Supporting Information

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Abbreviations.

DIPEA: diisopropylethylamine

DMF: dimethylformamide

EDCI: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride

HATU: 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate

NMM: N-methylmorpholine

Et_3N: triethylamine

TFA: trifluoroacetic acid

THF: tetrahydrofuran

MeOH: methanol

LiOH: lithium hydroxide

DMF: dimethylformamide

EtOAc: ethyl acetate

DCM: dichloromethane

EtOH: ethyl alcohol

DMAP: 4-dimethylaminopyridine

Pd/C: palladium on carbon

TMS: trimethylsilane

CAM: ceric ammonium molybdate

$KMnO_4$: potassium permanganate

H_2SO_4: sulfuric acid

PBS: phosphate buffered saline

Supplementary Figures and Schemes.

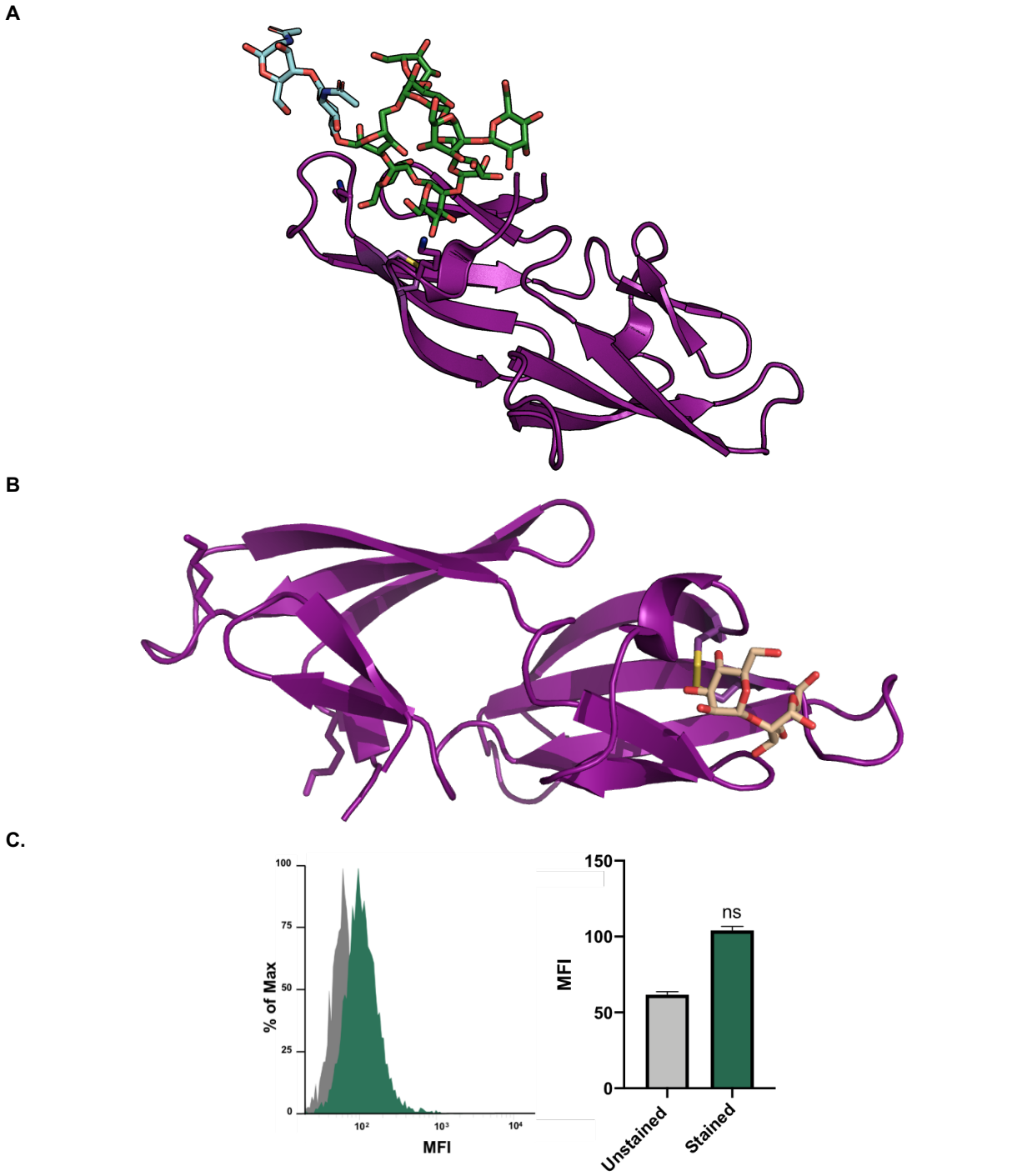


Figure S1. Mannose recognition domain highlighting the positioning of the lysine and cystine residues. A) Overlay of the Cyt-CVNH crystal structure (PBD: 5k79) with the Man-9 glycan bound to cyanovirin (PDB: 3GXZ) in domain