atoms and contraction of the piperidine ring to a 2,2-dimethyl pyrrolidine (2,2-DMPy). Three different compounds (I-III) with distinct structures, but all containing a 2,2,6,6-TMPi functionality. Additional accurate tandem mass spectrometry data indicated that one of the original two gem-dimethyl groups was intact in the metabolite structure. Proof of a ring-contracted 2,2-DMPy structure was obtained using 1H-NMR experiments on a metabolite purified from liver microsomal incubations, which showed only two geminal methyl groups, instead of four in the parent compound. Two-dimensional correlation spectroscopy and decoupling experiments established aliphatic protons arranged in a pyrrolidine ring pattern. The fact that the formation of 2,2-DMPy metabolites in human liver microsomes was NADPH-dependent suggested that this novel metabolic reaction was catalyzed by the cytochrome P450 (P450) enzyme(s). Immunoinhibition studies in human liver microsomal incubations revealed at least 48 compounds with a 2,2,6,6-TMPi moiety that have entered preclinical and/or clinical testing for various disease indications, suggesting that this is a viable and commonly used approach for overcoming limitations of the unsubstituted piperidine. This strategy has also been successful at obtaining improvements in pharmacokinetics and blocking iminium ion formation among our new drug candidates (unpublished observations). However, to our knowledge, the metabolic fate of the 2,2,6,6-TMPi moiety has not been examined. Here we describe a novel metabolic transformation of the 2,2,6,6-TMPi moiety in human liver microsomal incubations that results in the loss of three carbon atoms and contraction of the piperidine ring to a 2,2-dimethyl pyrrolidine (2,2-DMPy). Three different compounds (I-III) with distinct structures, but all containing a 2,2,6,6-TMPi moiety (Fig. 1), were found to undergo this metabolic reaction,

Piperidine is one of the most common alicyclic amines encountered in drugs and is frequently used in drug design for enhancing potency via introduction of basicity and/or lipophilicity into the chemical structure. However, piperidine suffers from the drawback of being susceptible to extensive metabolism, generally at the carbons alpha to the nitrogen, that leads to oxidation and/or ring opening (Oelschläger and Al Shaik, 1985). This frequently results in poor pharmacokinetic properties for the new drug candidates. The N-substituted piperidines are also prone to metabolic activation via the formation of reactive iminium ions that may covalently modify proteins and other cellular nucleophiles and potentially result in toxicity (Sayre et al., 1991; Gorrod and Aislaitner, 1994; Castagnoli et al., 1997). Iminium ion formation also presumably involves oxidation at the piperidine ring alpha-carbons (Castagnoli et al., 1997). Furthermore, in certain cases (e.g., haloperidol), the N-substituted piperidines are known to undergo oxidative conversion to potentially neurotoxic pyridinium species; this conversion may also occur via a pathway that initiates with an alpha-carbon oxidation (Subramanyam et al., 1991). One possible strategy to overcome these drawbacks of the unsubstituted piperidine is the use of a 2,2,6,6-tetramethyl-piperidine moiety (2,2,6,6-TMPi)1 that would block the oxidation at the alpha-carbons. A search of the MDL Drug Data Report database (MDL Information Systems, Inc., San Leandro, CA) revealed at least 48 compounds with a 2,2,6,6-TMPi moiety that have entered preclinical and/or clinical testing for various disease indications, suggesting that this is a viable and commonly used approach for overcoming limitations of the unsubstituted piperidine. This strategy has also been successful at obtaining improvements in pharmacokinetics and blocking iminium ion formation among our new drug candidates (unpublished observations). However, to our knowledge, the metabolic fate of the 2,2,6,6-TMPi moiety has not been examined. Here we describe a novel metabolic transformation of the 2,2,6,6-TMPi moiety in human liver microsomal incubations that results in the loss of three carbon atoms and contraction of the piperidine ring to a 2,2-dimethyl pyrrolidine (2,2-DMPy). Three different compounds (I-III) with distinct structures, but all containing a 2,2,6,6-TMPi moiety (Fig. 1), were found to undergo this metabolic reaction,

1 Abbreviations used are: 2,2,6,6-TMPi, 2,2,6,6-tetramethyl-piperidine; 2,2-DMPy, 2,2-dimethyl pyrrolidine; P450, cytochrome P450; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Q-Tof, quadrupole-time-of-flight; MS, mass spectrometry; CID, collision-induced dissociation; HPLC, high performance liquid chromatography.
suggesting that this may be a general biotransformation route for this moiety.

