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Synthesis of Substituted Phenyl Diaziridines and Characterization as Mechanism-Based Inactivators of Human Cytochrome P450 2B6

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Abbreviations: P450, cytochrome P450; 7EFC, 7-ethoxy-4-(trifluroromethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; BFC,7-benzyloxy-(4-trifluoromethyl) coumarin; Tscl, p-toulenesulfonyl chloride.

Abstract. The metabolism of arylhydrazines by cytochromes P450 has previously been shown to yield aryl-iron complexes that inhibit P450 enzymes due to heme modification. These modifications of the heme have been used to probe the topology of the active site of several P450s. Therefore, diaziridines containing one or more substitutions on the phenyl ring were synthesized and evaluated as potential mechanism-based inactivators of P450 2B enzymes that could be used to elucidate the active site topology. Five of the six trifluoroaryldiaziridines tested selectively inactivated P450 2B6 in the reconstituted system in a time-, concentration-, and NADPH-dependent manner as measured using the 7-ethoxy-4-(trifluoromethyl)coumarin *O*-deethylation (7-EFC) assay. The kinetic parameters for P450 2B6 inactivation by the five compounds were calculated. Analysis of the P450 heme from P450s inactivated by the five substituted diaziridines suggested that the activity loss was not due to heme destruction as measured by the reduced-CO spectrum or HPLC of the P450 heme. Dialysis experiments indicated the irreversible nature of the inactivation and the reaction between the diaziridine compounds and the P450 enzyme. Interestingly, a thiomethyl substituted phenyl diaziridine had no effect on the activity of P450 2B6 in the reconstituted system, but competitively inhibited the *O*-debenzylation activity of P450 3A4 with 7-benzyloxy-4-(trifluoromethyl)coumarin as substrate. Binding spectra suggest that this compound bound reversibly to P450 2B6, and preliminary results indicate that 3-(4-methylthiophenyl)-3-(trifluoromethyl)diaziridine is metabolized by P450 2B6.

Introduction.

The membrane-bound mammalian cytochrome P450s are hemeproteins that play an important role in the metabolism of endogenous compounds as well as in the oxidation of myriads of xenobiotics such as drugs and chemical carcinogens (Gonzalez, 1988;Porter and Coon, 1991). P450s catalyze a variety of reactions including aromatic and aliphatic hydroxylations, epoxidations, dealkylations and dehydrogenations (Silverman, 1995). Some xenobiotics are subject to specific catalytic bioactivation events by P450 enzymes that result in the production of reactive intermediates that modify and inactivate the P450s. Several structurally distinct groups of mechanism-based inactivators of P450 have been examined including, but not limited to, acetylenes, isothiocyanates, and xanthates. Loss of P450 function has been documented to proceed through either covalent modification of the apoprotein or alkylation of the prosthetic heme group. The development of selective mechanism-based inactivators for CYP450 is critical for probing individual isozyme function and structure, and for potentially modulating CYP450 activity in a therapeutic setting (Kent et al., 2001;Coon, 2005).

Diaziridines are three-membered ring heterocyclic compounds that exhibit chemical and physiochemical characteristics analogous to both amines and hydrazines. Diaziridines have been utilized as precursors for the preparation of diazirines, photolabile groups that serve as robust photoaffinity reagents for receptor mapping studies. A report has also described the incorporation of diaziridine groups as part of structure-activity relationship studies (Hartmann et al., 2000). An earlier study showed that pre-incubation of 1,2-dimethyl-3-p-chlorophenyldiaziridine with rat liver microsomes resulted in the formation of methylamine, *p*-chlorobenzaldehyde and *p*-chlorobenzylalcohol. The initial

step in the metabolism of the diaziridine ring was believed to be a consequence of reduction of this ring (Hata and Watanabe, 1982). Studies have also shown that incubation of various cytochrome P450s including 1A1, 2B1, 2B2 and 2E1 with phenylhydrazines or phenyldiazene yielded inactive protein complexes with absorption maxima at 474-480 nm (Swanson et al., 1991;Raag et al., 1990). Phenylethylhydrazine, an anti-depressive agent, has been shown to inhibit cytochrome P450 in rat microsomes by heme destruction (Tuck and Ortiz de Montellano, 1992). The inactivation of cytochrome P450 enzymes by hydrazines, olefins, and acetylenes has been shown to involve heme adduct formation (Muakkassah and Yang, 1981;Ortiz de Montellano et al., 1981;Blobaum et al., 2002)

Thus, in our attempts to design a new class of specific mechanism-based inhibitors for P450s, the diaziridine functional group containing two contiguous basic nitrogen atoms in a strained three-membered ring presented an attractive feature. A single electron transfer, occurring in the initial step of catalysis, may generate a reactive intermediate capable of inactivating the P450 (Scheme 1). We hypothesized that the prosthetic heme could possibly oxidize one of the equivalent diaziridine nitrogens to the corresponding amine radical cation, analogous to the oxidative catalytic mechanisms involving *N*-dealkylation reactions with CYP450 (Gonzalez, 1988;Silverman, 1995). Additional evidence for the practicality of using diaziridines as mechanism-based inactivators was found in a recent chemical model study that demonstrated that carbenes can be generated directly from their respective diaziridines (Post and Morrison, 1996). The steps leading to carbene formation were believed to involve an initial single electron transfer from a diaziridine to the carbonyl triplet exited state to form an initial diazirine

radical cation. Homolytic bond scission followed by deprotonation and hydrogen atom abstraction (or e^-, H^+ loss) afforded cyclohexanecarbene (identified by trapping with methanol-d_4) and N_2. Related bond scission reactions have been reported in the reaction of oxaziridines with ferrous sulfate. These chemical model studies support the hypothesis that diaziridine amine radical cation formation is both viable and favorable, and leads to highly reactive carbene or radical intermediates.

Thus, based on this hypothesis, six different diaziridines (**1a-f**) with substitutions on the phenyl ring were synthesized and assessed for their potential to inhibit different P450 isoforms *in vitro* using purified P450s in a reconstituted system. This study shows that a number of these diaziridine compounds form reactive intermediates that act as mechanism-based inactivators and are selective for human P450 2B6. The study further indicates that diaziridines with para-alkoxide substitutions inactivated P450 2B6 in a mechanism-based manner.

Materials and Methods

Materials. All 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 F_{254} plates. Column chromatography was performed using standard grade Sorbent Technologies silica gel with a particle size of 32-63 μM. 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 2400 CHNS/O Elemental Analyzer. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker Avance DXR 400, using $CDCl_3$ as a solvent. The following compounds were synthesized as previously described: (4-methyoxyphenyl series, **1a**, **3a**, **4a**, **5a** (Hatanaka Y et al., 1994)**;** (4-methylthio series, **1d**, **3d**, **4d**, **5d**; (Findlay BC et al., 1995).

Dilaurolyl-L-α-phosphatidylcholine, bovine serum albumin, NADPH, catalase, glutathione, sodium dithionite and Hepes used in the assays were purchased from Sigma-Aldrich (St. Louis, MO). BFC was obtained from BD Gentest (Woburn, MA). 7-EFC was obtained from Molecular Probes (Eugene, OR). HPLC-grade acetonitrile was purchased from Mallinckrodt (Chesterfield, MO). Slide-A-Lyzer cassettes were from Pierce Chemical (Rockford, IL).

