Hyperpolarized [1,4-^{13}C]-Diethylsuccinate: A Potential DNP Substrate for In Vivo Metabolic Imaging

(Supporting Information)

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General Methods

[1,4-^{13}C]-Succinate was purchased from Cambridge Isotopes (99% 1,4-^{13}C, CLM-1084, Andover, MA); all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. ^{1}H and ^{13}C NMR spectra for characterization of succinate-based metabolites were collected at Varian INOVA 500 (^{1}H at 500 MHz, ^{13}C at 125 MHz). All chemical shifts (δ) are reported in ppm. ^{1}H chemical shifts are reported relative to the residual solvent peak (chloroform = 7.26 ppm; deuterium oxide = 4.80 ppm). ^{13}C chemical shifts are reported relative to the residual deuterated solvent ^{13}C signals (chloroform = 77 ppm) or referenced to [1,4-^{13}C]-diethylsuccinate at 176.4 ppm.

Synthesis of [1,4-^{13}C]-Monoethylsuccinate. In order to confirm the identity of [^{13}C]-MES, the compound was prepared by an adaptation of a published procedure[1] (^{1}H and ^{13}C NMR spectra in agreement with previous report): In a 1 mL vial equipped with magnetic stir bar, 50 mg (0.49

mmol) of [1,4-^{13}C]-succinic anhydride was added. The solid was dissolved in a mixture of anhydrous ethanol (0.15 mL, 2.57 mmol) and anhydrous pyridine (0.10 mL). The vial was sealed and reaction was allowed to stir at 100 °C. After 60 min, water (0.25 mL) was added and the solution was acidified with HCl (aq) to achieve a pH = 2. The desired product was extracted from the aqueous solution with 3 x 0.10 mL of dichloromethane. Organic layers were combined, dried over anhydrous sodium sulfate, and filtered; the solvent was removed by evaporation and 60 mg (83% yield) of pure product was isolated as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.22 (bs, 1H), 4.12 (q, *J* = 7 Hz, 2H), 2.63 (t, *J* = 7 Hz, 2H), 2.58 (t, *J* = 7 Hz, 2H), 1.22 (t, *J* = 7 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.9, 172.4, 60.8, 29.0, 28.6, 14.0 ppm.

***In Vitro* Experiments**

In vitro experiments were performed in order to facilitate the identification of the metabolites observed from [^{13}C]-DES *in vivo* experiments. These experiments include exposure of [^{13}C]-DES to: (1) Pig Liver Esterase, (2) Rat Blood, (3) Homogenates of Rat Heart and (4) Human Prostate Cancer Cells (PC-3). Experimental protocols for (1) – (3) are detailed in the methods section of the manuscript, and results are discussed in the text. The procedure and results for (4) are detailed below:

(4) Human Prostate Cancer Cells (PC-3). In order to assign the products from [^{13}C]-DES that occur via intracellular metabolic processing, we performed various *in vitro* studies. Four plates of human prostate cancer PC-3 cells ($5x10^6$) were incubated with media containing [^{13}C]-DES (10 mM). At particular time points (1, 5, 20, 60 min), the medium was removed and quenched with methanol to provide four extracellular samples for analysis. In addition, the remaining adherent PC-3 cells were then treated with methanol, scraped from the flask to yield four

intracellular samples, which were subjected to further analysis. Examination of the intra- and extracellular fractions (eight total samples) by NMR revealed that [^{13}C]-DES was primarily present in the extracellular samples, and only minor quantities could be observed in intracellular ones. No metabolic products were observed. This result potentially suggests DES may be metabolized prior to entry into the intracellular environment.

In a related set of experiments, the media containing [^{13}C]-DES (10 mM) was initially supplemented with pig liver esterase (7.5 U/mL). Ideally, this procedure would mimic potential esterase found in the blood that may facilitate metabolic processing of [^{13}C]-DES and increase cellular entry. [^{13}C]-MES was observed in all extracellular samples. However, no metabolic products corresponding to the signal at 172.7 ppm was observed.

