Electronic Supplementary Material (ESI) for RSC Advances

Supporting information

Facile Functionalization of FK506 for Biological Studies by the Thiol-Ene 'Click' Reaction

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Fig. S1. TEC reaction setup. Reaction was carried out using handheld UV lamp at room temperature.

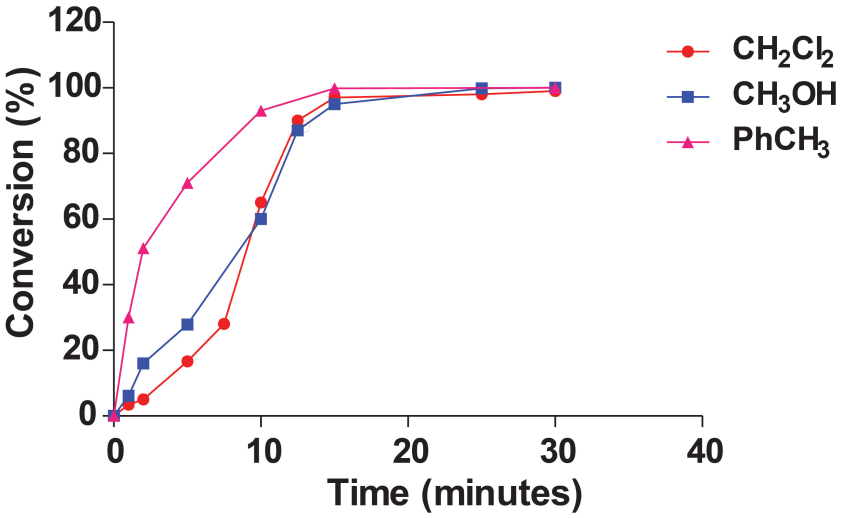


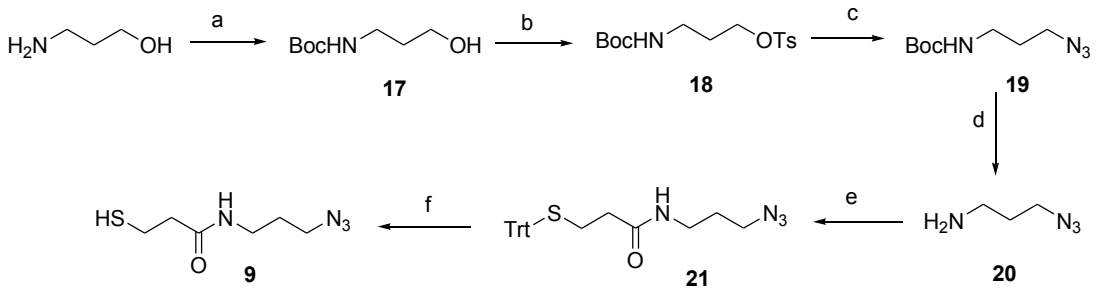
Fig. S2. TEC reaction time profile. The conversion was based on FK506 consumption followed under HPLC.

Experiment section

Chemicals and instruments:

Bulk solvents were obtained from EMD. Cysteamine, 3-thiopropanoic acid, Cysteine, Dithiothreitol, 5-hexynoic acid, *N*-Ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), Et_3N, 1-Hydroxybenzotriazole hydrate (HOBt), 3-Aminopropanol, di-tert-butyl dicarbonate (Boc_2O), 4-(Dimethylamino)pyridine (DMAP), *p*-Toluenesulfonyl chloride (TsCl), triethylsilane, NaN_3 and 2,2-Dimethoxy-2-phenylacetophenone (DPAP) were obtained from Sigma-Aldrich and Alfa-Aesar and were used directly without further purification. Other chemicals are commercially available. Boc-cysteamine was synthesized from Cysteamine following the general procedure. 3-(tritylthio)propinoic acid was synthesized following the reported literature (*Langmuir,* 2008, **24**, 13581). NMR spectra were recorded on a Bruker instrument (300 MHz). Mass and NMR spectra for new compounds were recorded at the Mass Spectrometry and NMR Facilities, Department of Chemistry and Chemical Biology, University of New Mexico.

Synthesis of compounds:



Scheme S1. a) $(Boc)_2O$, DIPEA, MeOH (99%); b) TsCl, DMAP, Et_3N, DCM (96%); c) NaN_3, DMF, rt, 13 h (89%); d) TFA/DCM, rt, 1 h (99%); e) HATU, DIPEA, DCM, rt, overnight (92%); f) TFA/DCM(v/v= 2: 8), Et_3SiH, rt, 1h (94%).

Prepare of compound **17**

3-Aminopropanol (7.0 g, 92 mmol) dissolved in methanol (100 mL) was successively treatd with di-tert-butyl dicarbonate (22.0 g, 101.2 mmol) and di-iso-propylethylamine (32 mL, 184 mmol) stir overnight at room temperature. After removal of the solvent under reduced pressure, the product was dissolved in DCM (100 mL). The organic layer was washed with 10 % citric acid (X 2). The aqueous layers were extracted with DCM. The combined organic layers were dried over sodium sulfate. Removal of the solvent under vacuum gave compound **17** as colorless viscous oil (17.5 g, yield: 99 %). ^{1}H NMR ($CDCl_3$, 300 MHz): 4.78 (bs, 1H), 3.67-3.63 (t, J = 11.4 Hz, 2H), 3.29-3.25 (t, J = 12.3 Hz, 2H), 1.69- 1.63 (m, 2H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$, 300 MHz): 157.50, 79.64, 59.26, 36.97, 32.89, 28.36.

Prepare of compound **18**

Compound **17** (3.50 g, 20 mmol), Et_3N (2.02 g, 20 mmol) and DMAP (0.244g, 2 mmol) were stirred in DCM about 5 min, then add TsCl (4.00 g, 21 mmol) and stirred further 2 h at room temperature. Colorless viscous solid was

obtained after purification by silica gel column chromatography using hexane/ethyl acetate (v/v= 2: 1) as an eluting solvent (R_f= 0.72). Yield: 96 %. ^{1}H NMR ($CDCl_3$, 300 MHz): 7.79-7.77 (d, *J* = 8.4 Hz, 2H), 7.36-7.33 (d, *J* = 8.1 Hz, 2H), 4.61 (bs, 1H), 4.09-4.05 (t, *J* = 12.0 Hz, 2H), 3.15 (s, 2H), 2.44 (s, 3H), 1.87- 1.79 (m, 2H), 1.41 (s, 9H). ^{13}C NMR ($CDCl_3$, 300 MHz): 155.87, 144.91, 129.92, 127.88, 79.36, 68.00, 36.82, 29.26, 28.35, 21.64.

Prepare of compound **19**

Compound **18** (3.29 g, 10 mmol) and NaN_3 (3.30 g, 30 mmol) were stirred in THF/H_2O (v/v= 5: 1) for 2 h. The mixture was extracted with ethyl acetate; the organic layers were combined, washed three times with $NaHCO_3$ and brine, and subsequently dried with anhydrous Na_2SO_4. Colorless viscous solid was obtained after the solvent was removed under reduced pressure. Yield: 89%. ^{1}H NMR ($CDCl_3$, 300 MHz): 4.66 (bs, 1H), 3.37-3.33 (t, *J* = 13.2 Hz, 2H), 3.22-3.18 (t, *J* = 12.9 Hz, 2H), 1.80- 1.71 (m, 2H), 1.43 (s, 9H). ^{13}C NMR ($CDCl_3$, 300 MHz): 155.94, 79.42, 49.11, 38.10, 29.29, 28.37. TOF-HRMS (m/z) found (calcd.) for $C_8H_{16}N_4OS$ (M): $[M+Na]^+$, 223.1171 (223.1171).

Prepare of compound **20**

Compound **19** (1g, 5 mmol) was stirred in DCM/TFA (v/v= 5: 1) for 1 h, white solid was obtained after the solvent was removed under reduced pressure. Yield: 99%. ^{1}H NMR ($CDCl_3$, 300 MHz): 8.30 (bs, 2H), 3.57 (s, 2H), 3.18 (s, 2H), 2.09 (s, 2H). ^{13}C NMR ($CDCl_3$, 300 MHz): 48.51, 37.81, 26.80. TOF-HRMS (m/z) found (calcd.) for $C_3H_8N_4$ (M): $[M+H]^+$, 101.0814 (101.0827).

Prepare of compound **21**

3-(tritylthio)propanoic acid (697 mg, 2.0 mmol), HATU (836 mg, 2.2 mmol), DIPEA (516 mg, 4.0 mmol), and compound **20** (220 mg, 2.2 mmol) were stirred at room temperature overnight in DCM. White solid was obtained after purification by silica gel column chromatography using hexane/ethyl acetate (v/v= 1: 1) as an eluting solvent (R_f= 0.65). Yield: 96 %. ^{1}H NMR (Acetone-d_6, 300 MHz): 7.41-7.39 (m, 6H), 7.34-7.28 (m, 6H), 7.26-7.20 (m, 3H), 7.14 (bs, 1H), 3.39-3.35 (t, *J*= 13.5 Hz, 2H), 3.27-3.20 (q, *J*= 19.2 Hz, 2H), 2.44-2.39 (t, *J*= 14.7 Hz, 2H), 2.22-2.17 (t, *J*= 14.7 Hz, 2H), 1.77- 1.68 (m, 2H). ^{13}C NMR (Acetone-d_6, 300 MHz): 171.11, 145.83, 130.34, 128.70, 127.47, 67.17, 49.66, 37.10, 35.40, 28.53. TOF-HRMS (m/z) found (calcd.) for $C_{25}H_{26}N_4OS$ (M): $[M+Na]^+$, 453.1717 (453.1725), $[2M+Na]^+$, 883.3400 (883.3552).

Prepare of compound **9**

To a solution of compound **21** (215 mg, 0.5 mmol) in trifluoroacetic acid (TFA, 1mL) and CH_2Cl_2 (2 mL) was added triethylsilane (174 mg, 1.5 mmol). The resulting mixture was stirred for a half hour at room temperature. After evaporating the reaction solvent, CH_2Cl_2 (20 mL) was added to the resulting residues. The organic layer was extracted with H_2O (10 mL). The aqueous layer was then evaporated to give the transparent oil product. Yield: 94 %. ^{1}H NMR ($CDCl_3$, 300 MHz): 5.93 (s, 1H), 3.41-3.36 (m, 4H), 2.84-2.80 (q, *J*= 12.9 Hz, 2H), 2.52-2.49 (t, *J*= 7.8 Hz, 2H), 1.84-1.79 (m, 2H), 1.62-1.59 (t, *J*= 9.9 Hz, 1H). ^{13}C NMR ($CDCl_3$, 300 MHz): 171.58, 49.45, 40.46, 37.48, 28.79, 20.54. TOF-HRMS (m/z) found (calcd.) for $C_6H_{12}N_4OS$ (M): $[M+Na]^+$, 211.0628 (211.0630), $[2M+Na-2H]^+$, 397.1207 (397.1207).

Synthesis of compound **3**

FK506 (201.0 mg, 0.25 mmol), Boc-cysteamine (46.0 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL dichloromethane were put in a vials, and stirred 15 min under UV light. White solid (246 mg, 98% yield) was

obtained after purification by silica gel column chromatography using ethyl acetate as an eluting solvent (R_f= 0.5). ^{1}H NMR (300 MHz, $CDCl_3$): 5.33-5.21(d, *J*= 36.8 Hz, 1H), 5.12-5.08 (m, 2H), 4.88-4.40 (m, 1H), 3.94-3.57 (m, 3H), 3.41-3.29 (m, 9H), 3.05-2.95 (m, 2H), 2.78-2.50 (m, 6H), 2.38-1.26 (m, 45H), 1.07-0.82(m, 13H). TOF-HRMS (m/z) found (calcd.) for $C_{51}H_{84}N_2O_{14}S$ (M): $[M+Na]^+$, 1003.5583 (1003.5541).

Synthesis of compound **4**

Method A: compound **3** disovled in DCM/TFA and stir 1 h at room temprature.