General procedure for the preparation of ketones (3a-f)

Mg turnings (2.41 g, 0.1 mol), substituted bromobenzenes, **2a-f** (0.1 mol) and anhydrous tetrahydrofuran (80 mL) were placed in a round bottom flask. The mixture was slowly heated to reflux and refluxing was maintained until all the Mg was consumed. The mixture was cooled in an ice bath and a solution of N-trifluoroacetylpiperidine (0.1 mol)

in anhydrous tetrahydrofuran (25 mL) was added slowly to the Grignard reagent over a period of 0.5 h with stirring at 0^{o}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 (10 mL). The precipitated solids were filtered, and the filtrate was dried over Na_2SO_4 and evaporated in *vacuo*, and the residual oil was purified by column chromatography (Si gel) eluting with hexanes/ CH_2Cl_2 (95:5) to give the product ketone.

2,2,2-Trifluoro-1-(4-methoxyphenyl)ethanone (3a). Yield, 14.6 g (72%); oil, ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 3H), 7.00 (d, 2H, *J*=8.4 Hz), 8.04 (d, 2H, *J*=8.4 Hz).

2,2,2-Trifluoro-1-(4-ethoxyphenyl)ethanone (3b). Yield, 15.2 g (70%); oil, ^{1}H NMR ($CDCl_3$): δ 1.45 (t, 3H, *J*=6.8 Hz), 4.12 (q, 2H, *J*=6.8 Hz), 6.93 (d, 2H, *J*=8.8 Hz), 8.02 (d, 2H, *J*=8.8 Hz).

2,2,2-Trifluoro-1-(3,4-dimethoxyphenyl)ethanone (3c). Yield, 18.2 g (78%); mp = 78 ^{o}C. ^{1}H NMR ($CDCl_3$): δ 3.95 (s, 3H), 3.99 (s, 3H), 6.95 (d, 2H, *J*=8.6 Hz), 7.56 (s, 1H), 7.72 (d, 2H, *J*=8.4 Hz).

2,2,2-Trifluoro-1-[4-(methylthio)phenyl]ethanone (3d).Yield, 14.3 g (65%); mp =46 ^{o}C (lit = 46.8-47.8) ^{1}H NMR ($CDCl_3$): δ 2.55 (s, 3H), 7.30 (d, 2H, *J*=8.4 Hz), 7.97 (d, 2H, *J*=8.4 Hz).

2,2,2-Trifluoro-1-(3-methyl-4-methoxyphenyl)ethanone (3e). Yield, 14.8 g (68%); mp= 30 ^{o}C. ^{1}H NMR ($CDCl_3$): δ 2.26 (s, 3H), 3.94 (s, 3H), 6.91 (d, 1H, *J*=8.4 Hz), 7.87 (s, 1H), 7.94 (d, 1H, J=8.4 Hz).

2,2,2-Trifluoro-1-(3,4,5-trimethoxyphenyl)ethanone (3f). Yield, 21.1g (80%); amorphous solid, ^{1}H NMR ($CDCl_3$): δ 3.85 (bs, 9H), 6.58 (s, 2H).

General Procedure for the Synthesis of Oximes (4a-f)

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Hydroxylamine hydrochloride (0.125 mol), added to a solution of ketones **3a-f** (0.05 mol) in absolute ethanol (25 mL) and dry pyridine (40 mL), was heated at 60 ºC for 8.0 h. After the solvent was removed the remaining residue was dissolved in diethyl ether (40 mL), and washed with 1N HCl. The organic layer was washed with water (50 x 2 mL), and dried over Na_2SO_4. After evaporation of the solvent, the crude oxime was purified by column chromatography (Si gel) eluting with hexanes/CH_2Cl_2 (90:10) to give white to off-white solids as mixtures of (E/Z) isomers.

2,2,2-Trifluoro-1-(4-methoxyphenyl)ethanone oxime (4a). Yield, 8.5 g (78%); mp 95-96 ºC; (lit. 78-79 ºC). ^{1}H NMR ($CDCl_3$): δ 3.85 (s, 3H), 6.99 (d, 2H, *J*=8.4 Hz), 7.54 (d, 1H, *J*=8.4 Hz), 8.42 (bs, 1H). HRMS: *m/z* $[MH]^+$ 220.0588. ($C_9H_9F_3NO_2$ requires 220.0585).

2,2,2-Trifluoro-1-(4-ethoxyphenyl)ethanone oxime (4b). Yield, 9.0 g (78%); mp 94-95 ºC; ^{1}H NMR ($CDCl_3$): δ 1.43 (t, 3H, *J*=6.8 Hz), 4.08 (q, 2H, *J*=6.8 Hz), 6.95 (d, 2H, *J*=8.8 Hz), 7.52 (d, 2H, *J*=8.4 Hz), 8.43 (bs, 1H). HRMS: *m/z* $[MH]^+$ 234.0734. ($C_{10}H_{11}F_3NO_2$ requires 234.0742).

2,2,2-Trifluoro-1-(3,4-dimethoxyphenyl)ethanone oxime (4c). Yield, 9.9 g (80%); mp 132-134 ºC; ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 3H), 3.92 (s, 3H), 6.95 (d, 1H, *J*=8.0 Hz), 7.08 (s,1H), 7.15 (d, 1H, *J*=8.0 Hz), 8.37 (bs,1H). HRMS: *m/z* $[MH]^+$ 250.0696. ($C_{10}H_{11}F_3NO_3$ requires 250.0691).

2,2,2- Trifluoro-1-[(4-methylthio)phenyl]ethanone oxime (4d). Yield, 7.2 g (62%); mp 78-80 ºC; (lit. 64.5-77.5 ºC). ^{1}H NMR ($CDCl_3$): δ 2.47 (s, 3H), 7.30 (d, 2H, *J*=8.4 Hz),

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7.47 (d, 2H, *J*=8.0 Hz), 8.60 (bs, 1H). HRMS: *m/z* $[MH]^+$ 236.0368. ($C_9H_9F_3NOS$ requires 236.0357).

2,2,2-Trifluoro-1-[(3-methyl-4-methoxyphenyl]ethanone oxime (4e). Yield, 7.5 g (65%); mp 96-99 ºC; ^{1}H NMR ($CDCl_3$): δ 2.25 (s, 3H) 3.87 (s, 3H), 6.88 (d, 1H, *J*=8.4 Hz), 7.36 (s, 1H), 7.40 (d, 1H, *J*=8.0 Hz), 8.79 (bs, 1H). HRMS: *m/z* $[MH]^+$ 234.0736. ($C_{10}H_{11}F_3NO_2$ requires 234.0742).

2,2,2-Trifluoro-1-(3,4,5-trimethoxyphenyl)ethanone oxime (4f). Yield, 10.0 g (72%); mp 177-179 ºC; ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 9H), 6.92 (d, 2H, *J*=8.6 Hz), 7.00 (m, 1H), 8.42 (bs, 1H). HRMS: *m/z* $[MH]^+$ 280.0788. ($C_{11}H_{13}F_3NO_4$ requires 280.0797).

