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**Structure-Activity Relationship and Elucidation of the Determinant Factor(s) Responsible
for the Mechanism-Based Inactivation of P450 2B6 by Substituted Phenyl Diaziridines.**

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Running Title: Determinant factors for 2B6 inactivation by phenyl diaziridines

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¹Abbreviations: P450, cytochrome P450; reductase, NAPDH-cytochrome P450 reductase; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; SAR, structure-activity relationship; MBI, mechanism-based inactivator; LC, liquid chromatography; GC, gas chromatography; HR, high resolution; MS, mass spectrometry; ESI, electrospray ionization; GSHEE, glutathione ethyl ester; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; r.t., retention time; APCI, atmospheric pressure chemical ionization.

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Abstract

It has been previously demonstrated that several 3-trifluoromethyl-3-(4-alkoxyphenyl) diaziridines inhibit the 7-ethoxy-4-(trifluoroethyl)coumarin (7-EFC) *O*-deethylation activity of P450 2B6 in a mechanism-based manner. In contrast, 3-trifluoromethyl-3-((4-methylthio)phenyl) diaziridine did not have any effect on the activity of P450 2B6. Interestingly, both the alkoxy and the thiophenyl compounds were metabolized by P450 2B6. In this report, the structure-activity relationships (SAR) for the mechanism-based inactivation of cytochrome P450 2B6 by a series of aryl diaziridines were investigated. Three diaziridines that did not contain a 4-alkoxy- substituent on their phenyl ring, namely, 3-trifluoromethyl-3-(3-methoxyphenyl)diaziridine, 3-trifluoromethyl-3-phenyl diaziridine and, 3-trifluoromethyl-3-(4-chlorophenyl)diaziridine had no effect on the P450 2B6 7-EFC activity. Another analog that did not contain a diaziridine substructure, 3-trifluoromethyl-3-(4-methoxyphenyl)ethanone, also had no effect on the activity of P450 2B6. Glutathione ethylester adducts of the phenyldiaziridine reactive intermediates were isolated from reaction mixtures of the inactivated samples and analyzed by LC-MS/MS. The structures of the conjugates suggested that the electrophilic reactive intermediate in each case was a quinone methide (quinomethane), 4-ethylidene-cyclohexa-2,5-dienone, generated from the 4-alkoxyphenyldiaziridines by removal of both of the diaziridine and the 4-alkyl groups. In

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conclusion, the determinant factor for the mechanism-based inactivator activity of the aryl diaziridines seems to be the formation of the reactive quinomethane intermediate, which is generated from the 4-alkoxyphenyl diaziridines by a P450-catalyzed metabolic reaction.

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An important part of drug design focuses on the characterization of the metabolic profile of the candidate drugs by the enzymes responsible for the metabolism and clearance of the new drugs to elucidate if metabolism may lead to the formation of reactive intermediates. Formation of reactive intermediates might lead to potentially dangerous side effects due to potent drug-drug interactions resulting from mechanism-based inactivation (Zhou et al, 2005; Jones and Hall, 2002), or due to idiosyncratic drug reactions caused by reactive metabolites that escaped from endogenous scavengers such as glutathione and reacted with various cellular macromolecules (Utrecht, 2003a; Seguin and Utrecht, 2003b; Nassar and Lopez-Anaya, 2004; Evans et al, 2004). Thus, precise structure-activity relationships (SAR) to understand the biochemical mechanisms of metabolism and the generation of reactive intermediates that may act as mechanism-based inactivators (MBIs) are required in the early stages of the contemporary drug discovery in order to provide safer drugs to the clinical stage.

Recent efforts to understand the SAR involved in mechanism-based inactivation of P450s have seen an intensified interest in elucidating the structural basis of P450 function, thus aiding in the discovery and design process of drugs metabolized by P450 enzymes. Several studies have reported a variety of different functional groups likely to cause MBI. These functional groups are easily transformed to radical or carbene species that can react with the apoprotein or the heme of the P450s (Correia M. et al, 2005). In some cases, the ability of a series of

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compounds to act as MBI varies depending on the structures of the analogs even though the compounds contain the same reactive functional group that theoretically could lead to the formation of a reactive intermediate. For instance, tienilic acid is a potent MBI of P450 2C9 (Lopez-Garcia et al, 1994; Lopez Garcia et al, 1993) and the inactivation is due to the metabolism of tienilic acid at its thiophenyl structure (Koenigs et al, 1999). However, ticlopidine and clopidogrel, which also contain the thiophenyl substructure, shows much weaker inhibition of P450 2C9, but inhibits P450s 2C19 and 2B6 in a mechanism-based manner (Ha-Duong et al, 2001; Richter et al, 2004). Similarly, the antidiabetic drugs troglitazone, pioglitazone, and rosiglitazone are all thiazolidinedione derivatives causing mechanism-based inactivation of P450 3A4. However, the potencies for inactivation by the three compounds varied 6-fold (Lim et al, 2005). Troglitazone, the most potent MBI, contained other substructures that could be responsible for production of reactive intermediates (Yamamoto et al, 2002; He et al, 2004; Reddy et al, 2005). This suggests that a single potential reactive functional group may not be the only contributing factor for inactivation and that other functional substructures within the compounds may also contribute to mechanism-based inactivation. Thus, in the drug discovery process it becomes necessary to investigate more precisely the “determinant factor(s)” in the structure of a compound which may be responsible for the mechanism-based inactivation rather than to investigate only the potential reactive group. It has

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been previously reported that several (4-alkoxyphenyl)diaziridines inactivated P450 2B6 in a mechanism-based manner whereas (4-(methylthio)phenyl)diaziridine had no effect on P450 2B6 (Sridar et al., 2006). These results are of interest since all of the alkoxy and the thiophenyl compounds contained a diaziridine moiety. The purpose of the current study was to investigate the SAR for mechanism-based inactivation by a series of these diaziridines in conjunction with additional aryl diaziridine analogs.

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Materials and Methods

Six of the phenyldiaziridines shown in Figure 1, namely, 3-trifluoromethyl-3-(4-methoxyphenyl) diaziridine (**1**), 3-(4-ethoxyphenyl)-3-(trifluoromethyl) diaziridine (**2**), 3-trifluoromethyl-3-(3,4-dimethoxyphenyl)diaziridine (**3**), 3-trifluoromethyl-3-(3,4,5-trimethoxyphenyl)diaziridine (**4**), 3-trifluoromethyl-3-(3-methyl-4-methoxyphenyl) diaziridine (**5**), 3-trifluoromethyl-3-(4-(methylthio)phenyl) diaziridine (**6**) and 2,2,2-trifluoro-1-(4-methoxyphenyl)ethanone (**10**) were synthesized as previously described (DMD/2006/010082). 3-(3-methoxyphenyl)-3-(trifluoromethyl)diaziridine (**7**) and 3-(trifluoromethyl)-3-phenyl-diaziridine (**9**) were also synthesized as previously described (Brunner et al.1980; Hatanaka et al, 1994), and the analytical and spectroscopic analysis were in accord with the published data. 4-Bromophenol-2,3,5,6-d₄ (CAS 152404-44-9) was purchased from C/D/N Isotopes (Quebec, Canada) and methylated according to the reported procedure for the synthesis of **12b** (Cho et al.,1993). All remaining chemicals and reagents for the synthesis of compounds used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Thin-layer chromatography was performed on Merck silica gel 60 F₂₅₄ plates. Column chromatography was performed using standard grade Sorbent Technologies silica gel with a particle size of 32-63 μ M. Mass spectral data was acquired using a Waters ZQ LCMS system (ESI+ mode), and HRMS data was acquired using a Micromass QTOF. Elemental Analysis was performed using a Perkin Elmer Series II

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2400 CHNS/O Elemental Analyzer. ^1H NMR spectra were recorded at 400 MHz or 500 MHz, and ^{13}C NMR spectra were recorded at 100 MHz or 125 MHz using Bruker Avance DXR systems. NADPH, bovine serum albumin (BSA), glutathione ethylester (GSHEE), and catalase were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl)coumarin (7-EFC) was obtained from Molecular Probes, Inc. (Eugene, OR). Bergamottin was purchased from Indofine Chemical Co., Inc. (Somerville, NJ). In order to better understand the contribution of the substituents at the 4-position of the phenyl ring to the inactivation of P450 2B6 by phenyldiaziridines, three additional phenyldiaziridine analogs were synthesized (Figure 1). They are: 3-(3-methoxyphenyl)-3-(trifluoromethyl)diaziridine (**7**), which contains a *meta*-methoxy substituent, 3-(4-chlorophenyl)-3-(trifluoromethyl)diaziridine (**8**) containing a metabolically inert functionality, and 3-trifluoromethyl-3-phenyl diaziridine (**9**), devoid of aryl substituents. In addition, in order to understand the importance of the diaziridine substructure, 2,2,2-trifluoro-1-(4-methoxyphenyl)ethanone (**10**), an analog of compound **1** that contains a ketone instead of the diaziridine substructure, was also investigated. A deuterated analog of compound **1**, 3-(4-methoxy-(2,3,5,6- $^2\text{H}_4$))phenyl-3-(trifluoromethyl)diaziridine (**11**), was synthesized and used for LC-MS/MS structural experiments.

