Supplemental Information

Identification of the Molecular Targets of Disulfide Bond Disrupting Agents

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Affiliations
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[#]Co-Contributing Corresponding Authors

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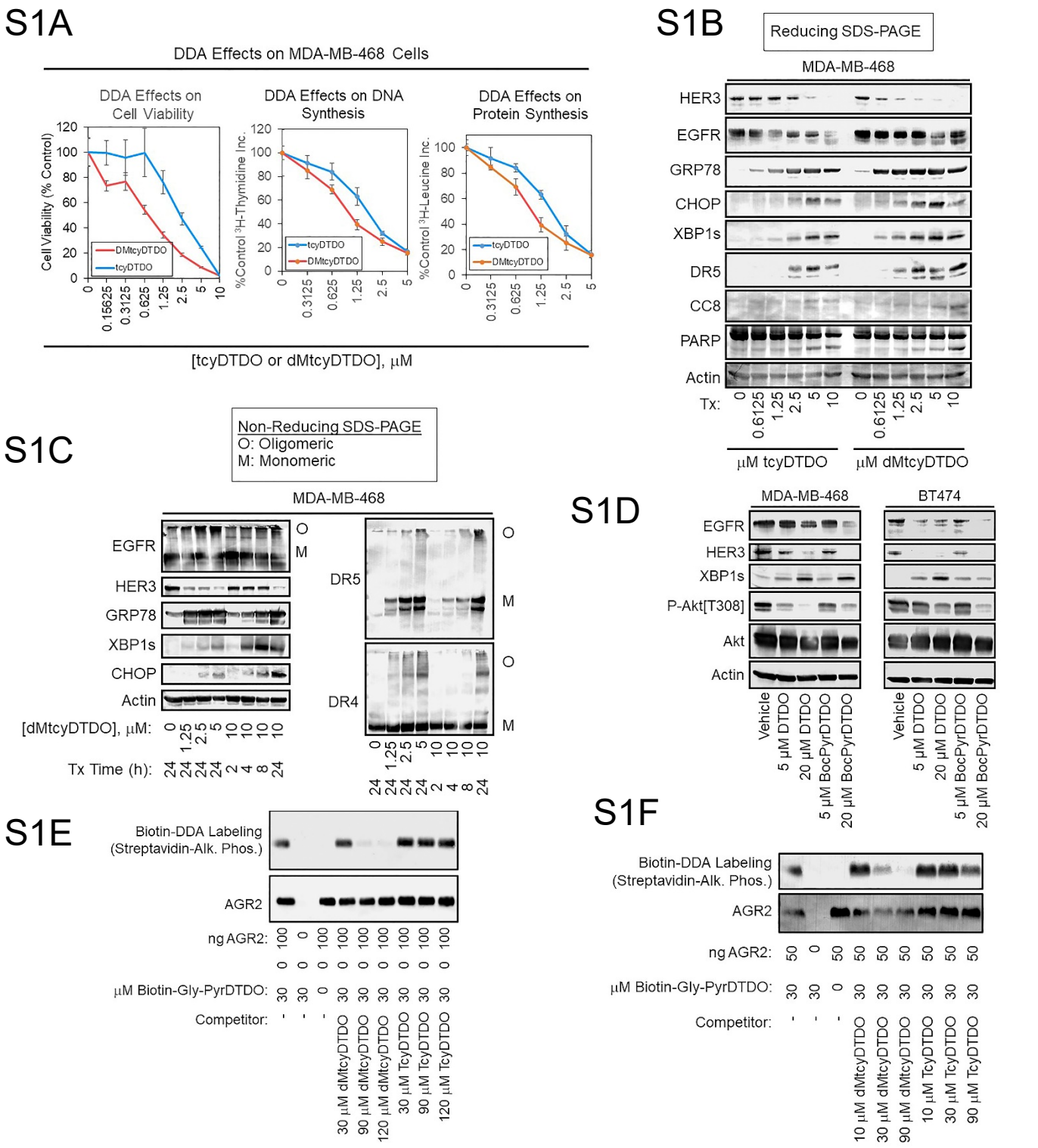
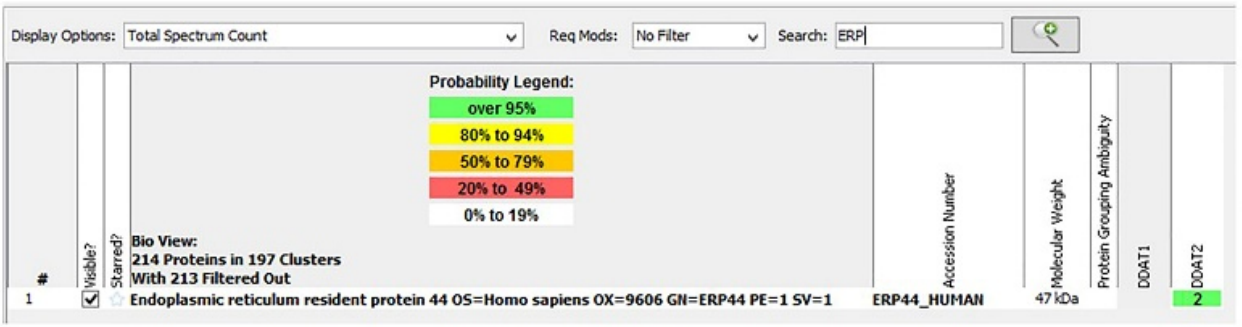
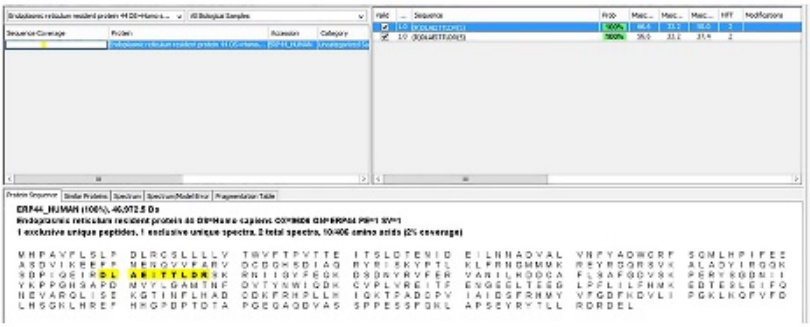


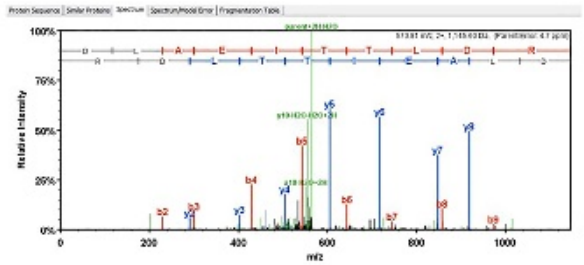
Fig. S1-Characterization of novel DDAs and an AGR2 DDA binding assay. S1A. MDA-MB-468 cells were treated for 24 h as indicated and assayed for viability, DNA synthesis, and protein synthesis in MTT assays, and tritiated thymidine and leucine incorporation assays, respectively. S1B. Reducing immunoblot analysis of MDA-MB-468 cells treated for 24 h as indicated. S1C. Non-reducing immunoblot analysis of MDA-MB-468 cells treated for 24 h as indicated. S1D. Immunoblot analysis of MDA-MB-468 and BT474 cells treated for 24 h as indicated. S1E. and S1F. AGR2 binding assays employing recombinant AGR2 and Biotin-GlyPyrDTDO under the indicated conditions followed by immunoblot analysis for AGR2 or blotting with Streptavidin-conjugated Alkaline Phosphatase.

S2A



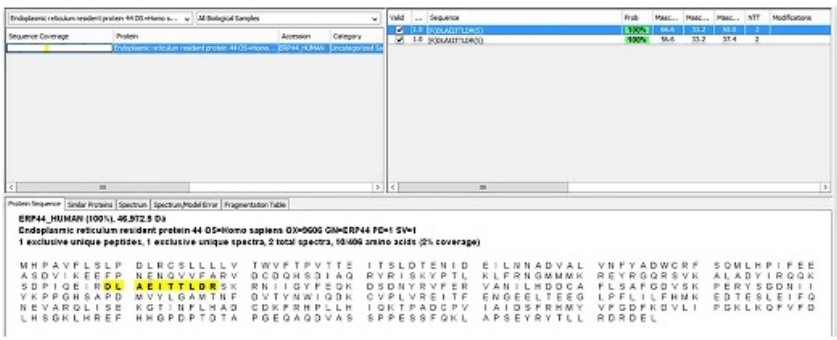
Unique Peptide Search: ERp44 (one peptide sequence; two peptides)

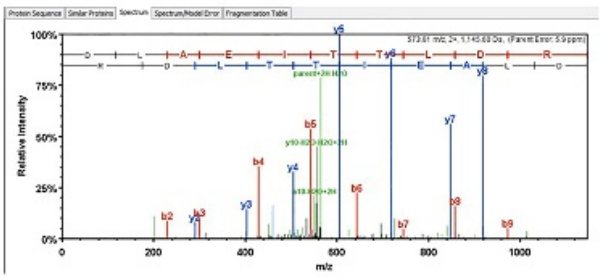




B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	116.0			98.0	D	1,146.6	573.8	1,129.6	1,128.6	10
2	229.1			211.1	L	1,031.6	516.3	1,014.5	1,013.6	9
3	300.2			282.1	A	918.5	459.7	901.5	900.5	8
4	429.2			411.2	E	847.5	424.2	830.4	829.4	7
5	542.3			524.3	I	718.4	359.7	701.4	700.4	6
6	643.3	322.2		625.3	T	605.3		588.3	587.3	5
7	744.4	372.7		726.4	T	504.3		487.3	486.3	4
8	857.5	429.2		839.5	L	403.2		386.2	385.2	3
9	972.5	486.7		954.5	D	290.1		273.1	272.1	2
10	1,146.6	573.8	1,129.6	1,128.6	R	175.1		158.1		1

Unique Peptide Search: ERp44 (one peptide sequence; two peptides)





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1	116.0			98.0	D	1,146.6	573.8	1,129.6	1,128.6	10
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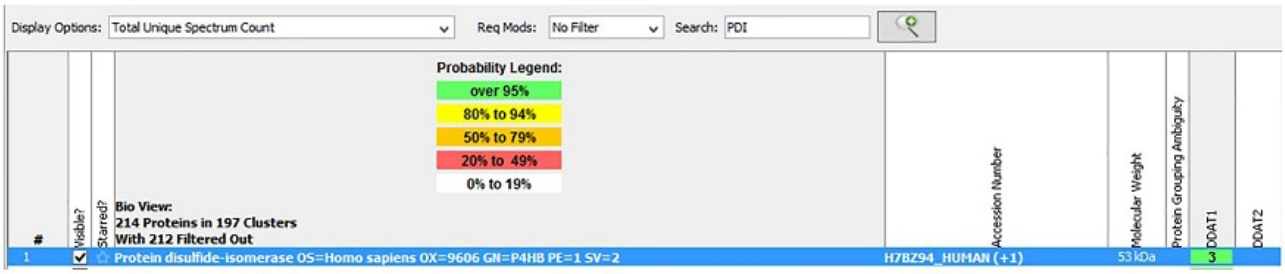
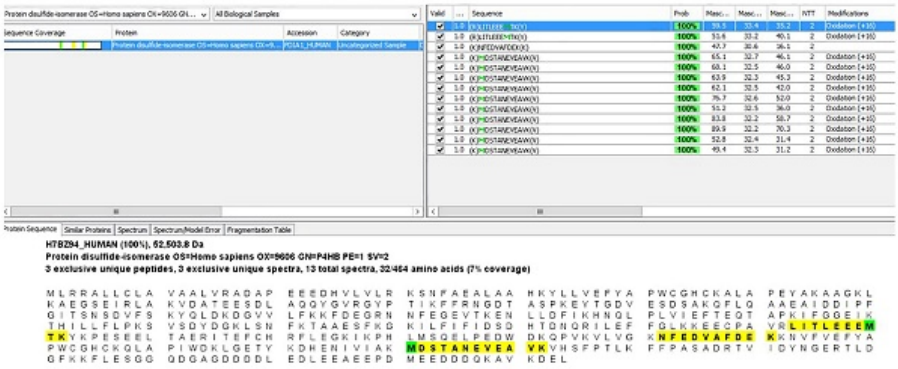
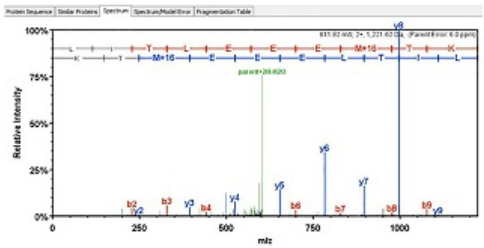
S2B



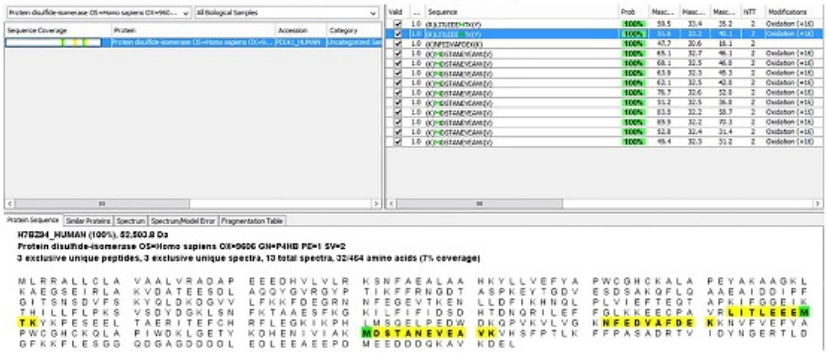
Unique Peptide Search: PDIs (three peptides sequences; 13 peptides)

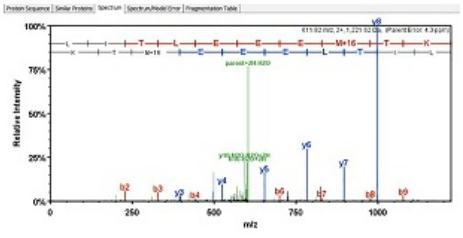




B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	114.1				L	1,222.6	611.8	1,205.6	1,204.6	10
2	227.2				I	1,109.5	555.3	1,092.5	1,091.5	9
3	328.2			310.2	T	996.5	498.7	979.4	978.4	8
4	441.3			423.3	L	895.4	448.2	878.4	877.4	7
5	570.3			552.3	E	782.3	391.7	765.3	764.3	6
6	699.4	350.2		681.4	E	653.3		636.3	635.3	5
7	828.4	414.7		810.4	E	524.2		507.2	506.2	4
8	975.5	488.2		957.5	M+16	395.2		378.2	377.2	3
9	1,076.5	538.8		1,058.5	T	248.2		231.1	230.1	2
10	1,222.6	611.8	1,205.6	1,204.6	K	147.1		130.1		1

Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)





B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	114.1				L	1,222.6	611.8	1,205.6	1,204.6	10
2	227.2				I	1,109.5	555.3	1,092.5	1,091.5	9
3	328.2			310.2	T	996.5	498.7	979.4	978.4	8
4	441.3			423.3	L	895.4	448.2	878.4	877.4	7
5	570.3			552.3	E	782.3	391.7	765.3	764.3	6
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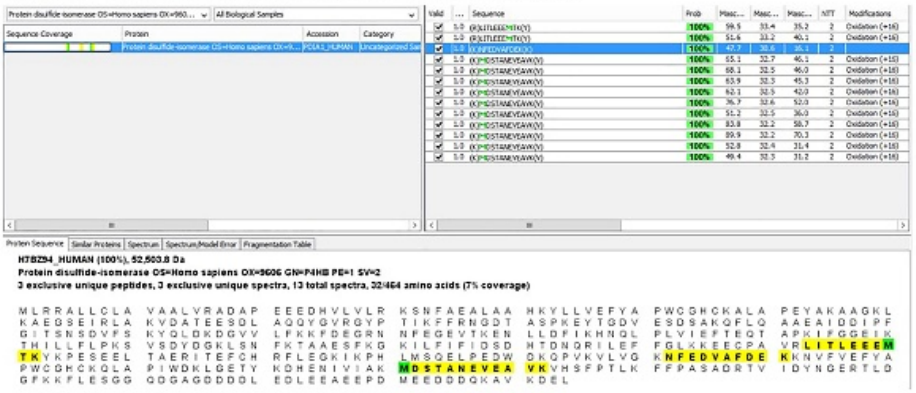
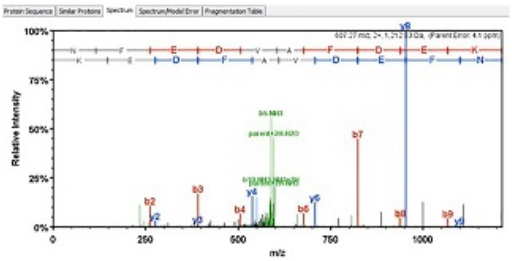
9	1,076.5	538.8		1,058.5	T	248.2		231.1	230.1	2
10	1,222.6	611.8	1,205.6	1,204.6	K	147.1		130.1		1