General Procedure for the preparation of Tosylates (5a-f).

To 40 mL of CH_2Cl_2 was added oximes **4a-f** (0.05 mol), triethylamine (0.09 mol), 4-dimethylaminopyridine (0.01 mol) and *p*-toluenesulfonyl chloride (0.12 mol) at 0 ºC. The reaction was allowed to warm to room temperature and stirring was continued for 2.0 h. After quenching with water, the organic phase was separated, washed successively with water, and dried over Na_2SO_4. The solvent was evaporated in *vacuo*, and the crude product was purified by column chromatography (Si gel) eluting with hexanes/CH_2Cl_2 (90:10) to give white to half-white solids.

2,2,2-Trifluoro-1-(4-methoxyphenyl)-1-ethanone-*O*-(*p*-toluenesulfonyl) oxime (5a). Yield, 13.0 g (70%); mp 112 ºC; ^{1}H NMR ($CDCl_3$): δ 2.47 (s, 3H), 3.85 (s, 3H), 6.95 (d 2H, *J*=8.4 Hz), 7.37 (d, 2H, *J*=8.0 Hz), 7.44 (d, 2H, *J*=8.4 Hz), 7.89 (d, 2H, *J*=8.0 Hz). Anal. Calcd for $C_{16}H_{14}F_3NO_3S$: C, 51.47; H, 3.78; N, 3.75. Found: C, 51.87; H, 3.75; N, 3.72.

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2,2,2-Trifluoro-1-(4-ethoxyphenyl)-1-ethanone-*O*-(*p*-toluenesulfonyl) oxime (5b). Yield, 13.9 g (72%); mp 113 ºC; ^{1}H NMR ($CDCl_3$): δ 1.46 (t, 3H, J=7.0 Hz), 2.50 (s, 3H), 4.10 (q, 2H, J=7.0 Hz), 6.96 (d, 2H, *J*=8.8 Hz), 7.40 (d, 2H, *J*=8.0 Hz), 7.46 (d, 2H, *J*=8.8 Hz), 7.91 (d, 2H, *J*=8.0 Hz). Anal. Calcd for $C_{17}H_{16}F_3NO_3S$: C, 52.71; H, 4.16; N, 3.62. Found: C, 52.91; H, 4.01; N, 3.60.

2,2,2-Trifluoro-1-(3,4-dimethoxyphenyl)-1-ethanone-*O*-(*p*-toluenesulfonyl) oxime (5c). Yield, 16.1 g (80%); mp 115 ºC; ^{1}H NMR ($CDCl_3$): δ 2.47 (s, 3H), 3.87 (s, 3H), 3.92 (s, 3H), 6.91 (d, 2H, *J*=8.4 Hz), 6.96 (s, 1H), 7.09 (d, 2H, *J*=8.4 Hz) 7.38 (d, 2H, *J*=8.0 Hz), 7.89 (d, 2H, *J*=8.0 Hz). Anal. Calcd for $C_{17}H_{16}F_3NO_4S$: C, 50.62; H, 4.00; N, 3.47. Found: C, 50.55; H, 3.80; N, 3.47.

2,2,2-Trifluoro-1-(4-methylthio)-1-ethanone-*O*-(*p*-toluenesulfonyl) oxime (5d). Yield, 14.5 g (75%); mp 100 ºC; (lit. 87.5-98 ºC). ^{1}H NMR ($CDCl_3$): δ 2.50 (s, 3H), 2.53 (s, 3H), 7.29-7.42 (m, 6H), 7.91 (d, 2H, *J*=8.4 Hz). Anal. Calcd for $C_{16}H_{14}F_3NO_2S_2$: C, 49.35; H, 3.62; N, 3.60. Found: C, 49.46; H, 3.62; N, 3.51.

2,2,2-Trifluoro-1-(3-methyl-4-methoxyphenyl)-1-ethanone-*O*-(*p*-toluenesulfonyl) oxime (5e). Yield, 13.2 g (70%); mp 75 ºC; ^{1}H NMR ($CDCl_3$): δ 2.18 (s, 3H), 2.52 (s, 3H), 3.90 (s, 3H), 6.89 (d, 1H, *J*=8.8 Hz), 7.24 (s, 1H), 7.35 (d, 1H, *J*=8.8 Hz), 7.40 (d, 2H, *J*=8.0 Hz), 7.91 (d, 2H, *J*=8.0 Hz). Anal. Calcd for $C_{17}H_{16}F_3NO_3S$: C, 52.71; H, 4.16; N, 3.62. Found: C, 52.69; H, 4.14; N, 3.56.

2,2,2-Trifluoro-1-(3,4,5-trimethoxyphenyl)-1-ethanone-*O*-(*p*-toluenesulfonyl) oxime (5f). Yield, 16.4 g (76%); mp 132 ºC; ^{1}H NMR ($CDCl_3$): δ 2.47 (s, 3H), 3.84 (s, 6H), 3.89 (s, 3H), 6.57 (s, 2H) 7.37 (d, 2H, *J*=8.0, Hz), 7.87 (d, 2H, *J*=8.0, Hz). Anal. Calcd for $C_{18}H_{18}F_3NO_5S$: C, 49.88; H, 4.19; N, 3.23. Found: C, 49.84; H, 3.97; N, 3.19.

General Procedure for the preparation of Diaziridines (1a-f)

Tosyl oximes (0.001 mol, **5a-f**) and diethyl ether (60 mL) were placed in a three-necked round bottom flask equipped with dry ice condenser and gas inlet. The reaction was cooled to –78 ºC and approximately 8.0 mL of anhydrous NH_3 was condensed into the flask. The reaction mixture was stirred for 1.0 h at –78 ºC. The cooling bath was removed and the gas inlet was replaced with a drying tube. The reaction mixture was stirred at room temperature while the NH_3 refluxed for 2.0 h. The condenser was removed and the ammonia was allowed to evaporate. The remaining residue was dissolved in diethyl ether and washed with water and brine. Subsequent drying over Na_2SO_4 and evaporation yielded the crude products, which were purified by column chromatography (Si gel), (1 to 10% EtOAc, $CHCl_3$) to yield the final diaziridines.

3-(4-methoxyphenyl)-3-(trifluoromethyl) diaziridine (1a). Yield, 0.15 g (72%); mp 72 ºC; (lit. 69-70 ºC). ^{1}H NMR ($CDCl_3$): δ 2.15 (bs, 1H), 2.75 (bs, 1H), 3.85 (s, 3H), 6.95 (d, 2H, *J*=8.0 Hz), 7.56 (d, 2H, *J*=8.4 Hz). HRMS: *m/z* $[MH]^+$ 219.0753. ($C_9H_{10}F_3N_2O$ requires 219.0745). Anal. Calcd for $C_9H_9F_3N_2O$: C, 49.55; H, 4.16; N, 12.84. Found: C, 49.58; H, 4.05; N, 12.84.

