SUPPORTING INFORMATION

Oxidation of Methyl and Ethyl Nitrosamines by Cytochromes P450 2E1 and P450 2B1

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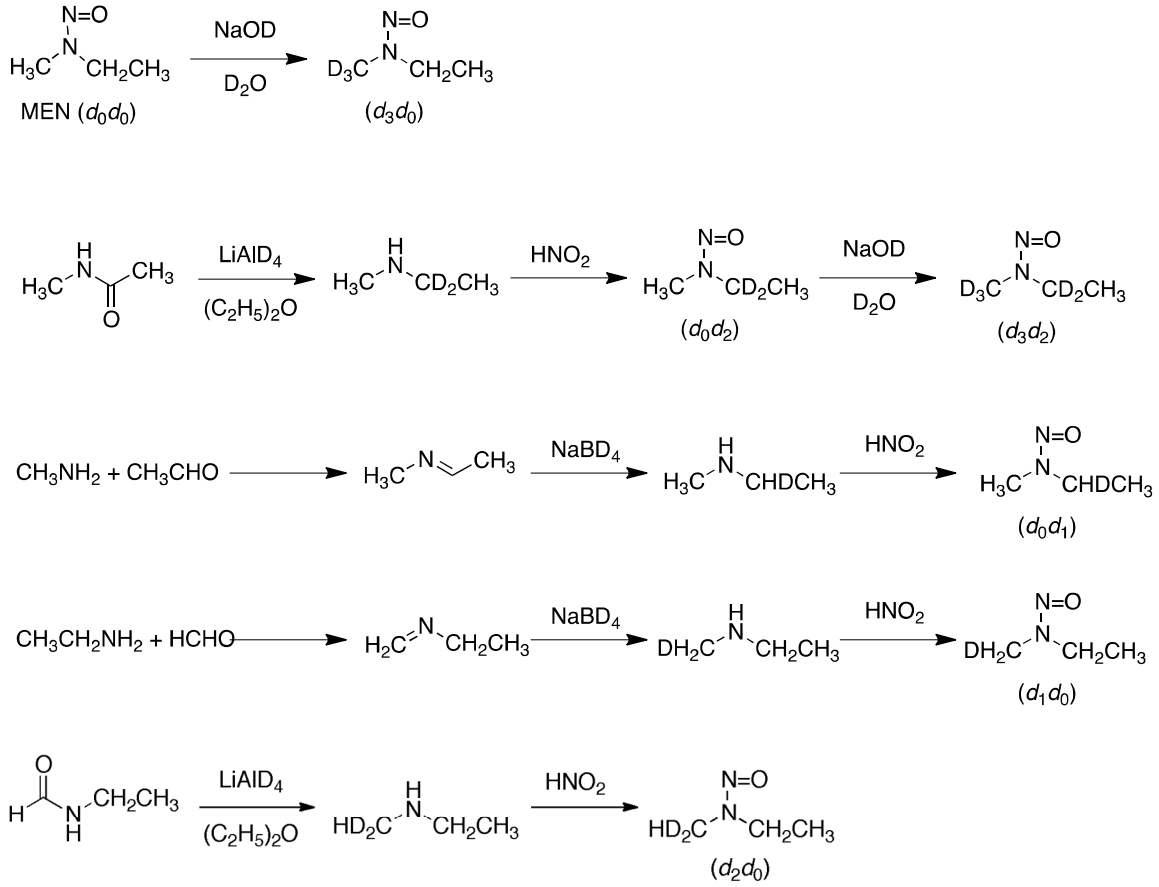
Figure S1. (A) Synthesis of deuterated MEN substrates; (B) ^{1}H-NMR spectra. Note splitting of signals in nitrosamines, as discussed earlier.[1]

A



[d_3d_0]-MEN. MEN (4.15 g, 47 mmol) was dissolved in 50 mL of 0.5 M NaOD in D_2O, which had been prepared by the addition of 0.57 g of Na° in 50 mL D_2O (at 0 °C), with a $CaSO_4$ drying tube attached to the reaction flask. The solution was stirred at 70 °C for 48 h. The mixture was cooled to room temperature and the pH (pD) was adjusted to ~ 4 (as judged by indicator paper) with D_3PO_4 (prepared by the careful addition of P_2O_5 to D_2O). NaCl (~12 g) was dissolved in the aqueous solution, and the solution was extracted three times with an equal volume of $CHCl_3$. The combined organic layers were dried with $MgSO_4$ and, after removal of $MgSO_4$ by filtration, the $CHCl_3$ was evaporated carefully under a stream of N_2 in a water bath. Yield 77%. MS: *m*/*z* 92.3 (MH^+ for d_3). NMR ($CDCl_3$) δ 1.04 (t, -CH_2CH_3), 1.95 (t, -CH_2CH_3), 1.33 (t, -CH_2CH_3), 2.96 (s, residual –CH_3, ≤ 3%), 3.56 (q, -CH_2CH_3), 4.14 (q, -CH_2CH_3).

[d_0d_2]-MEN. $LiAlD_4$ (10 g, 0.24 mol) was stirred in 300 mL of dry $(C_2H_5)_2O$ (freshly opened can) in a 500 mL-round bottom flask, which was stirred and cooled to 0 °C using an ice bath. (All glass had been heated to remove moisture, and a $CaSO_4$ drying tube was attached to the system.) *N*-Methylacetamide (17 g, 0.24 mol, dissolved in 60 mL of $(C_2H_5)_2O$), was added from an addition funnel over 1 h. The addition funnel was

replaced with a condenser and the solution was stirred and heated at reflux for 20 h. The reaction was cooled to 0 °C and quenched by the careful addition of 5 mL D_2O, followed by 5 mL of 15% NaOD in D_2O (*vide supra*) and then another 5 mL of D_2O. This mixture was stirred another 17 h. After cooling on ice, the solids were removed by filtration through Celite and washed with another cold 200 mL of $(C_2H_5)_2O$. The combined filtrate was chilled to 0 °C (ice) and sparged with HCl gas. The resulting $CH_3NHCH_2CH_3$-HCl was extracted from the $(C_2H_5)_2O$ layer three times with 25 mL of aqueous 2.5 M HCl. The water was removed *in vacuo* (rotary evaporator) to yield a solid, which was dried *in vacuo* (vacuum pump) overnight.