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Synthesis

Synthesis of 2,2,2-trifluoroacetophenones (13a and 13b). Mg turnings (1.2 g, 0.05 mol), substituted bromobenzenes, **12a** or **12b** (0.05 mol) and anhydrous THF (40 mL) were placed in a round bottom flask. The mixture was slowly heated to reflux and maintained until all the magnesium was dissolved. The mixture was cooled in an ice bath, and a solution of N-trifluoroacetyl piperidine (0.05 mol) in anhydrous THF (15 mL) was added slowly to the Grignard reagent over a period of 0.5 h with stirring at 0 °C. The reaction mixture was stirred for 2 h at ambient temperature, and the reaction was quenched by the addition of saturated aqueous ammonium chloride (5 mL). The precipitated solids were filtered, the filtrate dried over Na₂SO₄ and evaporated *in vacuo*, and the residual oil was purified by silica gel column chromatography eluting with hexanes/ CH₂Cl₂ (95:5) to give the product ketones as pale yellow to colorless oils.

1-(4-chlorophenyl)-2,2,2-trifluoroethanone (13a). Yield, 6.6 g (65%); ¹H NMR (CDCl₃): δ 7.50 (d, 2H, *J*=8.0 Hz), 8.07 (d, 2H, *J*=8.0 Hz) (Kesavan et al., 2000).

2,2,2-Trifluoro-1-(4-methoxy-(2,3,5,6-²H₄)phenyl)ethanone (13b). Yield, 6.3 g (62%); ¹H NMR (CDCl₃): δ 3.91 (s, 3H). ¹³C NMR (CDCl₃): δ 55.80, 114.03 (t, *J*= 25 Hz, C-D), 116.87 (q, *J*= 288 Hz, C-F₃), 122.50, 132.19 (t, *J*= 25 Hz, C-D), 165.14, 178.57 (q, *J*= 34 Hz, O=C-CF₃).

Synthesis of Oximes (14a and 14b). Hydroxylamine hydrochloride (0.0625 mol) was added

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to a solution of ketone **13a** or **13b** (0.025 mol) in absolute ethanol (15 mL) and dry pyridine (20 mL) and heated at 60 °C for 8.0 h. The solvent was then removed *in vacuo* and the remaining residue was dissolved in diethyl ether (40 mL) and washed with 1N HCl to remove residual pyridine. The organic layer was then washed successively with water (50 x 2 mL), brine, and dried over Na₂SO₄. After evaporation of the solvent, the crude oxime was purified by silica gel column chromatography eluting with ethyl acetate/dichloromethane (5:95) to yield product oximes as white solids.

1-(4-chlorophenyl)-2,2,2-trifluoroethanone oxime (14a). Yield, 4.3 g (78%); mp 65-67 °C; ¹H NMR (CDCl₃): δ 7.50 (d, *J*=8.5 Hz, 2H), 7.54 (d, *J*=8.5 Hz, 2H), 9.36 (bs, 1H). ¹³C NMR (CDCl₃): δ 120.37 (q, *J*= 272 Hz, CF₃), 123.90, 128.89, 130.06, 136.94, 146.51 (q, *J*= 32 Hz, N=C-CF₃). HRMS: *m/z* [MH]⁺ 224.0091. (C₈H₆ClF₃NO requires 224.0090).

2,2,2-Trifluoro-1-(4-methoxy-(2,3,5,6-²H₄)phenyl)ethanone oxime (14b). Yield, 3.7 g (68%); mp 100-103 °C; ¹H NMR (CDCl₃): δ 3.85 (s, 3H), 8.95 (bs, 1H). ¹³C NMR (CDCl₃): δ 55.71(OCH₃), 113.57 (t, *J*= 24 Hz, 2C-D), 117.70, 118.66 (q, *J*= 241 Hz, CF₃), 130.05 (t, *J*= 24 Hz, 2C-D), 146.90 (q, *J* = 66 Hz, N=C-CF₃), 160.87. HRMS: *m/z* [MH]⁺ 224.0830. (C₉H₅D₄F₃NO₂ requires 224.0836).

Synthesis of *p*-toluenesulfonyl oximes (15a and 15b). *p*-Toluenesulfonyl chloride (12.0 mmol) was added to a stirred solution of oxime **14a** or **14b** (5.0 mmol) in triethylamine (9.0

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mmol) and 4-dimethylaminopyridine (1.0 mmol) in methylene chloride (10 mL) at 0 °C in lot wise manner under N₂. The reaction was allowed to stir for 2.0 h at RT. The reaction mixture was quenched with water and the organic phase was separated, washed successively with water, brine, and dried over Na₂SO₄. The solvent was evaporated *in vacuo*, and the crude product was purified by silica gel column chromatography eluting with hexanes/CH₂Cl₂ (80:20) yielding product tosyl oximes as white solids.

1-(4-chlorophenyl)-2,2,2-trifluoro-1-ethanone-O-[(4-methylphenyl)sulfonyl] oxime (15a).

Yield, 1.35 g (72%); mp 130-131 °C; ¹H NMR (CDCl₃): δ 2.50 (s, 3H), 7.38 (d, *J*=8.5 Hz, 2H), 7.41 (d, *J*=8.0 Hz, 2H), 7.48 (d, 2H, *J*=8.5 Hz), 7.90 (d, 2H, *J*=8.0 Hz). ¹³C NMR (CDCl₃): δ 22.16, 119.42 (q, *J*=275 Hz, C_{CF3}), 122.80, 129.20 (4C), 129.80 (2C), 129.84 (2C), 130.93, 138.06, 146.15, 152.76 (q, *J*=33 Hz, N=C-CF₃). Anal. Calcd for C₁₅H₁₁ClF₃NO₃S: C, 47.69; H, 2.93; N, 3.71. Found: C, 47.58; H, 2.81; N, 3.64. HRMS: *m/z* [MH]⁺ 378.0176 (C₁₅H₁₂ClF₃NO₃S: requires 378.0179).

2,2,2-Trifluoro-1-(4-methoxy-(2,3,5,6-²H₄)phenyl)-1-ethanone-O-[(4-methylphenyl)

sulfonyl] oxime (15b). Yield, 1.22 g (65%); mp 114-115 °C; ¹H NMR (CDCl₃): δ 2.50 (s, 3H) 3.88 (s, 3H), 7.40 (d, 2H, *J*= 8.0 Hz), 7.91 (d, 2H, *J*= 8.0 Hz). ¹³C NMR (CDCl₃): δ 22.14, 55.66, 113.8 (t, *J*= 25 Hz , 2C-D), 116.22, 120.50 (q, *J*= 235 Hz, C_{CF3}), 129.18 (2C), 129.73 (2C), 130.27 (t, *J*= 25 Hz , 2C-D), 131.21, 145.85, 152.90 (q, *J* = 60 Hz, N=C-CF₃), 161.81.

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(Anal. Calcd for $C_{16}H_{10}D_4F_3NO_4S$: C, 50.92; (H+D) as H, 3.78; N, 3.71. Found: C, 50.90; H, 3.54; N, 3.64. HRMS: m/z $[MH]^+$ 378.0929 ($C_{16}H_{11}D_4F_3NO_4S$: requires 378.0925).

General Procedure for the Preparation of Diaziridines (8 and 11) Tosyl oximes (**15a** or **15b**,

1.0 mmol) and anhydrous diethyl ether (10 mL) were placed in a three-necked round bottom flask equipped with a dry ice condenser and a gas inlet. The solution was cooled to -78 °C and approximately 5 mL of anhydrous NH_3 was condensed into the flask. The solution was stirred for 1.0 h at -78 °C. The cooling bath was removed and the gas inlet was replaced with a drying tube. The solution was stirred at ambient temperature while NH_3 refluxed for 2.0 h. The condenser was removed and the ammonia was allowed to evaporate. The remaining residue was dissolved in ethyl ether, washed with water and brine, dried over Na_2SO_4 , and concentrated to afford the crude diaziridines, which were subsequently purified by column chromatography (Si gel), 1-5% ethyl acetate/99-95% $CHCl_3$) to yield product diaziridines as white solids.

3-(4-Chlorophenyl)-3-(trifluoromethyl)diaziridine (8). Yield, 0.16g (75%); amorphous solid,

1H NMR ($CDCl_3$): δ 2.22 (bs, 1H), 2.83 (bs, 1H), 7.43 (d, 2H, $J=8.5$ Hz), 7.58 (d, 2H, $J=8.5$ Hz).

Anal. Calcd for $C_8H_6ClF_3N_2$: C, 43.17; H, 2.72; N, 12.58. Found: C, 43.25; H, 2.78; N, 12.49.

HRMS: m/z $[MH]^+$ 223.0258. ($C_8H_7ClF_3N_2$ requires 223.0250).

3-(4-methoxy-(2,3,5,6- 2H_4)phenyl)-3-(trifluoromethyl)diaziridine (11). Yield, 0.14 g (65%);

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mp 74 °C; ¹H NMR (CDCl₃): δ 2.19 (bs, 1H), 2.78 (bs, 1H), 3.85 (s, 3H). Anal. Calcd for C₉H₅D₄F₃N₂O: C, 48.65; (H+D) as H, 4.16; N, 12.61. Found: C, 48.43; H, 3.89; N, 12.36.

HRMS: *m/z* [MH]⁺ 223.0987. (C₉H₆D₄F₃N₂O requires 223.0996).

Purification of Enzymes. P450 NADPH-reductase was expressed in *Escherichia coli* Topp3 cells and the purification was carried out as described previously (Hanna et al, 1998). P450 2B6 was expressed in *E. coli* Topp3 cells and purified as described previously (Hanna et al, 2000).