A. B) Overlay of the Cyt-CVNH crystal structure (PBD: 5K79) with the Man-2 disaccharide bound to cyanovirin (PDB: 2DRK) in domain B. Models were prepared as previously described.[1] C) Cyt-CVNH LSH was functionalized with MB 488 NHS ester through conjugation to nucleophilic residues, and the ability of the resulting construct to label high mannose expressing CHO K1 cell was assessed. Left – Histogram depicting the binding of this construct to CHO-K1 cells (Grey = unstained controls; Green = Cty-CVNH labelled with MB 488). Right – Bar chart depicting the binding of this construct to CHO-K1 cells (Grey = unstained controls; Green = Cty-CVNH labelled with MB 488). MFI = Median Fluorescence Intensity.

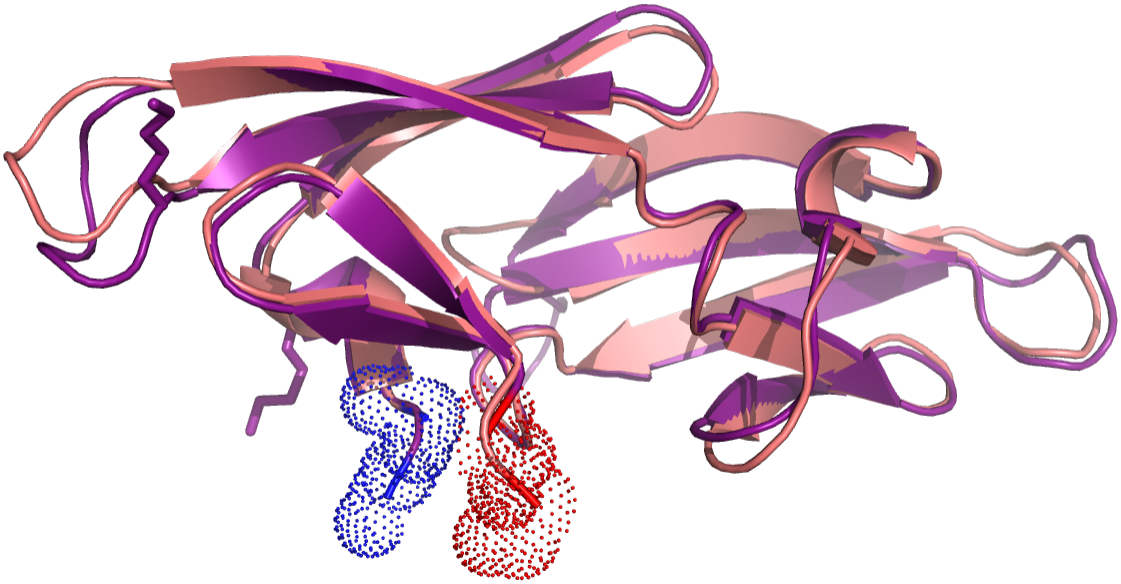


Figure S2. Structural similarities between Cyt-CVNH and Cyanovirin. Overlay of Cyt-CVNH crystal structure (Purple, PBD: 5k79) and the cyanovirin crystal structure (Peach, PDB: 3GXZ). The N- and C-termini are highlighted with blue and red spheres respectively.

