Supplementary Information for:

Non-invasive analysis of a protein-templated dynamic combinatorial library by ^{19}F NMR

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The raw data files associated with this paper can be accessed here.

http://dx.doi.org/10.7488/ds/2436

Contents:

Non-invasive DCL rationale

Methods of protein removal from a 3-membered DCL templated by ecFabH

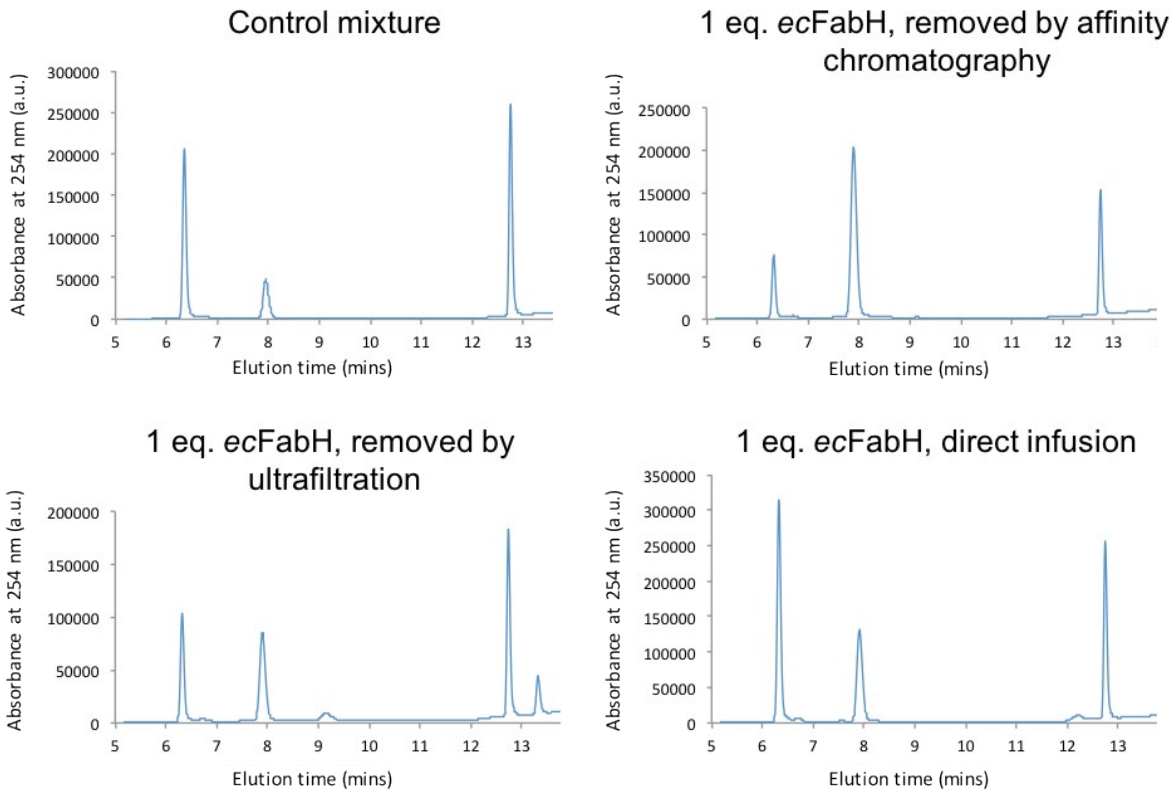


Figure S1 - HPLC chromatograms showing four aliquots of a 3-membered DCL containing NAHs **A2 (**6.23 mins), **A5** (7.59 mins), **A4** (12.52 mins) where ecFabH was removed by different methods prior to HPLC infusion.

The library was assembled with each of the hydrazides 2, 4 and 5, and aldehyde A at a final concentration of 200 µM, buffered at pH 6.2 using 50 mM sodium phosphate with 50% D_2O, 10% DMSO, 50 mM NaCl and 3 mM 4-APA. The library cocktail also contained either 200 µM *ec*FabH or an equivalent volume of enzyme purification buffer. The libraries were equilibrated at RT overnight and the histidine affinity tagged protein was removed by either affinity chromatography (HisTrap 1 mL GE Healthcare Ni^{2+}-affinity chromatography column pre-equilibrated in purification buffer), ultrafiltration (30 kDa MWCO Sartorius micro-spin filter) or injected directly onto the column without protein removal (note: this impacted the

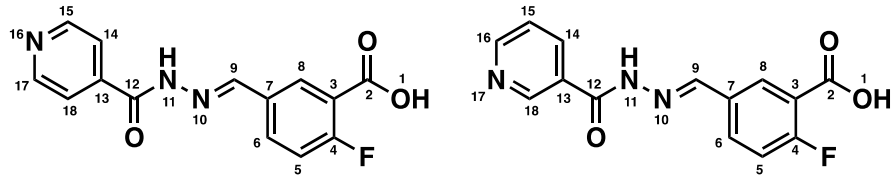
performance of the C18(2) column, requiring several gradient washes to restore optimal column back pressure). The control mixture was not templated by ecFabH and was injected directly.

Column: Luna 5µ C18(2) 50 mm x 4.6 mm, flow rate 1 mL/min, 30 °C

Solvents: A = Water (0.1% formic acid), B = Acetonitrile (0.1% formic acid)

Method: 5% B for 1 minute, 5-95% B over 7 minutes, 95% B for 1 minute, re-equilibrate at 5% B for 5 minutes.

^{19}F NMR sensitivity



^{19}F NMR (470 MHz, DMSO)
δ -110.58

^{19}F NMR (470 MHz, DMSO)
δ -110.75

Figure S2 - showing two very closely related NAHs with individually resolved ^{19}F NMR signals.

Nucleophilic catalysis of NAH exchange

NAH formation from 1-membered library

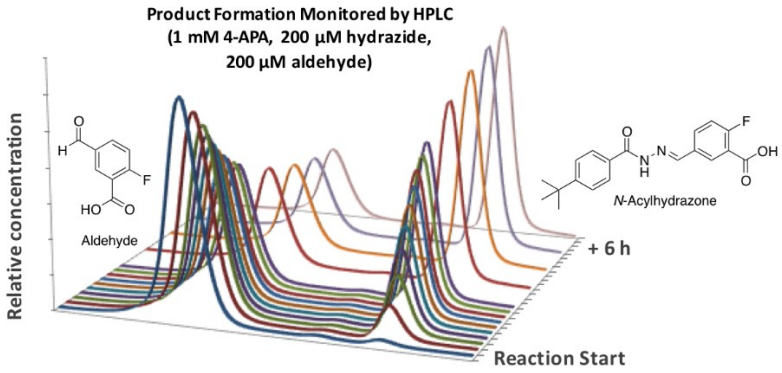


Figure S3 - HPLC chromatograms used to monitor the formation of NAH A4 over time. This example used NAH equilibration catalyst 4-APA at 1 mM. The area under each peak was used to plot a time course of the reaction at different concentrations of nucleophilic catalyst (see figure 3 in the main text).

HPLC chromatograms were collected at hourly intervals over a 9 h period. The following cocktail was prepared to characterise the catalysis of aniline and 4-APA at different concentrations:

2-Fluoro-5-formylbenzoic acid **A** (200 µM), 4-tert-butylbenzohydrazide **4** (200 µM), aniline (0 mM, 0.1 mM, 1 mM, 10 mM) *or* 4-aminophenylalanine (0 mM, 0.1 mM, 1 mM, 3 mM, 10 mM), sodium phosphate buffer (50 mM, pH 6.2), 10% DMSO.

Column: Luna 5µ C18(2) 50 mm x 4.6 mm, flow rate 1 mL/min, 30 °C

Solvents: A = Water (0.1% formic acid), B = Acetonitrile (0.1% formic acid)

Method: 5% B for 1 minute, 5-95% B over 7 minutes, 95% B for 1 minute, re-equilibrate at 5% B for 5 minutes.