3-(4-ethoxyphenyl)-3-(trifluoromethyl) diaziridine (1b). Yield, 0.17 g (76%); mp 64 ºC; ^{1}H NMR ($CDCl_3$): δ 1.42 (t, 3H, *J* = 6.8 Hz) 2.15 (bs, 2H), 4.05 (q, 2H, *J* = 6.8 Hz), 6.91 (d, 2H, *J*=8.4 Hz), 7.51 (d, 2H, *J*=8.4 Hz). HRMS: *m/z* $[MH]^+$ 233.0906. ($C_{10}H_{12}F_3N_2O$ requires 233.0902). Anal. Calcd for $C_{10}H_{11}F_3N_2O$: C, 51.73; H, 4.77; N, 12.06. Found: C, 51.59; H, 4.67; N, 11.81.

3-(3,4-dimethoxyphenyl)-3-(trifluoromethyl)diaziridine (1c). Yield, 0.19 g (80%); mp 66-67 ºC; ^{1}H NMR ($CDCl_3$): δ 2.17 (bs, 1H), 2.74 (bs, 1H), 3.91 (s, 6H), 6.88 (d, 1H,

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J=8.4 Hz), 7.10 (s, 1H), 7.19 (d, 1H, *J*=8.4 Hz). HRMS: *m/z* $[MH]^+$ 249.0863. ($C_{10}H_{12}F_3N_2O_2$ requires 249.0851). Anal. Calcd for $C_{10}H_{11}F_3N_2O_2$: C, 48.39; H, 4.47; N, 11.29. Found: C, 48.34; H, 4.37; N, 11.13.

3-(4-methylthiophenyl)-3-(trifluoromethyl)diaziridine (1d). Yield, 0.14 g (62%); mp 107-109 ºC; (lit. 105-107.5 ºC). ^{1}H NMR ($CDCl_3$): δ 2.18 (bs, 1H), 2.49 (s, 3H), 2.75 (bs, 1H), 7.26 (d, 2H, *J*=8.0 Hz), 7.51 (d, 2H, *J*= 8.0 Hz). HRMS: *m/z* $[MH]^+$ 235.0508. ($C_9H_{10}F_3N_2S$ requires 235.0517). Anal. Calcd for $C_9H_9F_3N_2S$: C, 46.15; H, 3.87; N, 11.96. Found: C, 46.30; H, 3.69; N, 11.90.

3-(3-methyl-4-methoxyphenyl)-3-(trifluoromethyl)diaziridine (1e). Yield, 0.15 g (65 %); yellow oil, ^{1}H NMR ($CDCl_3$): δ 2.15 (br d, 1H, *J* = 8.0 Hz), 2.27 (s, 3H), 2.72 (br d, 1H, *J* = 8.0 Hz), 3.85 (s, 3H), 6.82 (d, 1H, *J*=8.5 Hz), 7.36 (s, 1H), 7.40 (d, 1H, *J*=8.5 Hz) HRMS: *m/z* $[MH]^+$ 233.0910. ($C_{10}H_{12}F_3N_2O$ requires 233.0902). Anal. Calcd for $C_{10}H_{11}F_3N_2O$: C, 51.73; H, 4.77; N, 12.06. Found: C, 52.21; H, 4.94; N, 11.25.

3-(3,4,5-trimethoxyphenyl)-3-(trifluoromethyl)diazridine (1f). Yield, 0.19 g (68%); mp 110-112 ºC; ^{1}H NMR ($CDCl_3$): δ 2.22 (br d, 1H, *J*=8.4 Hz), 2.76 (br d, 1H, *J*=8.4 Hz), 3.85 (s, 3H), 3.88 (s, 6H), 6.84 (s, 2H). HRMS: *m/z* $[MH]^+$ 279.0967. ($C_{11}H_{14}F_3N_2O_3$ requires 279.0957). Anal. Calcd for $C_{11}H_{13}F_3N_2O_3$: C, 47.49; H, 4.71; N, 10.07. Found: C, 47.49; H, 4.57; N, 9.93.

Purification of enzymes. P450 NADPH-reductase from rat was expressed in *Escherichia coli* Topp3 cells. Expression and purification was carried out as described by Hanna et al (Hanna et al, 1998). P450 2B6 was expressed in JM109 cells and purified as described previously (Hanna et al, 2000). Expression and purification of P450s 2C9, 3A4,

2D6 and 2E1 was carried out according to previously published procedures (Gillam et al., 1993;Shimada et al., 1998).

Effect of trifluoroaryldiaziridines on human cytochromes P450. The effects of the substituted phenyl diaziridines on various human cytochrome P450s were tested. The activities of P450s 2B6, 2C9, 2D6 and 2E1 were determined by measuring the loss in the 7-EFC *O*-deethylation activity assay. Purified P450s (0.5 nmol) were reconstituted with reductase (1 nmol) and phospholipid (except in the case of 2B6) at 4 ºC for 45 min. The reconstituted enzymes were then supplemented with catalase (100 units/ml), and diluted with 50 mM potassium phosphate buffer, pH 7.4, to a final volume of 0.3 ml. The concentrations of the substituted diaziridine compounds used were 1, 10, and 100 μM in DMSO or DMSO was added in the control sample. Reactions were initiated by the addition of 1.2 mM NADPH or water. At 0, 10 and 16 min, 10 pmol of the reconstituted enzyme mixture were transferred into a secondary reaction buffer containing 50 mM KPi, pH 7.4, 1 mM NADPH, 100 μM 7-EFC, and 40 μg/ml BSA. The secondary reactions were incubated for 10 min and the reactions were stopped with ice-cold acetonitrile (334 μl). The amount of 7-hydroxy-(4-trifluoromethyl)coumarin formed was determined spectrofluorometerically on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia MD) at room temperature with excitation and emission wavelengths of 410 and 510 nm, respectively.

The activity of P450 3A4 was determined by measuring the loss of 7-BFC *O*-debenzylation activity. P450 3A4 was reconstituted with reductase and 20 μg of a lipid-mixture (1:1:1 of L-α-dilaurylphosphatidylcholine, L-α-dioleyl-sn-glycero-3-phosphocholine and phosphatidylserine) in the ratio of 1:2:1 at RT for 20 min. The

reconstitution mixture was then brought to a volume of 300 µl with 50 mM Hepes buffer, 2 mM GSH and 250 units catalase. At the three time points indicated, 9 pmol aliquots of the enzyme were transferred into a secondary reaction mixture containing 1mM NADPH, 50 µM BFC, 4 mM $MgCl_2$, and 40 µg/ml BSA in 200 mM potassium phosphate buffer (pH 7.4) and incubated for 10 min at 30°C with shaking. The reactions were quenched with a solution comprised of 80% acetonitrile and 20% 0.5 M Tris. The BFC *O*-debenzylation activity was measured using fluorescence as indicated above with excitation and emission wavelengths of 409 and 530 nm, respectively.