Method B: FK506 (201.0 mg, 0.25 mmol), cysteamine (19.3 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL methanol were put in a vials, and stirred 15 min under UV light. White solid (211 mg, 96% yield) was obtained after purification by silica gel column chromatography using ethyl DCM/methanol (v/v= 5: 1) as an eluting solvent (R_f= 0.56). ^{1}H NMR (300 MHz, Acetone-d_6): 6.93-6.80 (m, 1H), 6.45-6.29 (m, 1H), 5.30-4.98 (m, 3H), 4.66-4.34 (m, 1 H), 4.13-4.02 (m, 2H), 3.80-3.32 (m, 11H), 3.02-3.00 (m, 2H), 2.68-2.50 (m, 4H), 2.49-1.58 (m, 36 H), 1.21-0.88 (m, 13H). TOF-HRMS (m/z) found (calcd.) for $C_{46}H_{76}N_2O_{12}S$ (M): $[M+H]^+$, 881.5215 (881.5197).

Synthesis of compound **6**

5-hexynoic acid (28.0 mg, 0.25 mmol), EDCI (52.7 mg, 0.275 mmol), HOBt (37.1 mg, 0.275 mmol), and Et_3N (50.5 mg, 0.5 mmol), stir about 1 h in DCM, then add compound **4** (242 mg, 0.275 mmol) and stir overnight. White solid (190 mg, 78% yield) was obtained after purification by silica gel column chromatography using ethyl acetate as an eluting solvent (R_f= 0.42). ^{1}H NMR (300 MHz, $CDCl_3$): 6.80-6.69 (m, 1H), 6.34-6.10 (m, 1H), 5.97 (s, 1H), 5.26-4.85 (m, 2H), 4.75-4.72 (m, 1H), 4.49-4.16 (m, 1H), 3.92-3.72 (m, 1H), 3.59-3.26 (m, 11H), 3.06-2.92 (m, 2H), 2.63-2.61 (m, 2H), 2.49-2.23 (m, 6H), 2.12-1.23 (m, 39H), 1.80-0.83 (m, 13H). TOF-HRMS (m/z) found (calcd.) for $C_{52}H_{82}N_2O_{13}S$ (M): $[M+H]^+$, 979.5360 (976.2860).

Synthesis of compound **11**

FK506 (201.0 mg, 0.25 mmol), 3-thiopropanoic acid (28.0 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL dichloromethane were put in a vials, and stirred 15 min under UV light. White solid (222 mg, 98% yield) was obtained after purification by silica gel column chromatography using ethyl acetate/ acetone (v/v= 1: 1) as an eluting solvent (R_f= 0.51). ^{1}H NMR (300 MHz, $CDCl_3$): 5.32-5.20(d, *J*= 36.4 Hz, 1H), 5.10-5.00 (m, 2H), 4.75-4.30 (m, 1H), 3.94-3.54 (m, 3H), 3.40-3.29 (m, 11H), 3.05-2.95 (m, 2H), 2.75-2.51 (m, 6H), 2.38-1.25 (m, 36H), 1.06-0.82(m, 13H). TOF-HRMS (m/z) found (calcd.) for $C_{47}H_{75}NO_{14}S$ (M): $[M-H]^+$, 908.4821 (908.4830).

Synthesis of compound **12**

FK506 (201.0 mg, 0.25 mmol), compound **9** (48.9 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL DCM were put in a vials, and stirred 15 min under UV light. Pale yellow solid (235 mg, 95% yield) was obtained after purification by silica gel column chromatography using ethyl acetate/ acetone (v/v= 4: 1) as an eluting solvent (R_f= 0.56). ^{1}H NMR (300 MHz, $CDCl_3$): 6.50 (s, 1H), 5.32-4.95 (m, 3H), 4.74-4.22 (m, 2H), 3.91-3.53 (m, 2H), 3.38-3.27 (m, 17H), 2.98-2.95 (m, 4H), 2.80-2.64 (m, 4H), 2.58-2.39 (m, 4H), 2.15-1.34 (m, 34H), 0.98-0.80 (m, 13H). TOF-HRMS (m/z) found (calcd.) for $C_{50}H_{81}N_5O_{13}S$ (M): $[M+Na]^+$, 1014.5485 (1014.5449).

Synthesis of compound **13**

FK506 (201.0 mg, 0.25 mmol), Cysteine (31.5 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.5 mL methanol/water (1: 1) were put in a vials, and stirred 15 min under UV light. White solid (220 mg, 95% yield) was

obtained after purification by silica gel column chromatography using acetone/methanol (v/v= 1: 1) as an eluting solvent (R_f= 0.46). ^{1}H NMR (300 MHz, $CDCl_3$): 5.27-4.95 (m, 3H), 4.63-4.33 (m, 1H), 4.08-3.97 (m, 2H), 3.70-3.41 (m, 3H), 3.38-2.75 (m, 13H), 2.09-1.20 (m, 34H), 0.96-0.71(m, 13H). TOF-HRMS (m/z) found (calcd.) for $C_{47}H_{76}N_2O_{14}S$ (M): $[M+H]^+$, 925.5076 (925.5096).

Synthesis of compound **15**

FK506 (201.0 mg, 0.25 mmol), dithiothreitol (20.2 mg, 0.13 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL dichloromethane were put in a vials, and stirred 15 min under UV light. White solid (220 mg, 99% yield) was obtained after purification by silica gel column chromatography using ethyl acetate/acetone (v/v=1:1) as an eluting solvent (R_f= 0.56). ^{1}H NMR (300 MHz, $CDCl_3$): 5.33-5.20 (d, *J*= 36.5 Hz, 2H), 5.10-5.06 (m, 4H), 4.74-4.28 (m, 4H), 3.92-3.57 (m, 8H), 3.41-3.30 (m, 22H), 3.06-2.97 (m, 4H), 2.74-2.54 (m, 14H), 2.30-1.25 (m, 64H), 1.08-0.83 (m, 26H). TOF-HRMS (m/z) found (calcd.) for $C_{92}H_{148}N_2O_{26}S_2$ (M): $[M+Na]^+$, 1783.9602 (1783.9659).

Plasmid construction

All DNA fragments were amplified by PCR (Polymerase chain reaction) from other intermediate constructs. PCR was carried out with Phusion DNA Polymerase (New England Biolabs), PfuUltra II Fusion HotStart DNA Polymerase (Agilent Technologies) under S1000 thermal cycler with Dual 48/48 Fast Reaction Module (Bio-Rad).

1) For SV-ires-GalDBD-3FKBP12, a DNA construct SV-VP16-Frb-ires-GalDBD-FKBP12x3 (*Sci. Signal.,* 2011, **4**, rs2) was firstly digested by EcoRI and BamHI. The sticky ends of the vector were blunted by DNA Polymerase I Lg (Klenow) Fragment (New England Biolabs) under the present of dNTPs. Finally, the blunt ends were ligated by T4 DNA Ligase (New England Biolabs).
2) For SV-VP16-FKBP12x2, the DNA construct was first made as SV-VP16-FKBP12. SV-VP16-Frb-ires-GalDBD-FKBP12x3 was firstly digested by AscI and NotI. The PCR product of FKBP12 was inserted into the vector by recombination using the In-Fusion HD Enzyme Premix (ClonTech). The second copy of FKBP12 was inserted via the AcsI site using T4 DNA ligase.

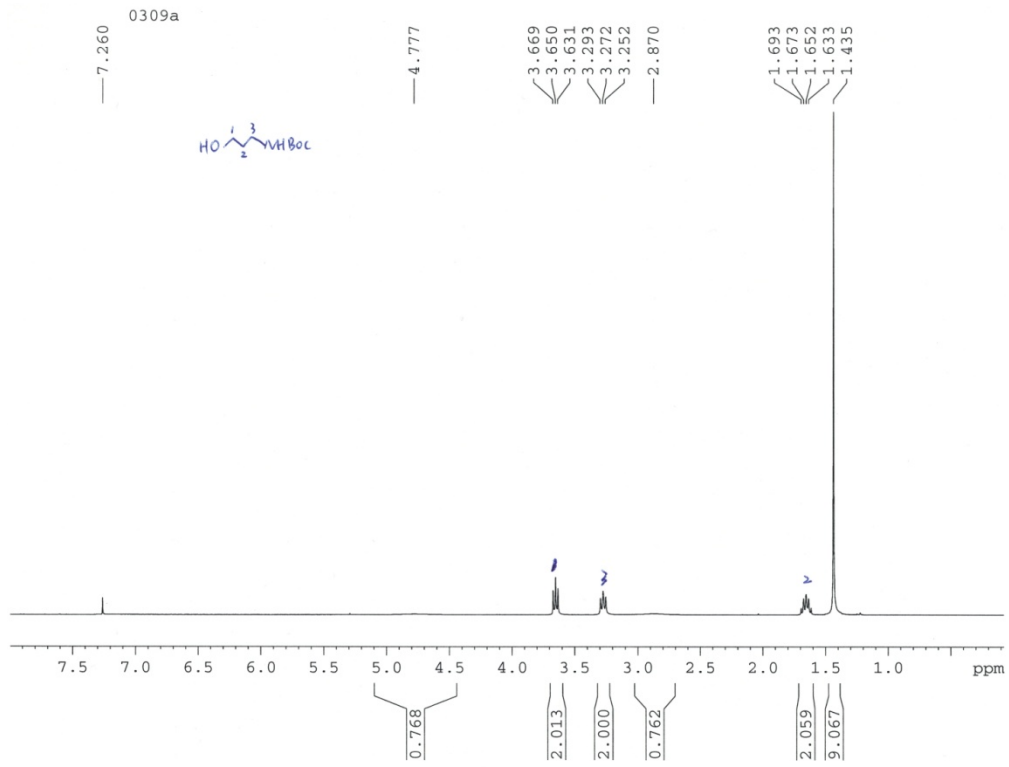
Cell Culture and transfection

CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals), 1×glutamate (100x from Gibco) and 1×penicillin/streptomycin (Pen/Strep, 100X from Gibco). Cells were plated with the starting concentration of 50,000 cells per well in a 24-well plate (Greiner Bio-one) the day before transfection. An amount of 0.2μg of each DNA construct was mixed with Opti-MEM (Gibco) and PEI. After incubation at room temperature for 15min, the mixture was added to the cells and cultured for 24h. Then FK506, FK1012-DT and FK1012-ZE dissolved in DMSO were added into the cell culture with the final concentration of 200nM, 100nM and 100nM respectively. Each type of the experiments, including the one with transfected DNA but without drugs, was carried out as triplets. After the incubation of 10 hours, the cells were harvested and washed by PBS buffer (Gibco) for 3 times.