NAH formation from 3-membered forward library

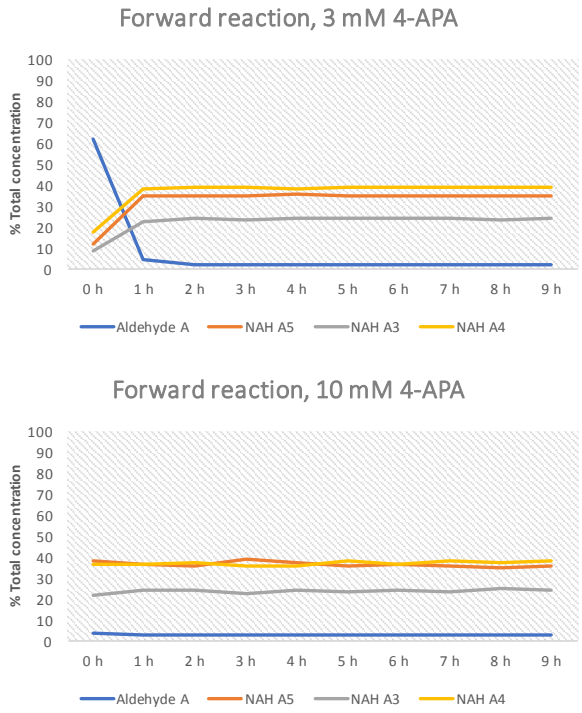


Figure S4 - HPLC chromatograms were collected every 60 minutes up to 9 h. The integral under each peak was calculated using LabSolutions (Shimadzu) and plotted as a percentage of total concentration for each time point. The following cocktail was prepared to characterise the catalysis of the forward reaction 4-APA at 3 mM and 10 mM: 2-Fluoro-5-formylbenzoic acid **A** (200 µM), 3-methoxybenzhydrazide **3** (200 µM), 4-tert-butylbenzhydrazide **4** (200 µM), 4-hydroxy-3-methoxybenzhydrazide **5** (200 µM), 4-aminophenylalanine (3 mM *or* 10 mM), sodium phosphate buffer (50 mM, pH 6.2), 10% DMSO.

Column: Luna 5µ C18(2) 50 mm x 4.6 mm, flow rate 1 mL/min, 30 °C

Solvents: A = Water (0.1% formic acid), B = Acetonitrile (0.1% formic acid)

Method: 5% B for 1 minute, 5-95% B over 7 minutes, 95% B for 1 minute, re-equilibrate at 5% B for 5 minutes.

NAH formation from 3-membered reverse library

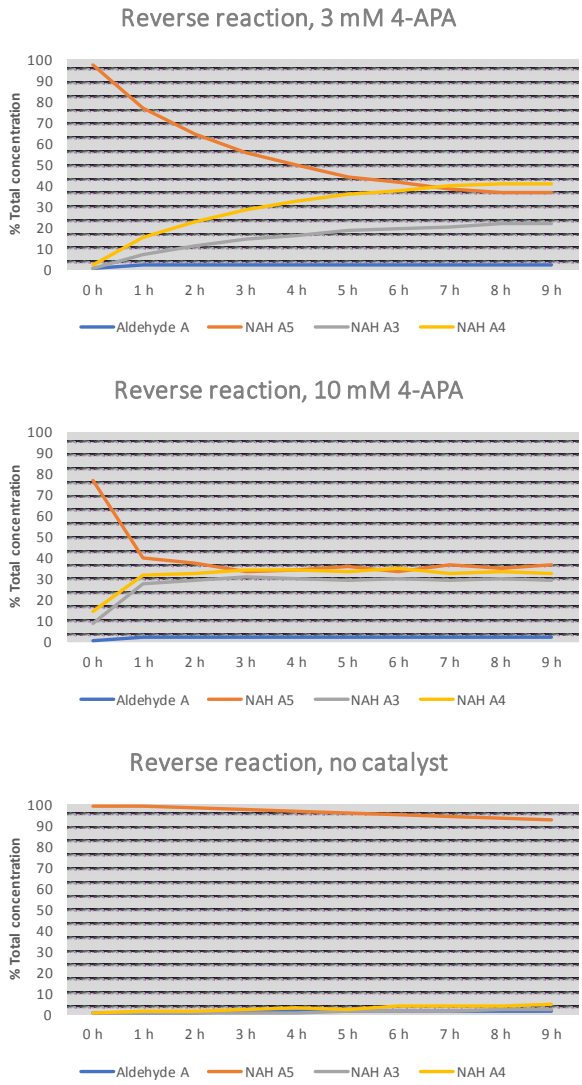


Figure S5 - HPLC chromatograms were collected every 60 minutes up to 9 h. The integral under each peak was calculated using LabSolutions (Shimadzu) and plotted as a percentage of total concentration for each time point. The following cocktail was prepared to characterise the catalysis of the forward reaction 4-APA at 3 mM and 10 mM:
NAH **A5** (200 µM), 4-tert-butylbenzhydrazide **4** (200 µM), 3-methoxybenzhydrazide **3** (200 µM), 4-aminophenylalanine (3 mM *or* 10 mM), sodium phosphate buffer (50 mM, pH 6.2), 10% DMSO.

Column: Luna 5µ C18(2) 50 mm x 4.6 mm, flow rate 1 mL/min, 30 °C

Solvents: A = Water (0.1% formic acid), B = Acetonitrile (0.1% formic acid)

Method: 5% B for 1 minute, 5-95% B over 7 minutes, 95% B for 1 minute, re-equilibrate at 5% B for 5 minutes.

Inhibitory activity of NAH DCL members

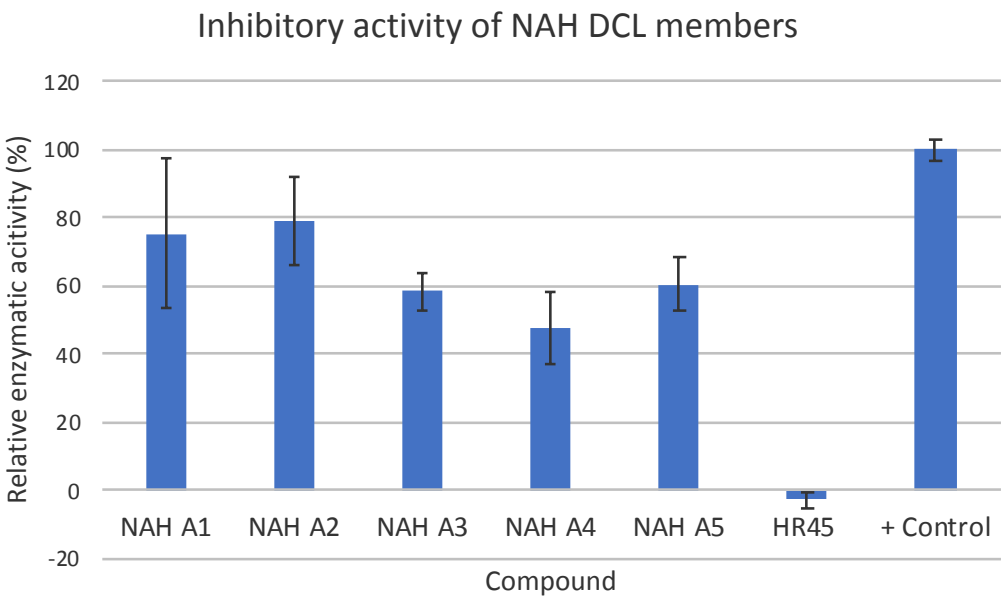
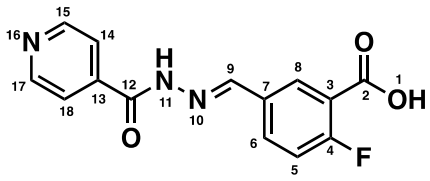


Figure S6 - The activity of FabH was quantified by 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) assay with the following procedure. An assay cocktail was prepared from DTNB (1 mL, 4 mM in 50 mM NH_4OAc), HEPES buffer (1 mL, 1 M, pH 8.0) and ddH_2O (8 mL). The assay was run in 96-well plate format with the following protocol (the following bracketed figures refer to final concentrations): 100 µL DTNB assay cocktail, 25 µL *ec*FabH (0.63 µM), 25 µL Acetyl-CoA (375 µM), 25 µL Malonyl-CoA (375 µM), 5 µL ddH_2O, 20 µL NAH A1-A5 (3 mM) *or* HR45 (200 µM) in DMSO *or* DMSO control. Following 20 mins incubation at 37 °C, the reaction was initiated by the addition of malonyl-CoA and followed by monitoring absorbance change at 412 nm over 20 mins at 37 °C. The DMSO concentration was maintained at 10% to aid the solubility of the NAH compounds.