The above compound (moist solid) was dissolved in 60 mL of cold glacial CH_3CO_2H and diluted with 30 g ice. The solution was stirred at 0 °C for 1 h, $NaNO_2$ (35 g, 0.51 mol) was added, and the reaction was stirred for 1.5 h at 0 °C and then overnight at 23 °C. Cold H_2O (25 mL) and NaCl (10 g) were added, and the product (d_0d_2-MEN) was extracted five times with 25 mL of $CHCl_3$. The combined $CHCl_3$ extracts were dried with $MgSO_4$ and filtered, and the $CHCl_3$ was carefully evaporated, under a stream of nitrogen at 23 °C, to give 9.7 g of [d_0d_2]-MEN (46% yield). NMR ($CDCl_3$) δ, 0.96 (s, -CD_2CH_3), 1.25 (s, -CD_2CH_3), 2.93 (s, CH_3-N), 3.62 (s, 3H, CH_3-N). The product was further purified by vacuum distillation (bp 50-55 °C, 12 Torr) (6.2 g).

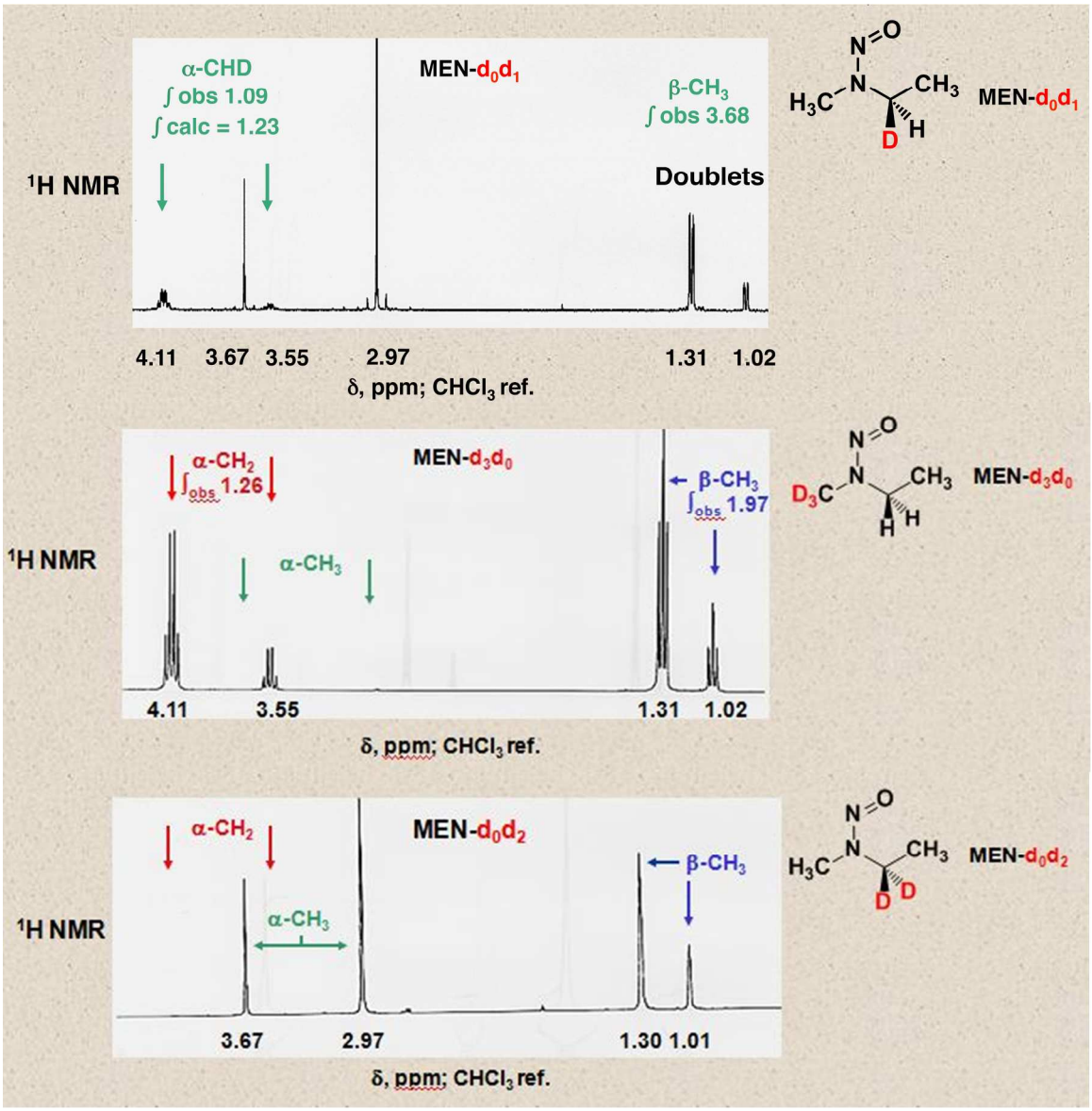
[d_3d_2]-MEN. The above product ([d_0d_2]-MEN, 5.7 g, distilled) was exchanged with 100 mL of 0.5 M NaOD in H_2O as described for [d_3d_0]-MEN (*vide supra*) for 130 h at 70 °C to yield [d_3d_2]-MEN with 98.5% excess atom D. MS: *m*/*z* 94.3 (MH^+). NMR ($CDCl_3$) δ 1.01 (s, -CD_2CH_3), 1.30 (s, -CD_2CH_3).