Inactivation of P450 2B6 by trifluoroaryldiaziridines . For the inactivation reactions, P450 2B6 (0.5 nmol) was reconstituted with reductase (1 nmol) at 4 ºC for 45 min. The reconstituted enzyme mixture was supplemented with catalase (100 units/ml) and diluted with 50 mM potassium phosphate buffer, pH 7.4, to a volume of 0.7 ml. Samples then received increasing concentrations of the trifluoroaryldiaziridines (**1a-f**) in DMSO or DMSO only (in the control sample). The concentration ranges used for all of the diaziridines were 0 µM to 50 µM, with the exception of 3-(trifluoromethyl)-3-(3,4-dimethoxyphenyl)diaziridine (**1c**) (1 to 20 µM). The reactions were initiated by the addition of 1.2 mM NADPH. Control samples received water. At selected time intervals, 10 pmol aliquots of the P450 enzyme mixture were transferred to a secondary reaction buffer containing 1 mM NADPH and 100 µM 7-EFC in 50 mM KPi, pH 7.4 with 40 µg/ml BSA. The secondary reactions were incubated for 10 min at 30 ºC and then stopped by the addition of ice-cold acetonitrile (334 µl) and assayed for residual activity as described above.

Determination of heme loss and irreversibility of the inactivation. Reconstitution and inactivation of P450 2B6 by the various trifluoroaryldiaziridines was carried out as described above. At 0 and 15 min following initiation of the inactivation reactions an aliquot of each of the samples was assayed for 7-EFC *O*-deethylation activity. A second aliquot (100 pmol) of each of the control (-inactivator, -NADPH), exposed (+inactivator, -NADPH) and inactivated samples (+inactivator, + NADPH) was then injected onto a C4 column (Phenomenox, 250 × 4.6 mm) equilibrated with 30% acetonitrile, 0.1% TFA. The components of the reconstituted system were resolved by linearly increasing the percentage of acetonitrile to 90 % over a period of 30 min. The effluent was monitored at 405 nm for intact heme and the areas under the heme peaks of the control, exposed, and inactivated samples were integrated and compared. Another portion of the primary reaction mixture was used to determine the reduced-CO spectra by transferring 100 pmol of the control, exposed, and inactivated samples into quench buffer containing 50 mM potassium phosphate, 40% glycerol, and 0.6% tergitol. The samples were bubbled with CO, sodium dithionite was added and the reduced-CO spectra were recorded between 400-500 nm on a DW2 UV/VIS spectrophotometer equipped with an OLIS operating system (On Line Instruments Systems, Bogart, GA). The remainder of each sample was dialyzed overnight in a Slide-A-Lyzer dialysis cassette at 4°C against 2×500 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol. Portions of the dialyzed samples were assayed for activity in the presence or absence of fresh reductase. The dialyzed samples were also assayed for reduced-CO spectra and for heme loss by HPLC.

Partition Ratio. The reconstituted mixture containing 0.5 µM P450 2B6 was incubated with concentrations ranging from 0 to 100 µM of the trifluoroaryldiaziridines (**1a-f**) and 1.2 mM NADPH. The reactions were allowed to go to completion by incubating for 30 min at 30° C. Aliquots of the reaction mixture were assayed for residual 7-EFC activity at 0 and 30 min as described above. Partition ratios were calculated as previously described (Kent, Juschyshyn, and Hollenberg, 2001).

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Results.

Synthesis of trifluoroaryldiaziridine compounds. Trifluoromethylaryldiaziridines (**1a-f**) were synthesized from a series of commercially available aromatic bromides in a convenient four step protocol as shown in Scheme 2. Treatment of the bromobenzenes (**2a-f**) with magnesium followed by reaction with N-trifluoroacetylpiperidine afforded the trifluoromethyl ketones (**3a-f**). The reaction of the ketones (**3 a-f**) with hydroxylamine yielded the corresponding oximes (**4 a-f**), which were converted to the tosyl oximes (**5 a-f**) by reaction with *p*-toluenesulfonyl chloride. Reaction of the tosyl oximes with ammonia yielded diaziridines as the final products (**1 a-f**). The diaziridines were characterized by analysis of the ^{1}H and ^{13}C NMR, in addition to MS and elemental analysis. NMR figures of the compounds can be found in the supplemental data section.

Effects of trifluoroaryldiaziridines on human cytochrome P450s. Six phenyl diaziridines with substitutions on the phenyl ring were incubated at concentrations of 1, 10 and 100 μM with human cytochromes P450 (2B6, 2C9, 2D6, 2E1 and 3A4) in the reconstituted system. Time-, and concentration dependent loss was seen with five of the six phenyl diaziridines when incubated with P450 2B6 as measured by 7-EFC O-deethylation activity. Table 1 shows the loss in the 7-EFC *O*-deethylation activity after 16 min when 100 μM of the phenyl diaziridines were incubated with P450 2B6. The phenyl diaziridine with the 4-methyl thio substitution (**1d**) did not affect the 7-EFC *O*-deethylation acitivity of P450 2B6. However, this diaziridine competitively inhibited P450 3A4 exhibiting a 50% decrease in the BFC *O*-debenzylation activity at a concentration of 100 μM. Less than 10% loss in the 7-EFC *O*-deethylation activity was seen with P450s 2E1, 2D6, and 2C9 when incubated with any of the substituted phenyl

diaziridines. Competitive inhibition of the BFC *O*-debenzylation activity of P450 3A4 was also seen when this enzyme was incubated with the dimethoxy substituted diaziridine (**1c**).

Effect of trifluoroaryldiaziridines on P450 2B6 inactivation. The kinetics for the inactivation of purified P450 2B6 by the substituted phenyl diaziridines in the reconstituted system were determined. At various time points, an aliquot of the primary reaction mixture was transferred into a secondary buffer containing EFC to measure the residual activity. Figure 2 shows the effect of 3-(trifluoromethyl)-3-methoxy-(4-methylphenyl)diaziridine (**1e**) on the 7-EFC *O*-deethylation activity of P450 2B6 as a representative experiment. Addition of this compound to the P450 2B6 reconstituted system in the absence of NADPH reduced the 7-EFC *O*-deethylation activity of P450 2B6 marginally, whereas the loss in activity ranged from 60-70 % in the presence of NADPH when incubated with up to 50 μM of 5 of the substituted phenyl diaziridines. The loss in activity increased with time and with increasing concentration with all trifluoroaryldiaziridines (data not shown), except with the thiomethyl substituted phenyl diaziridine (**1d**) which did not result in any inactivation of the enzyme. The time course studies demonstrated that the inactivation by the trifluoroaryldiaziridines resulted in pseudo-first order losses in enzymatic activity. The inactivation rates also exhibited the expected concentration dependence with respect to the inactivator as shown in the inset of Fig 2. The kinetic parameters for the five trifluoroaryldiaziridines are shown in Table 2 and were calculated from the double reciprocal plots of the rates of inactivation as a function of the inactivator concentrations.