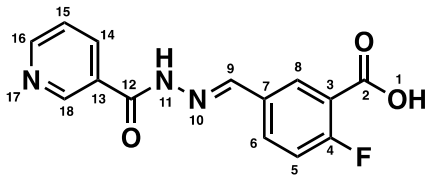
Synthetic Chemistry

Numbering of *N*-acylhydrazones

A1 - *2-fluoro-5-[(1E/Z)-[(pyridin-4-yl-formamido)imino]methyl]benzoic acid*



A2 - *2-fluoro-5-[(1E/Z)-[(pyridin-3-ylformamido)imino]methyl]benzoic acid*



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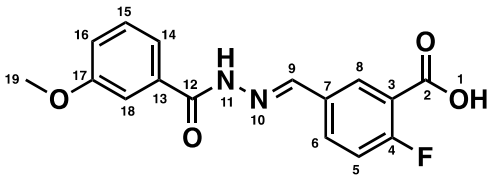
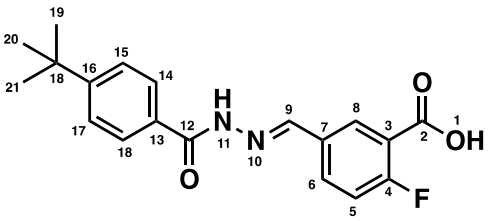
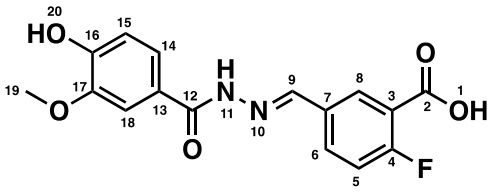


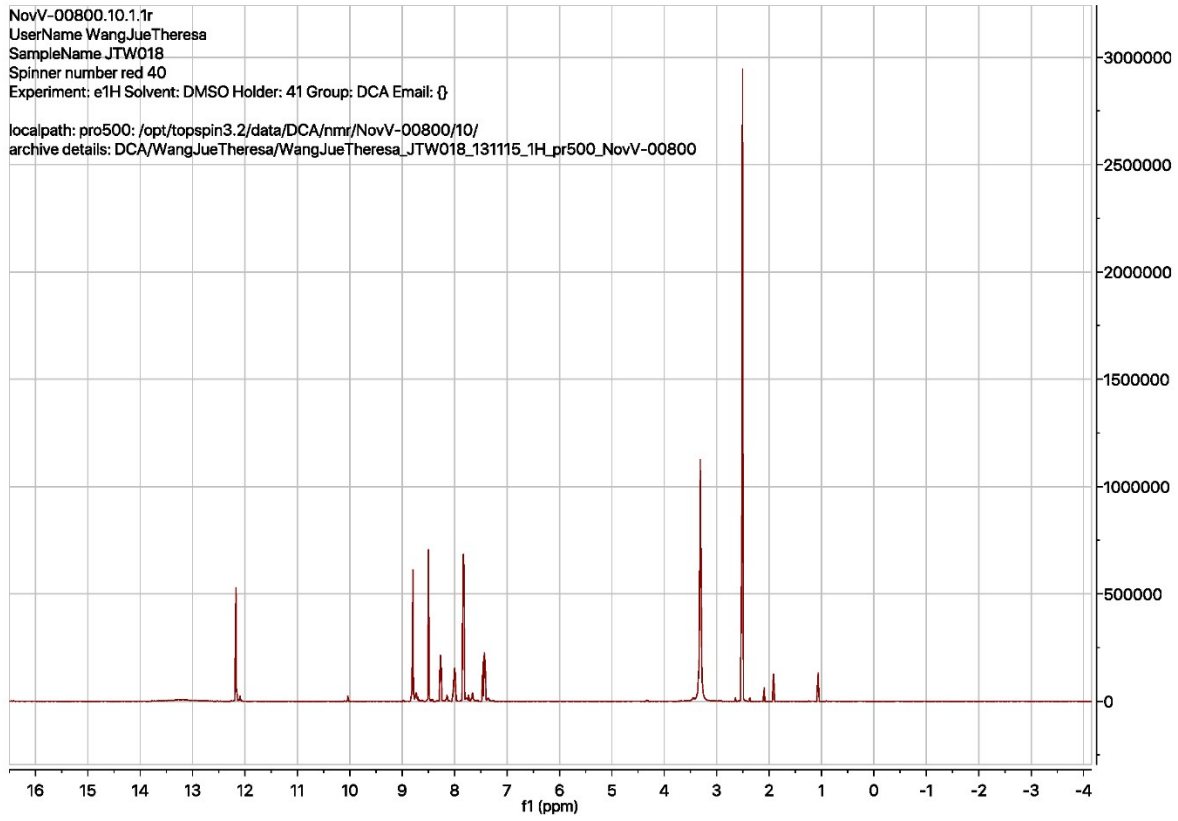
A4 - 5-[(1E/Z)-{[(4-*tert*-butylphenyl)formamido]imino}methyl]-2-fluorobenzoic acid



A5- *2-fluoro-5-[(1E/Z)-{[(4-hydroxy-3-methoxyphenyl)formamido]imino}methyl] benzoic acid*



NAH A1 ^{1}H NMR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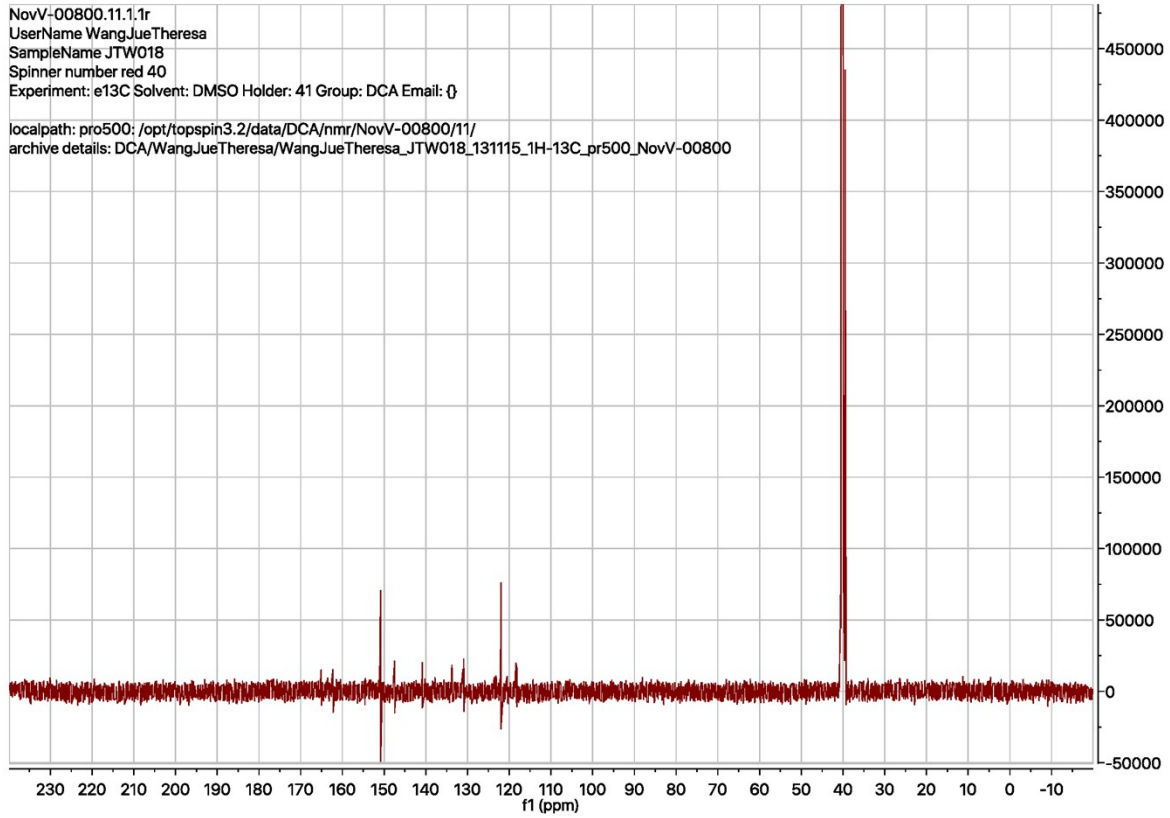
f1 (ppm)

