Supporting information

Mitigation of Doxorubicin-Induced Cardiotoxicity with an H_2O_2-Activated, H_2S-Donating Hybrid Prodrug

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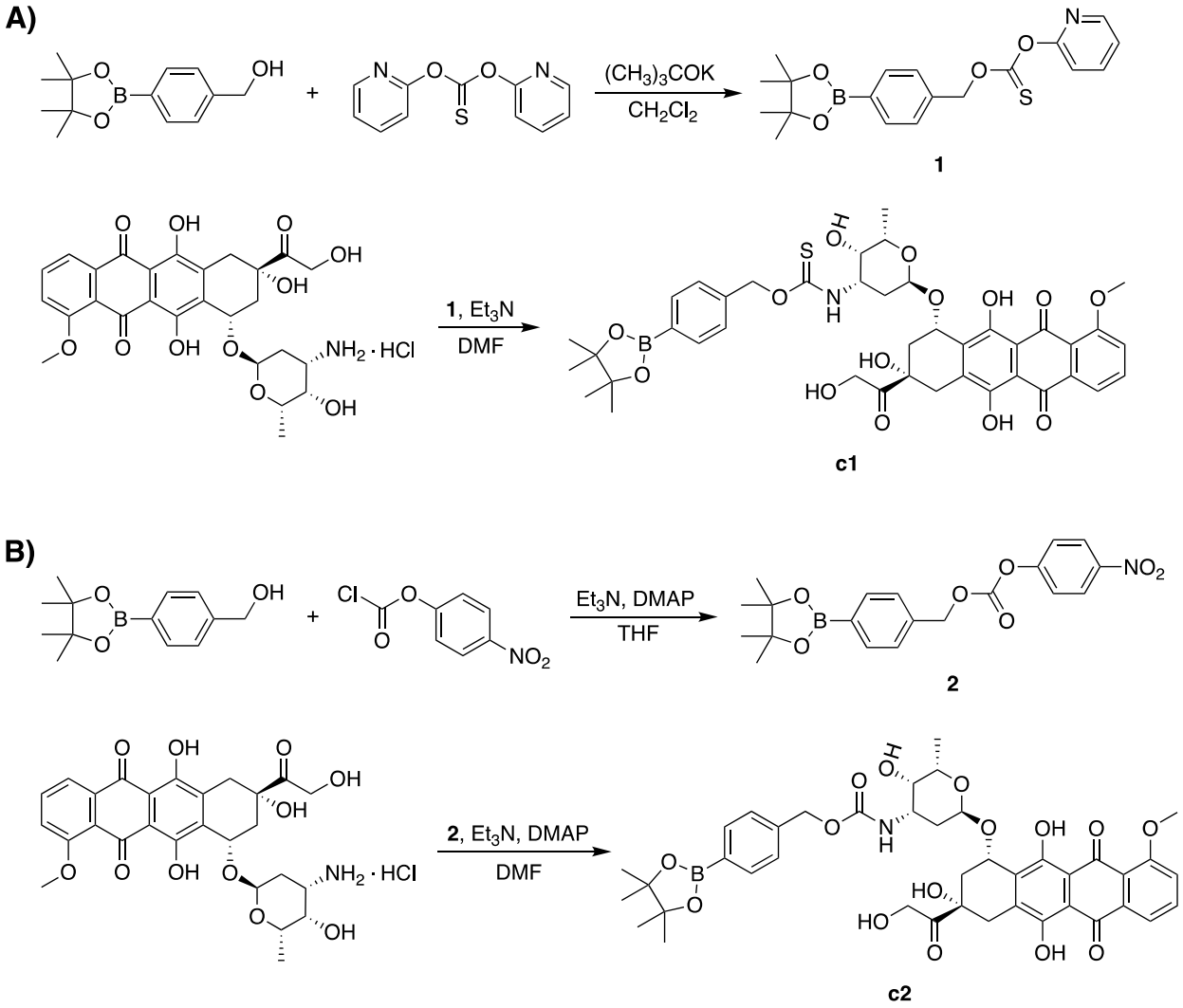
Synthetic schemes for c1 and c2



Scheme S1. Synthesis of doxorubicin prodrugs. (A) c1 and (B) c2.

Methylene Blue Assays

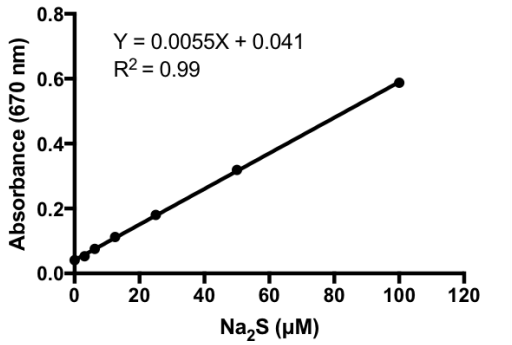


Figure S1. Calibration curve (with carbonic anhydrase) from a methylene blue assay using differing amounts of Na_2S. The resulting absorbance at each concentration of Na_2S was recorded at 670 nm.

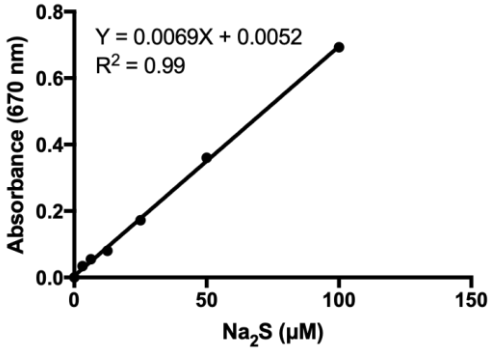


Figure S2. Calibration curve (without carbonic anhydrase) from a methylene blue assay using differing amounts of Na_2S. The resulting absorbance at each concentration of Na_2S was recorded at 670 nm.