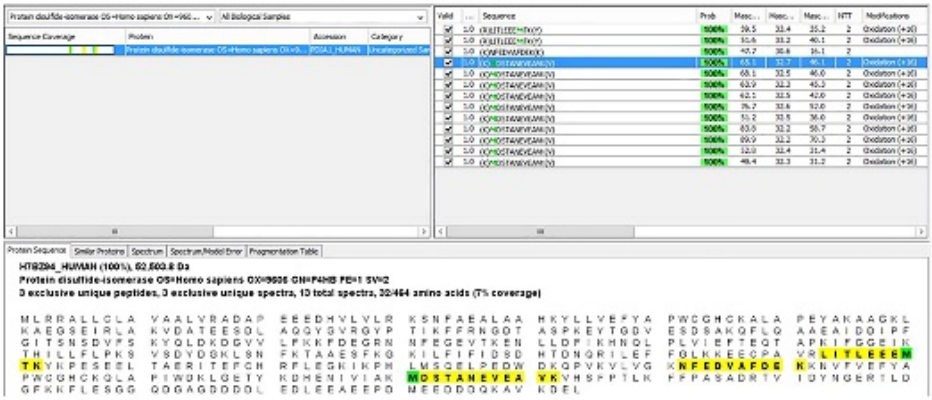
Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)

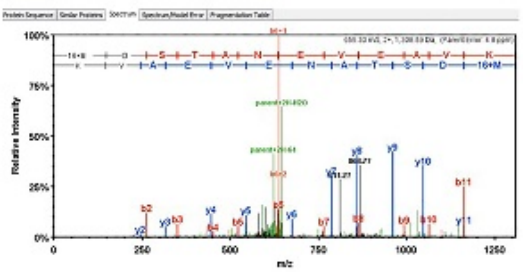




B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	115.1		98.0		N	1,213.5	607.3	1,196.5	1,195.5	10
2	262.1		245.1		F	1,099.5	550.3	1,082.5	1,081.5	9
3	391.2		374.1	373.2	E	952.4	476.7	935.4	934.4	8
4	506.2		489.2	488.2	D	823.4	412.2	806.4	805.4	7
5	605.3		588.2	587.2	V	708.4	354.7	691.3	690.3	6
6	676.3	338.7	659.3	658.3	A	609.3		592.3	591.3	5
7	823.4	412.2	806.3	805.4	F	538.3		521.2	520.2	4
8	938.4	469.7	921.4	920.4	D	391.2		374.2	373.2	3
9	1,067.4	534.2	1,050.4	1,049.4	E	276.2		259.1	258.1	2
10	1,213.5	607.3	1,196.5	1,195.5	K	147.1		130.1		1

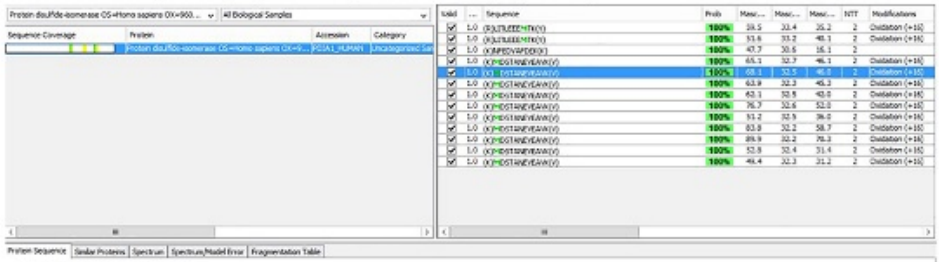
Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)

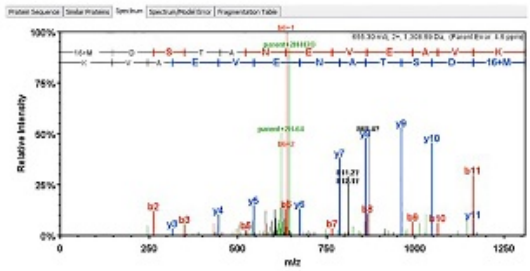




B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	148.0				M+16	1,309.6	655.3	1,292.6	1,291.6	12
2	263.1			245.1	D	1,162.6	581.8	1,145.5	1,144.5	11
3	350.1			332.1	S	1,047.5	524.3	1,030.5	1,029.5	10
4	451.1			433.1	T	960.5	480.8	943.5	942.5	9
5	522.2			504.2	A	859.5	430.2	842.4	841.4	8
6	636.2	318.6	619.2	618.2	N	788.4	394.7	771.4	770.4	7
7	765.3	383.1	748.2	747.3	E	674.4	337.7	657.3	656.4	6
8	864.3	432.7	847.3	846.3	V	545.3		528.3	527.3	5
9	993.4	497.2	976.4	975.4	E	446.3		429.2	428.3	4
10	1,064.4	532.7	1,047.4	1,046.4	A	317.2		300.2		3
11	1,163.5	582.2	1,146.5	1,145.5	V	246.2		229.2		2
12	1,309.6	655.3	1,292.6	1,291.6	K	147.1		130.1		1

Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)





Protein Sequence	Similar Proteins	Spectrum	Spectrum/Model Error	Fragmentation Table

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4	451.1			433.1	T	960.5	480.8	943.5	942.5	9
5	522.2			504.2	A	859.5	430.2	842.4	841.4	8
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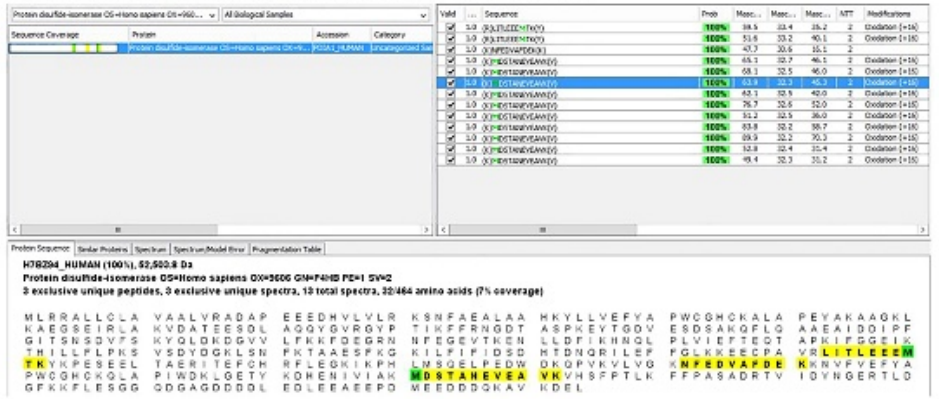

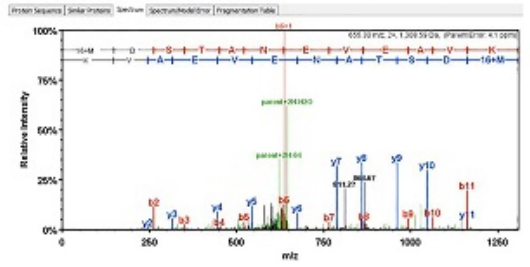

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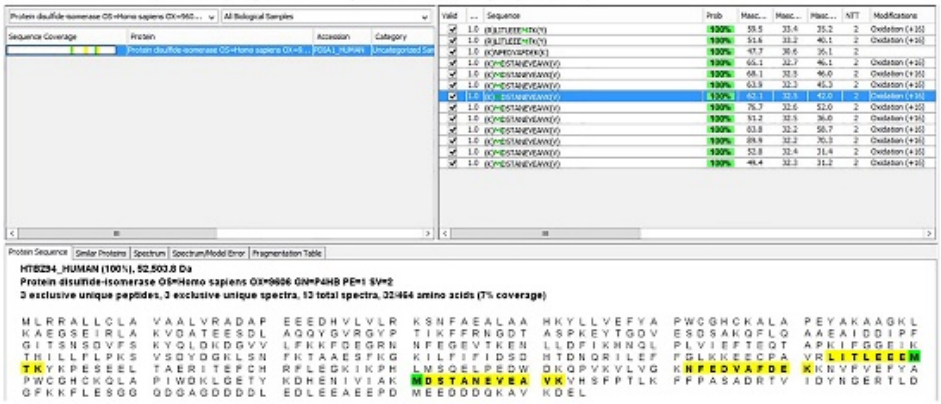

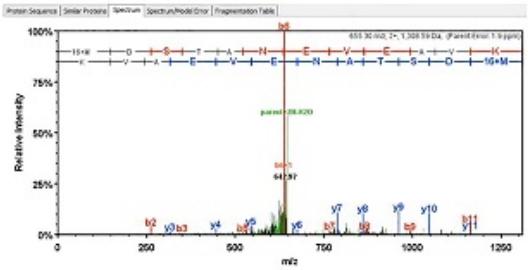

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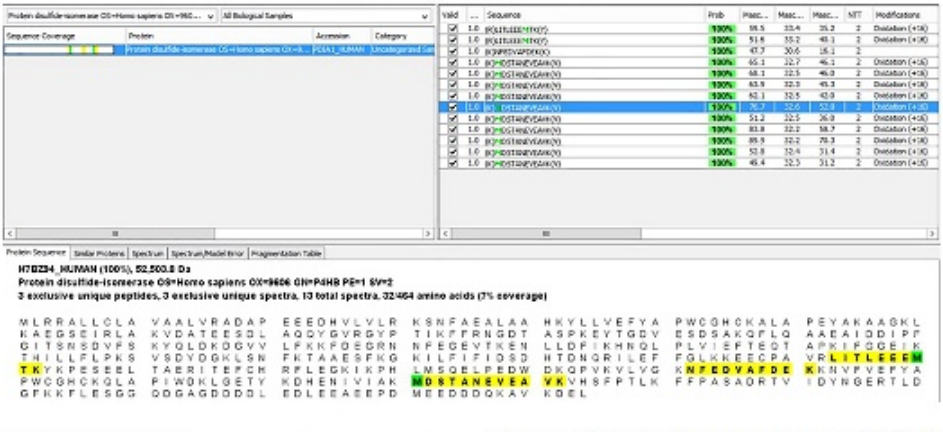

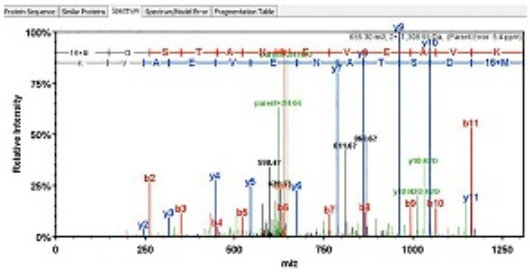

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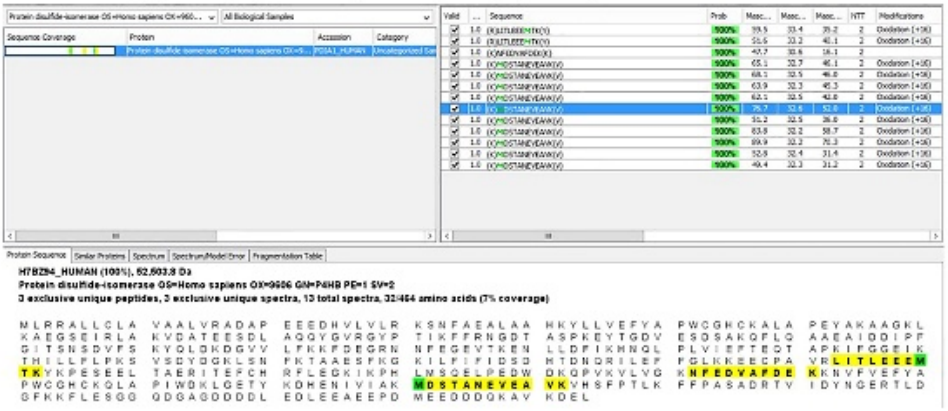
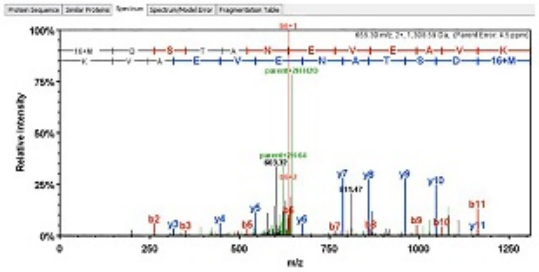
7	765.3	383.1	748.2	747.3	E	674.4	337.7	657.3	656.4	6
8	864.3	432.7	847.3	846.3	V	545.3		528.3	527.3	5
9	993.4	497.2	976.4	975.4	E	446.3		429.2	428.3	4
10	1,064.4	532.7	1,047.4	1,046.4	A	317.2		300.2		3
11	1,163.5	582.2	1,146.5	1,145.5	V	246.2		229.2		2
12	1,309.6	655.3	1,292.6	1,291.6	K	147.1		130.1		1

Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)





B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	148.0				M+16	1,309.6	655.3	1,292.6	1,291.6	12
2	263.1			245.1	D	1,162.6	581.8	1,145.5	1,144.5	11
3	350.1			332.1	S	1,047.5	524.3	1,030.5	1,029.5	10
4	451.1			433.1	T	960.5	480.8	943.5	942.5	9
5	522.2			504.2	A	859.5	430.2	842.4	841.4	8
6	636.2	318.6	619.2	618.2	N	788.4	394.7	771.4	770.4	7
7	765.3	383.1	748.2	747.3	E	674.4	337.7	657.3	656.4	6
8	864.3	432.7	847.3	846.3	V	545.3		528.3	527.3	5
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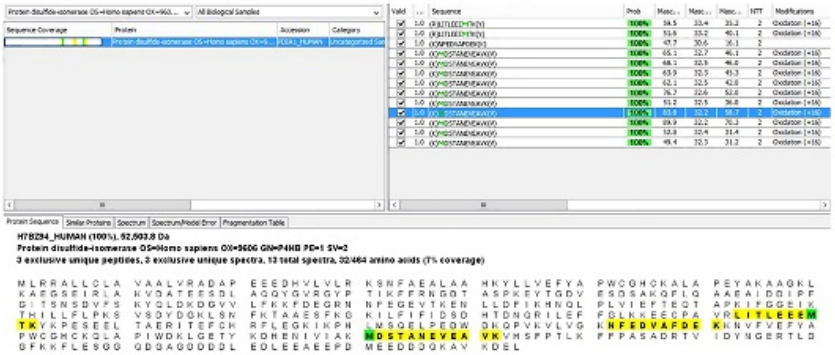
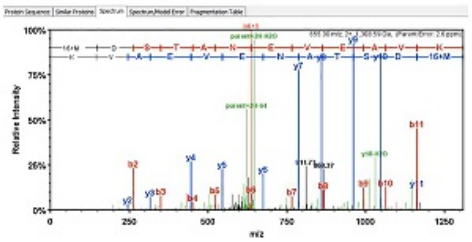
12	1,309.6	655.3	1,292.6	1,291.6	K	147.1		130.1		1

Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)





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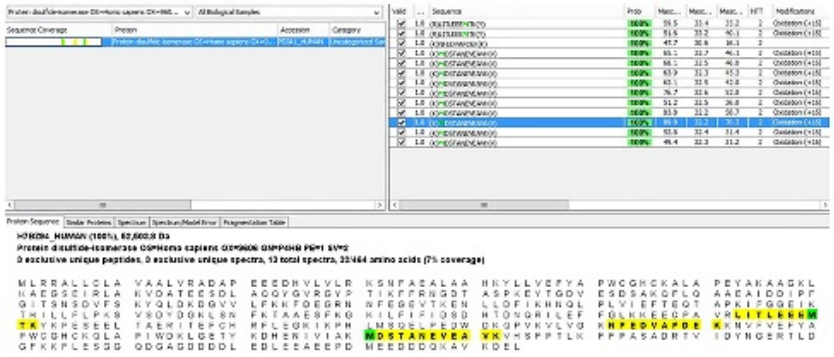