NAH A1 ^{13}C NMR



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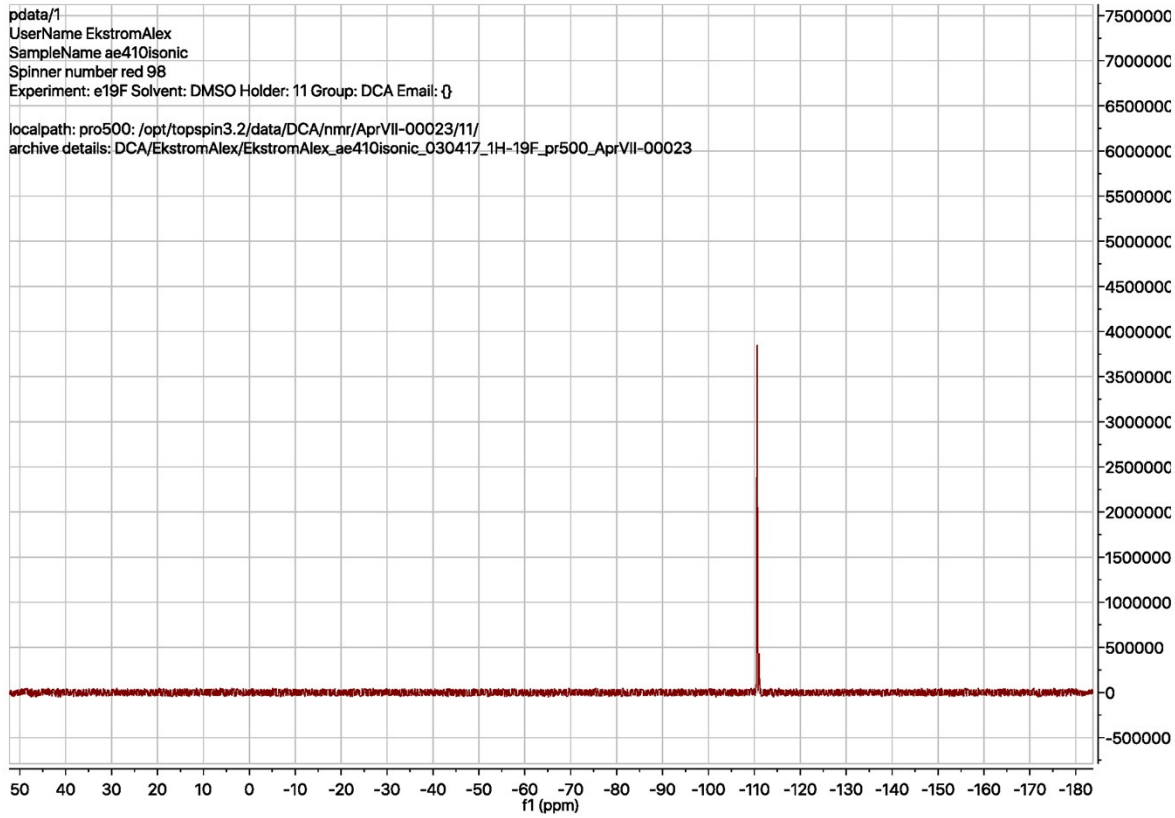

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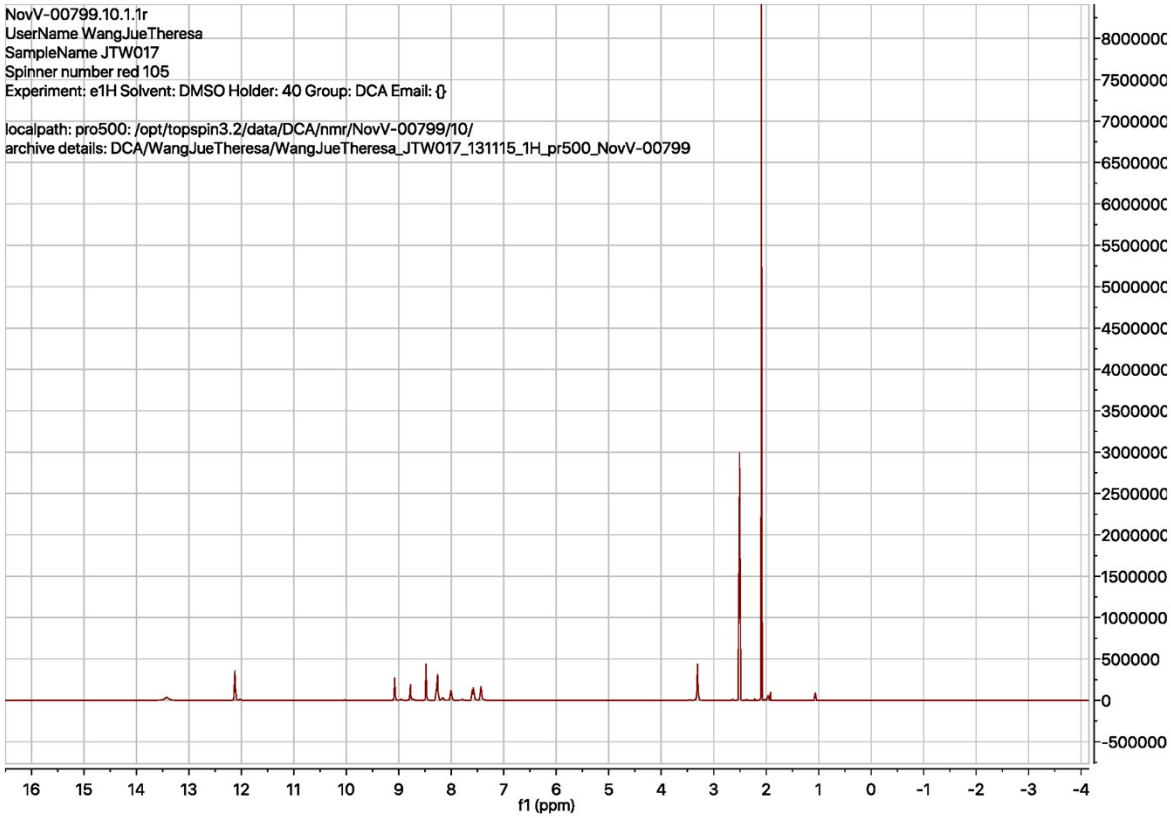
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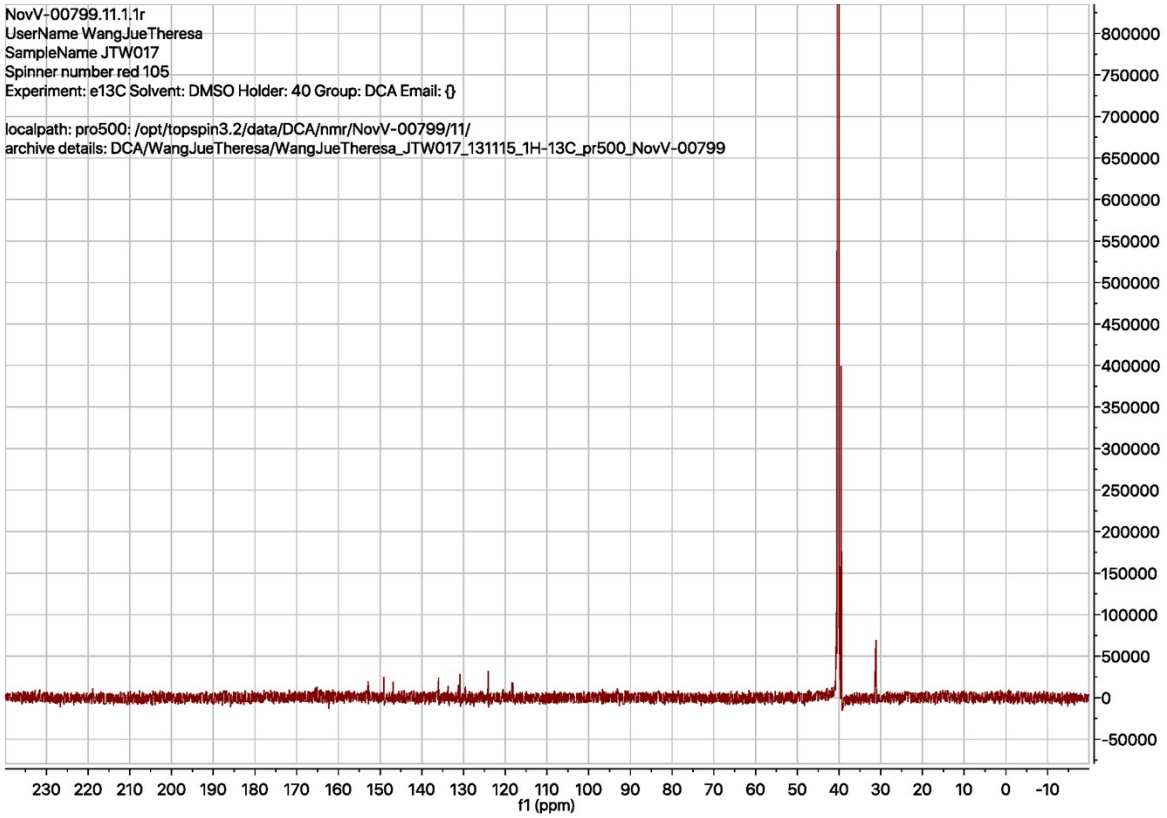
NAH A2 ^{13}C NMR