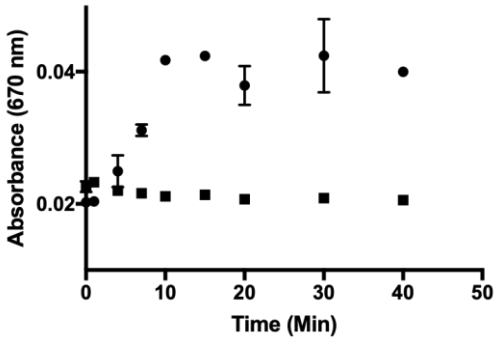


Figure S3. Methylene blue assay depicting the time-dependent release of H_2S from **c1** (40 μM) while in the presence of H_2O_2 (40 μM) but in the absence of carbonic anhydrase (CA). Plotted as the average ± SEM from three independent experiments. Data were collected in the presence (circles) or absence (squares) of $+H_2O_2$.

LC-MS Analysis

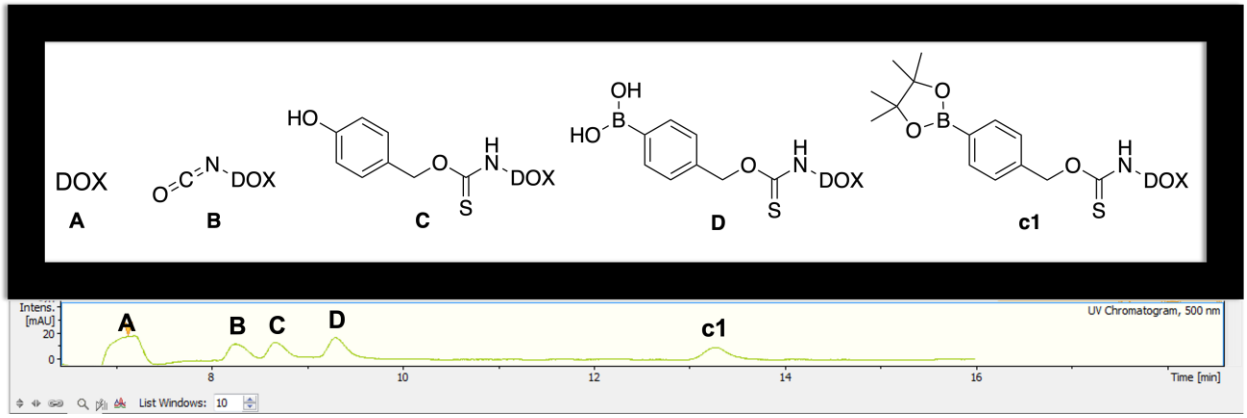


Figure S4. Representative LC-MS chromatogram for the reaction between c1 (10 μM) and H_2O_2 (10 μM) after an 80 min time period.

DOX and Prodrug uptake in H9C2 Cardiomyoblasts

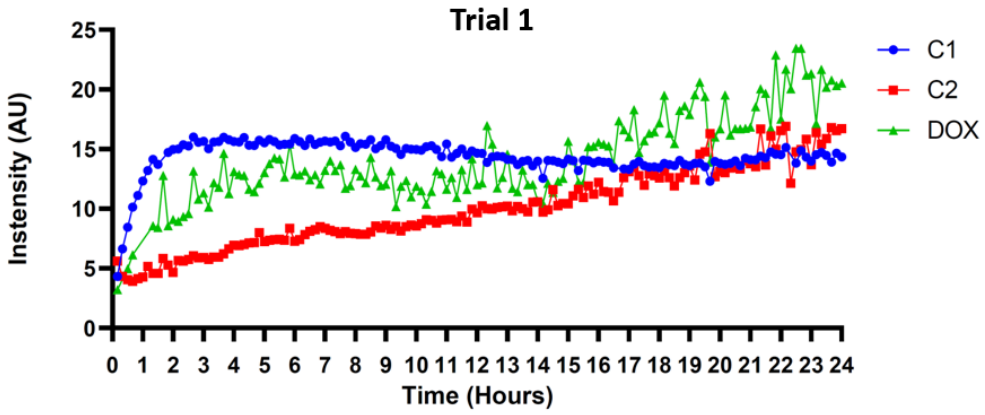


Figure S5. Uptake of DOX and prodrugs **c1** and **c2** by H9C2 cardiomyoblasts. H9C2 cells cultures grown overnight on chambered coverslips in media with 10% serum were switched to Fluorobrite DMEM imaging media supplemented with 5% serum and prepared for live cell imaging on a Zeiss LSM 880 confocal microscope, then 10 μM of **c1**, **c2**, or DOX was added and images taken every 10 min (averaged every 30 min) for 24 h. Approximately 20 cells present in each field of view were averaged for each sample. This experiment was conducted independently of and prior to the experimental work presented in Figure 6.

4T1 mouse breast cancer cells in culture exhibit substantial cytotoxicity when treated with c1.

