

## Short Communication

### Characterization of the Novel Benzisothiazole Ring-Cleaved Products of the Antipsychotic Drug Ziprasidone

#### ABSTRACT:

Characterization of two novel benzisothiazole ring cleaved metabolites of the antipsychotic drug, ziprasidone (ZIP), in rat has been described. Metabolites designated M6 and M9 were isolated from urine and bile of the rat dosed with radiolabeled ZIP and purified by reversed phase HPLC. The chemical structures of these metabolites were assigned based on tandem mass spectrometry in combination with chemical derivatization techniques. M6 and M9 were unaffected upon treatment with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide. Reaction of M9 with aqueous  $\text{TiCl}_3$  also did not change the HPLC retention time or the CID spectrum of

metabolite M9. These data excluded the possibility that these metabolites were owing to *N*-oxidation and/or aromatic hydroxylation. M6 and M9 were generated only when *in vitro* incubations of ZIP were conducted with human liver S-9 fraction in the presence of *S*-adenosyl-L-methionine. Based on these data, metabolites M6 and M9 were identified as *S*-methyl-dihydro-ZIP and *S*-methyl-dihydro-ZIP-sulfoxide, respectively. The structure of M9 was unambiguously confirmed by comparing the LC/MS retention time and mass spectral data with synthetic standard. A mechanism for the formation of these metabolites from ZIP is proposed.

ZIP<sup>1</sup> (fig. 1) is a substituted benzisothiazolypiperazine that exhibits potent and highly selective dopamine  $\text{D}_2$  and serotonin 5-HT<sub>2</sub> receptor antagonistic activities (1, 2). It is undergoing clinical trials for the treatment of schizophrenia. Its preclinical and clinical pharmacological profiles suggested that ZIP should be an efficacious antipsychotic with little or no liability for motor side effects (3). Previous studies have shown that ZIP is extensively metabolized in rats (4) and dogs, and in humans<sup>2</sup> after oral administration of radiolabeled ZIP. Only a very small amount of administered radioactivity (<1%) was excreted in urine as unchanged drug. Approximately 41% of the administered radioactivity was excreted in the urine and bile of rats (4). The major metabolites were due to oxidation on the benzisothiazole ring and *N*-dealkylation of the ethyl side chain attached to the piperazinyl nitrogen atom. Metabolism of benzisothiazoles typically involves oxidation at the sulfur, resulting in formation of sulfoxides and sulfones (5, 6). But, in addition to ZIP-SO and ZIP-SO<sub>2</sub>, two additional metabolites of ZIP were detected in the urine and bile of rats that seem to be modified on the benzisothiazole ring at sites other than sulfur oxidation (4). These metabolites accounted for 31 and 34% of the total radioactivity excreted in rat urine and bile, respectively. This short communication describes the characterization of these two novel metabolites of ZIP, resulting from the reductive cleavage of the

benzisothiazole ring. Metabolites were isolated from rat urine and bile and purified by HPLC, and characterized by: 1) LC/MS and LC/MS/MS; 2) derivatization with MTBSTFA or diazomethane; 3) treatment with aqueous  $\text{TiCl}_3$ ; and 4) comparison of chromatographic and spectral behaviors with the metabolites obtained from an *in vitro* incubation of <sup>14</sup>C-ZIP with human liver homogenate (S-9) in the presence of SAM. The structure of one metabolite was unambiguously confirmed by coelution on HPLC with synthetic standard.

**Materials and Methods.** <sup>14</sup>C-ZIP, ZIP-SO, ZIP-SO<sub>2</sub>, dihydro-ZIP, and *S*-methyl-dihydro-ZIP were synthesized at Pfizer Central Research (Groton, CT). <sup>14</sup>C-ZIP showed a specific activity of 9.0 mCi/mmol (21.8  $\mu\text{Ci}/\text{mg}$ ) and a radiochemical purity of  $\geq 98\%$ , as determined by radio-HPLC. MTBSTFA and  $\text{TiCl}_3$  were purchased from Aldrich Chemical Co. (St. Louis, MO) and Fisher Scientific (Springfield, NJ), respectively. SAM and <sup>3</sup>H-SAM were purchased from Sigma Chemical Co. (St. Louis, MO) and NEN-Research Products (Boston, MA), respectively. Diazomethane was generated just before use from *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide obtained from Aldrich (Milwaukee, WI).