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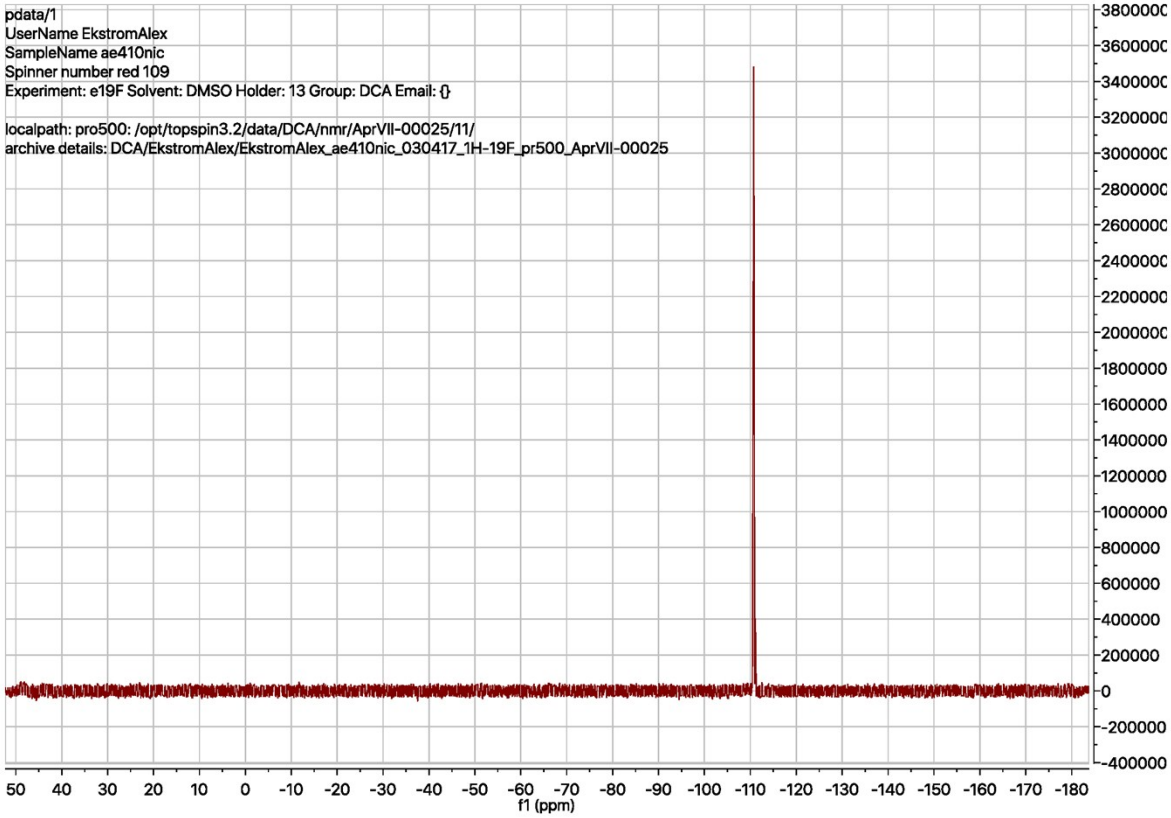