Determination of heme loss and irreversibility. The loss in the 7-EFC *O*-deethylation activity of P450 2B6 upon exposure to the five trifluoroaryldiaziridines in the presence of NADPH was compared to the amount of heme remaining and the results are shown in Table 3. The 7-EFC *O*-deethylation activity of P450 2B6 decreased by approximately 70% following incubation with 10 μM 3-(trifluoromethyl)-3-methoxy-(4-methylphenyl)diaziridine (**1e**) for 16 min in the presence of NADPH (Fig 2). The loss in P450 content of the same samples as measured by the reduced-CO spectra ranged from 0 – **25** % approximately. Minimal losses in the enzymatic activity and P450 content were seen in both the control and the exposed samples. Losses in intact heme up to approximately 40% were seen after inactivation with some of the substituted diaziridines when the samples were analyzed using the HPLC assay. However, no heme loss was observed when the heme content of the same samples was measured using the pyridine hemochrome assay (data not shown). These results suggest that the loss in heme seen by HPLC could be due to the acidic HPLC conditions or because of precipitation and retention on the column of a fraction of the heme from the inactivated P450s.

The 7-EFC *O*-deethylation activity of the trifluoroaryldiaziridine-inactivated P450 2B6 samples was not restored after overnight dialysis, suggesting that the inactivation was not due to competition but was irreversible under these conditions. Further, no recovery of the P450 activity was observed following addition of fresh reductase to the inactivated samples suggesting that the inactivation was due to covalent modification of the P450 and not the reductase (data not shown).

Partition Ratio. The number of molecules of the various substituted phenyl diaziridines metabolized per molecule of P450 2B6 inactivated was determined as described in

Materials and Methods. The reactions were allowed to go to completion (30 min) and the turnover number was estimated from a graph of the percentage of activity remaining as a function of the molar ratio of inactivator to P450 2B6 (Kent, Juschyshyn, and Hollenberg, 2001). The partition ratios for the five diaziridine compounds tested ranged from approximately 10 to 62 as shown in Table 4.

Discussion

We report here the synthesis of a new class of compounds containing a diaziridine moiety as a functional group and the evaluation of these compounds as mechanism-based inactivators of P450s. These compounds also have the potential to be used to target a variety of other oxidizing enzymes. It was anticipated that the heme containing P450s would oxidize one of the diaziridine nitrogens to an amine radical cation leading to the formation of benzylic radicals. Radicals of type **9** (R = benzyl) as shown in Scheme 3 have been implicated in the denaturation of hemoglobin by reacting with a cysteinyl thiol group and generation of a hemoglobin-thiyl free radical (Hb-S•) (Maples et al., 1988). Also, with diazirines, it is possible that complexes could occur between the partially oxidized diaziridines and the heme center of the enzymes analogous to the reaction of alkyldiazenes with cytochrome P450 leading to the formation of the [P450-Fe(II)(NH=NR)] complex (Battioni et al., 1983) or the formation of an alkylated porphyrin (Tuck and Ortiz de Montellano, 1992). It has also been shown that 1,2-disubstituted hydrazines lacking α-hydrogens lead to stable azo derivatives that may then be further oxidized to their corresponding azoxy derivatives (Lindeke, 1982). An accepted reaction mechanism for the N-oxidation of monosubstituted hydrazine is shown in Scheme 3. Starke et al. (1984) reported that hemoglobin exhibited monooxygenase activity *in vitro* with a variety of substrates in a system containing CYP450 reductase, NADPH, and O_2, closely resembling the reactions catalyzed by P450s. These studies revealed a novel strategy to synthesize a new class of mechanism-based inhibitors for heme containing enzymes utilizing diaziridines. As described here, six substituted phenyl

diaziridine compounds were synthesized and characterized using ^{1}H and ^{13}C NMR in addition to MS and elemental analysis.

The six trifluromethylaryldiaziridines were screened for their ability to inactivate a number of different recombinant human cytochrome P450s in the reconstituted system. The abilities of these compounds to inactivate various purified P450s in the reconstituted system are reported in Table 1. Five of the six trifluromethylaryldiaziridines showed time-, concentration-, and NADPH-dependent inactivation of the 7-EFC *O*-deethylation activity of P450 2B6 in a reconstituted system. These five diaziridines were further characterized and found to be mechanism-based inactivators of P450 2B6 based on the following criteria: 1. time-dependent losses in the 7-EFC *O*-deethylation activity of P450 2B6; 2. the inactivations of 2B6 showed saturation kinetics with respect to the concentrations of the inactivators; 3. the inactivations were NADPH-dependent; 4. the inactivations were irreversible. A representative figure for the inactivation of P450 2B6 by one of the trifluoroaryldiaziridines is shown in Figure 2. Similar results were obtained for the remaining trifluoroaryldiaziridine compounds except the 4-methylthio compound (**1d**). The nucleophilic reagent, GSH, did not significantly protect P450 2B6 from inactivation by trifluoroaryldiaziridines (data not shown) when added to the incubation mixture at a final concentration of 10 mM, suggesting that the enzyme was covalently modified by an electrophilic reactive intermediate formed from the inactivator before it could leave the active site.

The kinetic constants obtained for the 7-EFC inactivation of P450 2B6 by the trifluoroaryldiaziridines are summarized in Table 2. The concentration required for the half-maximal rate of inactivation (K_I) of P450 2B6 by 3-methyl-4-methoxy substituted

phenyl diaziridine (**1e**) was the lowest (1.7 μM) with respect to the other diaziridines. The range of K_I values varied by a factor of 4 and the k_{inact} values ranged from approximately 0.04 to 0.08 min^{-1} indicating that all five diaziridines are good mechanism-based inactivators. The partition ratio illustrates the efficiency of a particular inactivator for the enzyme. The partition ratio for the inactivation of 2B6 by the 3-methyl,4-methoxy compound (**1e**) was 29; approximately 3-fold higher than that observed for dimethoxy substituted phenyl diaziridine (**1c**) indicating that the dimethoxy substituted phenyl diaziridine was a more efficient inactivator for P450 2B6 compared to methyl substituted phenyl diaziridine (**1e**) or any of the other compounds tested (Table 4).

The five trifluoroaryldiaziridines that acted as mechanism-based inactivators of P450 2B6 did not inactivate P450s 2E1, 2C9, 2D6 or 3A4. Although the 4-methyl thio diaziridine (**1d**) did not lead to inactivation of P450 2B6, it competitively inhibited the BFC activity of P450 3A4. Thus, these data indicate that substitution of a thiomethyl for a methoxy at the ring position leads to a compound that has no *in vitro* inactivation activity. In order to investigate whether reversible binding of the 4-methyl thio diaziridine compound (**1d**) occurs in the enzyme active site without subsequent inactivation of the enzyme, spectral binding studies were performed. The spectral binding data revealed reversible binding of compound 1d to P450 2B6 (data not shown). Preliminary results also indicate that the compound is readily metabolized by P450 2B6. These data suggest that the methoxy group may play an important role in forming the reactive intermediate that covalently binds to the P450 resulting in inactivation.