Urine and bile (0–24 hr) from a previous study (4) were used for the isolation and purification of metabolites. The mixture of metabolites was subjected to chromatography on an HPLC system. Chromatography was conducted on a YMC basic HPLC column (4.6 mm × 250 mm, 5  $\mu\text{m}$ ) with a binary mixture of 20 mM ammonium acetate (pH 5.0, solvent A) and methanol (solvent B), as described previously (4). Several injections were made, and the radioactive fractions corresponding to metabolites M6 and M9 (with retention times of ~32.2 and ~40.1 min, respectively) were collected and concentrated on a

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<sup>1</sup> Abbreviations used are: ZIP, ziprasidone (5-[2-(4-(1,2-benzisothiazol-3-yl)piperazin-1-yl)ethyl]-6-chloro-1,3-dihydro-indol-2-one) hydrochloride hydrate; 5-HT, 5-hydroxytryptamine; ZIP-SO, ziprasidone sulfoxide; ZIP-SO<sub>2</sub>, ziprasidone sulfone; MTBSTFA, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoro-acetamide; SAM, *S*-adenosyl-methionine; dihydro-ZIP, 6-chloro-5-(2-(4-[imino-(2-mercapto-phenyl)-methyl]-piperazin-1-yl)-ethyl)-1,3-dihydro-indol-2-one; *S*-methyl-dihydro-ZIP, 6-chloro-5-(2-(4-[imino-(2-methylsulfanyl-phenyl)-methyl]-piperazin-1-yl)-ethyl)-1,3-dihydro-indol-2-one; CID, collision-induced dissociation; TMT, thiol methyltransferase; TPMT, thiopurine methyltransferase.

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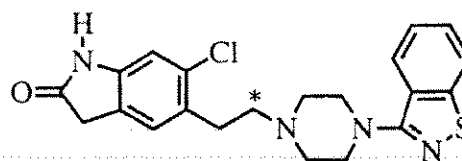


FIG. 1. Structure of ZIP.

Asterisk indicates position of the <sup>14</sup>C-label.

nitrogen evaporator. Derivatization of the purified metabolites **M6** and **M9** (each ~5000 dpm and 100 ng) was conducted by reaction with MTBSTFA or diazomethane using standard procedures (4, 7). The reaction with  $\text{TiCl}_3$  was performed as described (8).

*In vitro* incubations of ZIP with human liver microsomes or S-9 were conducted using standard procedures. The typical incubation mixture (1 ml total volume) contained 100 mM potassium phosphate buffer (pH 7.4), 0.5  $\mu\text{M}$  cytochrome P450 (microsomes) or protein equivalent to 32 mg/ml of liver (S-9),  $^{14}\text{C}$ -ZIP (50  $\mu\text{M}$ , 0.2% dimethylsulfoxide), SAM or  $^3\text{H}$ -SAM (50  $\mu\text{M}$ ), and an NADPH-generating system (9 mM  $\text{MgCl}_2$ , 0.54 mM  $\text{NADP}^+$ , 6.2 mM DL-isocitric acid, and 0.5 units/ml isocitric dehydrogenase). The reactions were initiated by addition of an NADPH regenerating system after a 5-min preincubation period. Reactions were incubated at 37°C for 30 min and were stopped by addition of methanol (2 ml).

Analysis of metabolites was performed on a SCIEX API III HPLC/MS/MS system using ion spray. The ion spray interface was operated at 6000 V, and the mass spectrometer was operated in the positive ion mode. CID studies were performed using argon gas at a collision energy of 25–30 eV and a collision gas thickness of  $3.5 \times 10^{14}$  molecules/cm<sup>2</sup>.