Experimental Procedures

Materials and Methods. Compounds I-III were synthesized and purified at Merck Research Laboratories (Hunt et al., 2002). Microsomes containing individual recombinant human P450 isozymes and monoclonal antibodies against human P450 isozymes were obtained from Drs. Magang Shou and Thomas H. Rushmore (Department of Drug Metabolism, Merck Research Laboratories, West Point, PA). Recombinant P450 microsomes were prepared from Sf21 insect cells infected with recombinant baculoviruses encoding individual P450 cDNA’s and cytochrome P450 reductase (Mei et al., 1999). Monoclonal antibodies against human CYP3A4, 2D6, 2C8/9, and 2C19 were prepared in mice following immunization with individual recombinant isozymes as described previously (Mei et al., 1999). Protein assay reagent kit was purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of reagent grade.

Microsomal Incubations. Liver microsomes from five individual male human livers were prepared by differential centrifugation of the homogenized liver tissue (Rauch and Lasker, 1991). Before experimentation, equal amounts of microsomal protein from the five individual microsomal preparations were pooled to provide a representative average preparation. Microsomal protein concentrations were measured with bichinonic acid according to the instructions of the manufacturer for the use of protein assay kit.

All microsomal incubations (1-ml total volume) were conducted at 37°C in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM MgCl₂, and with or without (controls) an NADPH-regenerating system that consisted of 10 mM glucose 6-phosphate, 1 mM NADP⁺, and 0.7 u/ml glucose-6-phosphate dehydrogenase. Substrate and microsomal protein concentrations for all incubations were 20 μM and 2 mg/ml, respectively. Incubations were conducted for 2 h, and the reactions were quenched by the addition of 2 ml of acetonitrile. Samples were centrifuged, the supernatant was separated from precipitated protein and concentrated by evaporation under a gentle stream of nitrogen for analysis by LC-MS/MS.

Structure Elucidation of Metabolites Using LC-MS/MS on a Q-Tof II

High Resolution Mass Spectrometer. The Q-Tof II mass spectrometer (Micromass, Inc., Cheshire, UK) had a minimum mass-resolution of 6000 (measured as full width at half maximum) during all experiments. The mass spectrometer was operated in positive ion electrospray mode and was calibrated on each experiment day using a mixture solution of polyethylene glycols with known exact molecular masses (approximate range 100-1000 Da). Electrospray capillary voltage and cone voltage were set at 3400 V and 40 V, respectively. Source and desolvation temperatures were 100 and 300°C, respectively. Nitrogen was used as the desolvation and nebulizing gas at flow rates of 400- and 50-l/h, respectively. Full scan Tof MS spectra were first acquired for measurement of accurate masses of molecular ions of parent compounds and relevant metabolites. Subsequently, MS/MS fragmentation spectra were acquired after CID of appropriate molecular ions that were selected using the quadrupole mass filter before the Tof analyzer. Argon was used as the collision gas and the collision energy was set at 30 eV; this permitted substantial fragmentation of compounds but still retained some precursor ion to be used as a lock mass for accurate mass measurement of fragment ions. Separation of metabolites of interest was achieved on a Zorbax SB-Phenyl HPLC column (250 × 4.6 mm, 5 μm) using a Hewlett Packard 1100 HPLC system (Hewlett Packard, Palo Alto, CA) consisting of two pumps, an autosampler, and a column oven. Mobile phase A consisted of a 2 mM ammonium acetate buffer and mobile phase B of a 70:30 (v/v) mixture of acetonitrile and methanol, respectively; both mobile phases contained 0.1% formic acid. HPLC run began with the solvent composition at 10% mobile phase B for the first 3 min, and then increased to 50 and 90% B at 50 and 51 min, respectively, using linear gradients. After 60 min, the mobile phase composition was returned to the initial conditions for column re-equilibration. HPLC flow rate was 1 ml/min, and the column effluent was split 1:5 between mass spectrometer and waste, respectively. Leucine-enkephalin (1 μg/ml) was introduced into the column effluent at a rate of 5 μl/min via a T-joint to act as an internal mass calibrant (lock-mass). Acquisition and analysis of the data were performed using the MassLynx software (version 3.5, Micromass Inc.).