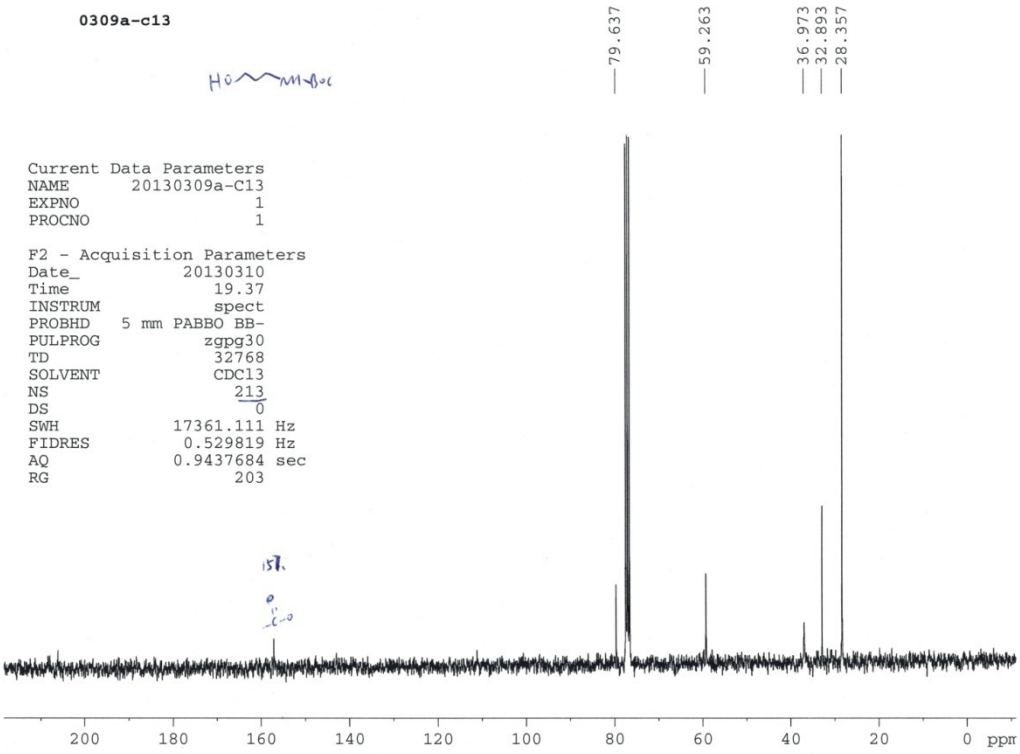
Luciferase assay

Cells in 24-well plates were frozen under the temperature of -80°C at first and then lysed with 100 μL of 1×Passive Lysis Buffer (Promega Corporation) at room temperature for 10min on a shaker. Cell lysates were then collected and centrifuged in tubes and 10μL of supernatant was added separately into a 96 well plate for Luciferase assay. 90μL

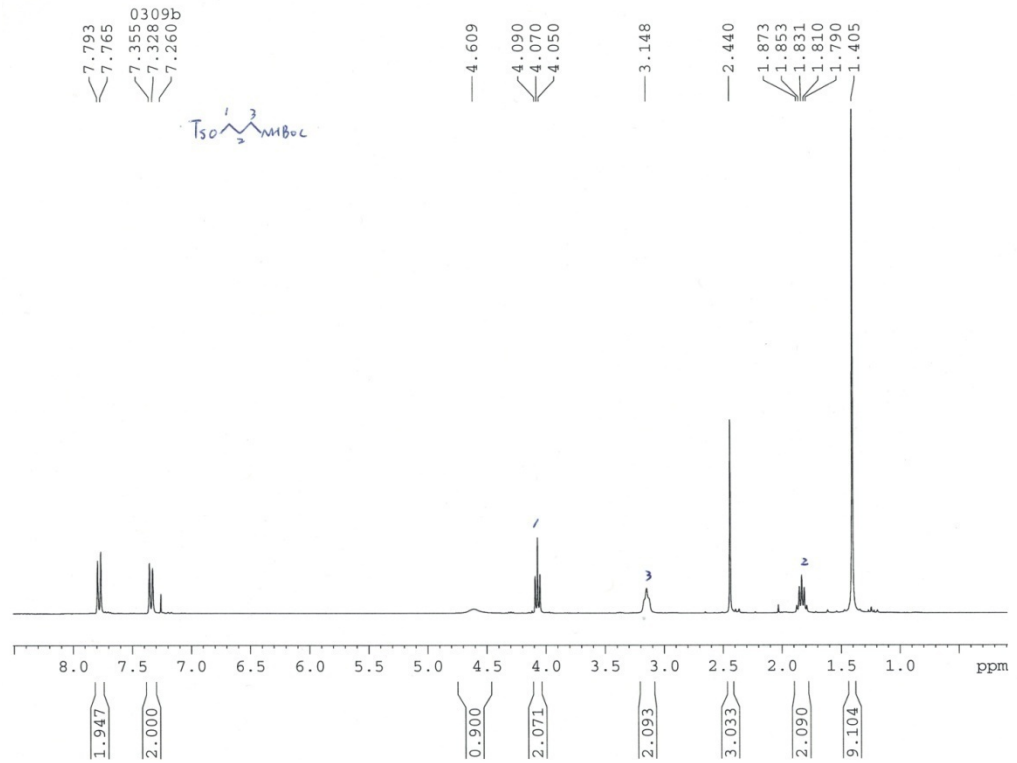
Luciferase substrate solution (5mg of D-luciferin and 7mg of coenzyme A in 33mL of Luciferase reading buffer, which includes 20mM tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67mM $MgSO_4$, 0.1mM EDTA, 33.3mM dithiothreitol and 0.53mM ATP in water) was added into each well with cell lysates. The signal was read with a 3s delay and 1s integration with Clomax Multi Detection System (Promega). Obtained data were analyzed by KaleidaGraph. The shown results are from 3 experimental repeats.



^{1}H NMR of compound **17**



^{13}C NMR of compound **17**



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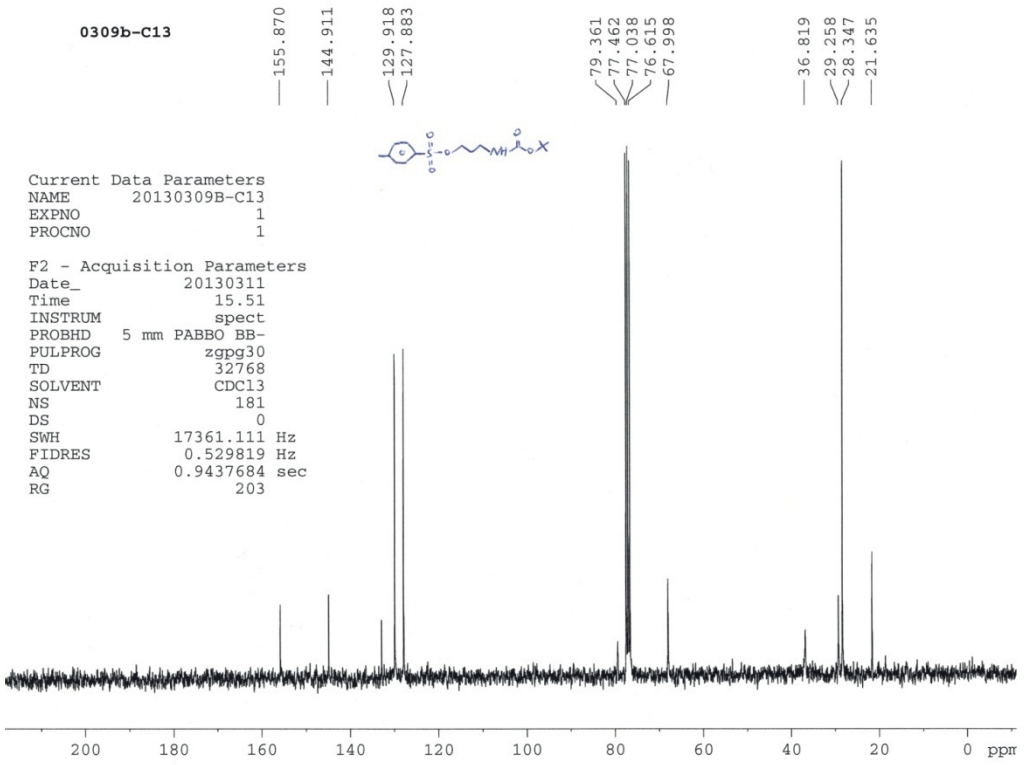

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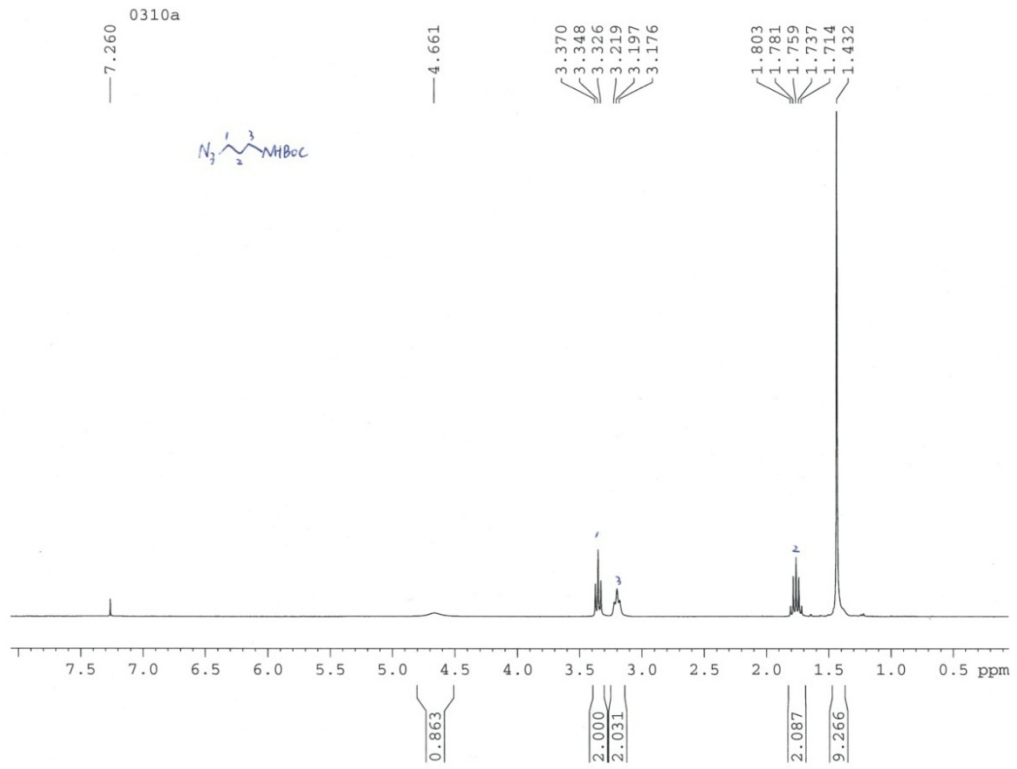

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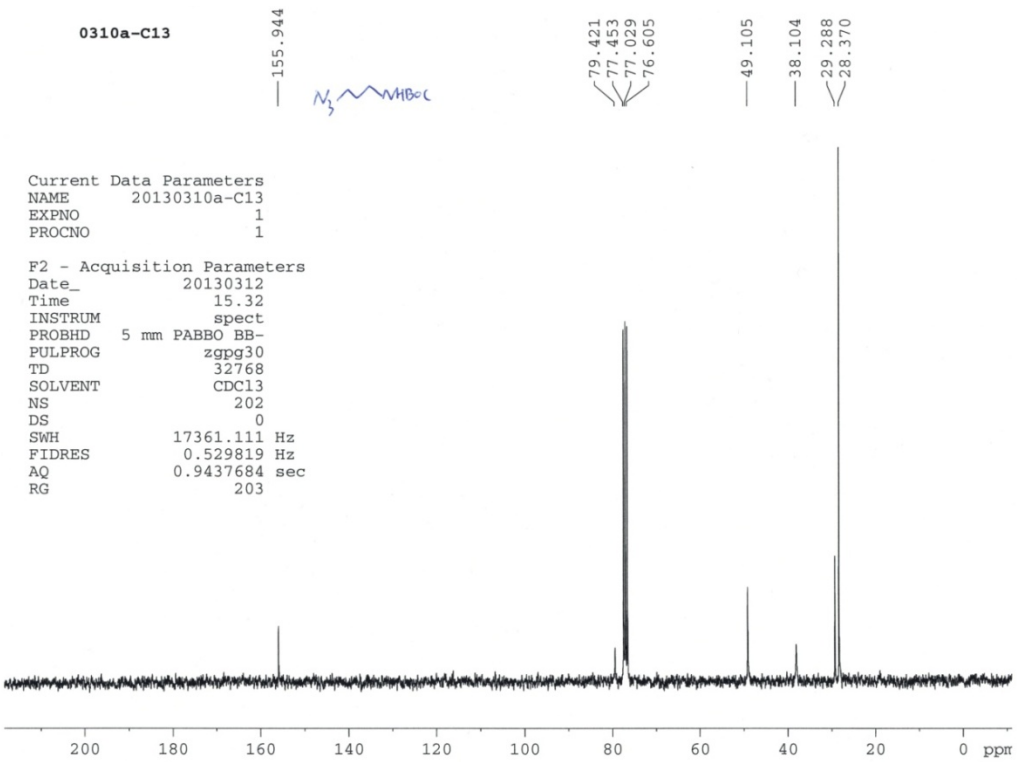