**Results and Discussion.** Representative reversed-phase HPLC tracings showing the metabolite profiles from rat urine and bile are shown in fig. 2, *a* and *b*, respectively. The presence of two metabolites designated **M6** and **M9**, along with several additional metabolites of ZIP, was established in both urine and bile (4). Metabolite **M9** showed a protonated molecular ion at  $m/z$  429, 16 mass units higher than the parent drug. Its CID spectrum ( $m/z$  429) showed fragment ions at  $m/z$  280, 263, 219, 194, 150, and 123 (fig. 3*a*). The ion at  $m/z$  280 corresponds to a charge initiated fragmentation of the piperazinyl nitrogen—benzothiazole carbon bond with the expulsion of the benzothiazole + 16 amu moiety as a neutral molecule. The ion at  $m/z$  150 resulted from the cleavage of the same nitrogen—carbon bond with charge retention on the benzothiazole + 16 amu moiety and suggested the addition of one oxygen atom to the benzothiazole ring. The presence of other characteristic fragment ions at  $m/z$  194 and 263 further suggested that the addition of an oxygen atom had occurred remote from the oxindole part of the molecule. The assignment of these ions was confirmed by a parallel CID spectrum of  $m/z$  431 ( $\text{MH}^+$ ,  $^{37}\text{Cl}$ ), which gave fragment ions at  $m/z$  282, 265, 196, 150, and 123. These results strongly suggested that the oxidation had occurred at the benzothiazole moiety. However, the retention time of **M9** was different from that of the synthetic ZIP-SO standard, suggesting a modification of the benzothiazole other than sulfur oxidation.

Based on addition of 16 mass units to the benzothiazole moiety, three structures were originally considered for **M9** (fig. 4): oxidation at the nitrogen of the benzothiazole ring to form *N*-oxide **1**; aromatic hydroxylation of the benzothiazole moiety **2**; and reductive cleavage of the benzothiazole followed by methylation of the resulting thiophenol to form **3**.

Treatment of the purified metabolite **M9** with aqueous  $\text{TiCl}_3$  did not change the HPLC retention time, the molecular ion, or the CID spectrum of metabolite **M9**. This eliminated an *N*-oxide as a possible structure for **M9**. Reaction of the purified metabolite **M9** with MTBSTFA or diazomethane also resulted in no change in the retention time or MS data, suggesting that a free hydroxyl group was not present. Based on these data, **M9** was tentatively identified as *S*-methyl-dihydro-ZIP **3** (fig. 4).

Metabolite **M6** showed a protonated molecular ion at  $m/z$  445, 32 mass units higher than the parent drug. Its CID spectrum showed fragment ions at  $m/z$  280, 263, 209, 194, 177, 166, and 109, suggesting

