Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

A Trifluoromethylphenyl Diazirine-Based SecinH3 Photoaffinity Probe

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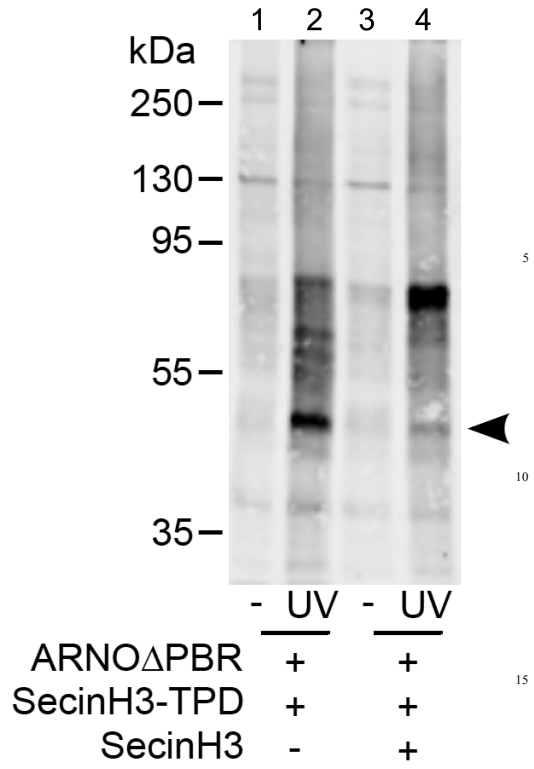


Figure S1: SecinH3 competes with SecinH3-TPD for the binding to ARNOΔPBR. H460 cells were harvested, permeabilised and the soluble proteins collected by centrifugation. The labelling reaction was done in proteome diluted to 2 mg/ml total protein. 1 µM ARNOΔPBR, 5 µM SecinH3-TPD and 15 µM SecinH3 in 10% DMSO were added as indicated. After separation on a 7.5% SDS-PAGE and Western blotting, the labelled proteins were detected with NeutrAvidin DyLight 800 fluorescent conjugate. 1 µM ΔPBR, 5 µM SecinH3-TPD and 15 µM SecinH3 in 10% DMSO were used. Arrowhead: ARNOΔPBR. Similar to Figure 2D, labelling of ARNOΔPBR was possible in cell lysate (lane 2). Competition with SecinH3 drastically reduced the amount of labelled ARNOΔPBR, and a new band between 70 and 80 kDa was observed (lane 4).

At present, we cannot attribute the ~70-80 kDa band to any specific interaction with SecinH3-TPD. A possible explanation is that SecinH3-TPD could interact via its reporter group with biotin binding protein. Searching the UniProtKB database for human proteins with a biotinyl-binding domain gave 5 hits, two of which with a size in the range of 70-80 kD. The QuickGO[S2] browser delivered an additional 80 kDa human protein with biotin binding function (search ID: GO:0009374). These proteins are:

UniProt accession number	Protein	Size (kDa)
P50747	Biotin-protein ligase	80.8
Q96RQ3	Methylcrotonyl-CoA carboxylase, subunit α, mitochondrial	80.5
P05165	Propionyl-CoA carboxylase α-chain, mitochondrial	80.1

Experimental

Materials and Methods

Chemicals were bought from *Alfa Aesar*, *Aldrich*, *Bachem* and *Fluka* and used without further purification. For synthesis, solvents of the grade per analysis from *Fluka*, *Merck* and *Riedel de Haen* were used.

For column chromatography, solvents of technical grade were distilled before use and silica gel from *Acros*, with particle dimension of 35-70 µm, was employed. Thin layer chromatography were carried out on Merck pre-coated silica gel plates (60F-254) and visualised using ultra violet light, $KMnO_4$ solution or *p*-anisaldehyde solution.

^1H and ^{13}C-NMR spectra were recorded on a Bruker AM 400 (^1H: 400 MHz, ^{13}C: 100 MHz) and Bruker AM 300 (^1H: 300 MHz) at room temperature. Chemical shifts are expressed in parts per million (ppm) and the spectra calibrated to residual solvent signals of $CDCl_3$ (7.27 ppm (1H) and 77.0 ppm (13C)).

Coupling constants are given in hertz (Hz) and the following notations indicate the multiplicity of the signals: s (singlet), d (doublet), brs (broad singlet), t (triplet), q (quartet), m (multiplet), ap. (apparent).

Synthesis

Compound 5

Potassium thiocyanate (4.49 g, 46.2 mmol) was added in one portion to a stirred solution of Piperonyloyl chloride (7.10 g, 38.5 mmol) in acetone (200 mL) at 55°C. The reaction mixture was stirred at this temperature for 1h. Subsequently, 2-(2-Boc-aminoethoxy)ethanol[S1] (11.8 g, 57.6 mmol in 60 mL acetone) was added at 55°C dropwise over 2 min with stirring. The reaction mixture was stirred at 55°C for 12 h. The reaction mixture was concentrated under reduced pressure and the remaining residue was partitioned between ethyl acetate (200 mL) and water (100 mL). The organic layer was washed with saturated sodium chloride (50 mL), dried and concentrated under reduced pressure. The remaining residue was purified using column chromatography (SiO_2) using ethyl acetate/ cyclohexane (1:1) as eluent to give **5** as a yellow oil (6.66g, 42%).

R_f (EtOAc/cyclohexane 1:1): 0.64

^1H NMR (DMSO): δ ppm 11.87 (1H, br. s), 7.48 (1H, d, J = 8.2 Hz),7.36 (1H, d, J = 1. Hz), 7.03-6.98

(1H, m), 6.77 (1H, t, J = 5.25), 6.15 (2H, s), 4.54-4.44 (2H, m), 3.73-3.67 (2H, m), 3.42 (2H, ap. t, J = 6.1 Hz), 3.07 (2H, ap. q, J = 5.9 Hz), 1.38 (9H, s)

^{13}C NMR (DMSO): 186.0, 170.4, 167.2, 155.2, 151.2, 147.4, 124.8, 108.6, 107.9, 102.0, 77.7, 69.2 67.5, 59.8, 28.2

HRMS (EI+) Cald for $C_{18}H_{24}N_2O_7SNa$: 435.1196 ($[M+Na]^{\cdot+}$), Found: 412.1191.

Compound 6

5 (1.60 g, 3.88 mmol) and 4-nitrophenylhydrazine (0.7 g, 4.57 mmol) were suspended in EtOH (22 mL) and stirred at 80 °C under reflux for 8 h. The reaction mixture was cooled and concentrated under reduced pressure. The remaining residue was purified using column chromatography (SiO_2) using (cyclohexane / EtOAc100:0 →45:55) as eluent to give **6** as a red solid (1.20 g, 60%).

R_f (EtOAc/cyclohexane 1:1): 0.83

^{1}H NMR ($CDCl_3$): δ ppm 8.28 (2H, d, J=8.8 Hz), 7.57 (2H, d, J=8.8 Hz), 6.96 - 7.00 (1H, m), 6.95 (1H, s), 6.82 (1H, d, J=7.8 Hz), 6.06 (2H, s), 4.96 (1H, br. s), 4.50 - 4.56 (2H, m), 3.84 - 3.90 (2H, m), 3.63 (2H,t, J=5.05 Hz), 3.36 (2H, t, J=4.53 Hz) 1.87 (2H, br. s) 1.46 (9H, s)

^{13}C NMR ($CDCl_3$): 167.9, 156.0, 153.8, 149.8, 148.1, 146.6, 142.8, 124.9, 124.7, 123.8, 120.7, 109.1, 108.8, 101.8, 79.2, 70.4, 69.1, 69.0, 40.4, 28.4

HRMS (ESI-TOF) Calculated for $C_{24}H_{27}N_5O_8Na$: 536.1752 ($[M+Na]^+$), Found: 536.1753.

Compound 7

6 (800 mg, 1.6 mmol) was added to formic acid (14.5 mL) at RT with stirring. The reaction mixture was stirred at RT for 2.5 h. The reaction was concentrated under reduced pressure and redissolved in formic acid (4 mL) and evaporated again. The remaining residue was co-evaporated twice with toluene (20 mL). The remaining residue was purified using column chromatography (SiO_2) using (CH_2Cl_2/MeOH 99:1→CH_2Cl_2/MeOH/triethylamine 98:1:1 → 96:3:1) as eluent to give **7** as a red solid (433 mg, 67%).

^{1}H NMR ($CDCl_3$): δ ppm 8.21 (2H, d, *J*=8.8 Hz), 7.53 (2H, d, *J*=8.8 Hz), 7.27 - 7.30 (1H, m), 6.99 - 7.41 (4H, m), 6.89 - 6.94 (1H, m), 6.89 (1H, s), 6.77 (1H, d, *J*=8.06 Hz), 6.01 (2H, s), 4.47 (2H, br. s), 3.86 (2H, br. s), 3.80 (2H, br. s) 3.17 (2H, br. s)

^{13}C NMR ($CDCl_3$): δ ppm 168.9, 153.8, 149.8, 148.1, 146.6, 142.7, 124.9, 124.7, 123.8, 120.7, 109.0, 108.7, 101.8, 72.6, 69.2, 69.0, 41.4

HRMS (ESI-TOF) Calculated for $C_{19}H_{19}N_5O_6Na$: 436.1228 ($[M+Na]^+$), Found: 436.1230.

Compound 8

N,N-Diisopropylethylamine (830 µl, 4.8 mmol) was added to a solution of HBTU (359 mg, 0.9 mmol) and D(-)-desthiobiotin (223 mg, 1.0 mmol) in DMF (4 ml). The mixture was stirred for 10 min at room temperature. A solution of **7** (in DMF 10 ml) was added dropwise over 5 min and the reaction stirred at RT for 1 h. The reaction mixture was partitioned between saturated sodium chloride and CH_2Cl_2. The organic layer was separated, dried and concentrated under reduced pressure. The remaining residue was purified using column chromatography (SiO_2) using ethyl acetate/ cyclohexane (1:1) as eluent to give **8** as a pale yellow solid (698 mg, 99%.)