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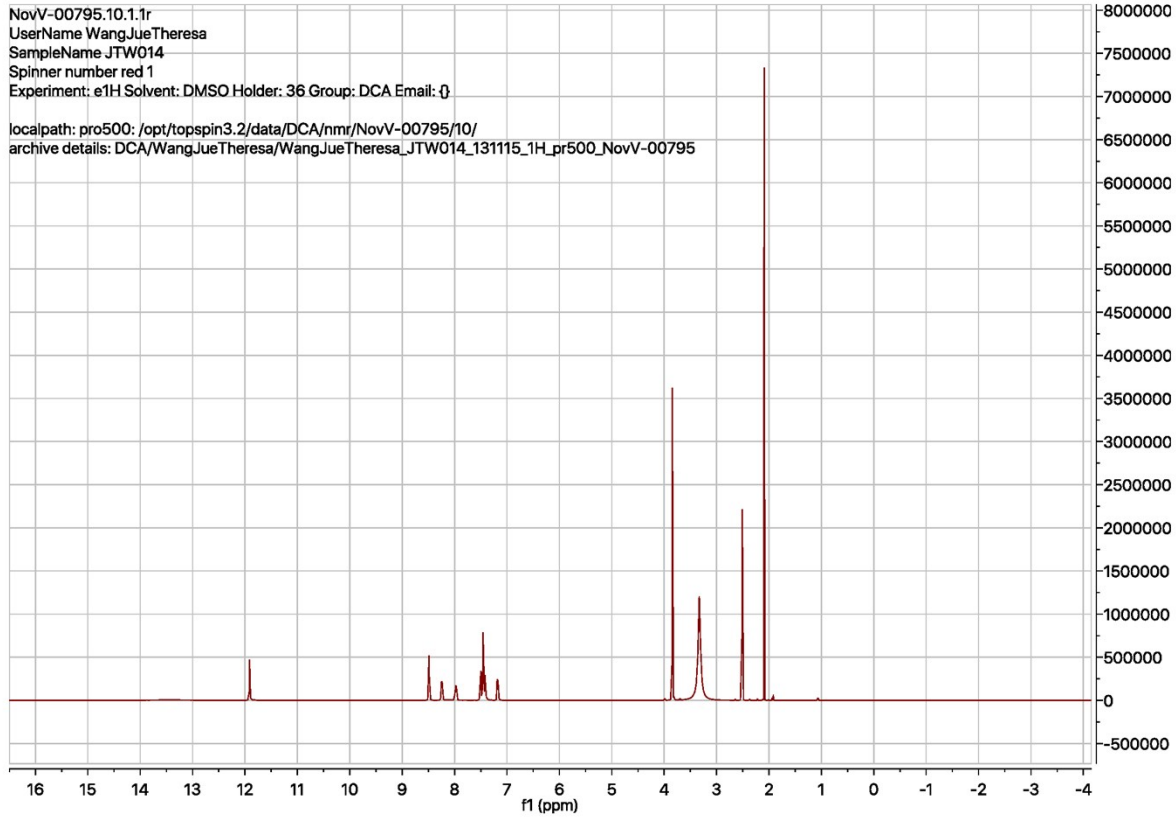
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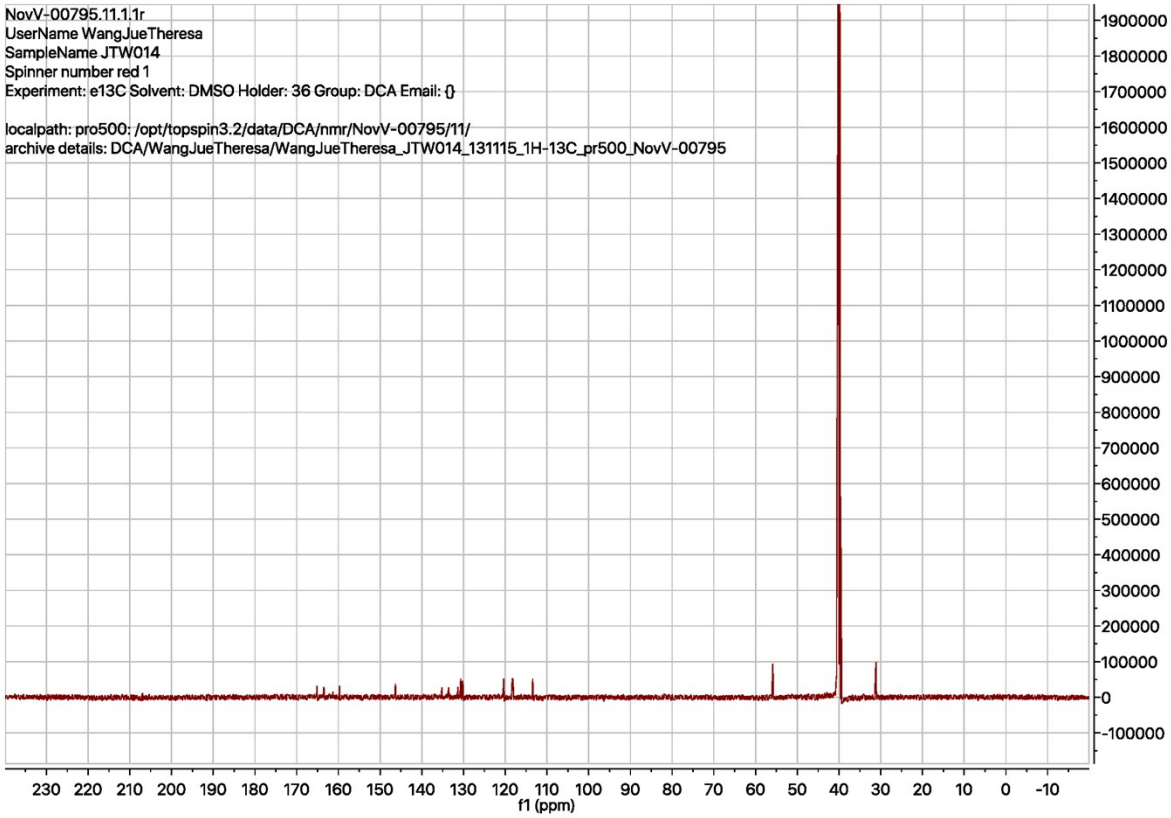

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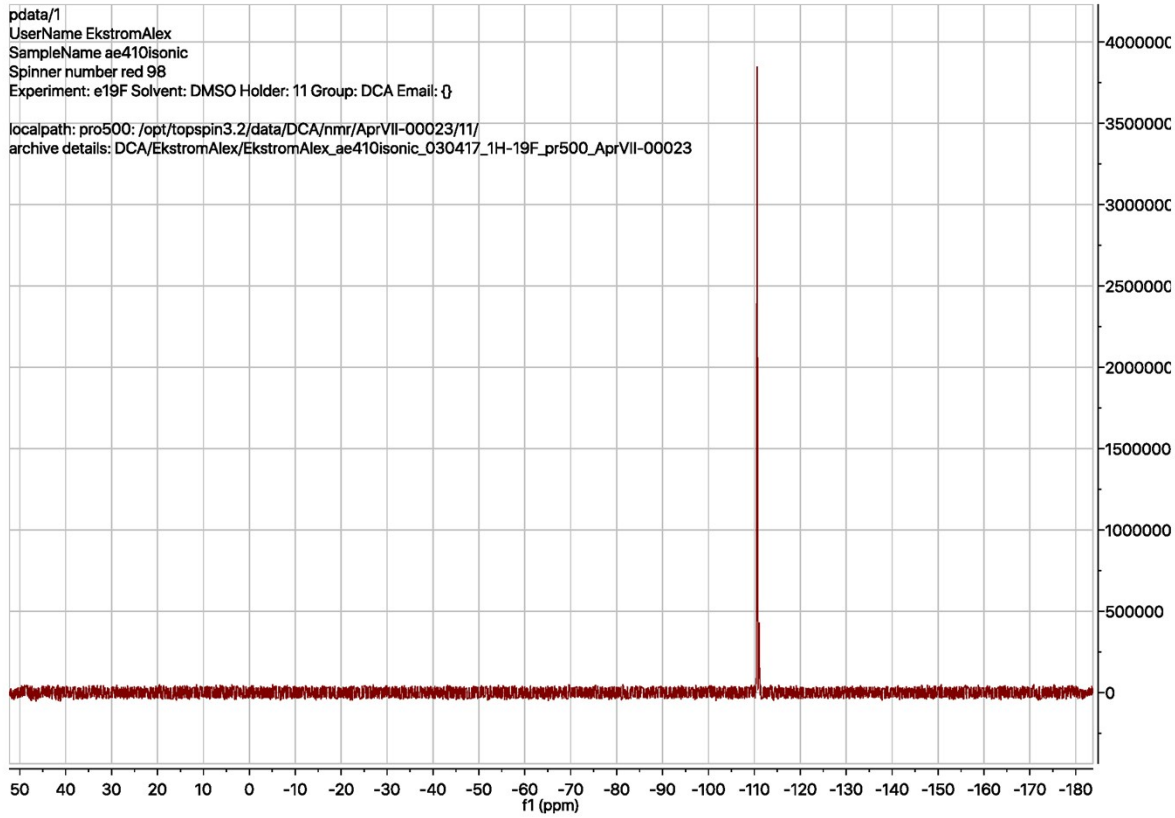