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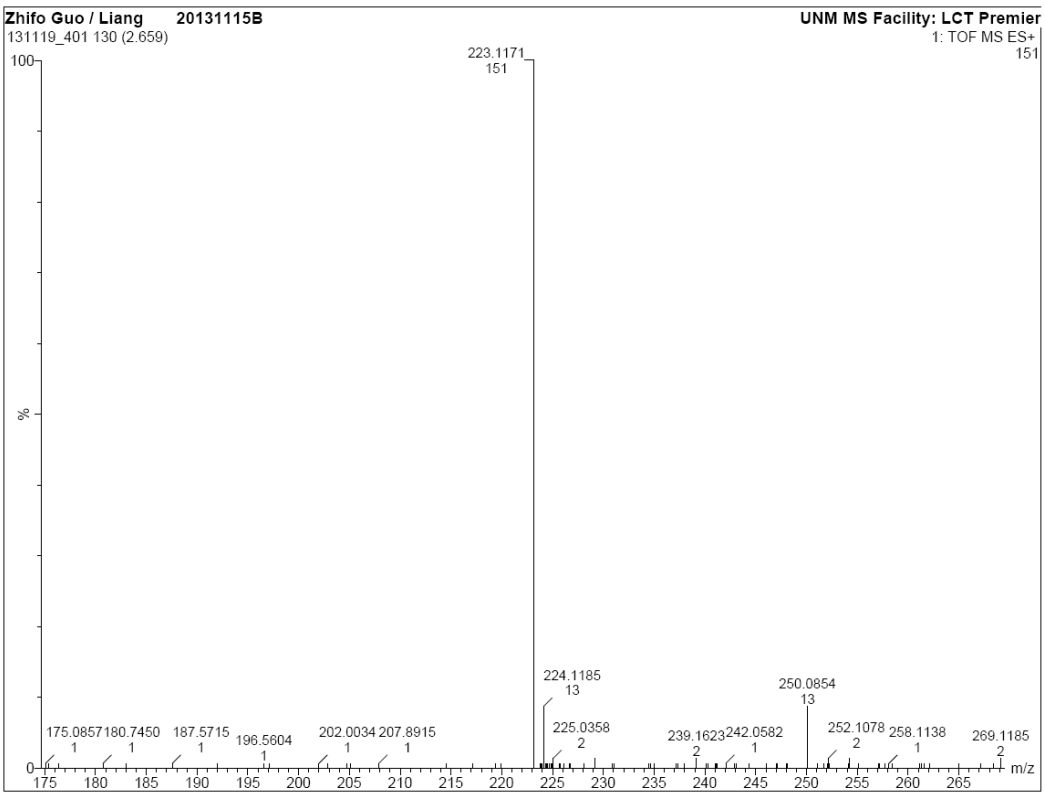
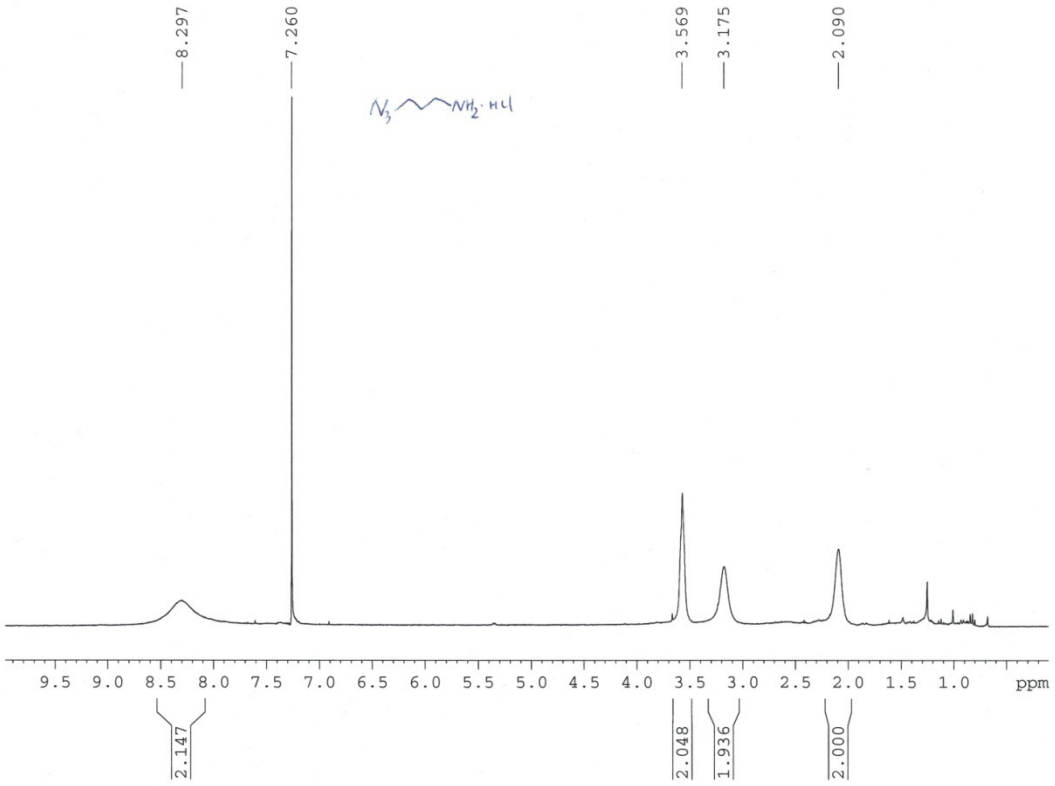


HRMS of compound **19**



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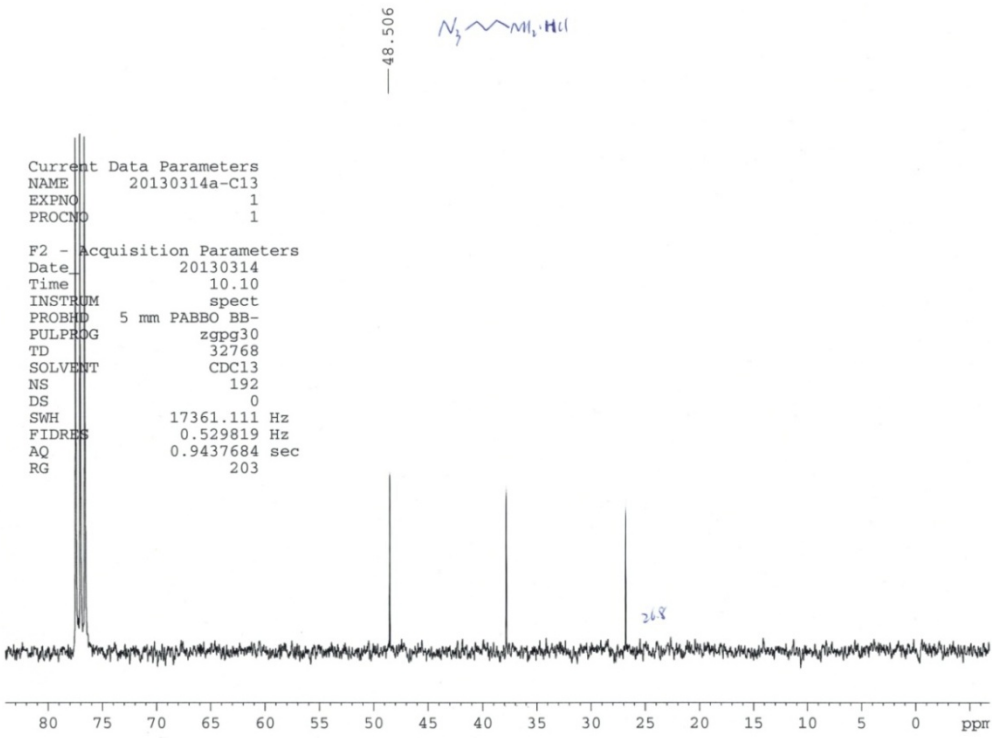

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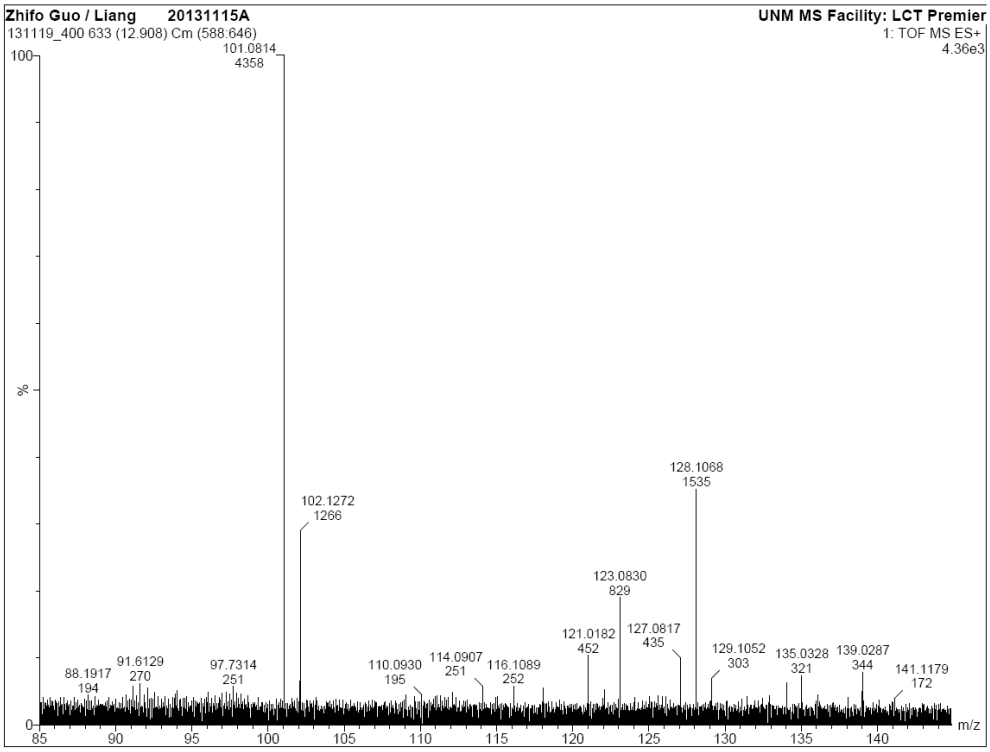

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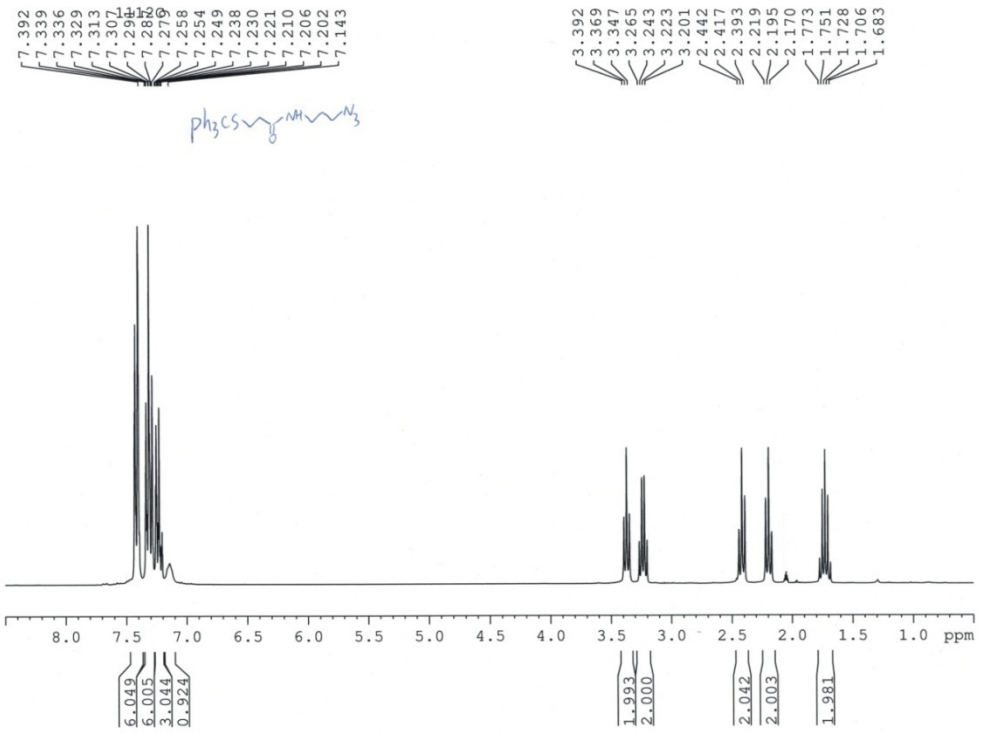