Time-Dependent Inactivation of P450 2B6 7-EFC Activity. P450 2B6 was reconstituted with reductase at 4 °C for 45 min as previously described (Sridar et al., 2006). The primary reaction mixture contained 0.05 μM P450 2B6, 0.1 μM NADPH-reductase, 50 units of catalase, and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.1 mL. The phenyl diaziridine analogs or the ketone analog of compound **1** in DMSO were added to each sample. The final concentrations were 20 μM for compounds **1**, **7**, **8**, and **9**, and 100 μM for compounds **6** and **10**. Control samples received only DMSO. The final concentration of DMSO in each sample was 1% (v/v). After the reaction mixtures were allowed to equilibrate at 30 °C for 15 min, the reactions were initiated by the addition of 1.2 mM NADPH (primary mixture). Aliquots (8 μL, 0.4 pmol of P450 2B6) were removed at 0, 2, 4, 10, and 16 min and added to 988 μL of a secondary reaction mixture containing 1 mM NADPH, 100 μM 7-EFC, and 40 μg/mL BSA in

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50 mM potassium phosphate buffer, pH 7.4. The secondary reaction was allowed to proceed at 30 °C for 10 min and was then stopped with 334 μ L of cold acetonitrile. The amount of 7-(hydroxy-4-trifluoromethyl) coumarin that was formed was determined spectrofluorometrically on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD) with excitation at 410 nm and emission at 510 nm.

Metabolism. Reaction mixtures contained 0.08 nmol of purified P450 2B6, 0.16 nmol of reductase and 50 units of catalase in a total volume of 1 mL of 50 mM potassium phosphate buffer, pH 7.4. The compounds (**1** and **6-10** dissolved in DMSO) were added to each sample. The final concentration of the compounds was 200 μ M and the final concentration of the DMSO was 1% (v/v). The reactions were initiated by the addition of 1.2 mM NADPH to the 50 μ L of incubation mixtures. The control sample received the same amount of water instead of NADPH. The samples were incubated at 30 °C for 90 min and the reactions were terminated by the addition of 1 mL of acetonitrile. Bergamottin (10 μ M) was added as an internal standard. The mixtures were centrifuged at 13,200 x g at room temperature for 30 min and 100 μ L of the supernatants was injected onto a 4.6 x 150-mm Waters Symmetry C18 reverse phase column. HPLC was performed using a Waters 600E HPLC system with Waters 501 series pumps, a Waters 996 photodiode array detector, and a Waters 717 autosampler (Waters Corporation, Millford, MA). The initial conditions were 98 % solvent A (0.1% (v/v) acetic acid in water)

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and 2 % solvent B (0.1% (v/v) acetic acid in acetonitrile) for 2 min at a flow rate of 0.8 mL/min.

The percentage of B was increased by a linear gradient to 70% B from 2 to 15 min and 70% B was maintained for 5 min. The percentage of B was then increased linearly to 95% B from 20 to 25 min and maintained from 25 to 35 min. The percentage of B was then decreased to the initial conditions over the course of 5 min and the column was equilibrated at 2% B for 10 min prior to a new injection. The peak areas of the compounds were integrated at their maximum absorbance wavelength between 210-300 nm: i.e. 270 nm for compound **1**, 260 nm for compound **6**, 274 nm for compound **7**, 220 nm for compound **8**, 260 nm for compound **9**, and 297 nm for compound **10**. The internal standard bergamottin was integrated at 308 nm. The amount of the compound metabolized in each sample was estimated from the ratio of the peak area of the compound of interest compared to the area of the internal standard. The metabolic ratio of each compound after incubation with P450 was estimated from the ratio of the amount of the compound in each sample compared to the amount in the control sample.

Metabolite Identification using GC-MS. The reaction mixtures containing compounds **1** or **6** were prepared as above except that methanol was used as the solvent in the stock solutions for these compounds. The reaction mixtures were quenched with 1 mL of ethyl acetate and extracted twice with ethyl acetate. After extraction, the organic phases were evaporated under a stream of nitrogen to approximately 20 uL and 2 uL of each sample was injected onto the

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GC-MS system. Metabolites were analyzed on a HP6890/MSD5973 gas chromatography-mass spectrometer (Agilent Technologies, USA). The metabolites were separated on a DB-210 capillary column (20 m x 0.18 mm x 0.3 μ m, J&W Scientific, USA) with helium as the carrier gas. 2 μ L aliquots of the ethyl acetate extracts were injected by pulsed splitless injection. The injector temperature was 250 $^{\circ}$ C. The pulse pressure was set at 40 psi for 1 min. The initial oven temperature was 50 $^{\circ}$ C and it was increased to 200 $^{\circ}$ C at 20 $^{\circ}$ C/min after injection. After each run, the oven temperature was increased to the isothermal temperature of the DB-210 column (240 $^{\circ}$ C) and held for 5 min to clean the column. The mass spectra for the compounds eluting from the DB-210 column were obtained by scanning the MS detector in the range of 35-550 amu.

Structure Elucidation of GSHEE Conjugates of the Metabolites of the Phenyldiaziridines.

The reaction mixtures contained 0.25 nmol of purified P450 2B6, 0.5 nmol of reductase, 120 units of catalase, and 10 mM of GSHEE (Soglia et al, 2004) in a total volume of 0.125 mL of 50 mM potassium phosphate buffer, pH 7.4. Each compound tested was added from a stock solution prepared in DMSO. The final concentration of each compound was 400 μ M. The metabolic reaction was initiated by the addition of 1.2 mM NADPH. Control samples received the same amount of water instead of NADPH. The samples were incubated at 30 $^{\circ}$ C for 80 min and the reactions were terminated by the addition of 1 mL of acetonitrile. The mixtures were centrifuged at 13,200 x g at room temperature for 30 min. The supernatants were transferred to

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new tubes and dried down under a stream of nitrogen. The residues were dissolved in 150 μ L of 50% (v/v) acetonitrile in water and injected (70 μ L) onto a Phenomenex Luna C18 reverse phase column (4.6 x 100 mm). ESI-LC-MS/MS was carried out using a ThermoQuest LCQ ion trap mass spectrometer interfaced with a Hewlett Packard 1100 series HPLC system. The sheath gas was set at 80 (arbitrary units) and the auxiliary gas was set at 15 (arbitrary units). The spray voltage was 5kV and the capillary temperature was 200 °C. The flow rate was 0.3 mL/min. Initial conditions were 95 % of 0.1% (v/v) acetic acid in water (solvent A) and 5 % of 0.1% (v/v) acetic acid in acetonitrile (solvent B). The percentage of B was maintained at 5% for 5 min followed by a linear gradient to 30% B from 5 to 15 min, to 80% B from 15 to 35 min, and to 90% B from 35 to 40 min. The column was washed with 90% B for 15 min before returning to the initial conditions and equilibrating the column for 10 min at the initial conditions prior to the next injection.

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Results

Effect of Phenyldiaziridines without a 4-Alkoxy Group on the Activity of P450 2B6.

Purified reconstituted cytochrome P450 2B6 was incubated for 30 min at 30 °C with NADPH and 1 mM of each of the compounds **6**, **7**, **8** or **9** that did not contain a 4-alkoxy group and compound **10**, which contained the 4-alkoxy group and the diaziridine substructure replaced with the ketone. No significant loss in the 7-EFC *O*-deethylation activity of P450 2B6 was observed with any of these phenyldiaziridines (Figure 2). However, incubation of P450 2B6 with 20 μ M of compound **1** in the presence of NADPH resulted in a time-dependent loss in enzymatic activity as reported previously (Sridar et al., 2006). After 16 min of incubation, only 33% of the activity remained (Figure 2). It has been reported previously that compounds **2-5** also cause mechanism-based inactivation of P450 2B6 (Sridar et al., 2006).

Metabolic Stability of the Phenyldiaziridines without a 4-Alkoxy Group. The metabolic stability of the various phenyldiaziridines was evaluated to determine the rate of disappearance of the parent compounds due to metabolism. In our studies, HPLC–UV was used to estimate the amount of the phenyl diaziridines remaining after incubation with recombinant P450 2B6 in a reconstituted system. Purified reconstituted cytochrome P450 2B6 was incubated for 90 min at 30 °C with NADPH and 1 mM of each of the compounds (**1**, **6**, **7**, **8**, **9**, or **10**). The percentage of each compound remaining was estimated from the ratio of the area of the peak of the LC-UV

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chromatogram compared to the same peak in control samples and was found to be 93%, 40%, 85%, 79%, 98% and 79% for compounds **1**, **6-10**, respectively (Figure 3). In addition to the parent compounds, peaks corresponding to metabolites were also observed for compounds **6**, **7**, **8**, and **10** (data not shown). Compound **6**, the 4-(methylthio)phenyl analog, was extensively metabolized.

Structural Elucidation of Metabolites of the Phenyldiaziridines by GC-MS. Purified, reconstituted cytochrome P450 2B6 was incubated with compounds **1** and **6** and the metabolites were identified by GC-MS. For each of the phenyldiaziridines, a metabolite with a m/z 14 mass units smaller than its respective parent (204 amu for compound **1** and 220 amu for compound **6**) was found as the major peak in each sample (Figure 4 (a) and Figure 4 (c)), respectively. To confirm the structure assignment of the metabolites, the ketone standards **10** and **16** (the structures are shown in Figure 1 and Scheme 1, respectively), were subjected to GC-MS using the same conditions. The results reported in Figure 4 reveal that the retention times and the fragmentation patterns for the metabolite of **1** (Figure 4 (a)) and that of **6** (Figure 4 (c)) on GC-MS are identical with those of the authentic standards **10** (Figure 4 (b)) and **16** (Figure 4 (d)), respectively. Although some additional peaks were observed in the range of $m/z < 61$, they are thought to be derived from the glycerol contained in the P450 2B6 stock solution.