NMR Spectra

Spectra not displayed as figures in the body of the manuscript are displayed below. Experimental descriptions are listed in Methods and Materials section. The following are items are shown:

Figure S1: ^{1}H NMR of [^{13}C]-DES,

Figure S2: ^{13}C NMR of [^{13}C]-DES,

Figure S3: ^{1}H NMR of [^{13}C]-MES,

Figure S4: ^{13}C NMR of [^{13}C]-DES

Figure S5: Spectra of Hyperpolarized [^{13}C]-DES post-dissolution (no in vivo injection),

Figure S6: ^{13}C NMR of [^{13}C]-DES with Pig Liver Esterase after 5 min,

Figure S7: ^{13}C NMR of [^{13}C]-DES (100 mM) with Rat Heart Homogenate,

Figure S8: ^{13}C NMR of [^{13}C]-DES with Succinic Anhydride.

Figure S1: *Characterization of [^{13}C]-DES*

^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.15 (q, J = 7 Hz, 4H, -OCH_2-), 2.60 (s, 4H, -C(O)CH_2-), 1.12 (t, J = 7 Hz, 6H, -CH_3) ppm.

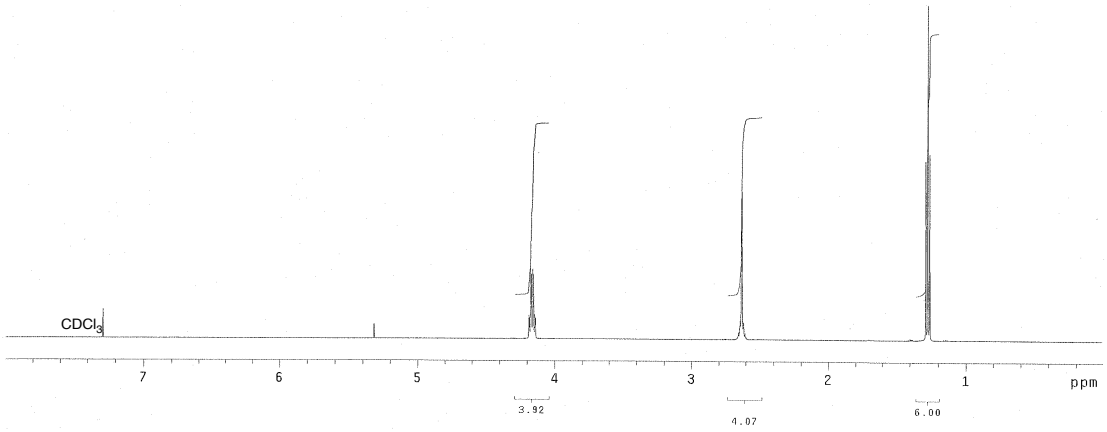


Figure S2: *Characterization of [^{13}C]-DES*

^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.4 (-C(O)-), 60.8 (-OCH$_2$-), 29.1 (-C(O)CH$_2$-), 14.1 (-CH$_3$) ppm.