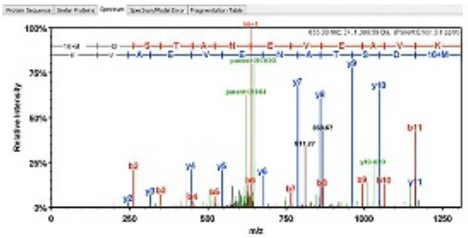

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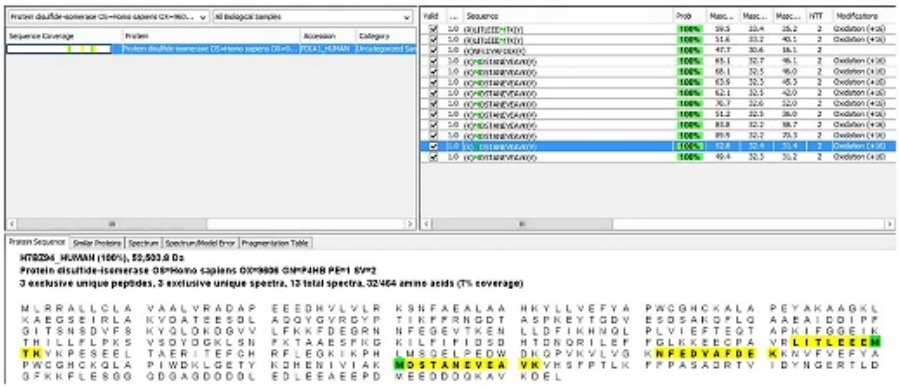
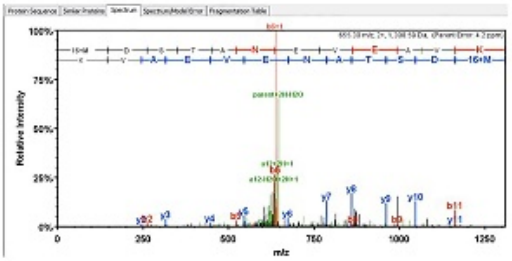
11	1,163.5	582.2	1,146.5	1,145.5	V	246.2		229.2		2
12	1,309.6	655.3	1,292.6	1,291.6	K	147.1		130.1		1

Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)





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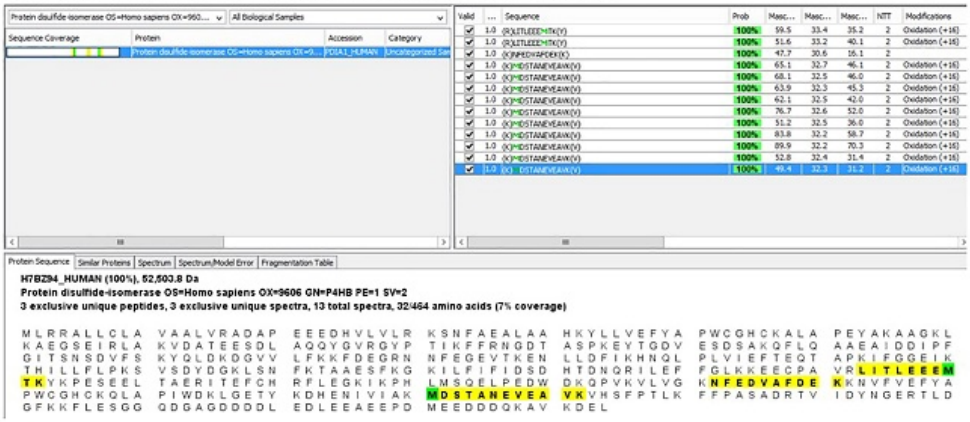
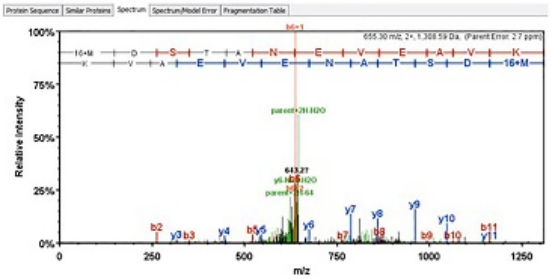
12	1,309.6	655.3	1,292.6	1,291.6	K	147.1		130.1		1

Unique Peptide Search: PDIs (three peptide sequences; 13 peptides)





Protein Sequence | Similar Proteins | Spectrum | Spectrum/Model Error | Fragmentation Table

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	148.0				M+16	1,309.6	655.3	1,292.6	1,291.6	12
2	263.1			245.1	D	1,162.6	581.8	1,145.5	1,144.5	11
3	350.1			332.1	S	1,047.5	524.3	1,030.5	1,029.5	10
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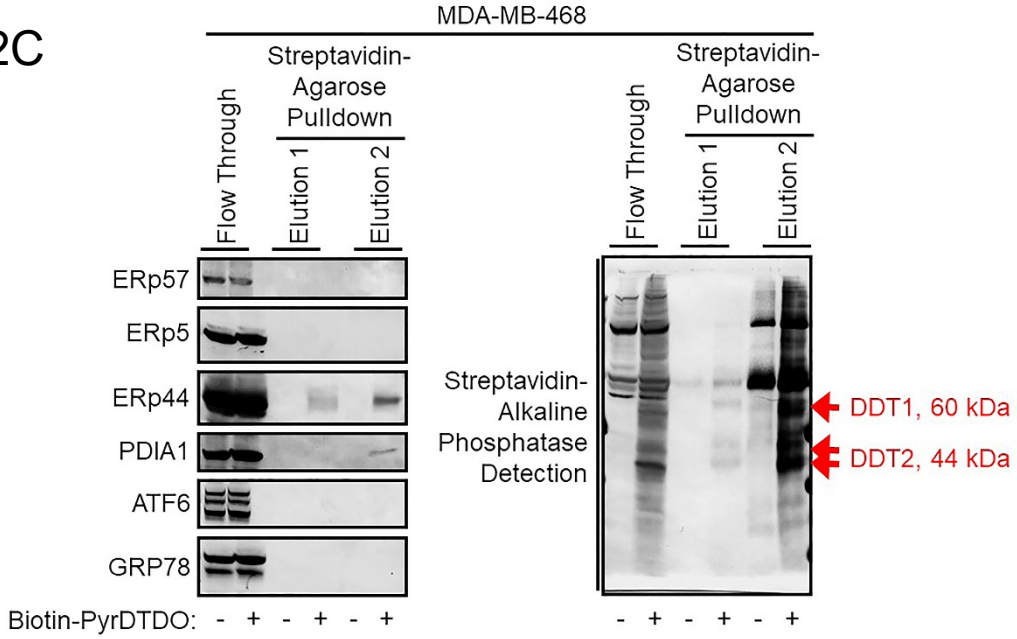
10	1,064.4	532.7	1,047.4	1,046.4	A	317.2		300.2		3
11	1,163.5	582.2	1,146.5	1,145.5	V	246.2		229.2		2
12	1,309.6	655.3	1,292.6	1,291.6	K	147.1		130.1		1

S2C



S2D

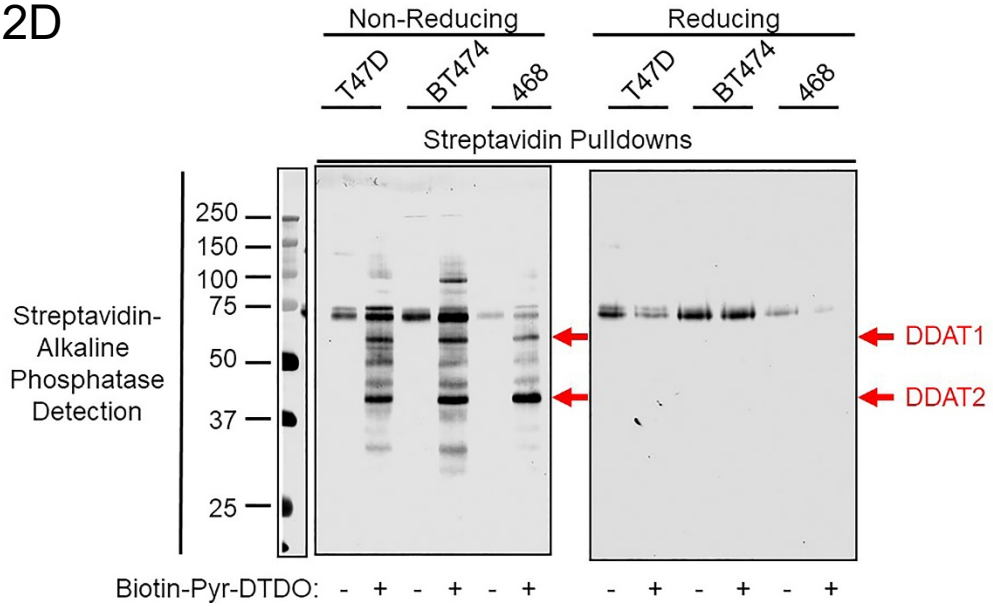
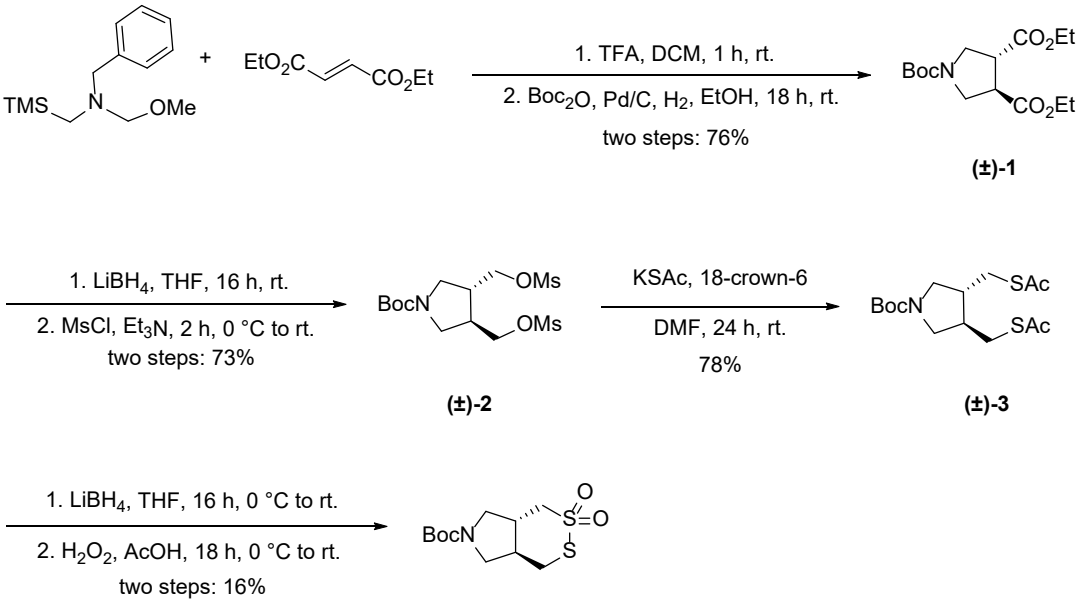


Fig. S2-Confirmation of ERp44 and PDIA1 as DDA targets. S2A. Peptide coverage of ERp44, and S2B. Peptide coverage of PDIA1 identified in Biotin-PyrDTDO/Streptavidin-Agarose pulldowns from MDA-MB-468 cells by tandem mass spectrometry. S2C. Streptavidin-Agarose pulldowns of Biotin-PyrDTDO treated cells in which the flow through, elution one (250 μM dMtcyDTDO + 100 μM 2-Mercaptoethnol), and elution 2 (2X SDS-PAGE Laemmli sample buffer + 2 mM Biotin) were analyzed by immunoblot and Streptavidin-Alkaline Phosphatase detection. S2D. Streptavidin-Agarose pulldowns of Biotin-PyrDTDO treated cells performed in the presence of 100 mM NEM. Half of each sample was brought to 1 M 2-mercaptoethanol and boiled for 20 minutes to generate reduction samples. These non-reducing (left panel) or reducing samples (right panel) were analyzed by blotting with Streptavidin-Alkaline Phosphatase. Molecular weight markers are indicated in kiloDaltons.

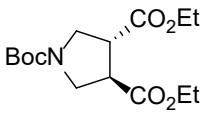
General Methods

Reagents and solvents were purchased from commercial sources and used without further purification unless otherwise specified. Anhydrous solvents were obtained using a commercial solvent drying system (using activated alumina for THF, diethyl ether, dichloromethane; molecular sieves for DMF) and transferred via syringe to flame-dried glassware that had been cooled under an argon atmosphere. ^{1}H and ^{13}C NMR spectra were recorded using commercially-obtained deuterated solvents on Bruker-600 (^{1}H at 600 MHz; ^{13}C at 151 MHz) and Varian Inova-500 (^{1}H at 500 MHz; ^{13}C at 125 MHz) spectrometers. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS and referenced to residual protonated solvent ($CDCl_3$: δ_H 7.26 ppm, δ_C 77.16 ppm; CD_3OD: δ_H 4.87 ppm, δ_C 49.00 ppm; DMSO-d_6: δ_H 2.50 ppm, δ_C 39.52 ppm; D_2O: δ_H 4.79 ppm). Coupling constants (J) are reported in Hz. Spin multiplicities are presented by the following symbols: s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), p (pentet), and m (multiplet). Electrospray ionization (ESI) high-resolution mass spectra (HRMS) were recorded on an ESI-TOF instrument, operating in positive mode as stated, with methanol as the carrier solvent otherwise as mentioned.

Synthesis of (±)-BocPyrDTDO:



1-(*tert*-Butyl)-3,4-diethyl-(3*S*,4*S*)- and (3*R*,4*R*)-pyrrolidine-1,3,4-tricarboxylate, (±)-1



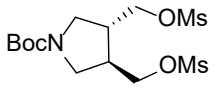
(±)-1

Intermediate **(±)-1** was synthesized following a procedure described in a patent.[1a] To a stirred solution of diethyl fumarate (3.8 mL, 23 mmol) in DCM (50 mL) at 0 °C was added *N*-benzyl-1-methoxy-*N*-((trimethylsilyl)methyl)methanamine (5.4 mL, 21 mmol) followed by dropwise addition of a solution of TFA in DCM (0.1 mL of TFA in 1 mL of DCM) over 10 min. After completion of addition, the cold bath was removed, and the reaction mixture was stirred at rt for 1 h. The reaction mixture was diluted with DCM (20 mL), washed with a saturated solution of sodium bicarbonate (20 mL), and the organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (25 mL) and to this was added Boc_2O (8.00 g, 36.6 mmol) followed by 10% palladium on carbon (1 g, wet). The resulting reaction mixture was subjected to catalytic hydrogenation using hydrogen gas for 18 h with stirring. The catalyst was filtered through a pad of celite, and the filtrate was concentrated. The residue was purified by flash silica gel column chromatography (0–25% EtOAc/hexanes) to provide the Boc-protected diester **(±)-1** (5.1 g, 16 mmol, 76% yield) as a pale-yellow oil. The ^{1}H NMR data matches the literature.[1]

^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.16 (q, J = 7.1 Hz, 4H), 3.78–3.69 (m, 2H), 3.57–3.44 (m, 2H), 3.42–3.29 (m, 2H), 1.45 (s, 9H), 1.26 (t, J = 7.1 Hz, 6H).

^{13}C NMR ($CDCl_3$, 125 MHz): δ 171.81, 154.02, 80.02, 61.44, 48.12, 48.02, 46.12, 45.39, 28.54, 14.23.

tert-Butyl (3*S*,4*S*)- and (3*R*,4*R*)-3,4-bis(((methylsulfonyl)oxy)methyl)pyrrolidine-1-carboxylate, (±)-2



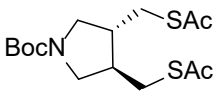
(±)-2

To an ice-cooled solution of the diester **(±)-1** (5.0 g, 16 mmol) in anhydrous THF (135 mL) was added dropwise a solution of $LiBH_4$ in THF (16 mL of a 2.0 M $LiBH_4$ solution in THF diluted with an additional 20 mL of anhydrous THF). The reaction mixture was stirred overnight at rt and quenched with an aqueous 2 M NaOH solution. Ether was added, the layers were separated, and the aqueous phase was back extracted twice with ether. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated to give the diol which was used without further purification. To a stirred solution of the diol in DCM (110 mL) cooled to 0 °C was added triethylamine (11 mL, 80 mmol) followed by methanesulfonyl chloride (3.1 mL, 40 mmol). The reaction mixture was stirred at 0 °C for 15 min, then at rt for 1.5 h. The reaction mixture was poured into water (76 mL) and the phases were separated. The aqueous phase was extracted with DCM (2 × 76 mL). The combined organics were dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (0–100% EtOAc/DCM) to afford the desired dimesylate **(±)-2** (4.5 g, 12 mmol, 73% yield) as a white solid.[2]

^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.25 (m, 4H), 3.65 (m, 2H), 3.24 (m, 2H), 3.05 (s, 6H), 2.53 (m, 2H), 1.46 (s, 9H).