Signals corresponding to metabolites of compound **1** were not found using ESI-LC-MS

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analysis in either the positive or negative modes. The standard solutions of **10** or **16** also did not give any peaks on ESI-LC-MS. However, we were able to observe these ions using electron ionization in the GC-MS system. The metabolite ions were also not observed on an APCI instrument. Similarly, no metabolites were observed for compounds **2-5** and **7-9** when the reaction mixtures were analyzed using ESI-LC-MS. In contrast, two metabolite peaks were observed for compound **6** on the ESI-LC-MS system. The m/z values of these peaks were not identical with **16** (data not shown). Attempts at structure identification of these compounds are in progress.

Glutathione Ethylester Conjugates of the Metabolites of Phenyldiaziridines. The electrophilic reactive metabolites that were generated by incubating compounds **1-5** with recombinant 2B6 in the reconstituted system in the presence of NADPH were trapped using the nucleophilic trapping reagent, GSHEE, and analyzed by LC-MS (Soglia et al, 2004). GSHEE has been reported to be a more useful trapping reagent than glutathione for monitoring trapped adducts of electrophilic intermediates by LC-MS analysis. Figure 5 shows a representative extracted ion chromatogram obtained from a reaction mixture where P450 2B6 was incubated with compound **1** and NADPH in the presence of GSHEE. The peak eluting at approximately 25 minutes with $m/z=510$ (Figure 5 (a)) was observed only in samples incubated in the presence of NADPH. Figure 5 (a) also shows the MS/MS fragmentation pattern for the ion with m/z 510.

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The MS/MS spectrum of the adduct exhibits prominent ions at m/z 381 and 407 which are characteristic of a loss of 129 and 103 mass units corresponding to loss of the pyroglutamate and glycylolether ester of the GSHEE moiety, respectively. These results indicate that the peak corresponds to a GSHEE adduct generated by the attack of an electrophilic intermediate produced during metabolism of **1** by P450 2B6 on GSHEE. The fragmentation pattern of the GSHEE adduct (Figure 5 (a)) also suggests that the diaziridine substructure of compound **1** was metabolized to form the reactive intermediate. Interestingly, the MS/MS results also indicate that the methoxy group of compound **1** has been converted to a hydroxyl group, presumably by O-demethylation. The exact position where the GSHEE was adducted to the reactive diaziridine intermediate could not be determined from the MS spectrum.

Similar adducts were observed for compounds **2-5** when they were incubated with recombinant 2B6 in a reconstituted system. Their MS/MS spectra were similar to that observed with compound **1** (data not shown). The structures of the GSHEE adducts of compounds **1-5** are summarized in Figure 6. All of the adduct structures indicate that the reactive intermediates are generated by the metabolism-dependent loss of both the diaziridine moiety and the alkoxy group from the parent compounds **1-5**.

Determination of the Position at which GSHEE Reacts to Form the Adduct. In order to identify the position where the GSHEE reacts to form a covalent adduct with the reactive

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intermediate formed by compound **1**, the deuterated compound **11** (Figure 1) was incubated with 2B6 and the GSHEE-adduct was analyzed by MS in the same way as compound **1** was. Figure 5 (b) shows the extracted ion chromatogram. A peak with an m/z of 514 was observed having essentially the same retention time as the GSHEE-adduct of compound **1** (Figure 5 (b)). The fragmentation pattern indicates that the peak with an m/z of 514 shown in the chromatogram corresponds to a GSHEE-adduct produced from compound **11**. The MS/MS fragmentation of the adduct exhibits prominent ions at m/z 385 and 411 which are characteristic of a loss of 129 and 103 mass units corresponding to the GSHEE moiety. The m/z of the GSHEE adduct generated with the deuterated compound **11** was 514, exactly 4 mass units higher than the adduct formed by reaction of GSHEE with the reactive intermediate formed by compound **1** (m/z 510). Although another adduct with m/z 513 was also detected, the amount of this adduct was less than one tenth of the adduct with m/z 514, based on estimates from their peak areas. This result indicates that all four of the deuterium atoms on the phenyl ring were retained in this GSHEE adduct, indicating that GSHEE was not conjugated to the phenyl ring of the deuterated compound **11**.

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Discussion

The SAR study presented here, which focused on identifying those determinant factors in the compound structures of the phenyl diaziridines responsible for mechanism-based inactivation was prompted by the initial studies (Sridar et al., 2006) that demonstrated that a series of diaziridine compounds inactivated P450 2B6 in a time-, concentration, and NADPH-dependent fashion in the reconstituted system. The five 3-trifluoromethyl-3-(4-alkoxyphenyl)diaziridines labeled **1-5** in Figure 1 all inhibited P450 2B6 in a mechanism-based manner. Kinetic parameters for the inactivation of P450 2B6 by these five compounds were determined. Analysis of the inactivated samples by determination of changes in the reduced-CO spectrum or HPLC of the heme suggested that the primary loss in activity was not due to heme destruction. Replacement of the methoxy moiety on the diaziridine compounds with a methylthio group abolished the mechanism-based inactivation of P450 2B6 as measured using the 7-EFC assay.

In this study, a series of analogs of the diaziridines have been used to understand the structural determinants involved in the mechanism of inactivation. The presence of a trifluoromethyl group in each of the diaziridine analogs served to enhance the reactivity of the carbene, if formed via the CYP catalyzed reaction. Carbene formation from trifluoromethylaryl diazirine photolysis is well established, and leads to O-H, N-H and C-H insertion with no intramolecular rearrangements (Hatanaka et al., 1996). In addition, GSHEE was used as a

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nucleophilic trapping agent to identify the pathway leading to the formation of the reactive intermediate. An analog of compound **1** containing a ketone moiety instead of the diaziridine substructure, 3-trifluoromethyl-3-(4-methoxyphenyl)ethanone (**10**), was synthesized (Figure 1). Interestingly, no inactivation of 7-EFC activity was seen when compound **10** was incubated with P450 2B6 in the presence of NADPH. Although compound **10** was presumably produced from compound **1** as a result of the metabolism by 2B6 (Figure 4), compound **10** itself is not the reactive metabolite responsible for inactivating the enzyme. This suggests that the diaziridine structure is essential but not sufficient for mechanism-based inactivation of the P450s. 3-Trifluoromethyl-3-phenyl diaziridine (**9**), an analog that does not contain another substituent on the phenyl ring, also did not inactivate P450 2B6, indicating the need for a substituted phenyl ring for the inactivation. 3-Trifluoromethyl-3-(3-methoxyphenyl)diaziridine (**7**), an analog in which the methoxy group was placed at the 3-position of the phenyl ring, also did not inactivate P450 2B6. Another analog of compound **1**, 3-trifluoromethyl-3-(4-chlorophenyl)diaziridine (**8**), in which a chlorine- was substituted for the methoxy- at the 4-position also lacked the ability to inactivate P450 2B6. Taken together, these data clearly demonstrate the combined importance of the 4-alkoxy substructure in addition to the diaziridine substructure for the mechanism-based inactivation of P450 2B6 by these compounds.

If those compounds that did not lead to mechanism-based inactivation were not metabolized

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by the P450, SAR determinant factors affecting metabolism alone would be easy to consider. However, as shown by the results in Figure 3 and 4, the reason for the inability of these compounds to inactivate was not because they were not metabolized by P450 2B6. These results indicate that these MBI-negative compounds were, in fact, metabolized by 2B6 but did not form a reactive intermediate capable of inactivating the P450; whereas, the MBI-positive 4-alkoxyphenyl diaziridines, even though they were metabolized to a lesser extent (Figure 3), generated sufficient levels of reactive intermediates to inactivate the enzyme.

In order to investigate why only the 4-alkoxyphenyl diaziridines are metabolized to reactive intermediates by P450 2B6, the reactive metabolites were trapped using GSHEE and their structures analyzed using LC-MS/MS. GSHEE was used for these studies since the presence of the less polar ethyl ester moiety on the glycine molecule has been reported to make GSHEE more sensitive to MS detection (Soglia et al, 2004). GSHEE adducts were observed using LC-MS for all five of the MBI-positive diaziridines **1-5**, which indicated that an electrophilic reactive intermediate was indeed generated during the metabolism of these compounds by P450 2B6. Interestingly, MS/MS analysis of all of the GSHEE conjugates revealed that not only was the diaziridine substructure lost during metabolism but that the alkoxy group was also removed from the respective parent compounds (Figures 5, 6). MS/MS analysis of the GSHEE adduct formed from a deuterated analog (**11**) of compound **1** demonstrated that the GSHEE moiety was

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not adducted to the phenyl ring (Figure 5) and suggested that this was in all probability also the case for all the other MBI-positive diaziridines **2-5**. Thus, it appears that the most probable structures for the GSHEE adducts are related to structure **18** in Scheme 1.

The most plausible sequence of reactions that takes into consideration all of these results is depicted in Scheme 1. The structure of the reactive intermediate that is ultimately responsible for inactivation and adduct formation must be a *p*-quinone methide (quinomethane), namely the 4-ethylidene-cyclohexa-2,5-dienone (**17**) (Scheme 1a). The sequence of steps leading to its formation involves the initial abstraction of a hydrogen radical from the parent compound (**1-5**) by a one electron oxidation by 2B6. When the second oxidation step occurs at the 3-carbon of the diaziridine, the final metabolite will be a ketone **10** which does not inactivate the enzyme. However, when the second oxidation occurs at the 4-carbon of the phenyl ring, the alkoxy group is lost and the quinone methide **17** is formed. The quinone methide, trapped as a GSHEE adduct **18**, is the most likely candidate to be the intermediate that results in the inactivation of P450 2B6. It is well established that quinone related compounds can form potent reactive intermediates (Fan and Bolton, 2001, Yan et al, 2001, Monks et al, 2002, Utrecht, 2003a, Utrecht, 2003b).