^{1}H NMR ($CDCl_3$): δ ppm 8.27 (2H, d, *J*=8.8 Hz), 7.56 (2H, d, *J*=8.8 Hz), 6.96 (1H, d, *J*=1.26 Hz), 6.82 (1H, d, *J*=7.8 Hz) 6.94 (1H, s), 6.51 (1H, t, *J*=4.8 Hz), 6.05 (2H, s), 3.85 - 3.89 (2H, m), 4.50 - 4.54 (2H, m) 3.80 - 3.85 (1H, m), 3.68 - 3.74 (1H, m), 3.65 (2H, t, *J*=5.05 Hz), 3.48 (2H, q, *J*=5.05 Hz), 2.21 (2H, t, *J*=7.30 Hz), 1.66 (2H, quin, *J*=6.85 Hz), 1.18 - 1.54 (9H, m), 1.12 (3H, d, *J*=6.55 Hz),

^{13}C NMR ($CDCl_3$): 173.5, 171.8, 167.8, 164.0, 163.9, 153.8, 149.8, 148.1, 146.6, 142.7, 125.0, 124.7, 123.7, 120.6, 108.9, 108.7, 101.83, 70.0, 69.0, 68.9, 56.0, 51.4, 45.5, 39.1, 35.8, 35.1, 29.4, 29.1, 28.4, 26.1, 25.6, 25.1, 20.9, 20.6, 18.9

HRMS (ESI-TOF) Calculated for $C_{29}H_{35}N_7O_8Na$: 632.2439 ($[M+Na]^+$), Found: 632.2447.

Compound 9

10% palladium on carbon (126 mg) was added to a stirred solution of **8** (530 mg, 0.9 mmol) in THF (10 ml) and EtOH (10 ml) in an autoclave. The reactor was purged with argon and pressurized with hydrogen (9 bar). After 2 h the reaction was filtered through a pad of celite and concentrated under reduced pressure. The remaining residue was purified using column chromatography (SiO_2) using (CH_2Cl_2/MeOH 98:2 →95:5) as eluent to give **9** as a colorless solid (235 mg, 50%).

R_f (MeOH/CH_2Cl_2/triethylamine 10:89:1): 0.66

^{1}H NMR ($CDCl_3$): 7.88 (1H, t, J = 8.7 Hz), 6.61 (2H,d, J = 8.7 Hz), 6.30 (1H, s), 6.12 (1H, s), 6.06 (2H, s), 5.52 (2H, s), 4.36-4.33 (2H, m), 3.76-3.73 (3H, m), 3.65-3.55 (1H, m), 3.49 (2H, ap.t, J = 5.65 Hz), 3.23 (2H, ap.t, J = 5.65 Hz), 2.08 (2H, t, J = 7.2 Hz), 1.54-1.44 (1H, m), 1.34-1.21 (8H, m), 0.96 (3H, d, J = 6.4 Hz)

^{13}C NMR ($CDCl_3$): 173.6, 167.8, 164.1, 153.2, 150.9, 149.8, 148.5, 128.4, 127.4, 124.3, 122.6, 115.0, 109.7, 109.5, 102.9, 70.5, 69.8, 69.5, 56.3, 51.5, 36.5, 30.8, 30.0, 26.9, 26.5,16.8

HRMS (ESI+-TOF) Calculated for $C_{29}H_{37}N_7O_6Na$: 580.2878 ($[M+Na]^+$), Found: 580.2871.

SecinH3-TPD 3

4-(1-Azi-2,2,2-trifluoroethyl)benzoic acid (25 mg, 0.100 mmol) was dissolved in THF (300 µl), and 2 drops of DMF added. The flask was flushed with argon and a solution of oxalyl chloride (92 µl, 1.00 mmol) in THF (250 µl) added dropwise. The mixture was stirred for 2.5 h at room temperature and the solvent evaporate under reduced pressure. The oxalyl chloride was removed by co-evaporation with THF (1 x 10 mL) and using toluene (2 x 10 mL). The crude product was dissolved in CH_2Cl_2 (500 µl) and the flask purged with argon. **6** (63.2 mg, 0.100 mmol) and *N,N*-Diisopropylethylamine (38 µl, 0.200 mmol) in CH_2Cl_2 (1.30 ml) were added dropwise and the reaction stirred for 2 h. The reaction mixture was concentrated under reduced pressure. Purification by preparative TLC (CH_2Cl_2/ MeOH 9:1) gave **SecinH3-TPD 3** as an colorless solid (35 mg, 41%).

^{1}H NMR ($CDCl_3$): δ ppm 9.42 (1H, s), 8.02 (2H, d, J = 5.95 Hz), 7.86 (2H, br.s), 7.39-7.19 (3H, m), 6.90-6.89 (3H, m), 6.82 (1H, br.s), 6.72 (2H, m), 5.98 (2H, s), 5.58 (1H, s), 4.95 (1H, s), 4.50 (3H, br.s), 3.84 (3H, br.s), 3.61 (2H, br.s), 3.42 (2H, br.s), 2.17 (2H, br.s), 1.62 (2H, br.s), 1.54-1.25 (6H,

m), 1.06 (3H, br.s).

^{13}C NMR ($CDCl_3$): 173.6, 166.6, 165.0, 152.5, 149.5, 147.8, 139.0, 135.5 133.6, 132.6, 128.1, 126.5, 125.9, 123.7, 121.2, 120.4, 108.9, 108.5, 101.7, 69.8, 69.1 69.0, 56.2, 53.8, 51.6, 42.0, 39.2, 35.8, 29.3, 28.2, 26.2, 25.7, 25.3, 18.6, 17.3, 15.6.

HRMS (ESI+-TOF) Calculated for $C_{38}H_{41}F_3N_9O_7$: 792.3076 ($[M+H]^+$), Found: 792.3067.

dt-SecinPP 4

4-Benzoylbenzoic acid (122 mg, 0.500 mmol) was dissolved in THF (1.5 mL), and 2 drops of DMF added. The flask was flushed with argon and a solution of oxalyl chloride (846 µl, 5.0 mmol) in THF (2.5 mL) added dropwise. The mixture was stirred for 2.5 h at room temperature and the solvent evaporate under reduced pressure. The oxalyl chloride was removed by co-evaporation with THF (1 x 10 mL) and toluene (2 x 10 mL). The crude product was dissolved in CH_2Cl_2 (5 mL) and the flask purged with argon. **6** (290 mg, 0.500 mmol) and *N,N*-Diisopropylethylamine (172 µL, 1.00 mmol) in CH_2Cl_2 (5.0 ml) were added dropwise and the reaction stirred for 2 h. The reaction mixture was concentrated under reduced pressure. Purification by using column chromatography (SiO_2) using (CH_2Cl_2/ MeOH 9:1) as eluent gave **4** as a colorless solid (146 mg, 41%).

^{1}H NMR ($CDCl_3$): δ ppm 9.24 (1H, s), 7.74 (4H, d, J = 8.6 Hz), 7.65-7.55 (3H, m), 7.50-7.40 (4H, m), 7.28-7.23 (2H, m), 6.93 (1H, dd, J = 8.2 Hz, J = 1.6 Hz), 6.89 (1H, d, J = 1.6 Hz), 6.71 (1H, d, J = 8.2 Hz), 6.63 (1H, t, J = 5.4 Hz), 5.96 (2H, s), 5.58 (1H, s), 4.95 (1H, s), 4.49-4.42 (3H, m), 3.81 (3H, m), 3.61 (2H, ap.t J = 4.85 Hz), 2.24-2.14 (2H, m), 1.64-1.54 (2H, m), 1.43-1.34 (2H, m), 1.30-1.15 (4H, m), 1.05 (3H, d, J = 6.45 Hz)

^{13}C NMR ($CDCl_3$): 195.6, 173.5, 167.3, 166.3, 163.8, 152.8, 149.3, 147.7, 140.9, 138.1, 137.3, 135.3, 134.0, 132.5, 130.9, 129.8, 128.3, 126.5, 126.0, 123.5, 120.9, 120.4, 108.8, 108.5, 101.6, 69.9, 69.0, 68.9, 65.8, 56.0, 51.4, 39.1, 37.2, 36.0, 29.3, 29.2, 28.6, 25.8, 25.2, 15.7, 15.2.

HRMS (ESI+-TOF) Calculated for $C_{44}H_{47}N_7O_8SNa$: 856.3099 ($[M+Na]^+$), Found: 856.3081.

General procedure for the labelling reaction

SecinH3-TPD was conserved dried at 4°C in the absence of light. Aliquots reconstituted to 25 mM in DMSO were stored at room temperature for several months. Further dilutions were done directly prior to use and discarded after the experiment.

All the labelling reactions were performed on ice with pre-chilled labelling buffer (50 mM Tris pH 7.65, 300 mM NaCl, 3 mM $MgCl_2$, 10 % Glycerol, 0.005 % Triton X-100). The proteins were prediluted in labelling buffer and aliquoted in 1.5 ml test tubes. The compounds were pre-diluted in DMSO, added to the protein dilution and mixed by pipetting.

The samples were incubated in the dark for 10 min and then irradiated for 10 min with UV light at 365 nm (3UV Lamp, bulb power 8 W, UVA output at 1 cm: 2800 µW/cm^2).

For western blot analysis aliquots were taken before and after irradiation, mixed with 1/6 volume of 6X loading buffer and boiled for 5 min. The samples were separated by PAGE and transferred on membrane by western blotting. The membrane was blocked for 1 h with 5 % BSA/TBS-T w/v.