^{13}C NMR ($CDCl_3$, 125 MHz): δ 154.24, 80.23, 68.86, 47.95, 47.46, 40.52, 39.42, 37.70, 28.58.

tert-Butyl-(3*S*,4*S*)- and (3*R*,4*R*)-3,4-bis((acetylthio)methyl)pyrrolidine-1-carboxylate, (±)-3



(±)-3

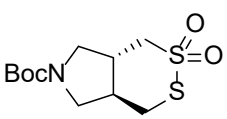
A mixture of the dimesylate **(±)-2** (7.8 g, 20 mmol), KSAc (4.6 g, 40 mmol), and 18-crown-6 (1.3 g, 5.0 mmol) in anhydrous DMF (392 mL) was stirred for 24 h at rt. After completion of the reaction, water (392 mL) was added and the mixture was extracted with EtOAc (3 × 392 mL). The combined organic phases were washed with water (392 mL) and brine (392 mL), dried (Na_2SO_4), concentrated, and purified by column chromatography (0–10% DCM/EtOAc) to afford the dithioacetate **(±)-3** (5.4 g, 16 mmol, 78% yield) as a colorless oil.

^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.69–3.50 (m, 2H), 3.20–3.09 (m, 2H), 3.09–2.93 (m, 2H), 2.85 (dd, J = 13.7, 7.3 Hz, 2H), 2.34 (s, 6H), 2.22–2.05 (m, 2H), 1.44 (s, 9H).

^{13}C NMR ($CDCl_3$, 125 MHz): δ 195.26, 154.37, 79.63, 50.64, 50.29, 43.13, 42.40, 30.74, 30.65, 28.60.

HRMS (ESI-TOF): Calculated for $[C_{15}H_{25}NO_4S_2 + Na]^+$: 370.1117; found: 370.1104.

tert-Butyl-(4a*S*,7a*S*)- and (4a*R*,7a*R*)-tetrahydro-1*H*-[1,2]dithiino[4,5-*c*]pyrrole-6(4*H*)-carboxylate 2,2-dioxide, (±)-BocPyrDTDO



(±)-BocPyrDTDO

To an ice-cooled solution of the dithioacetate **(±)-3** (5.42 g, 15.6 mmol) in anhydrous THF (132 mL) was added dropwise a solution of $LiBH_4$ in THF (7.8 mL of 4.0 M $LiBH_4$ in THF diluted with additional 27 mL of anhydrous THF). The reaction mixture was stirred overnight at rt and quenched with an aqueous 2 M NaOH solution. Ether (25 mL) was added, the layers were separated, and the aqueous phase was back extracted with ether (2 × 25 mL). The combined organic extracts were dried over Na_2SO_4, filtered, and

concentrated to give the crude dithiol (3.81 g, 14.5 mmol, 93% yield), which was used without further purification for the next step. A solution of the crude dithiol (1.91 g, 7.23 mmol) in AcOH (9.0 mL) was cooled in an ice bath and a solution of H_2O_2 in water/AcOH (2.59 mL of 30% H_2O_2 in water diluted with 3.0 mL of AcOH) was added slowly such that the reaction temperature did not rise above 35 °C. After stirring for 18 h, the solvent was removed under vacuum, and the residue was diluted with water (15 mL), neutralized with $NaHCO_3$, and extracted with EtOAc (3 × 50 mL). The organic extract was dried with Na_2SO_4, filtered, and concentrated under vacuum. The crude material was purified by column chromatography (0–100% EtOAc/DCM) to afford the desired **(±)-BocPyrDTDO** (0.66 g, 2.25 mmol, 16% yield).

^{1}H NMR ($CDCl_3$, 500 MHz): *δ* 3.75 (m, 1H), 3.67 (m, 1H), 3.52 (m, 1H), 3.41 (t, *J* = 12.6 Hz, 1H), 3.33 (t, *J* = 12.6, 1H), 3.14 (m,1H), 3.07 (t, *J* = 10.8 Hz, 1H), 3.01 (t, *J* = 10.8 Hz, 1H), 2.8–2.67 (m, 1H), 2.40–2.28 (m, 1H), 1.45 (s, 9H).

^{13}C NMR ($CDCl_3$, 125 MHz): *δ* 154.09, 80.27, 62.62, 62.50, 50.16, 49.76, 49.63, 49.19, 43.14, 43.01, 42.44, 42.36, 35.43, 35.30, 28.55.

HRMS (ESI-TOF): Calculated for $[C_{11}H_{19}NO_4S_2 + Na]^+$: 316.0648; found: 316.0646.

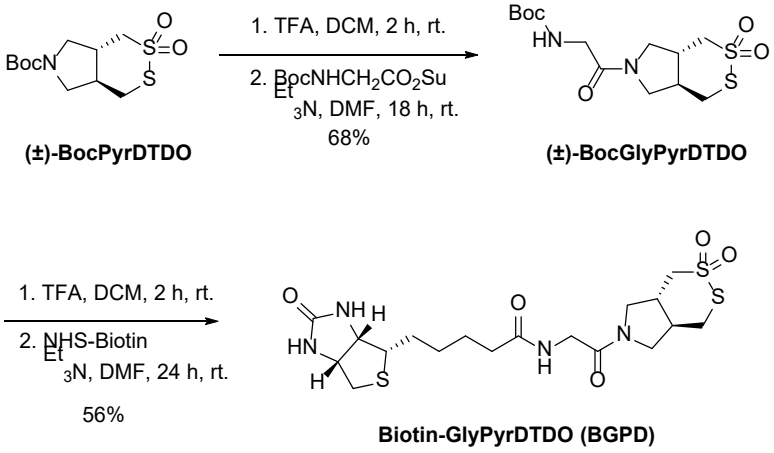
Slow *s*-cis/*s*-trans interconversion with respect to the *N*-acylpyrrolidine ring[3] produces two conformational diastereomers and additional NMR signals at room temperature.

The interconversion is fast on the NMR timescale at 75 °C in DMSO-d_6:

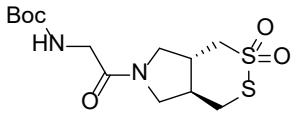
^{1}H NMR (DMSO-d_6, 500 MHz) at 75 °C: *δ* 3.80 (dd, *J* = 13.1, 2.8 Hz, 1H), 3.63–3.53 (m, 3H), 3.43 (dd, *J* = 13.5, 2.9 Hz, 1H), 3.32 (dd, *J* = 13.5, 11.4 Hz, 1H), 3.03 (t, *J* = 10.6 Hz, 1H), 2.97 (t, *J* = 10.6 Hz, 1H), 2.65–2.53 (m, 1H), 2.41–2.31 (m, 1H), 1.43 (s, 9H).

^{13}C NMR (DMSO-d_6, 125 MHz) at 75 °C: *δ* 153.87, 79.12, 62.83, 50.04, 49.58, 42.78, 42.01, 35.55, 28.70. High temperature 1D and 2D NMR spectra for this compound are included.

Synthesis of BioGlyPyrDTDO (BGPD):



***tert*-Butyl-(2-((4a*S*,7a*S*)- and (4a*R*,7a*R*)-2,2-dioxidotetrahydro-1*H*-[1,2]dithiino[4,5-*c*]pyrrol-6(4*H*)-yl)-2-oxoethyl)carbamate, (±)-BocGlyPyrDTDO**



BocGlyPyrDTDO

A solution of **(±)-BocPyrDTDO** (0.10 g, 0.34 mmol) was stirred in TFA:DCM (3.4 mL, 1:1) for 2 h, at rt. The reaction mixture was then concentrated under vacuum to provide the deprotected ammonium trifluoroacetate salt **HPyrDTDO.TFA** as a brown solid, which was used in the next step without further purification.

^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.25 (bs, 2H), 3.91 (dd, J = 13.0, 2.9 Hz, 1H), 3.68 (t, J = 12.6 Hz, 1H), 3.53–3.42 (m, 3H), 3.33 (dd, J = 13.6, 11.5 Hz, 1H), 2.95 (tq, J = 13.4, 6.6 Hz, 2H), 2.62–2.53 (m, 1H), 2.40–2.30 (m, 1H).

^{13}C NMR (DMSO-d_6, 125 MHz): δ 60.97, 47.55, 47.30, 41.98, 40.64, 33.97.

To the solution of the resulting solid in DMF (4.8 mL) was added Et_3N (0.10 mL, 0.68 mmol) and 2,5-dioxopyrrolidin-1-yl (*tert*-butoxycarbonyl)glycinate (0.09 g, 0.34 mmol), respectively, and the reaction mixture was stirred at rt for 24 h. After this time the solvent was evaporated, and the crude product was purified by flash chromatography (0–1% MeOH/DCM) to afford **(±)-BocGlyPyrDTDO** (0.08 g, 0.23 mmol 68%) as a viscous white solid.

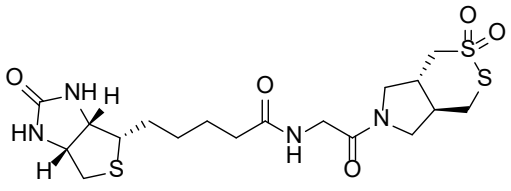
^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.38 (s, 1H), 4.01–3.95 (m, 1H), 3.93–3.79 (m, 2H), 3.74 (dt, J = 16.0, 8.5 Hz, 1H), 3.58 (t, J = 13.9 Hz, 1H), 3.48–3.34 (m, 2H), 3.26–3.04 (m, 3H), 2.9–2.7 (m, 1H), 2.53–2.29 (m, 1H), 1.44 (s, 9H).

^{13}C NMR ($CDCl_3$, 125 MHz): δ 167.38, 155.92, 80.06, 62.42, 62.14, 49.89, 49.38, 48.94, 43.39, 43.14, 42.96, 42.88, 41.72, 41.60, 35.18, 34.95, 28.46.

HRMS (ESI-TOF): Calculated for $[C_{13}H_{22}N_2O_5S_2 + Na]^+$: 373.0862; found: 373.0863.

Slow *s*-cis/*s*-trans interconversion with respect to the *N*-acylpyrrolidine ring[3] produces two diastereomers and additional NMR signals (e.g., PyrDTDO ring peak doubling) at room and even elevated (in DMSO-d_6) temperature.

_N_-(2-((4a_S_,7a_S_)- and (4a_R_,7a_R_)-2,2-Dioxidotetrahydro-1_H_-[1,2]dithiino[4,5-_c_]pyrrol-6(4_H_)-yl)-2-oxoethyl)-5-((3a_S_,4_S_,6a_R_)-2-oxohexahydro-1_H_-thieno[3,4-_d_]imidazol-4-yl)pentanamide, Biotin-GlyPyrDTDO (BGPD)



Biotin-GlyPyrDTDO (BGPD)

A solution of **(±)-BocGlyPyrDTDO** (0.08 g, 0.23 mmol) was stirred in TFA:DCM (6 mL, 1:2) for 2 h at rt. After this time the reaction was concentrated under vacuum to provide the deprotected ammonium trifluoroacetate salt as a brown oily solid, which was used in the next step without further purification. To a solution of the resulting solid and (+)-biotin *N*-succinimidyl ester (0.08 g, 0.23 mmol) in DMF was added Et_3N (0.06 mL, 0.46 mmol) and the reaction mixture was stirred at rt for 24 h. Then the solvent was evaporated and the crude product was purified by flash chromatography (0–10% MeOH/DCM) to afford **Biotin-GlyPyrDTDO** (0.06 g, 0.13 mmol, 56%) as a white solid.

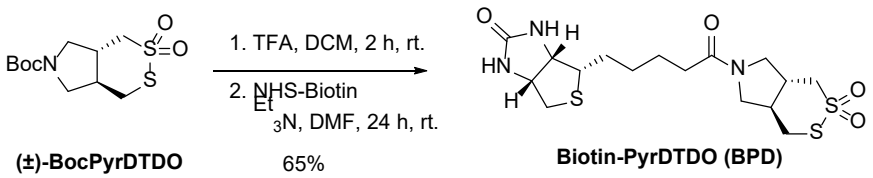
^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.94 (s, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.33–4.27 (m, 1H), 4.14–4.11 (m, 1H), 3.90–3.68 (m, 5H), 3.64 (td, J = 12.9, 2.0 Hz, 1H), 3.46 (ddd, J = 13.5, 6.4, 2.8 Hz, 1H), 3.35–3.30 (m, 1H), 3.24–3.17 (m, 1H), 3.11–3.05 (m, 1H), 3.02–2.91 (m, 1H), 2.82 (dd, J = 12.4, 5.1 Hz, 1H), 2.69–2.47 (m, 2H), 2.46–2.25 (m, 1H), 2.14 (t, J = 7.0 Hz, 2H), 1.65–1.58 (m, 1H), 1.55–1.42 (m, 3H), 1.39–1.25 (m, 2H).

^{13}C NMR (DMSO-d_6, 125 MHz): δ 172.31, 166.93, 166.86, 162.68, 61.79, 61.65, 61.00, 59.18, 55.41, 49.25, 48.76, 48.31, 42.72, 41.67, 41.05, 40.87, 40.78, 40.11, 34.94, 34.85, 34.80, 28.14, 28.02, 25.23.

HRMS (ESI-TOF): Calculated for $[C_{18}H_{28}N_4O_5S_3 + H]^+$: 477.1295; found: 477.1287.

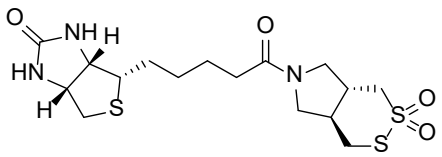
Slow *s*-cis/*s*-trans interconversion with respect to the *N*-acylpyrrolidine ring[3] produces two diastereomers and additional NMR signals (e.g., PyrDTDO ring peak doubling).

Synthesis of Biotin-PyrDTDO (BPD):



(±)-BocPyrDTDO 65% **Biotin-PyrDTDO (BPD)**

(3aS,4S,6aR)-4-(5-((4aR,7aR) and (4aS,7aS)-2,2-dioxidotetrahydro-1H-[1,2]dithiino[4,5-c]pyrrol-6(4H)-yl)-5-oxopentyl)tetrahydro-1H-thieno[3,4-d]imidazol-2(3H)-one, Biotin-PyrDTDO (BPD)



Biotin-PyrDTDO (BPD)

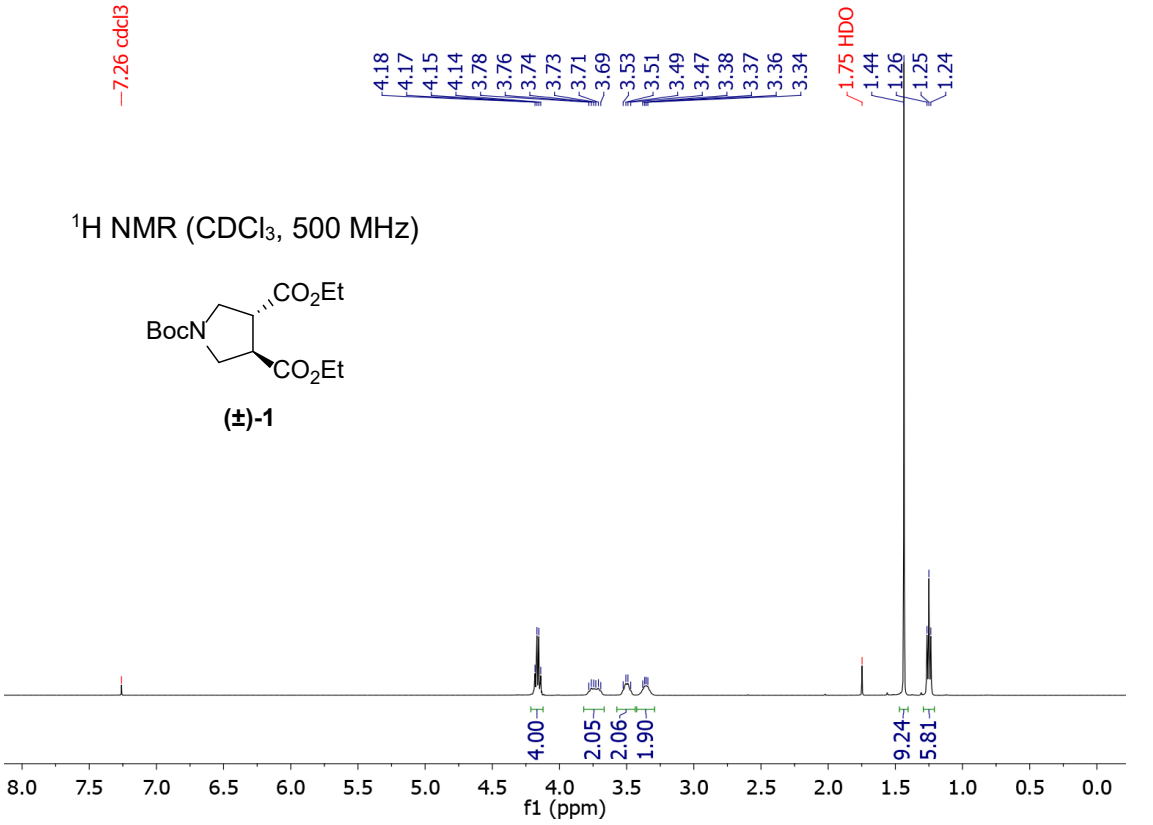
A solution of **(±)-BocPyrDTDO** (0.20 g, 0.68 mmol) was stirred in TFA:DCM (6 mL, 1:1) for 2 h at rt, monitoring by TLC. After reaction completion, the mixture was concentrated under vacuum to provide the deprotected ammonium trifluoroacetate salt as a brown solid, which was used in the next step without further purification. To a solution of the resulting solid and (+)-biotin *N*-succinimidyl ester in DMF was added Et_3N (0.20 mL, 1.40 mmol) and the reaction mixture was stirred at rt for 24 h. Then the solvent was evaporated and the crude product was purified by flash chromatography (0–10% MeOH/DCM) to afford **Biotin-PyrDTDO** (0.19 g, 0.45 mmol, 65%) as a white solid.