[d_0d_1]-MEN. A cold aqueous solution of 40% CH_3CHO (w/v, 20 mL, 0.18 mol) was added dropwise to 24 mL of a 40% aqueous solution of CH_3NH_2 on ice, with vigorous stirring. After 2 h, NaOH pellets were added to the mixture until two layers formed, and then a few excess NaOH pellets were added. The mixture, containing the imine (Fig. 2), was transferred to a pre-chilled separatory funnel. The lower aqueous layer was discarded and the upper layer (imine, 16.9 g crude weight) was transferred to a clean round bottom flask and distilled (bp 26-32 °C, 760 Torr) to give 9.2 g of the imine: NMR ($CDCl_3$) δ 0.88 (s, 3H, CH_3-N), 1.81 (s, 3H, =CH-CH_3), 2.35 (s, residual CH_3NH_2), 3.12 (s, unknown). The imine was dissolved in 50 mL C_2H_5OH and reduced with 2.0 g of $NaBD_4$ (0.048 mol, in 100 C_2H_5OH), beginning at 0 °C (during addition), heating at 50 °C for 3 h, and then stirring at 23 °C overnight. The resulting *N*-methylethylamine was recovered and nitrosated as described above for [d_0d_2]-MEN. MS: *m*/*z* 90.2 (MH^+). NMR ($CDCl_3$) δ 1.32 (d, 3H, -CHDCH_3), 2.98 (s, 3H, CH_3-N), 3.67 (s, 3H, CH_3-N), 4.10 (m, 1H, -C*H*DCH_3). See Part B for spectrum.

[d_1d_0]-MEN. The procedure was similar to that used above for [d_1d_0]-MEN, except that HCHO (20 mL of a 30% solution, w/v) and $C_2H_5NH_2$ (10.3 mL of a 70% aqueous solution) were used as starting materials (Fig. 2). The imine intermediate (crude yield 7.1 g, 45%) was not distilled but was reduced directly with $NaBD_4$ (4.0 g) in C_2H_5OH, with the workup of *N*-methylethylamine and nitrosation as before to yield the final product, d_1d_0-MEN. MS: *m*/*z* 90.2 (MH^+).

[d_2d_0]-MEN. The procedure was similar to that used above for [d_0d_2]-MEN (*vide supra*). *N*-Ethylformamide was reduced with $LiAlD_4$ and the product was nitrosated in the usual manner. MS: *m*/*z* 91.2 (MH^+). NMR ($CDCl_3$) δ 1.05, 1.34 (t, 3H, N-CH_2CH_3); 2.97, 3.67 (m, 1H, CD_2H-N); 3.58, 4.14 (q, 2H, N-CH_2CH_3).