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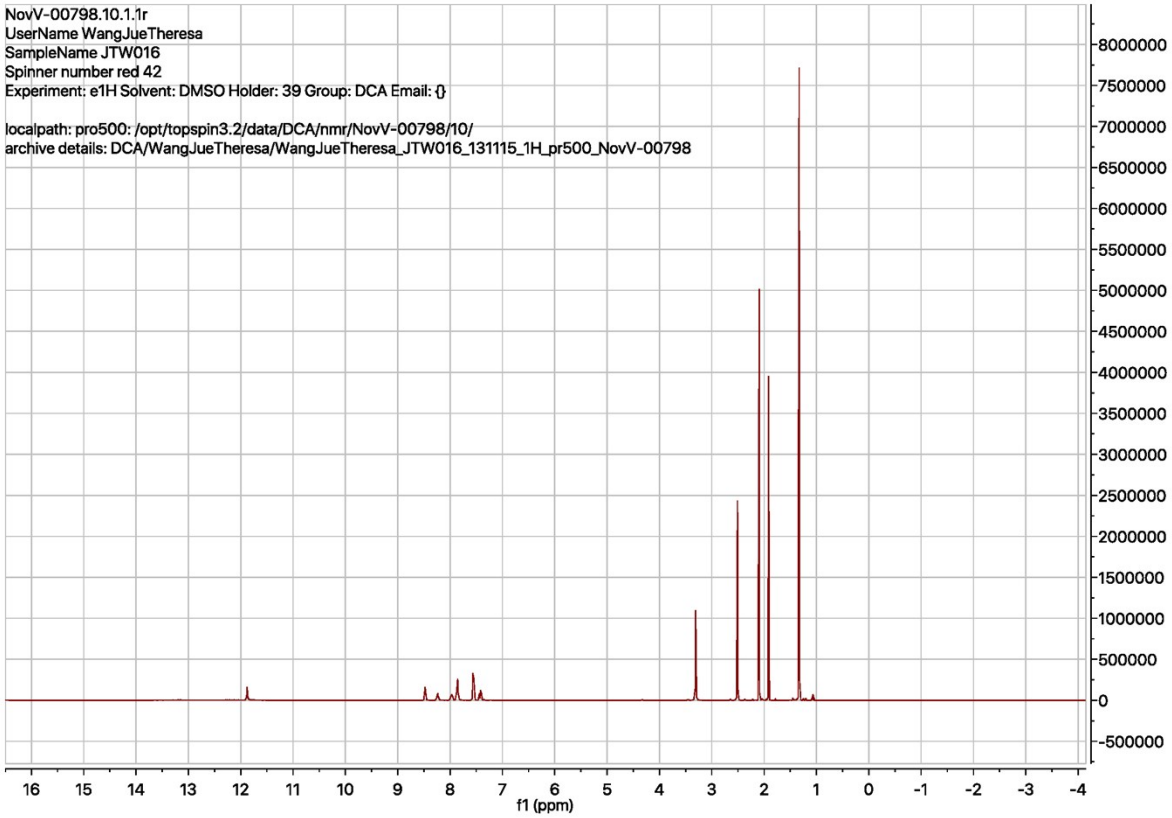
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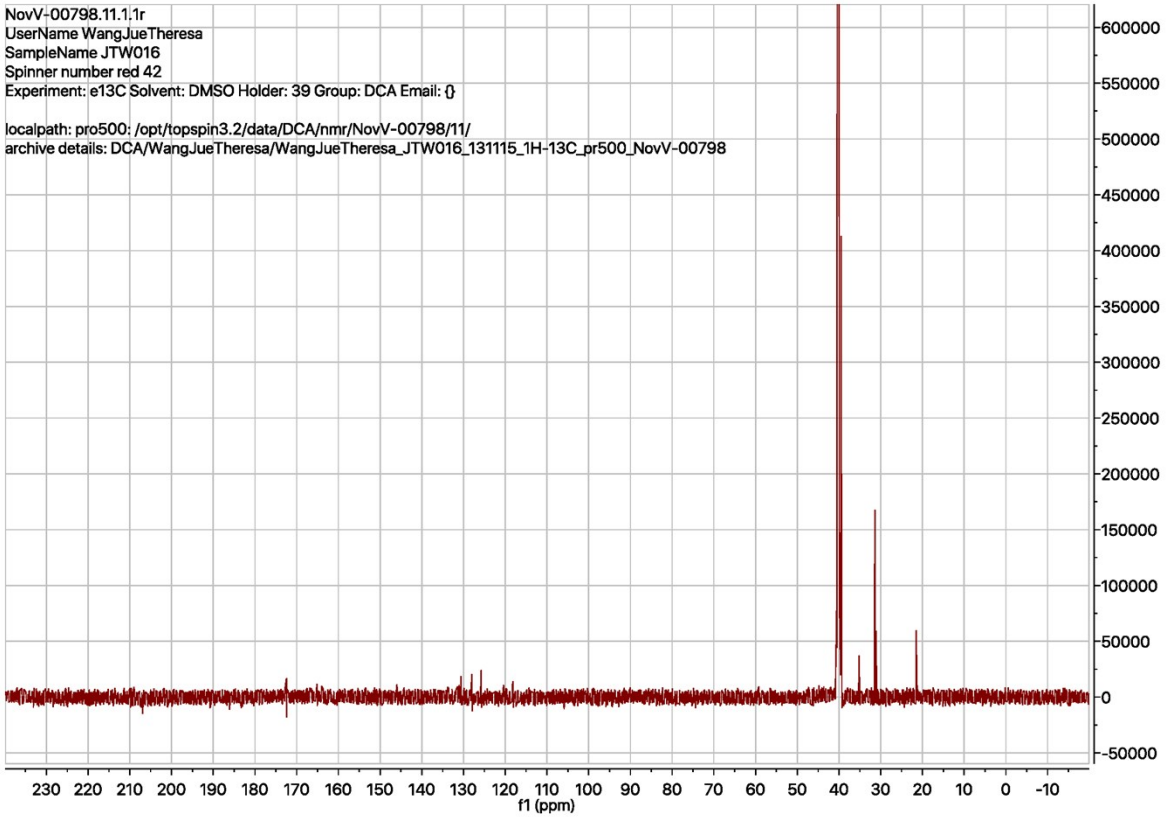
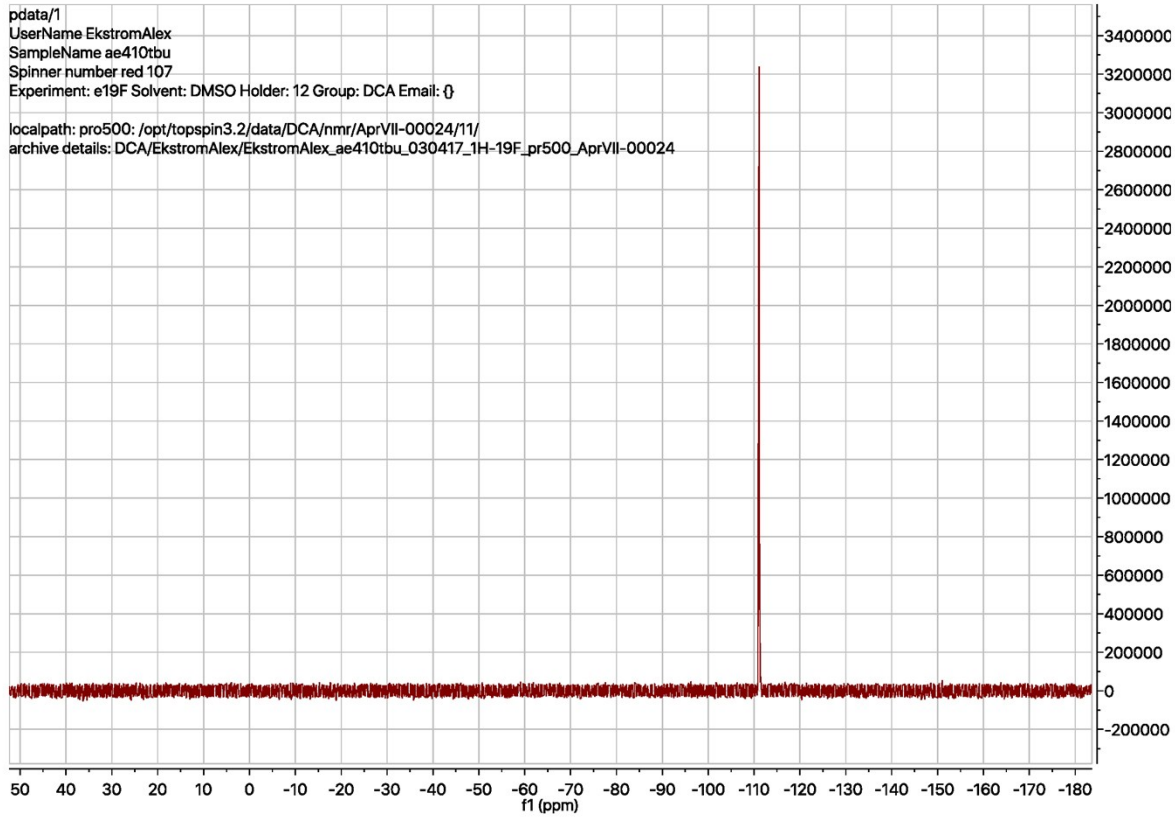


