Mechanism-Based Inactivation of Human Cytochrome P450s

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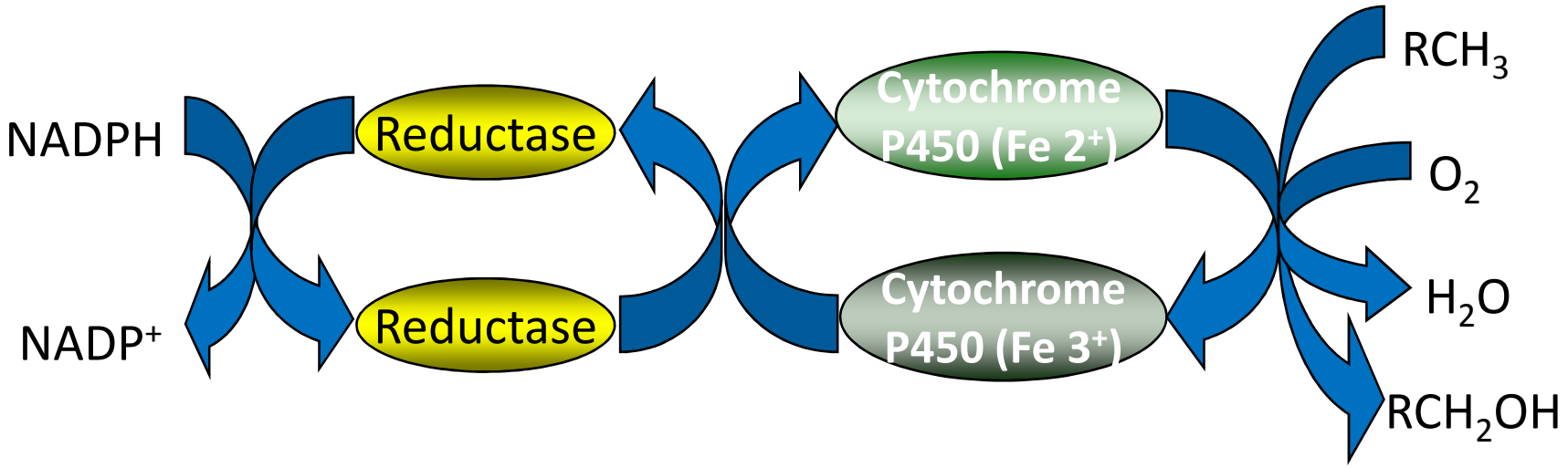
P450 Substrate Hydroxylation



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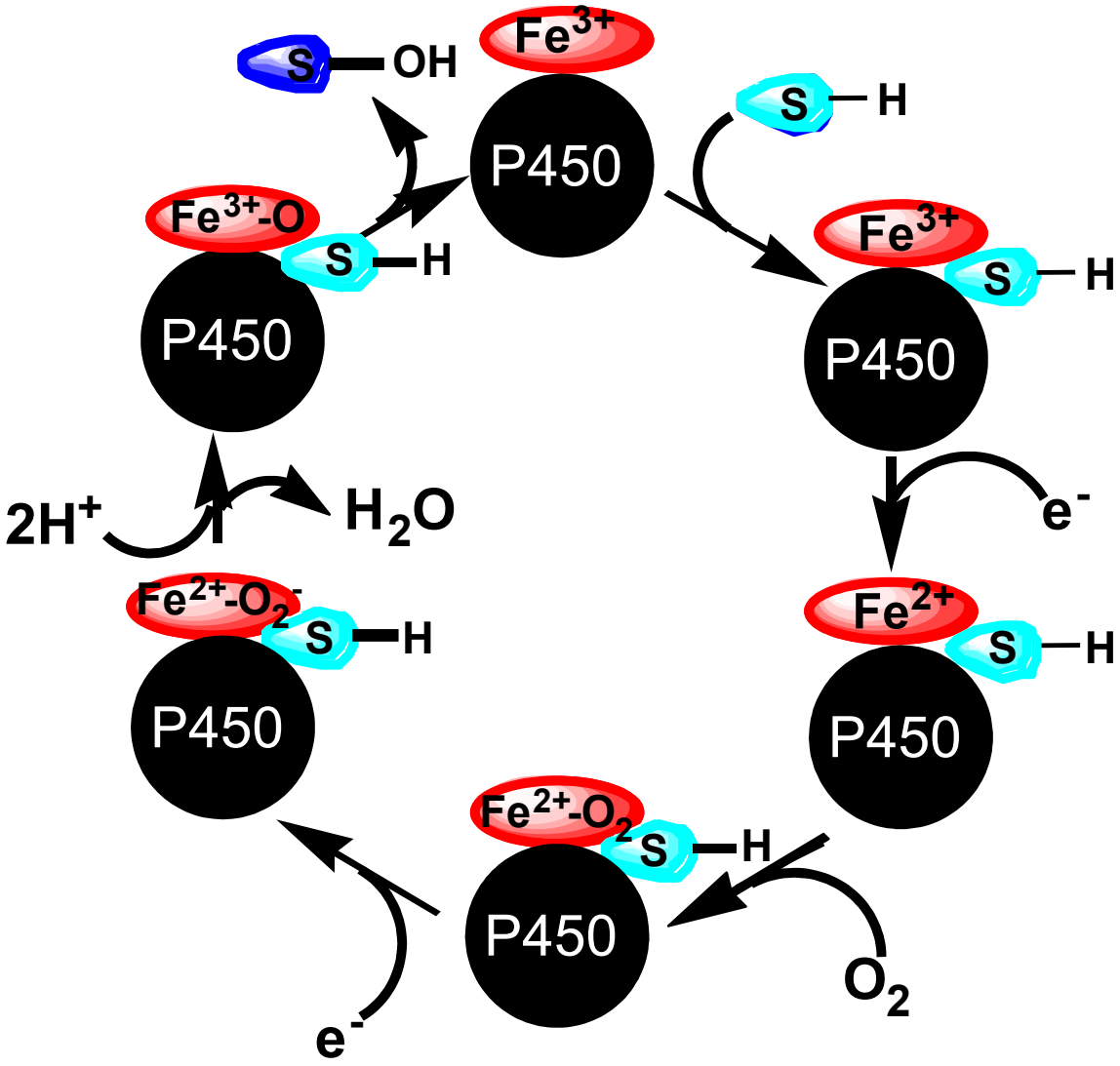

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Mechanism-Based Inactivator

- **Terminology:**
  - **suicide inactivator**
  - **enzyme-activated irreversible inhibitor**
  - **time-dependent inhibitor**

- **Definition: A substrate that in the process of catalytic turnover is metabolized to a reactive intermediate which inactivates the enzyme.**

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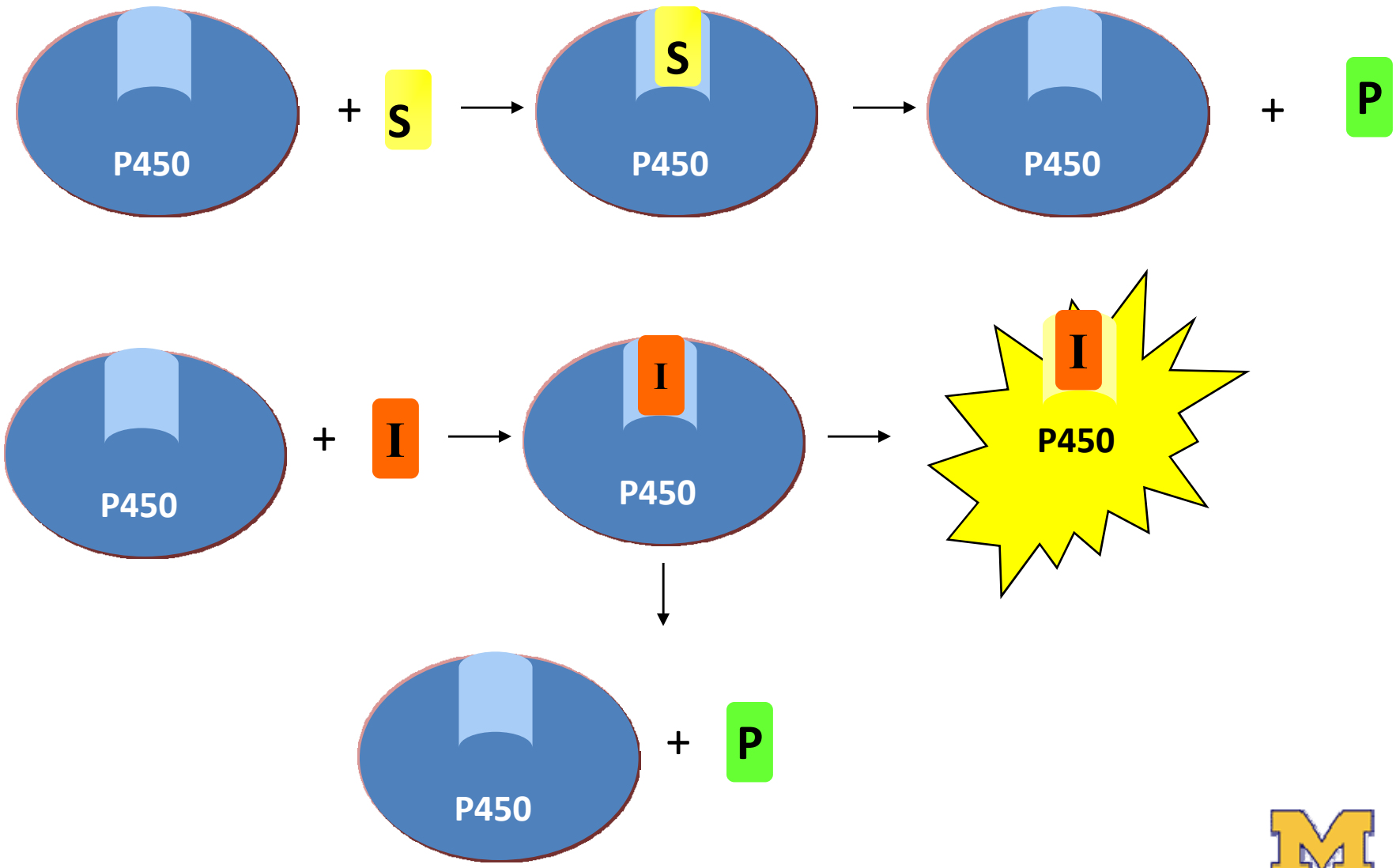

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Mechanism-Based Inactivators

Enzyme substrates

Require all coenzymes and substrates

Activity loss is first-order with enzyme

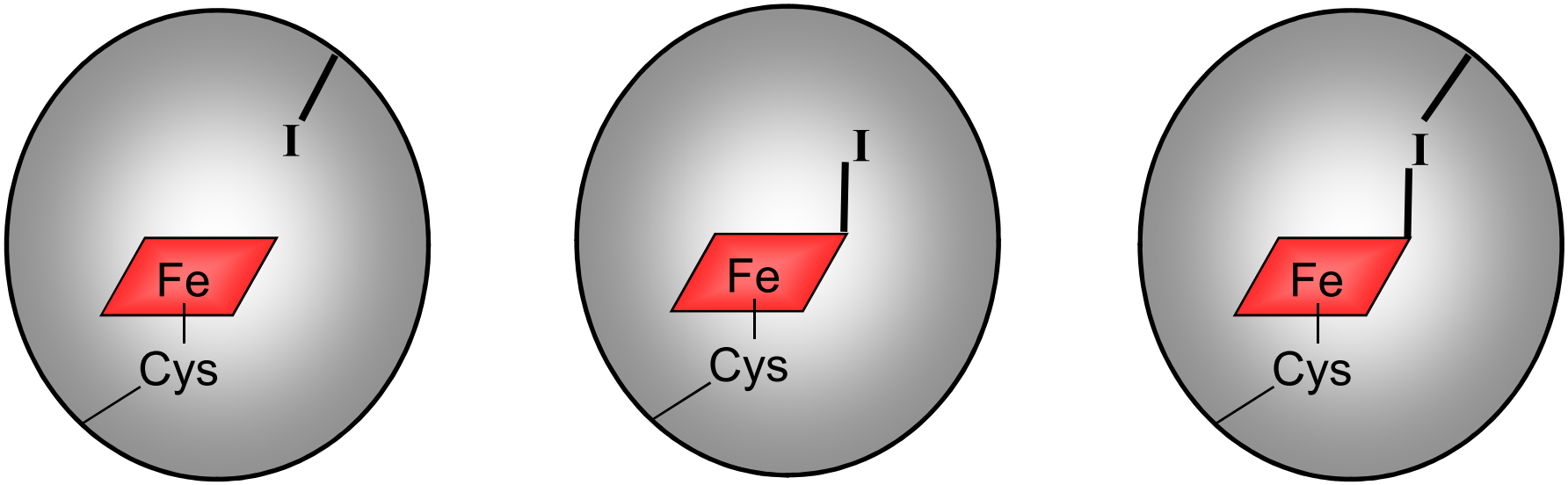
Exhibit saturation kinetics

Inactivation is stoichiometric

GSH and DDT do not protect against inactivation

Inactivation is irreversible

Three Pathways for Mechanism-Based Inactivation



Apoprotein Heme Crosslinked

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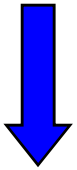
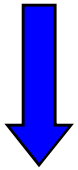
Information that Can be Obtained with Mechanism-Based Inactivators:





Structural Studies

a) Site of adduct binding:

- heme
- protein
  - i.d. adducted peptide
  - i.d. adducted amino acid

b) site-directed mutagenesis

Mechanistic Studies

a) Identify the step(s) in the P450 reaction that are compromised and result in the loss in activity