DOX is a known cytotoxic agent used against 4T1 triple-negative breast cancer cells grown in culture or injected orthotopically into mouse mammary fat pads to initiate the development of tumors.[1,2] As an initial test to see if **c1** could retain the cytotoxic properties of DOX toward tumor-forming 4T1 cells, we conducted LDH assays to assess cytotoxicity of DOX, **c1** and **c2** treatments. Other than the lowest dose and shortest time, depending on the concentration and time of exposure, **c1** provokes comparable, sometimes less and sometimes more, toxicity relative to DOX in 4T1 cells, perhaps owing to the anticancer activity of H_2S in combination with DOX (Figure S6).

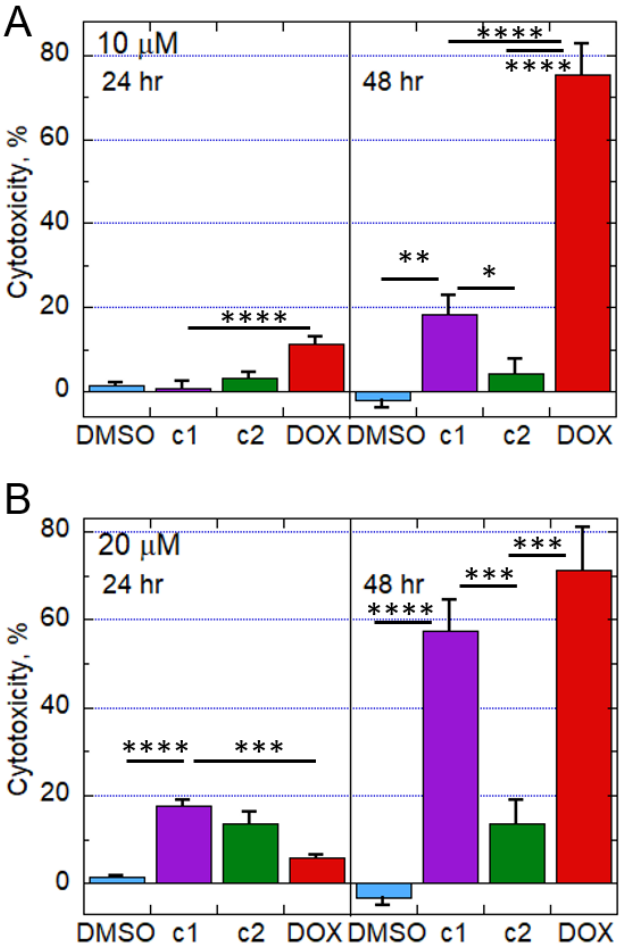


Figure S6. Cytotoxicity of DOX and prodrugs in 4T1 mouse breast cancer cells. As in Figure 7 depicting studies of H9C2 cells, supernatants of media from cells exposed for 24 or 48 h to 10 or 20 μM of **c1**, **c2**, DOX or vehicle) were assessed spectrophotometrically by lactate dehydrogenase (LDH) assay to evaluate release into the media as a measure of cytotoxicity (n=8 or more). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

