**Supplementary Information**

Semisynthetic Analogues of Anhydrotetracycline as Inhibitors of Tetracycline Destructase Enzymes

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This file includes Supplementary Results:

## Supplementary Results

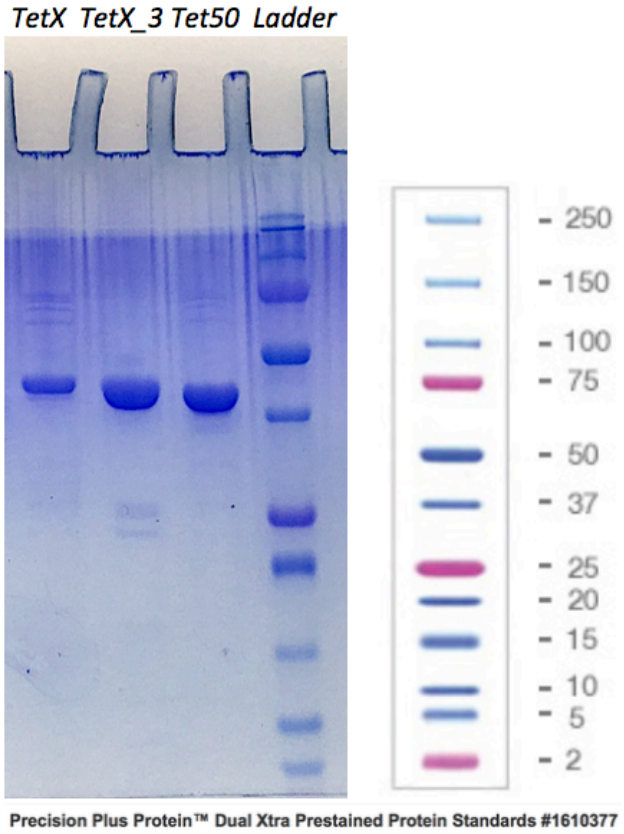
### I. Tables and Figures for Biological Evaluation of aTc and aTc analogues.

**Supplementary Table 1 | Relevant Sequences, Strains, Plasmids, and Primers for Tet(X)_3 [TE_7F_Contig_3].**

| *Sequences, Strains, Plasmids, Primers* | *Reference or source* |
|---|---|
| **Strains** | |
| MegaX DH10B | Invitrogen BioSciences, ThermoScientific |
| **Sequence** | |
| TE_7F_Contig_3 [Tet(X)_3] | NCBI GenBank Accession Number: KU547176.1:452..1588 |
| **Plasmids** | |
| pET28b(+) | Novagen, Merck Biosciences |
| pZE21 | (Lutz and Bujard, 1997)[1] |
| **Primers (5' → 3')** | |
| TE_7F_Contig_3 (452_1588) – ATGACTTTGCTAAAAAATAAAAAAATTA | Integrated DNA Technologies, Inc. (IDT) |
| TE_7F_Contig_3 (452_1588) – TTATAGATTCATTAGTTTTTGGAATGA | Integrated DNA Technologies, Inc. (IDT) |

[1]Lutz, R.; Bujard, H. Independent and Tight Regulation of Transcriptional Units in *Escherichia Coli* Via the LacR/O, TetR/O and AraC/I1-I2 Regulatory Elements. *Nuc. Acids Res.* **1997**, *25*, 1203–1210.

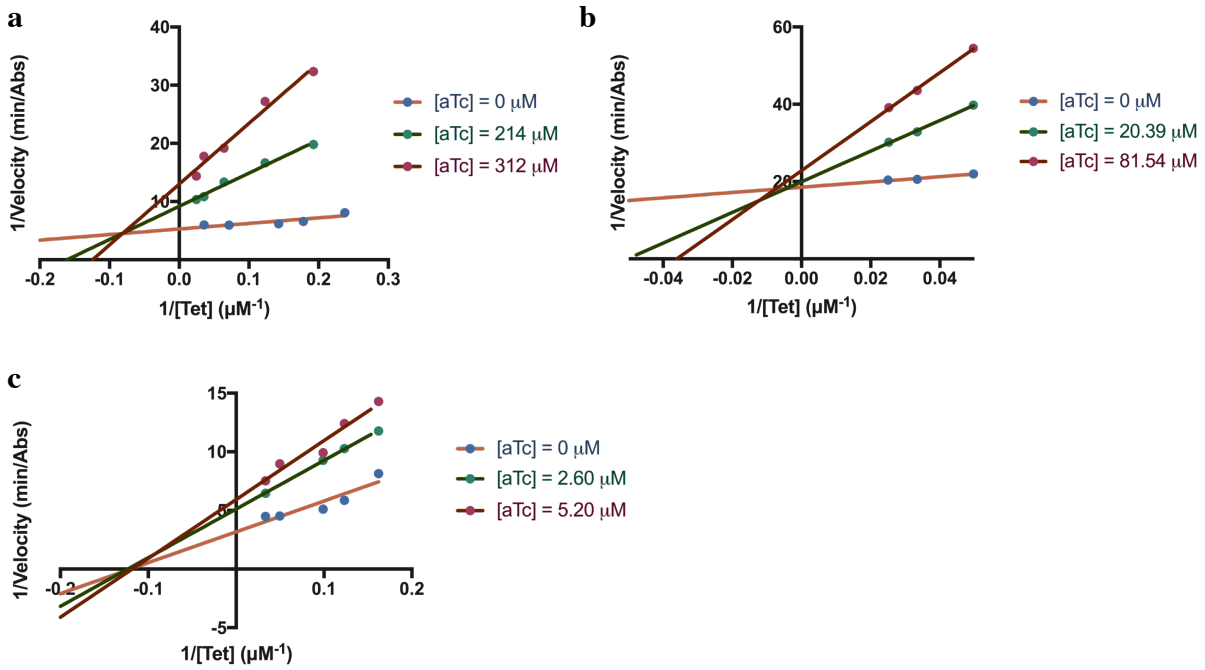
**Supplementary Figure 1 | SDS-Page Gel Image – Purified Tetracycline Destructase Enzymes**



**Supplementary Figure 2 | Lineweaver-Burk Plots for the *in vitro* inhibition of the tetracycline destructase-mediated degradation of tetracycline by anhydrotetracycline**

Reaction samples were prepared in TAPS buffer (100 mM, pH 8.5) with 504 μM NADPH, varying concentrations of tetracycline substrate (typically, 0–40 μM), a set amount of inhibitor (varied by enzyme, usually 0 μM to twice $IC_{50}$), and 0.4 μM enzyme. After the addition of enzyme, the reactions (in duplicate or triplicate) were mixed, manually by pipette, and the reaction was monitored continuously in a single frame by UV-Visible spectroscopy (absorbance at 380 nm, Carey UV-Visible spectrophotometer) for 3–4 minutes. Initial enzyme velocities were determined by linear regression using Agilent Cary WinUV Software over the linear range of the reaction, and the inverse of the velocities were plotted against the inverse of the substrate concentration and fitted to a line (linear regression) using GraphPad Prism 6. Extrapolation of each individual line (for set inhibitor concentrations) allowed for the visualization of line convergence.

**a,** Lineweaver-Burk plot for aTc inhibition of Tet(50) degradation of tetracycline.
**b,** Lineweaver-Burk plot for aTc inhibition of Tet(X) degradation of tetracycline.
**c,** Lineweaver-Burk plot for aTc inhibition of Tet(X)_3 degradation of tetracycline.



**Supplementary Tables 2 | Unabridged aTc analog inhibition of whole cell *E. coli* expressing tetracycline destructase enzymes.**

**a**, aTc analog inhibition of whole cell *E. coli* expressing Tet(50)
**b**, aTc analog inhibition of whole cell *E. coli* expressing Tet(X)
**c**, aTc analog inhibition of whole cell *E. coli* expressing Tet(X)_3

**a**

| *Enzyme* | *Substrate* | *Inhibitor* | *$IC_{50}$ ($\mu$M)* | *Substrate MIC ($\mu$g/mL)* | *Inhibitor MIC ($\mu$g/mL)* | *Substrate + Inhibitor MIC ($\mu$g/mL) [I] ($\mu$g/mL)* |
|---|---|---|---|---|---|---|
| Tet(50) | Tetracycline | aTc | 210 ± 25 | 128 | 8 | 64 [1] |
| | | aCTc | 37 ± 5 | 128 | 32 | 64 [4] |
| | | aDem | 300 ± 100 | 128 | 16 | 64 [4] |
| | | 7-I-aTc | 54 ± 5 | 128 | 128 | 64 [32] |
| | | 9-Br-aTc | 12 ±1 | 128 | 16 | 32 [8] |
| | CTc | aTc | 210 ± 52 | 128 | 8 | 64 [4] |
| | | aCTc | 86 ± 17 | 128 | 32 | 64 [4] |
| | | 7-I-aTc | 94 ± 16 | 128 | 128 | ---- |
| | | 9-Br-aTc | 60 ± 16 | 128 | 16 | 32 [8] |
| | Demeclocycline | aTc | 120 ± 25 | 64 | 8 | 16 [2] |
| | | aDem | 190 ± 47 | 64 | 16 | 32 [4] |

**b**

| *Enzyme* | *Substrate* | *Inhibitor* | *$IC_{50}$ ($\mu$M)* | *Substrate MIC ($\mu$g/mL)* | *Inhibitor MIC ($\mu$g/mL)* | *Substrate + Inhibitor MIC ($\mu$g/mL) [I] ($\mu$g/mL)* |
|---|---|---|---|---|---|---|
| Tet(X) | Tetracycline | aTc | 41 ± 5 | 32 | 16 | 16 [2] |
| | | aCTc | 1.4 ± 0.2 | 32 | 32 | 16 [4] |
| | | aDem | 1.8 ± 0.4 | 32 | 16 | 16 [2] |
| | | 7-I-aTc | 16 ± 3 | 32 | 256 | ---- |
| | | 9-Br-aTc | 5.6 ± 0.8 | 32 | 128 | ---- |
| | CTc | aTc | 75 ± 21 | 32 | 16 | 16 [8] |
| | | aCTc | 13 ± 3 | 32 | 32 | 8 [4] |
| | | 7-I-aTc | 19 ± 4 | 32 | 256 | 8 [128] |
| | | 9-Br-aTc | 11 ± 3 | 32 | 128 | ---- |
| | Demeclocycline | aTc | 41 ± 9 | 8 | 16 | 4 [8] |
| | | aDem | 4 ± 1 | 8 | 16 | 4 [1] |

**Supplementary Tables 2 (continued) | Unabridged aTc analog inhibition of whole cell *E. coli* expressing tetracycline destructase enzymes.**

**a**, aTc analog inhibition of whole cell *E. coli* expressing Tet(50)
**b**, aTc analog inhibition of whole cell *E. coli* expressing Tet(X)
**c**, aTc analog inhibition of whole cell *E. coli* expressing Tet(X)_3