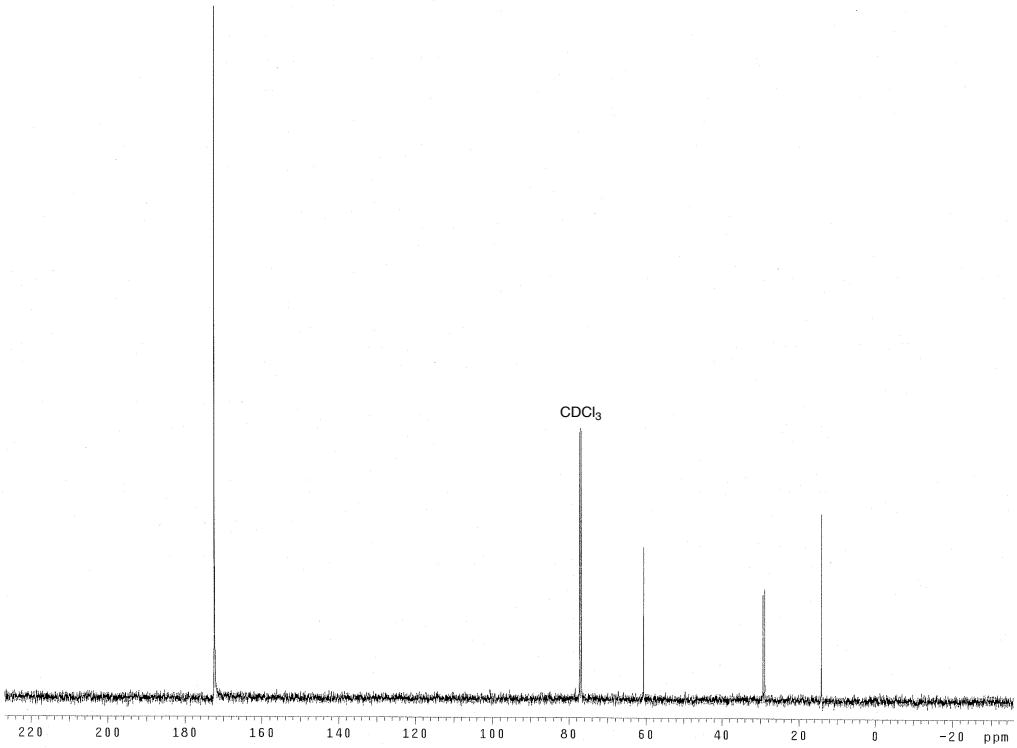


Figure S3: *Characterization of [^{13}C]-MES*

^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.58 (bs, 1H, -CO_2*H*), 4.18 (q, *J* = 7 Hz, 2H, -OC*H*$_2$-), 2.57-2.71 (m, 4H, -C(O)C*H*$_2$C*H*$_2$C(O)-), 1.19 (t, *J* = 7 Hz, 3H, -C*H*$_3$) ppm.

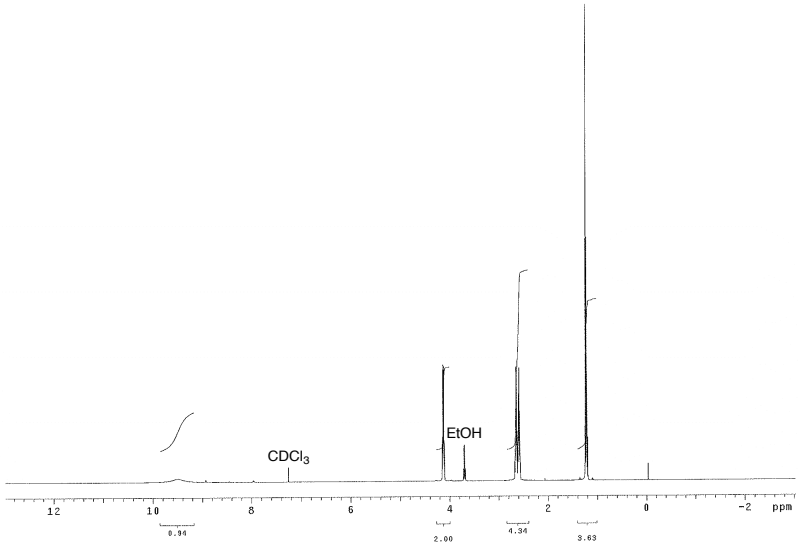


Figure S4: *Characterization of [^{13}C]-MES*

^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.2 (-*C*O_2H), 172.2 (-*C*O_2Et), 60.3 (-O*C*H$_2$-), 28.7 (-C(O)*C*H$_2$-), 14.0 (-*C*H$_3$) ppm.