For detection of total protein, the membrane was incubated with His5-antibody (diluted 1:2 000 in 5 % BSA/TBS-T w/v with 0.02 % thimerosal) for 1 h, washed 3-times 5 min with TBS-T and then incubated for 30-60 min with near infrared (NIR) dye coupled secondary antibody. After washing 3-times 5 min with TBS-T, visualisation was done by NIR immunofluorescence on an Odyssey scanner.

For detection of labelled protein, the membrane was incubated with NeutrAvidin DyLight 800 (1:40 000 in 5 % BSA/TBS-T w/v) for 30-60 min, washed at least 4-times 5 min with TBS-T and visualised as above.

6 X Loading buffer
Tris pH 6.8 49.5 mM
DTT 600 mM
SDS 15 % w/v
glycerin 30 %
bromophenolblue

Labeling of ARNO-Sec7 and ARNOΔPBR in cell lysates

HEK (Human Embryonic Kidney 293) or H460 cells, respectively, were harvested, permeabilised and the soluble proteins collected by centrifugation. The labelling reaction was performed in diluted proteome (1.1 mg/ml total protein) with or without addition of purified ARNO-Sec7 (1 µM) or ARNOΔPBR, respectively, and with 20 µM SecinH3-TPD in 20% diglyme. Labelling of the proteome without addition of purified ARNO-Sec7 is shown in lane 4. After separation on a 10% SDS-PAGE and Western blotting, the labelled proteins were visualised with horseradish peroxidase streptavidin conjugate.

Supporting Information References

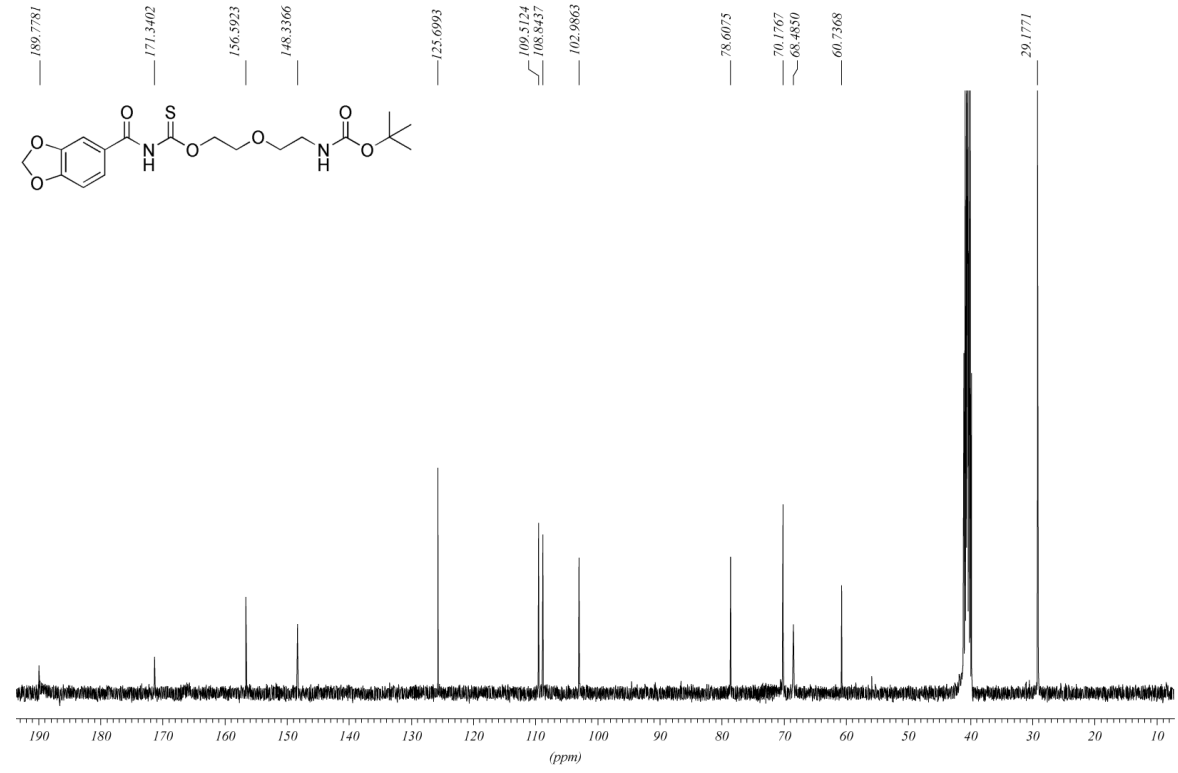
S1) Y. Zhang, M.-k. So, A. M. Loening, H. Yao, S. S. Gambhir and J. Rao, *Angew. Chem., Int. Ed.*, 2006, 45, 4936

S2) QuickGO is a fast web-based browser for Gene Ontology terms and annotations, which is provided by the UniProtKB-GOA group at the European Bioinformatics Institute (EBI). Adress: http://www.ebi.ac.uk/QuickGO/

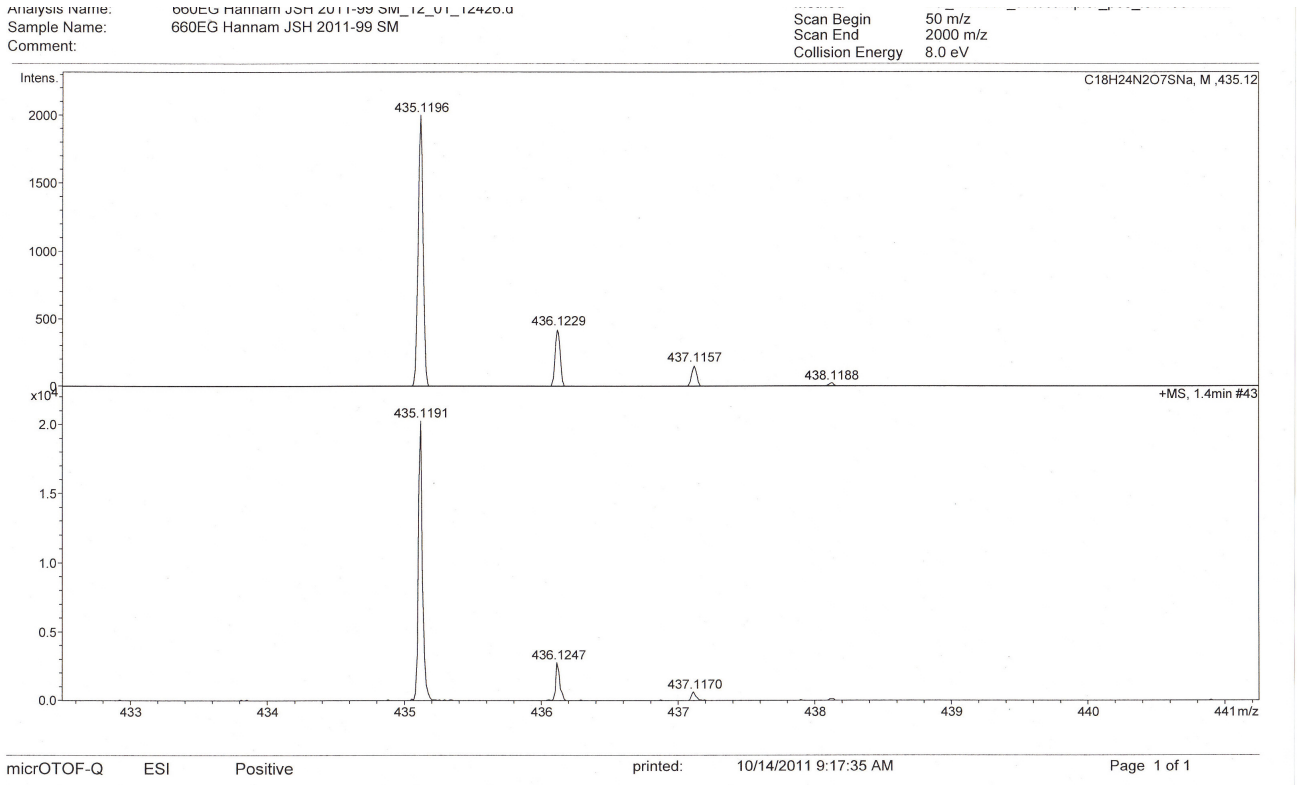
Copies of Spectral Data

^{1}H NMR of Compound (5)

^{13}C NMR of Compound (5)



Accurate Mass Spectra of Compound (5)