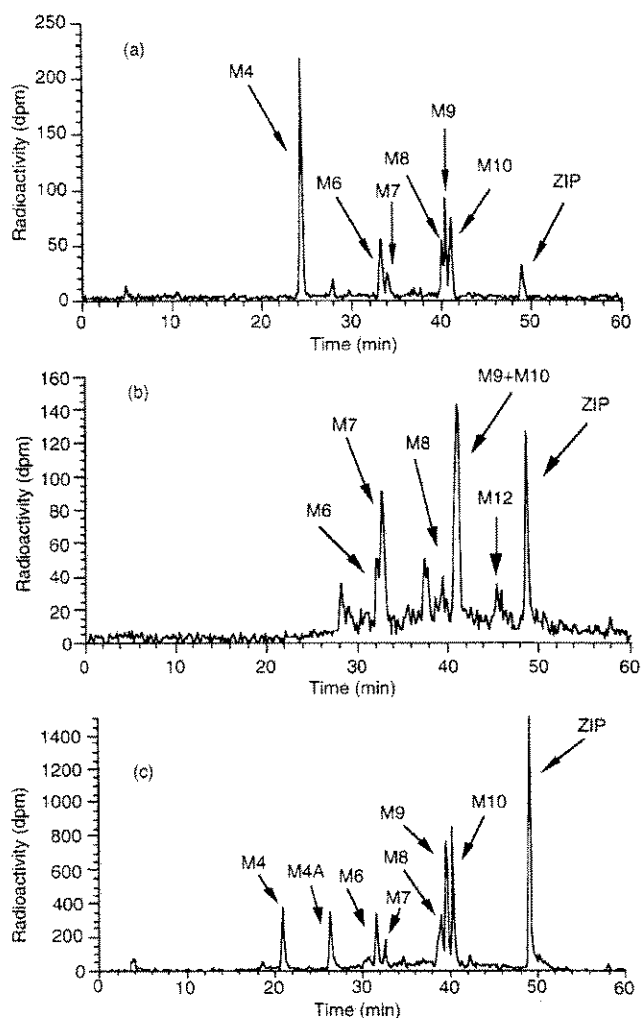


FIG. 2. HPLC radiochromatograms of ZIP metabolites from (a) rat urine, (b) rat bile, and (c) human liver homogenate (S-9).

that the 32 mass units were added on the benzothiazole moiety (fig. 3*b*). **M6** did not coelute with synthetic ZIP-SO<sub>2</sub> on HPLC and thus sulfur dioxygenation of the intact molecule was eliminated as a possible structure. Treatment of **M6** with MTBSTFA or diazomethane did not change the HPLC retention time, or the molecular ion of metabolite **M6**, indicating the absence of a free hydroxyl group. Treatment of **M6** with aqueous  $\text{TiCl}_3$  resulted in the disappearance of **M6** and the appearance of a new peak that had a retention time and MS data similar to those of **M9**. Based on these results, redox relationship between metabolite **M6** and **M9** was established. It was thus concluded that **M6** was a sulfur oxidation product of **M9** and was tentatively identified as *S*-methyl-dihydro-ZIP-sulfoxide.

In an attempt to confirm further the structures of **M6** and **M9**, a series of *in vitro* investigations were attempted. Incubations of ZIP with rat or human liver microsomes did not generate these metabolites (9). However, when microsomes were fortified with SAM, a small amount of metabolite **M9** was detected. Incubation of  $^{14}\text{C}$ -ZIP with S-9 plus an NADPH regenerating system and SAM resulted in the formation of both metabolites **M6** and **M9** (fig. 2*c*). These metabolites showed the HPLC retention times and mass spectral characteristics similar to those of **M6** and **M9** obtained from rat urine as shown in fig. 3*c* for **M9**. Additional experiments using  $^3\text{H}$ -SAM and unlabeled ZIP revealed that the formation of these metabolites required the presence