In order to further understand the mechanism of inactivation, spectral and HPLC analyses were performed following the inactivation of 2B6 by the various diaziridines. The 7-EFC

O-deethylation activity of P4502B6 in the reconstituted system decreased by approximately 60-70% with all five trifluoroaryldiaziridines following incubation with NADPH. In all cases the degree of inactivation was always significantly larger than the loss spectral P450 indicating there is no significant modification in the native heme. Although HPLC analysis showed some losses in heme with all of the trifluoroaryldiaziridines, the losses in heme as measured by the pyridine hemochrome assay (data not shown) were minimal (less than 10 %) suggesting the loss observed with HPLC analysis could result from the acidic conditions used in the mobile phase that could have caused the inactivated protein to precipitate. These results suggested that the inactivation of P450 2B6 was not due to heme destruction but due to covalent modification of the apoprotein. In addition, incubation of the trifluoroaryldiaziridines with recombinant P450 2B6 did not result in the appearance of a peak at 478 nm or a decrease in the native heme peak at 418 nm as would be expected for a phenyl-iron complex (data not shown) (Raag, Swanson, Poulos, and Ortiz de Montellano, 1990;Yamaguchi et al., 2004)

Extensive dialysis of P450 2B6 following inactivation by the five trifluoroaryldiaziridines did not result in recovery of any of the enzyme activity, suggesting that the reactive species formed during the inactivation covalently modified the enzyme. To verify that the loss in enzymatic activity resulted from covalent modification of P450 2B6 and not modification of reductase, fresh reductase was added to the dialyzed samples and the samples were checked for P450 catalytic activity. No gain in activity was observed, suggesting that the inactivation was due to modification of the P450 2B6 and not the NADPH-reductase.

In conclusion, these studies describe the synthesis of novel mechanism-based inhibitors for heme containing enzymes and evaluate their ability to inhibit human cytochrome P450s. The results show that several substituted phenyl diaziridines are relatively potent mechanism-based inactivators of human cytochrome P450 2B6 and that minor changes in the chemical structures of the compounds can have a significant influence on their ability to inactivate that P450. The most potent inhibitors were those derivatives that contained methoxy groups at the four position of the phenyl ring. Replacement of the methoxy moiety at position four with a methylsulfide (**1d**) resulted in a compound that had no ability to act as a mechanism-based inactivator of P450 2B6. Spectral binding studies indicated that this was not due to an inability of this compound to bind to the P450 active site. Preliminary studies also indicated that the methylsulfide substituted phenyl diaziridine **(1d)** was metabolized by P450 2B6. This result suggests that metabolism of the phenyl diaziridine to a reactive intermediate capable of inactivating P450 2B6 requires the presence of an alkoxide at position 4 on the phenyl ring.

A preliminary mechanistic interpretation, consistent with the data presented, invokes the formation of a reactive metabolite that may be formed as a result of an initial hydroxylation event catalyzed by P450 2B6 (Scheme 4). Reaction of para-methoxy substituted diaziridines with P450 2B6 would generate an N-hydroxy diaziridine, which we have not detected in the incubation mixtures, presumably due to its inherent reactivity. Although N-hydroxylation reactions of diaziridines have not been described in the literature, oxidation reactions of the counterpart acyclic hydrazines have been documented. Heterolytic bond scission of this initial oxidation product would result in the formation of a benzylic carbocation, further stabilized by electron donating substituents

(O-R groups) at the para position. Carbene formation concomitant with expulsion of nitrogen would afford the reactive species capable of enzyme inactivation (Scheme 4). Additional studies aimed at characterizing the metabolites formed and the reactive intermediates generated during the inactivation in order to better understand the mechanism of inactivation are in progress.

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Footnotes.

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Figure Legends.

Figure 1. Structures of substituted phenyl diaziridines.

Figure 2. Time- and concentration dependent inactivation of recombinant P450 2B6 as measured by 7-EFC *O*-deethylation activity after incubation with 3-(trifluromethyl)-3-methoxy-(4-methylphenyl)diaziridine. The reaction mixtures were incubated in the reconstituted system with NADPH and 0 μM (■), 0.1 μM (△), 1 μM (▲), 5 μM (◇), 10 μM (◆) or 50 μM (○) of substituted phenyl diaziridines. At the indicated time points, aliquots of the reaction mixture were assayed for residual activity as described in Materials and Methods. The inset shows a double reciprocal plot of the rate of inactivation of the 7-EFC *O*-deethylation activity as a function of the inactivator concentration. The data represent the mean and standard error from three separate experiments.

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Table 1. Effect of six substituted phenyl diaziridines on the 7-EFC O-deethylation activity of P450s 2B6, 2E1, 2C9 and 2D6 or the BFC *O*-debenzylation activity of P450 3A4.

	Activity Loss (% Control)*				
Substituted phenyl diaziridines	**P450 2B6**	**P450 2C9**	**P450 2D6**	**P450 2E1**	**P450 3A4**
4-methoxy (1a)	**65 %**	No loss	No loss	No loss	No loss
4-ethoxy(1b)	**62 %**	No loss	No loss	No loss	No loss
3,4-dimethoxy(1c)	**70 %**	No loss	No loss	No loss	**Competitive Inhibition**
4-methylthio (1d)	**No loss**	No loss	No loss	No loss	**Competitive Inhibition**
3-methyl,4-methoxy (1e)	**70 %**	No loss	No loss	No loss	No loss
3,4,5-trimethoxy (1f)	**70 %**	No loss	No loss	No loss	No loss

0.5 nmol of P450 was incubated with 100 µM of the substituted phenyl diaziridine indicated in the reconstituted system at 30 °C with or without NADPH. At different time points the residual activity was determined using the 7-EFC/BFC assay as described in Methods. The results represent an average of two sets of experiments done in duplicate. No loss indicates < 10 % loss.

Table 2. The kinetic parameters for the inactivation of the 7-EFC *O*-deethylation activity of P450 2B6 when incubated with the six different phenyl diaziridine compounds.

Substituted phenyl diaziridines	K_I μM	k_{inact} min^{-1}	$t_{1/2}$ min
4-methoxy (1a)	7.1 ± 1.9	0.042	16.5
4-ethoxy (1b)	2 ± 0.7	0.079	8.8
3,4-dimethoxy (1c)	2.5 ± 1.2	0.06	11.4
4-methylthio (1d)	No inactivation		
3-methyl,4-methoxy (1e)	1.7 ± 0.2	0.066	10.5
3,4,5-trimethoxy (1f)	2.7 ± 0.9	0.05	14

The kinetic constants were derived from the double reciprocal plots of the rates of inactivation of 7-EFC *O*-deethylation activity as a function of the inactivator concentrations. The kinetic constants represent the mean and standard error from three different experiments.

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Table 3. Effect of trifluoroaryldiaziridines on P450 2B6 residual activity, reduced CO spectrum and heme remaining by HPLC.

Substituted phenyl diaziridines	Activity Remaining	P450 Remaining by reduced CO	Heme Remaining by HPLC
4-methoxy (1a)	**35 ± 6**	**87 ± 2**	**68 ± 3**
4-ethoxy (1b)	**35 ± 20**	**99 ± 11**	**81 ± 4**
3,4-dimethoxy (1c)	**45 ± 4**	**73 ± 7**	**79 ± 5**
3-methyl,4-methoxy (1e)	**30 ± 7**	**90 ± 4**	**63 ± 2**
3,4,5-trimethoxy (1f)	**39 ± 1**	**81 ± 2**	**64 ± 4**

Assay conditions are described under Materials and Methods. Activity remaining was determined using a concentration of 10 μM of each diaziridine and an incubation time of 16 min. The amount P450 remaining was determined from the reduced CO spectrum. The control sample was 100 %. The amount of heme remaining by HPLC was calculated after integrating the area under the peak at 405 nm from the HPLC profile. The area obtained from the control sample was set to 100 %. The data represent the mean and standard deviation from 3 to 5 experiments.