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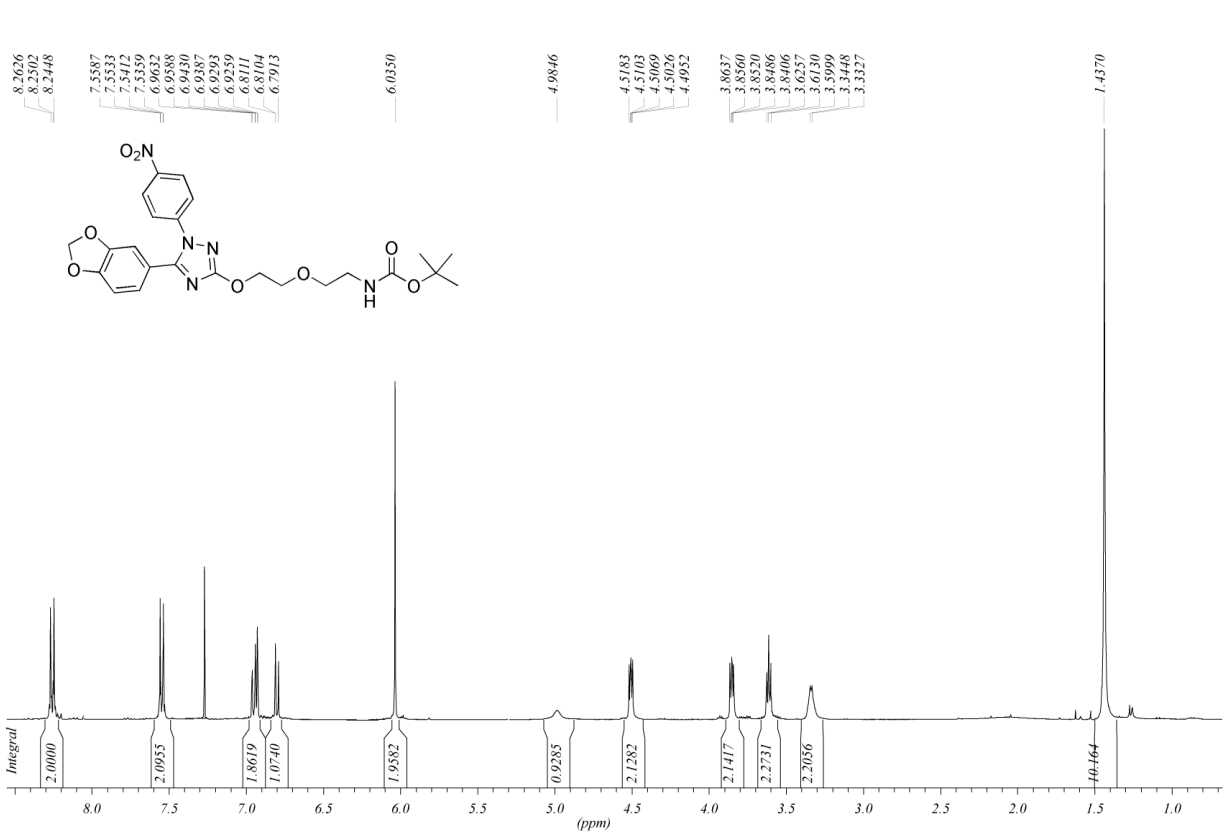

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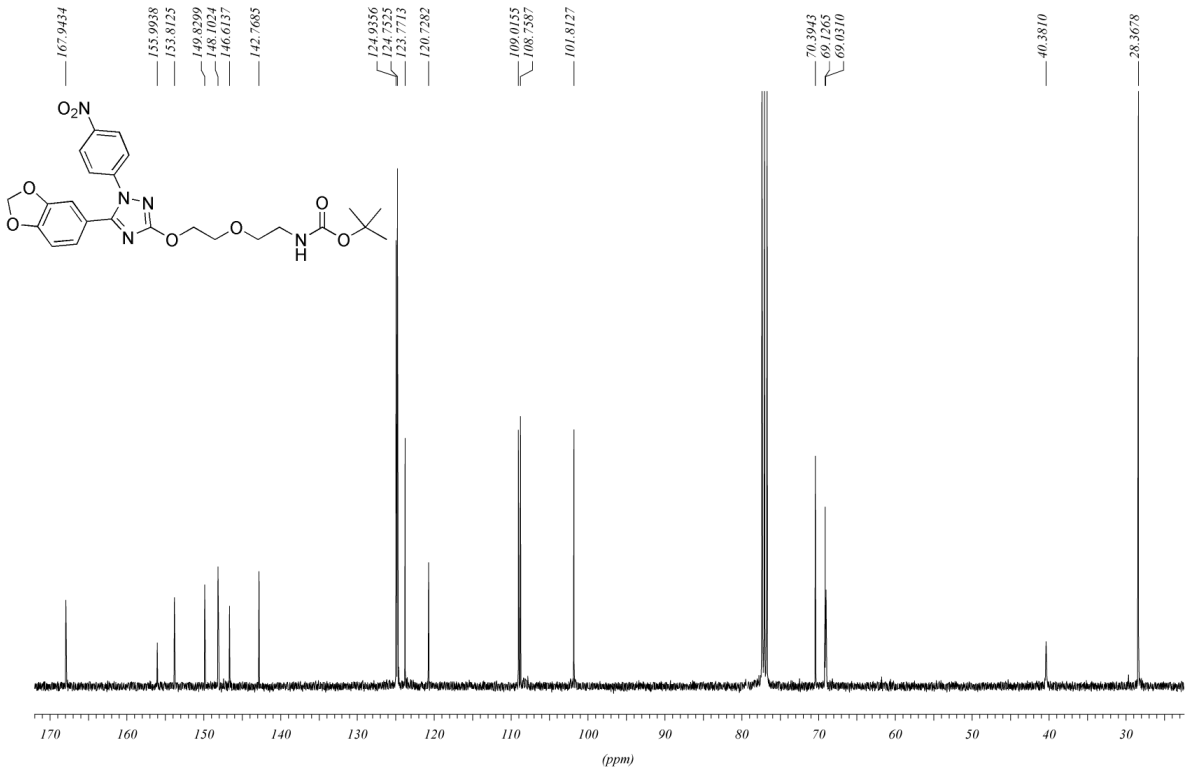

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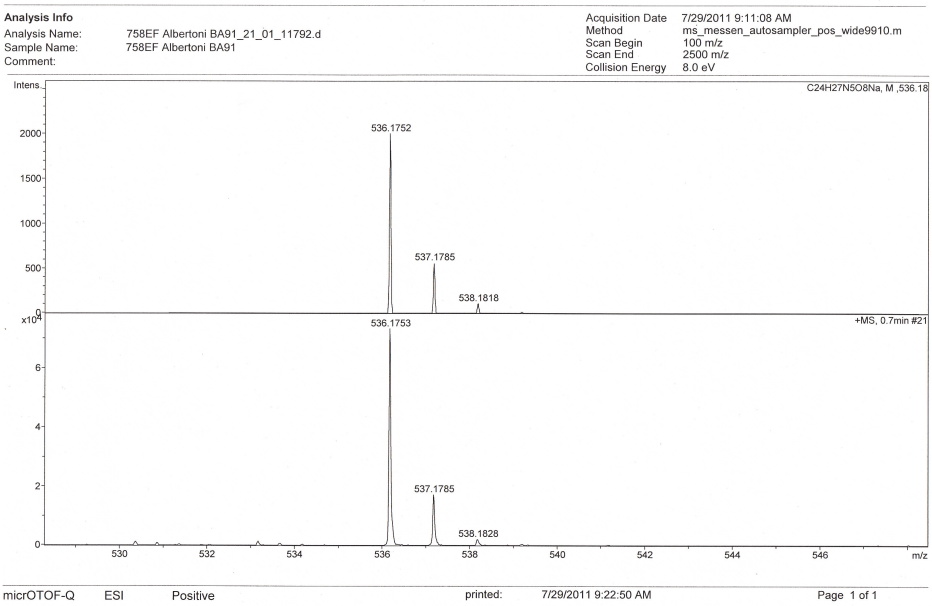

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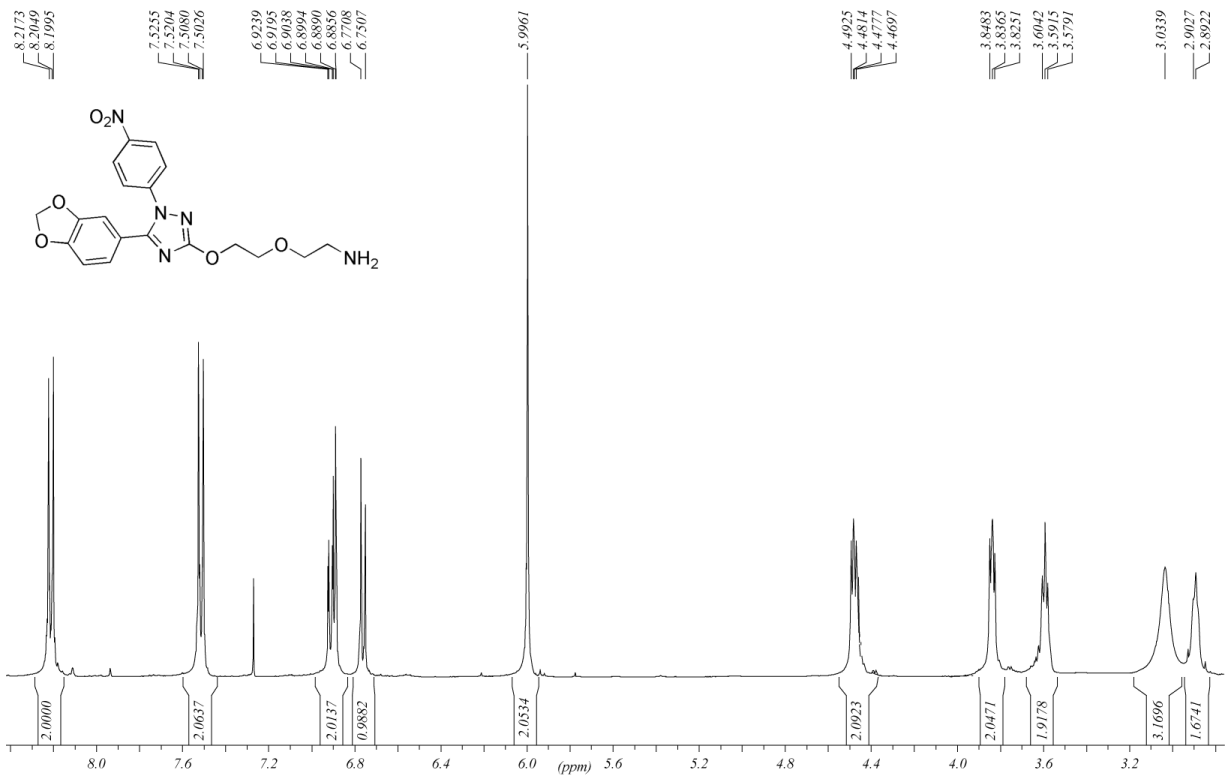