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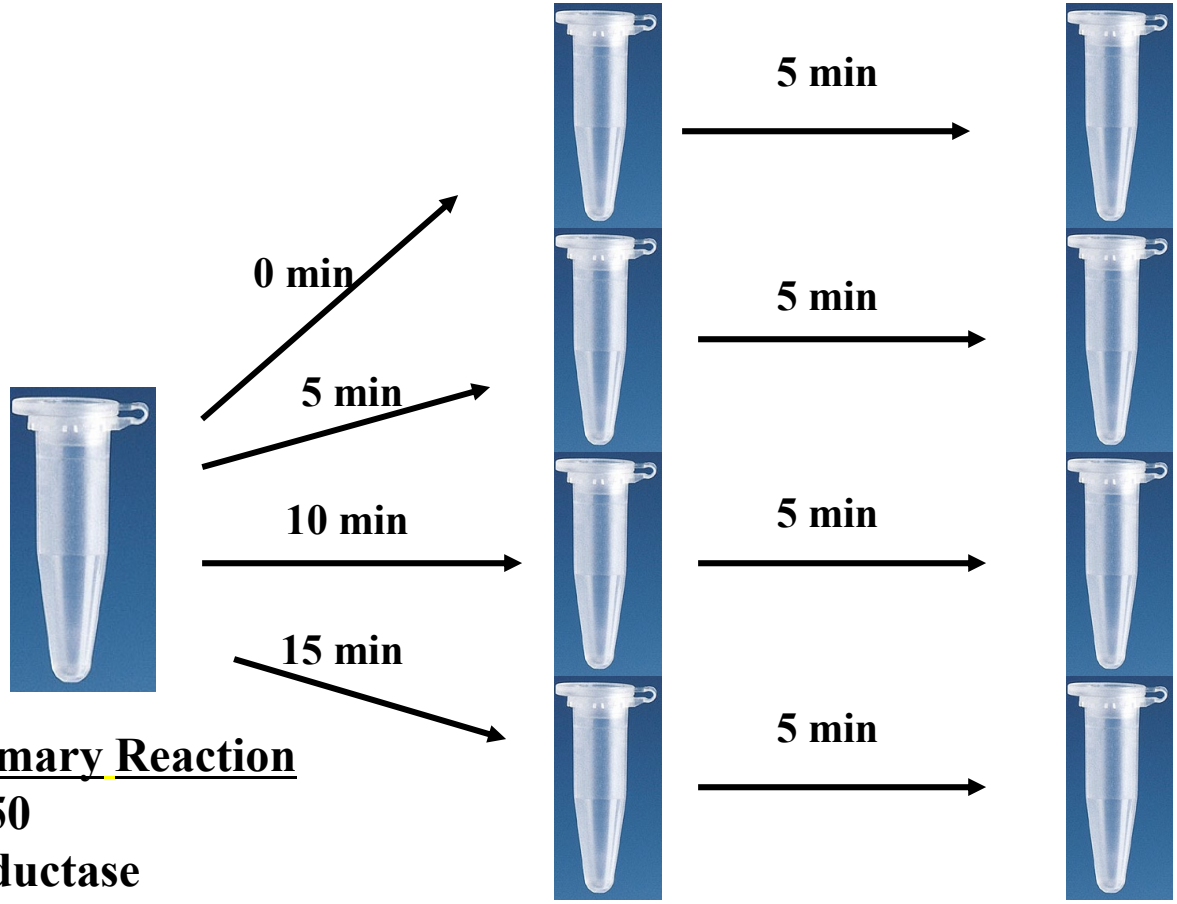
Method



Primary Reaction
P450
Reductase
NADPH
Inactivator

Secondary Reaction
Primary rxn mix
7-EFC
NADPH

HFC product formation

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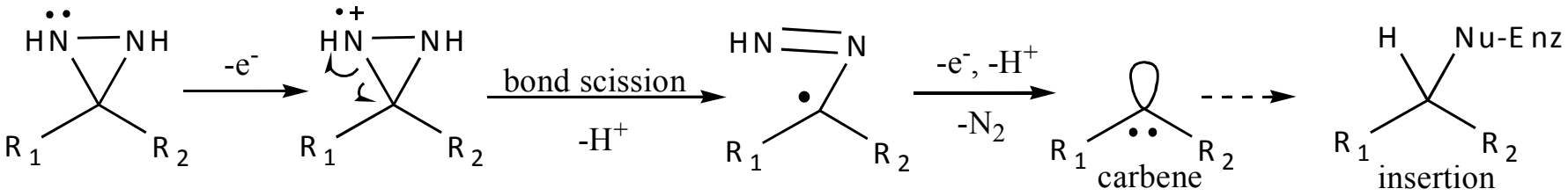
Proposed Mechanism for Diaziridine Oxidation



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Structures of Substituted Aryl Diaziridines

(1) $R_1 = H,\ R_2 = OCH_3,\ R_3 = H$

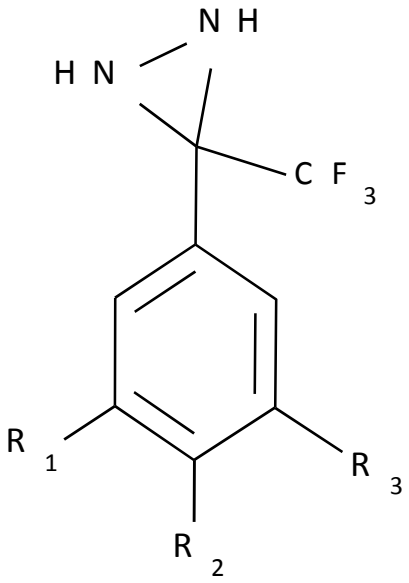
(2) $R_1 = H,\ R_2 = OCH_2CH_3,\ R_3 = H$

(3) $R_1 = H,\ R_2 = OCH_3,\ R_3 = OCH_3$

(4) $R_1 = H,\ R_2 = OCH_3,\ R_3 = CH_3$

(5) $R_1 = OCH_3,\ R_2 = OCH_3,\ R_3 = OCH_3$

(6) $R_1 = H,\ R_2 = SCH_3,\ R_3 = H$



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Inactivation of P450 2B6 by the Substituted Aryl Diaziridines

Activity Loss (% of Control)	
Substitution	**P450 2B6**
4-methoxy (1)	65 %
4-ethoxy(2)	62 %
3,4-dimethoxy(3)	70 %
3-methyl,4-methoxy (4)	70%
3,4,5-trimethoxy (5)	70 %
4-methylthio (6)	No loss

No inactivation was observed with P450s 2C9, 2D6, 2E1, or 3A4

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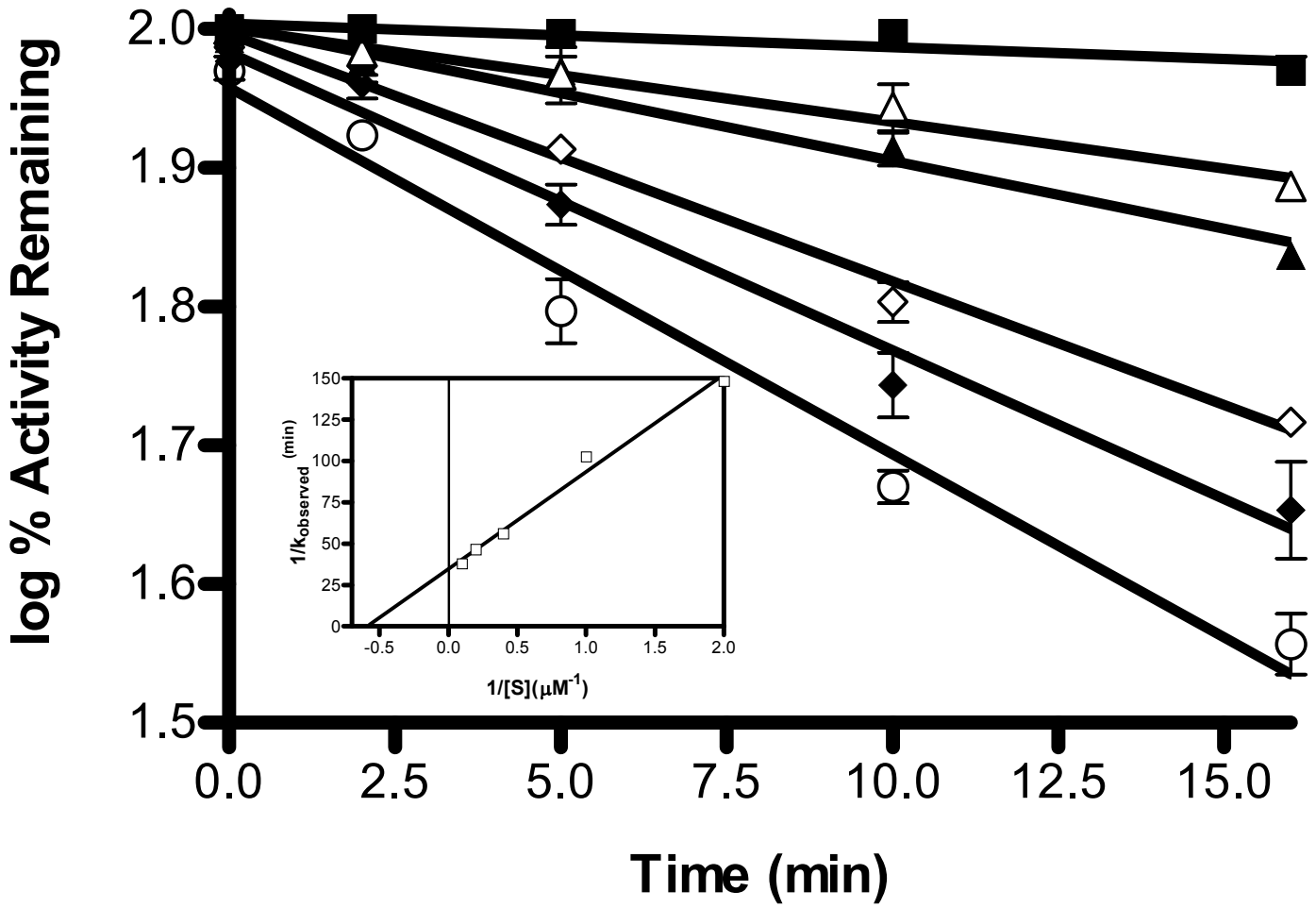
Time- and Concentration Dependent Inactivation of P450 2B6 by 3-(Trifluoromethyl)-4-methoxy(3-methylphenyl)diaziridine



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Kinetic Parameters for Inactivation of P450 2B6 by the Substituted Aryl Diaziridines

Substituted aryl diaziridine	K_I μM	k_{inact} min^{-1}	$t_{1/2}$ min
4-methoxy (1)	7.1 ± 1.9	0.042	16.5
4-ethoxy (2)	2 ± 0.7	0.079	8.8
3,4-dimethoxy (3)	2.5 ± 1.2	0.06	11.4
3-methyl,4-methoxy (4)	1.7 ± 0.2	0.066	10.5
3,4,5-trimethoxy (5)	2.7 ± 0.9	0.05	14
4-methylthio (6)		No inactivation	

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Partition Ratios for the Inactivation of P450 2B6 by the Substituted Aryl Diaziridines

Substituted aryl diaziridine	4-methoxy (1)	4-ethoxy (2)	3,4-dimethoxy (3)	3-methyl,4-methoxy (4)	3,4,5-trimethoxy (5)
Partition Ratio	41	62	9.6	29	45

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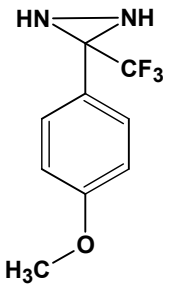
Other Properties for the Inactivation of P450 2B6 by the Substituted Aryl Diaziridiens

- Addition of reductase to the inactivated protein does not lead to recovery of activity
- Inactivation is irreversible
- There is no significant heme modification
- 10 mM GSH does not protect against inactivation

Structures of the Aryl Diazidirines



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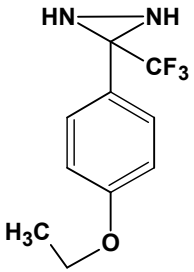

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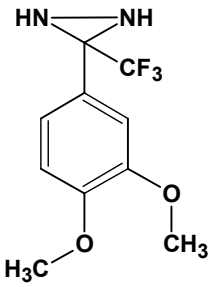

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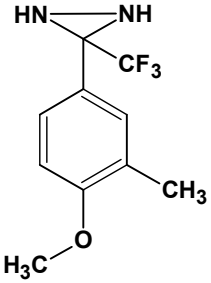

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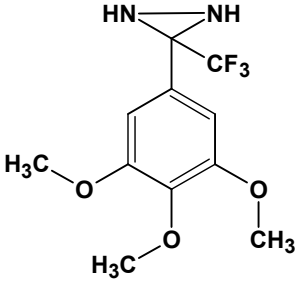

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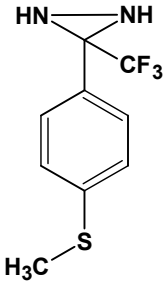

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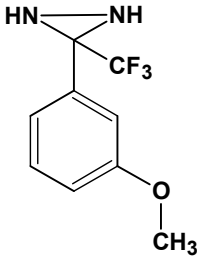

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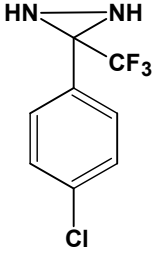

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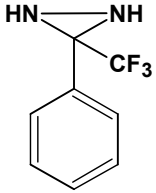

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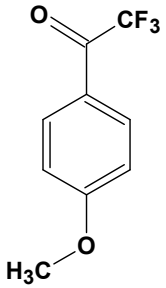

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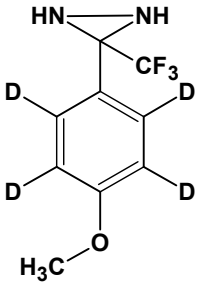

11

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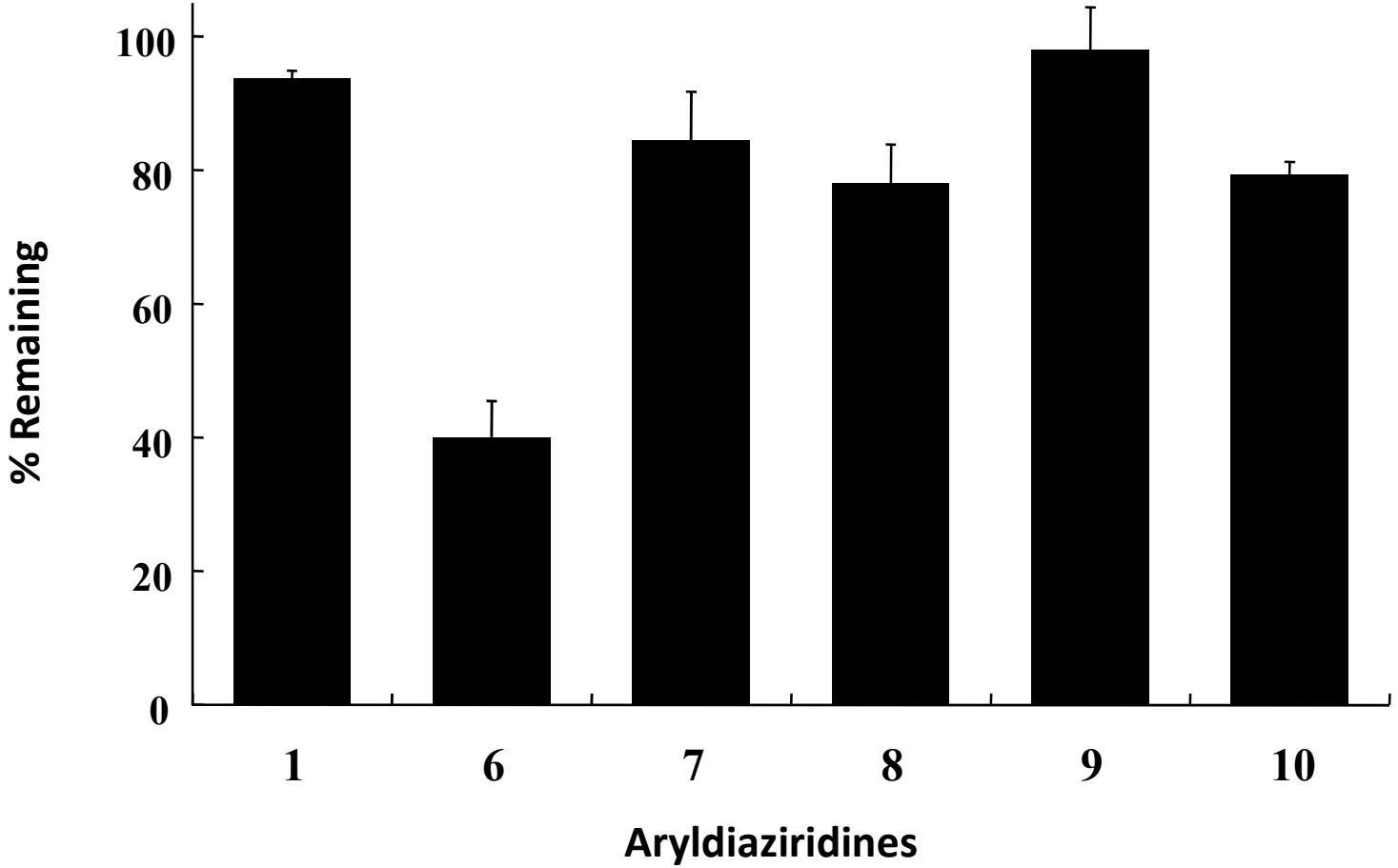
Metabolic Stability of the Aryl Diaziridines