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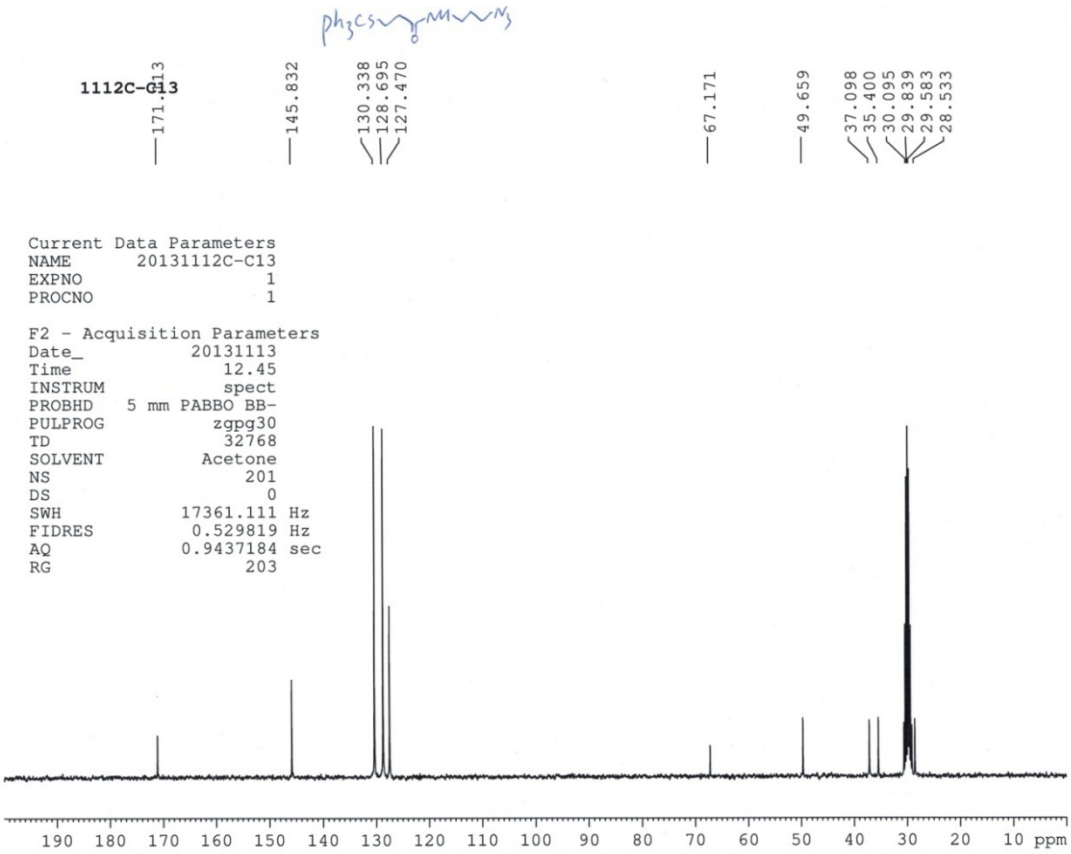

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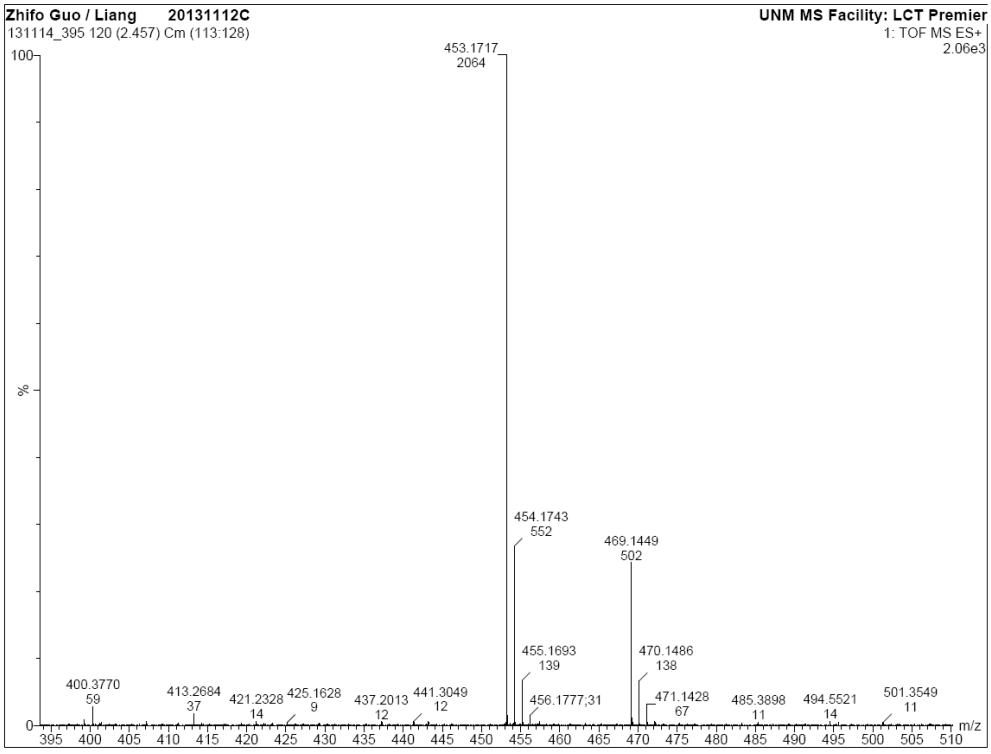

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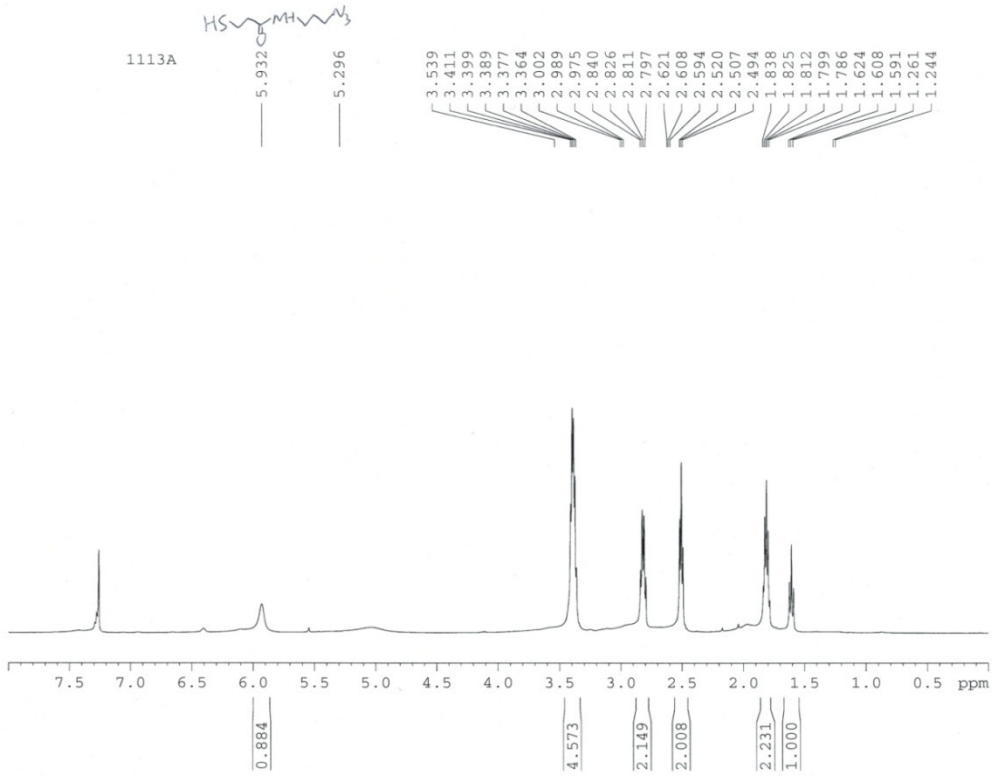
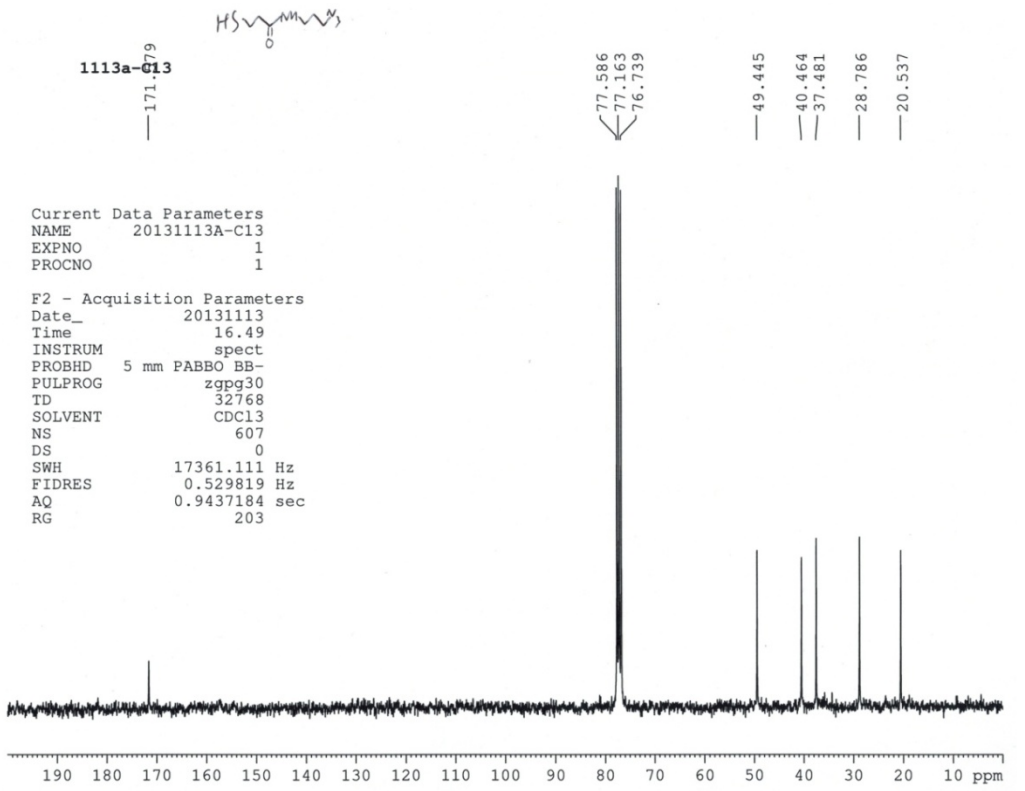


^{1}H NMR of compound **9**



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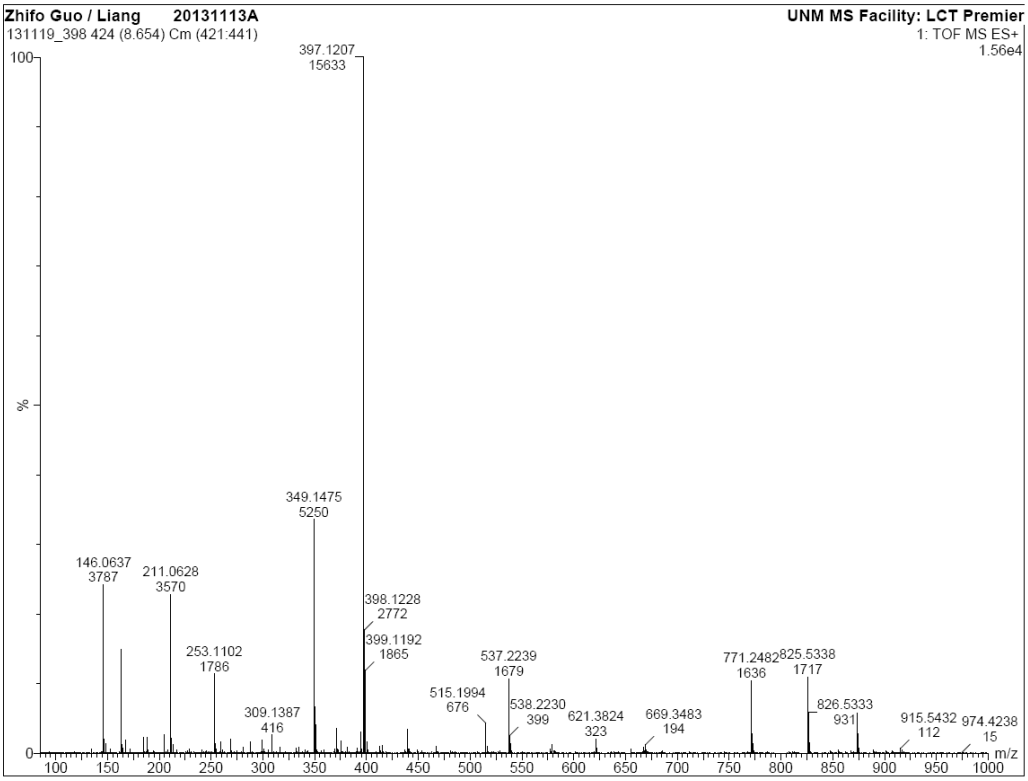