Because the diaziridines **6-9** do not have a 4-alkoxy group, they are much less likely to be transformed into quinone methide reactive intermediates during metabolism by P450s than are compounds **1-5**. A suggested pathway for the metabolism of diaziridine **6** to give the ketone

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metabolite, compound **16**, is shown in Scheme 1b. The inability of compounds **6-9** to act as MBIs is further explained by the inability to trap a reactive intermediate with GSHEE. Therefore, the possibility of metabolic transformation of compounds **6-9** into quinone methides such as compound **17** should be considered to be a critical determinant that decides whether or not a given compound in the series of aryl diaziridines is capable of being an MBI.

Diaziridine substructures tend to be easily metabolized to form radical or carbene intermediates by a one-electron oxidation. This was indeed the initial reason why a series of these phenyldiaziridine compounds was designed to serve as molecular probes that were expected to attack and modify the P450 heme. It was therefore surprising that these diaziridines did not modify the heme of P450 2B6 but rather the apoprotein (Sridar et al., 2006) and that the reactive intermediate responsible for MBI was not a carbene or a radical but rather an electrophile. However, at this point it is not possible to rule out completely that the other reactive species such as carbenes or radicals may be involved in the inactivation but that they could not be trapped by GSHEE. However, if carbenes or radicals were responsible for mechanism-based inactivation then the diaziridines **6-9** should also have been MBIs of P450 2B6. An alternative mechanism that accounts for both inactivation and the glutathione trapping experiments implicates an initial O-dealkylation, with the direct formation of 4-hydroxy diaziridine **19**, and its subsequent oxidation to the electrophilic p-quinone methide **20** (Scheme 2).

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However, our attempts at synthesizing **19** in order to test its role in inactivation highlighted the instability of this compound. Reaction of a suitably protected precursor (*p*-*O*-*tert*-butyldihphenylsilyl diaziridine) with tetrabutylammonium fluoride (well established protocols for deprotection of *O*-silyl phenols) led to extensive decomposition (unpublished data).

In conclusion, these results suggest that the determinant factor for mechanism-based inactivation by the series of aryl diaziridines investigated here is the formation of the reactive *p*-quinone methide intermediate, namely 4-ethylidene-cyclohexa-2,5-dienone, which can be generated during the metabolism of 4-alkoxyphenyldiaziridines by P450 2B6. It is also important to note that it was shown here that more than one substructure may be required for a compound to be an MBI of P450 2B6. For the phenyldiaziridine compounds studied in this report, the expected diaziridine substructure was not the only essential component but a 4-alkoxy group on the phenyl ring was also necessary to cause inactivation.

The importance of drug design that avoids the formation of potential reactive intermediates during metabolism has recently been emphasized during various stages of drug discovery in order to prevent potentially serious drug side effects. The studies discussed here strongly suggest that these drug design studies must be done with extreme care because the SARs for the formation of reactive intermediates may be much more complicated than expected from the apparent chemical structures. These studies also demonstrate that to better understand the

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structure-activity relationships for mechanism-based inactivators an entire series of analog structures may need to be taken into consideration.

This report also demonstrates that the elucidation of the structures of reactive intermediates by mass spectrometry is an effective method of investigating SAR of mechanism-based inactivators. The synthesis of various analogs and the structural elucidation of the reactive intermediates should complement each other in order to clarify the more complicated SAR of inactivators. In order to create safer drugs, a close collaborative effort between the fields of synthetic chemistry and metabolism appears to be increasingly more essential during the early stages of drug discovery.

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Figure 1. Structures of Aryl Diaziridines and their Synthetic Precursors.

Figure 2. Time-Dependent Inactivation of the 7-EFC O-Deethylation Activity of P450 2B6

by the Aryl Diaziridine Analogs 1, 6, 7, 8, 9, and 10 in a Reconstituted System. Incubation

conditions were as described under *Materials and Methods*. The concentrations of the analogs

were 20 μM for compounds **1 and 7-9**, and 100 μM for **6 and 10**. The symbols represent:

Control (■) without analog, compounds **1** (◆), **6** (×), **7** (○), **8** (△), **9** (□), and **10** (●).

Figure 3. Metabolic Stability of the Aryl Diaziridines 1, 6, 7, 8, 9, and 10 after Incubation

in a P450 2B6 Reconstituted System. Analysis conditions were as described under *Materials*

and Methods. The percentage of each compound remaining was estimated from the LC-UV

peak area ratio of samples incubated in the presence to those in the absence of NADPH, after

normalizing for recovery using bergamottin as the internal standard. The initial concentration

of each compound was 20 μM and the incubation time was 90 min (n=3).

Figure 4. GC-MS Results for Samples where Aryl Diaziridines 1 and 6 were Incubated in

P450 2B6 Reconstituted Systems. Details of the conditions used are described in *Materials*

and Methods. GC-MS spectra of (a) a metabolite of compound **1** (1 mM of **1** incubated for 45

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min with NADPH) that eluted with a r.t. of 6.6 min. (b) a solution of compound **10**, a ketone standard that eluted with a r.t. of 6.6 min, (c) a metabolite of compound **6** (1 mM of **6** incubated for 45 min with NADPH) that eluted with a r.t. of 7.5 min, and (d) a solution of compound **16**, a ketone standard that eluted with a r.t. of 7.5 min.

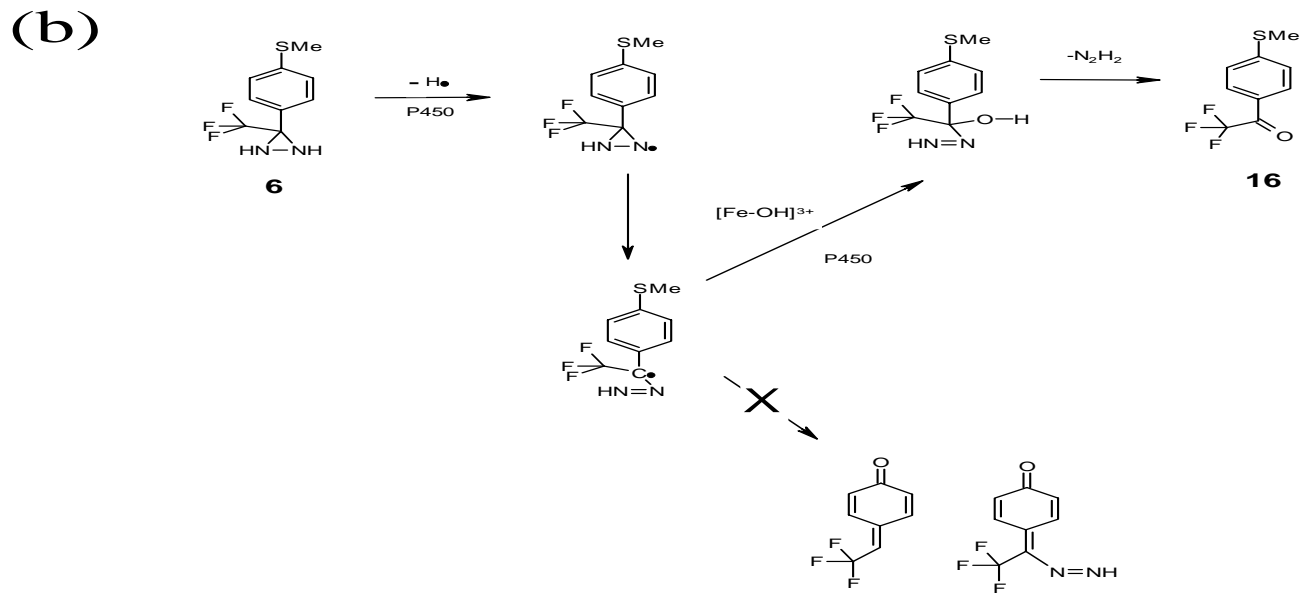
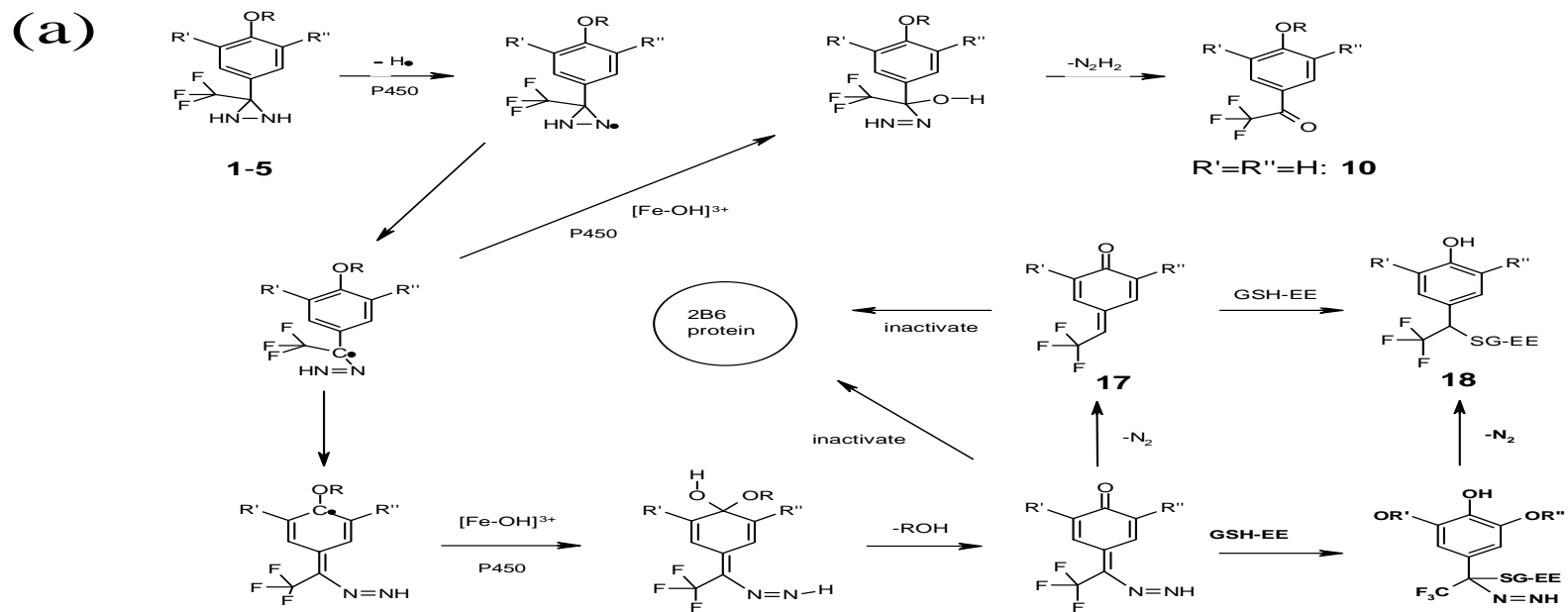
Figure 5. LC-MS/MS Analysis of GSHEE Adducts of 1 and 11. Representative extracted LC-MS chromatograms for samples where each of the two aryl diaziridines was incubated in the P450 2B6 reconstituted system together with GSHEE and NADPH are shown. Details of the conditions are described under *Materials and Methods*. (a) MS/MS spectra and proposed structure for the peak shown in the extracted ion chromatogram eluting at 25 min with an $m/z = 510$ of a sample incubated with **1**, (b) LC-MS/MS spectrum and proposed structure for the peak shown in the extracted ion chromatogram eluting at 25 min with an $m/z = 514$ of a sample incubated with **11**.