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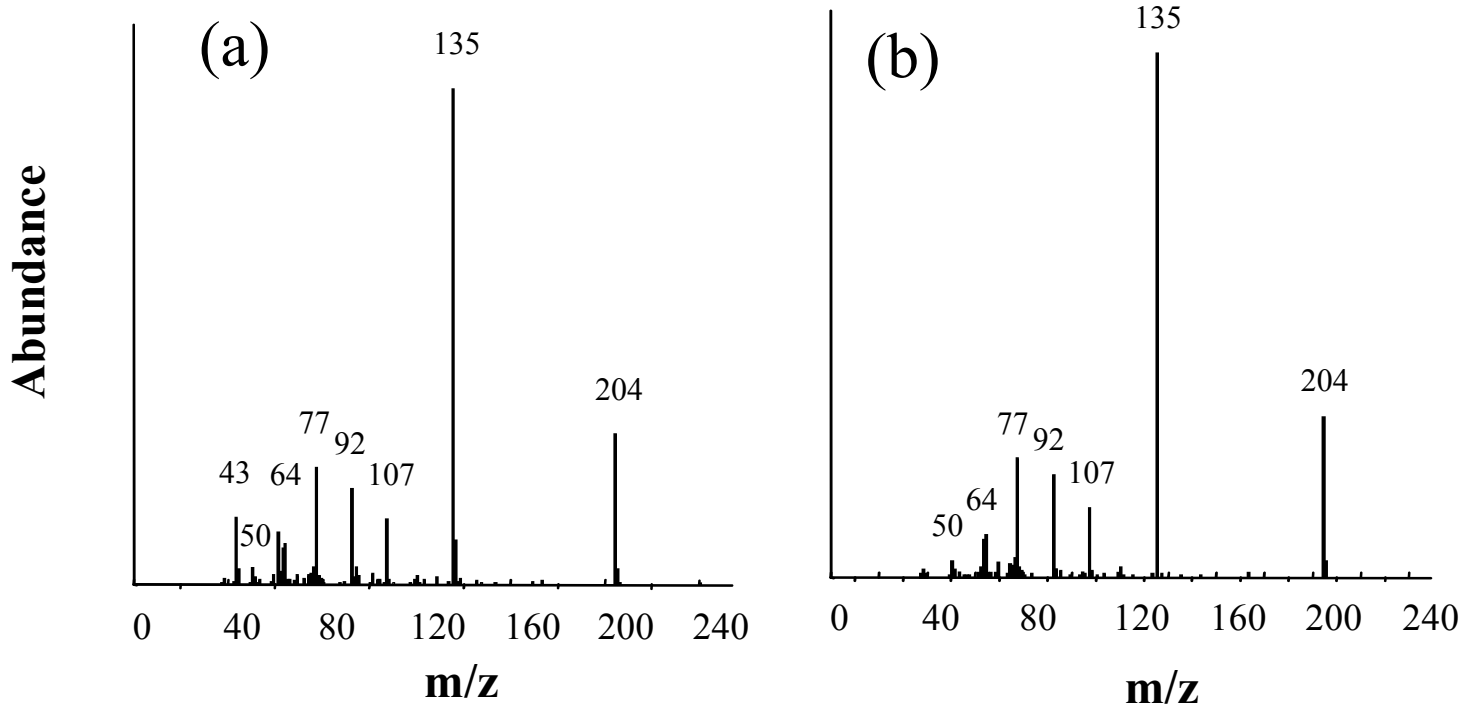
GC-MS Spectrum of the Metabolite of Aryl Diaziridine 1 (a) and its Ketone Standard (b)



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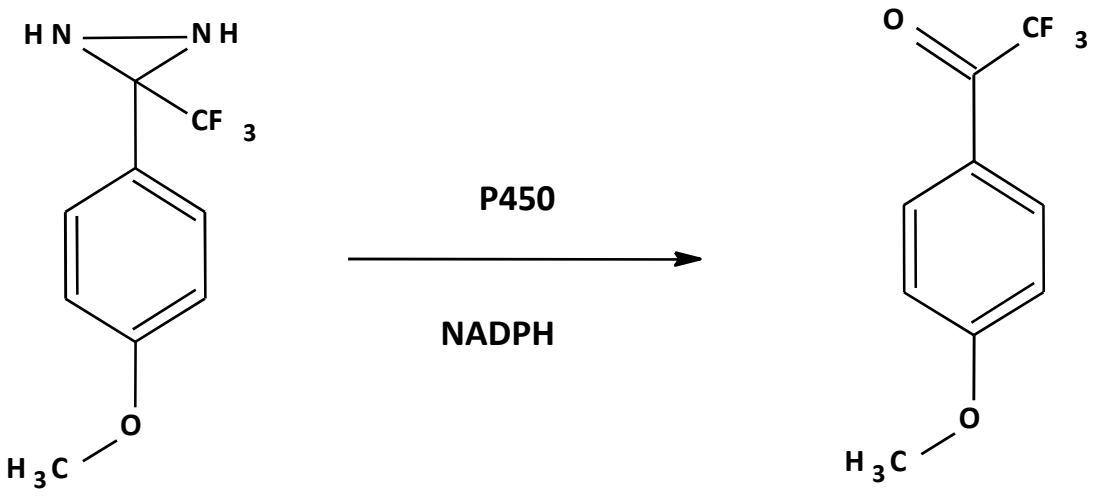
Metabolism of an Aryl Diaziridine to a Ketone



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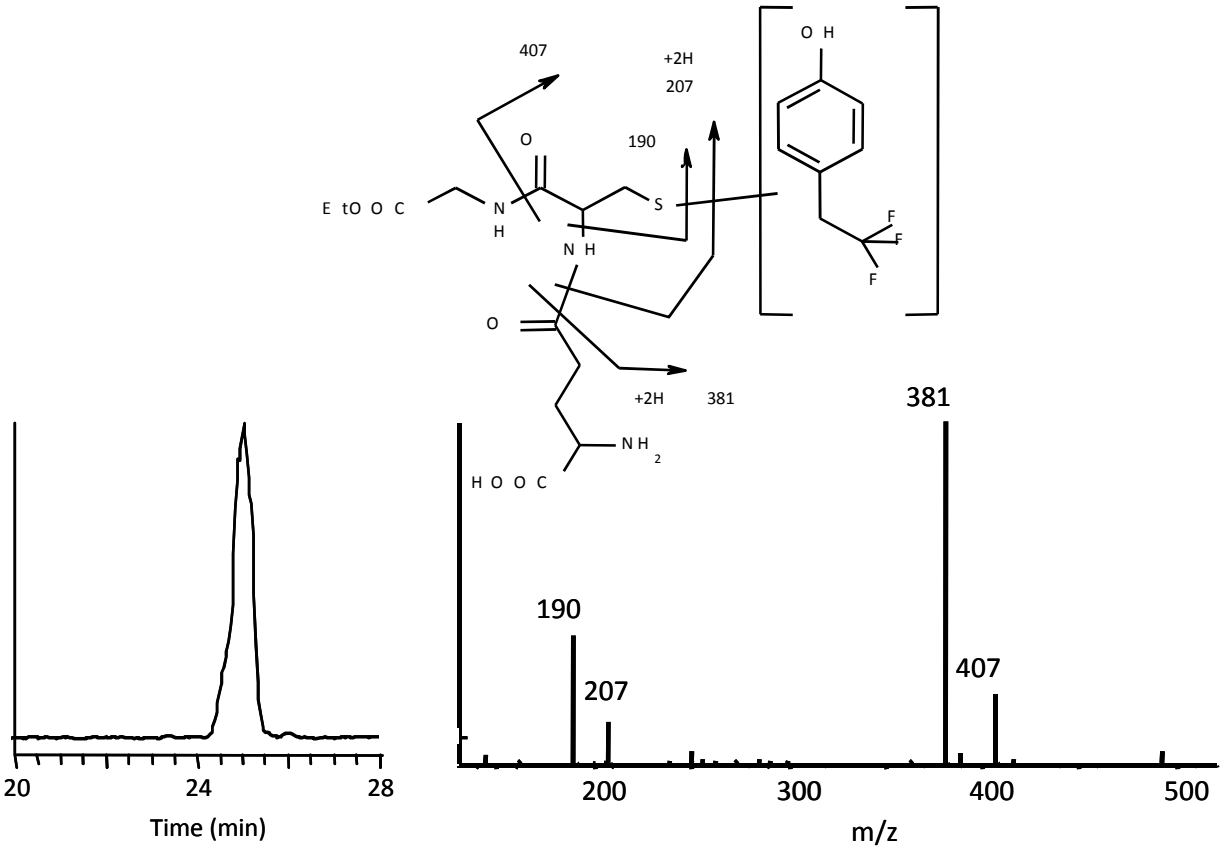
LC-MS/MS Analysis of GSHEE Adducts of Aryl Diaziridine 1



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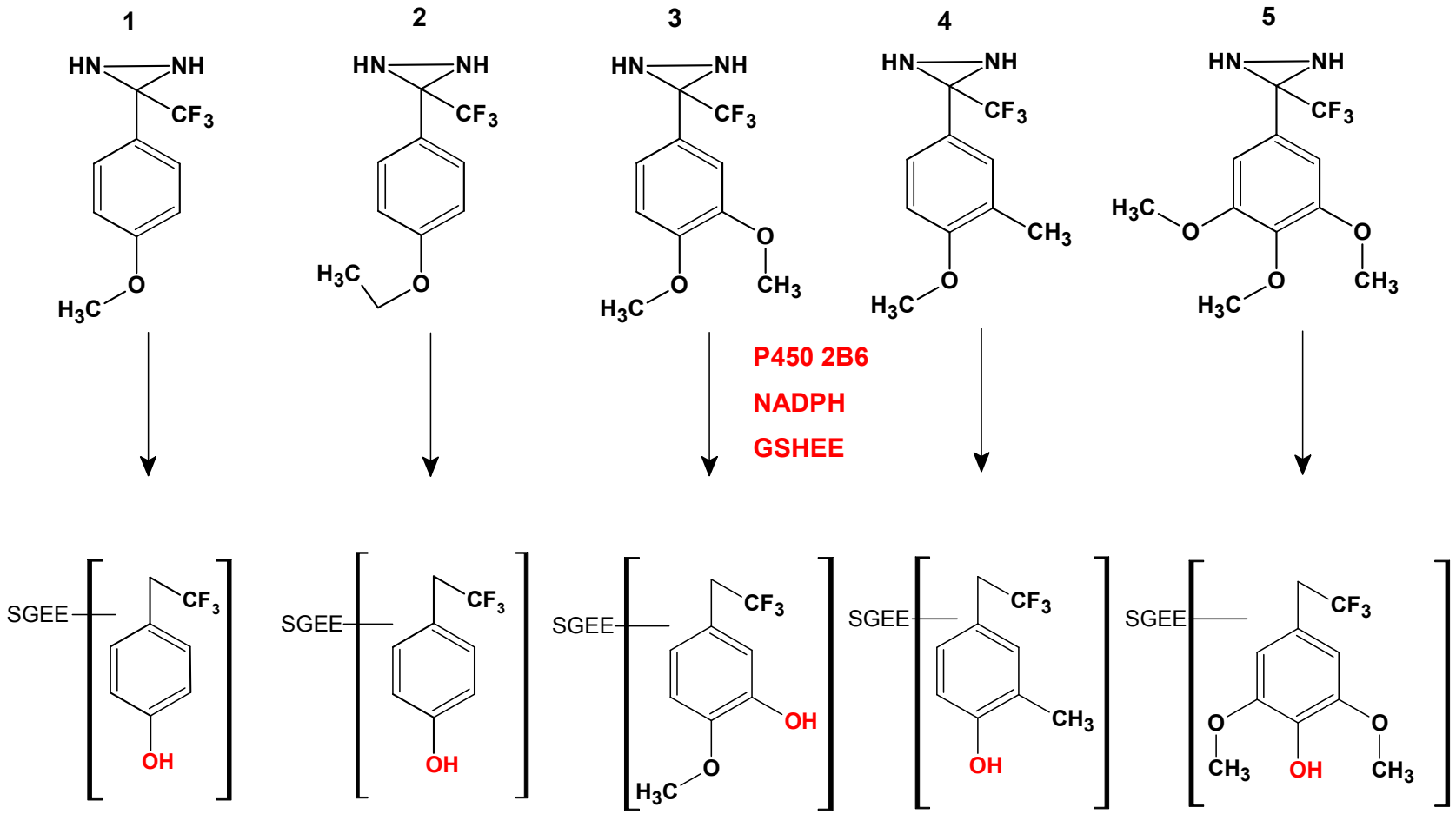
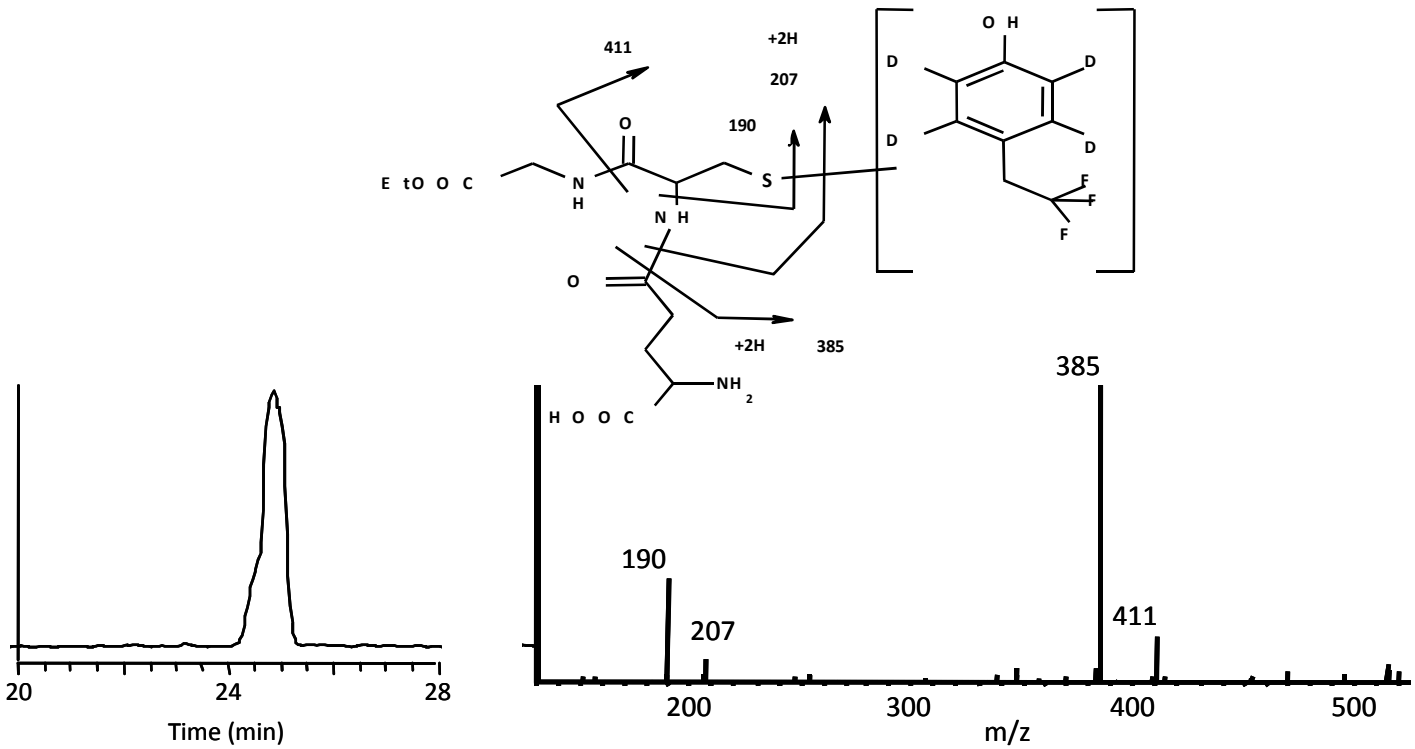
Proposed Chemical Structures for the GSHEE-Adducts formed by P450 2B6