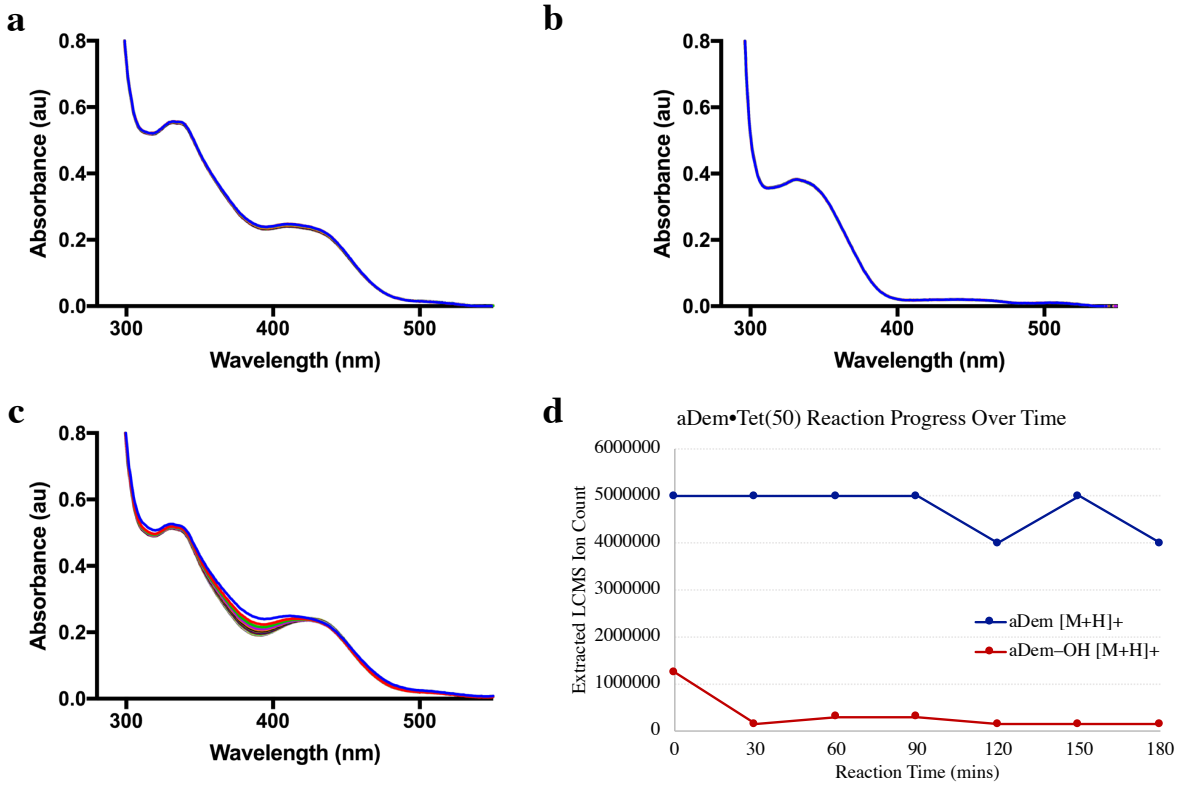
**c**

| *Enzyme* | *Substrate* | *Inhibitor* | *$IC_{50}$ ($\mu$M)* | *Substrate MIC ($\mu$g/mL)* | *Inhibitor MIC ($\mu$g/mL)* | *Substrate + Inhibitor MIC ($\mu$g/mL) [I] ($\mu$g/mL)* |
|---|---|---|---|---|---|---|
| Tet(X)_3 TE_7F_3 | Tetracycline | aTc | 2.7 ± 0.5 | 512 | 64 | 64 [16] |
| | | aCTc | 1 ± 0.1 | 512 | 128 | -- |
| | | aDem | 1.4 ±0.2 | 512 | 16 | 64 [4] |
| | | 7-I-aTc | 18 ± 3 | 512 | 256 | -- |
| | | 9-Br-aTc | 5 ± 0.6 | 512 | 128 | -- |
| | CTc | aTc | 26 ± 3 | 256 | 64 | 64 [16] |
| | | aCTc | 2.6 ± 0.4 | 256 | 128 | 64 [16] |
| | | 7-I-aTc | 67 ± 11 | 256 | 256 | -- |
| | | 9-Br-aTc | 9 ± 2 | 256 | 128 | -- |
| | Demeclocycline | aTc | 7 ±1 | 64 | 64 | 32 [16] |
| | | aDem | 2.4 ± 0.3 | 64 | 16 | 32 [4] |

**Supplementary Figure 3 | *in vitro* Characterization of the reaction of aDem and Tet(50) in the presence of NADPH.**

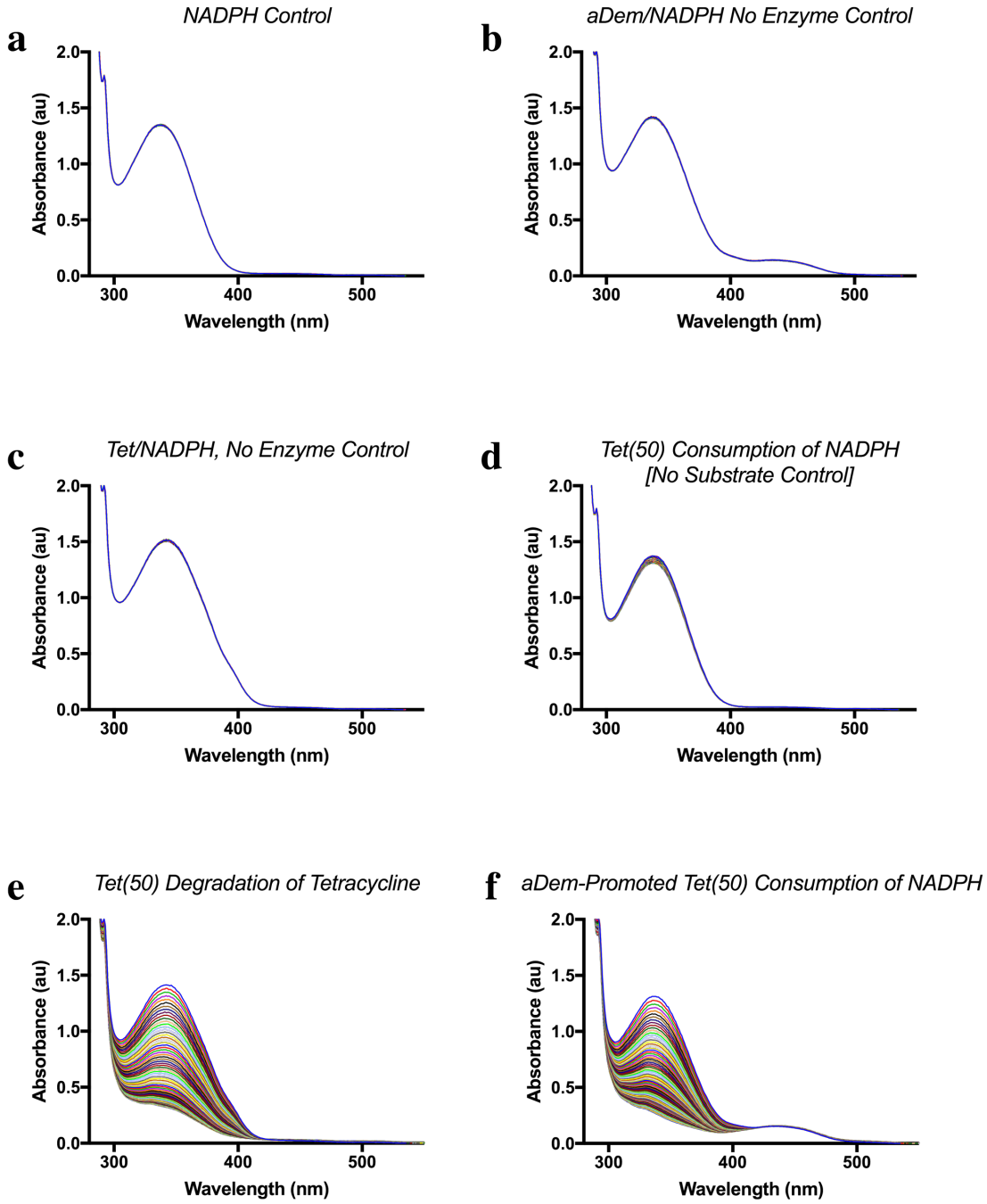
Reactions (in duplicate) were prepared in 100 mM TAPS buffer (pH 8.5) with an NADPH regenerating system (40 mM glucose-6-phosphate, 4 mM NADP, 1 mM MgCl2, 4 U/mL glucose-6-phosphate dehydrogenase), 28.0 μM aDem, and 0.24 μM enzyme. Reaction progress was monitored by UV-Visible spectroscopy (280–550 nm, 1 nm and 30 min intervals) over 3.0 hours, where 150 μL of reaction sample were removed at 30-minute intervals and quenched with 600 μL volumes of quench solution (1:1 acetonitrile:0.25M aqueous HCl). The quenched samples were centrifuged (5000 rpm, room temperature) for 5 minutes, and 600 μL of supernatant was transferred to an LCMS-compatible vial containing Fmoc-alanine internal standard (2.21 μM final concentration) and analyzed by LC-MS (reverse-phase HPLC, C18-silica, gradient 0–95% $CH_3CN/H_2O$, 0.5 mL/min flow rate). Substrate masses $[M+H]^+$ and hydroxylated product masses $[M–OH+H]^+$ were extracted from the crude mass chromatogram and plotted as a function of time.

**a**, NADPH-aDem, No enzyme control
**b**, Tet(50)-NADPH, No aDem control
**c**, Tet(50)-NADPH-aDem reaction
**d**, Tet(50)-NADPH-aDem Reaction, Plot of extracted masses over time



**Supplementary Figure 4 | Broad-scan detection of aDem- and Tet-promoted consumption of NADPH by Tet(50).**

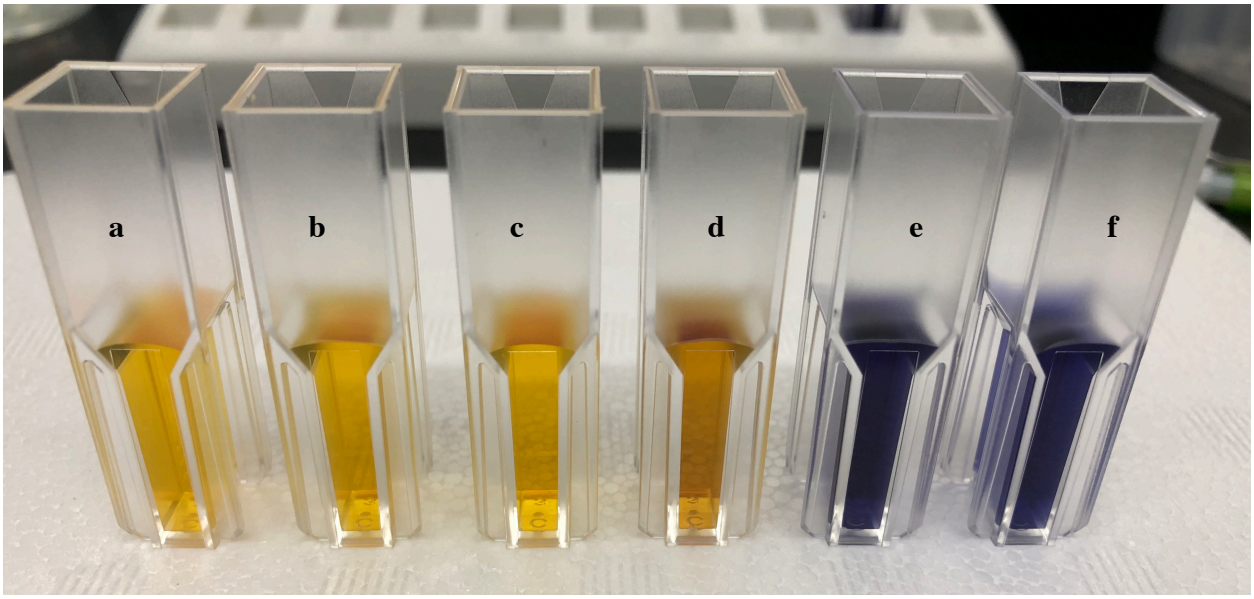
**a**, NADPH control: no enzyme, no substrate
**b**, aDem-NADPH, no enzyme control
**c**, Tet-NADPH, no enzyme control
**d**, Tet(50)-NADPH, no substrate control
**e**, Tet(50)-Tet-NADPH reaction
**f**, Tet(50)-aDem-NADPH reaction



**Supplementary Figure 5 | Qualitative detection of aDem- and Tet-promoted hydrogen peroxide formation by Tet(50).**

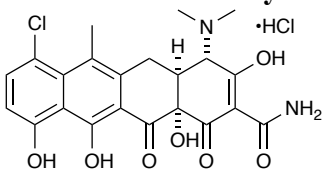
As described in the Experimental Section of the manuscript, aualitative colorimetric detection of aDem- and Tet-promoted hydrogen peroxide formation by Tet(50) was performed using an aqueous Pierce Quantitative Peroxide Assay kit (ThermoScientific). Reaction samples were prepared in 100 mM TAPS buffer (pH 8.5) with 252 μM NADPH, 25 μM substrate (either aDem or Tet), and 0.4 μM enzyme. After the addition of enzyme, the reaction was mixed manually by pipette, and the reaction was monitored by UV-Visible spectroscopy (280–550 nm, 1nm and 0.1 min scan intervals) over 8 minutes. At 8 minutes, 100 μL of reaction solution was added to a detection Eppendorf containing 1000 μL of Working Reagent (prepared according to specifications for Pierce Quantitative Peroxide Assay kit). The detection Eppendorf was incubated for 20 minutes at room temperature to result in the observed color changes, shown in the image below.