![Figure 1](https://example.com/image1)

**FIG. 1.** Structures of the three compounds that were examined for the transformation of 2,2,6,6-TMPi moiety to 2,2-DMPy. The peaks corresponding to the metabolites in question for the three compounds are depicted with arrow marks.
Identification of P450 Isomers Involved in the Formation of 2,2-DMPy Metabolites in Human Liver Microsomes. Compounds I-III were incubated with HLM in the presence or absence of monoclonal antibodies against CYP2C8/9, CYP2C19, CYP2D6, and CYP3A4 isoforms. Each incubation contained 2 mg/ml microsomal protein and 5 μl of the antibody preparation, along with the above described buffer and NADPH-regenerating system, in a total volume of 200 μl. The formation of the respective ring contracted 2,2-DMPy metabolite for all compounds in the incubation was determined by LC-MS/MS on a triple-quadrupole mass spectrometer (see below).

Further confirmation of the P450 isomorph(s) responsible for the formation of 2,2-DMPy metabolites was obtained from incubations with microsomes prepared from baculovirus-infected cells containing individually expressed human P450 isoforms (P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) and cytochrome P450 reductase. Each incubation contained 20 μM substrate, 250 pmol/ml P450 protein, an NADPH-regenerating system, and magnesium chloride (10 mM) in 100 mM potassium phosphate buffer (pH 7.4). Incubations were carried out for 1 h at 37°C, after which time the reaction was halted by the addition of an equal volume of acetonitrile. Following centrifugation, the supernatant was analyzed by LC-MS/MS.

LC-MS/MS analyses for the above samples were carried out using a PerkinElmer series 200 HPLC system (PerkinElmer Life Sciences, Boston, MA) coupled to a SCIEX API3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments, Boston, MA). Mass spectrometer was operated in positive ion electrospray mode, and analyte detection was performed using multiple reaction monitoring. Electrospray voltage and source temperature were 4500 V and 250 °C, respectively. Chromatography was carried out on a Zorbax RX-C8 column (9.4 × 250 mm, 5 μm). A variety of gradient elution profiles, with various proportions of 1 mM ammonium acetate buffer, acetonitrile and methanol (each with or without 0.1% formic acid), were used to resolve the metabolite from endogenous biological materials. Appropriate fractions containing the metabolite of interest were collected, dried under a gentle stream of nitrogen, and subjected to 1H-NMR analysis.

1H-NMR Analysis of the Purified 2,2-DMPy Metabolite of Compound I. The 1H NMR spectra of the purified 2,2-DMPy metabolite of compound I were acquired in CD3CN:D2O (9:1) at 25°C in 3-mm NMR tubes using a 600 MHz Varian spectrometer (Varian Medical Systems Inc., Palo Alto, CA). Chemical shifts are reported on the δ scale (parts per million) downfield from tetramethylsilane using the CD3CN lock signal for reference at 1.93 ppm.

Results

Our initial studies involving the incubation of compounds I-III with human liver microsomes and an NADPH-regenerating system revealed the presence of metabolites with a nominal mass that was either 56 Da less than the parent compounds I and III or 42 Da less than the parent compound II (Fig. 2). The mass-spectral abundance of these metabolites suggested that these were prominent metabolic products of the 2,2,6,6-TMPi moiety.