LC-MS/MS Analysis of GSHEE Adducts of Aryl Diaziridine 11



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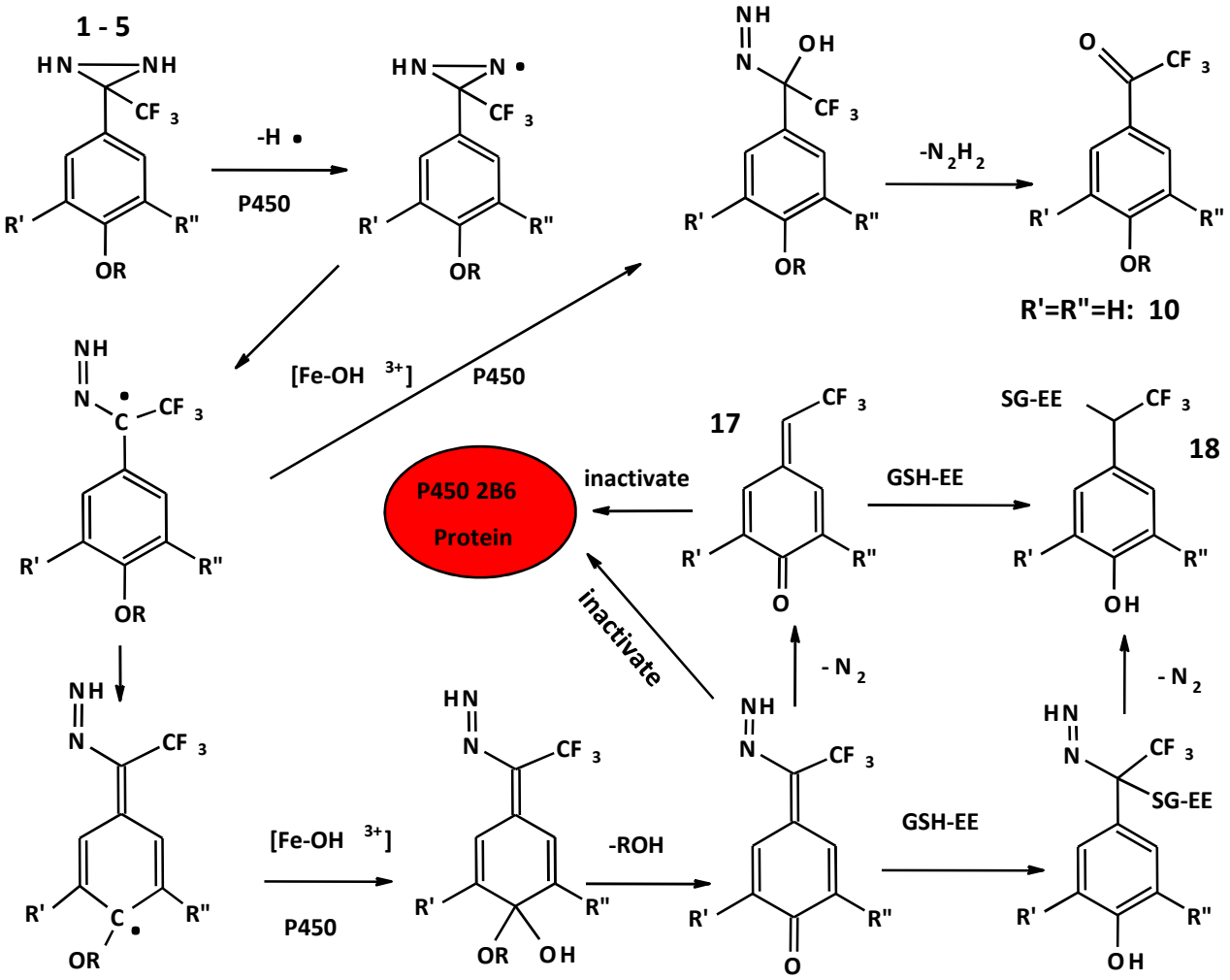
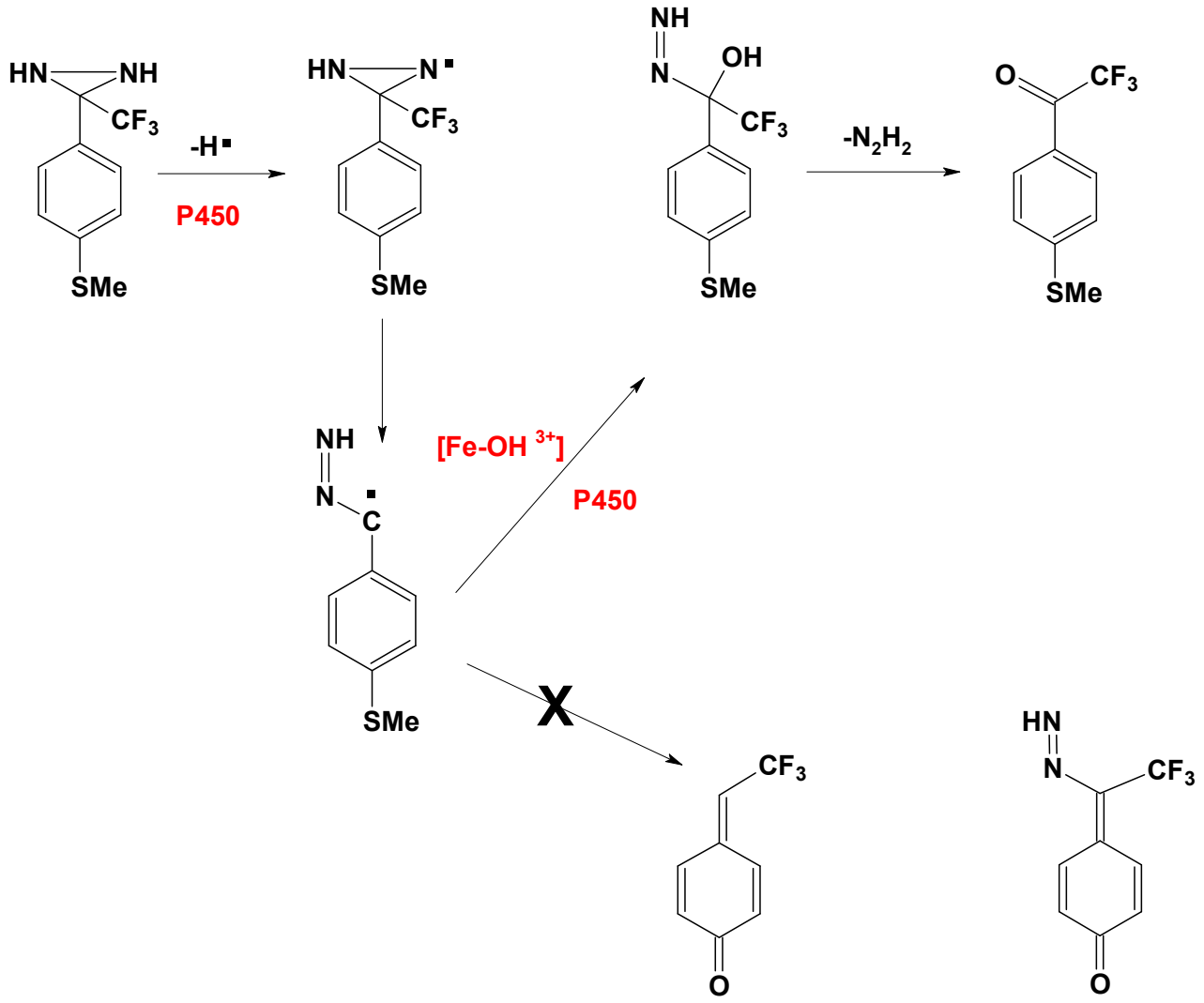
Proposed Mechanism for the Inactivation of P450 2B6 by Aryl Diaziridines 1-5



Pathway for the Metabolism of Compound 6 without Formation of a Reactive Intermediate



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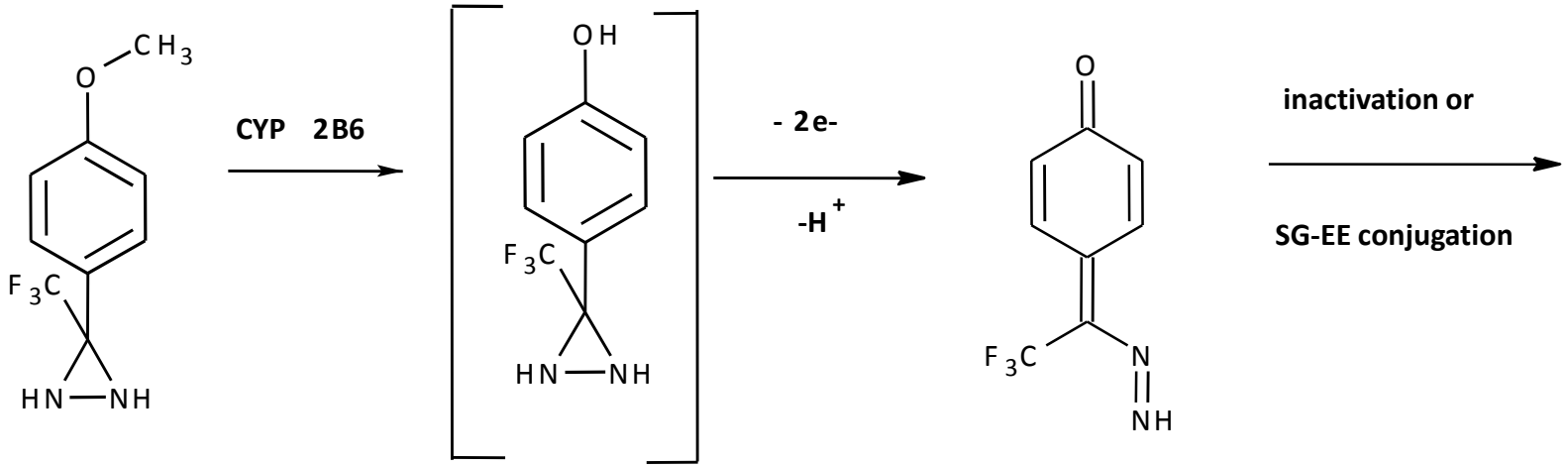
An Alternative Mechanism for the Inactivation of P450 2B6 by Aryl Diaziridines



1

19

unstable intermediate

20

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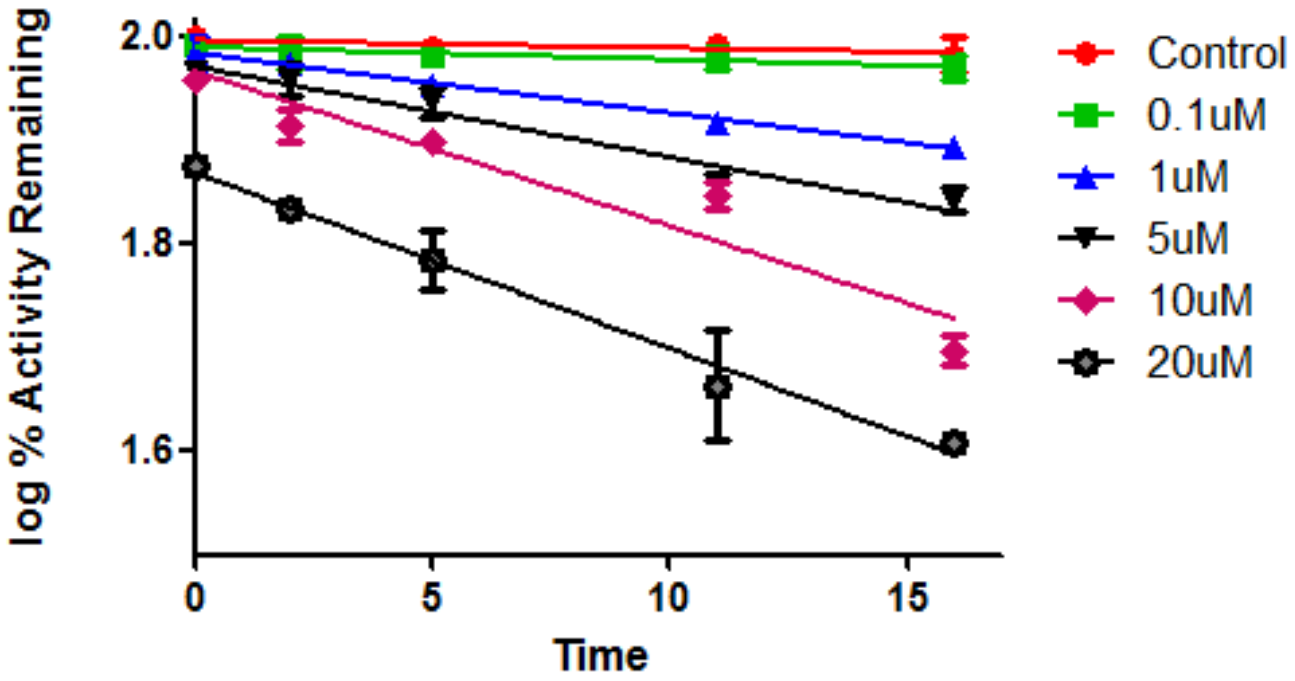
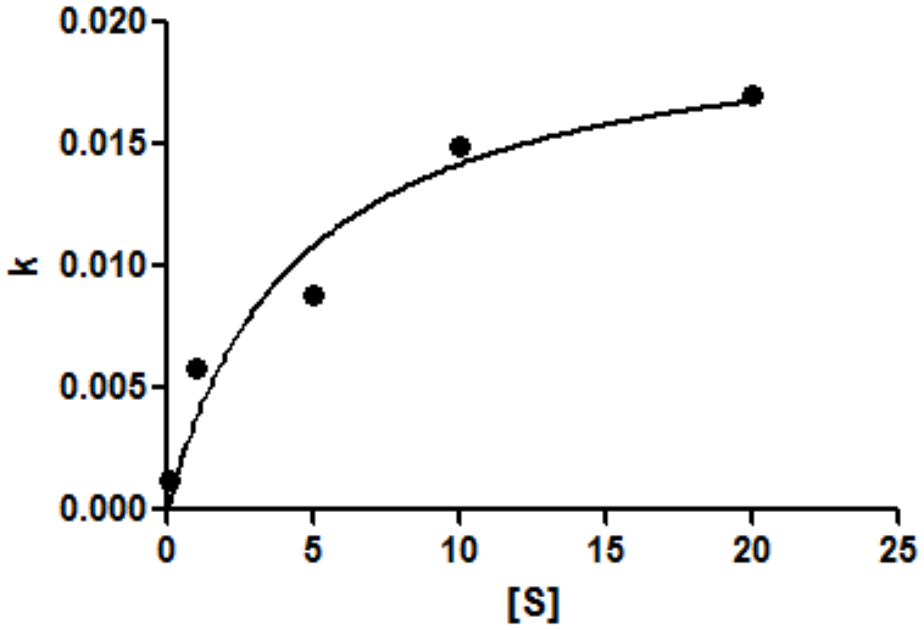
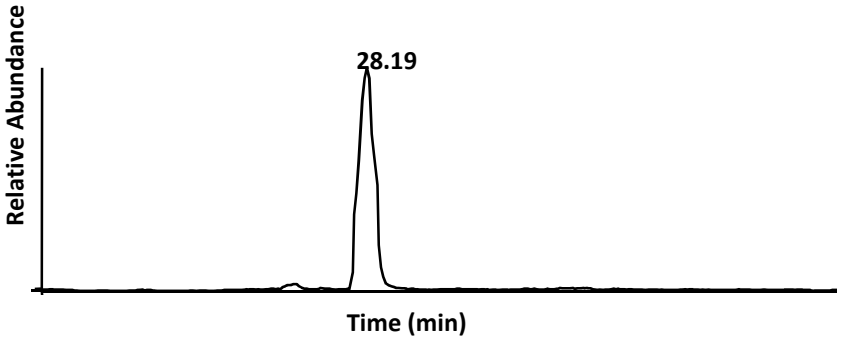
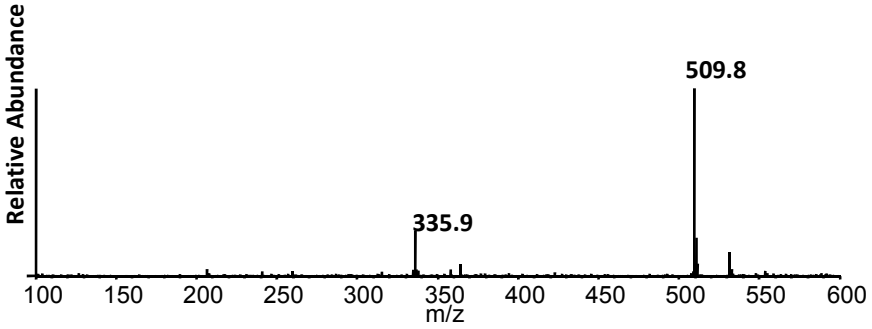
P450 2B6 and 4-hydroxy phenyl diaziridine