^{1}H NMR (DMSO-d_6, 600 MHz) δ 6.44 (s, 1H), 6.36 (s, 1H), 4.31 (dd, J = 7.8, 5.0 Hz, 1H), 4.14 (ddd, J = 7.1, 4.5, 1.8 Hz, 1H), 3.88–3.82 (m, 1H), 3.76–3.66 (m, 3H), 3.65–3.60 (m, 1H), 3.49–3.40 (m, 1H), 3.28–3.35 (m, 1H), 3.23–3.13 (m, 1H), 3.13–3.07 (m, 1H), 2.99–2.87 (m, 1H), 2.83 (dd, J = 12.4, 5.1 Hz, 1H), 2.58 (d, J = 12.4 Hz, 1H), 2.67–2.47 (m, 1H), 2.45–2.28 (m, 1H), 2.26–2.13 (m, 2H), 1.68–1.57 (m, 1H), 1.56–1.42 (m, 3H), 1.40–1.27 (m, 2H).

^{13}C NMR (DMSO-d_6, 151 MHz): δ 170.42, 170.35, 162.71, 61.89, 61.69, 61.05, 59.18, 55.45, 49.67, 49.22, 49.02, 48.52, 42.70, 41.71, 41.23, 40.34, 39.87, 35.06, 34.86, 33.18, 33.11, 28.29, 28.11, 24.27.

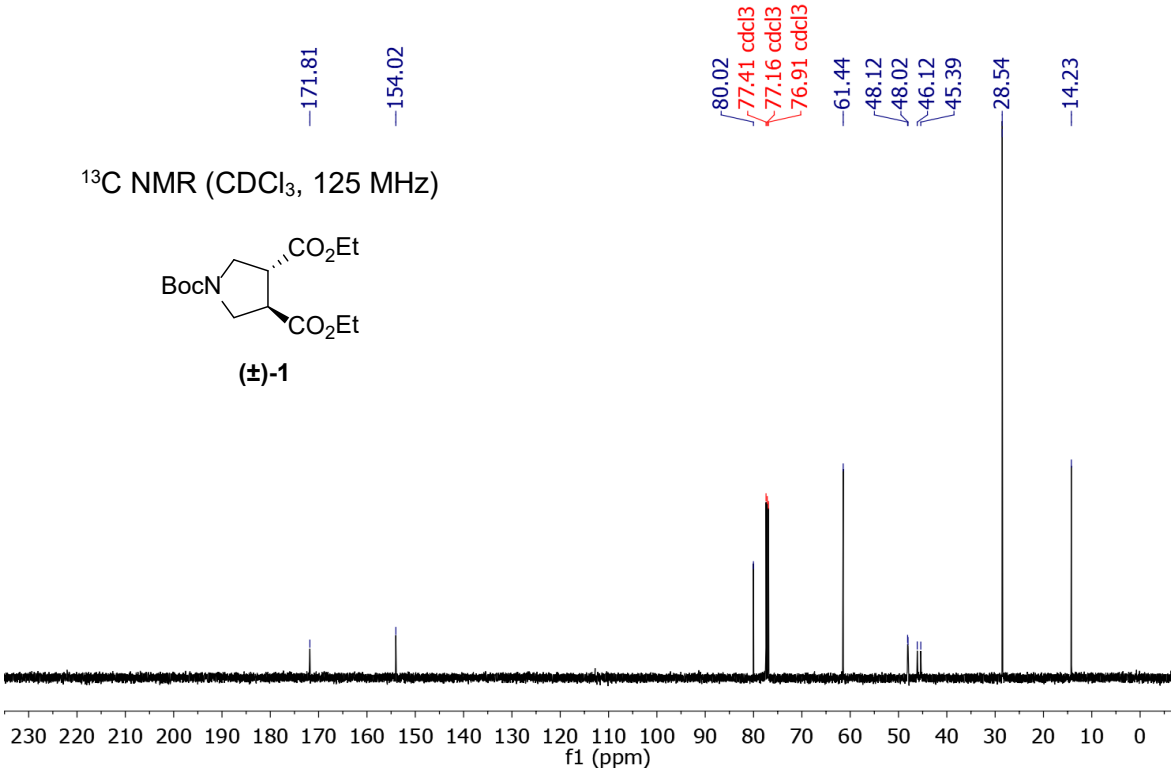
HRMS (ESI-TOF): Calculated for $[C_{16}H_{25}N_3O_4S_3 + Na]^+$: 442.0899; found: 442.0899.

Slow *s*-cis/*s*-trans interconversion with respect to the *N*-acylpyrrolidine ring[3] produces two diastereomers and additional NMR signals (e.g., PyrDTDO ring peak doubling).



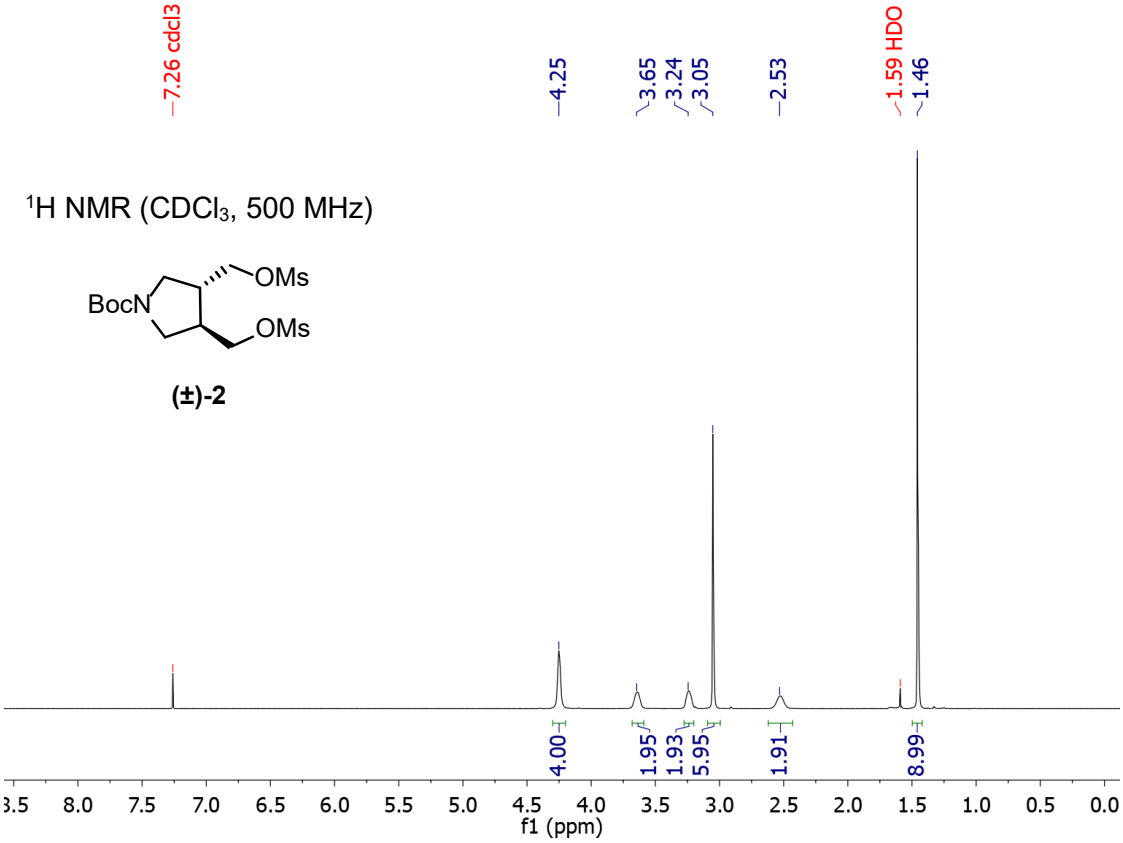
^{1}H NMR ($CDCl_3$, 500 MHz)

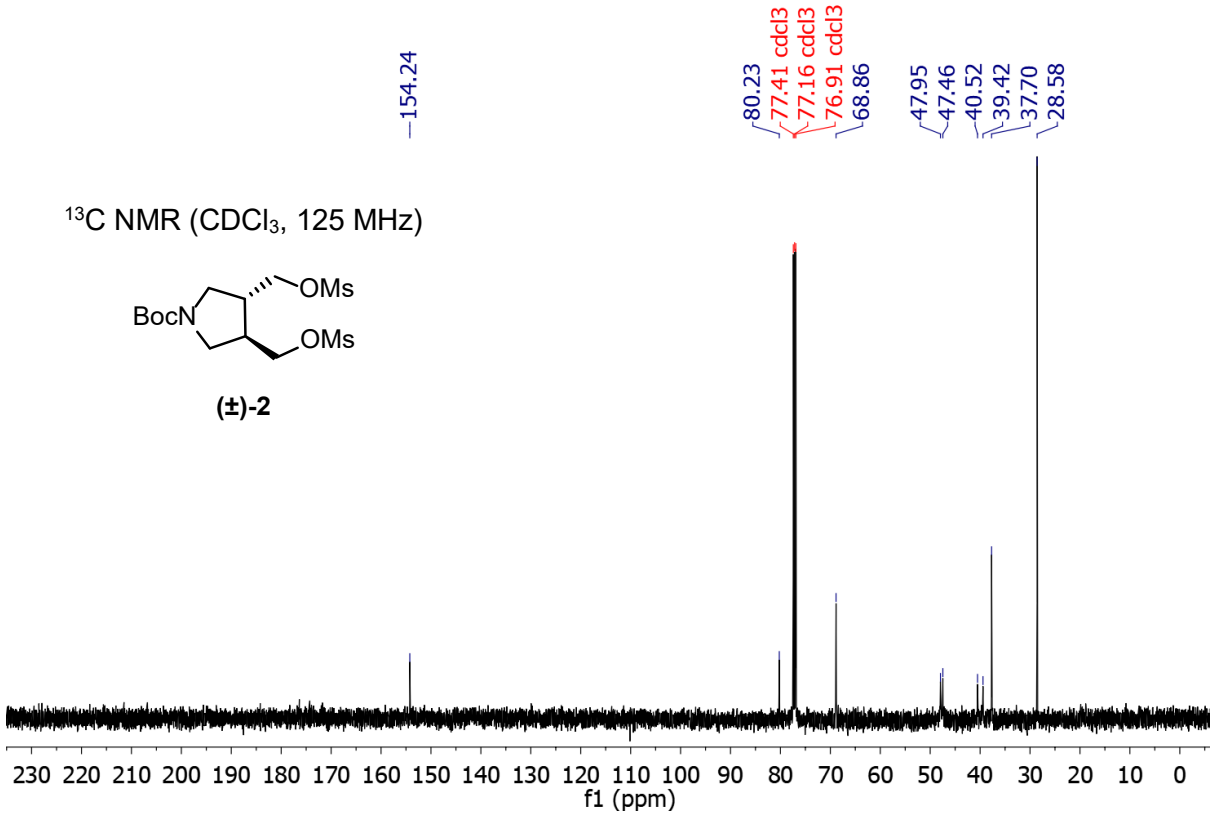
(±)-1

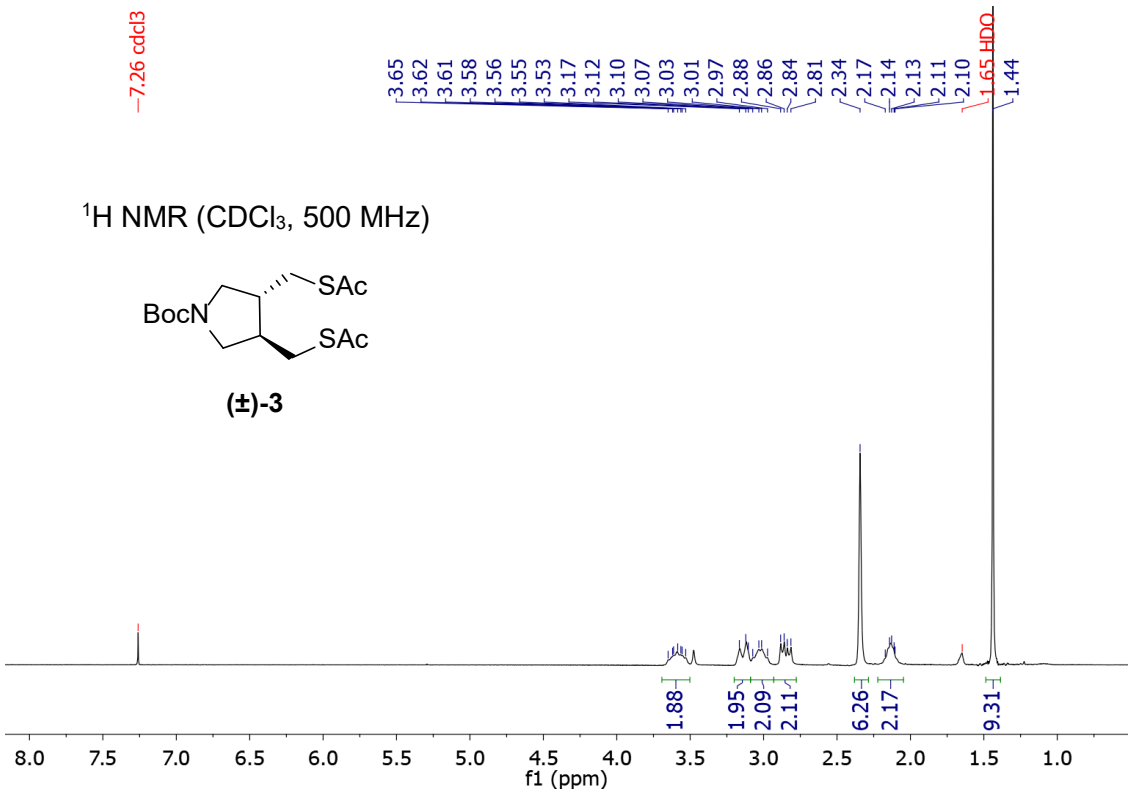


^{13}C NMR ($CDCl_3$, 125 MHz)