Isolation and Purification of the 2,2-DMPy Metabolite of Compound I. Compound I (25 nM) was incubated with human liver microsomes (2 mg of protein/ml) in the above described buffer and NADPH-regenerating system for 4 h. The incubation was terminated by the addition of an equal volume of acetonitrile, and the supernatant was separated from the precipitated protein by centrifugation. The 2,2-DMPy metabolite was isolated by semipreparative HPLC on a Zorbax RX-C8 column (9.4 × 250 mm, 5 μm). A variety of gradient elution profiles, with various proportions of 1 mM ammonium acetate buffer, acetonitrile and methanol (each with or without 0.1% formic acid), were used to resolve the metabolite from endogenous biological materials. Appropriate fractions containing the metabolite of interest were collected, dried under a gentle stream of nitrogen, and subjected to 1H-NMR analysis.

Table 1 presents the accurate mass and deviations of the measured molecular mass from the calculated mass of the proposed elemental composition. All mass deviations are less than the parent compounds I and III or 42 Da less than the parent compound II.
2,2,6,6-TMPi, and the core structure of the molecules was intact (data not shown). Similarly, the proposed molecular formulae for the 2,2-DMPy metabolites indicate the loss of a mass equivalent to $\text{C}_3\text{H}_6$ relative to the corresponding 2,2,6,6-TMPi derivatives (Table 1).

Figure 3 shows the comparative accurate mass MS/MS spectra of the 2,2,6,6-TMPi and 2,2-DMPy metabolites of compound I in human liver microsomes obtained by CID of the corresponding molecular ions. All relevant fragment ion assignments, suggested elemental composition of the fragment ions, and deviations of the measured mass of the fragment ions relative to the theoretically calculated mass.
are presented in Table 2. A close agreement between the measured and calculated masses of these fragment ions lends support to the proposed fragment ion structures for these two compounds. Comparison of the MS/MS spectra of the 2,2,6,6-TMPi and 2,2-DMPy derivatives suggests that in the latter the core structure of the molecule stays intact (see fragment ions at \( m/z \) 416.0139 and 416.0125 in Fig. 3), and the metabolic transformation (i.e., the loss of a mass equivalent to \(-\text{C}_2\text{H}_8\) relative to the 2,2,6,6-TMPi derivative) has occurred at the 2,2,6,6-TMPi moiety. A further characteristic feature of the MS/MS spectrum of the 2,2-DMPy metabolite was the fragment ion at \( m/z \) 457.0411 with an indicated elemental composition of \( \text{C}_{22}\text{H}_{16}\text{N}_4\text{OCl}_3\). This fragment ion suggests the loss of a \(-\text{NH}_3\) moiety from the molecular ion of this metabolite upon CID (i.e., \( m/z \) 513.1010, \( \text{C}_{26}\text{H}_{24}\text{N}_4\text{OCl}_3 \); Table 1). This provides evidence that at least one of the gem-dimethyl moieties is intact in the 2,2-DMPy metabolite structure. The fragment ions with measured masses of \( m/z \) 496.0752 and 440.0130 suggest the loss of a \(-\text{NH}_3\) moiety from the ions at \( m/z \) 513.1010 (precursor ion) and 457.0411, respectively (Fig. 3 and Table 2). This indicates that the original piperidine nitrogen is still present within the 2,2-DMPy metabolite structure. Based on these data, a ring contracted 2,2-DMPy structure shown in Fig. 3 was proposed for this metabolite. The other fragment ions in the accurate mass product ion spectrum of the 2,2-DMPy metabolite of compound I appeared to be consistent with the proposed structure (Table 2). The 2,2-DMPy metabolites of compounds II and III also underwent analogous fragmentation upon CID, and the measured accurate masses of fragment ions were consistent with a ring contracted pyrrolidine structure.