Figure 6. Proposed Chemical Structures of the GSHEE-Adducts Formed by Incubation of Compounds 1-5 with P450 2B6 in the Reconstituted System with NADPH and GSHEE. Details of the experimental conditions are as described in *Materials and Methods*. Each of structures shown was determined based on analysis of the LC-MS/MS data.

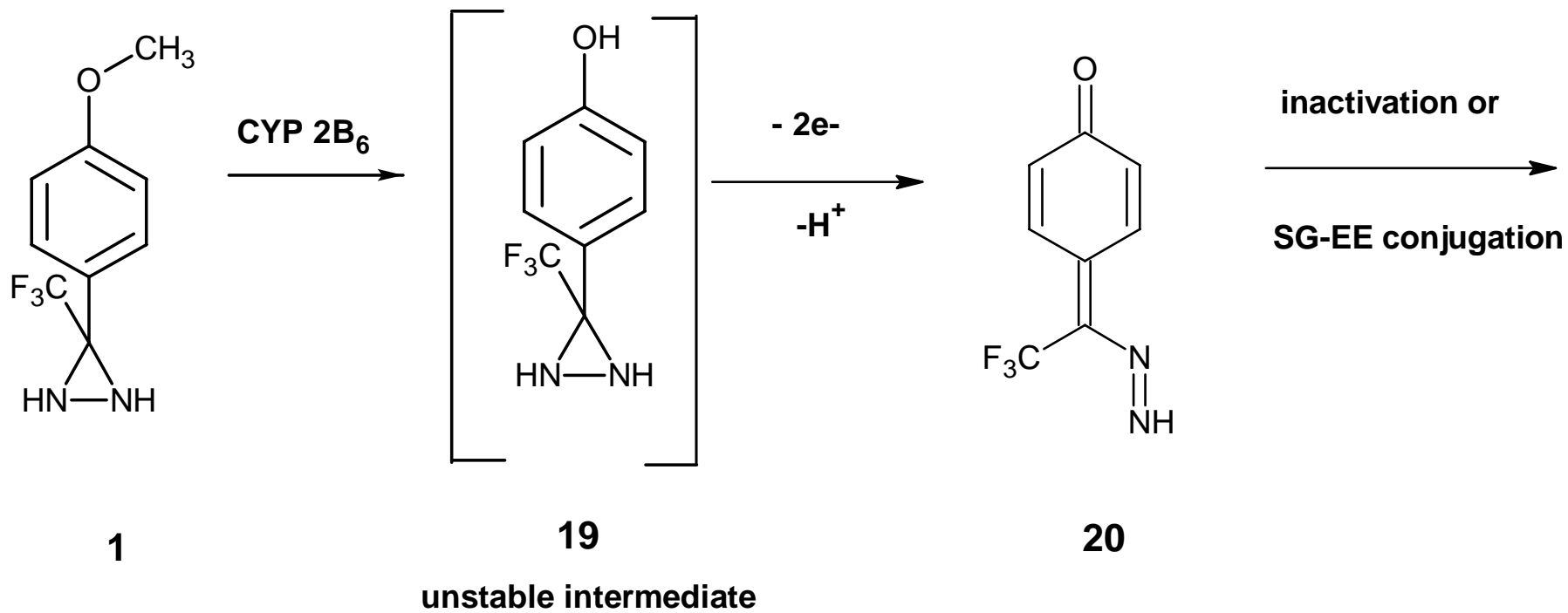
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Scheme 1. (a) Proposed Mechanism for the Inactivation of P450 2B6 by Aryl Diaziridines 1-5 and (b) the Pathway for the metabolism of Compound 6 without Formation of a Reactive Intermediate Leading to Inactivation.

Scheme 2. An alternative mechanism for the inactivation of P450 2B6 by aryl diaziridines.