(±)-1

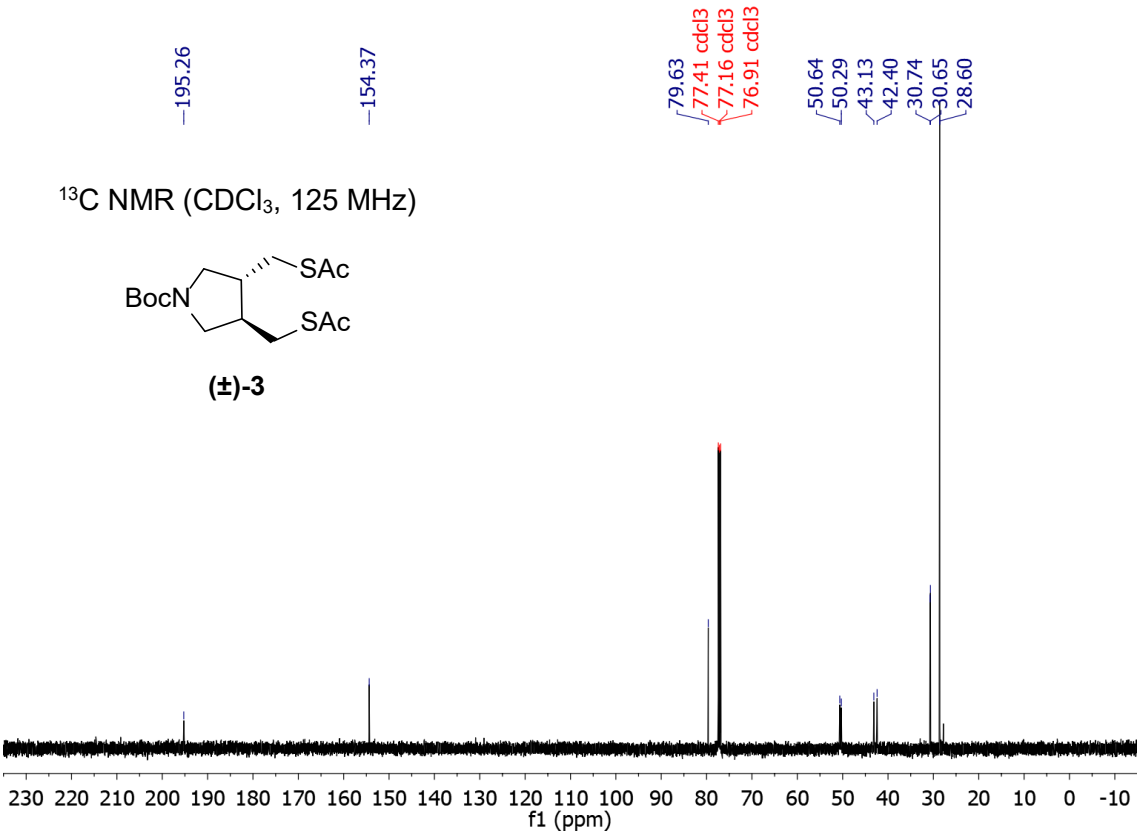

7.26 cdcl3
4.25
3.65
3.24
3.05
2.53
1.59 HDO
1.46
1H NMR (CDCl3, 500 MHz)
BocN
OMs
OMs
(±)-2
4.00
1.95
1.93
5.95
1.91
8.99
f1 (ppm)


154.24
80.23
77.41 cdcl3
77.16 cdcl3
76.91 cdcl3
68.86
47.95
47.46
40.52
39.42
37.70
28.58
13C NMR (CDCl3, 125 MHz)
BocN
OMs
OMs
(±)-2
f1 (ppm)



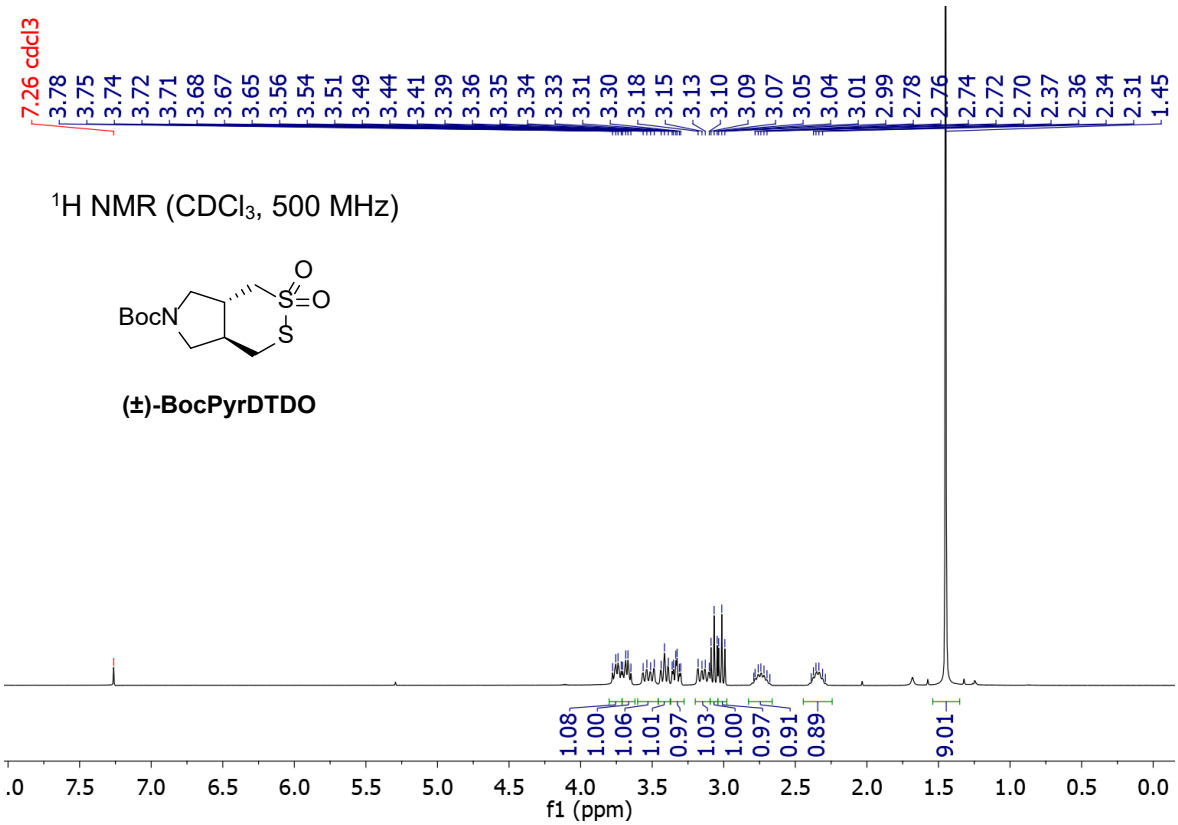
^{1}H NMR ($CDCl_3$, 500 MHz)

(±)-3



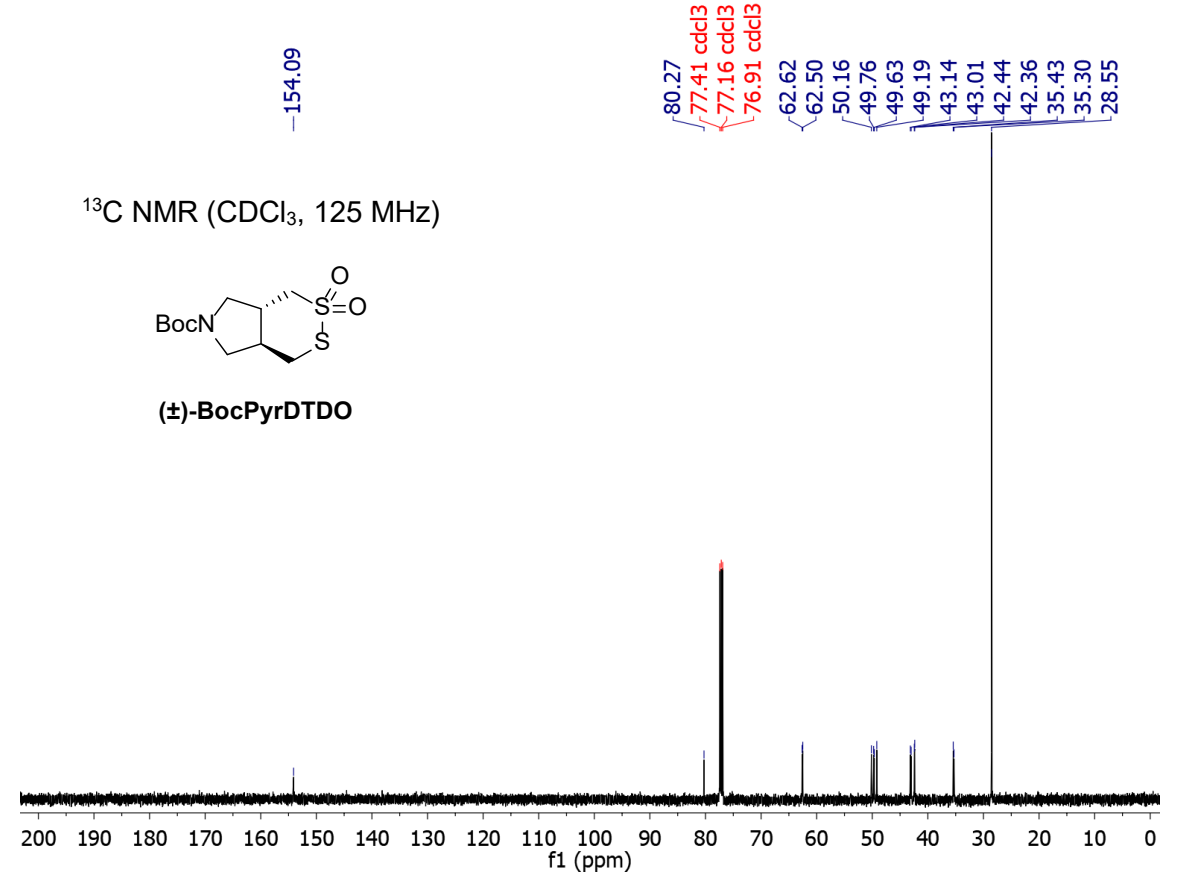
^{13}C NMR ($CDCl_3$, 125 MHz)

(±)-3



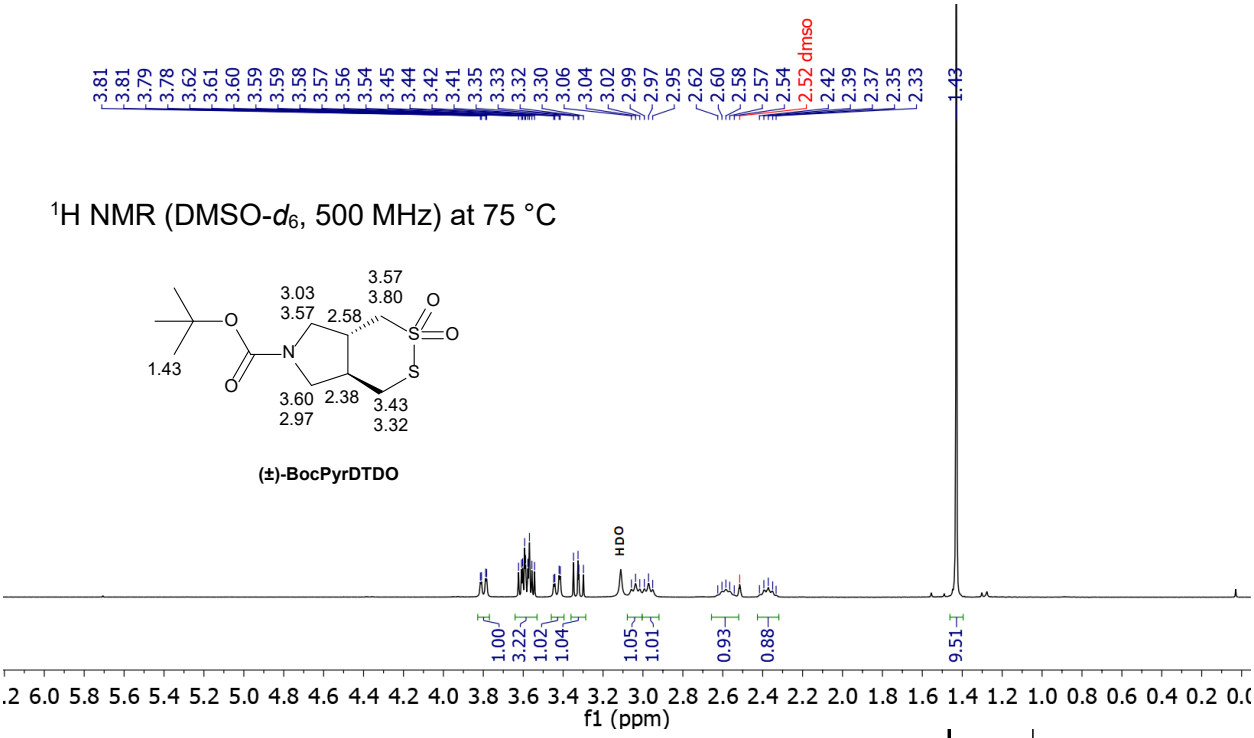
^{1}H NMR ($CDCl_3$, 500 MHz)

(±)-BocPyrDTDO



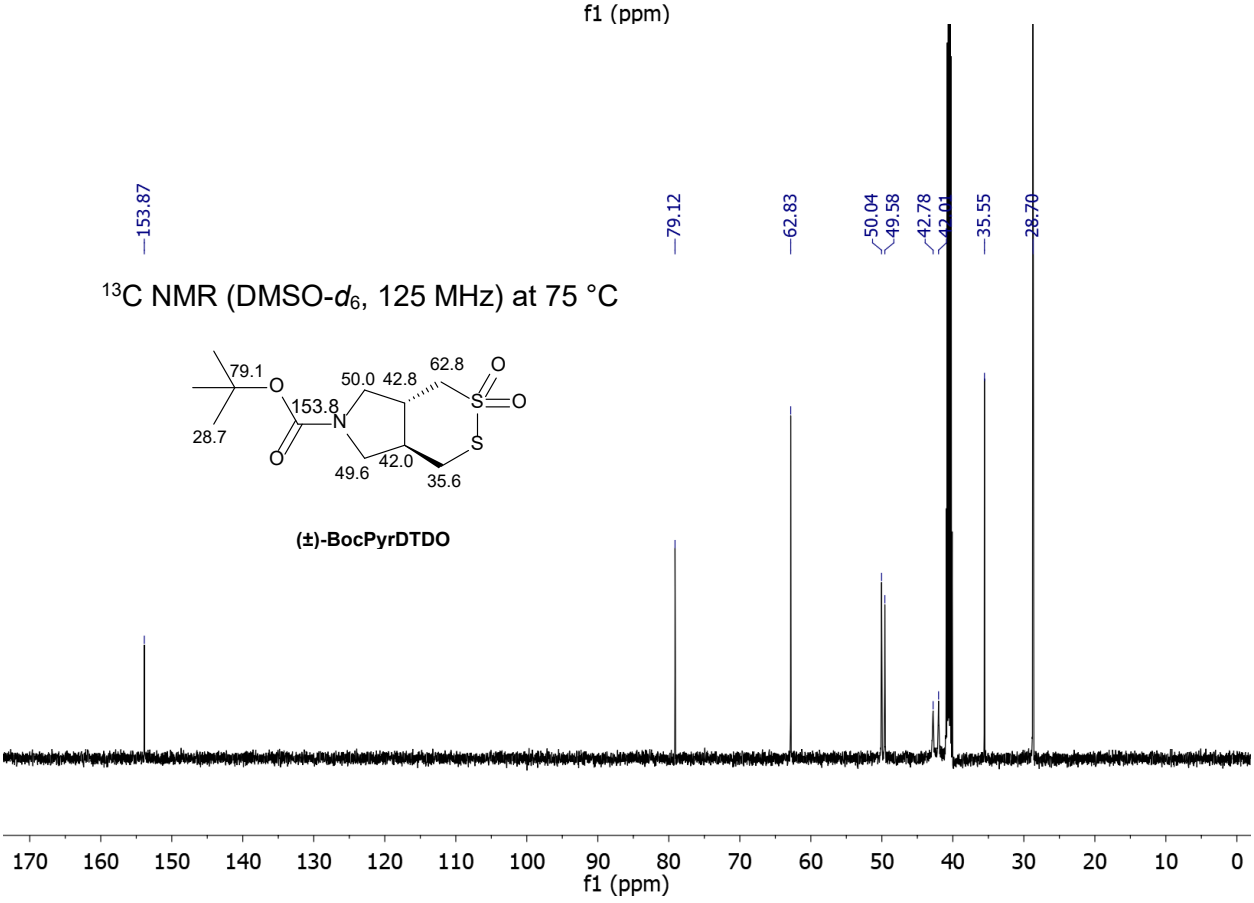
^{13}C NMR ($CDCl_3$, 125 MHz)