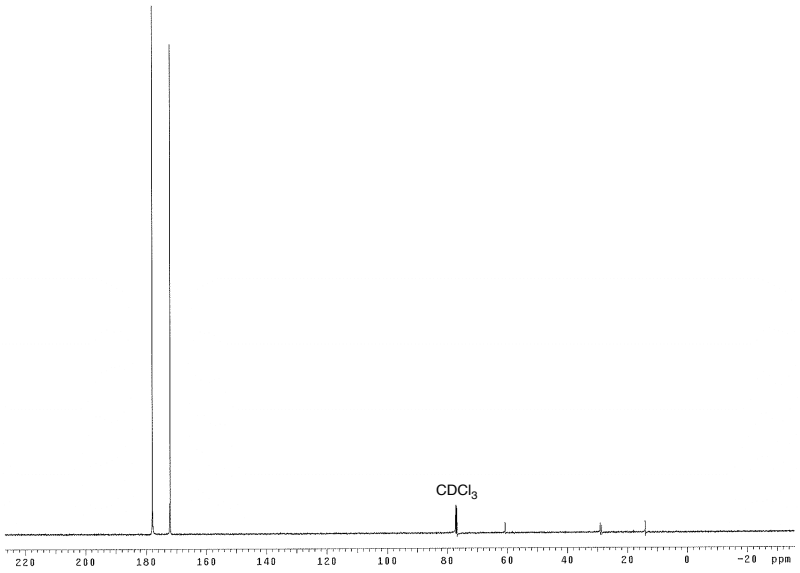


Figure S5: *[^{13}C]-DES (post-dissolution with no in vivo administration, pH = 7.4)*

^{13}C NMR: δ 176.4 (-*C*O$_2$Et), ^{13}C labeled carbonyl observed

Stack plot for first 175 min of observation – No hydrolysis products observed

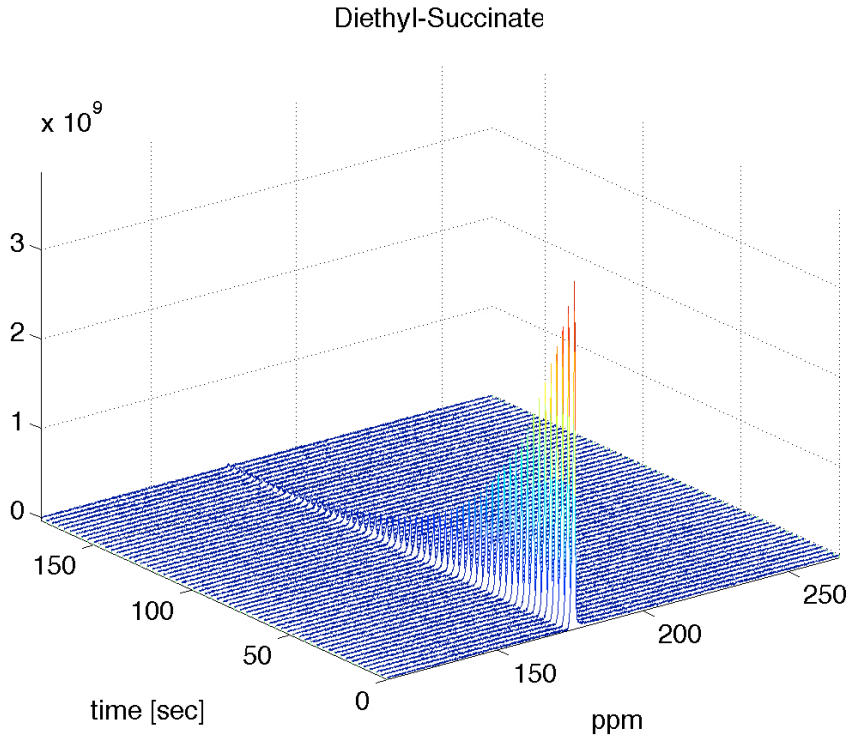


Figure S6: *Pig Liver Esterase + [^{13}C]-DES (pH = 7.4)*

^{13}C NMR (D$_2$O, pH = 7.4): δ 182.4 (monoethylsuccinate, -*C*O$_2$H), 177.6 (monoethylsuccinate, -*C*O$_2$Et), 176.4 (diethylsuccinate, -*C*O$_2$Et) ppm.