Scheme 1



Scheme 2

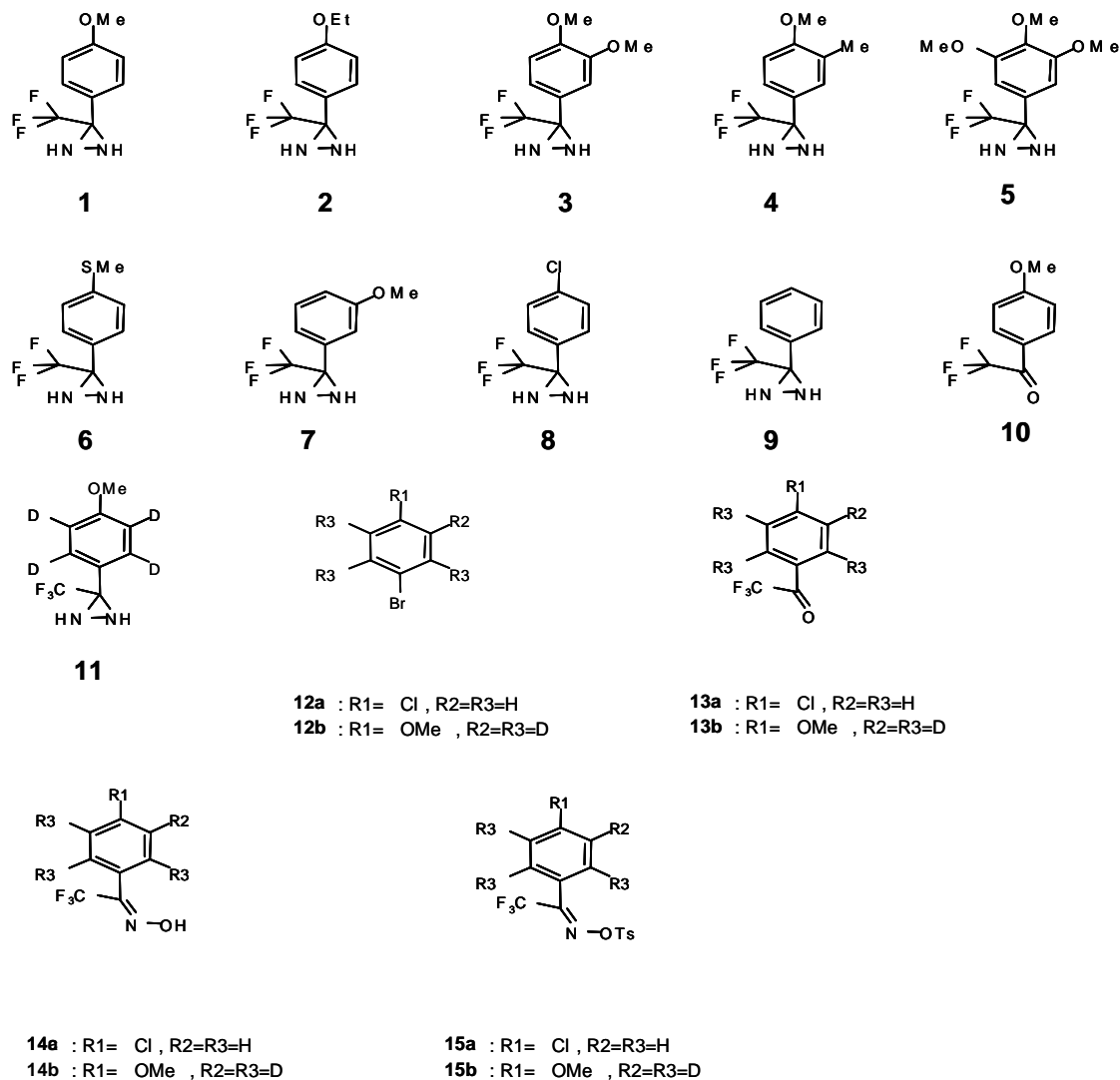


Figure 1

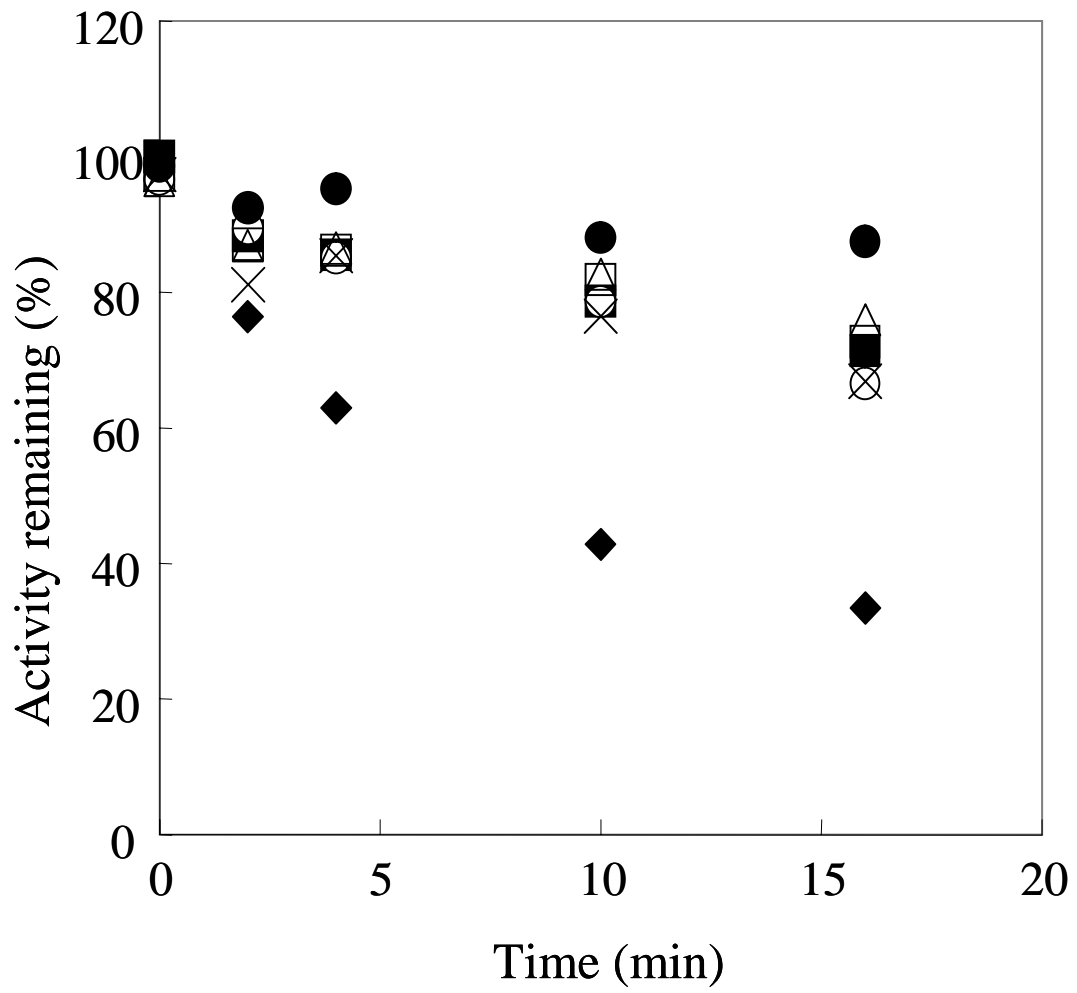


Figure 2

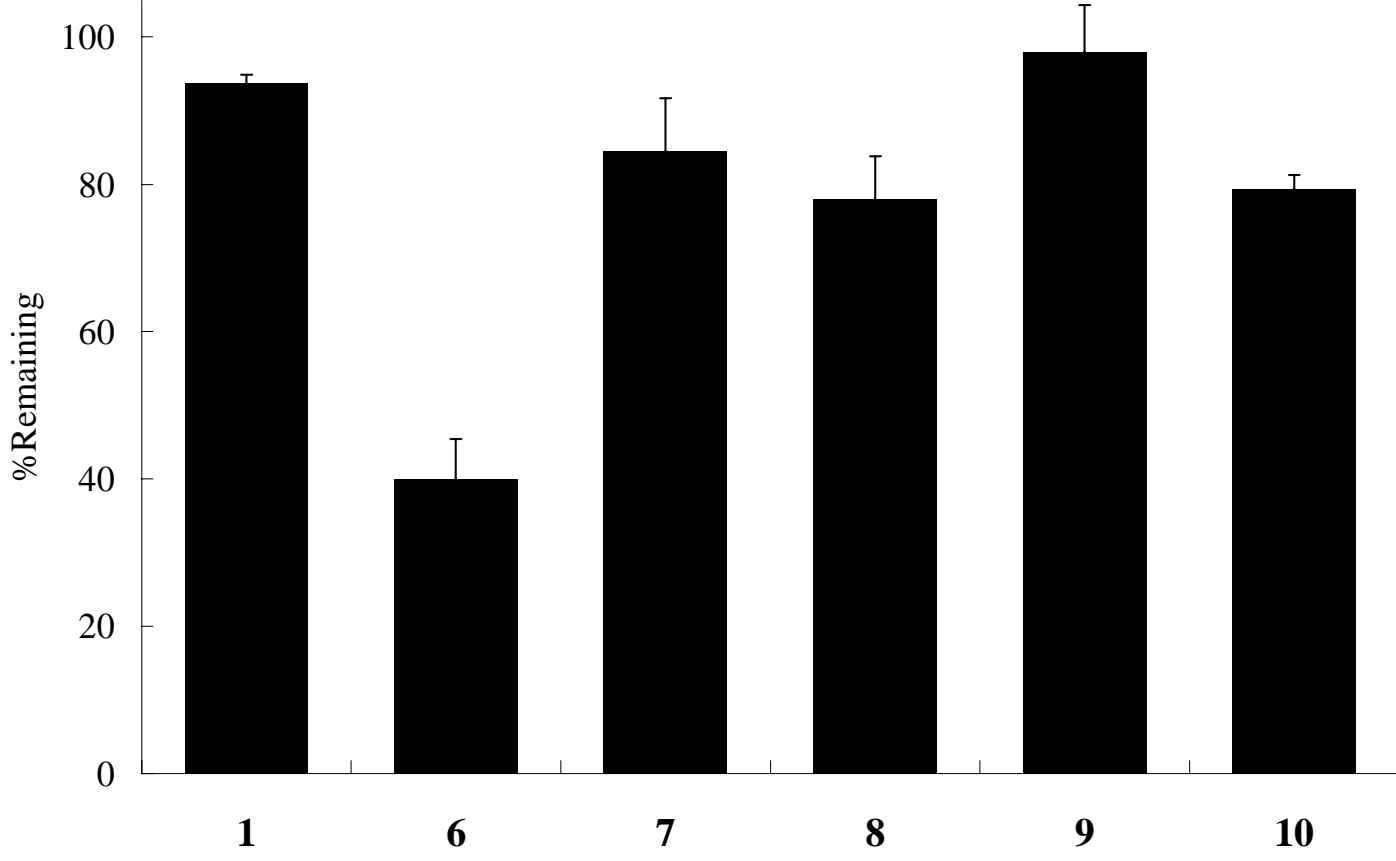