B



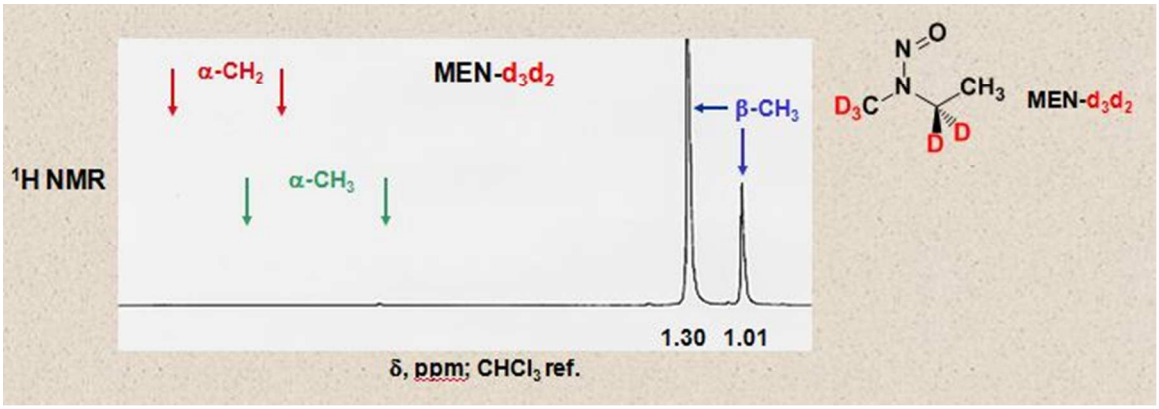
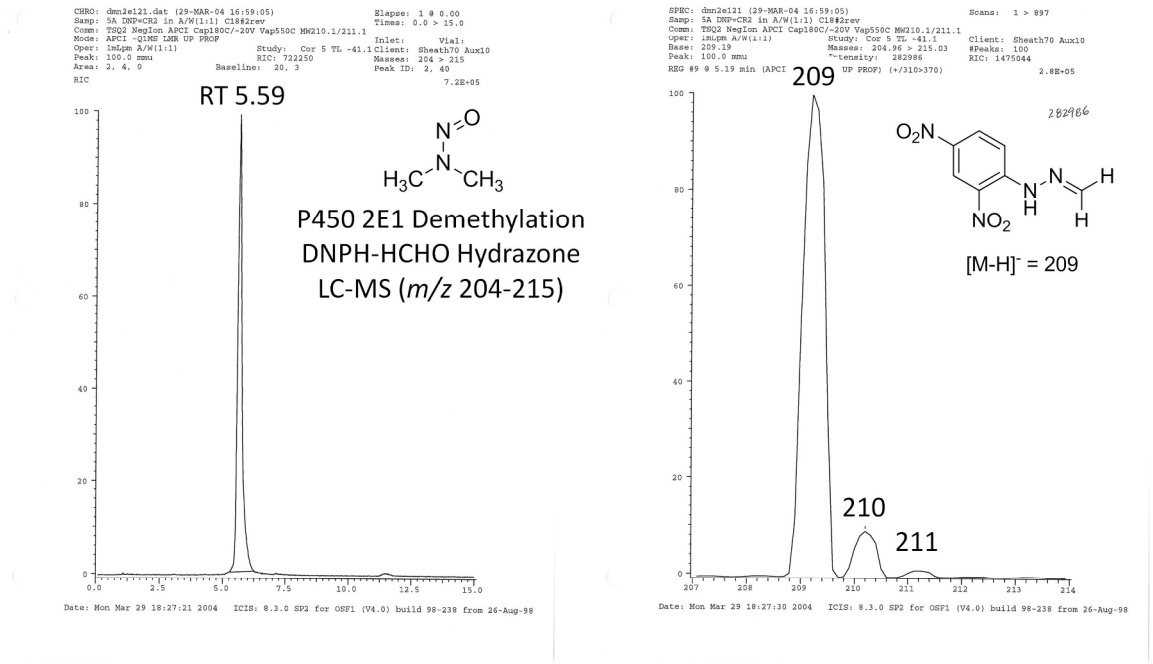
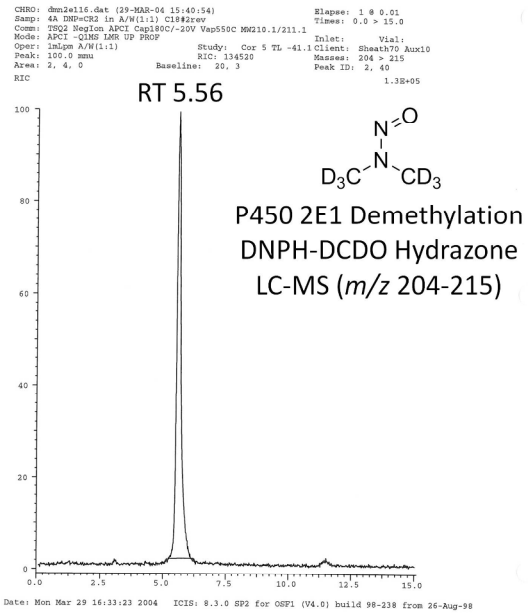

¹H NMR
α-CH2
MEN-d3d2
α-CH3
β-CH3
1.30 1.01
δ, ppm; CHCl3 ref.
N
O
N
D3C
CH3
D
D
MEN-d3d2

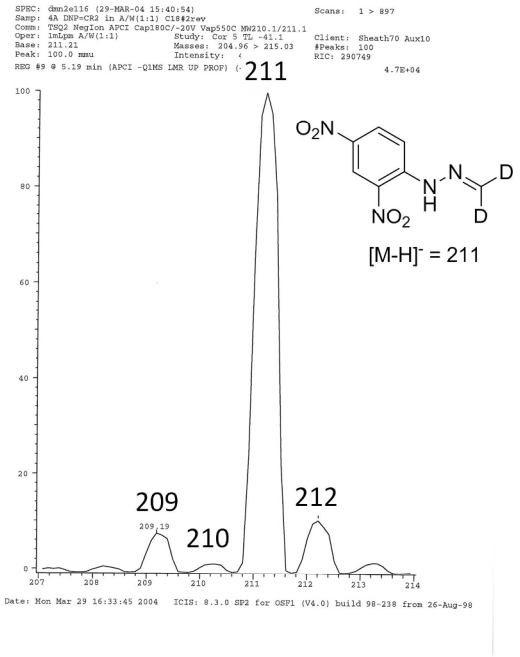
Figure S2. LC-MS of 2,4-dintirophenyl hydrazone of CH_3CHO formed from DMN. (A) LC-MS chromatogram and mass spectrum of product obtained from an incubation with d_0 DMN. (B) LC-MS chromatogram and mass spectrum of product obtained from an incubation with d_6 DMN. (C) LC-MS chromatogram and mass spectrum of product obtained from an incubation with a 1/1 molar mixture of d_0 and d_6 DMN (competition experiment).

A

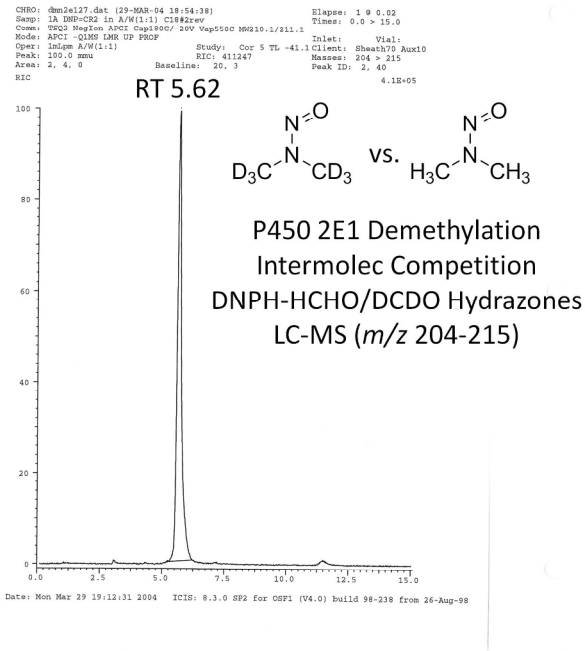


B


RT 5.56
P450 2E1 Demethylation
DNPH-DCDO Hydrazone
LC-MS (m/z 204-215)