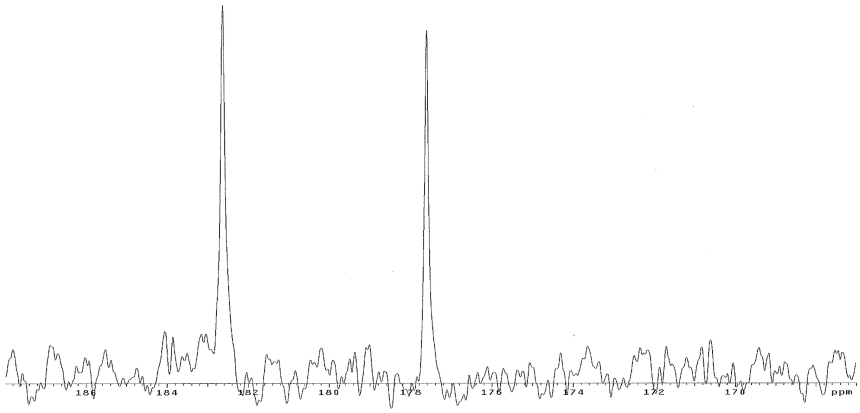
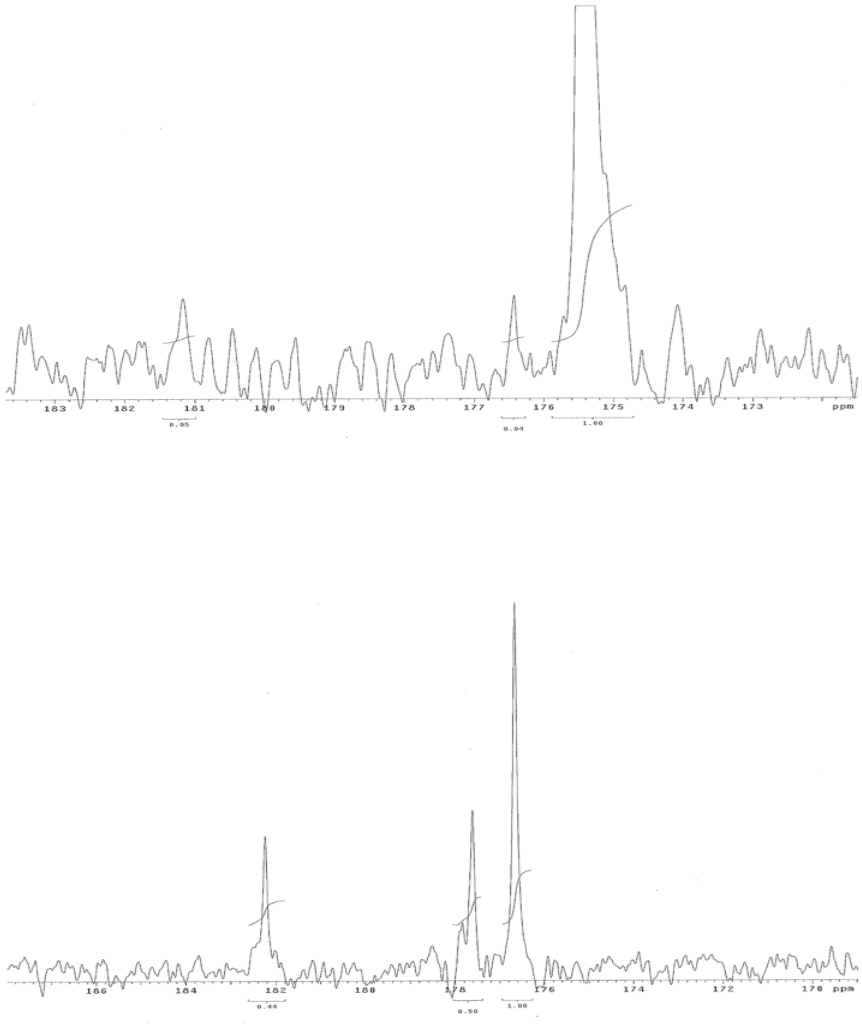


Figure S7: *Rat Heart Homogenate + [^{13}C]-DES* (100 mM, pH = 7.4);

Spectrum after 2, 20 and 60 min time points shown

^{13}C NMR (D_2O, pH = 7.4, 2 min): 182.4 (monoethylsuccinate, -*C*O_2H), 177.6 (monoethylsuccinate, -*C*O_2Et), 176.4 (diethylsuccinate, -*C*O_2Et) ppm. ^{13}C NMR (D_2O, pH = 7.4, 20 min): δ 182.3 (monoethylsuccinate, -*C*O_2H), 177.6 (monoethylsuccinate, -*C*O_2Et), 176.4 (diethylsuccinate, -*C*O_2Et) ppm. ^{13}C NMR (D_2O, pH = 7.4, 60 min): δ 181.9 (monoethylsuccinate, -*C*O_2H), 177.6 (monoethylsuccinate, -*C*O_2Et), 176.4 (diethylsuccinate, -*C*O_2Et) ppm.