Figure 3

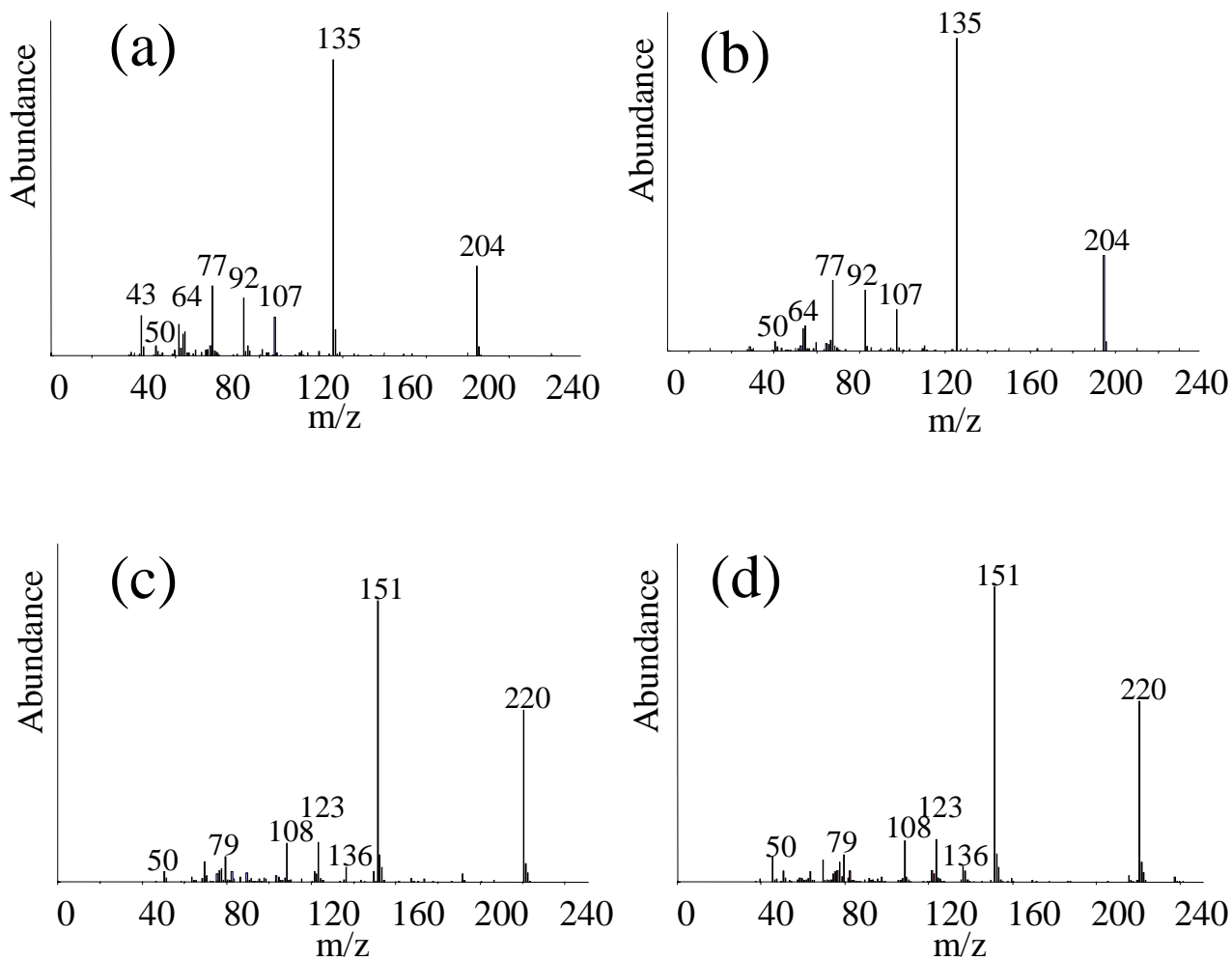
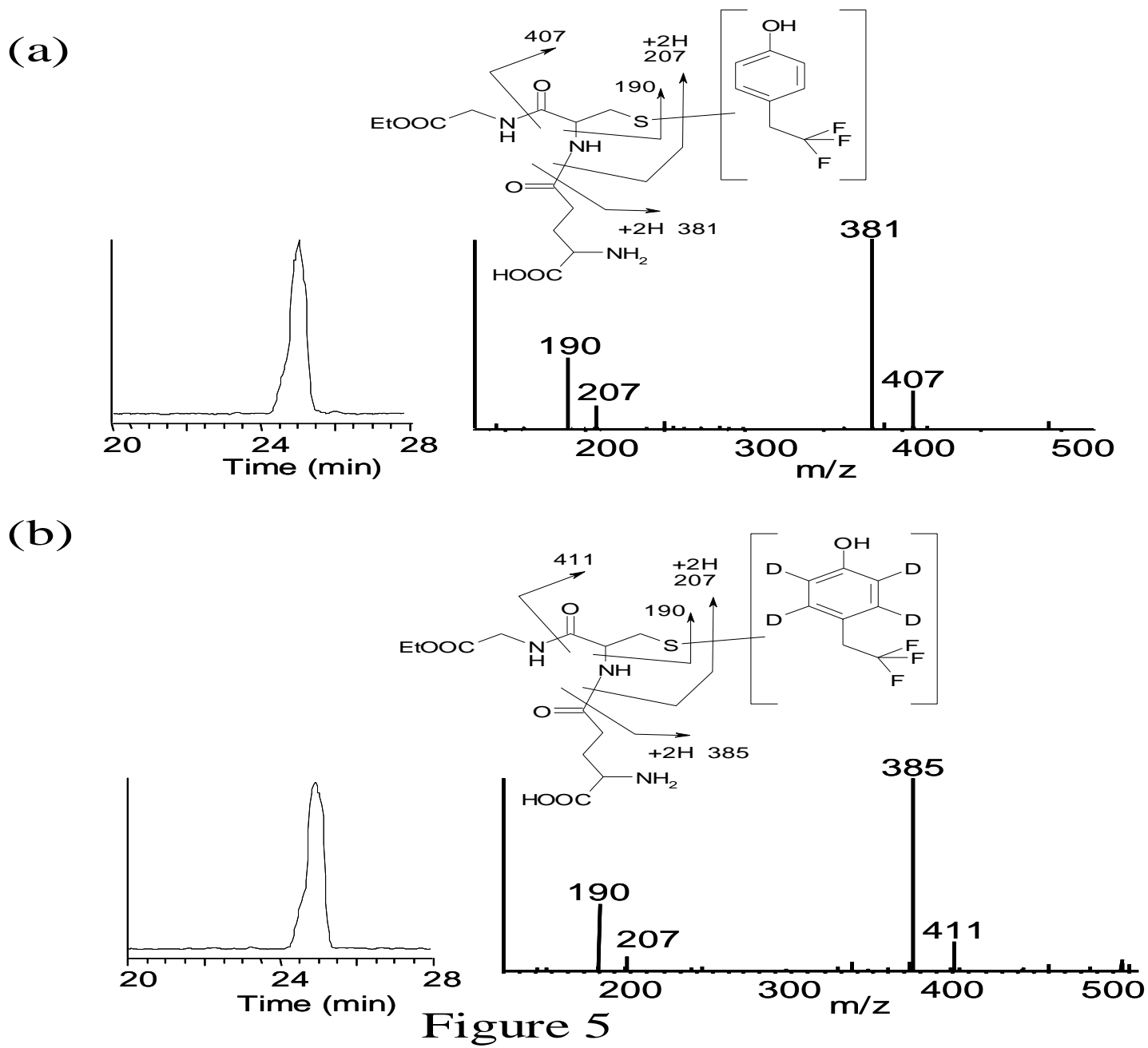


Figure 4



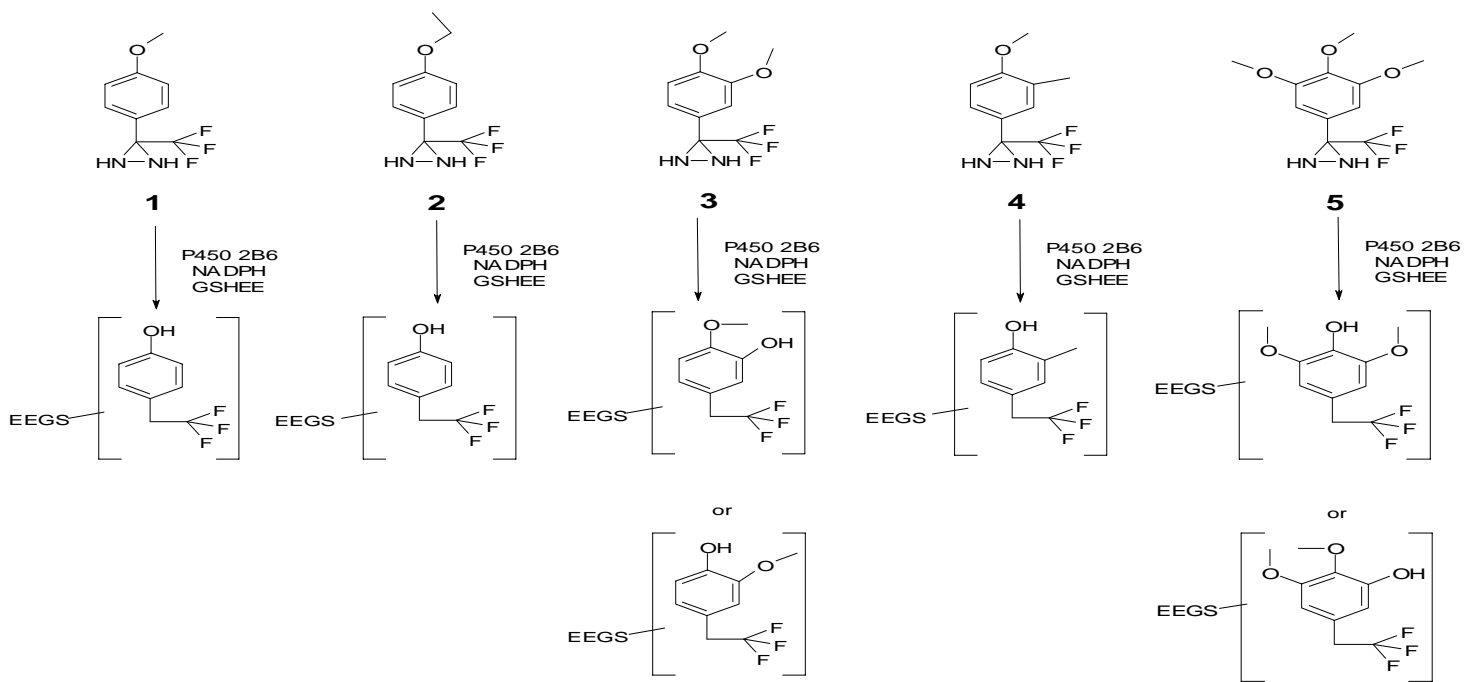


Figure 6