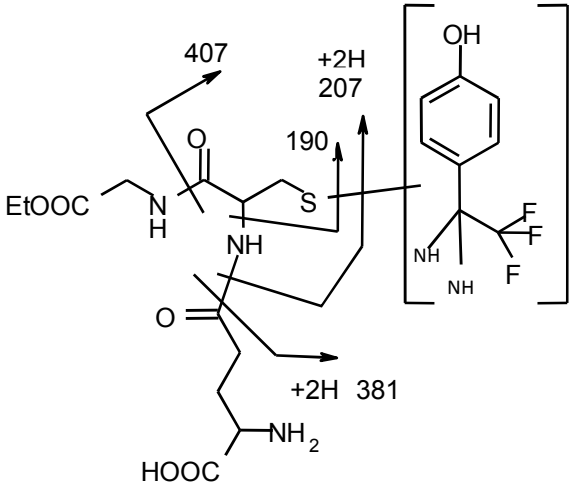


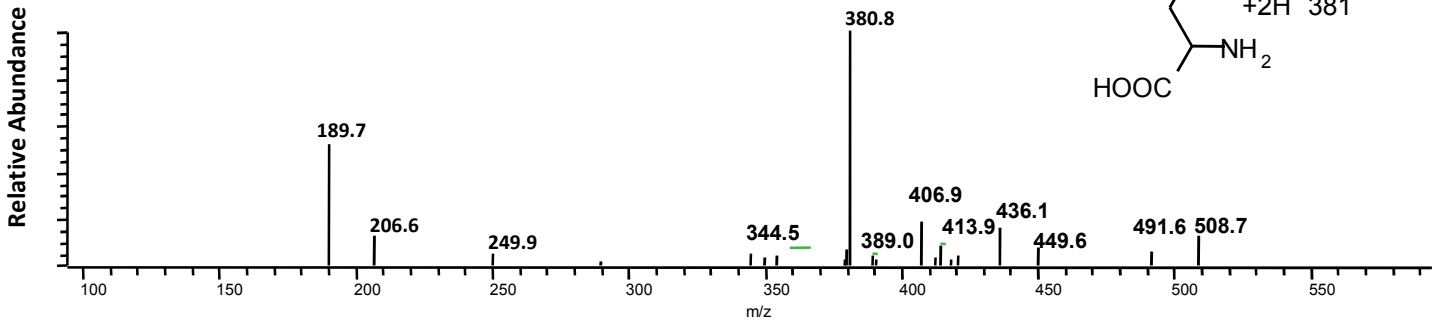


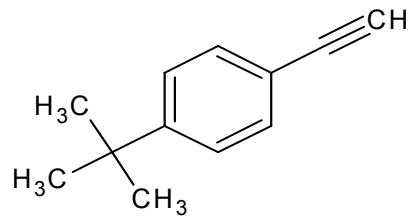
Km = 4.4μM and Vmax = 0.02


Relative Abundance
28.19
Time (min)

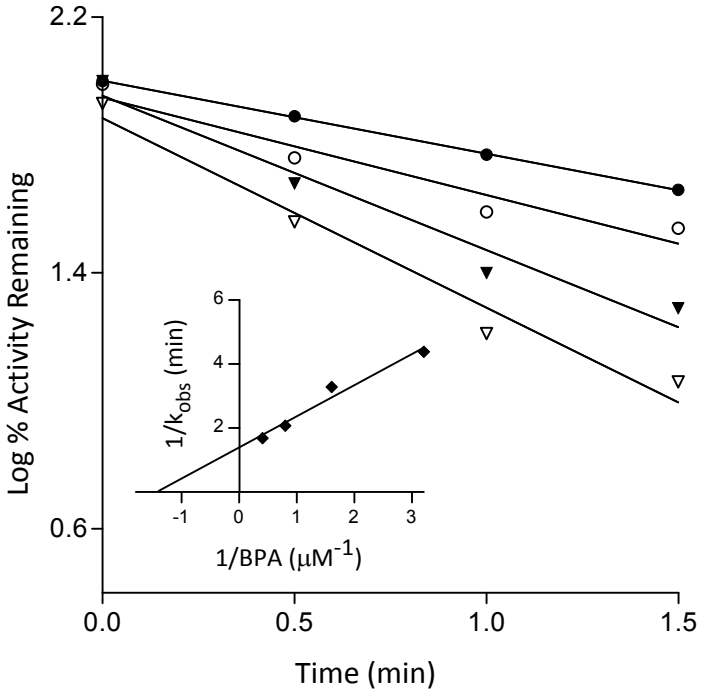

Relative Abundance
509.8
335.9
100 150 200 250 300 350 400 450 500 550 600
m/z


407
+2H
207
190
OH
O
EtOOC
N
H
S
NH
F
F
F
NH
NH
O
+2H 381
NH2
HOOC

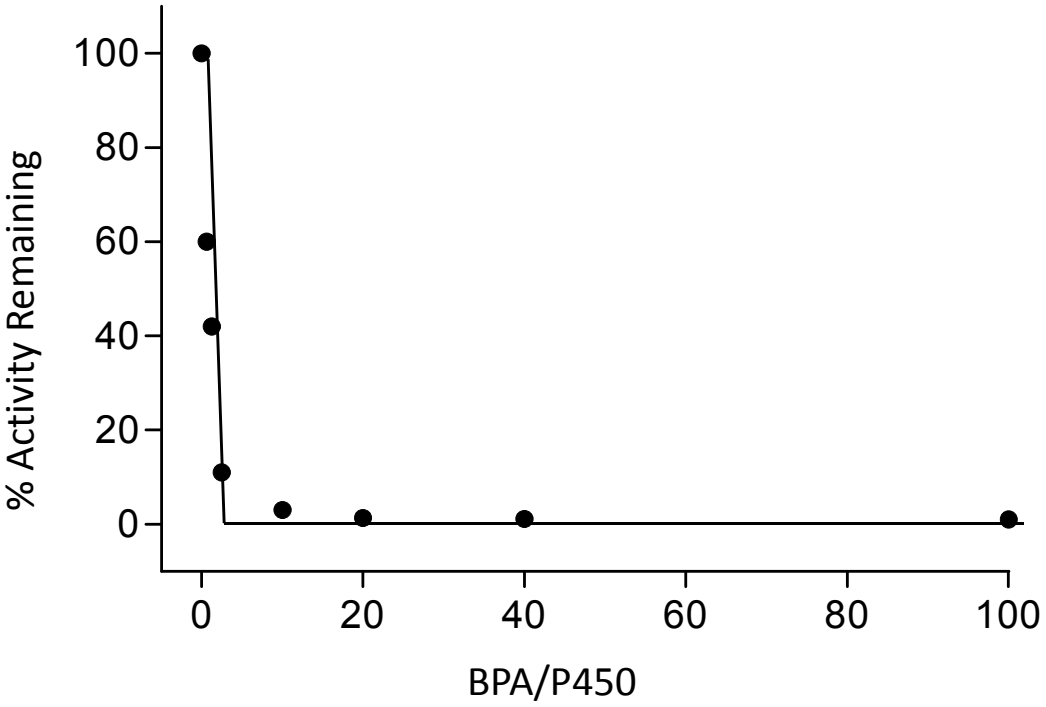

Relative Abundance
380.8
189.7
406.9
436.1
206.6
413.9
249.9
344.5
389.0
449.6
491.6
508.7
100 150 200 250 300 350 400 450 500 550
m/z

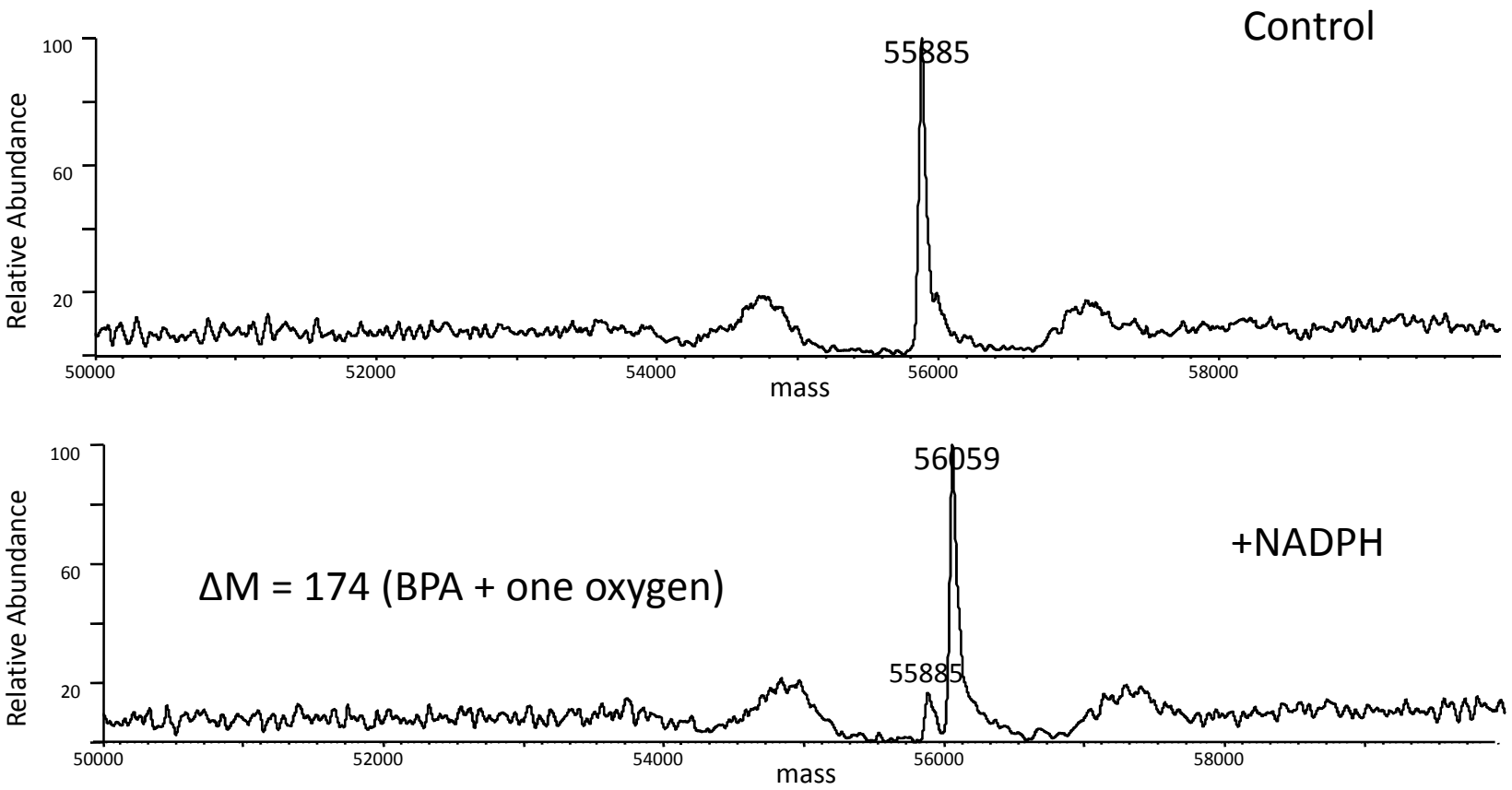


4-*tert*-butylphenylacetylene (BPA)
MW = 158 g/mol

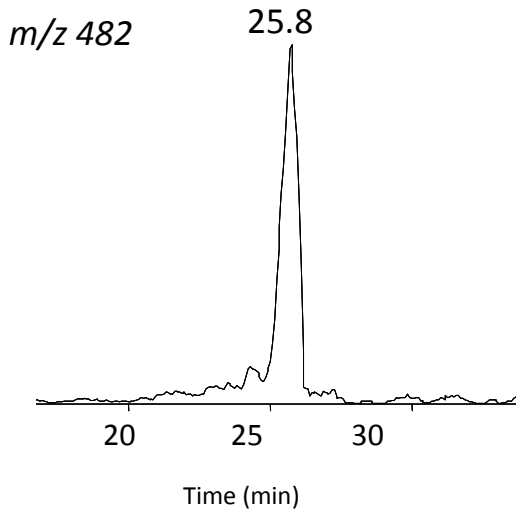

2.2
1.4
0.6
Log % Activity Remaining
0.0
0.5
1.0
1.5
Time (min)
6
4
2
1/k_{obs} (min)
-1
0
1
2
3
1/BPA (μM^{-1})