211
209
210
212
[M-H]- = 211

C


RT 5.62
vs.
P450 2E1 Demethylation
Intermolec Competition
DNPH-HCHO/DCDO Hydrazones
LC-MS (m/z 204-215)

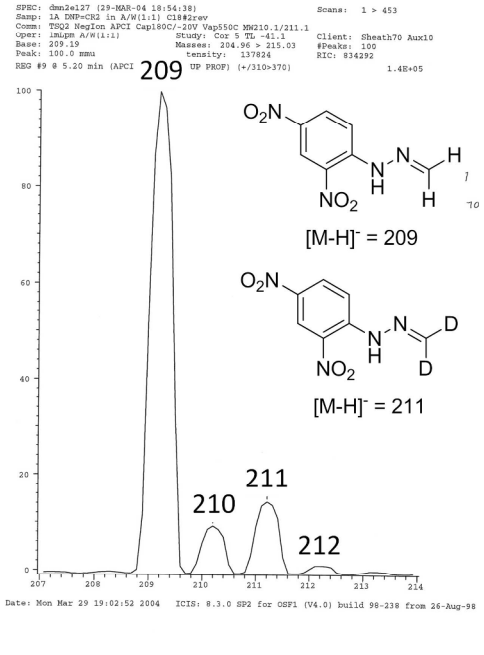
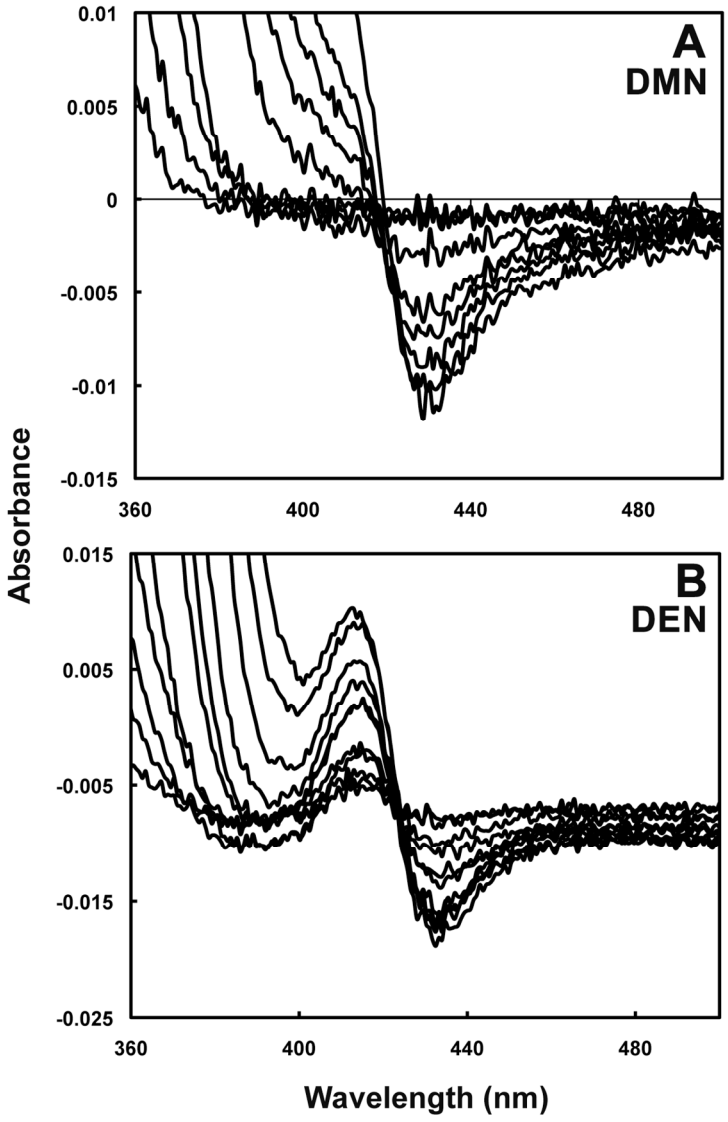
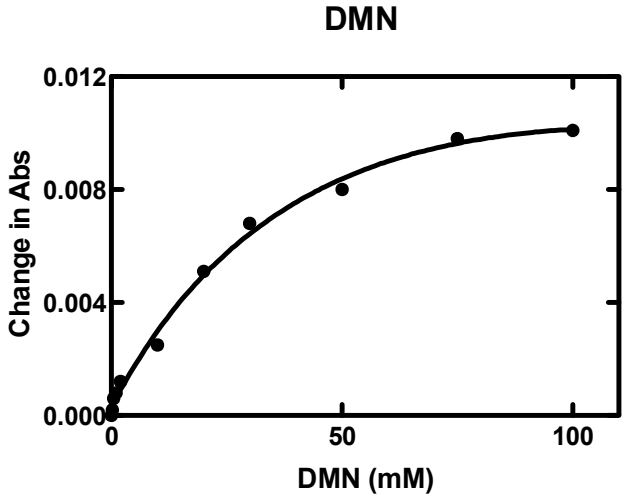

209
[M-H]- = 209
211
210
212
[M-H]- = 211

Figure S3. Binding spectra of P450 2E1 with DMN and DEN.[4] Two cuvettes each contained 1.0 μM P450 in 0.10 M potassium phosphate buffer. A baseline was recorded and varying concentrations of the nitrosamine (in H_2O) were added to the sample cuvette, with the same amount of H_2O added to the reference cuvette. (A) DMN spectra; (B) DEN spectra; (C) plot of absorbance change (A_{430}) vs. [DMN]; (D) plot of absorbance change (ΔA_{413}-A_{431}) vs. [DEN]. The K_d values for DMN and DEN were 69 (± 41) and 0.99 (± 0.20) mM, respectively.