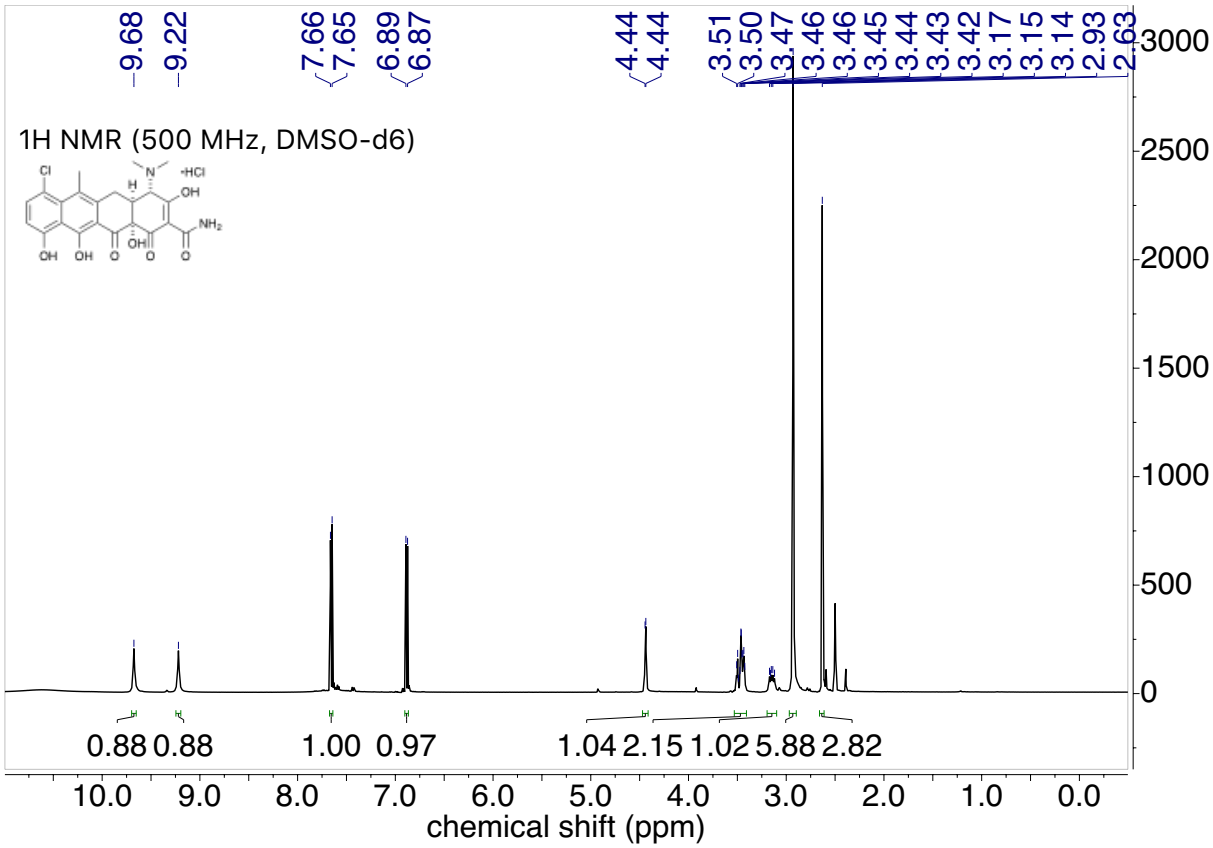
**a**, NADPH control: no enzyme, no substrate
**b**, aDem-NADPH, no enzyme control
**c**, Tet-NADPH, no enzyme control
**d**, Tet(50)-NADPH, no substrate control
**e**, Tet(50)-Tet-NADPH reaction
**f**, Tet(50)-aDem-NADPH reaction

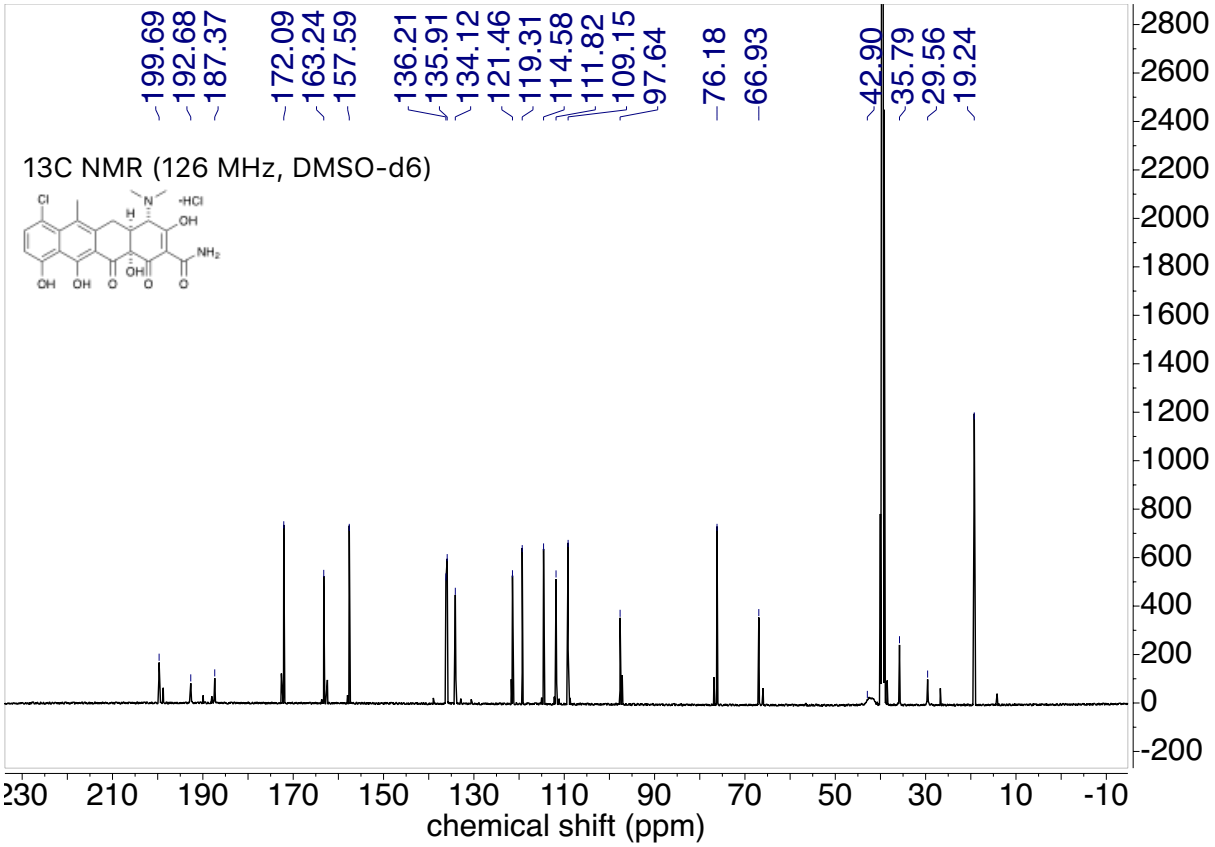


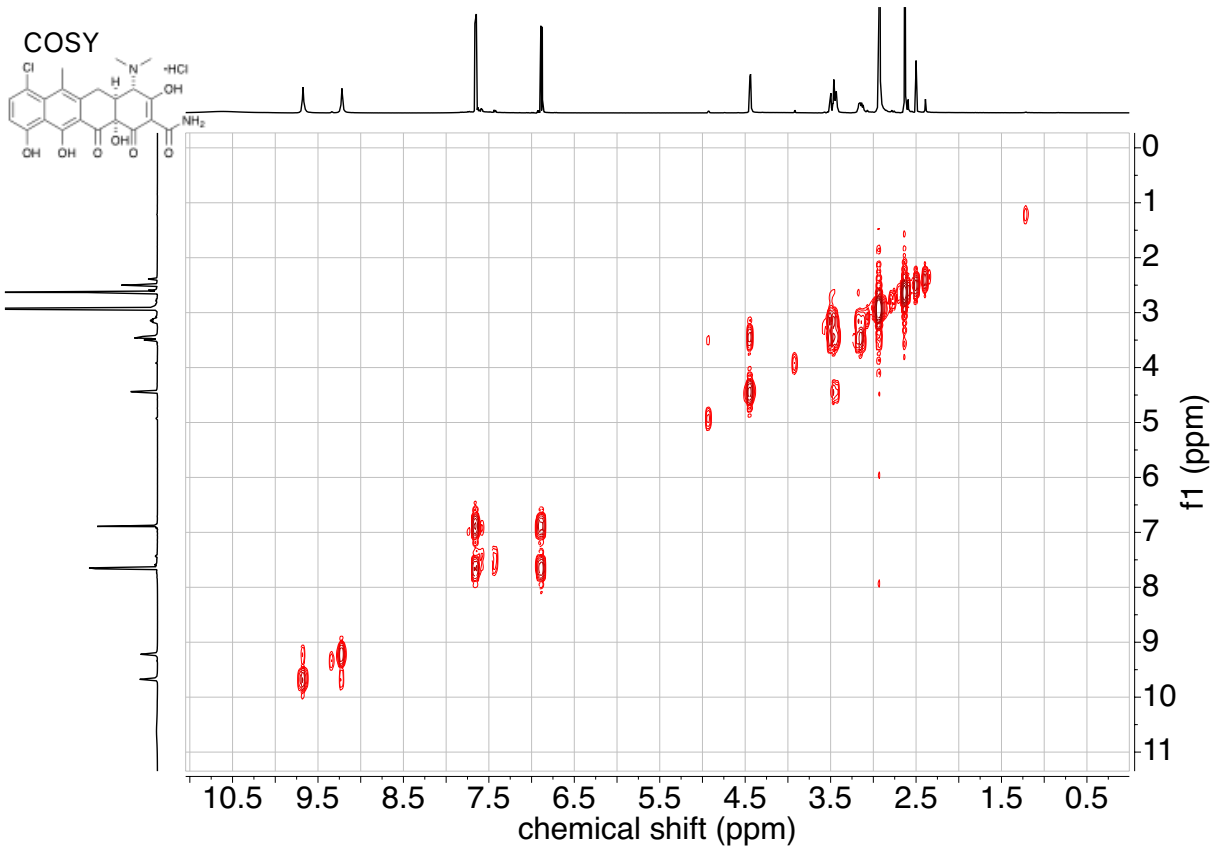
## II. Applicable NMR Spectra of aTc analogues 6–9.

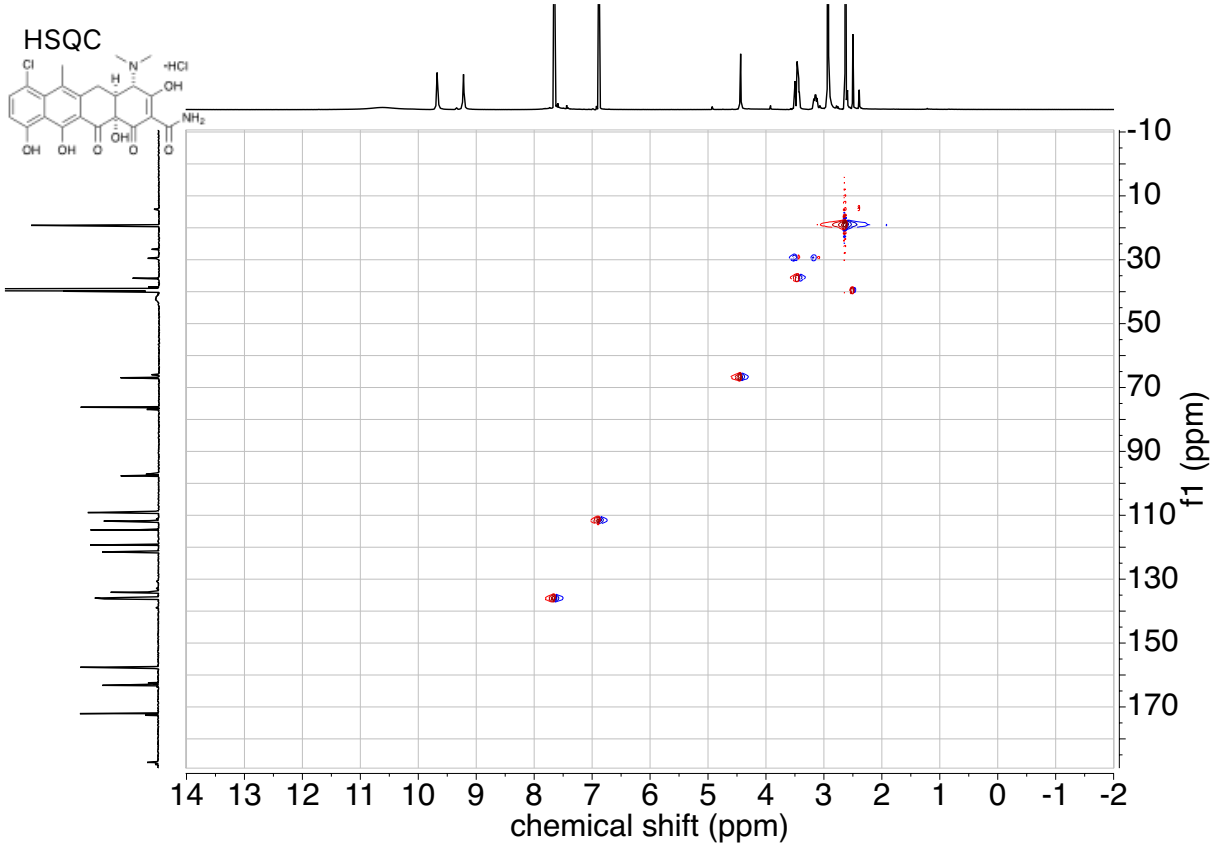
**(4*S*,4a*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride** (aCTc, **6**)