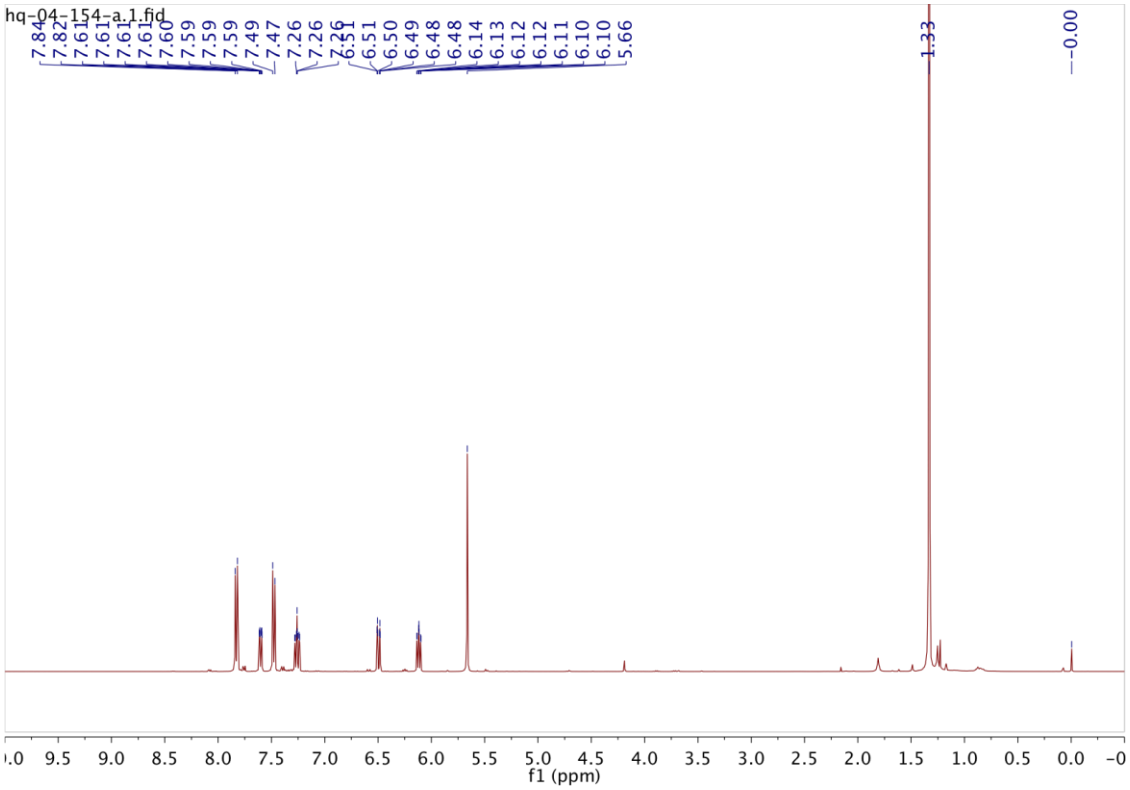
References

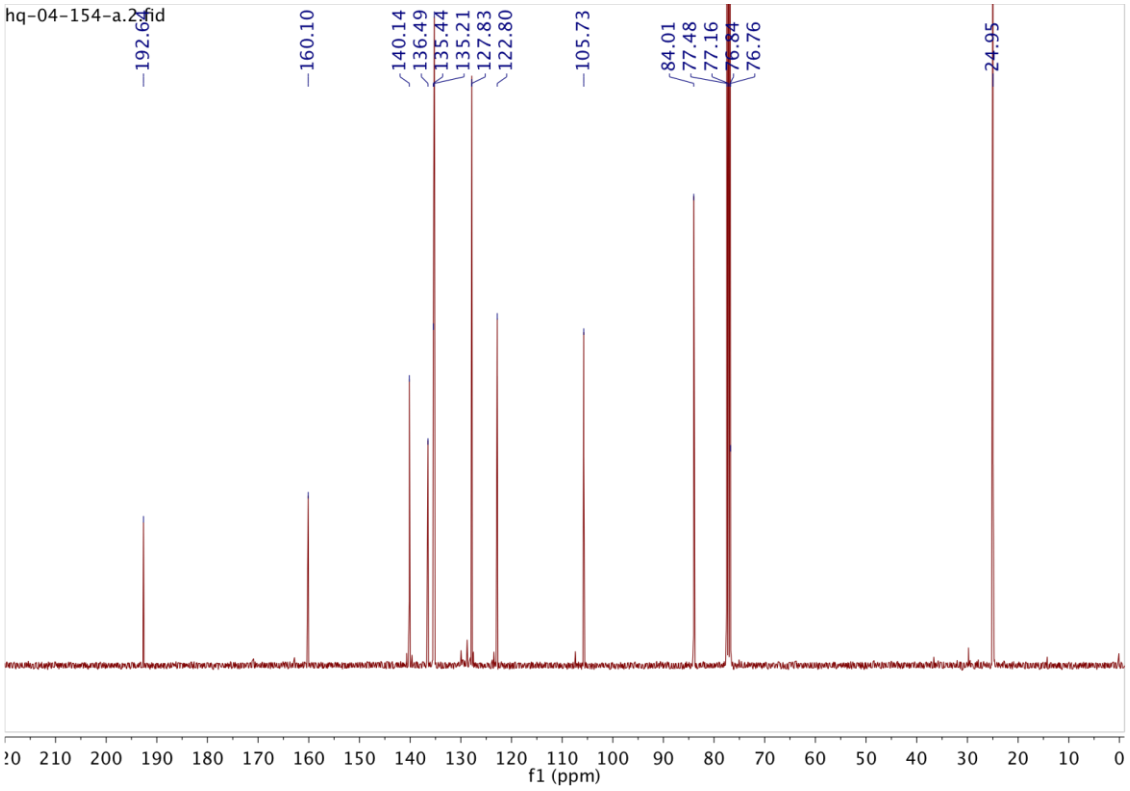
(1) Yang, S.; Zhang, J. J.; Huang, X.-Y. Mouse Models for Tumor Metastasis. *Methods Mol. Biol. Clifton NJ* 2012, *928*, 221–228. https://doi.org/10.1007/978-1-62703-008-3_17.

(2) Feliz-Mosquea, Y. R.; Christensen, A. A.; Wilson, A. S.; Westwood, B.; Varagic, J.; Meléndez, G. C.; Schwartz, A. L.; Chen, Q.-R.; Mathews Griner, L.; Guha, R.; Thomas, C. J.; Ferrer, M.; Merino, M. J.; Cook, K. L.; Roberts, D. D.; Soto-Pantoja, D. R. Combination of Anthracyclines and Anti-CD47 Therapy Inhibit Invasive Breast Cancer Growth While Preventing Cardiac Toxicity by Regulation of Autophagy. *Breast Cancer Res. Treat.* 2018, *172* (1), 69–82. https://doi.org/10.1007/s10549-018-4884-x.