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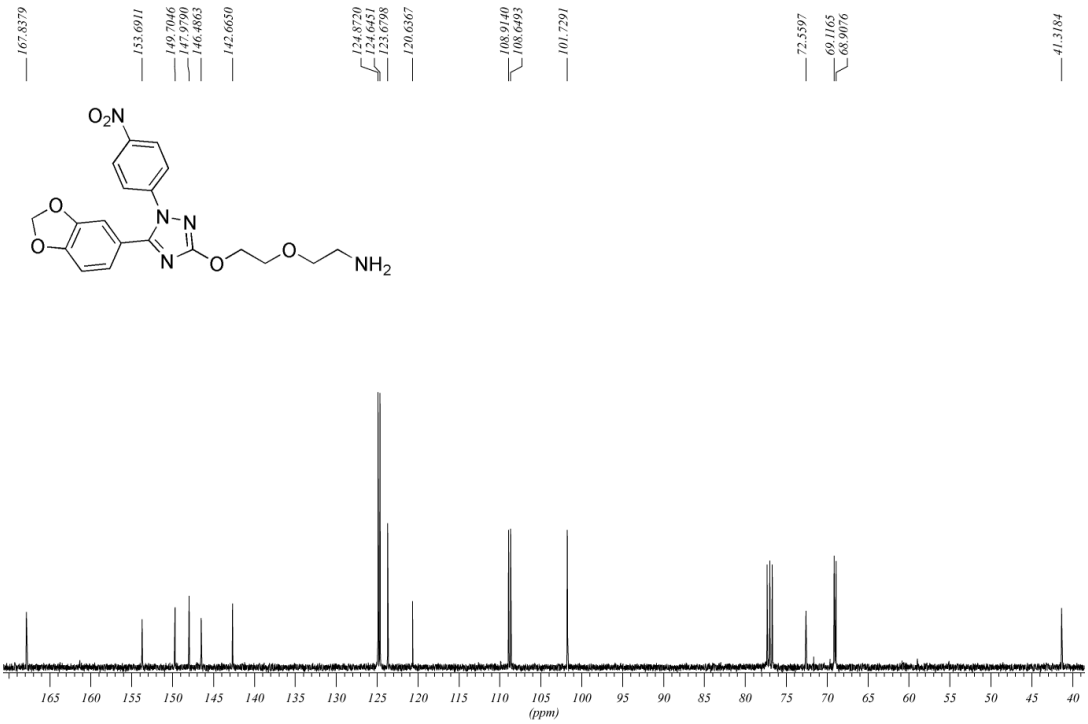

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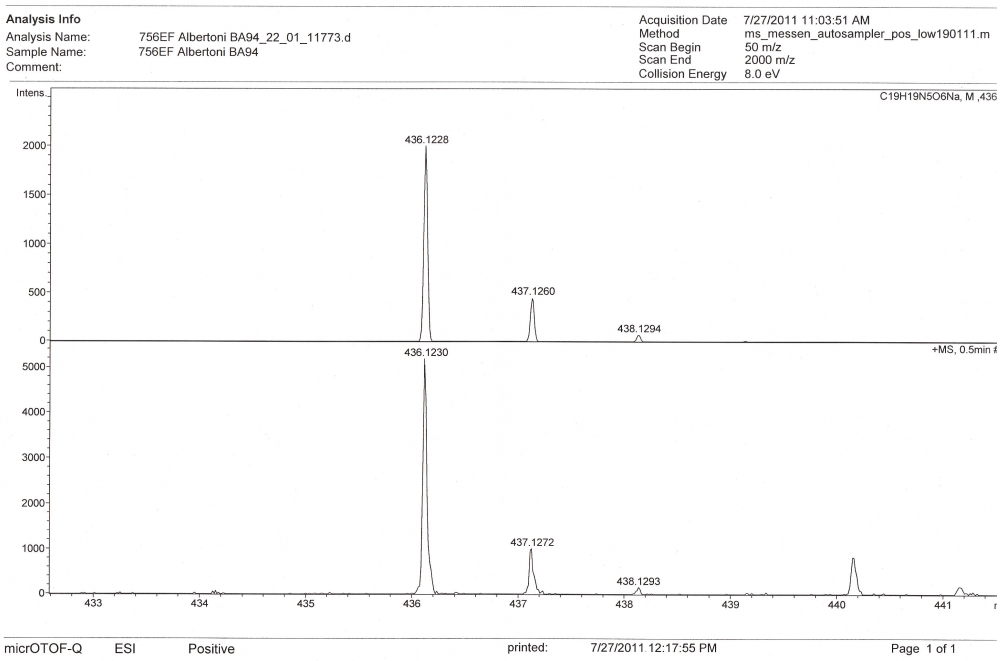

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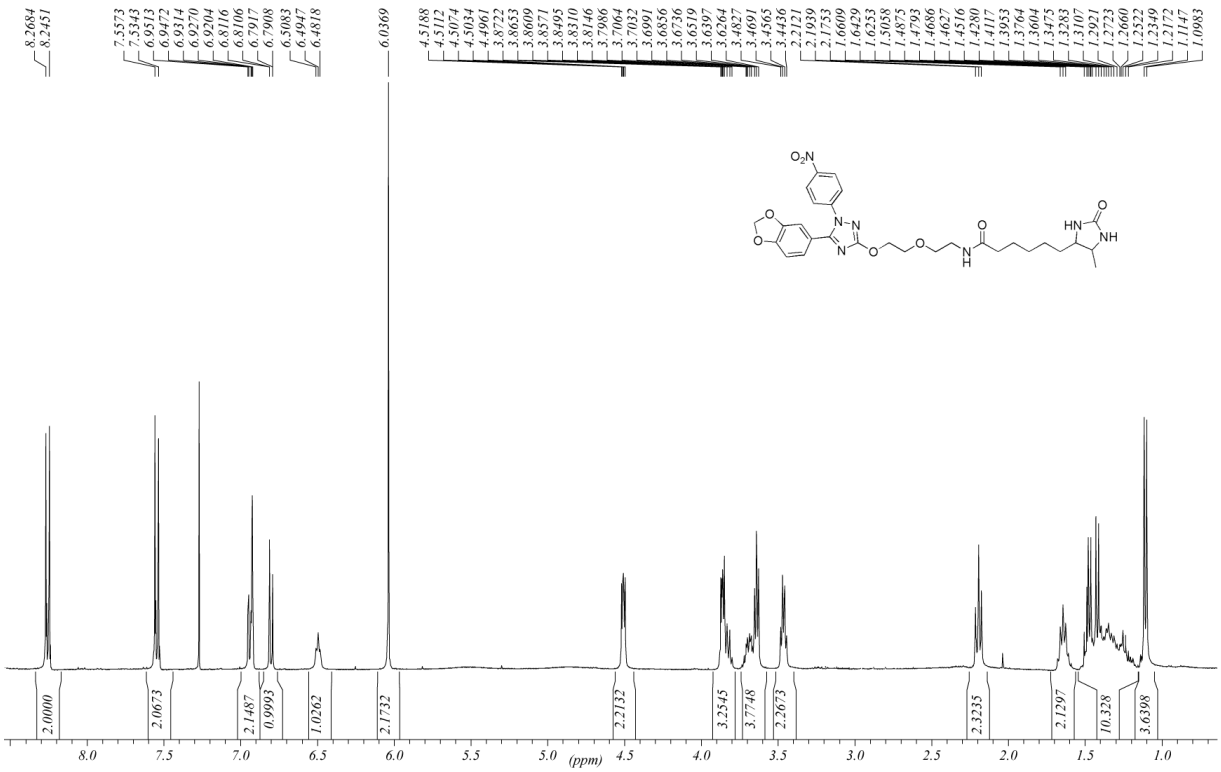

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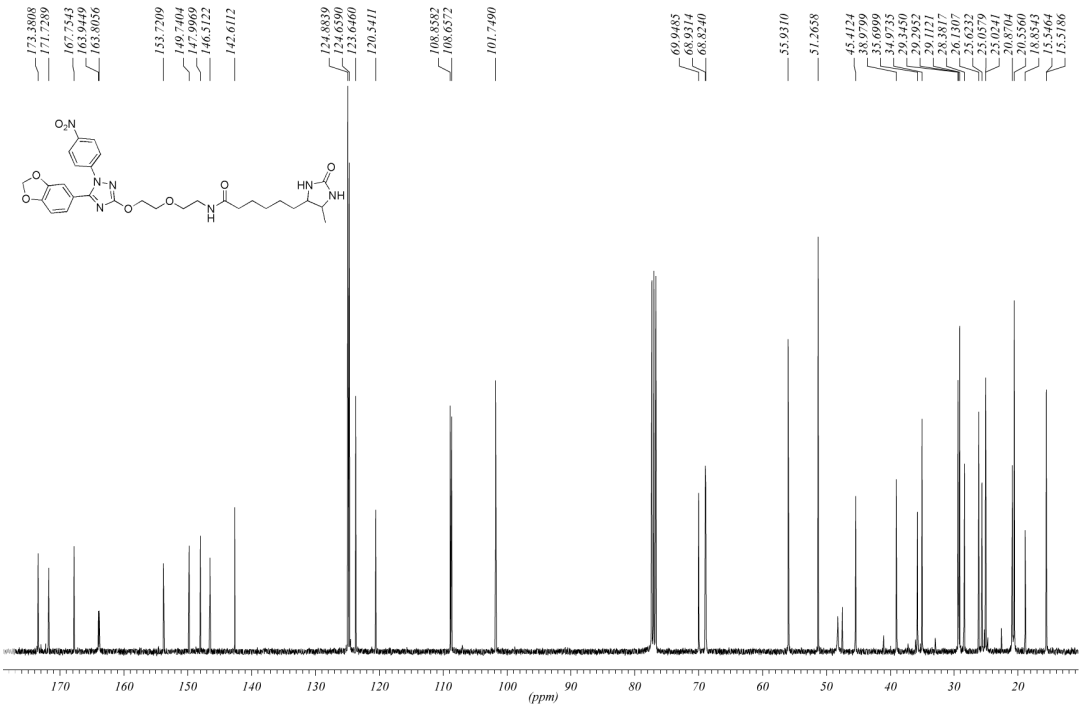