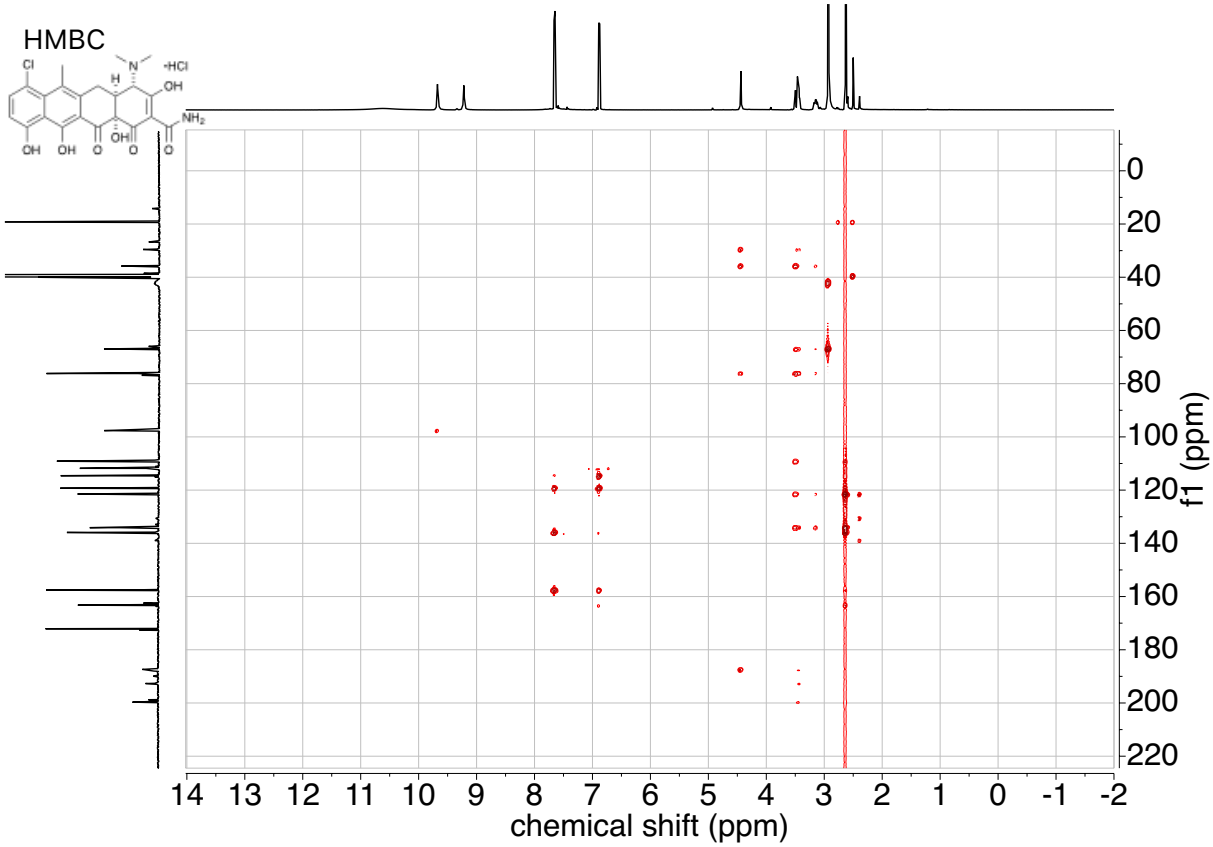


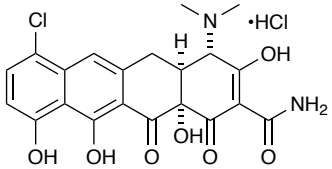

13C NMR (126 MHz, DMSO-d6)
199.69
192.68
187.37
172.09
163.24
157.59
136.21
135.91
134.12
121.46
119.31
114.58
111.82
109.15
97.64
76.18
66.93
42.90
35.79
29.56
19.24
chemical shift (ppm)

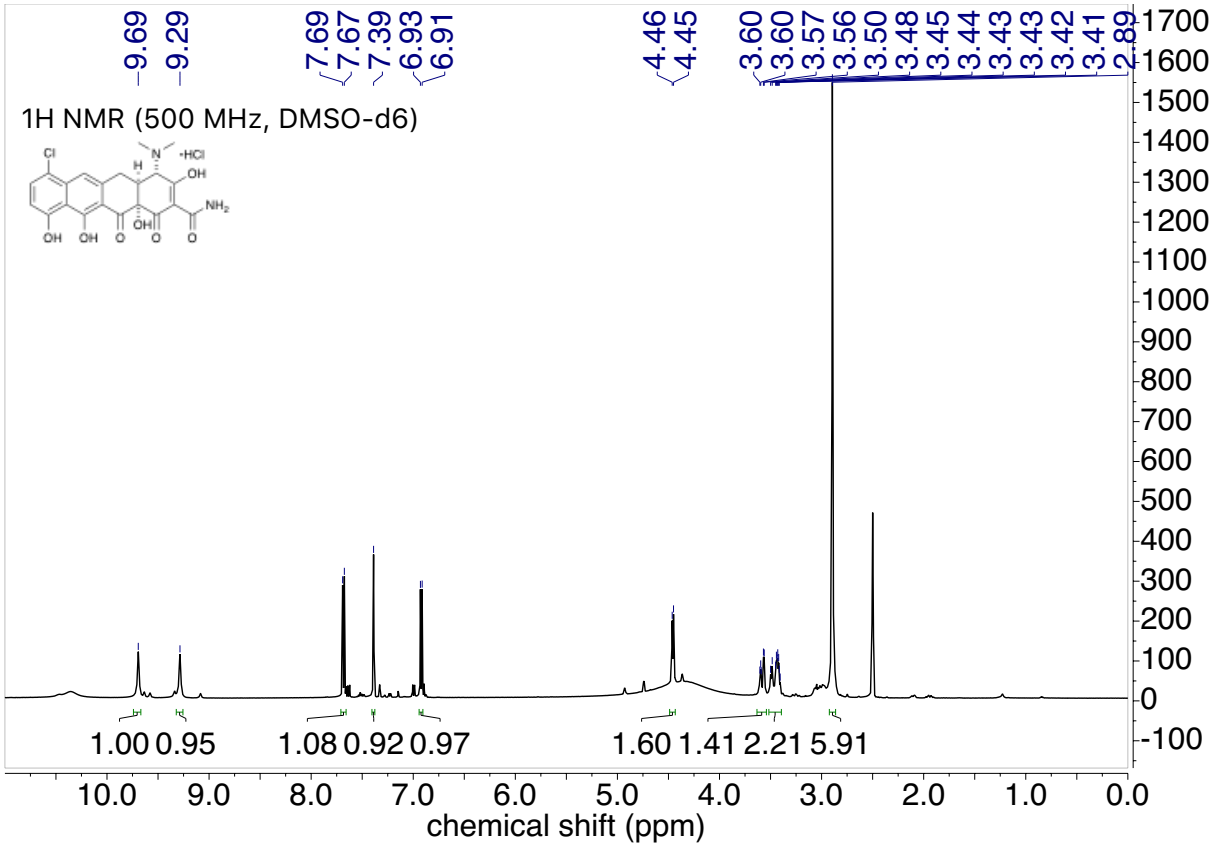

COSY
Cl
N
H
·HCl
OH
NH2
OH
OH
OH
O
O
O
0
1
2
3
4
5
6
7
8
9
10
11
f1 (ppm)
10.5
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
chemical shift (ppm)

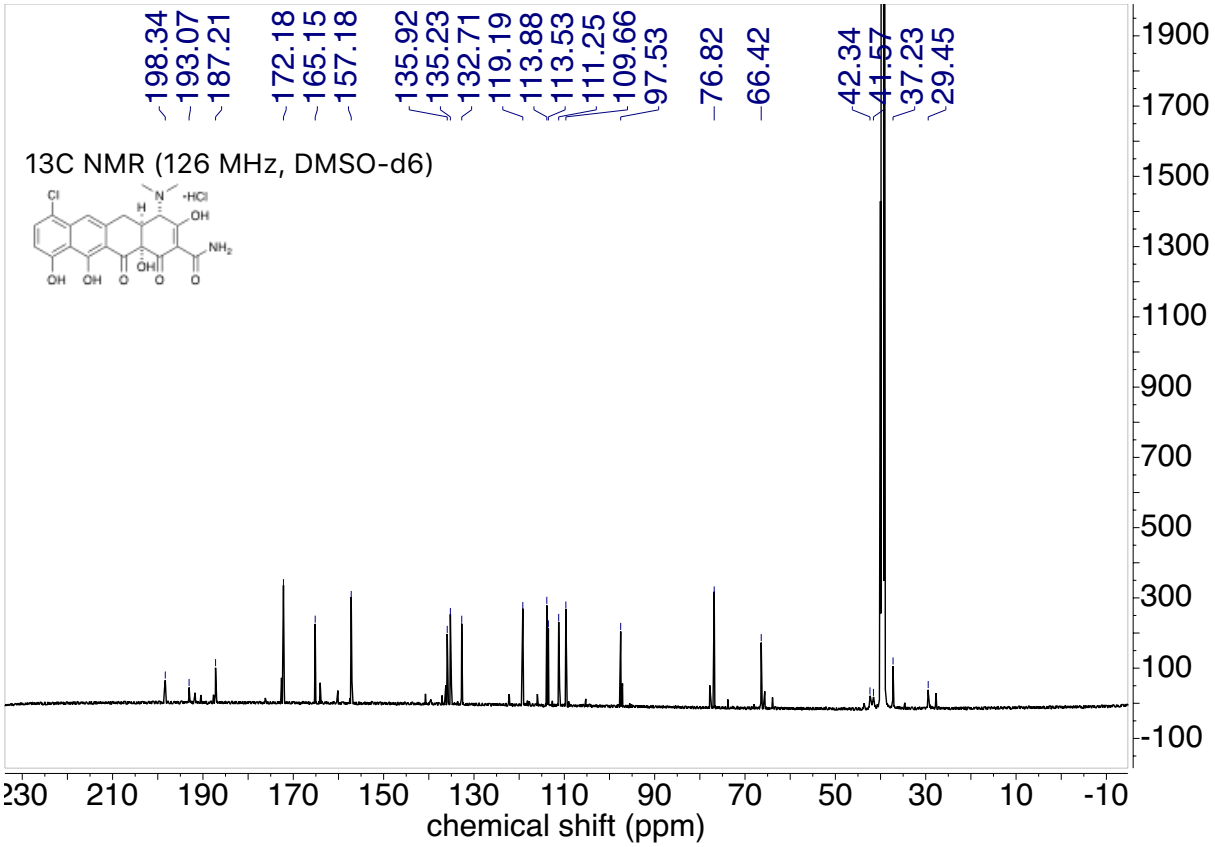

HSQC
chemical shift (ppm)
f1 (ppm)

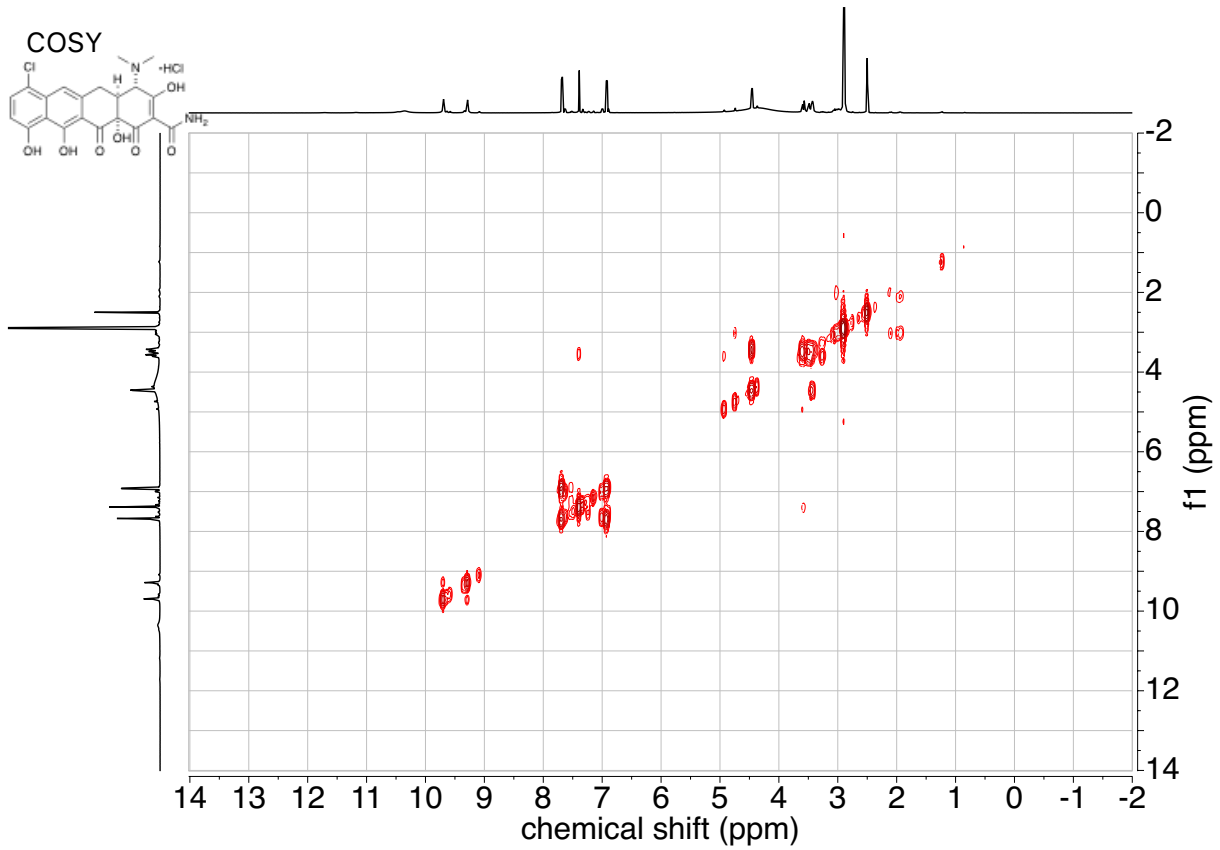

HMBC
chemical shift (ppm)
f1 (ppm)