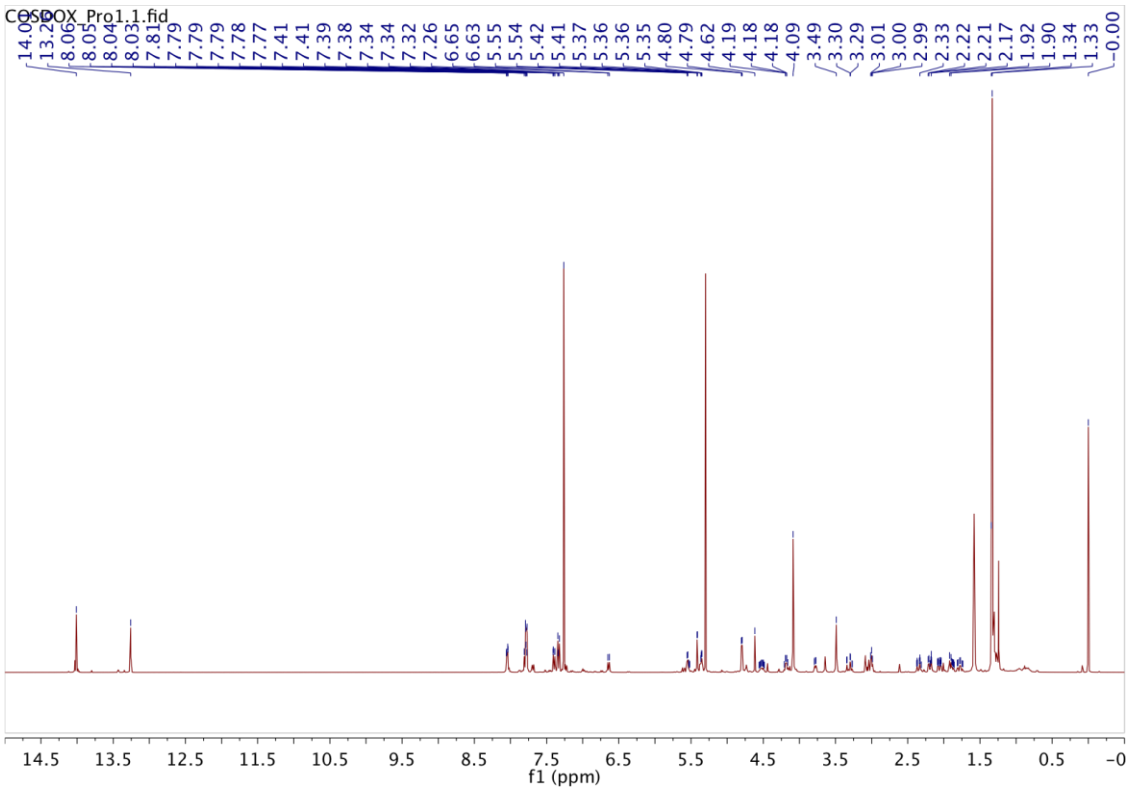
NMR Spectra

1





c1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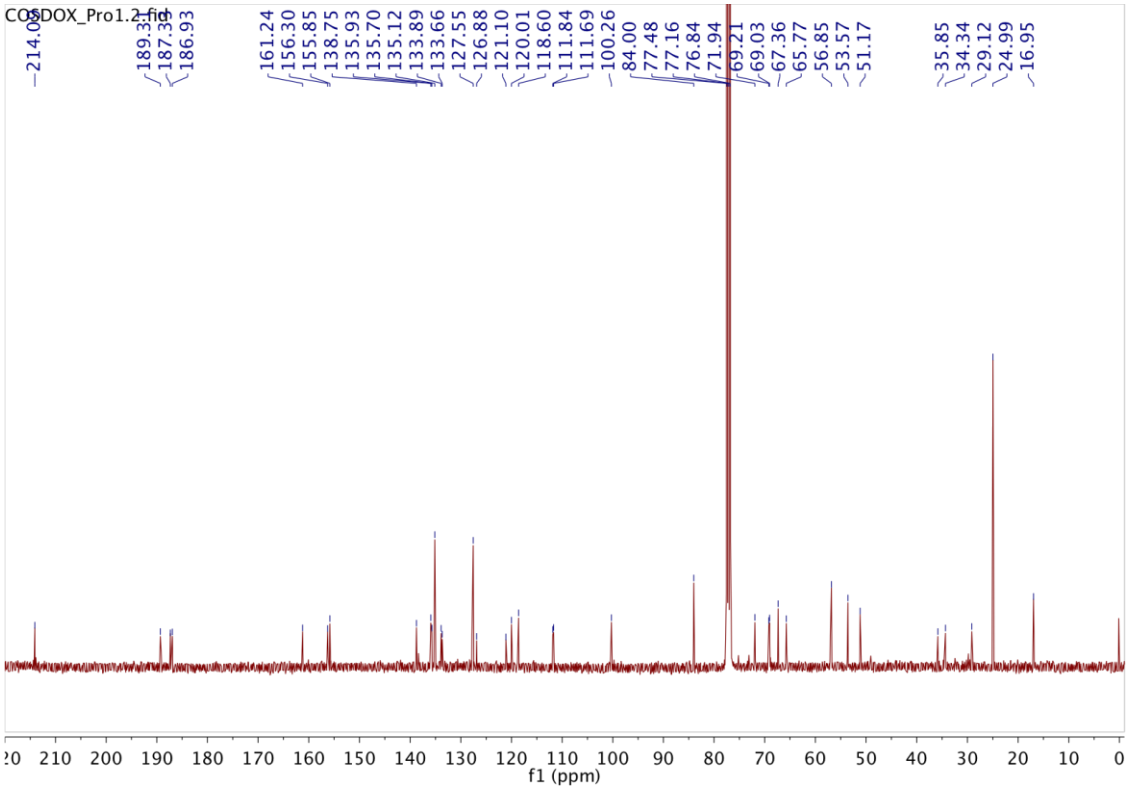
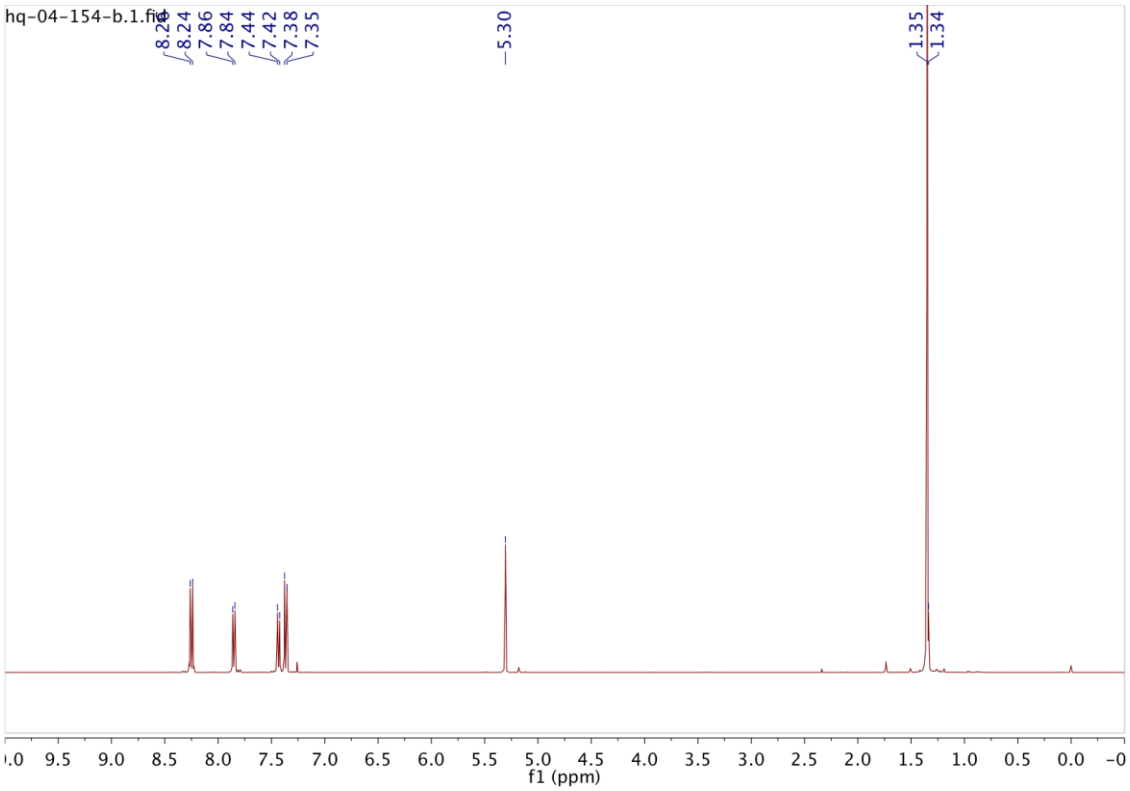
13.20
8.06
8.05
8.04
8.03
7.81
7.79
7.79
7.79
7.78
7.77
7.41
7.41
7.39
7.38
7.34
7.34
7.32
7.26
6.65
6.63
5.55
5.54
5.42
5.41
5.37
5.36
5.36
5.35
4.80
4.79
4.62
4.19
4.18
4.18
4.09
3.49
3.30
3.29
3.01
3.00
2.99
2.33
2.22
2.21
2.17
1.92
1.90
1.34
1.33
0.00
14.5
13.5
12.5
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0
f1 (ppm)