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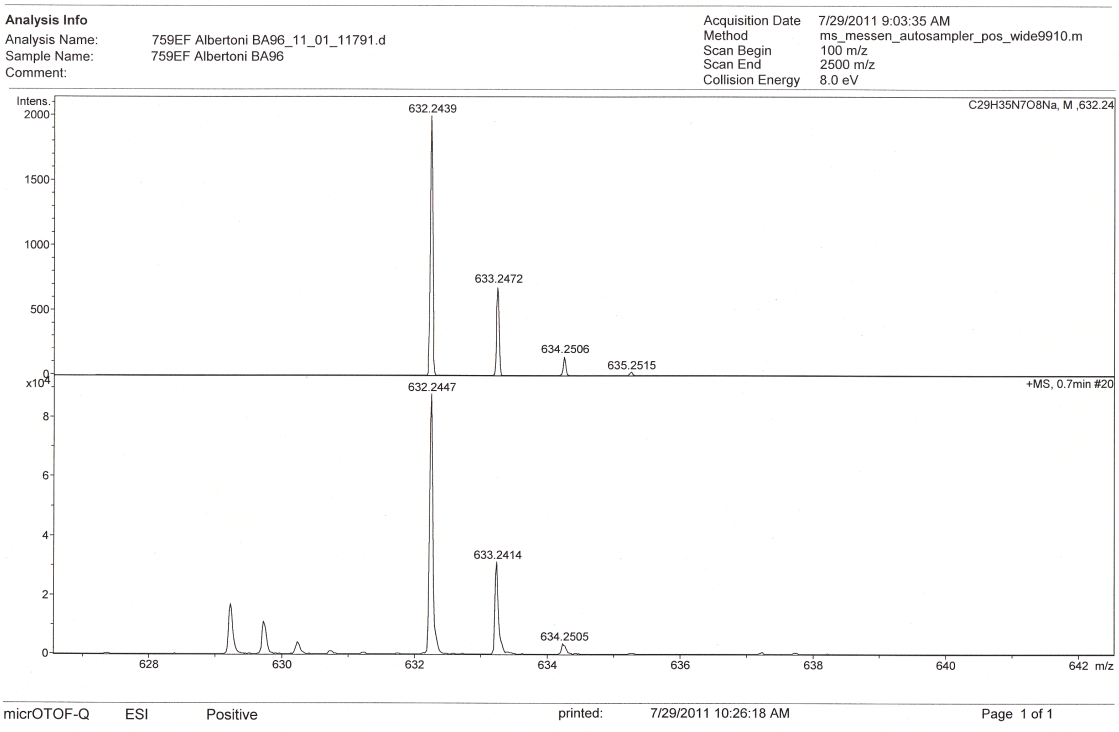

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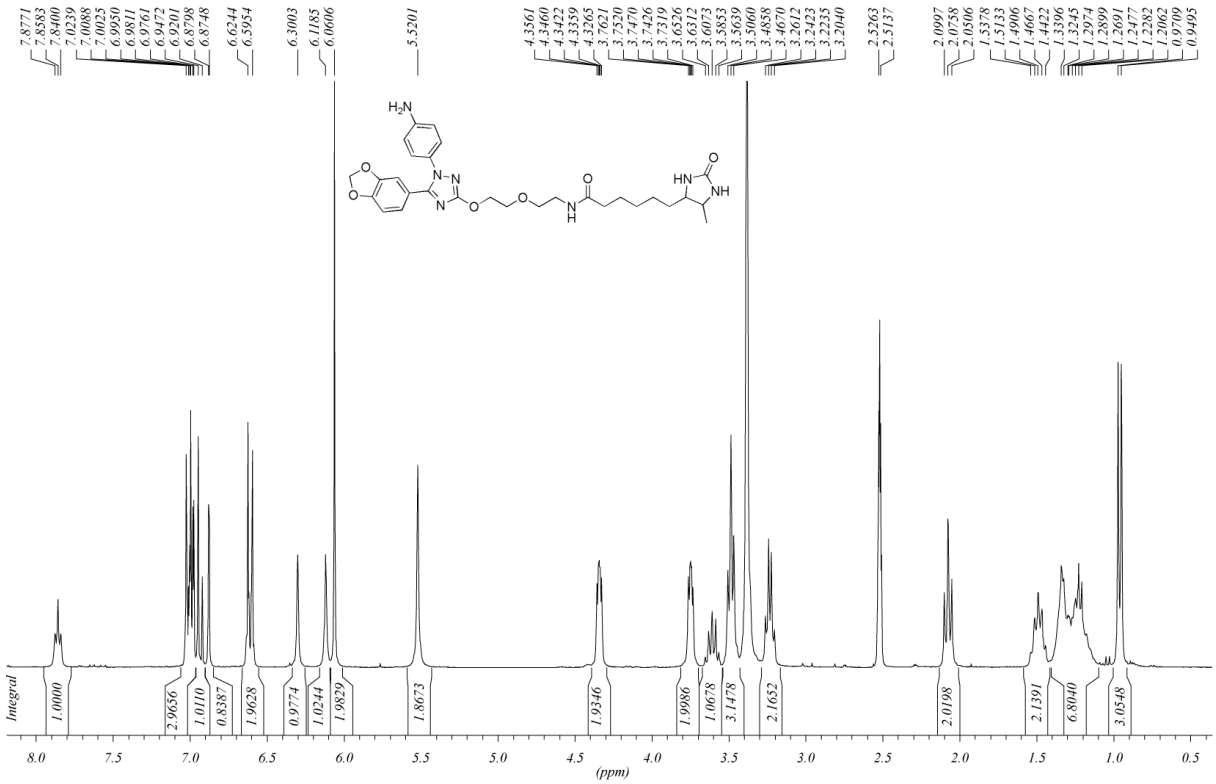

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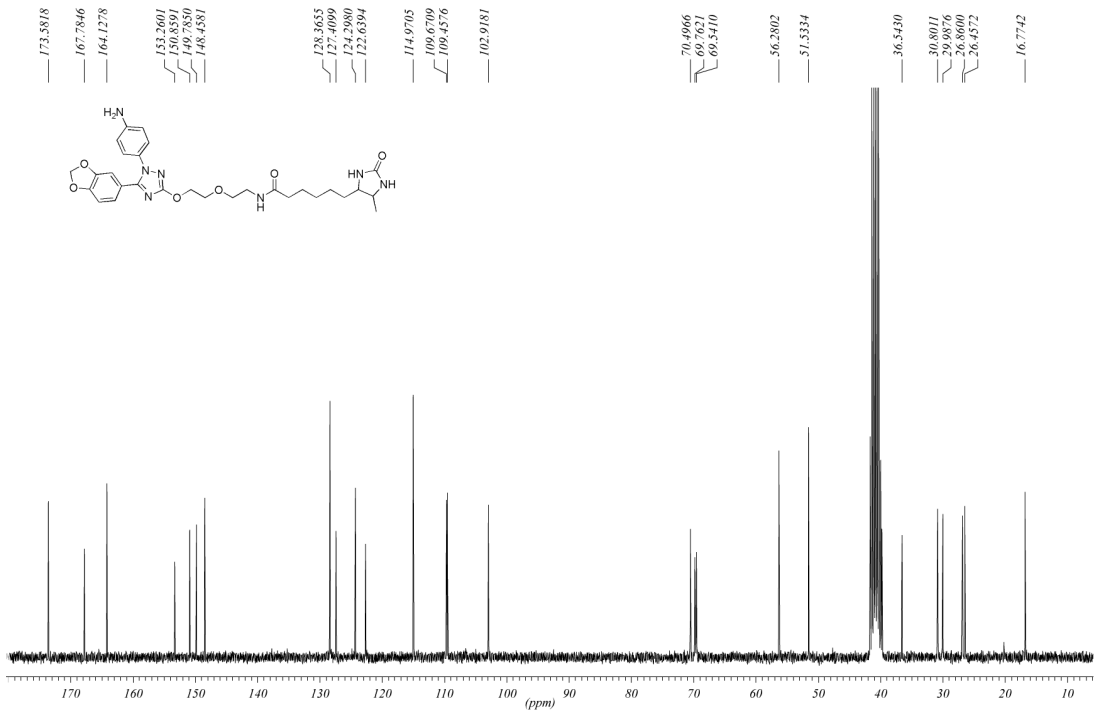