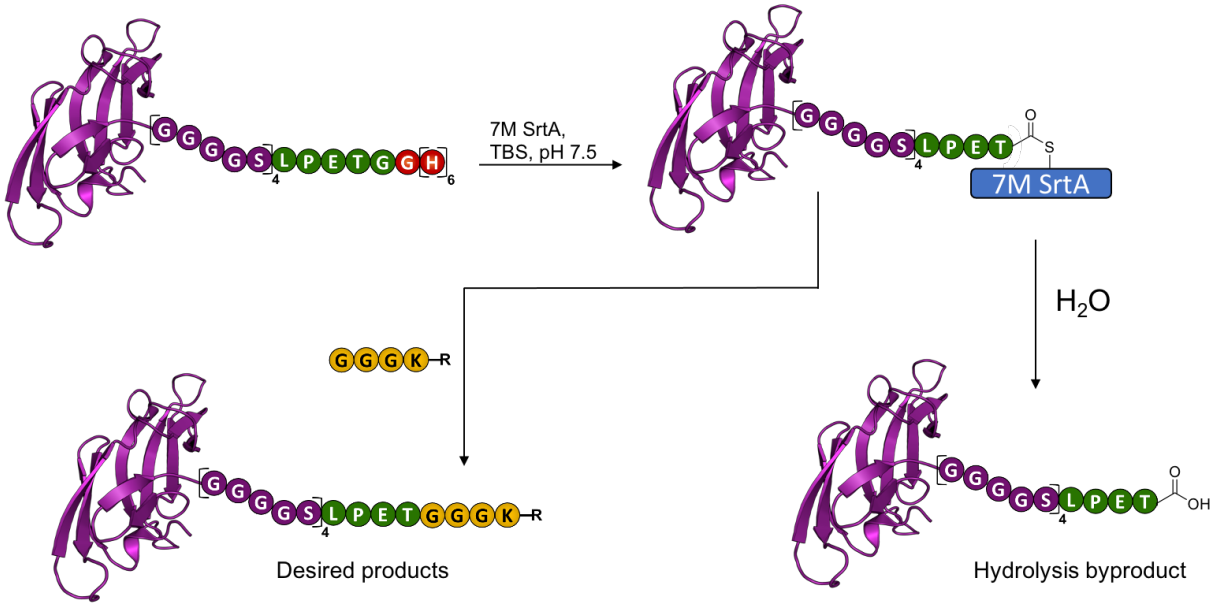
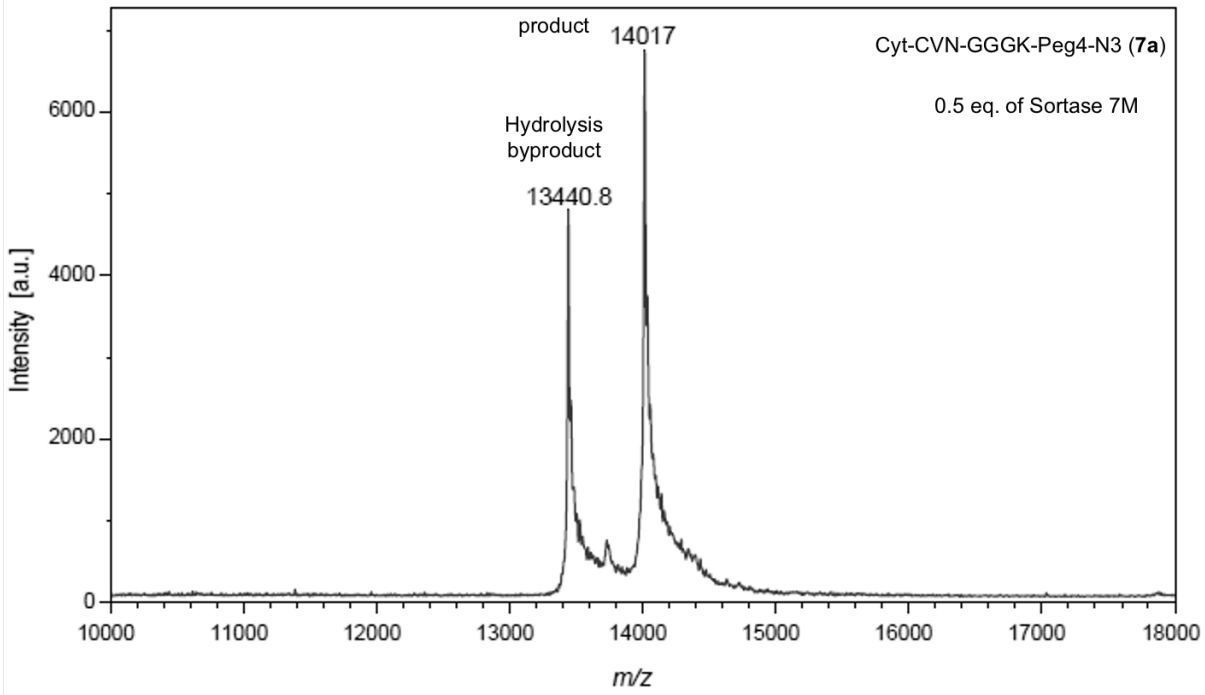