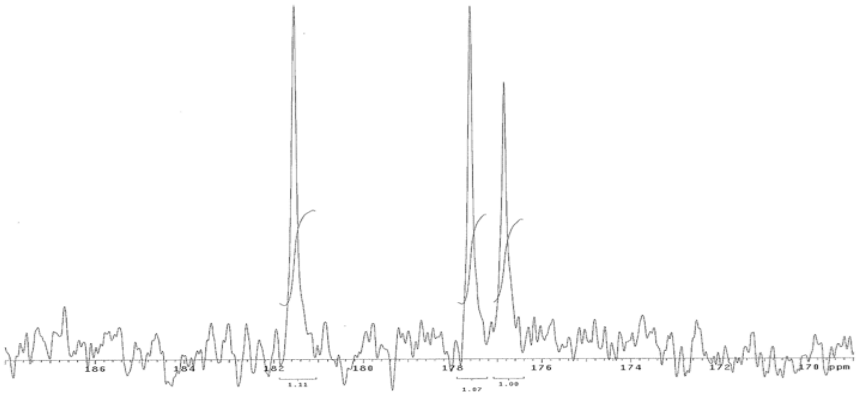
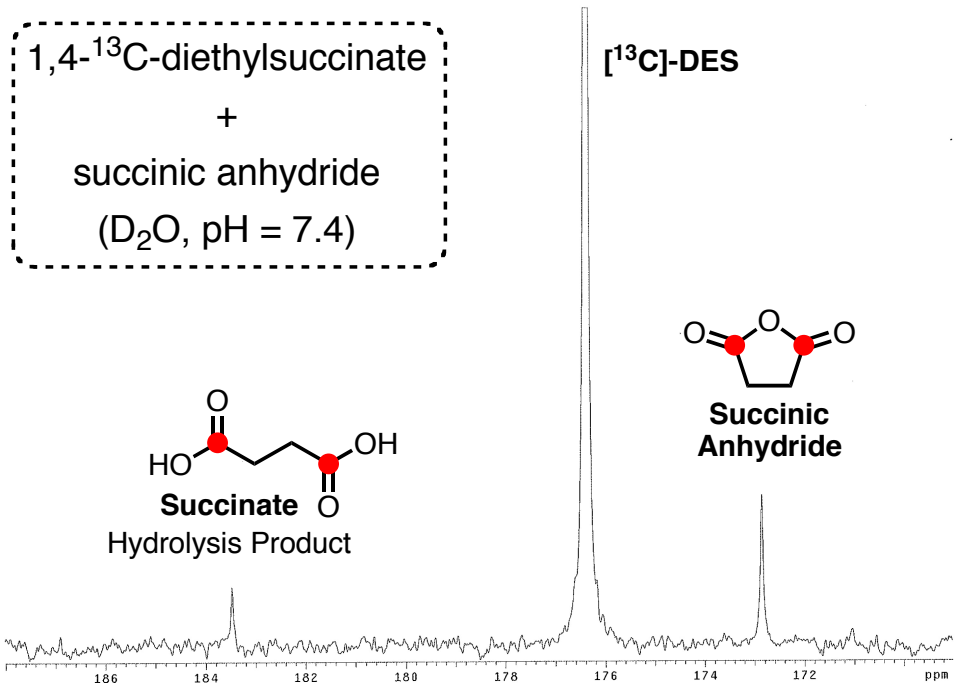


Figure S8: *Succinate Anhydride Reference*

^{13}C NMR (D_2O, pH = 7.4): δ 182.5 (succinate, -CO_2H), 176.4 (diethylsuccinate, -CO_2Et), 172.8 (succinic anhydride, -CO_2-) ppm.



References

1. Eisenführa A, Arora PS, Sengle G, Takaoka LR, Nowick JS, Famulok M. *Bioorg. Med. Chem.* 2003; **11**: 235-249.