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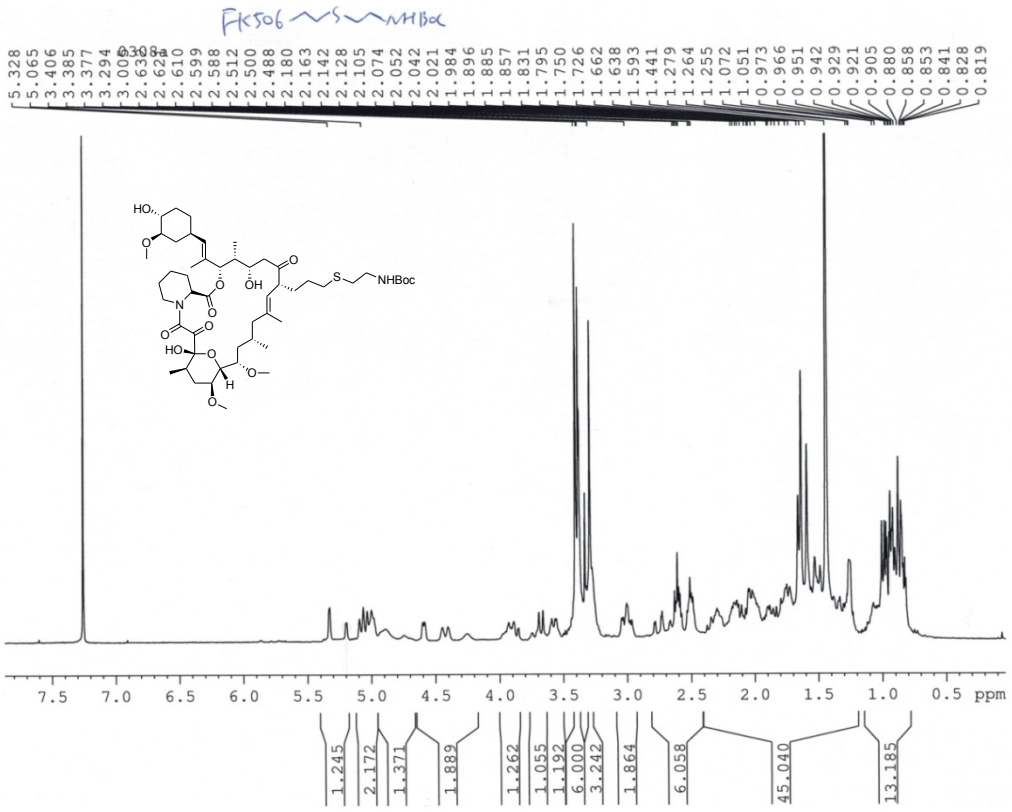
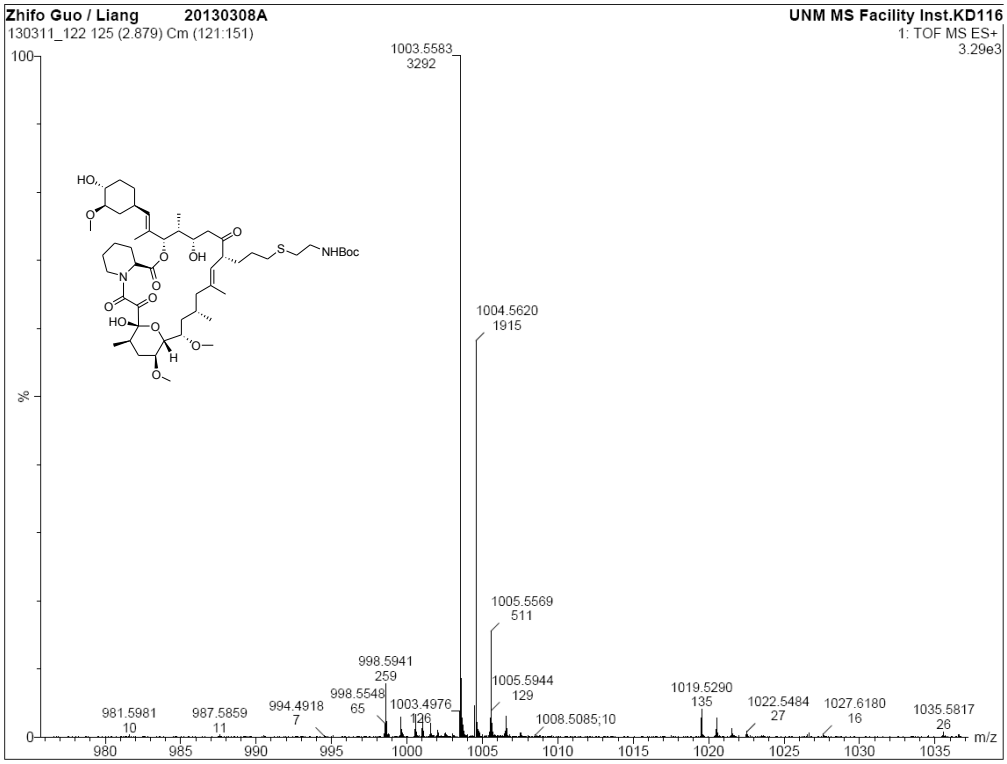
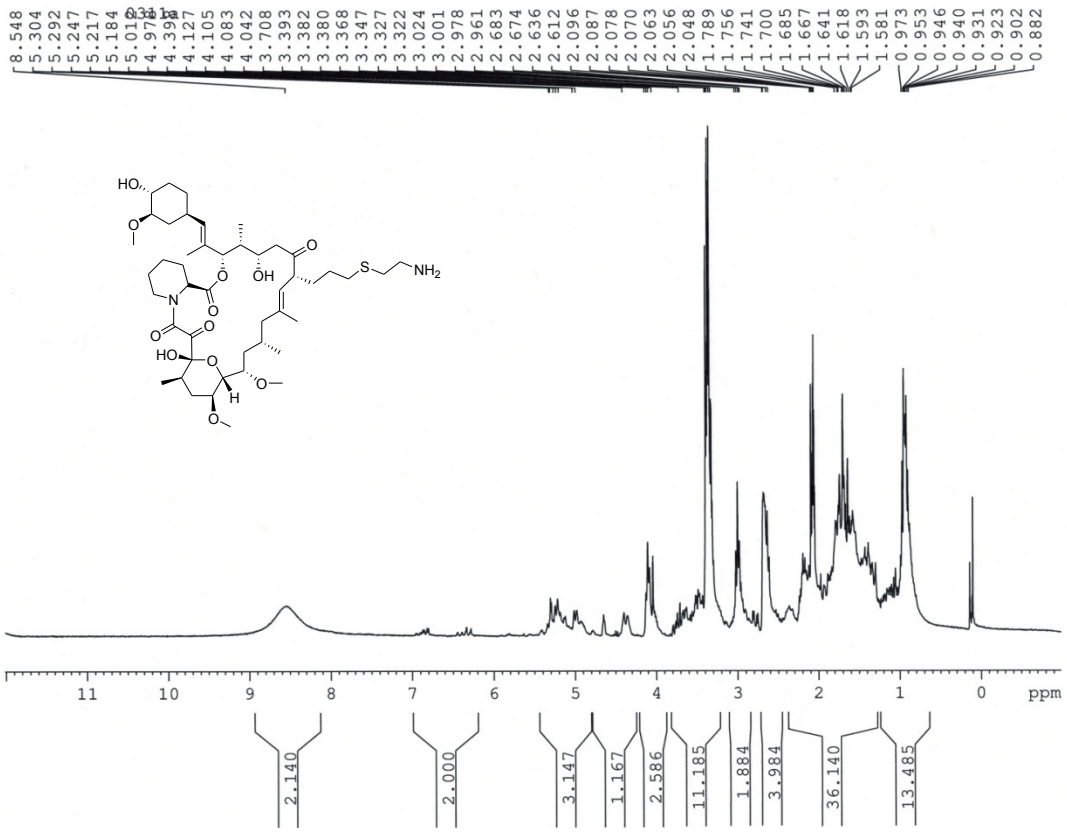