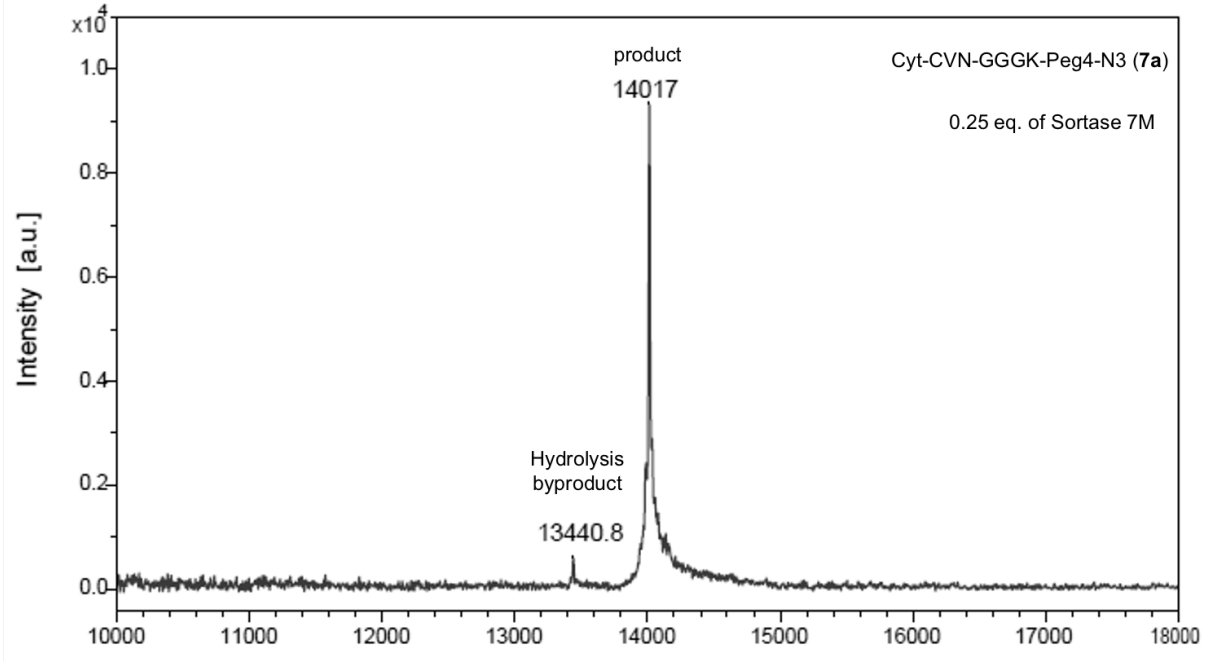