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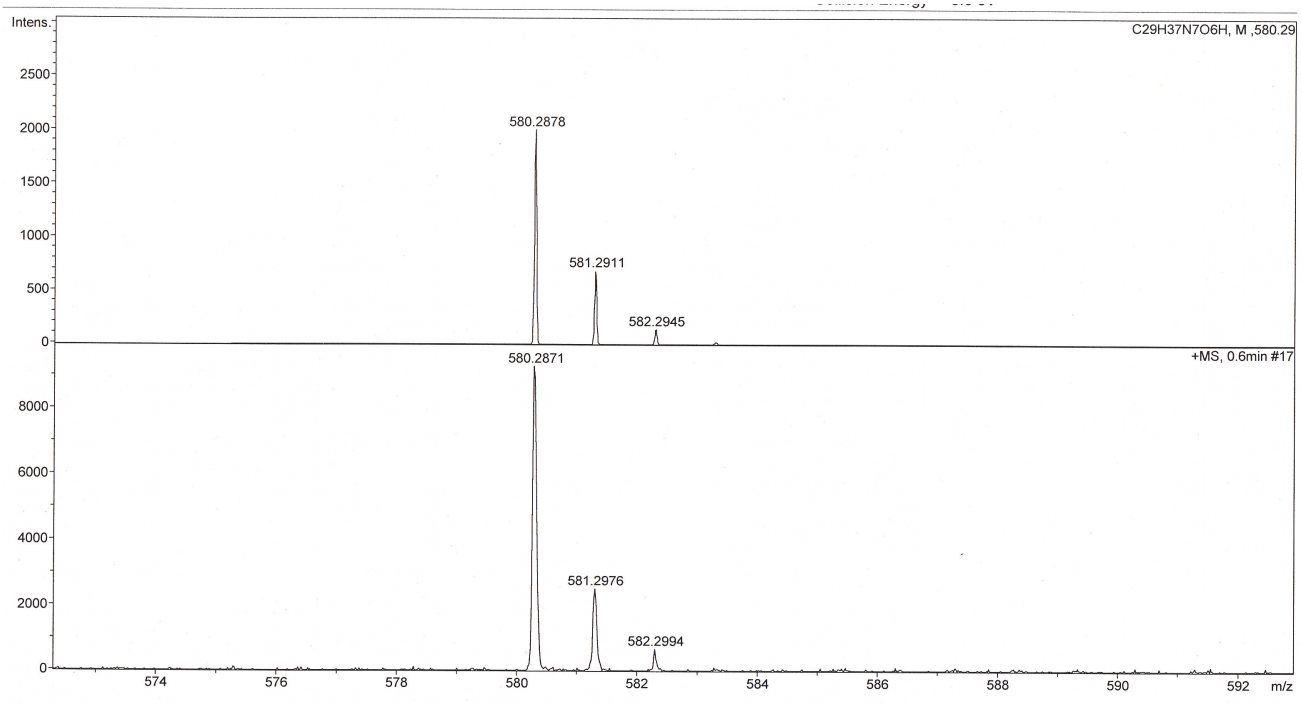

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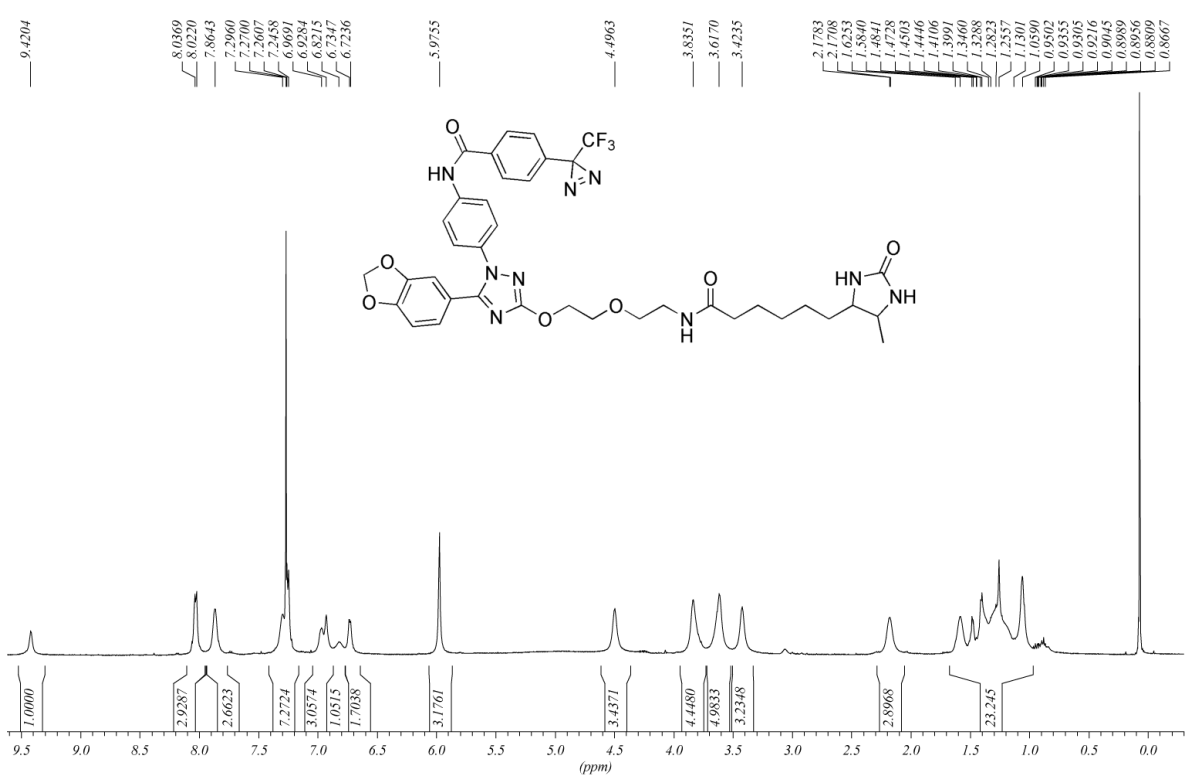
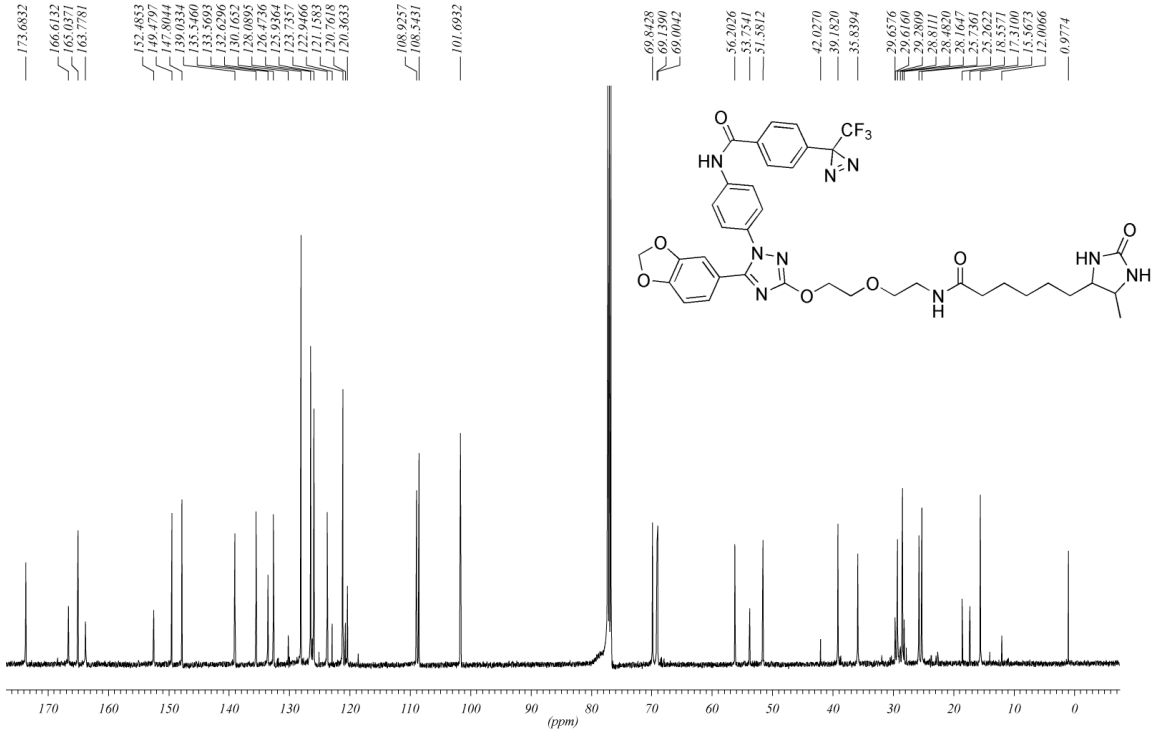
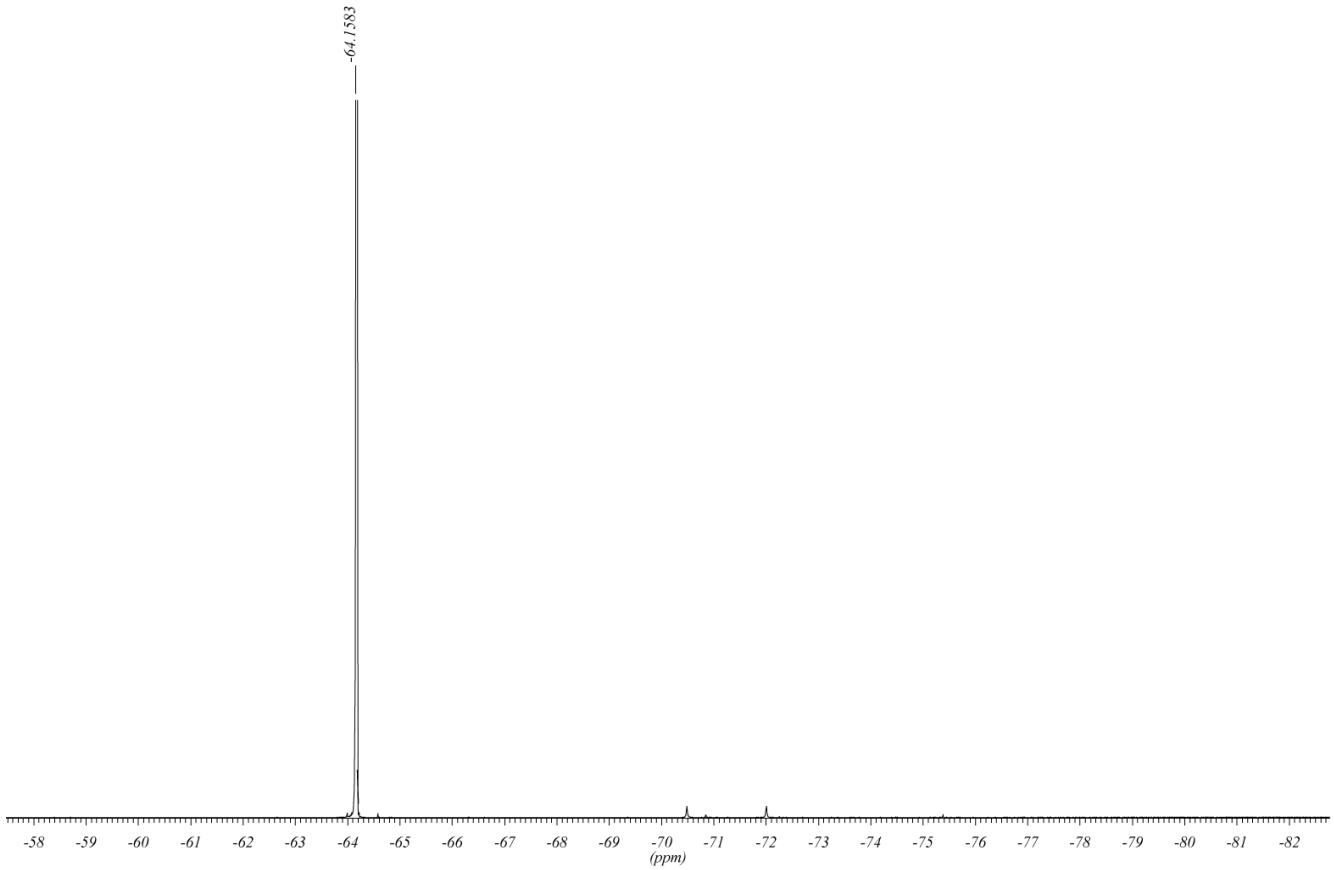