C


DMN
0.012
0.008
0.004
0.000
Change in Abs
0
50
100
DMN (mM)

D

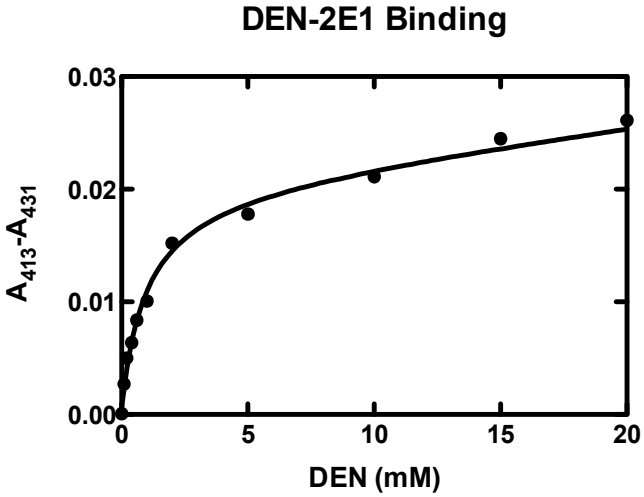

DEN-2E1 Binding
0.03
0.02
0.01
0.00
A_{413}-A_{431}
0
5
10
15
20
DEN (mM)

Figure S4. DynaFit modeling[5] of theoretical time courses of formation of aldehydes and carboxylic acids based on k_{cat} and K_m values for separate reactions.

Script

```
;2E1DAN RUN 3
;This is a situation in which Km & kcat results are available for several
;pathways.  The goal is to simulate the time course of different products as
;a function of time.

; units are min & nM
; E = P450
; S = DMN
; Q = aldehyde
; P = carboxylic acid
; Use k = Vmax = kcat
; Use Km = Kdissoc

[task]

data = progress
task = fit

[mechanism]

E + S  <==> ES :          K1      dissoc
ES --> EQ      :          k2
E  +   Q <==> EQ :        K3      dissoc
EQ ---> EP :              k4
E  +  P <==> EP :         K5      dissoc

 [constants]

K1 = 69000000
k2 =  13.8
K3 = 1300000
k4 = 0.72
K5 = 10000000000

[sweep]

[concentrations]

        E  = 2500
        S  = 1000000
 [progress]
```

```
        mesh linear from 0 to 10 step 0.1

        directory ./scripts

        ;variable S
        ;file     DMNHCO2Htime.txt
        ;response S = 1

        variable Q
        file     DMNHCO2Htime.txt
        response Q = 1

        variable P
        file     DMNHCO2Htime.txt
        response P = 1

[output]
  directory ./projects/P4502E1DAN/output/run3

[end]
```

Data file

DMNHCO2Htime.txt
(time, in min; concentration, in nM)

```
0       0
1       48
2       53
3       185
4       395
5       280
6       368
6       380
```

Differential equations

```
d[E]/dt =  +K1[ES]-dissoc[E][S]+K3[EQ]-dissoc[E][Q]+K5[EP]-dissoc[E][P]
d[S]/dt =  +K1[ES]-dissoc[E][S]
d[ES]/dt =  -K1[ES]+dissoc[E][S]-k2[ES]
d[EQ]/dt =  +k2[ES]-K3[EQ]+dissoc[E][Q]-k4[EQ]
d[Q]/dt =  +K3[EQ]-dissoc[E][Q]
d[EP]/dt =  +k4[EQ]-K5[EP]+dissoc[E][P]
d[P]/dt =  +K5[EP]-dissoc[E][P]
```

Predicted traces (for HCHO (1) and HCO_2H (2, with data points shown)):