The 2,2-DMPy metabolite of compound I was isolated from microsomal incubations and analyzed by 600 MHz NMR to confirm its structure (Fig. 4, Table 3). The NMR data fully supported the proposed 2,2-DMPy structure as follows. The aromatic protons were all intact. The proton signals from the \( N \)-methyl group were clearly absent. The aliphatic region showed only two geminal methyl groups, instead of four as in the parent compound. A two-dimensional NMR correlation spectroscopy experiment (Fig. 5) showed signals from five aliphatic protons arrayed in a \( \text{CH}_2\text{–CH–CH}_2 \) spin system; these were assigned to the pyrrolidine ring protons. Doubling of signals was notable in the NMR spectrum and is due to the presence of two diastereomers arising from chirality (atropisomerism) in the core structure of this metabolite (likely due to the restricted rotation of the “bottom” Cl-phenyl ring around the biaryl bond). Thus, two diastereomers are possible for this metabolite since a new chiral center was formed due to loss of symmetry of the original tetramethyl piperidine ring. The parent compound exhibited the same atropisomerism, which rendered the two sides of the piperidine ring diastereotopic, thus resulting in multiple signals in the NMR spectrum.

**TABLE 2**

**Accurate mass fragment assignments for the MS/MS spectra of 2,2,6,6-TMPi and 2,2-DMPy metabolites of compound I**

The core structure of compound I is unaltered in these metabolites and is represented by the molecular formula \( \text{C}_{20}\text{H}_{10}\text{Cl}_3\text{N}_2\text{O} \).

<table>
<thead>
<tr>
<th>Measured Fragment Mass</th>
<th>Suggested Elemental Composition for the Fragment Ion</th>
<th>Proposed Fragment Ion Structure</th>
<th>Deviation from the Calculated Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 416.0139 )</td>
<td>( \text{C}<em>{20}\text{H}</em>{13}\text{N}_3\text{OCl}_3^+ )</td>
<td>( \text{C}<em>{20}\text{H}</em>{10}\text{Cl}_4\text{O}_4\text{N}_4\text{H}_3^+ )</td>
<td>+1.5 + 3.6</td>
</tr>
<tr>
<td>( 442.0278 )</td>
<td>( \text{C}<em>{22}\text{H}</em>{14}\text{N}_3\text{OCl}_3^+ )</td>
<td>( \text{C}<em>{22}\text{H}</em>{10}\text{Cl}_4\text{O}_4\text{N}_4\text{H}_3^+ )</td>
<td>+0.3 + 0.6</td>
</tr>
<tr>
<td>( 482.0574 )</td>
<td>( \text{C}<em>{22}\text{H}</em>{14}\text{N}_3\text{OCl}_3^+ )</td>
<td>( \text{C}<em>{22}\text{H}</em>{10}\text{Cl}_4\text{O}_4\text{N}_4\text{H}_3^+ )</td>
<td>+0.6 + 1.3</td>
</tr>
<tr>
<td>( 416.0125 )</td>
<td>( \text{C}<em>{20}\text{H}</em>{13}\text{N}_3\text{OCl}_3^+ )</td>
<td>( \text{C}<em>{20}\text{H}</em>{10}\text{Cl}_4\text{O}_4\text{N}_4\text{H}_3^+ )</td>
<td>+0.1 + 0.2</td>
</tr>
<tr>
<td>( 440.0130 )</td>
<td>( \text{C}<em>{22}\text{H}</em>{13}\text{N}_3\text{OCl}_3^+ )</td>
<td>( \text{C}<em>{22}\text{H}</em>{10}\text{Cl}_4\text{O}_4\text{N}_4\text{H}_3^+ )</td>
<td>+2.1 + 4.7</td>
</tr>
<tr>
<td>( 457.0411 )</td>
<td>( \text{C}<em>{22}\text{H}</em>{16}\text{N}_4\text{OCl}_3^+ )</td>
<td>( \text{C}<em>{22}\text{H}</em>{10}\text{Cl}_4\text{O}_4\text{N}_4\text{H}_3^+ )</td>
<td>+0.2 + 0.4</td>
</tr>
</tbody>
</table>
The formation of 2,2-DMPy metabolites from compounds I-III in incubations with human liver microsomes was NADPH-dependent, suggesting possible involvement of P450 isozymes in this metabolic reaction. Figure 6A shows the amounts of 2,2-DMPy metabolites formed from compounds I-III upon incubation with insect cell microsomes containing individually expressed recombinant human P450 isozymes. All data are normalized to the amounts formed in incubations with CYP3A4-containing microsomes. It appears from these data that CYP3A4 is the major P450 isoform catalyzing the biotransformation of 2,2,6,6-TMPi moiety to 2,2-DMPy for these three compounds; the amounts of metabolite detected in microsomes containing other isozymes were <10% relative to those in CYP3A4 incubations. Figure 6B shows the effect of monoclonal antibodies against various human P450 isozymes on the formation of 2,2-DMPy metabolite when compounds I-III were incubated with human liver microsomes. Antibodies against CYP3A4 had a potent inhibitory effect on the formation of this metabolite for all three compounds and inhibited metabolite formation by >90%. In contrast, antibodies against the other P450 isozymes had negligible or weak inhibitory effects on the formation of 2,2-DMPy metabolites for compounds I and III; this further substantiates that CYP3A4 is the major isozyme involved in this metabolic transformation for these compounds. For compound II, antibodies against the members of the CYP2C and CYP2D families also exhibited significant inhibitory effects on the formation of 2,2-DMPy metabolite (Fig. 6B) suggesting that in addition to CYP3A4 these other isozymes may also be involved in one or more intermediate steps of this metabolic reaction (see Discussion).