Inactivator	P450	K_I	k_{inact}	k_{inact} / K_I	Partition ratio
		μM	min^{-1}	$min^{-1}mM^{-1}$	
BPA	WT	0.7	1.64	2343	1
	T205A	16	0.36	23	9
BMP	WT	17	0.56	33	10
	T205A	16	0.14	9	35

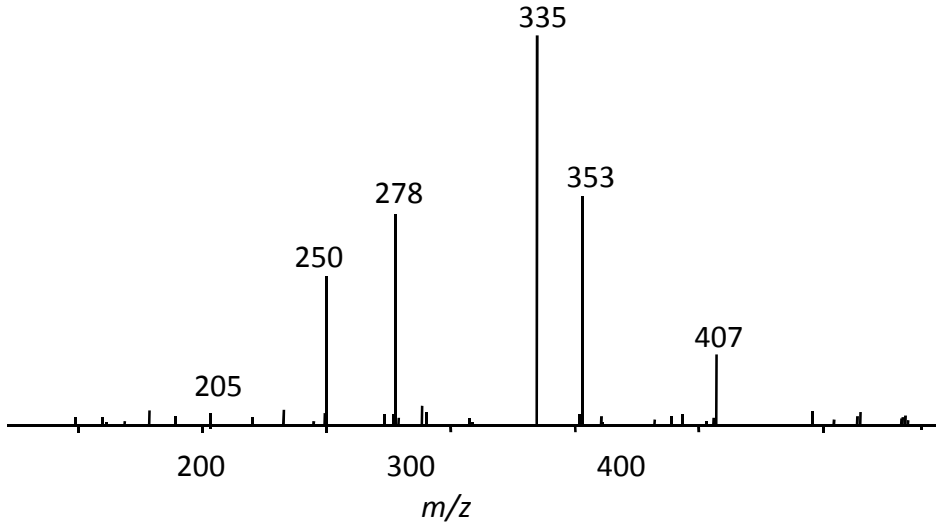

% Activity Remaining
100
80
60
40
20
0
0
20
40
60
80
100
BPA/P450


Control
55885
100
60
20
Relative Abundance
50000
52000
54000
56000
58000
mass
56059
+NADPH
ΔM = 174 (BPA + one oxygen)
55885
100
60
20
Relative Abundance
50000
52000
54000
56000
58000
mass

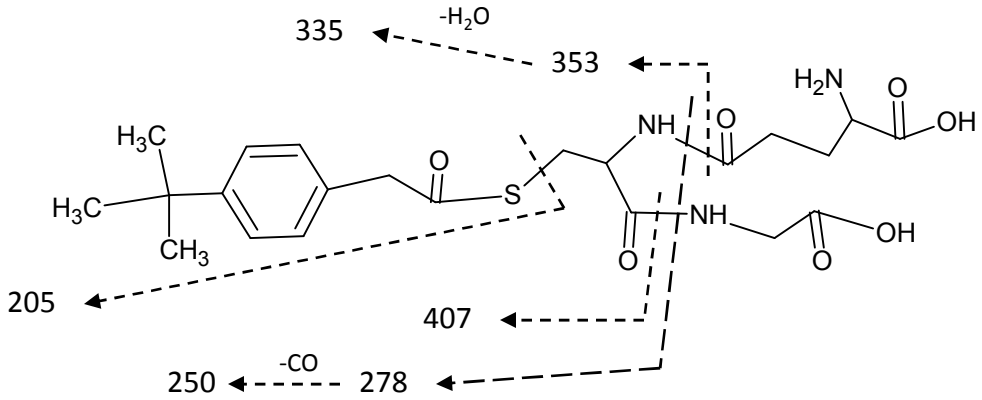
A Extracted ion chromatogram



B MS/MS spectrum



C



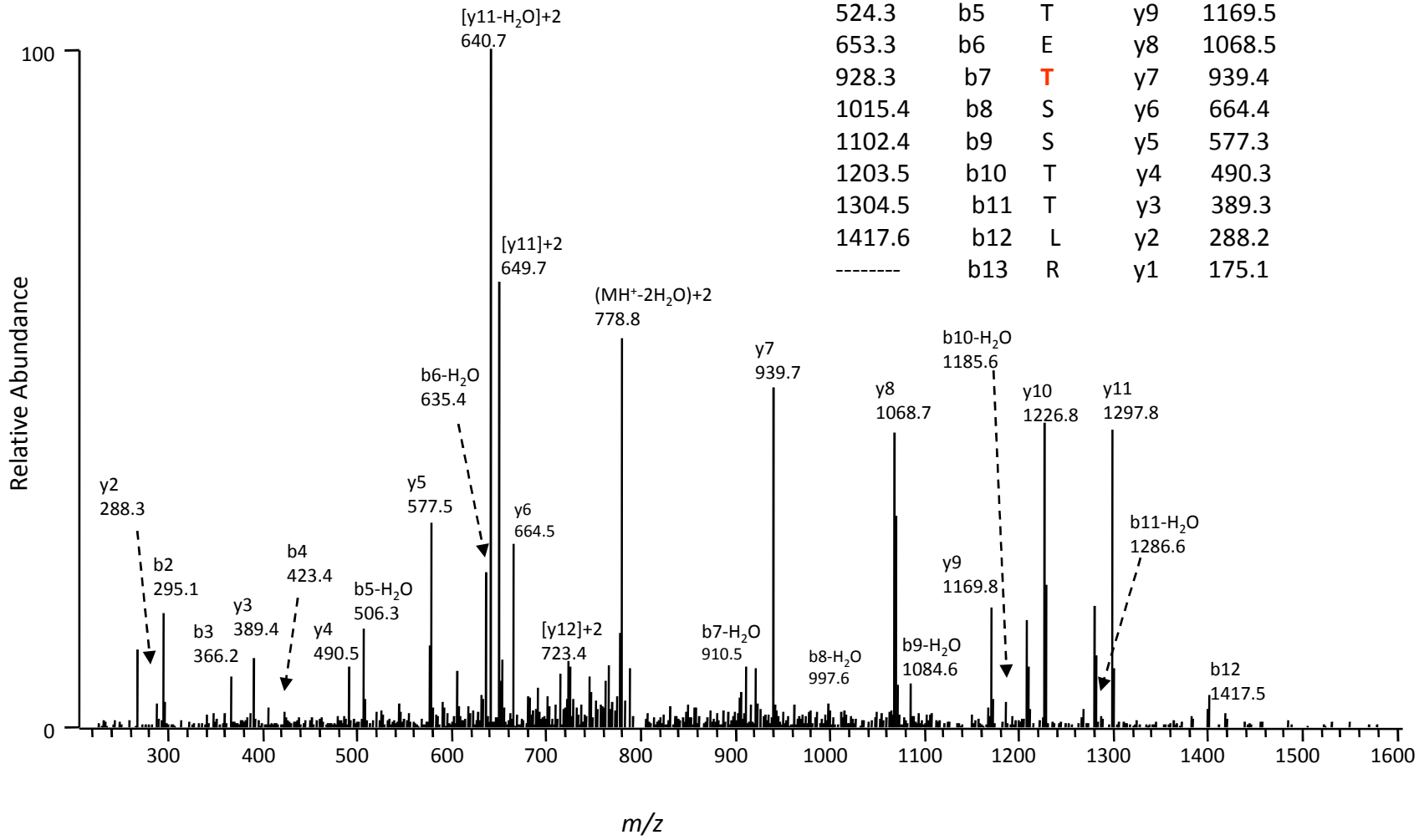
482 Da – 308 Da = 174 Da

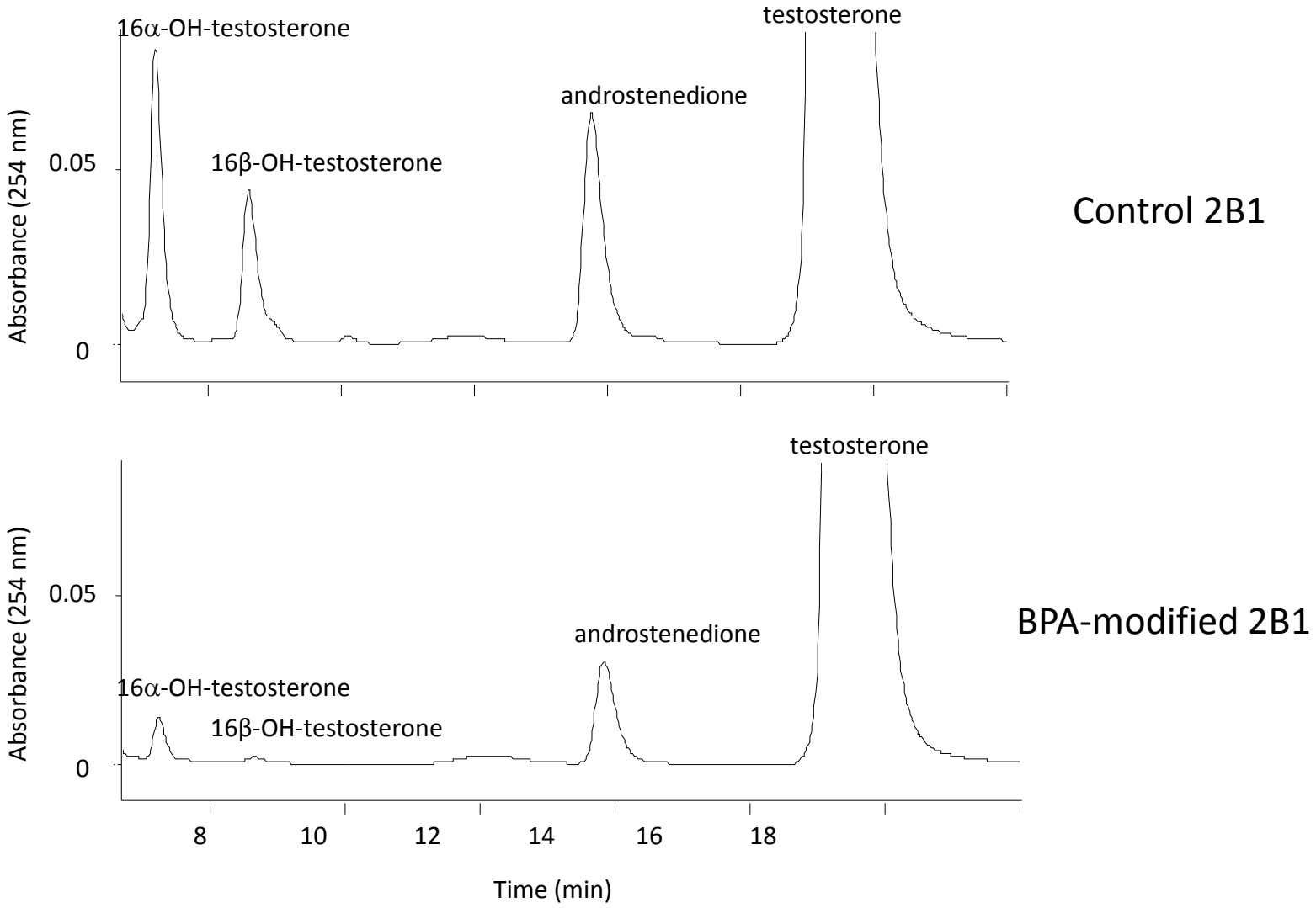
SEQUEST database search results

Modified peptide positions and sequence	Modified residue	Precursor ion charge	XCorr	Probability
^{296}FFAGTE**T**SSTTLR^{308}	Thr302	2	3.62	1.7×10^{-6}
^{296}FFAGTET**S**STTLR^{308}	Ser303	2	3.48	1.1×10^{-4}
^{100}**T**IAVIEPIFK^{109}	Thr100	2	2.90	8.0×10^{-5}

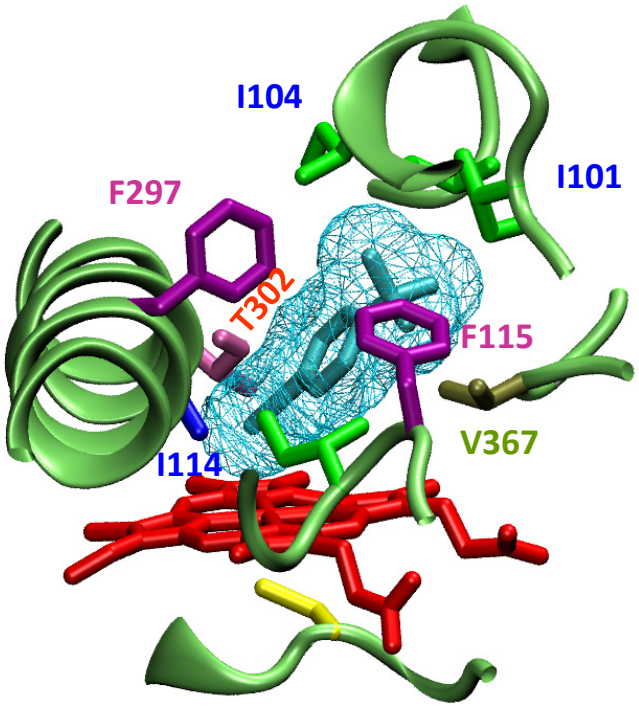
Xcorr: cross-correlation value between the observed peptide fragment mass spectrum and the one theoretically predicted.