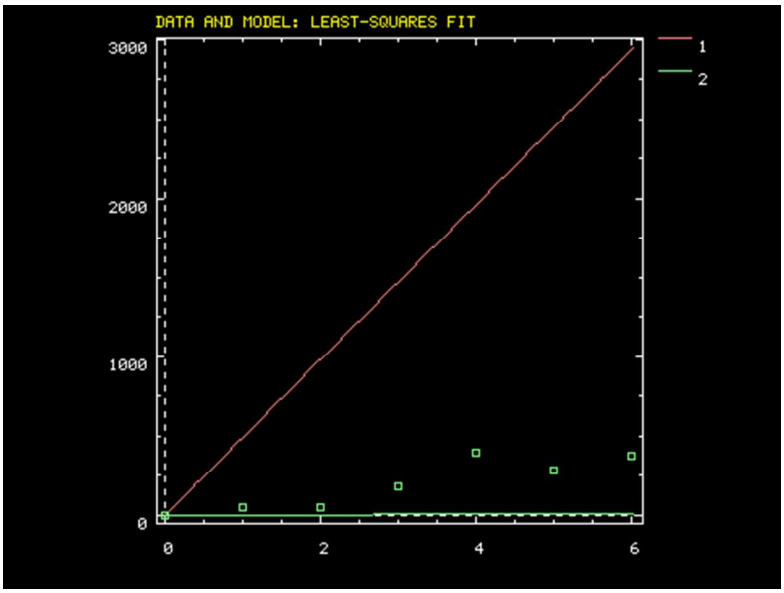
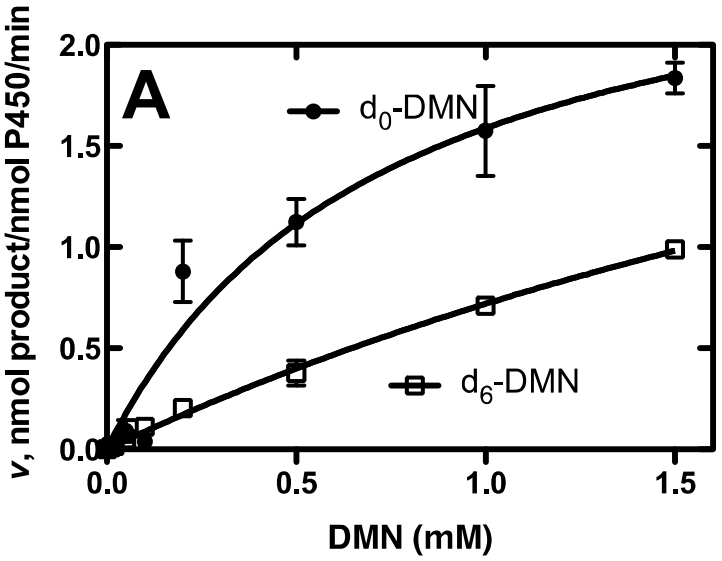
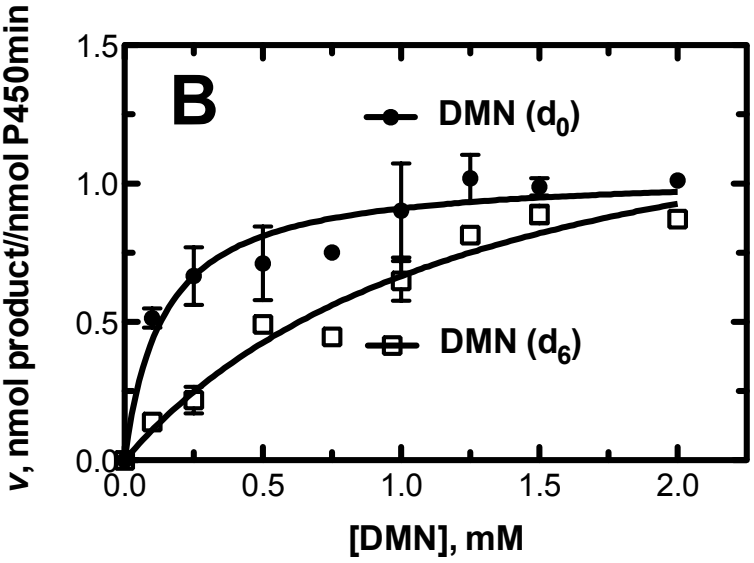
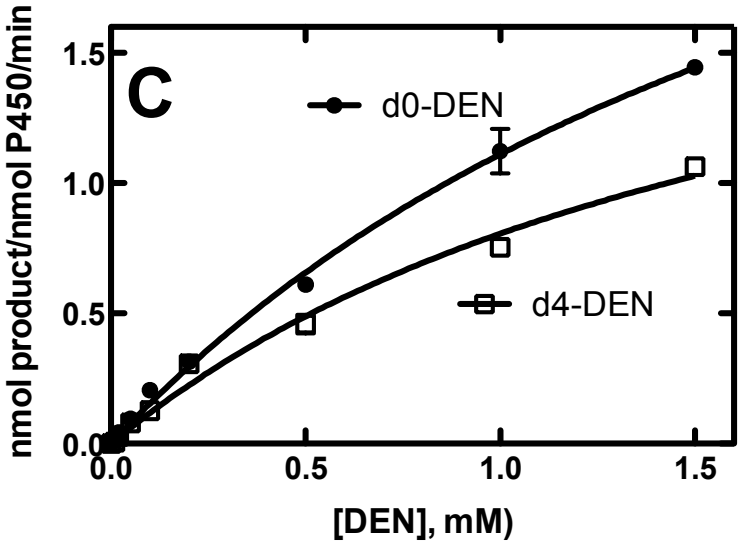


Figure S5. Steady-state kinetics of denitrosation products. Incubations were done for 15 min at 37 °C with 1 μM human P450 2E1 in the typical reconstituted system containing NADPH-P450 reductase and b_5. Analyses of alkylamines[2] and NO_2^- [3] are described in more detail under Experimental Procedures. (A) Formation of CH_3NH_2 from d_0 and d_6 DMN. (B) Formation of NO_2^- from d_0 and d_6 DMN. (C) Formation of $CH_3CH_2NH_2$ from d_0 and d_4 DEN ($CH_3CD_2N(NO)CD_2CH_3$). (D) Formation of NO_2^- from d_0 and d_4 DEN ($CH_3CD_2N(NO)CD_2CH_3$).