(±)-BocPyrDTDO



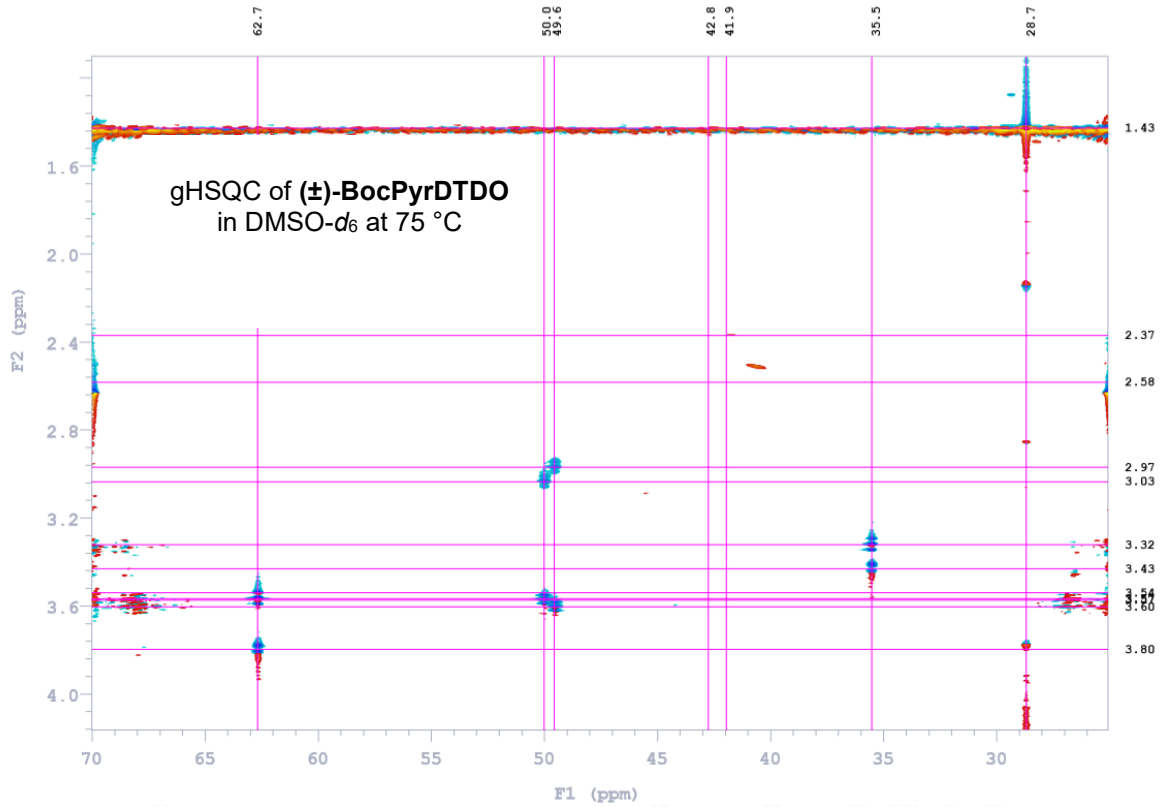
^{1}H NMR (DMSO-d_6, 500 MHz) at 75 °C

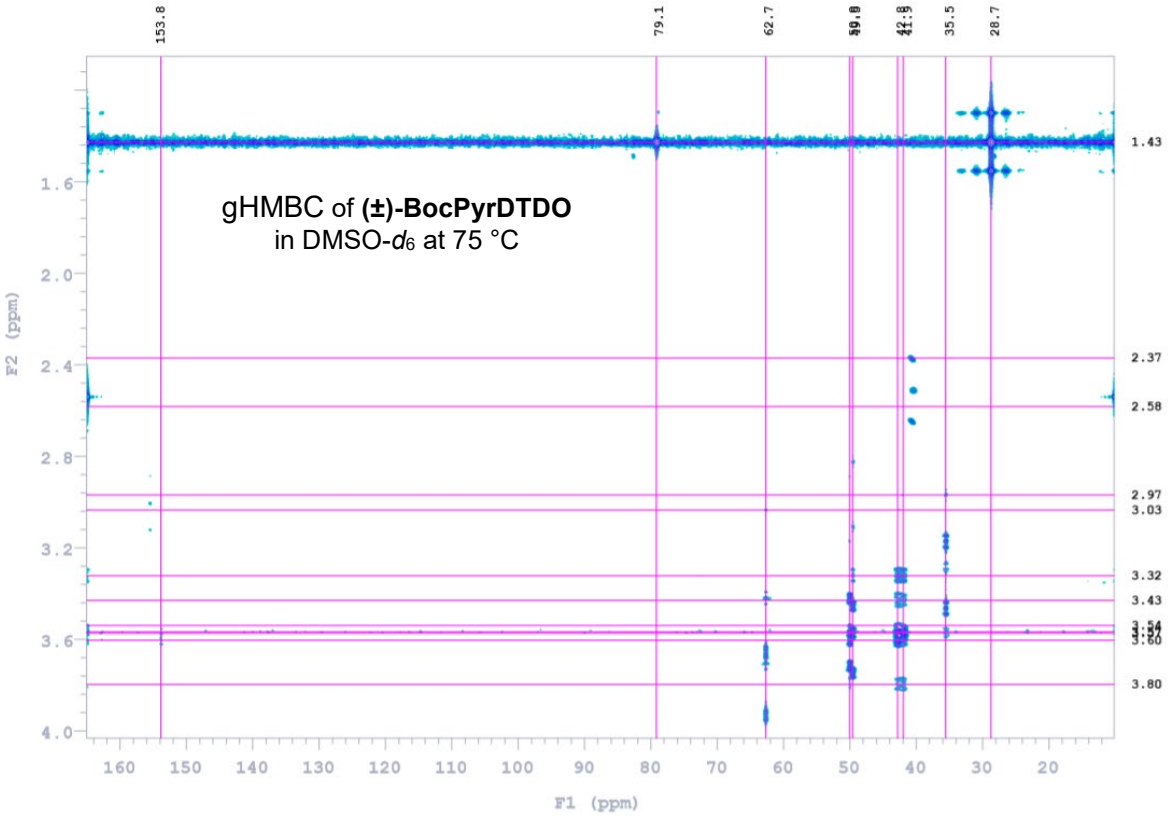
(±)-BocPyrDTDO

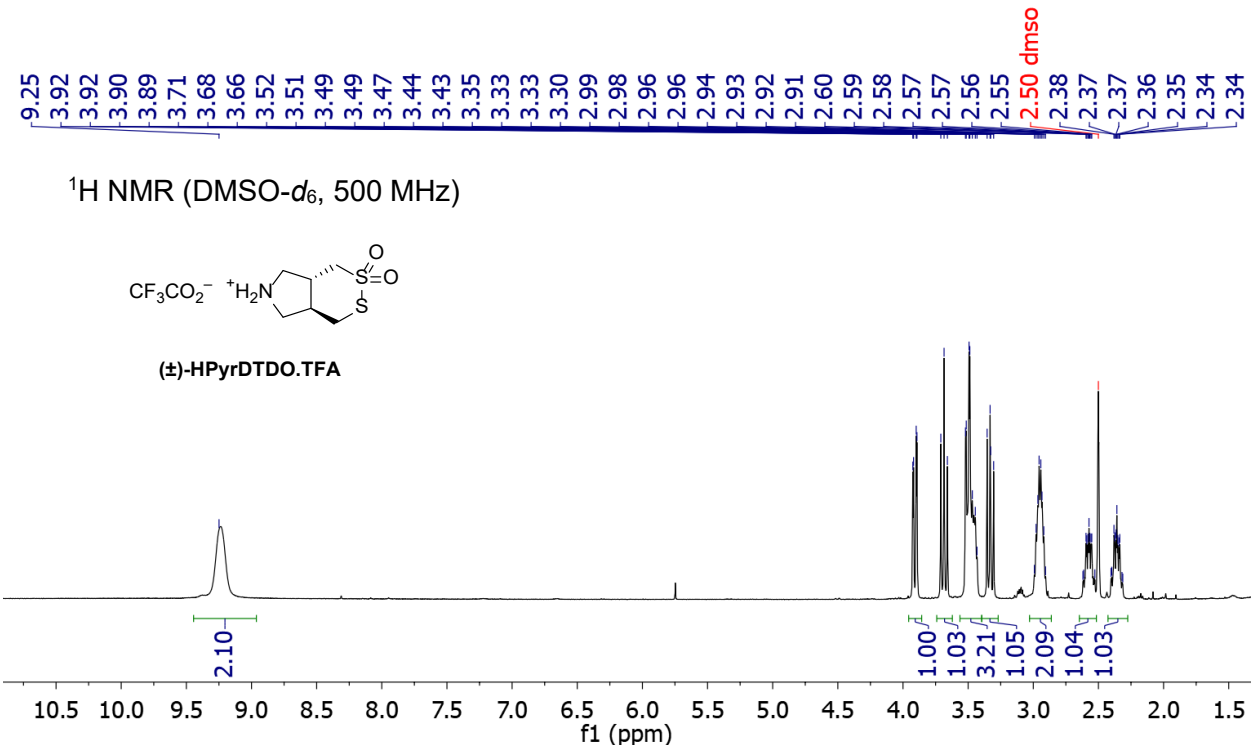


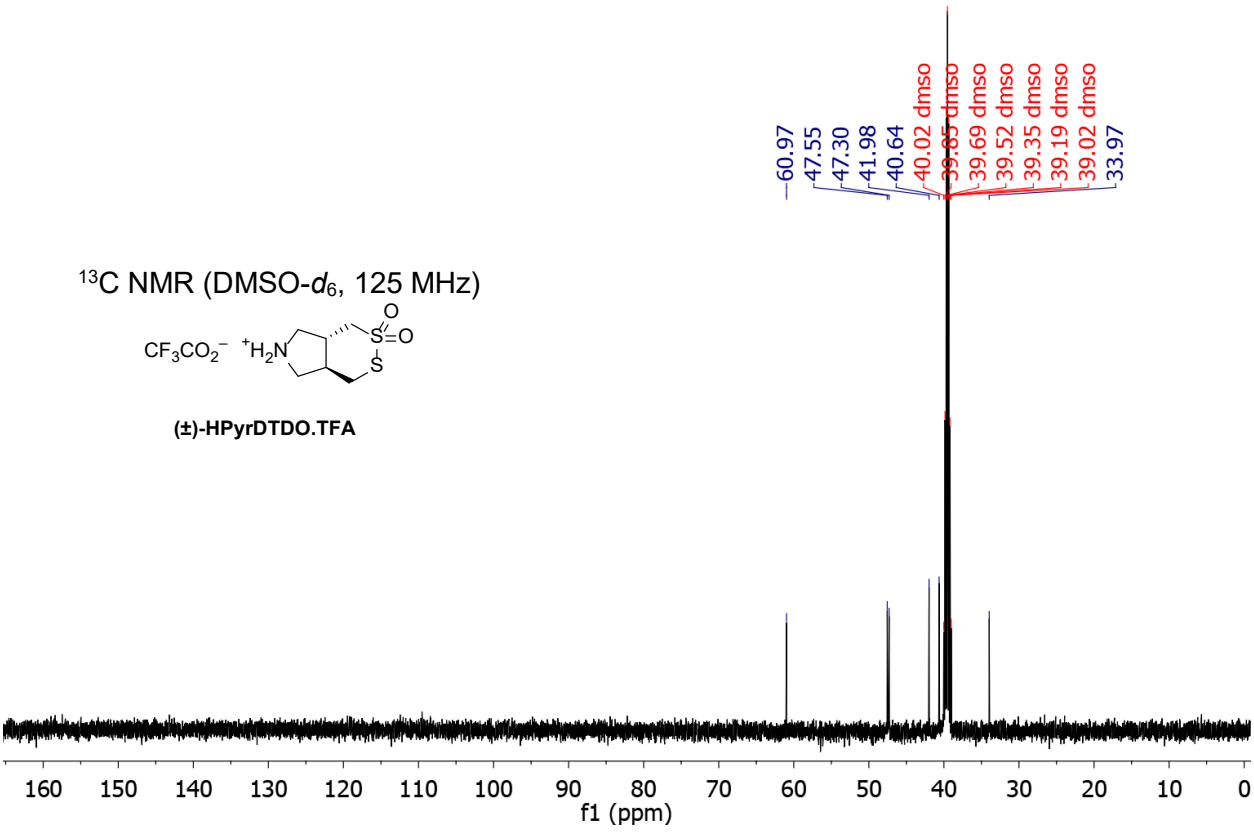
^{13}C NMR (DMSO-d_6, 125 MHz) at 75 °C

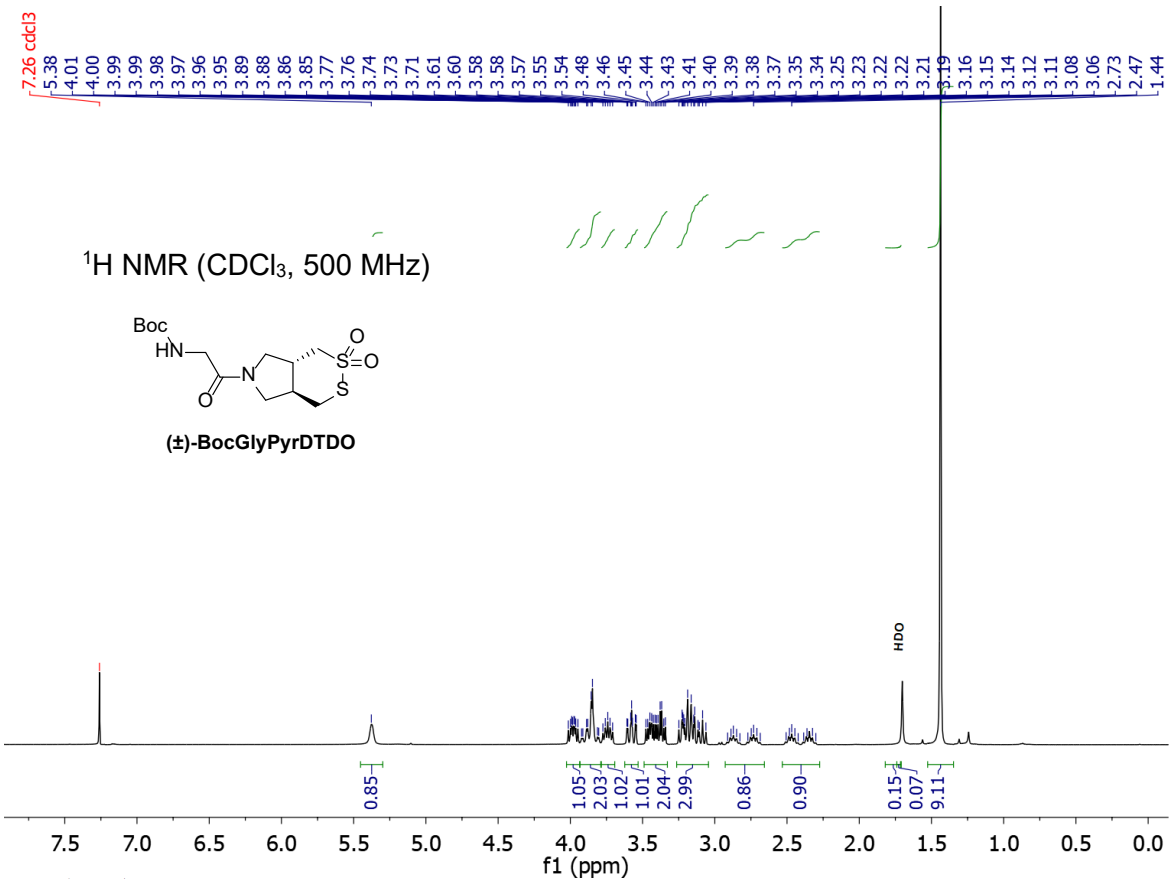
(±)-BocPyrDTDO


gHSQC of **(±)-BocPyrDTDO**
in DMSO-d_6 at 75 °C
62.7
50.0
49.6
42.8
41.9
35.5
28.7
1.43
2.37
2.58
2.97
3.03
3.32
3.43
3.60
3.80
F2 (ppm)
F1 (ppm)


gHMBC of **(±)-BocPyrDTDO**
in DMSO-d_6 at 75 °C
153.8
79.1
62.7
35.5
28.7
1.43
2.37
2.58
2.97
3.03
3.32
3.43
3.60
3.80
F2 (ppm)
F1 (ppm)