Discussion

Quadrupole mass spectrometry with its unit mass resolution makes it difficult to distinguish metabolites and fragment ions with the same nominal mass. This limitation becomes especially significant while examining unusual metabolic pathways that result in unique and intuitively unapparent metabolite structures. In many such cases, multiple fragmentation pathways can be proposed that result in fragment ions with the same nominal mass which are indistinguishable with a unit mass resolution. A hybrid quadrupole-time-of-flight mass spectrometric technique offers advantages of high resolution accurate
TABLE 3

Proton NMR data for compound I and its 2,2-DMPy metabolite

<table>
<thead>
<tr>
<th>Chemical shift in parts per million, assignment, integral, multiplicity, coupling constants in Hertz.</th>
<th>Compound I</th>
<th>2,2-DMPy Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.66</td>
<td>a</td>
<td>2H d</td>
</tr>
<tr>
<td>7.55</td>
<td>b</td>
<td>1H t</td>
</tr>
<tr>
<td>7.54</td>
<td>e</td>
<td>1H dt</td>
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<td>7.48</td>
<td>c</td>
<td>1H dd</td>
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<tr>
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<td>f</td>
<td>1H m</td>
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<td>4.11</td>
<td>4'</td>
<td>1H m br</td>
</tr>
<tr>
<td>2.64</td>
<td>NMe</td>
<td>3H</td>
</tr>
<tr>
<td>2.09</td>
<td>3'/5' eq</td>
<td>2H m</td>
</tr>
<tr>
<td>1.60</td>
<td>3'/5' ax</td>
<td>1H m</td>
</tr>
<tr>
<td>1.33</td>
<td>Me</td>
<td>6H s</td>
</tr>
<tr>
<td>1.24</td>
<td>Me</td>
<td>6H s</td>
</tr>
</tbody>
</table>

Fig. 5. Two-dimensional correlation spectroscopy NMR spectrum of the 2,2-DMPy metabolite of compound I.
mass measurement capability and increased sensitivity in the full scan mode because of its Tof analyzer. Thus, it is possible to measure routinely masses of precursor and product ions to an accuracy error of $\pm 5$ ppm (Hopfgartner et al., 1999; Watt et al., 2001). The accurate mass measurements can then be used to derive possible elemental compositions of these ions with the aid of computer software and known structural features of the molecule. Thus, accurate mass measurements frequently are invaluable in arriving at less ambiguous mass-spectral and structural assignments (Pillard et al., 2000; Zhang et al., 2000; Bobeldijk et al., 2001; Watt et al., 2001; Lopes et al., 2002).