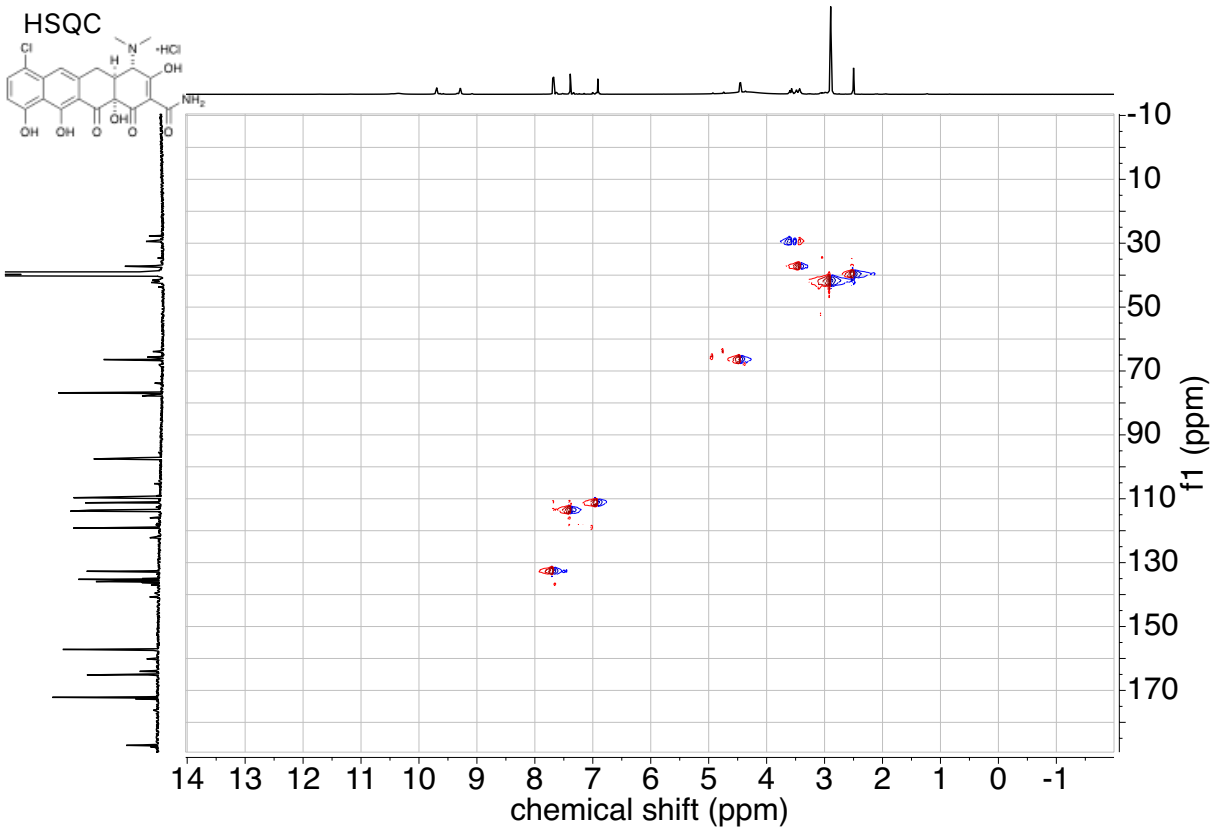
**(4S,4aS,12aS)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride** (aDem, **7**)




198.34
193.07
187.21
172.18
165.15
157.18
135.92
135.23
132.71
119.19
113.88
113.53
111.25
109.66
97.53
76.82
66.42
42.34
41.57
37.23
29.45
13C NMR (126 MHz, DMSO-d6)
chemical shift (ppm)


COSY
chemical shift (ppm)
f1 (ppm)


HSQC
Cl
N
•HCl
H
OH
NH2
OH
OH
O
OH
O
O
-10
10
30
50
70
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f1 (ppm)
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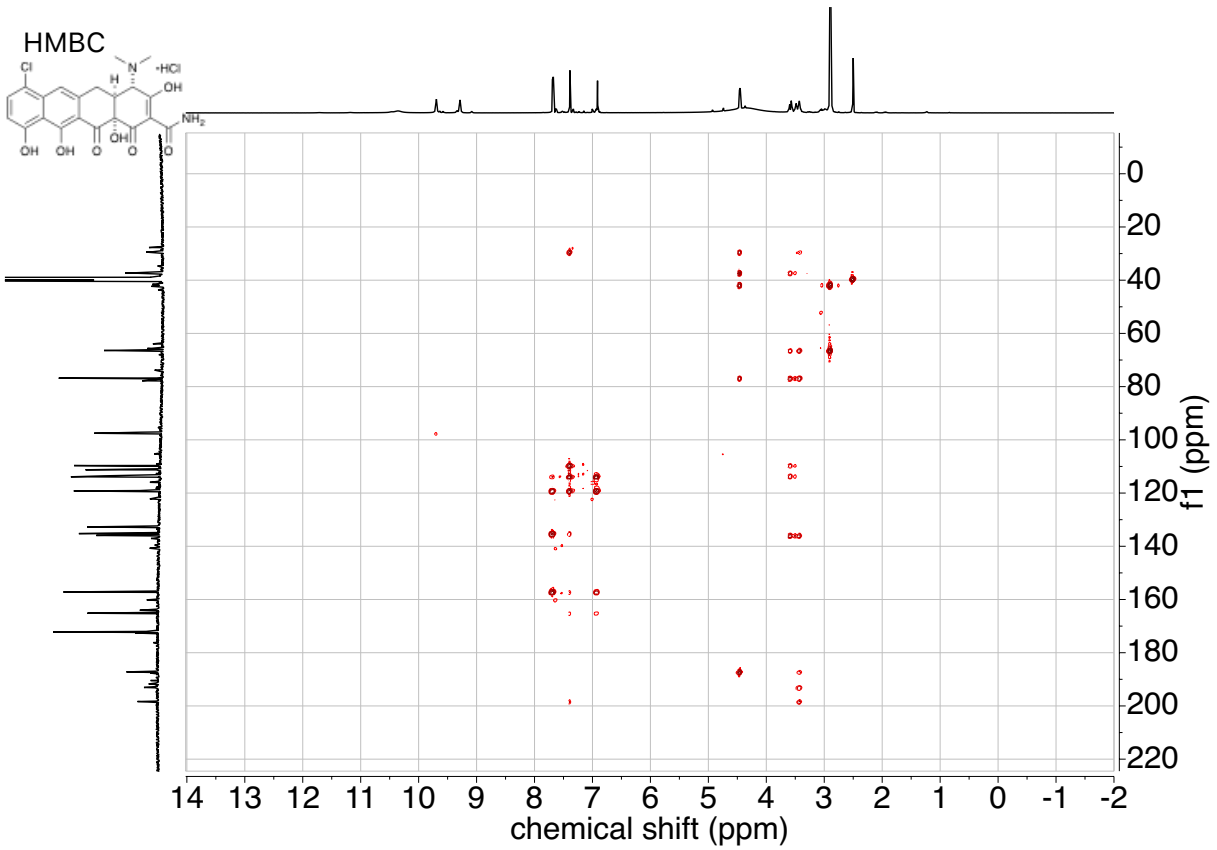
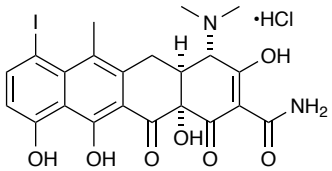
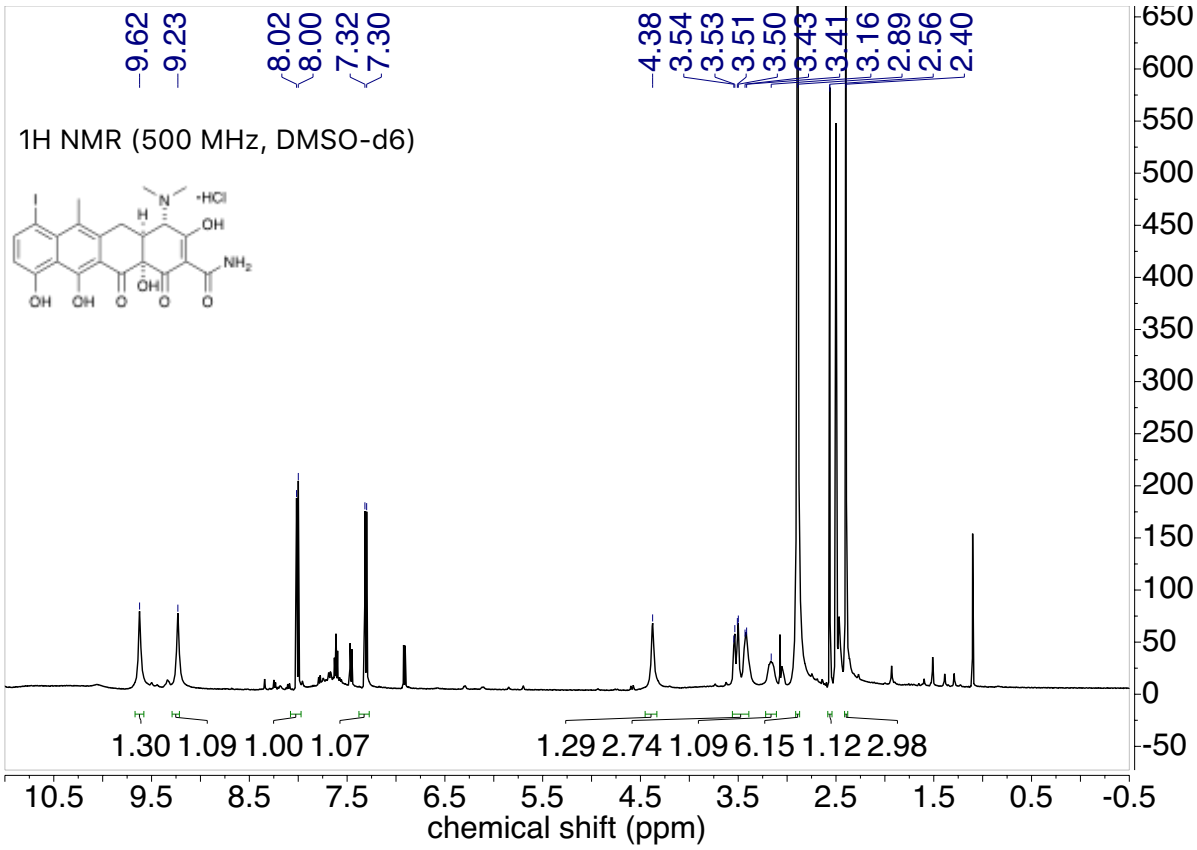
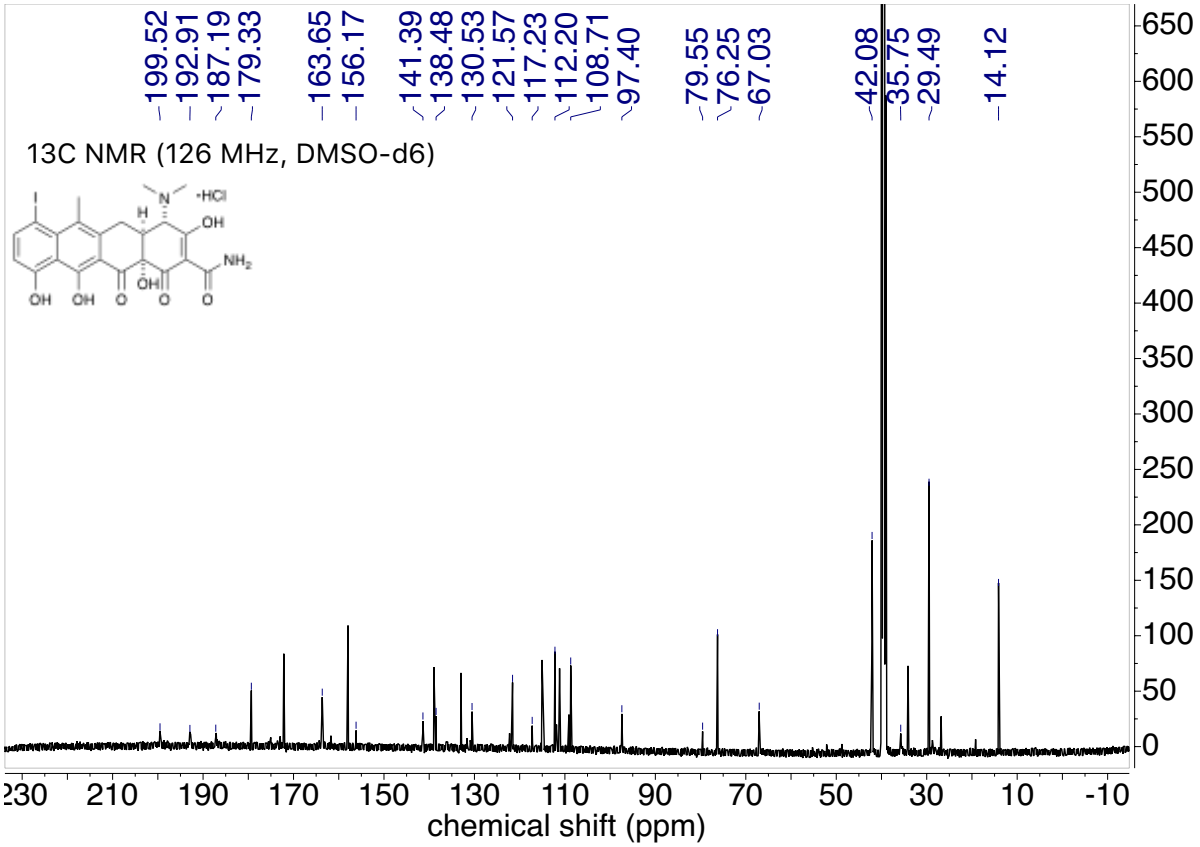
chemical shift (ppm)