NAH A4 ^{19}F NMR



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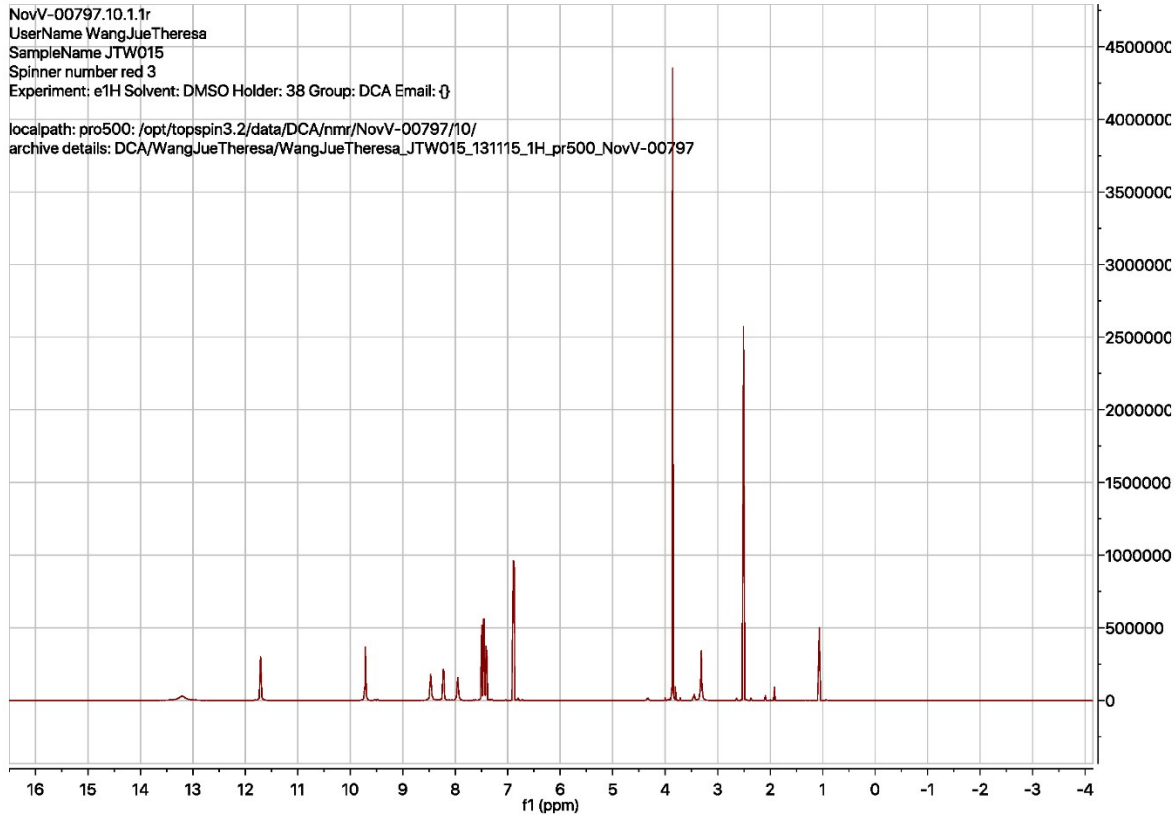
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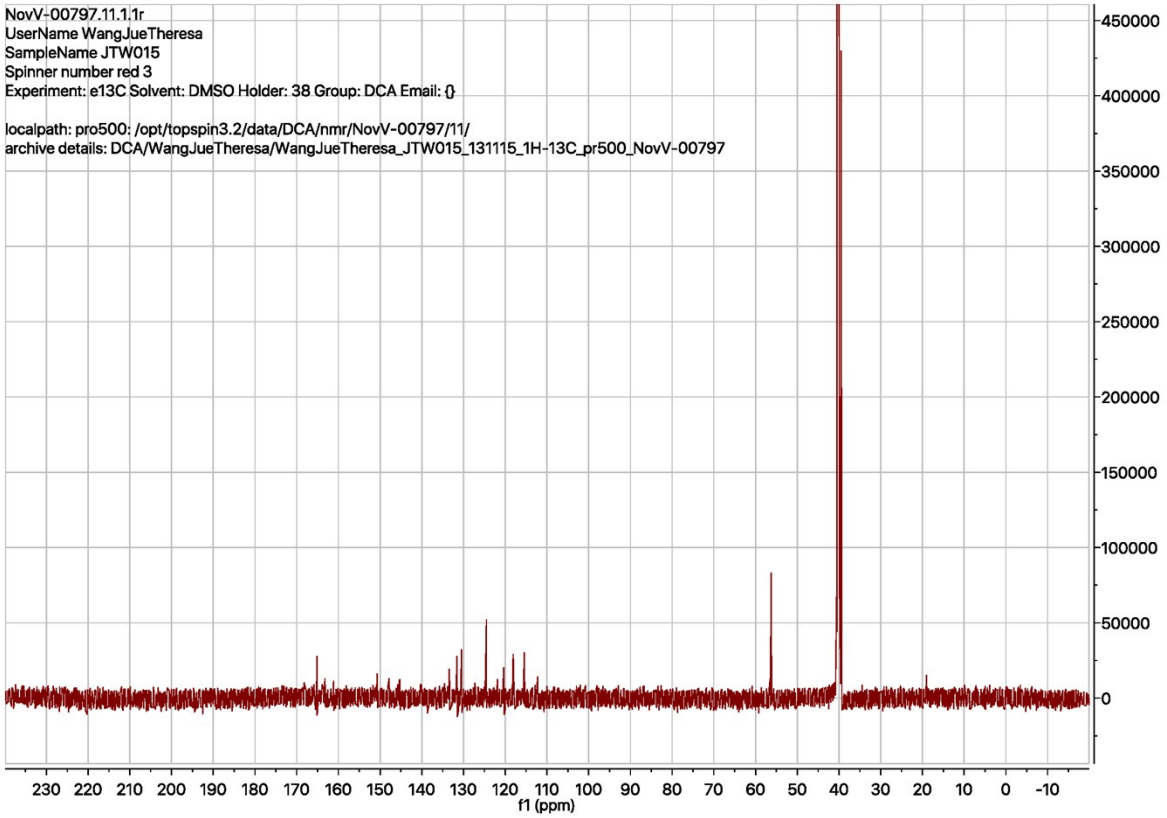
f1 (ppm)

NAH A5 ^{13}C NMR



NAH A5 ^{19}F NMR