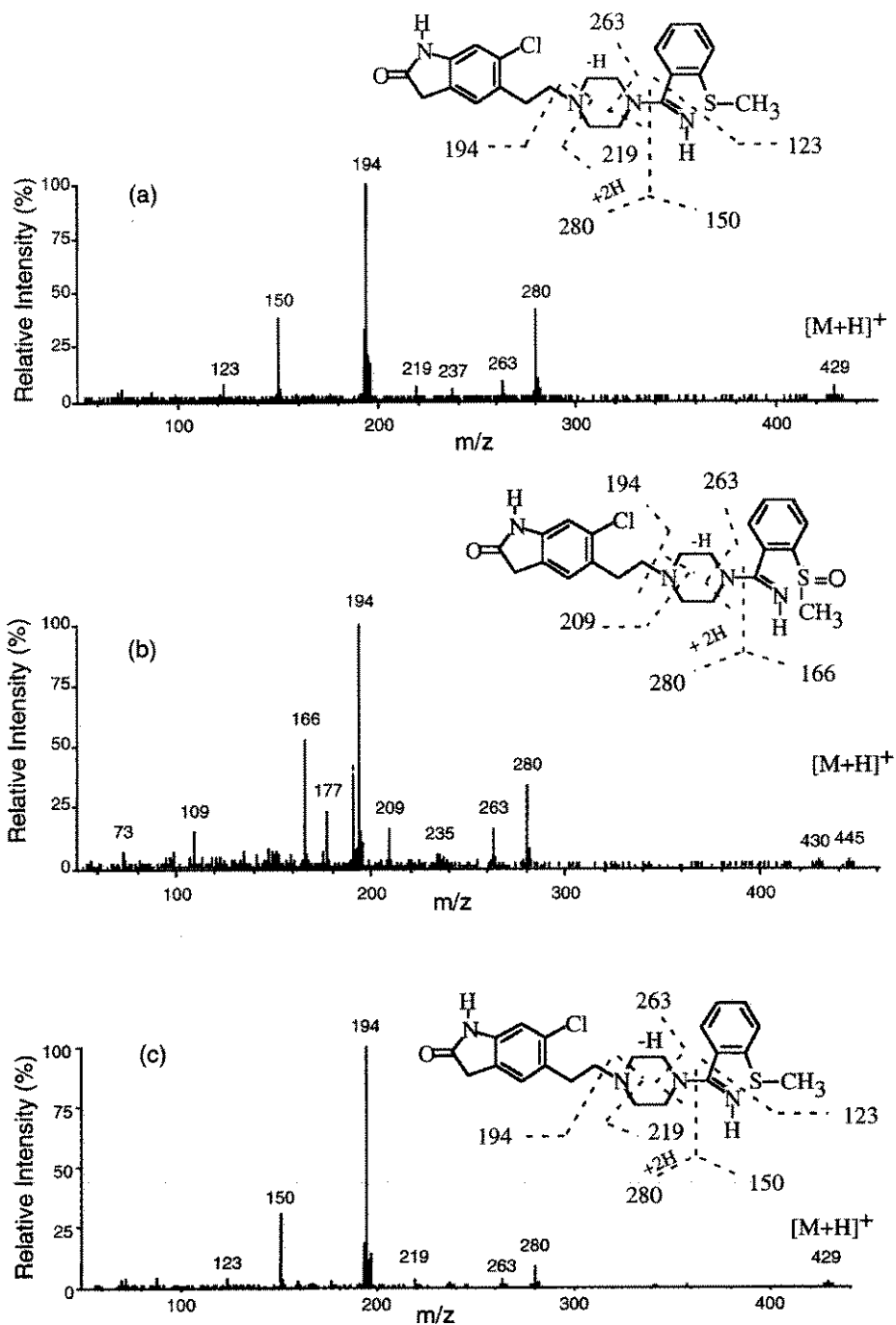


FIG. 3. CID product ion spectra of (a) urinary metabolite **M9**, (b) urinary metabolite **M6**, and (c) **M9** from in vitro incubations.

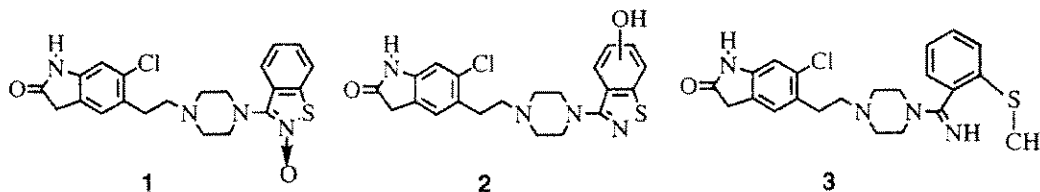


FIG. 4. Possible structures for metabolite **M9**.

of SAM, an essential cofactor for the methylation of thiols, in the incubation medium (10). Attempts to isolate enough amounts of **M6** and **M9** for NMR were unsuccessful. Therefore, **M9** was synthesized

by treatment of dihydro-ZIP with diazomethane or CH<sub>3</sub>I/KOH. **M9** coeluted with synthetic standard on HPLC and had an identical CID spectrum.