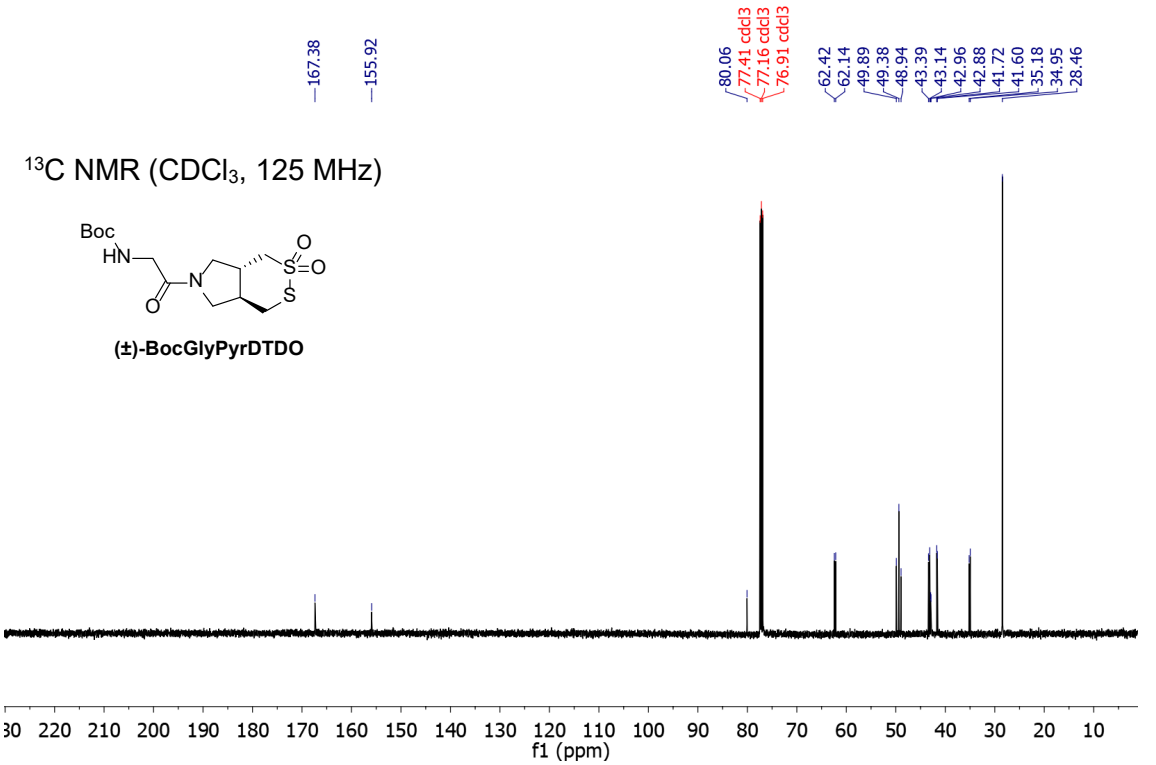

9.25 3.92 3.92 3.90 3.89 3.71 3.68 3.66 3.52 3.51 3.49 3.49 3.47 3.44 3.43 3.35 3.33 3.33 3.30 2.99 2.98 2.96 2.96 2.94 2.93 2.92 2.91 2.60 2.59 2.58 2.57 2.57 2.56 2.55 2.50 dmso 2.38 2.37 2.37 2.36 2.35 2.34 2.34
^{1}H NMR (DMSO-d_6, 500 MHz)
$CF_3CO_2^-$ ^+H_2N
(±)-HPyrDTDO.TFA
2.10 1.00 1.03 3.21 1.05 2.09 1.04 1.03
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5
f1 (ppm)


60.97 47.55 47.30 41.98 40.64 40.02 dmso 39.85 dmso 39.69 dmso 39.52 dmso 39.35 dmso 39.19 dmso 39.02 dmso 33.97
^{13}C NMR (DMSO-d_6, 125 MHz)
$CF_3CO_2^-$ ^+H_2N
(±)-HPyrDTDO.TFA
160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)



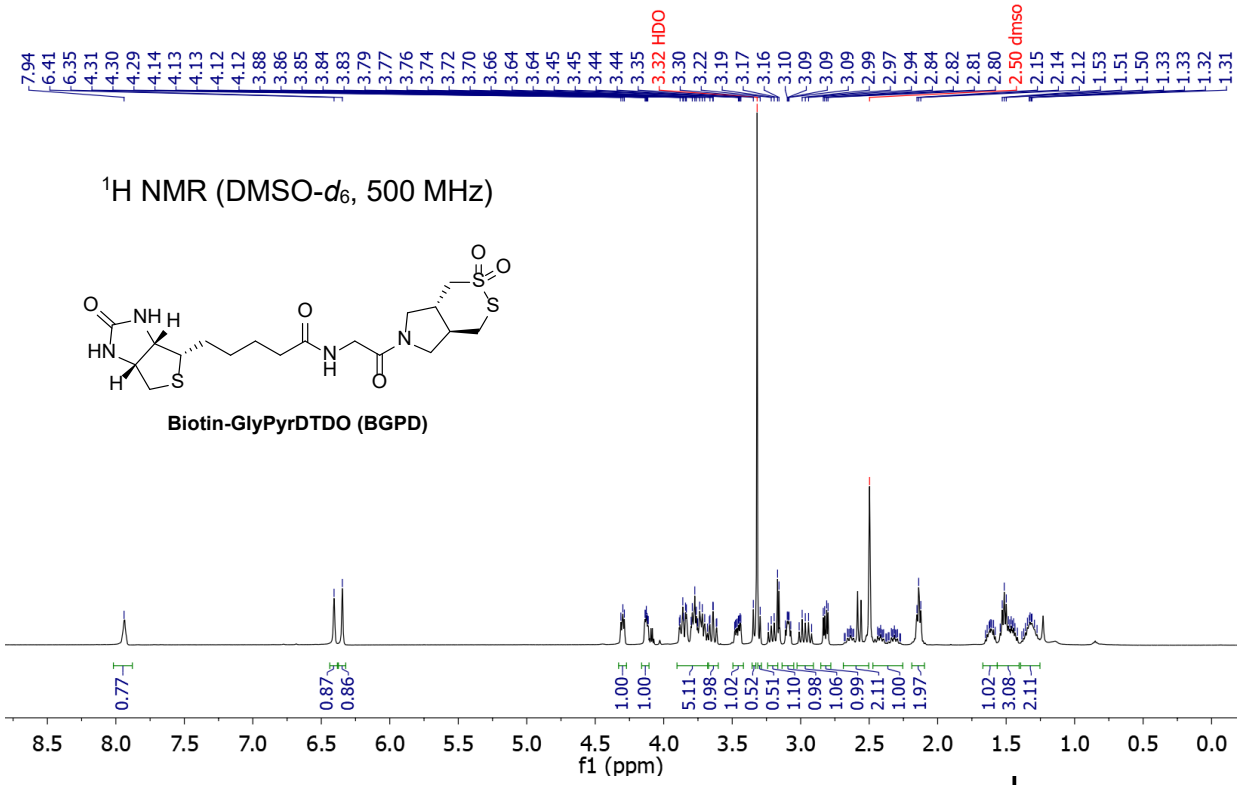
^{1}H NMR ($CDCl_3$, 500 MHz)

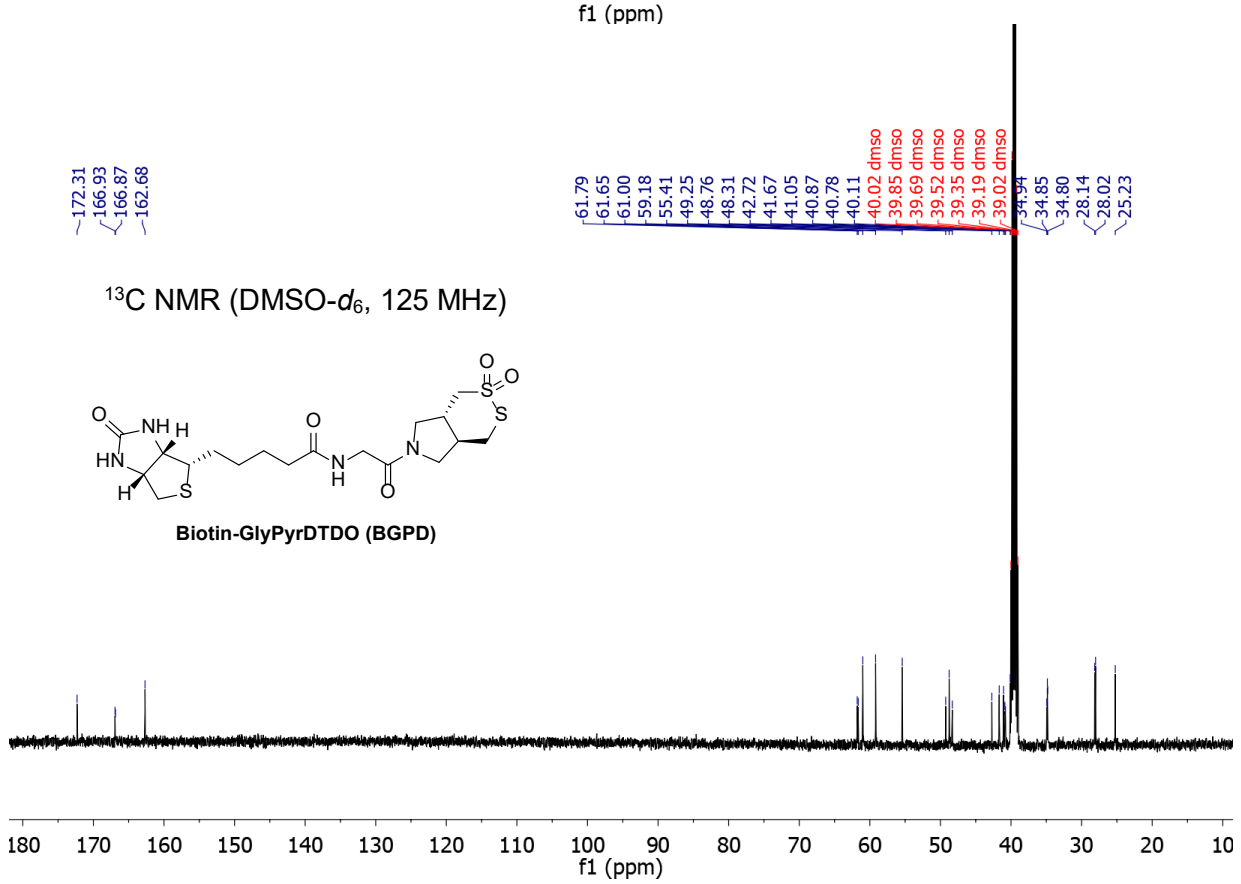
(±)-BocGlyPyrDTDO

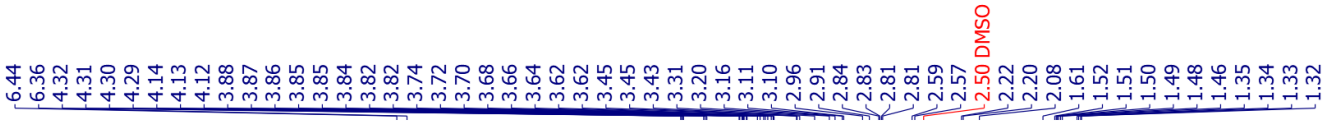


^{13}C NMR ($CDCl_3$, 125 MHz)

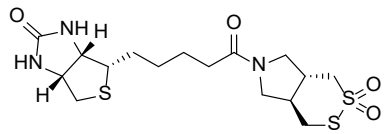
(±)-BocGlyPyrDTDO


7.94 6.41 6.35 4.31 4.30 4.29 4.14 4.13 4.13 4.12 4.12 3.88 3.86 3.85 3.84 3.83 3.79 3.77 3.76 3.74 3.72 3.70 3.66 3.64 3.64 3.45 3.45 3.44 3.44 3.35 3.32 HDO 3.30 3.22 3.19 3.17 3.16 3.10 3.09 3.09 3.09 2.99 2.97 2.94 2.84 2.82 2.81 2.80 2.50 dmso 2.15 2.14 2.12 1.53 1.51 1.50 1.33 1.32 1.31
1H NMR (DMSO-d6, 500 MHz)
Biotin-GlyPyrDTDO (BGPD)
0.77 0.87 0.86 1.00 1.00 5.11 0.98 1.02 0.52 0.51 1.10 0.98 1.06 0.99 2.11 1.00 1.97 1.02 3.08 2.11
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

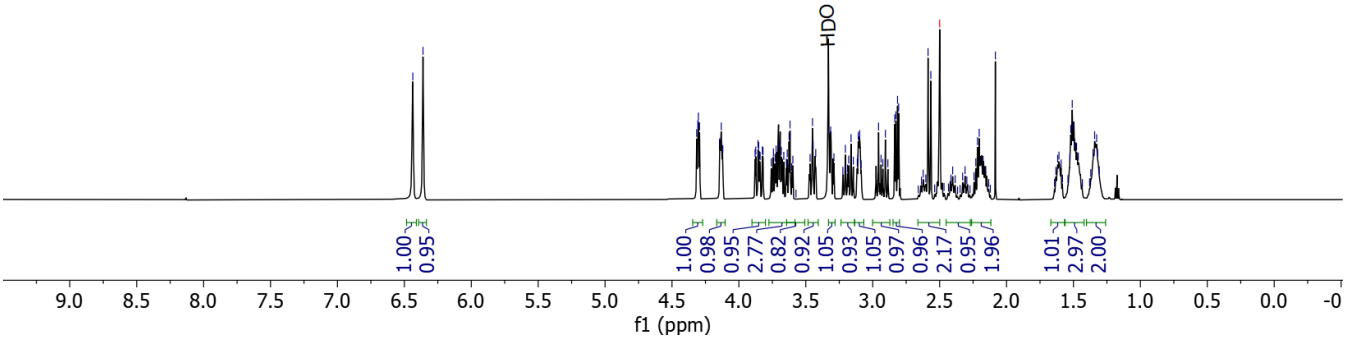

172.31 166.93 166.87 162.68
61.79 61.65 61.00 59.18 55.41 49.25 48.76 48.31 42.72 41.67 41.05 40.87 40.78 40.11 40.02 dmso 39.85 dmso 39.69 dmso 39.52 dmso 39.35 dmso 39.19 dmso 39.02 dmso 34.94 34.85 34.80 28.14 28.02 25.23
13C NMR (DMSO-d6, 125 MHz)
Biotin-GlyPyrDTDO (BGPD)
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
f1 (ppm)

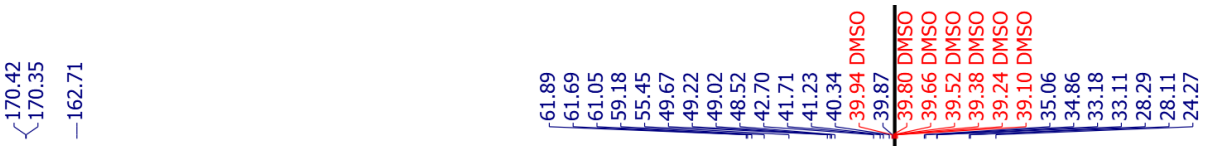


^{1}H NMR (DMSO-d_6, 600 MHz)

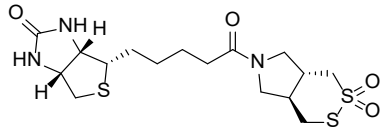


Biotin-PyrDTDO (BPD)

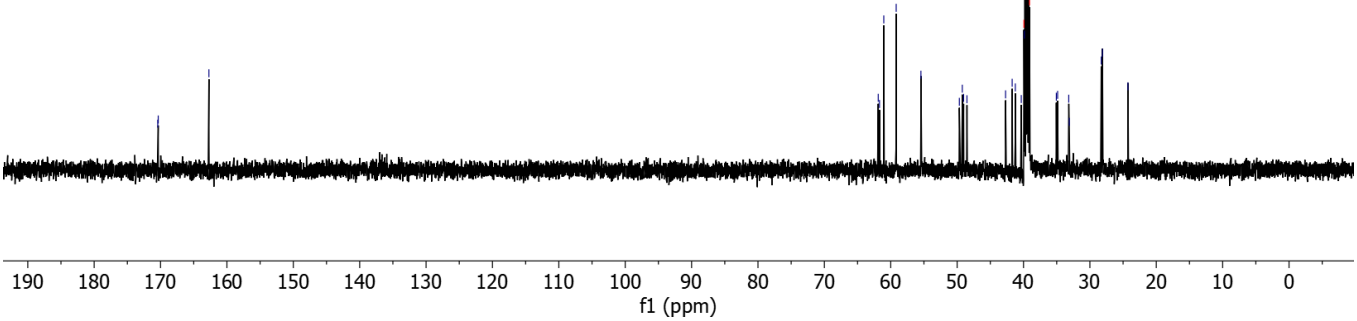




^{13}C NMR (DMSO-d_6, 151 MHz)



Biotin-PyrDTDO (BPD)



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3. For some case studies of N-acyl-prolyl isomerization please see: a) Kubyshkin, V.; Durkin, P.; Budisa, N., Energetic contribution to both acidity and conformational stability in peptide models. *New J. Chem.,* **2016,** *40* (6), 5209. b) Kubyshkin, V.; Budisa, N., Amide rotation trajectories probed by symmetry. *Org. Biomol. Chem.,* **2017,** *15* (32), 6764.