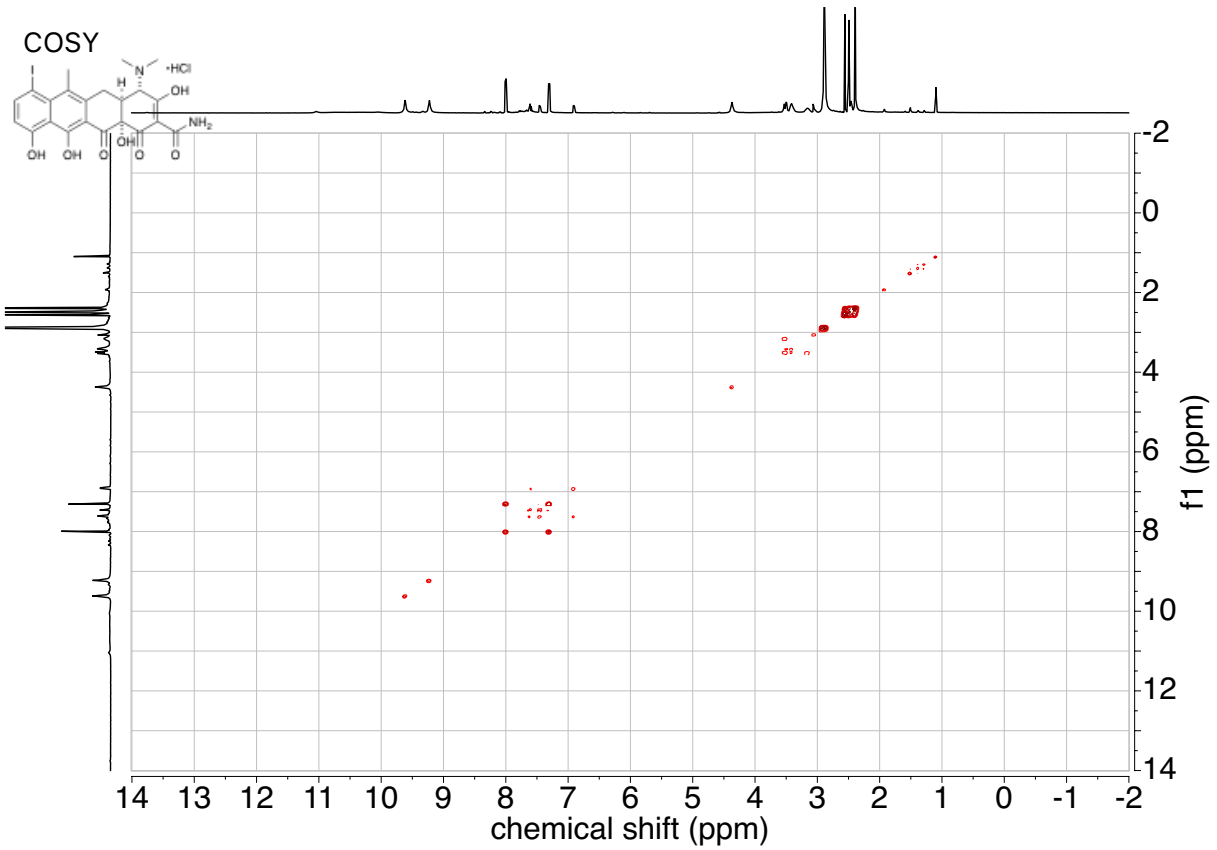

HMBC
chemical shift (ppm)
f1 (ppm)

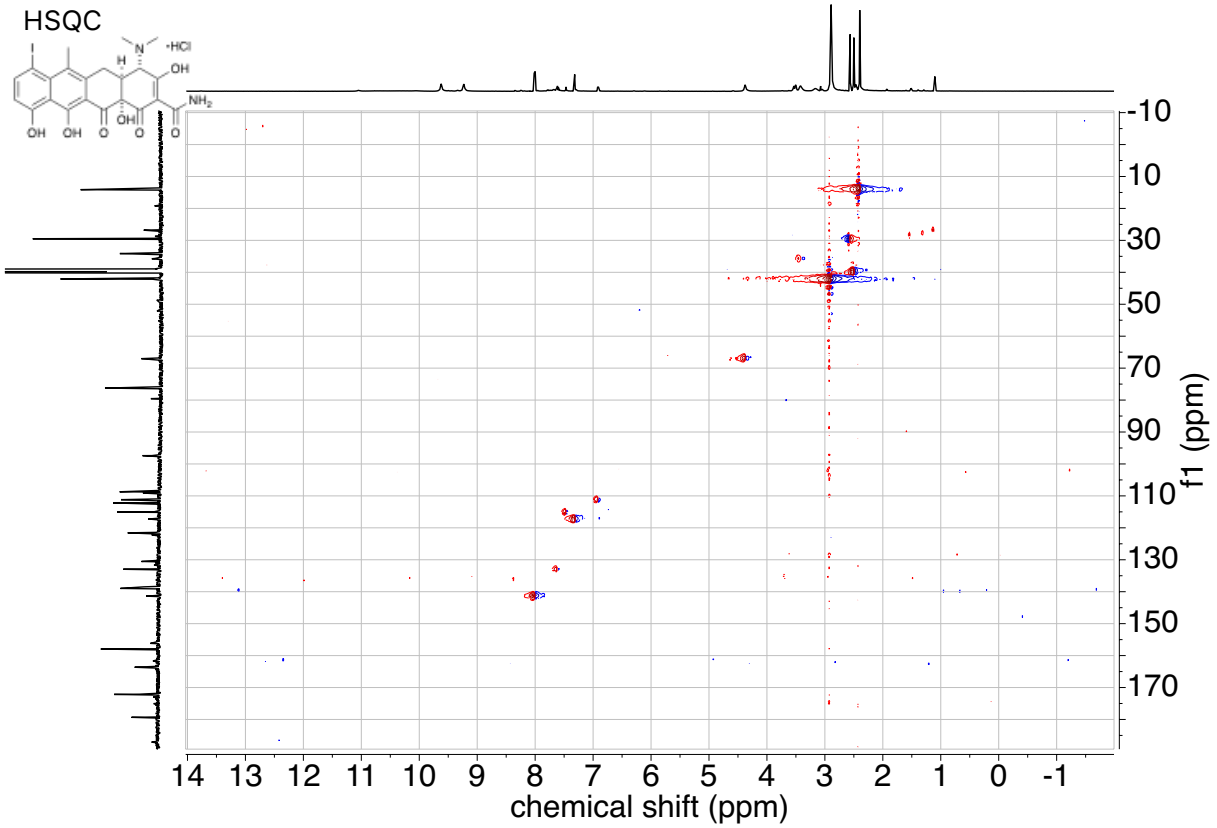
**(4S,4aS,12aS)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-7-iodo-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride** (7-I-aTc, **8**)

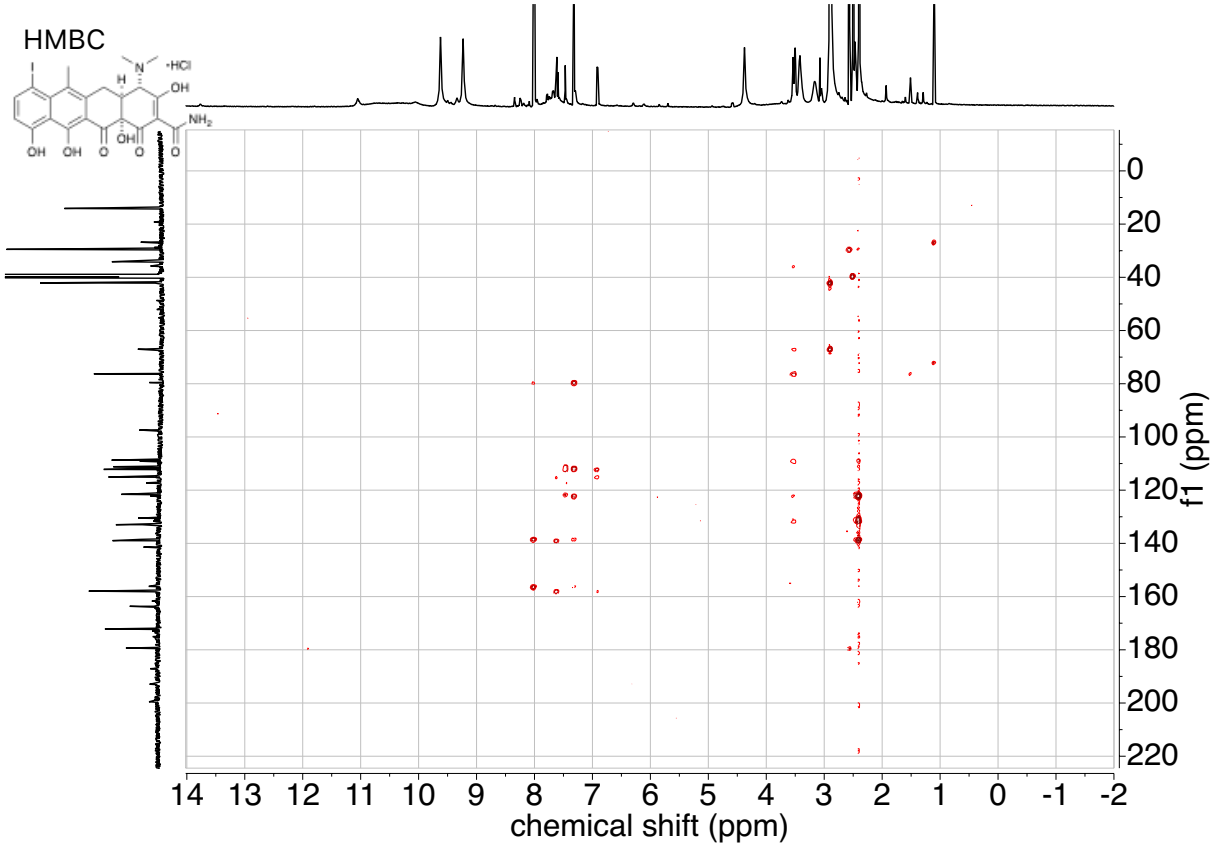


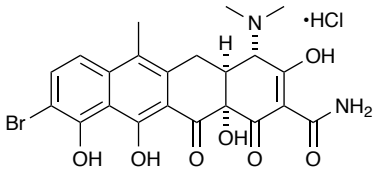

199.52
192.91
187.19
179.33
163.65
156.17
141.39
138.48
130.53
121.57
117.23
112.20
108.71
97.40
79.55
76.25
67.03
42.08
35.75
29.49
14.12
13C NMR (126 MHz, DMSO-d6)
chemical shift (ppm)

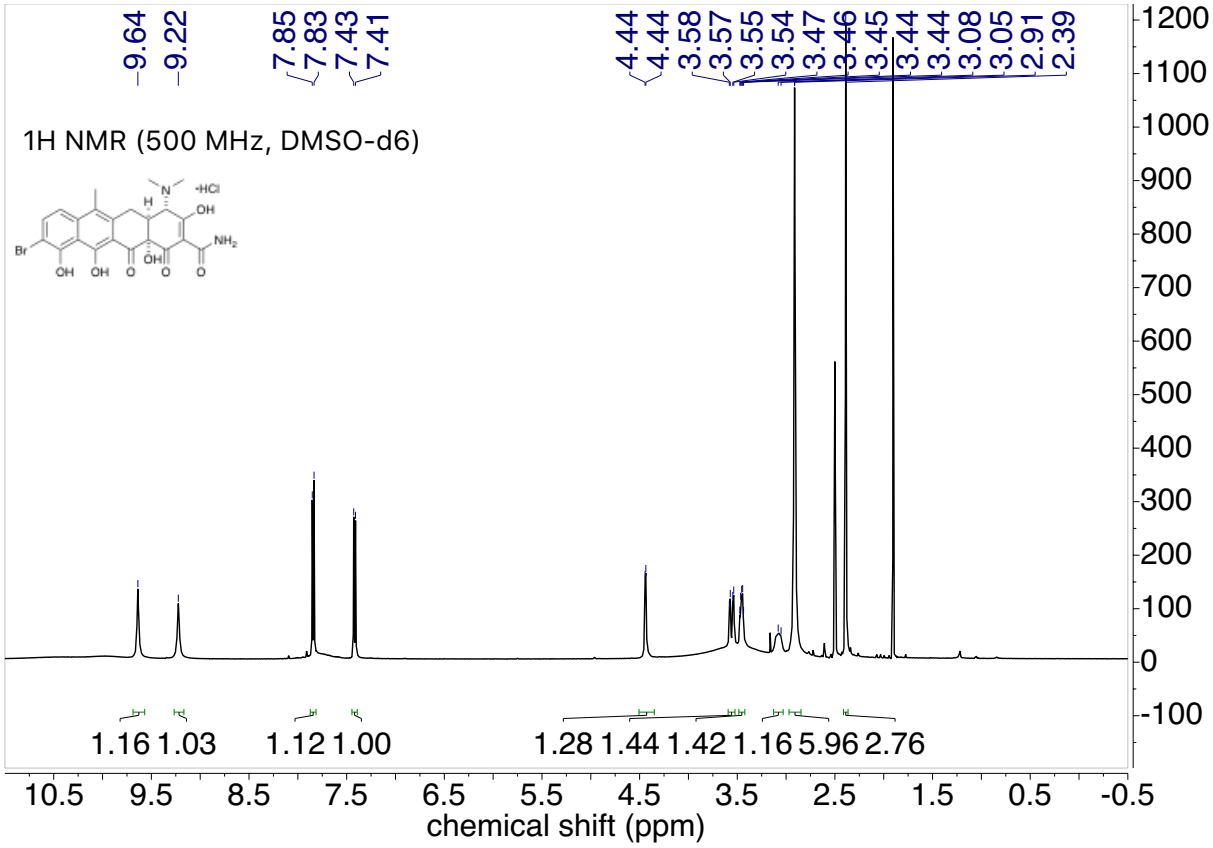

COSY
chemical shift (ppm)
f1 (ppm)