In our studies, the measurement of accurate masses of the molecular ions of the parent compounds and metabolites were useful at unambiguously establishing that the 2,2-DMPy metabolites had lost a mass equivalent to a $-\text{C}_6\text{H}_4$ moiety relative to the corresponding 2,2,6,6-TMPi analogs. Furthermore, the accurate mass product ion spectrum of the 2,2-DMPy metabolite of compound I revealed that at least one gem-dimethyl moiety was intact within this metabolite structure. These data provided important clues toward identification of the structure of this unusual metabolite and aided in the interpretation of the NMR data. This illustrates that online LC-MS/MS using high resolution time-of-flight mass spectrometry is a fast and powerful tool for the identification of unusual metabolite structures and can be extremely useful in supplementing the information obtained from NMR studies.

The data from experiments with individual recombinant human \( \text{P}450 \) isozymes and immuno-inhibition studies with human liver microsomes indicate that \( \text{CYP}3\text{A}4 \) is the major isozyme involved in the conversion of 2,2,6,6-TMPi moiety of compounds I-III to the corresponding 2,2-DMPy. However, it appears that at least for compounds II and III, members of the other \( \text{P}450 \) families are also capable of catalyzing this metabolic transformation. Our recent studies have indicated that the 2,2-DMPy metabolites of 2,2,6,6-TMPi-containing compounds are also formed in rat, dog, and monkey liver microsomes and hepatocytes in vitro as well as in rats in vivo (unpublished observations). Thus, this unique biotransformation pathway may represent a general metabolic fate for the 2,2,6,6-TMPi moiety in mammalian species. It is of note that the metabolic transformation in question involves the cleavage of a carbon-carbon (C-C) bond. Examples of \( \text{P}450 \)-catalyzed metabolic transformations that involve C-C bond cleavage are relatively rare. Some well characterized cases include oxidation of strained hydrocarbons such as quadricyclane, 4-dealkylation reactions of 4-alkyl-1,4-dihydropyridines, and ring opening of cyclopropyl- and cyclobutylamines. In all these cases, the intermediate radical or radical cation species formed following electron or hydrogen abstraction are hypothesized to undergo rearrangements involving C-C bond cleavage (Ortiz de Montellano, 1995; Guengerich, 2001). Other examples of \( \text{P}450 \)-catalyzed metabolic reactions involving C-C bond cleavage include the cholesterol side chain cleavage reaction catalyzed by mitochondrial \( \text{P}450_{\text{ccc}} \) that results in the cleavage of the C20-C22 bond to form pregnenolone, 14α-dehydroxylation of lanosterol, which occurs via multistep oxidation of the 14α-methyl group to a carboxylic acid and subsequent decarboxylation, and \( \text{CYP}17 \)-catalyzed C17-C20 bond cleavage in 17α-hydroxy derivatives of pregnenolone and progesterone to form dehydroepiandrosterone and androstenedione, respectively (Ortiz de Montellano, 1995; Guengerich, 2001). The exact mechanism of the formation of the 2,2-DMPy metabolites from their respective 2,2,6,6-TMPi analogs, as observed in our studies, is not fully clear at present and is the focus of our current investigations. Preliminary data suggest that oxidation of the 2,2,6,6-TMPi moiety to a hydroxylamine derivative and its further one-electron oxidation to a nitroxy radical are the intermediate steps in the final contractions of the 2,2,6,6-TMPi moiety to a 2,2-DMPy functionality.

In conclusion, we have identified a unique \( \text{P}450 \)-catalyzed conversion of the 2,2,6,6-TMPi moiety to a 2,2-DMPy in human liver microsomes with the aid of \( ^1\text{H}-\text{NMR} \) and online accurate mass MS and MS/MS measurements using a high resolution hybrid quadrupole-time-of-flight mass spectrometer. This metabolic pathway further adds to the long list of diverse xenobiotic metabolism pathways catalyzed by the cytochrome \( \text{P}450 \) superfamily of enzymes.

References


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