COSDOX_Pro1.2.fid
214.05
189.30
187.39
186.93
161.24
156.30
155.85
138.75
135.93
135.70
135.12
133.89
133.66
127.55
126.88
121.10
120.01
118.60
111.84
111.69
100.26
84.00
77.48
77.16
76.84
71.94
69.21
69.03
67.36
65.77
56.85
53.57
51.17
35.85
34.34
29.12
24.99
16.95
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

2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8.26
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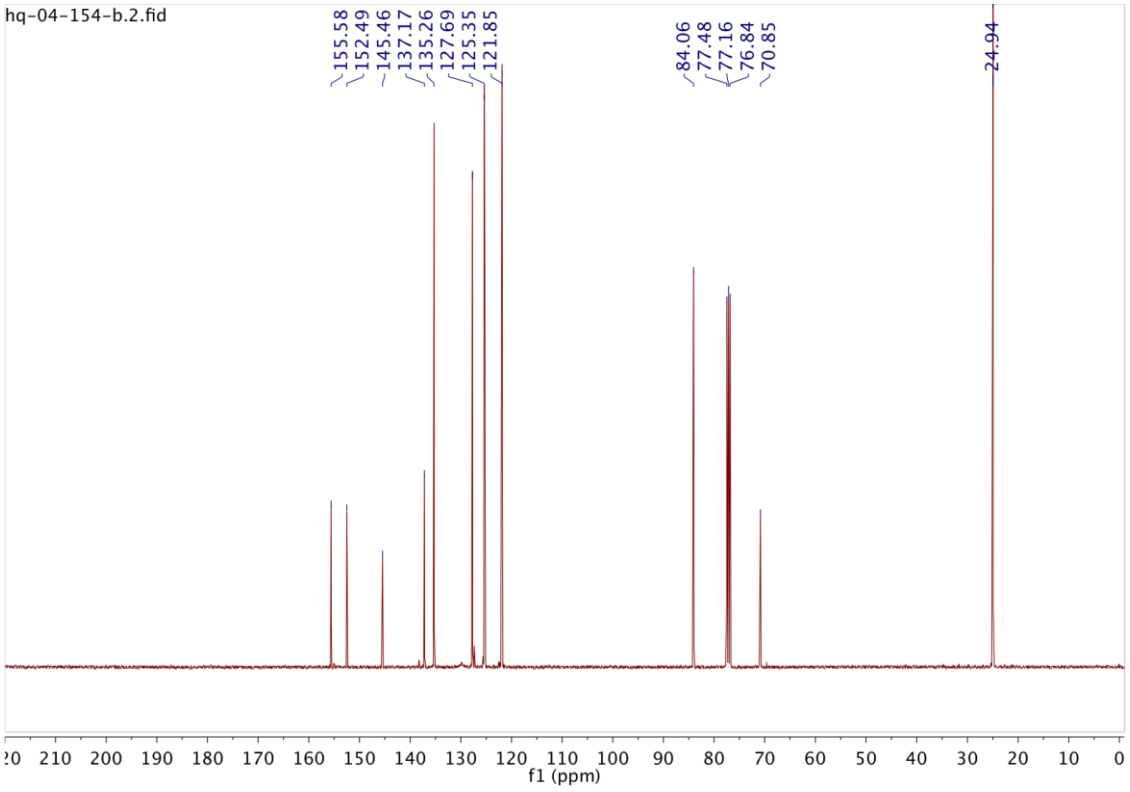
7.86
7.84
7.44
7.42
7.38
7.35
5.30
1.35
1.34
f1 (ppm)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152.49
145.46
137.17
135.26
127.69
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121.85
84.06
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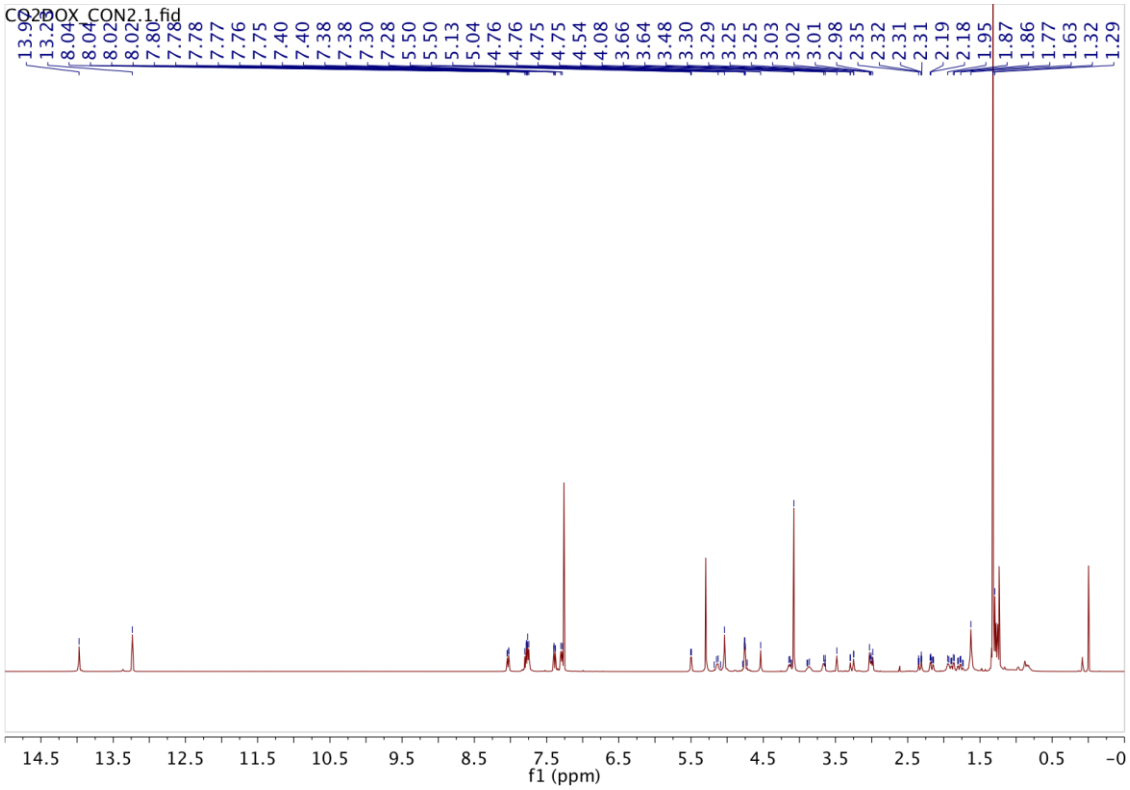
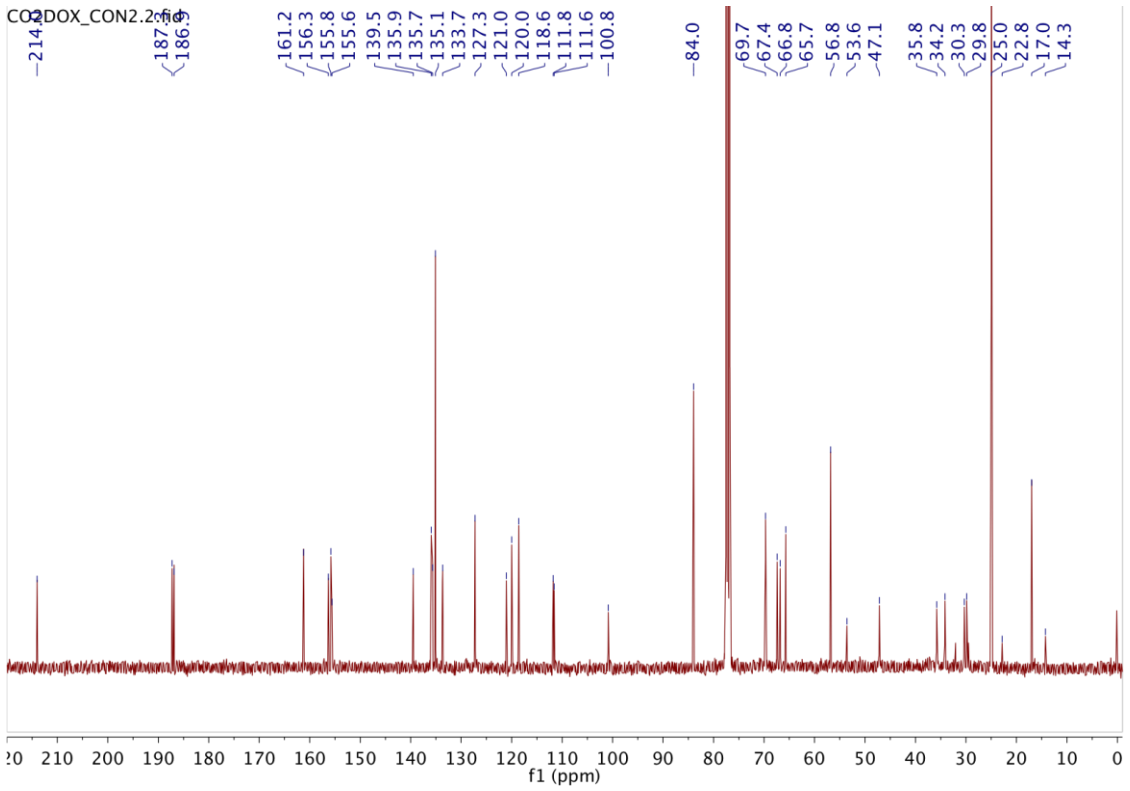
77.16
76.84
70.85
24.94
f1 (ppm)