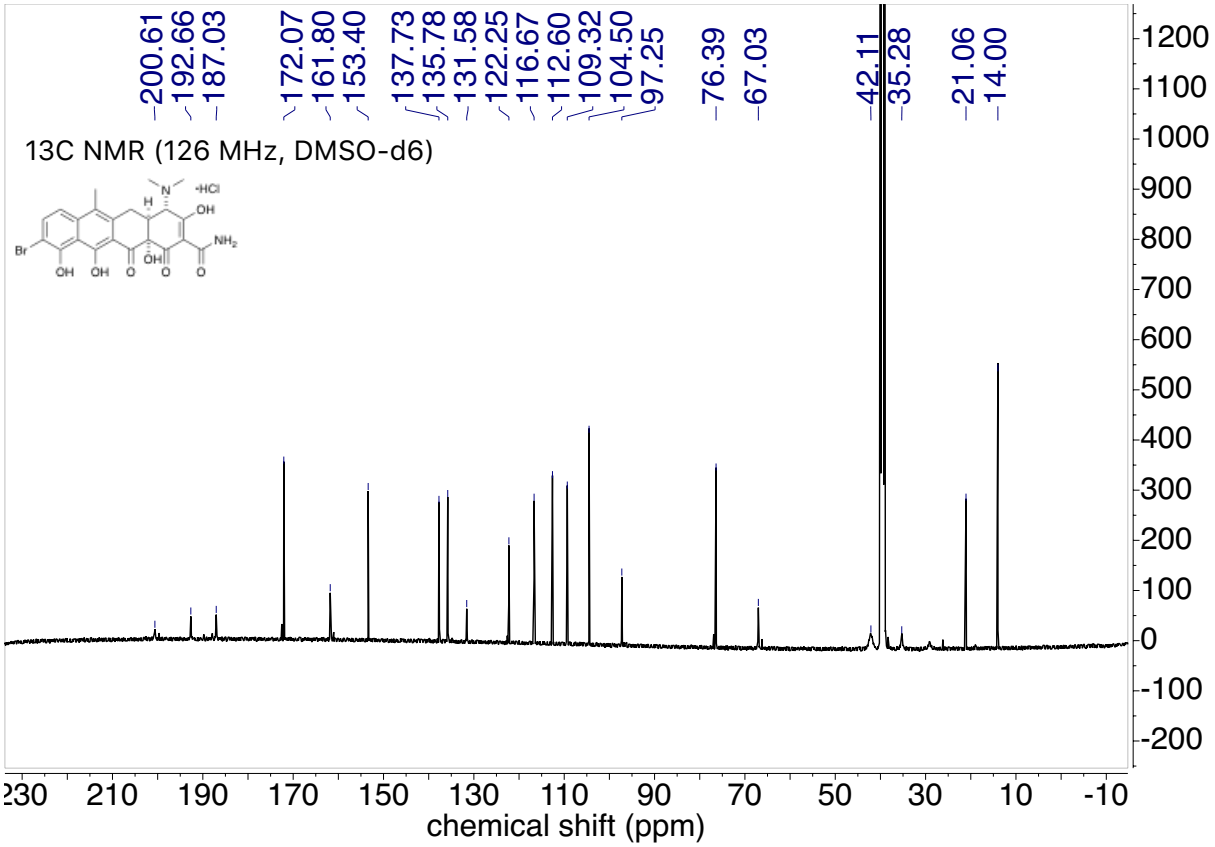

HSQC
chemical shift (ppm)
f1 (ppm)

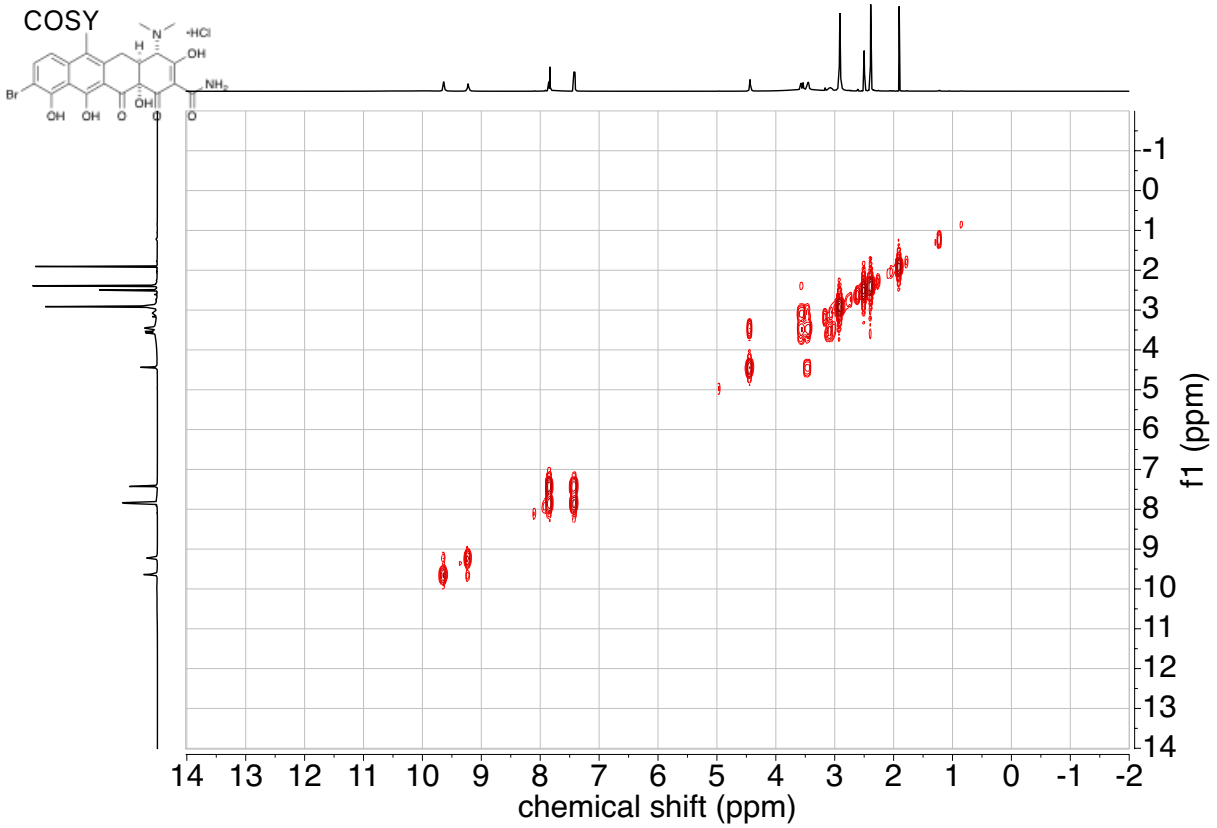

HMBC
chemical shift (ppm)
f1 (ppm)

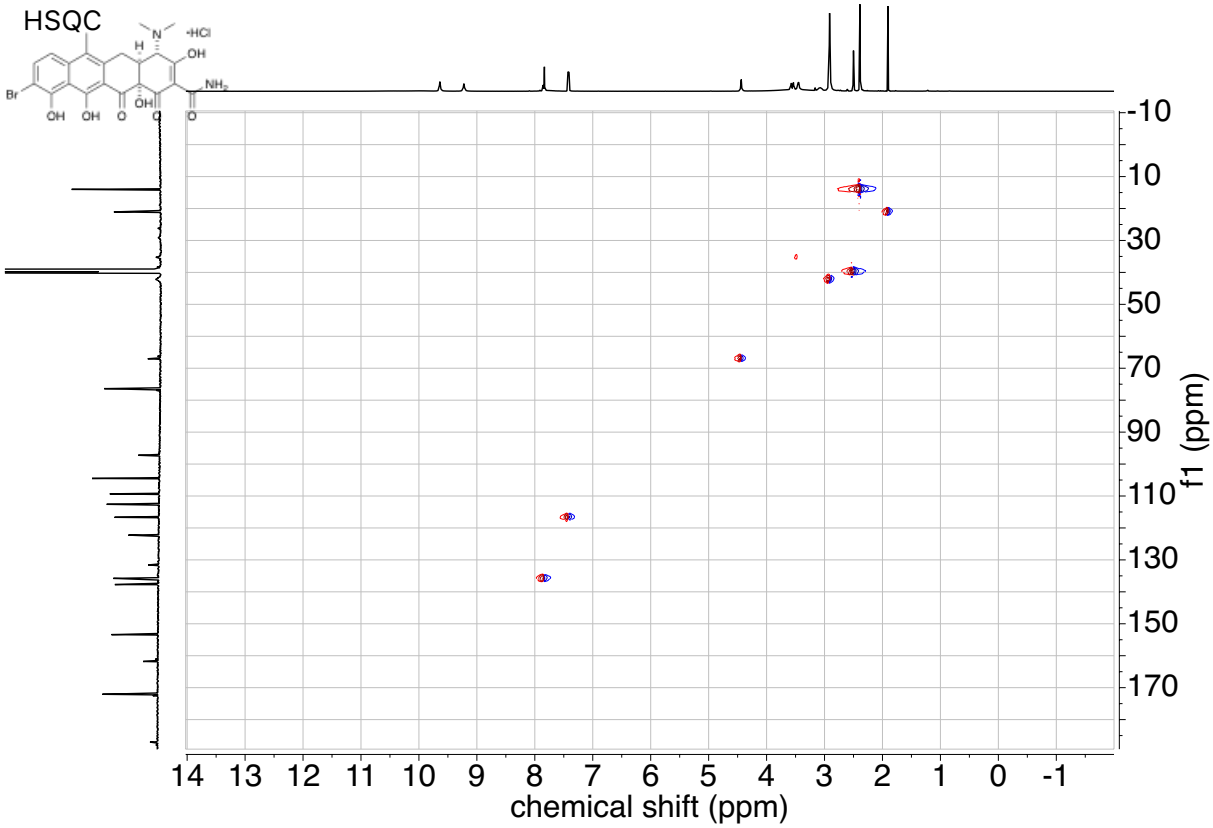
**(4S,4aS,12aS)-9-bromo-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide hydrochloride** (9-Br-aTc, **9**)