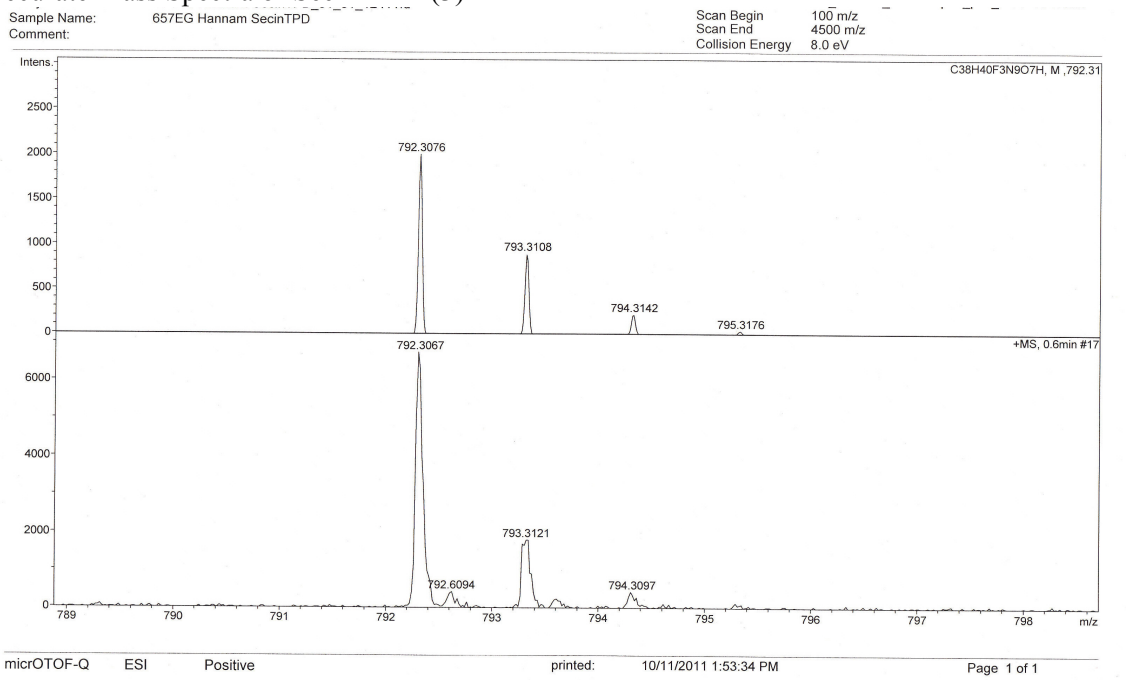
^{13}C NMR of Secin-TPD (3)



^{19}F NMR of Secin-TPD (3)



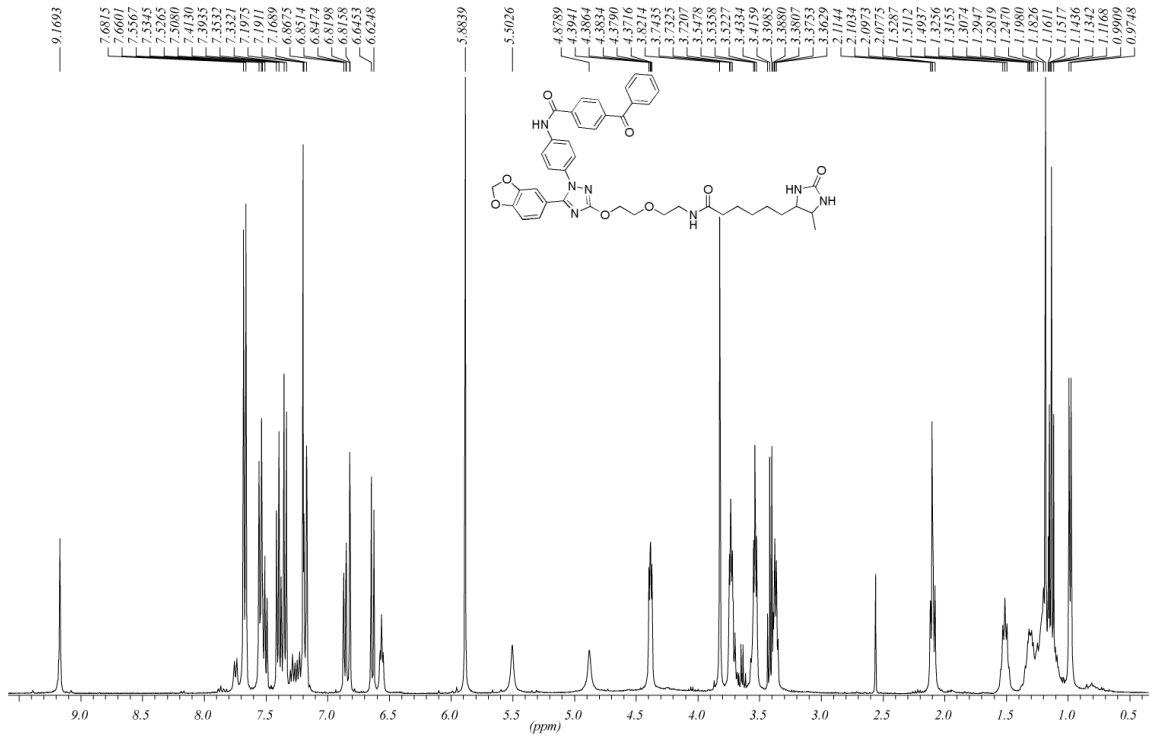
Accurate Mass Spectra of Secin-TPD (3)



1

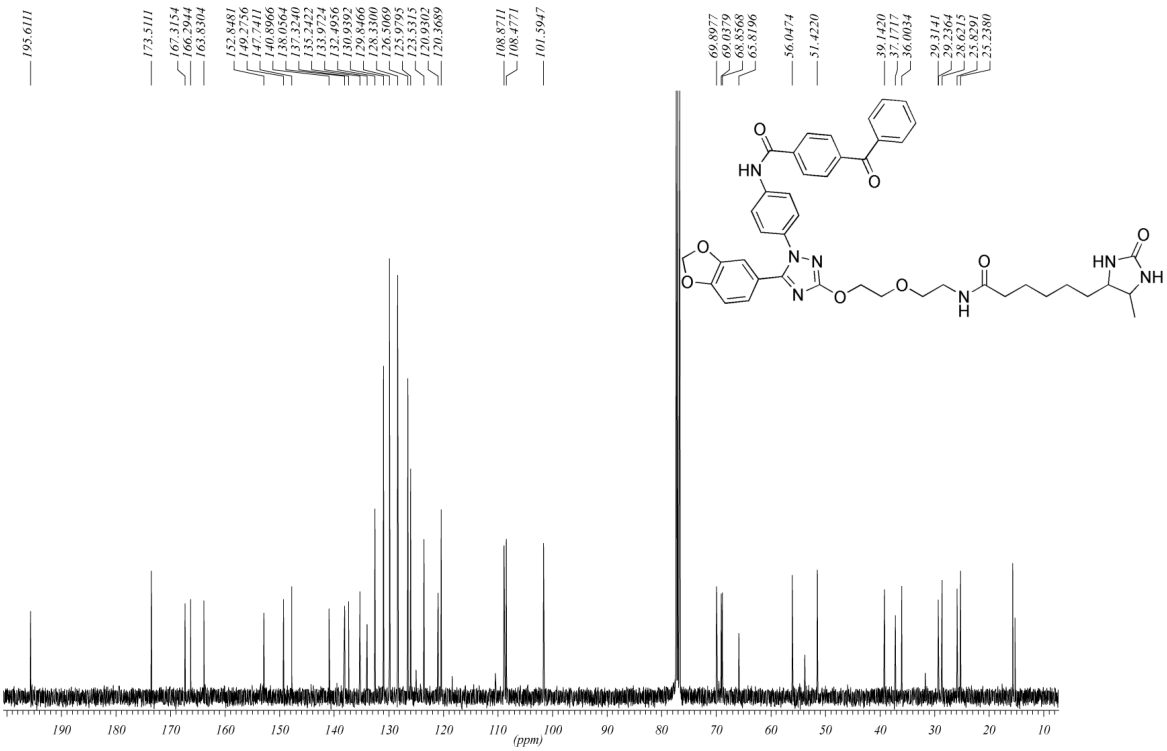
5

H NMR of dt-Secin PP (4)



10

^{13}C NMR of dt-Secin PP (4)



5

Accurate Mass Spectra of dt-Secin PP (4)