Probability: scoring algorithm in BioWorks based on the probability that the peptide is a random match to the spectral data


b ion
residue
y ions
148.1 b1 F y13 -----
295.1 b2 F y12 1444.6
366.1 b3 A y11 1297.6
423.2 b4 G y10 1226.5
524.3 b5 T y9 1169.5
653.3 b6 E y8 1068.5
928.3 b7 T y7 939.4
1015.4 b8 S y6 664.4
1102.4 b9 S y5 577.3
1203.5 b10 T y4 490.3
1304.5 b11 T y3 389.3
1417.6 b12 L y2 288.2
-------- b13 R y1 175.1
[y11-H2O]+2 640.7
[y11]+2 649.7
(MH+-2H2O)+2 778.8
b6-H2O 635.4
y7 939.7
b10-H2O 1185.6
y8 1068.7
y10 1226.8
y11 1297.8
y2 288.3
y5 577.5
y6 664.5
b4 423.4
b2 295.1
b5-H2O 506.3
y3 389.4
b3 366.2
y4 490.5
[y12]+2 723.4
b7-H2O 910.5
b8-H2O 997.6
b9-H2O 1084.6
y9 1169.8
b11-H2O 1286.6
b12 1417.5
Relative Abundance
100
0
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
m/z

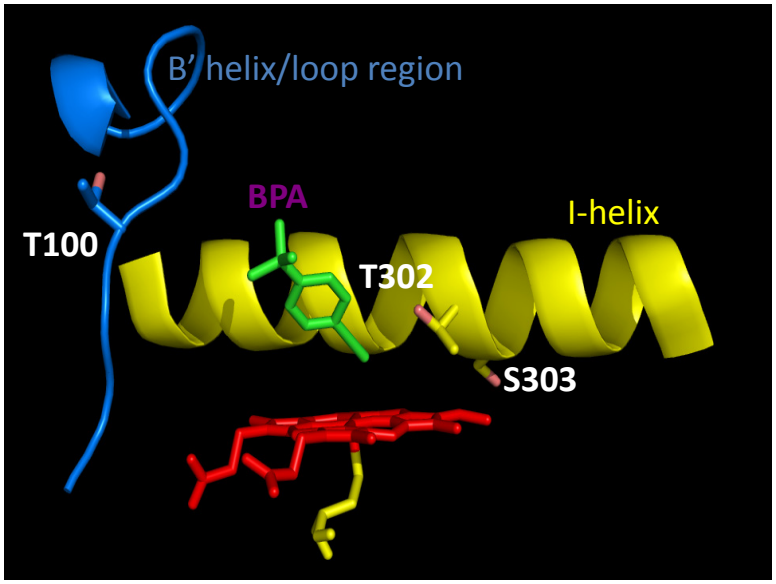

16α-OH-testosterone
16β-OH-testosterone
androstenedione
testosterone
Absorbance (254 nm)
0.05
0
Control 2B1
16α-OH-testosterone
16β-OH-testosterone
androstenedione
testosterone
Absorbance (254 nm)
0.05
0
BPA-modified 2B1
8
10
12
14
16
18
Time (min)

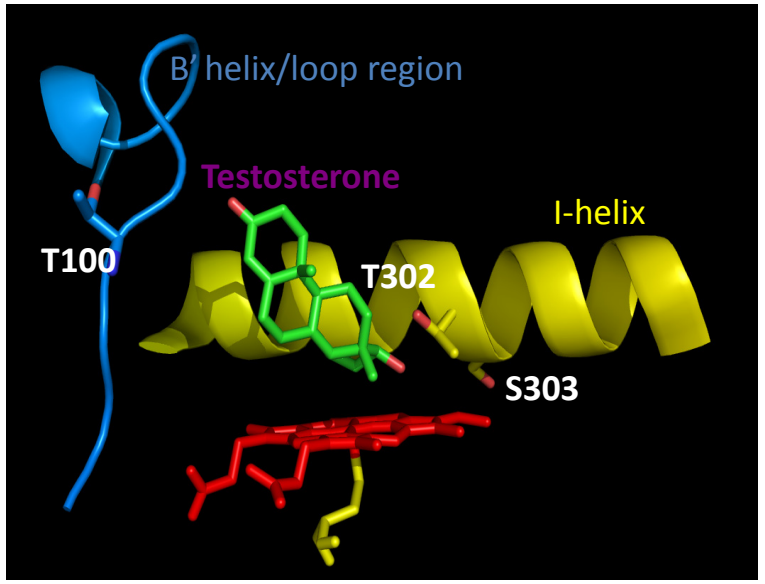
Reversible Docking of BPA in the CYP2B1 Active Site



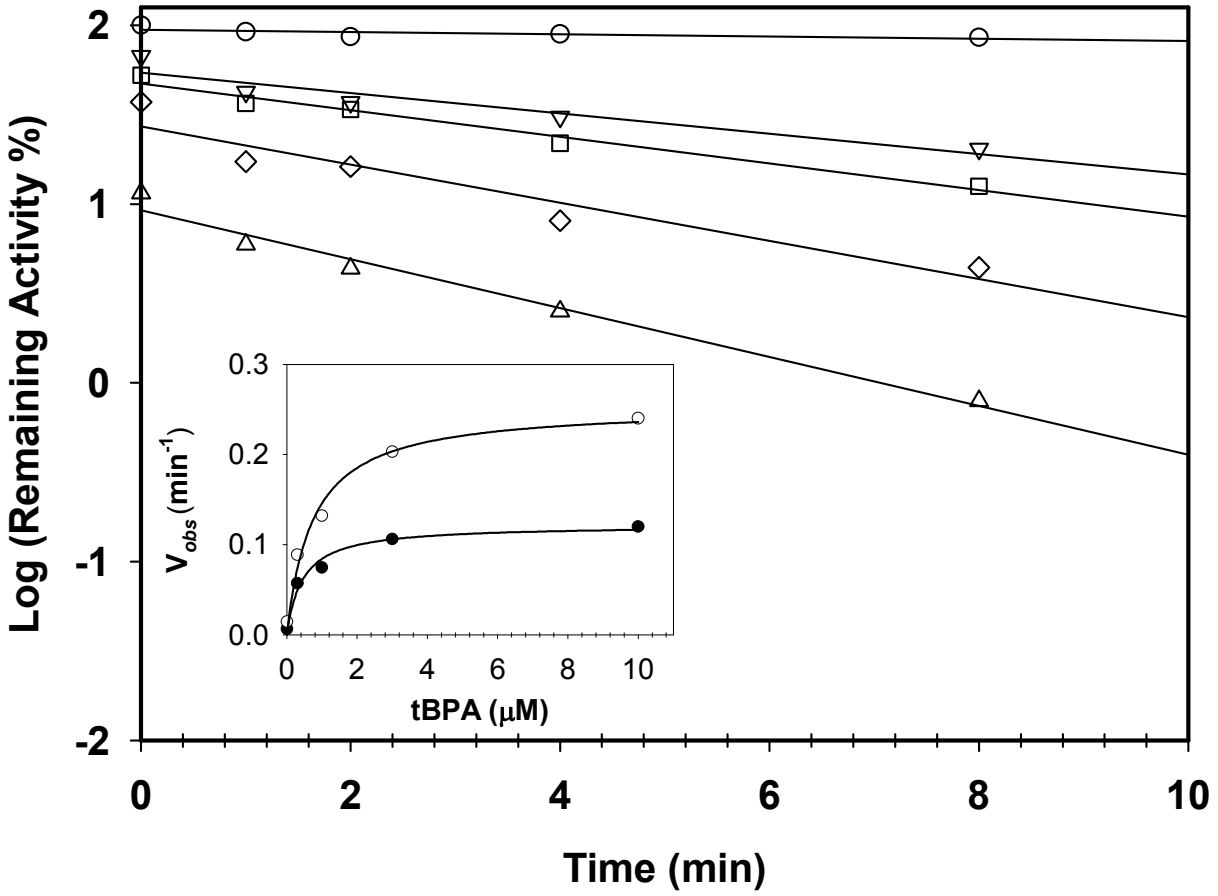
Modified residue	location	Distance to heme iron (Å)[a]	Distance to BPA (Å)[a]	Distance to testosterone (Å)[a]
Thr100	B' helix/loop	15.44	8.31	6.94
Thr302	I-helix	6.22	3.42	2.42
Ser303	I-helix	8.57	7.67	7.18

[a]Distance between the nearest atom of each residue and the heme iron, BPA, and testosterone based on CYP2B1 homology modeling.


B' helix/loop region
BPA
I-helix
T100
T302
S303


B' helix/loop region
Testosterone
I-helix
T100
T302
S303

Time- and Concentration Dependent Inactivation of P450 2B4 by *tert*-butylphenylacetylene



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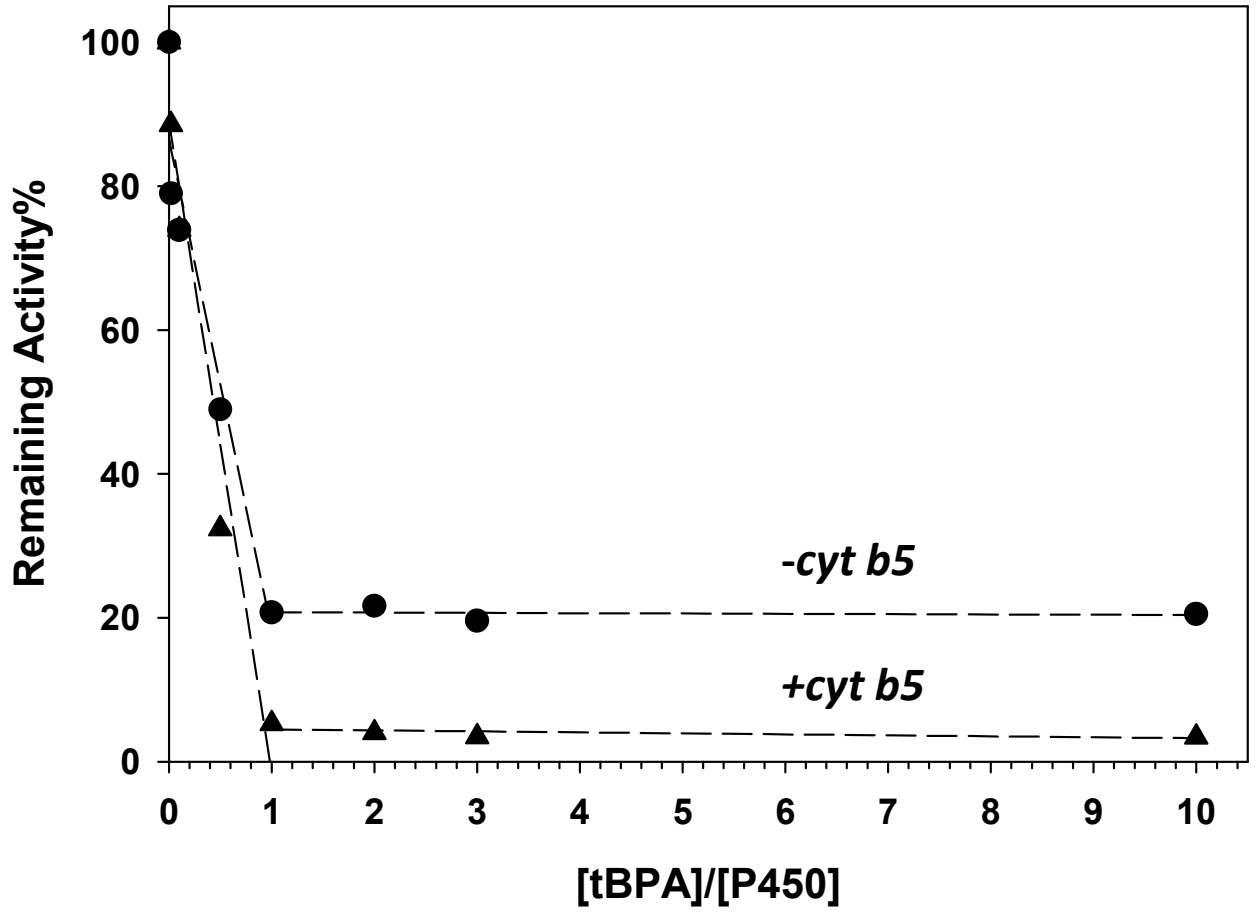
Partition Ratio for Mechanism-based Inactivation of P450 2B4 by *tert*-butylphenylacetylene



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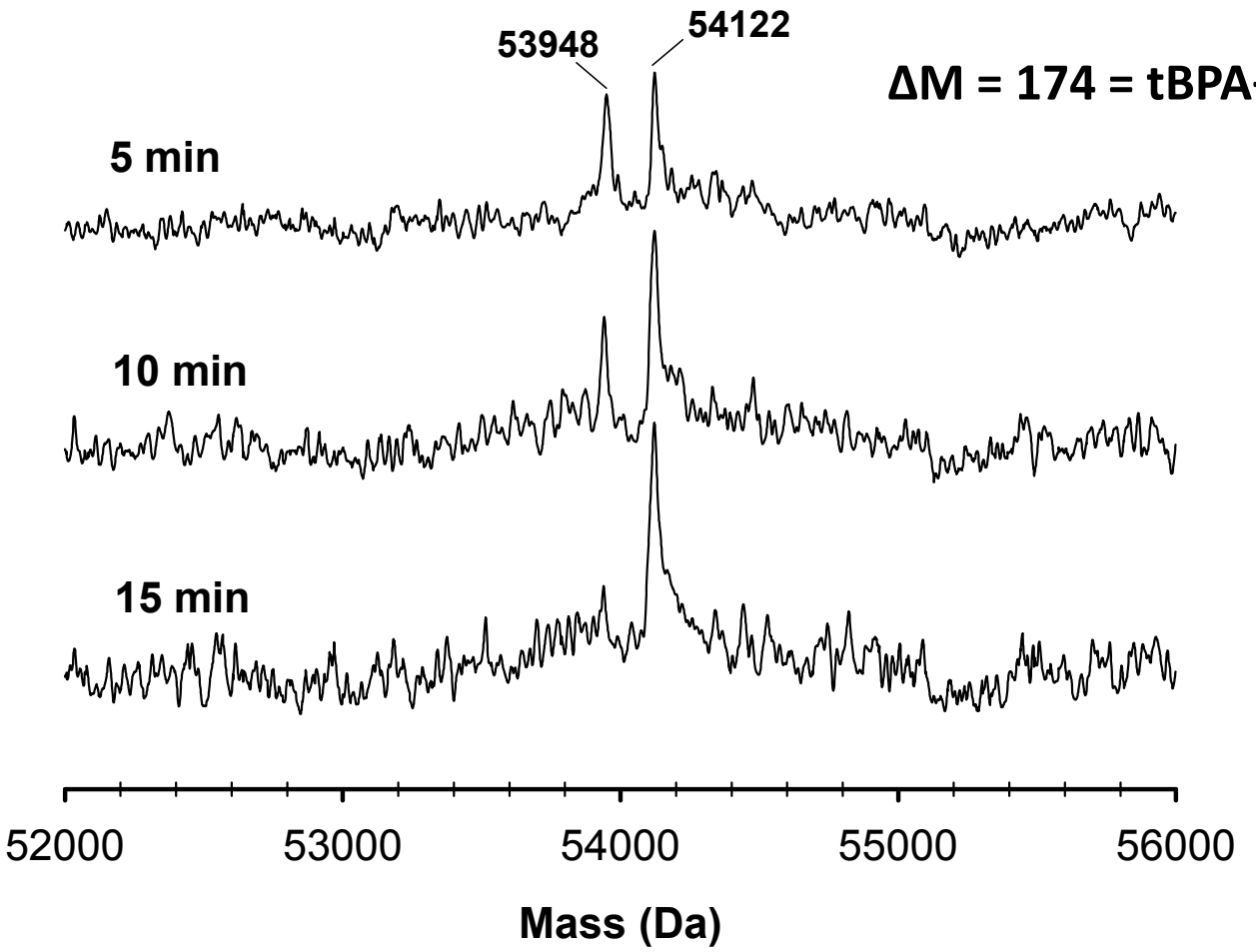
P450 2B4-tBPA Adduct Formation as Revealed by LC-MS Analysis