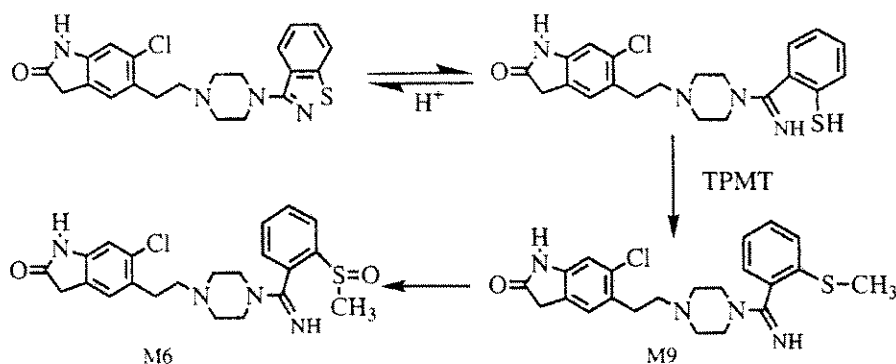


FIG. 5. Proposed pathway for the formation of **M6** and **M9**.

Based on these data, metabolites **M6** and **M9** were identified as benzisothiazole ring-cleaved products. The formation pathway of metabolites **M6** and **M9** is speculated to involve an initial reductive cleavage of the benzisothiazole moiety to give an intermediate with a free thiol function and an amidine. The thiol can be further converted to a methyl thioether by methylation with TMT or TPMT to form **M9** (fig. 5). Confirmation of this hypothesis was obtained by incubation of dihydro-ZIP with S-9 in the presence of SAM, resulting in the formation of **M9**. **M6** was formed by oxidation of **M9** at the sulfur of the S-methyl moiety of benzisothiazole ring.

Although there are several investigational pharmaceutical compounds containing a benzisothiazole structure (11–13), aromatic hydroxylation, N-oxidation, or cleavage of the benzisothiazole have not been reported. N-oxides of 1,2-benzisothiazoles have, however, been prepared by the reaction of 1,2-benzisothiazolium chloride with hydroxylamine (14). The common pathway for the metabolism of benzisothiazole analogs involves oxidation at the sulfur resulting in the sequential formation of sulfoxides and sulfones (5, 6), which suggests that the benzisothiazole moiety is more susceptible to oxidation at the sulfur than it is to aromatic hydroxylation. We have also recently demonstrated that oxidation at the sulfur of the benzisothiazole was one of the major pathways for metabolism of ZIP in rats (4). The other pathways involve N-dealkylation of the ethyl side chain attached to the piperazinyll nitrogen atom and hydration of C—N double bond of the benzisothiazole ring. Two metabolites, also modified on the benzisothiazole ring, were not fully characterized earlier (4). We have now identified these two novel metabolites of ZIP, which resulted from the modification of the benzisothiazole ring via a biotransformation not involving oxidation of the sulfur. These metabolites were formed by the reductive cleavage of benzisothiazole moiety. Similar cleavage has been reported for the compounds possessing a 1,2-benzisoxazole ring structure, risperidone (15), zonisamide (16), and iloperidone (17). Reductive cleavage of benzisoxazoles resulted in the formation of an intermediate imine that is hydrolyzed nonenzymatically to a stable ketone. But, in ZIP, the intermediate amidine was not identified due to its instability under acidic conditions.

The formation of metabolite **M9** involves two steps that are catalyzed by two different enzymes. Our studies speculate that soluble TPMT enzyme might be involved in the methylation of the intermediate thiophenol, similar to that reported for aromatic sulfhydryl compounds (18). The trace amount of **M9** formed in the incubation of ZIP with microsomes may be due to the presence of a small contamination of soluble TPMT in the microsomal preparations. But, we do not presently know which enzymes are involved in reduction of the benzisothiazole. The opening of the benzisoxazole ring of risperidone was attributed to the reduction by gut microflora (15). The reductive

ring opening is a reaction of dihydrogenation and as such must involve transfer of two electrons. It has recently been shown that the reductive ring opening in zonisamide is catalyzed mainly by a specific cytochrome P450 enzyme (CYP3A), in human and rat liver microsomes under anaerobic conditions (19, 20). Because ring-cleaved metabolites of ZIP were found both in urine and bile, we believe that the opening of the benzisothiazole ring is also mediated by liver enzymes. Further studies to determine the enzymes involved in the formation of these metabolites are currently under investigation. Finally, the identification of this novel metabolic pathway of ZIP will have relevance to understanding the metabolism of other benzisothiazole-piperazine drugs.

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