Table 4. Partition ratios for the inactivation of P450 2B6 by the substituted phenyldiaziridines.

Substituted phenyl diaziridines	**4-methoxy (1a)**	**4-ethoxy (1b)**	**3,4-dimethoxy (1c)**	**3-methyl,4-methoxy (1e)**	**3,4,5-trimethoxy (1f)**
Partition Ratio	**41**	**62**	**9.6**	**29**	**45**

Assay conditions are described in the Materials and Methods. The partition ratio is determined by incubating various concentrations of the inactivator molecules with P450 2B6 in the reconstituted system and allowing the reaction to go to completion. The partition ratio was then calculated from the graph of the percent activity remaining as a function of the ratio of the inactivator to enzyme concentration (Kent, Juschyshyn, and Hollenberg, 2001). The numbers represent the average of the results from three experiments with each substituted phenyl diaziridine.

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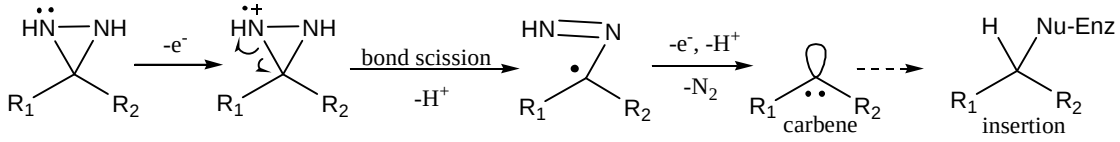
Scheme1. Proposed mechanism of a chemical model of diaziridine oxidation involving the single electron transfer pathway (Post and Morrison, 1996).



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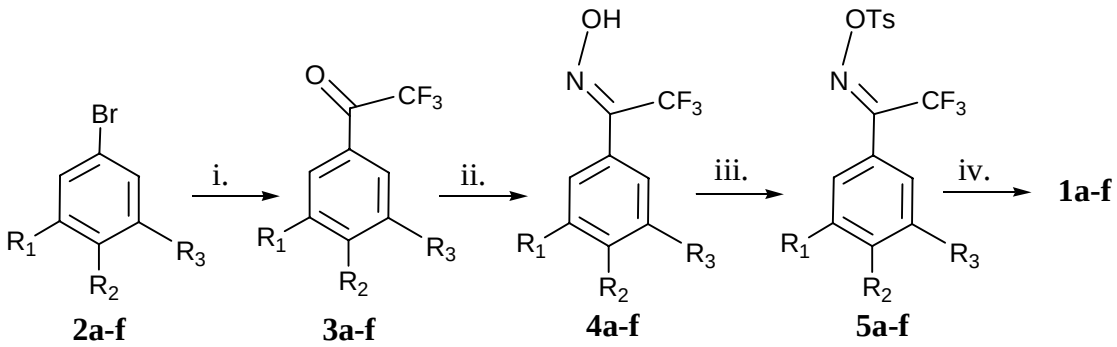
Scheme 2. Synthesis of diaziridines **1a-f**.



i.) Mg, trifluoroacetylpiperidine. ii.) NH_2OH, pyridine. iii.) TsCl, pyridine. iv.) NH_3

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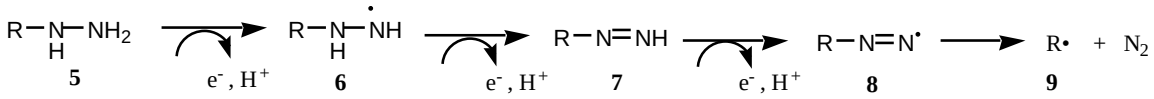
Scheme 3. Accepted reaction mechanism for the *N*-oxidation of monosubstituted hydrazines.



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Scheme 4: Proposed mechanism involving the hydroxylation event

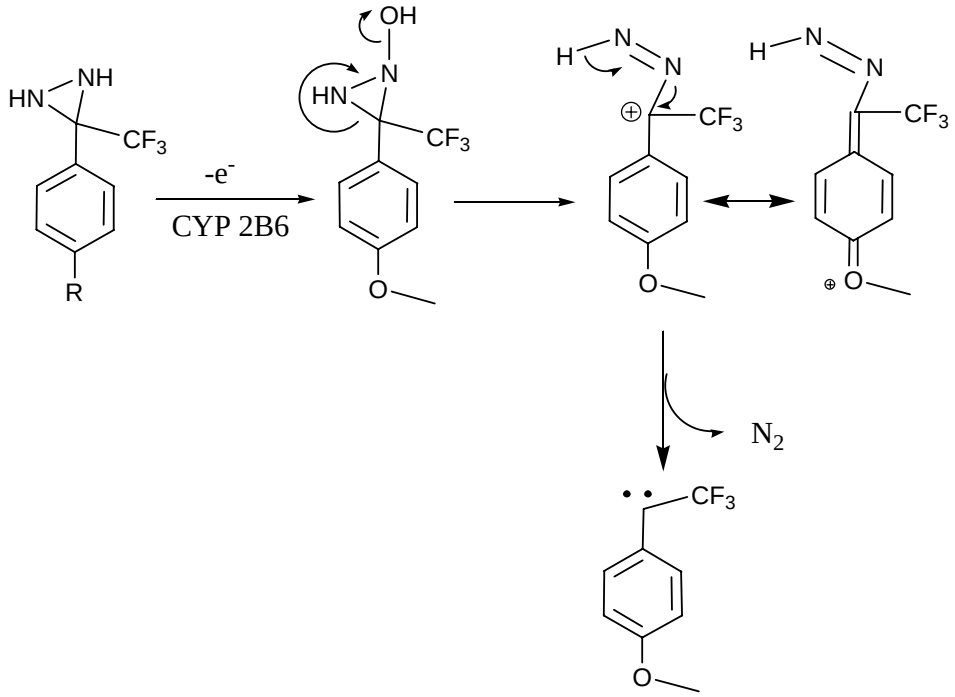

Figure 1

(**1a**) R_1=H, R_2 = OCH_3, R_3= H
(**1b**) R_1=H, R_2 = OCH_2CH_3, R_3= H
(**1c**) R_1=H, R_2 = OCH_3, R_3= OCH_3
(**1d**) R_1=H, R_2 = SCH_3, R_3=H
(**1e**) R_1=H, R_2 = OCH_3, R_3= CH_3
(**1f**) R_1=OCH_3, R_2 = OCH_3, R_3= OCH_3

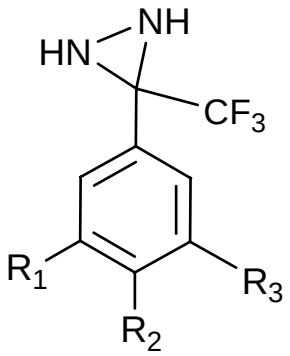

Figure 2