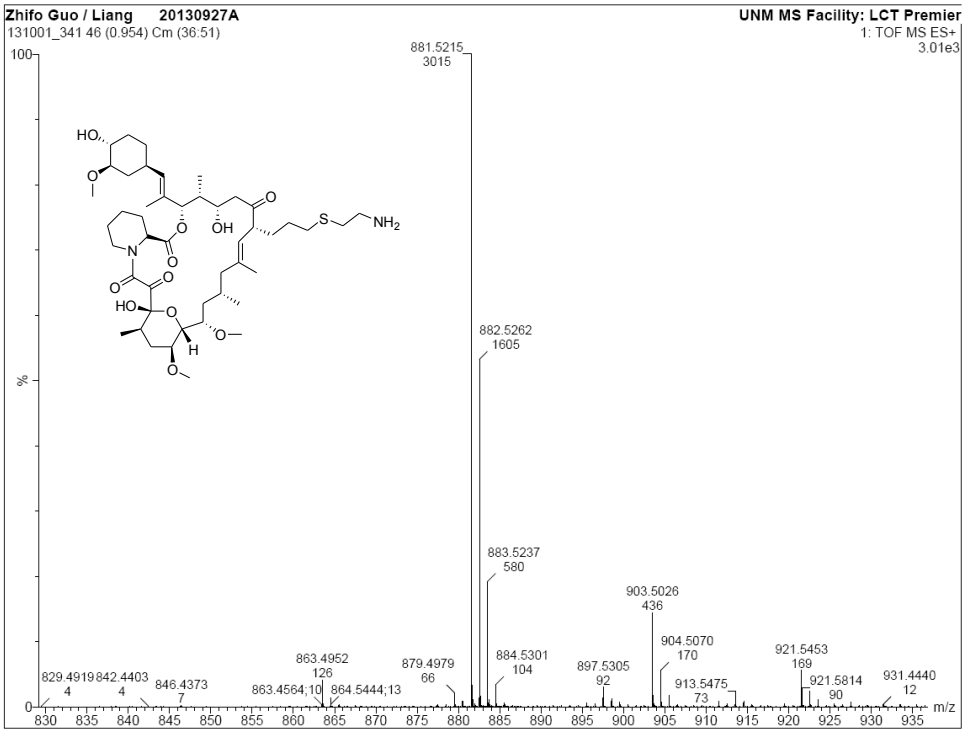
^{1}H NMR of compound **3**



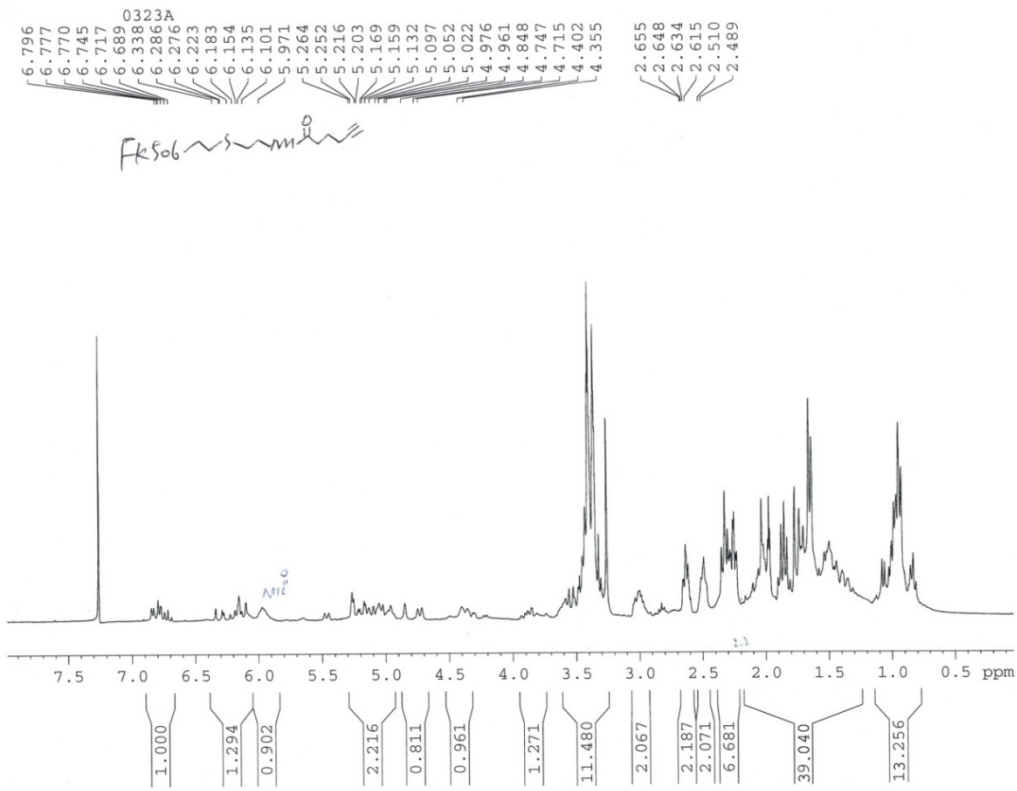
HRMS of compound **3**



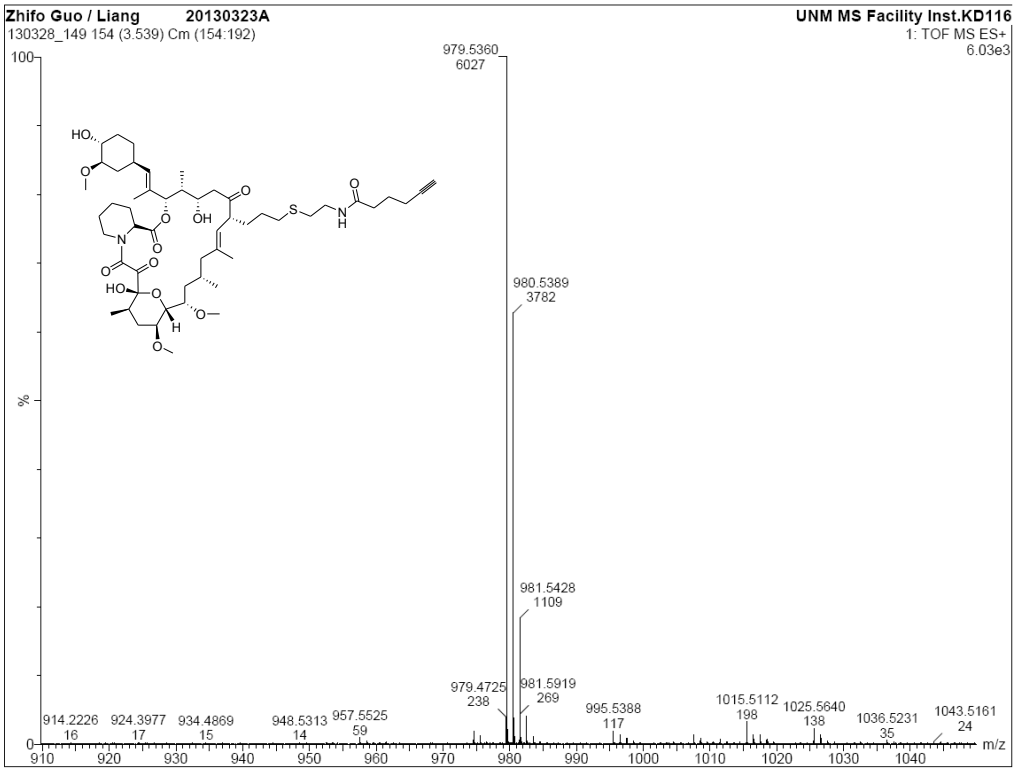
^{1}H NMR of compound **4**



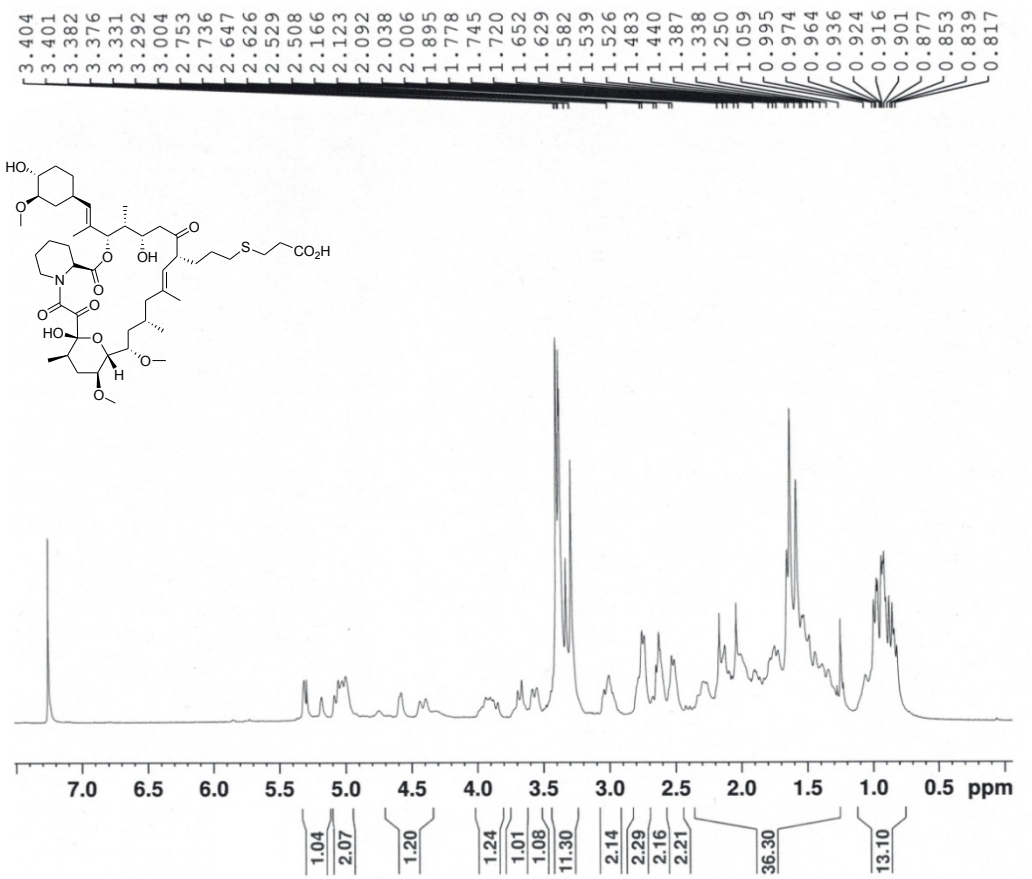
HRMS of compound **4**



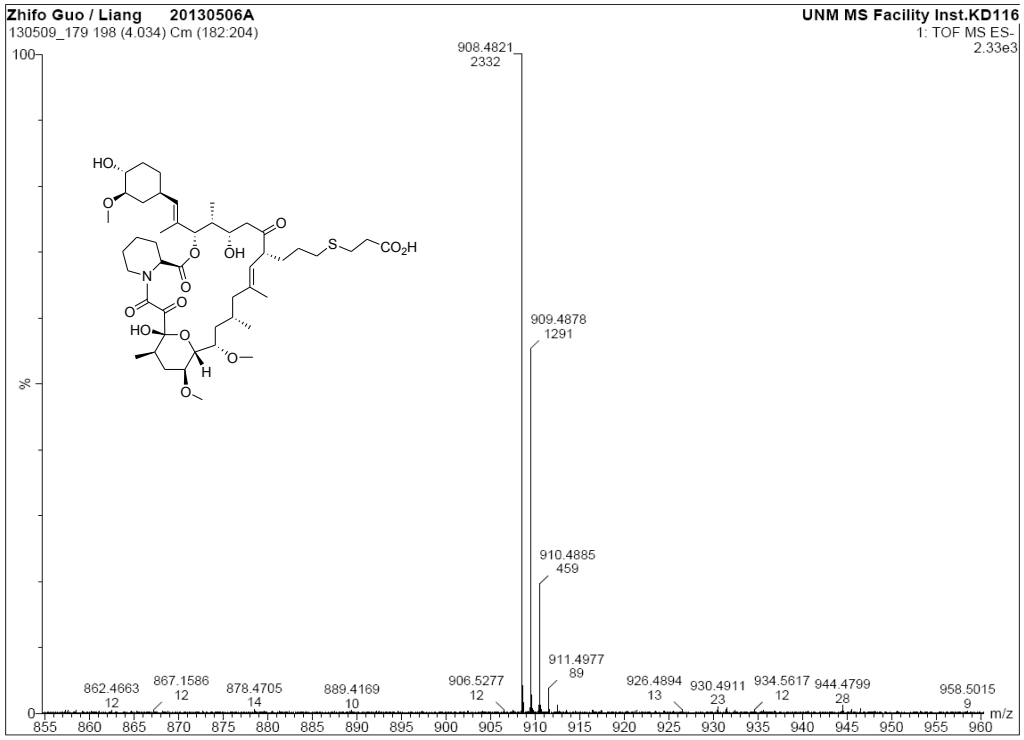
^{1}H NMR of compound **6**



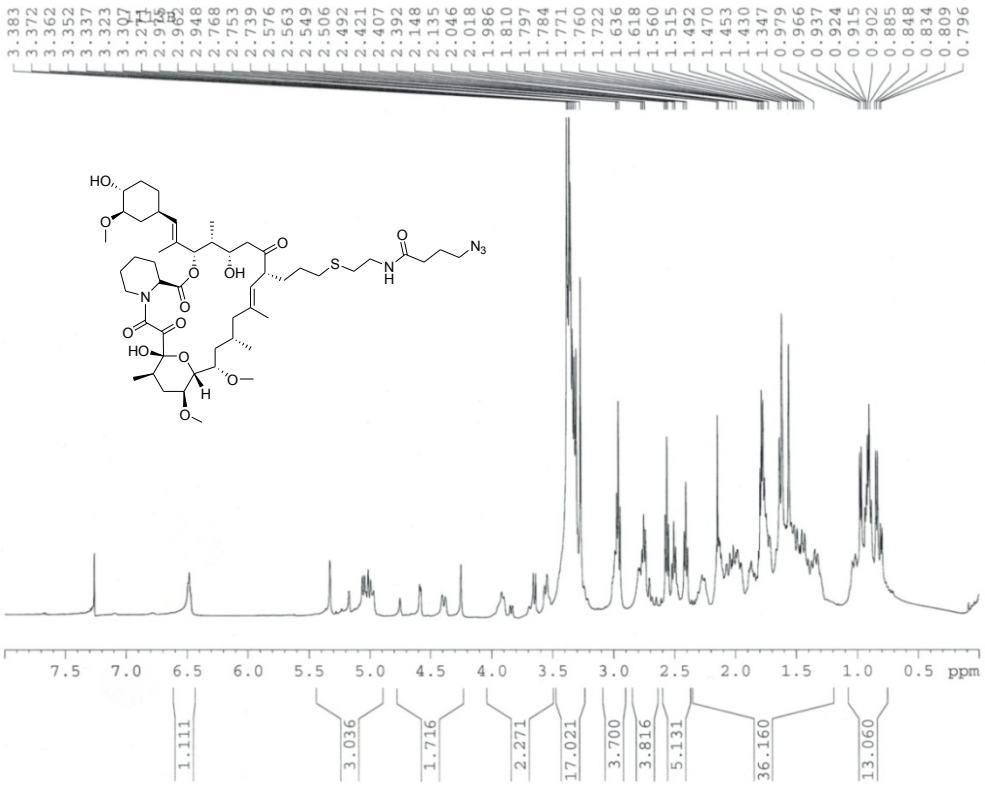
HRMS of compound **6**



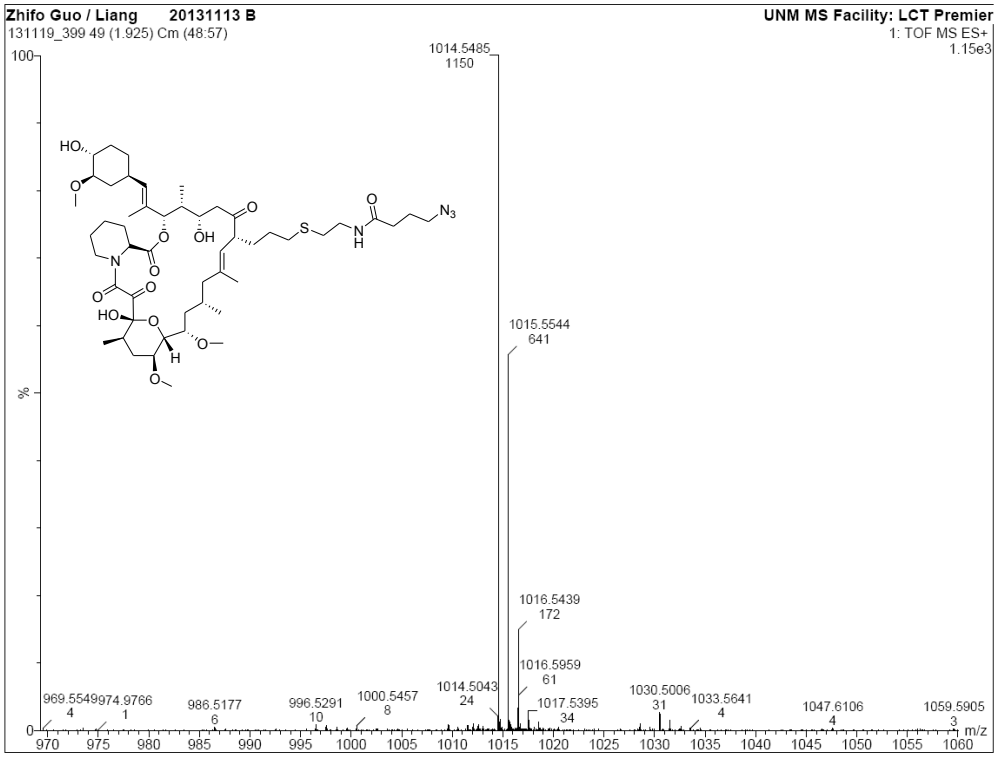
^{1}H NMR of compound **11**



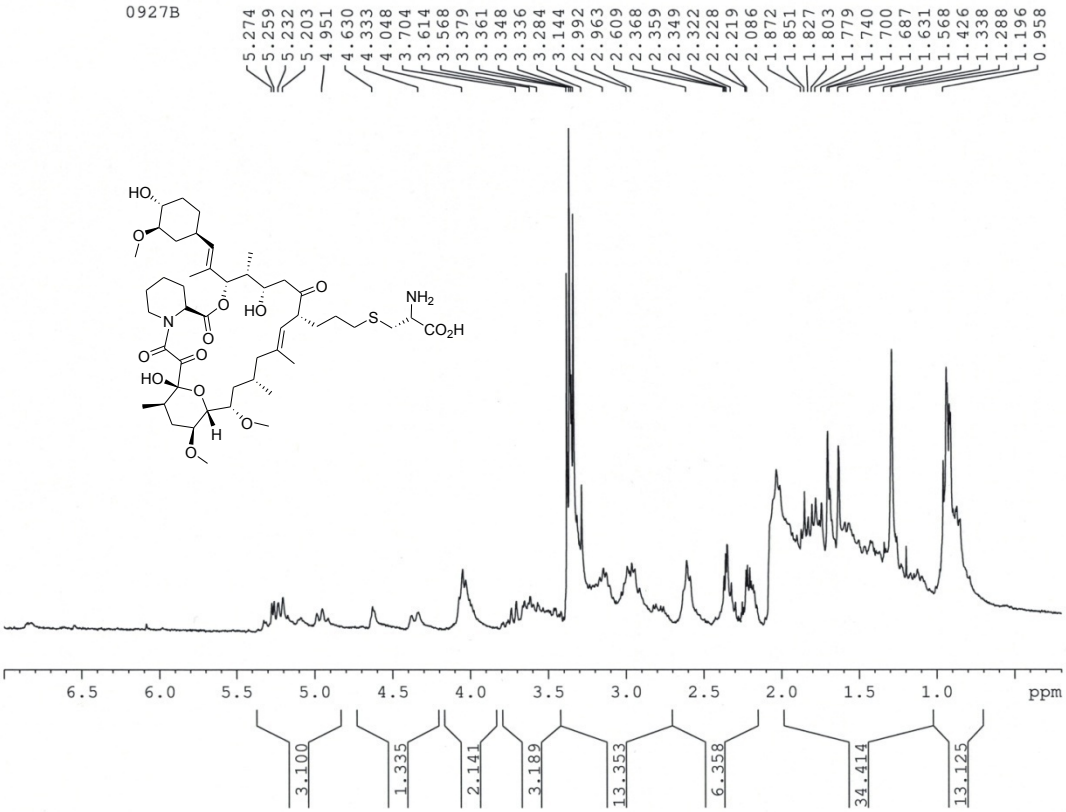
HRMS of compound **11**



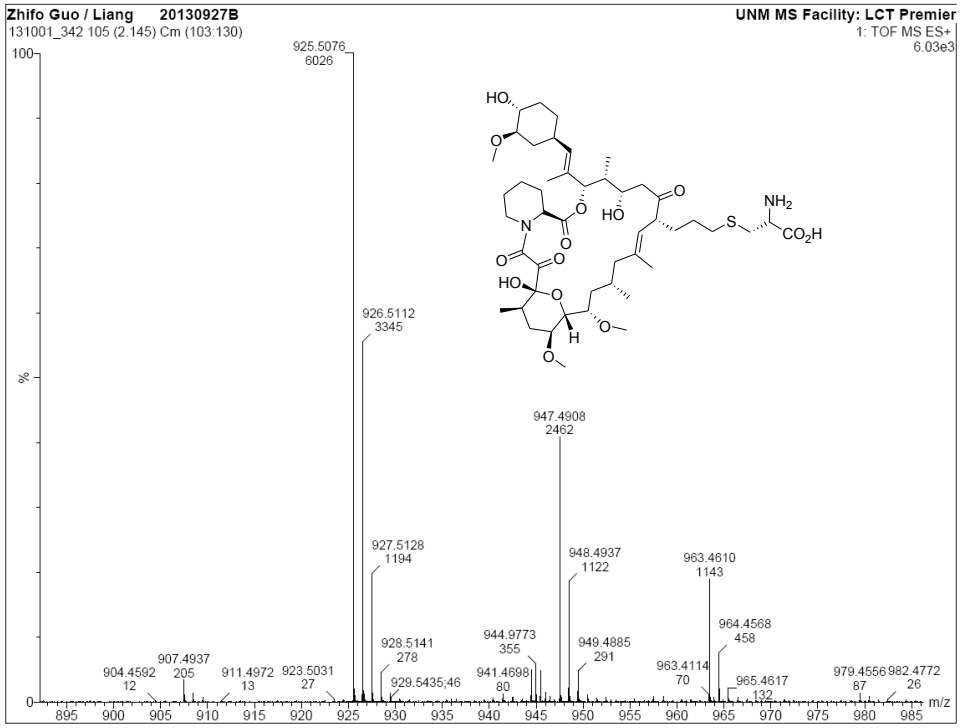
^{1}H NMR of compound **12**