B
DMN (d0)
DMN (d6)
v, nmol product//nmol P450min
[DMN], mM
0.0
0.5
1.0
1.5
2.0


C
d0-DEN
d4-DEN
nmol product/nmol P450/min
[DEN], mM)
0.0
0.5
1.0
1.5

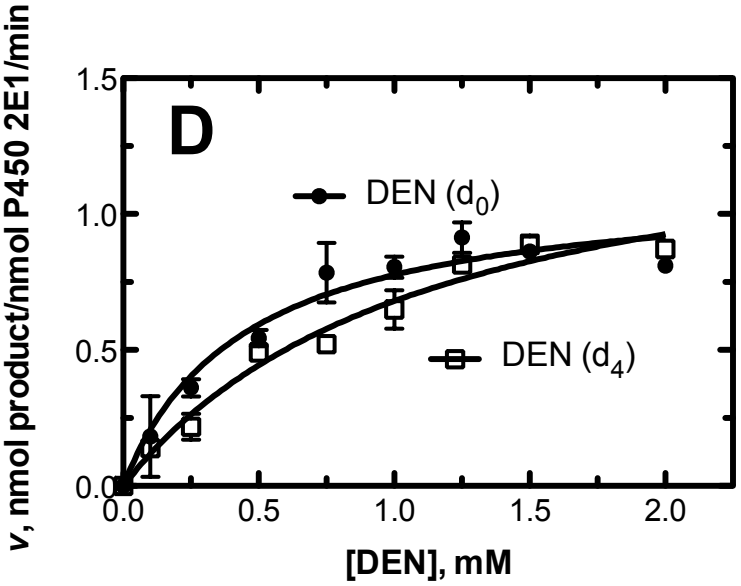

D
DEN (d0)
DEN (d4)
v, nmol product/nmol P450 2E1/min
[DEN], mM
0.0
0.5
1.0
1.5
2.0

Figure S6. Steady-state kinetics of *N*-demethylation of [d_4]-DMN to formaldehyde by rat P450 2B1. Apparent k_{cat} 1.6 ± 0.2 min^{-1} (see text for correction for isotopes), K_m 2.5 ± 0.5 mM. Dansyl hydrazone derivatives were analyzed by LC-MS, and the fit is to a hyperbolic (Michaelis-Menten) plot.

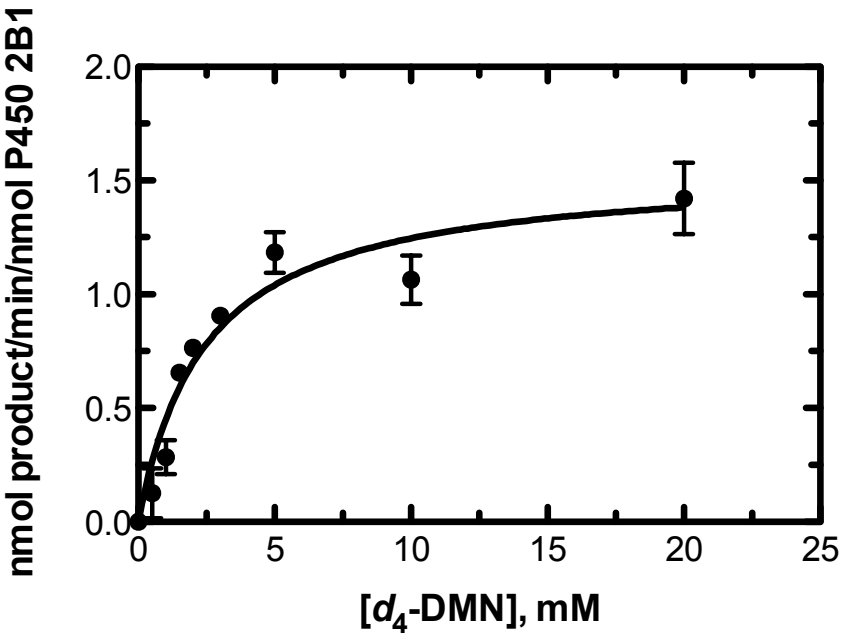
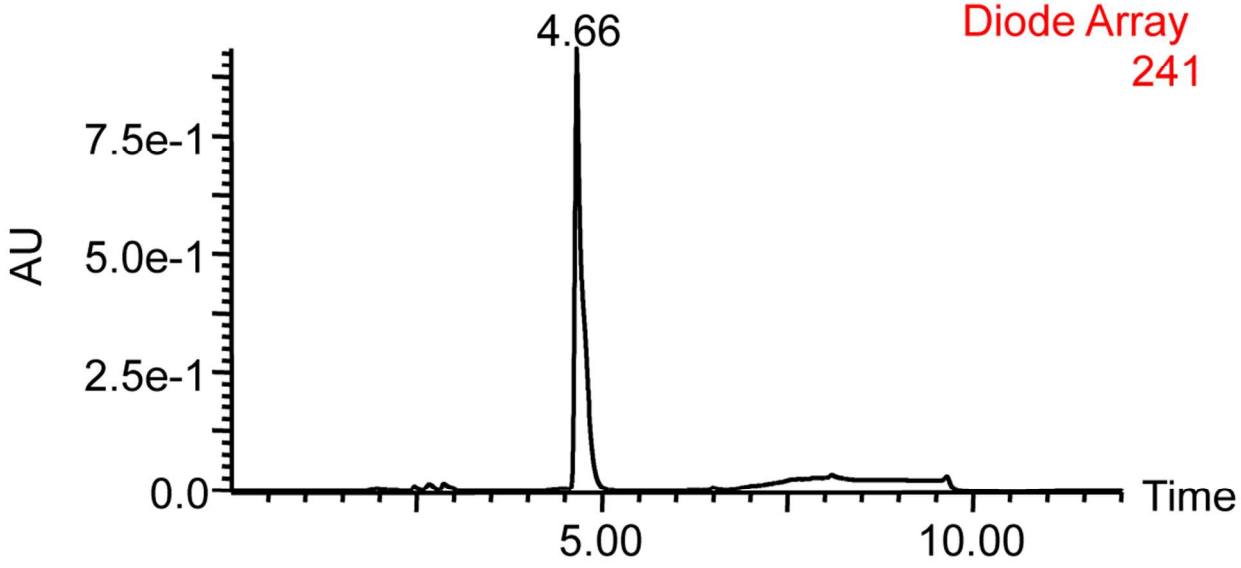


Figure S7. HPLC-UV of *N*-nitroso-*N*-ethylacetamide. UV absorbance at 241 nm was collected and is plotted on the y-axis.



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