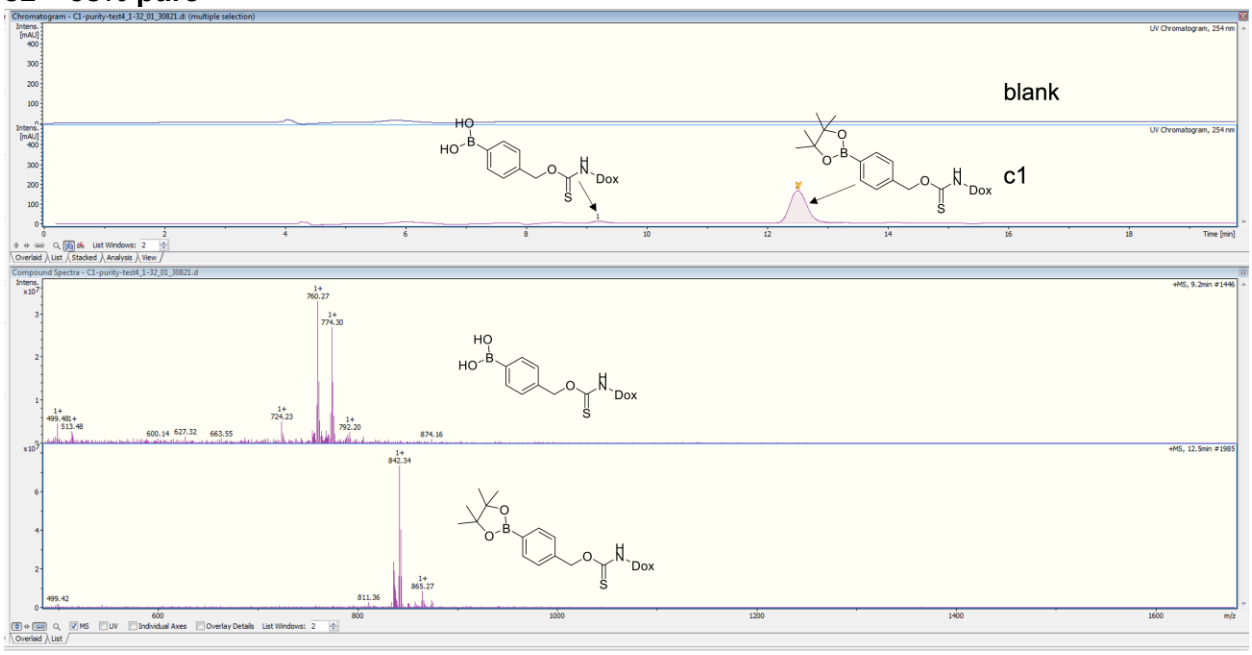
c2


CO2DOX_CON2_1.fid
13.92
13.20
8.04
8.04
8.02
8.02
7.80
7.78
7.78
7.77
7.76
7.75
7.40
7.40
7.38
7.38
7.30
7.28
5.50
5.50
5.13
5.04
4.76
4.76
4.75
4.75
4.54
4.08
3.66
3.64
3.48
3.30
3.29
3.25
3.25
3.03
3.02
3.01
2.98
2.35
2.32
2.31
2.31
2.19
2.18
1.95
1.87
1.86
1.77
1.63
1.32
1.29
14.5
13.5
12.5
11.5
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
−0
f1 (ppm)


CO2DOX_CON2.2.fid
214.2
187.9
186.9
161.2
156.3
155.8
155.6
139.5
135.9
135.7
135.1
133.7
127.3
121.0
120.0
118.6
111.8
111.6
100.8
84.0
69.7
67.4
66.8
65.7
56.8
53.6
47.1
35.8
34.2
30.3
29.8
25.0
22.8
17.0
14.3
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

LC-MS Traces

c1 > 95% pure



c2 > 95% pure