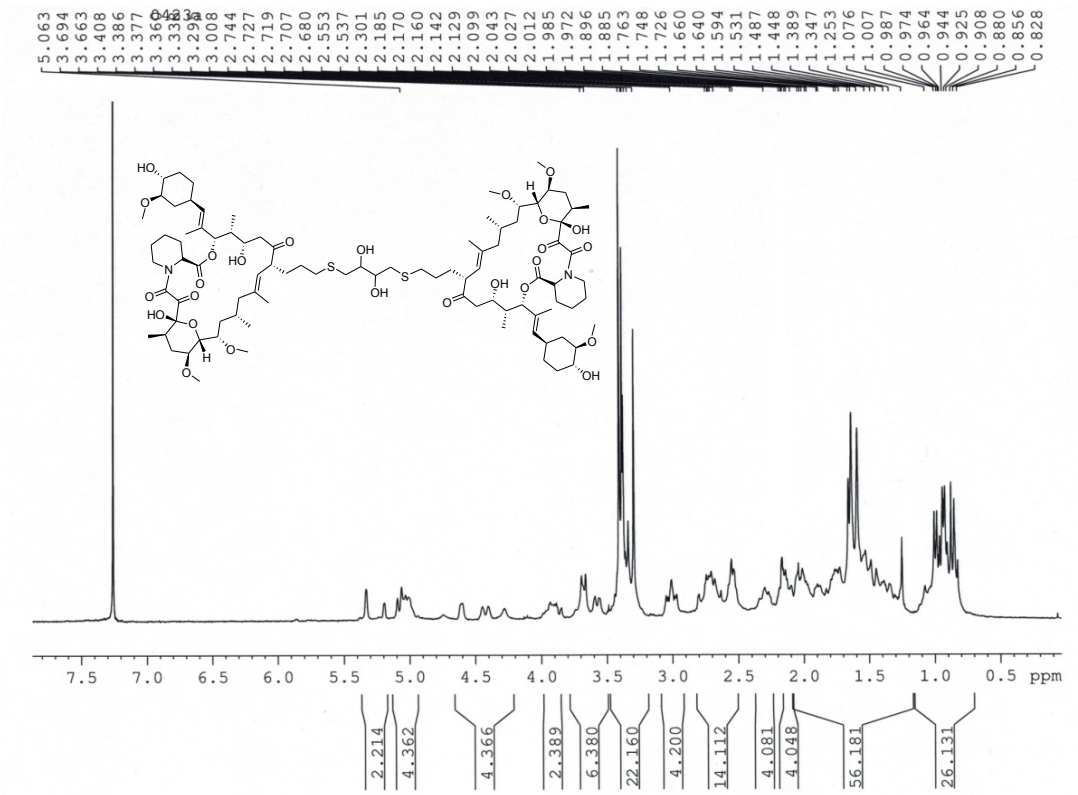
HRMS of compound **12**



^{1}H NMR of compound **13**



HRMS of compound **13**



^{1}H NMR of compound **15**

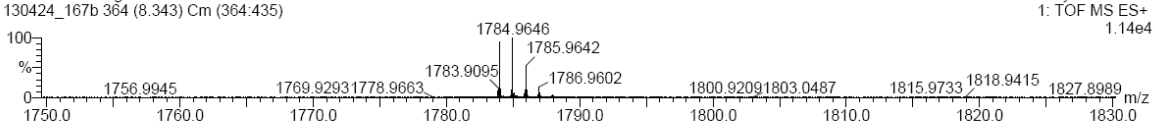
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)

Zhifo Guo / Liang 20130423A
130424_167b 364 (8.343) Cm (364:435)

UNM MS Facility Inst.KD116
1: TOF MS ES+
1.14e4



Minimum:				-1.5		
Maximum:		200.0	20.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
1783.9602	1783.9659	-5.7	-3.2	19.5	2	C92 H148 N2 O26 Na 32S2
	1783.9320	28.2	15.8	23.5	3	C93 H143 N2 O27 32S2
	1783.9296	30.6	17.2	20.5	1	C91 H144 N2 O27 Na 32S2

HRMS of compound **15**