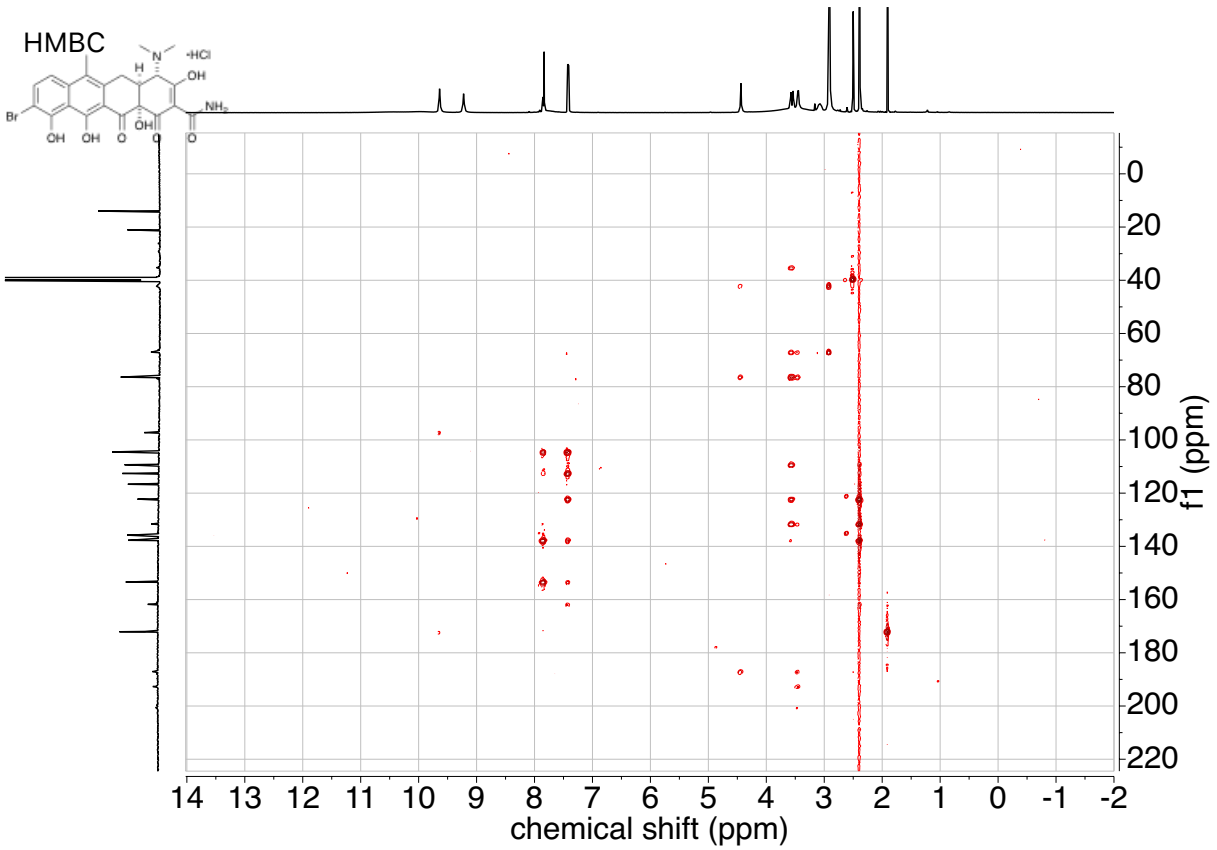


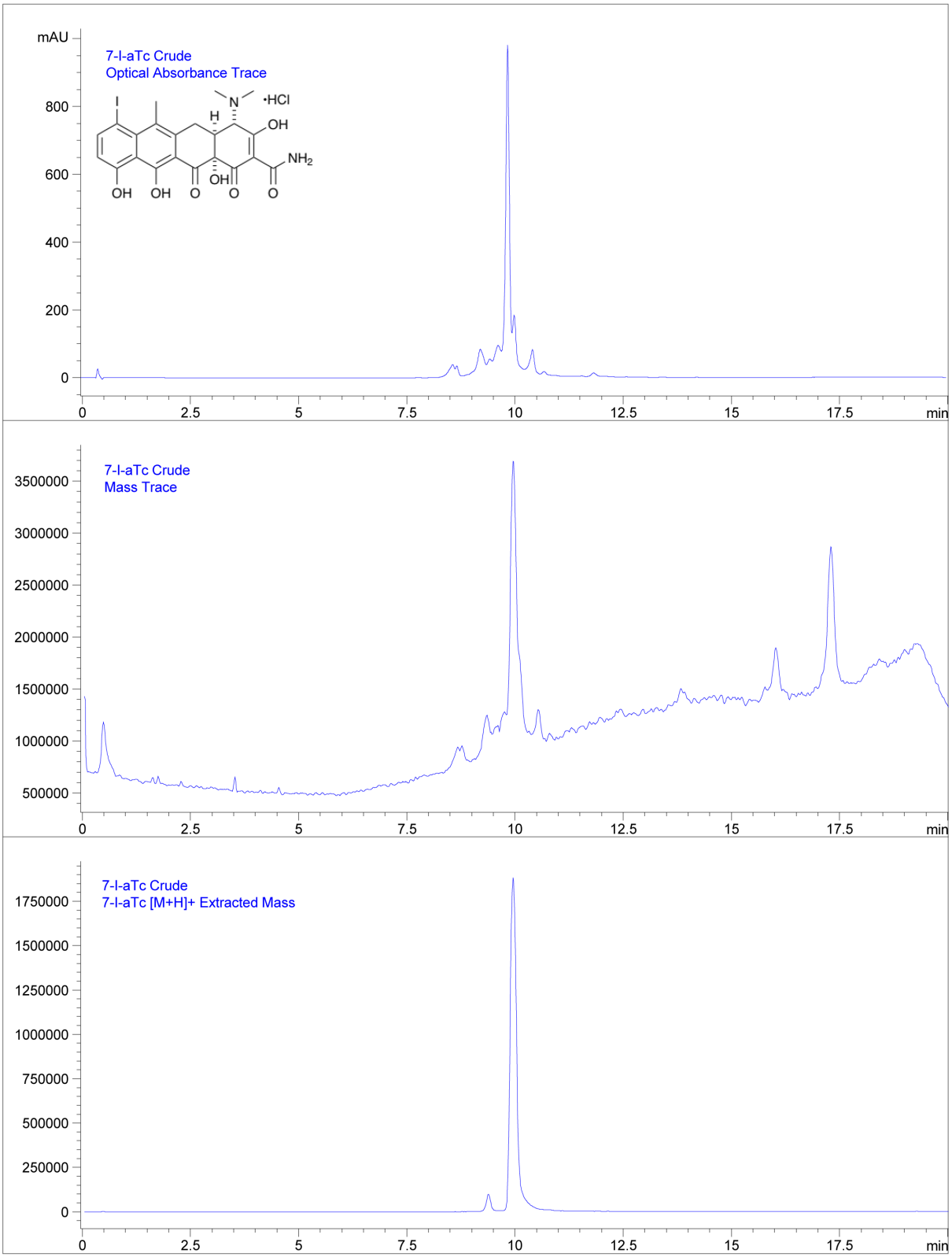

13C NMR (126 MHz, DMSO-d6)
200.61
192.66
187.03
172.07
161.80
153.40
137.73
135.78
131.58
122.25
116.67
112.60
109.32
104.50
97.25
76.39
67.03
42.11
35.28
21.06
14.00
chemical shift (ppm)

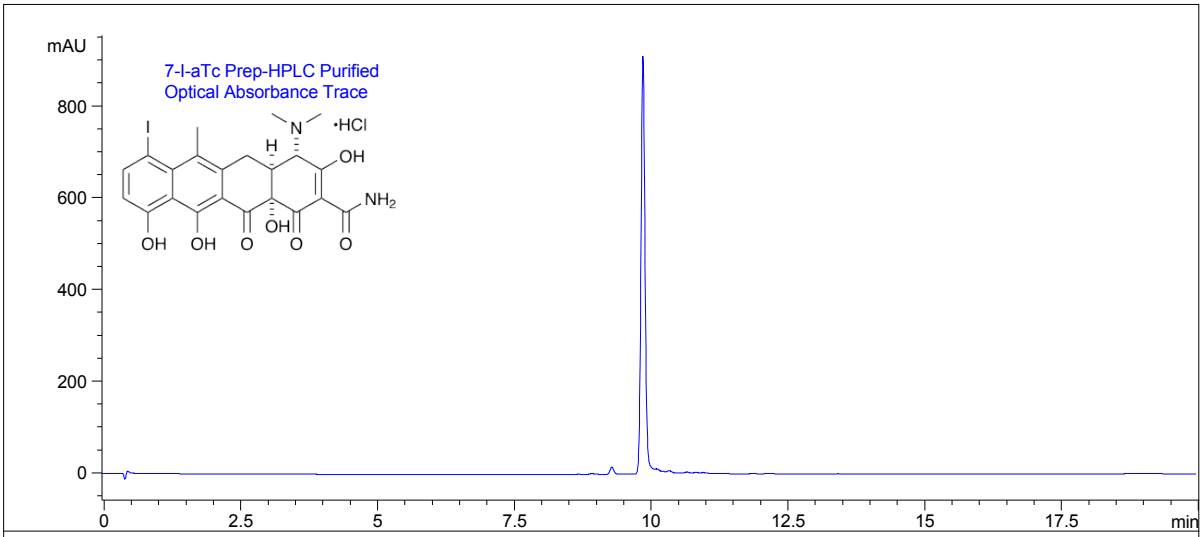

COSY
chemical shift (ppm)
f1 (ppm)

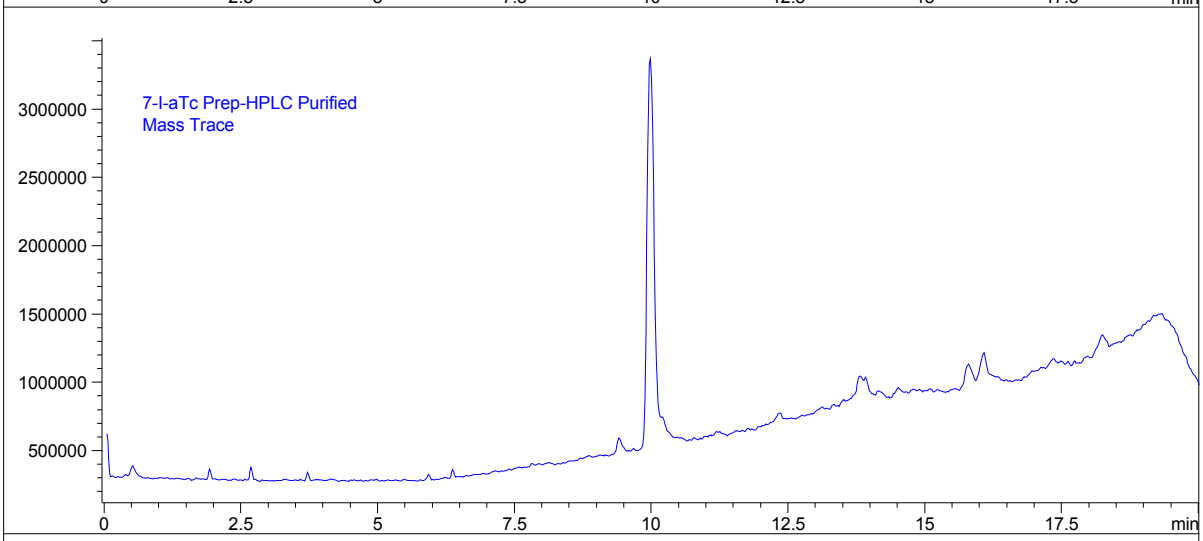

HSQC
chemical shift (ppm)
f1 (ppm)

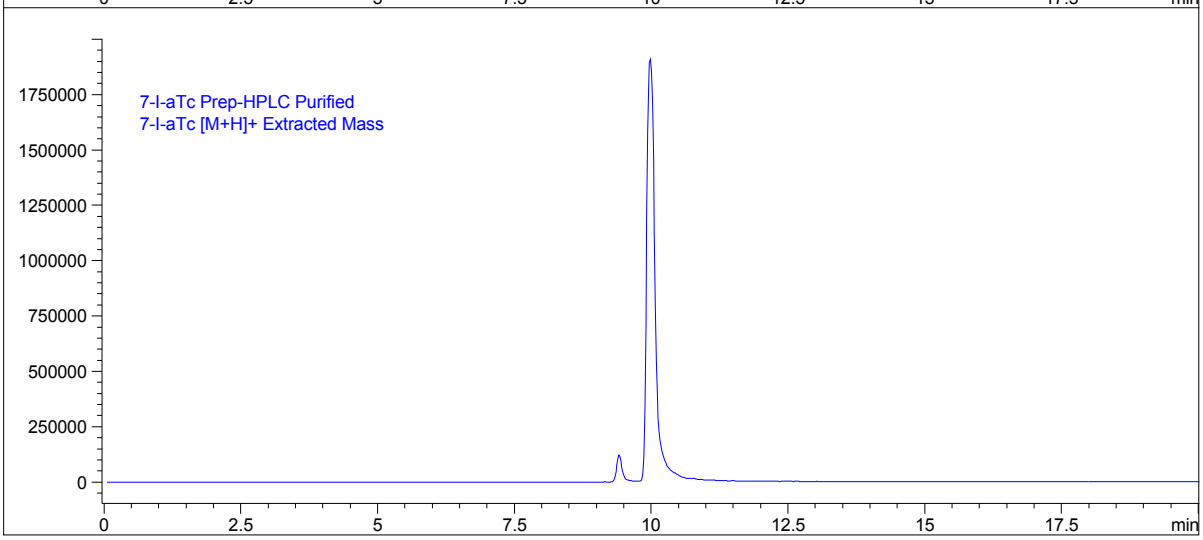

HMBC
chemical shift (ppm)
f1 (ppm)

## III. LCMS Traces for crude and preparative HPLC purified 7-I-aTc, 8.




mAU
7-I-aTc Prep-HPLC Purified
Optical Absorbance Trace
800
600
400
200
0
0
2.5
5
7.5
10
12.5
15
17.5
min


7-I-aTc Prep-HPLC Purified
Mass Trace
3000000
2500000
2000000
1500000
1000000
500000
0
2.5
5
7.5
10
12.5
15
17.5
min


7-I-aTc Prep-HPLC Purified
7-I-aTc [M+H]+ Extracted Mass
1750000
1500000
1250000
1000000
750000
500000
250000
0
2.5
5
7.5
10
12.5
15
17.5
min