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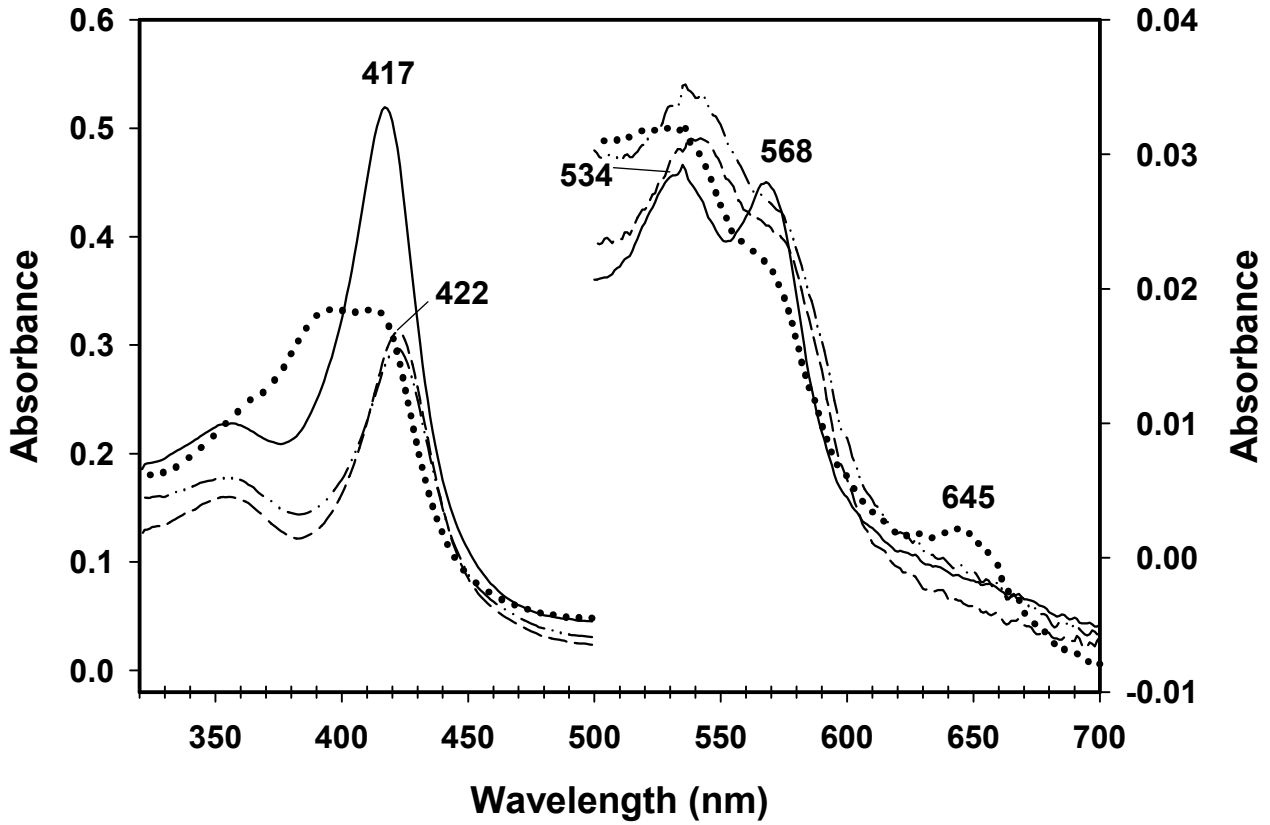
UV-visible Spectra of tBPA-modified P450 2B4



— P450 2B4

P450 2B4 + BNZ

--- modified P450 2B4

P450 2B4 + BNZ

•••

-••- modified

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Catalytic Activity of tBPA-modified P450 2B4

Substrates	Relative Turnover Rates (% of unmodified 2B4)
7-EFC	30
BNZ	21
Testosterone	9.6

Compounds	Volume (Å^3)
tBPA	198.7
7-EFC	226.6
BNZ	289.1
Testosterone	313.9

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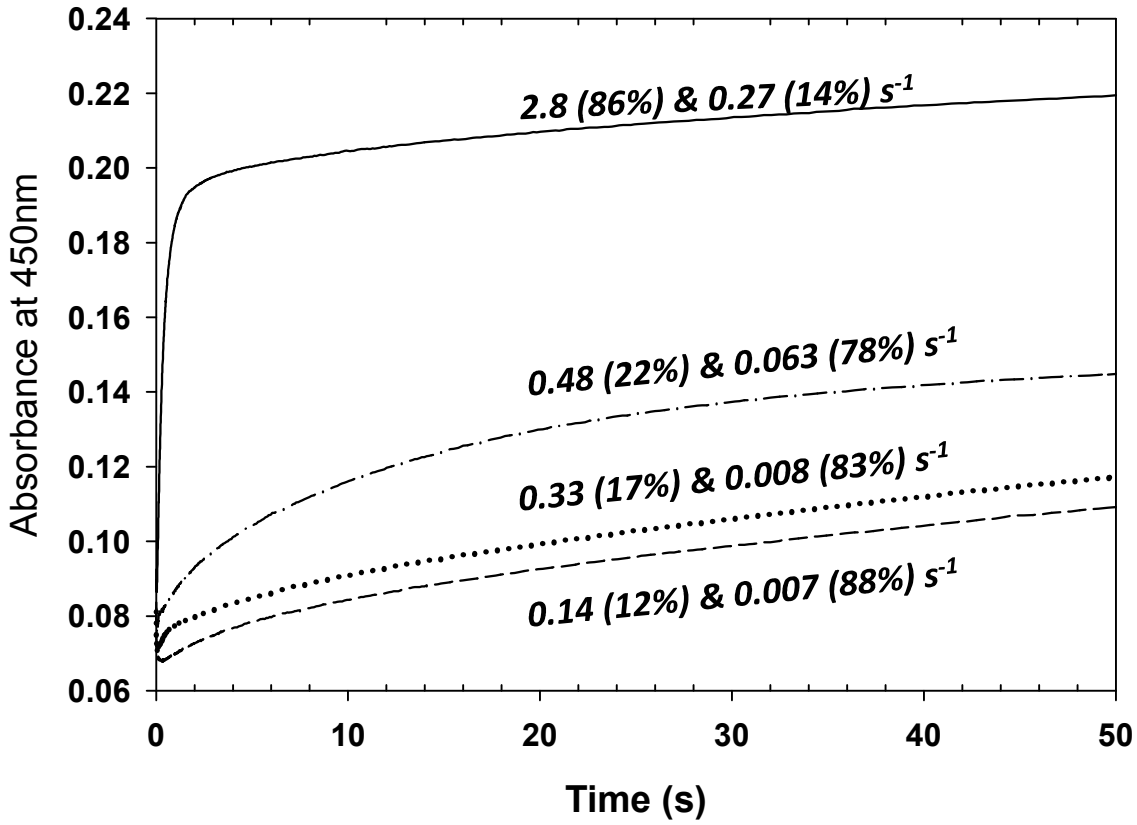
Rates of Electron Transfer from P450 Reductase to tBPA-modified Ferric P450 2B4



--- P450 2B4 — P450 2B4 + BNZ

••• Modified P450 2B4 -•- modified P450 2B4 + BNZ

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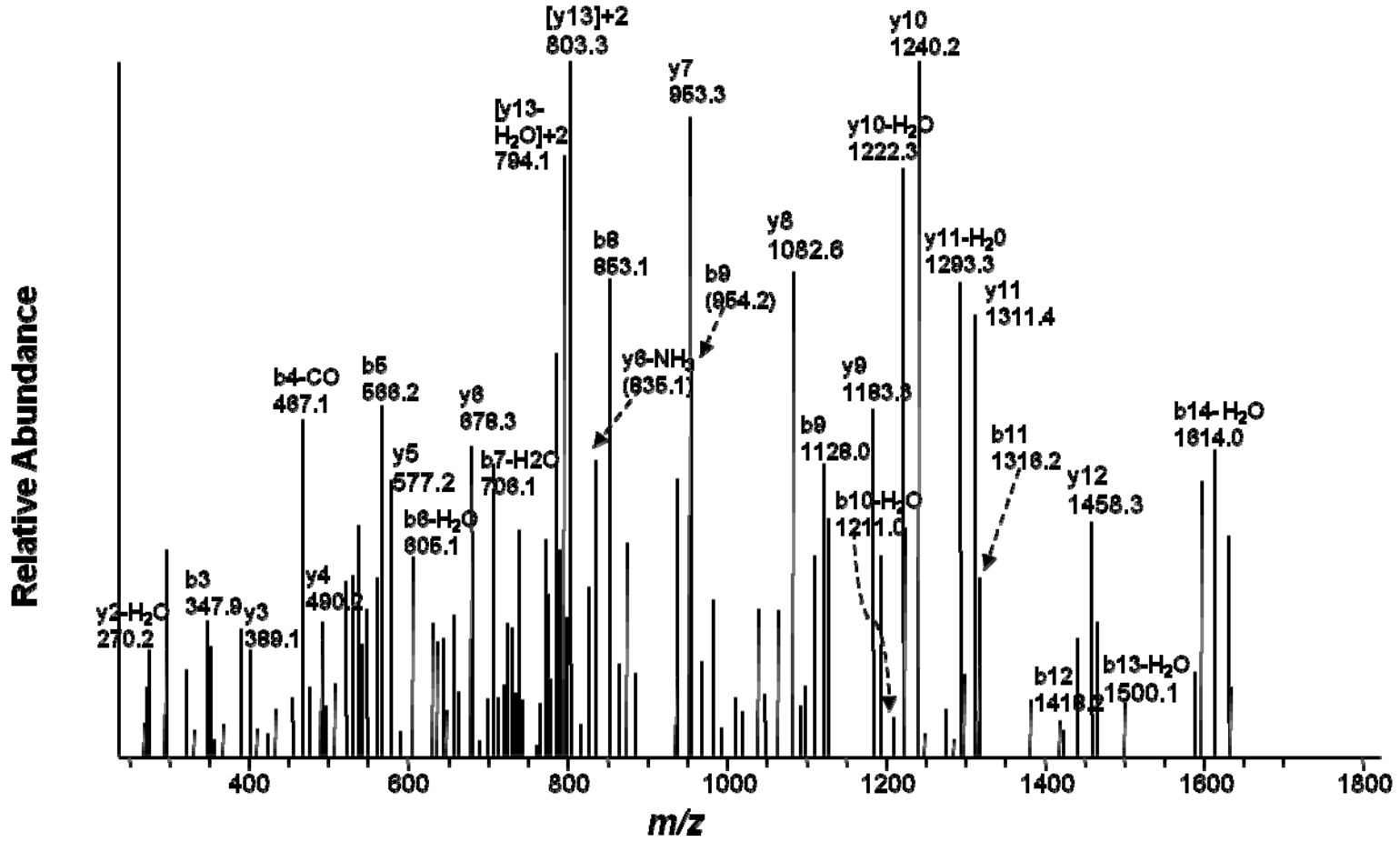
Peptide Mapping to Identify Site of Covalent Binding



B-Ions

	S	L	F	F	A	G	T	E	T*	T	S	T	T	L	R
		1544.8	1431.7	1284.6	1137.6	1066.5	1009.5	908.5	779.4	678.3	577.3	490.3	389.2	288.2	175.1
	88.0	201.1	348.2	495.3	566.3	623.3	724.4	853.4	954.4	1055.5	1142.5	1243.6	1344.6	1457.7	
			347.9		566.2			853.1	1128.0		1316.1	1418.2			

1458.5 1311.4 1240.3 1183.3 1082.8 953.0 678.3 577.2 490.2 389.1

Y-Ions

unlabeled

labeled

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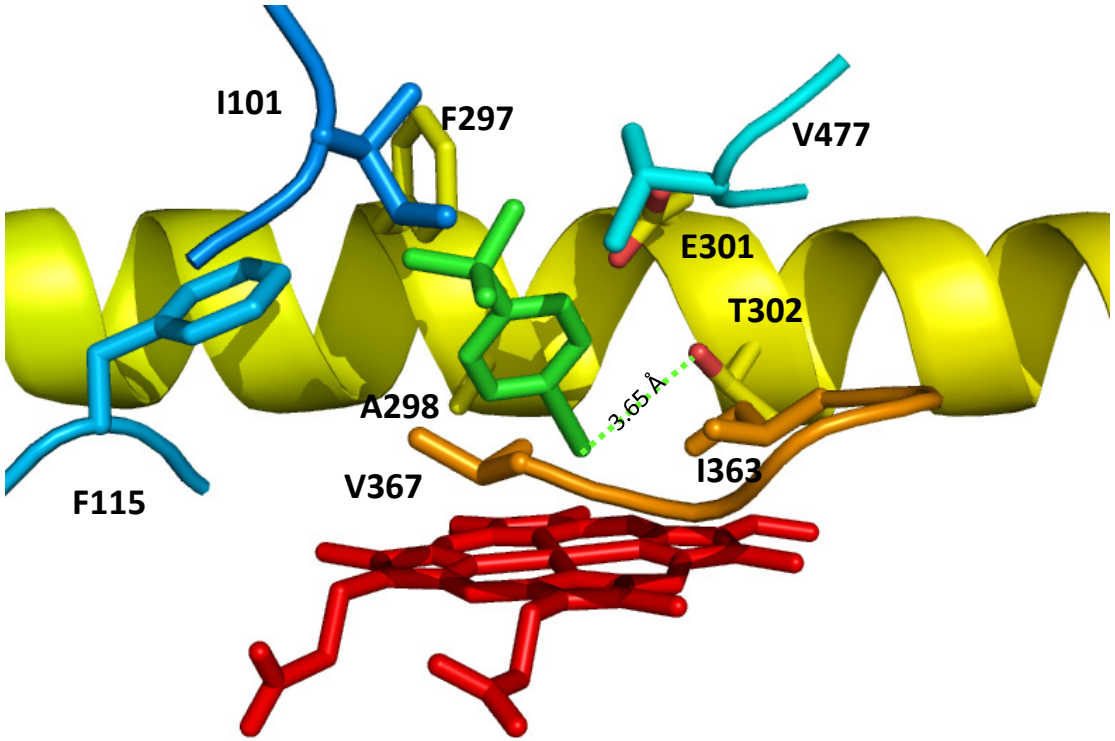
Molecular Modeling Showing the Binding of tBPA in the Active Site of P450 2B4



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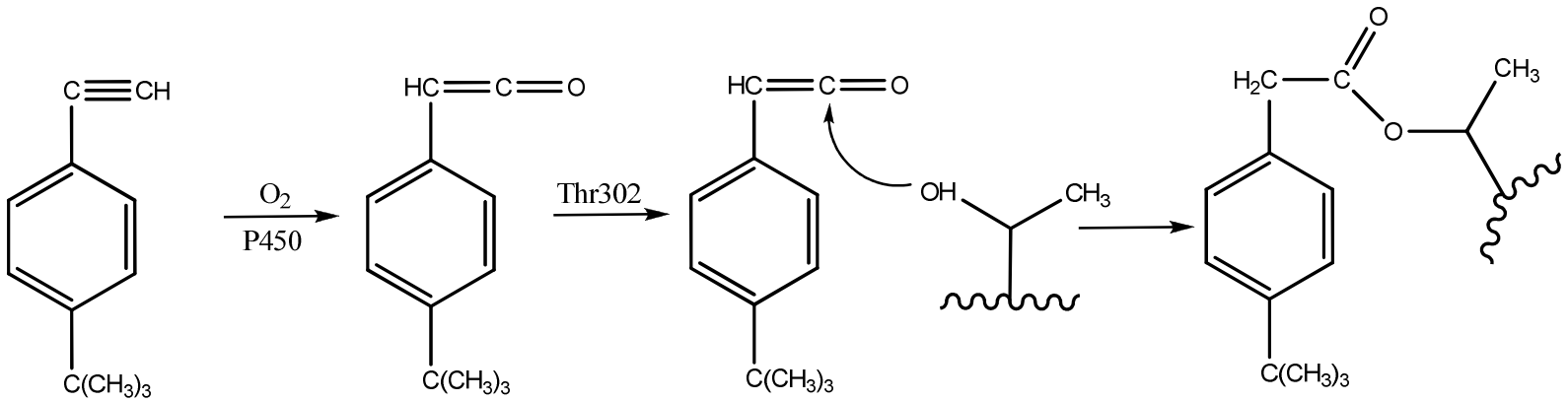
Proposed Mechanism for Mechanism-based Inactivation of P450 2B4 by *tert*-butylphenylacetylene



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