Figure S3. Hydrolysis byproduct formation. Sortase mediated ligations proceed via a thioester intermediate that can react with a functionalized triglycine motif to form desired products (bottom left) or with water to from a truncated byproduct.

A



B



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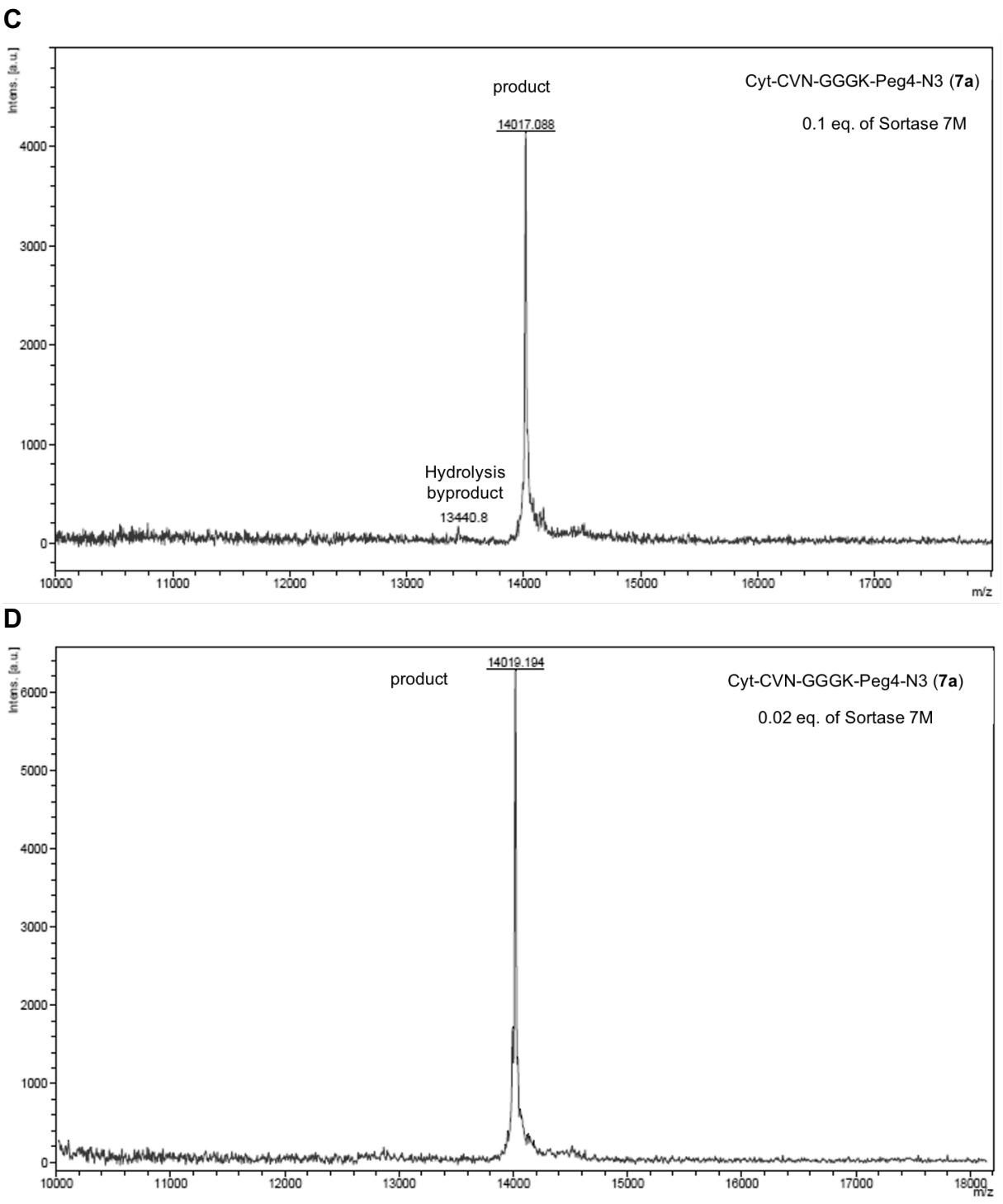


Figure S4. Altering ligation conditions to circumvent the formation of the hydrolysis by product. A-D) conditions are noted on each spectrum.

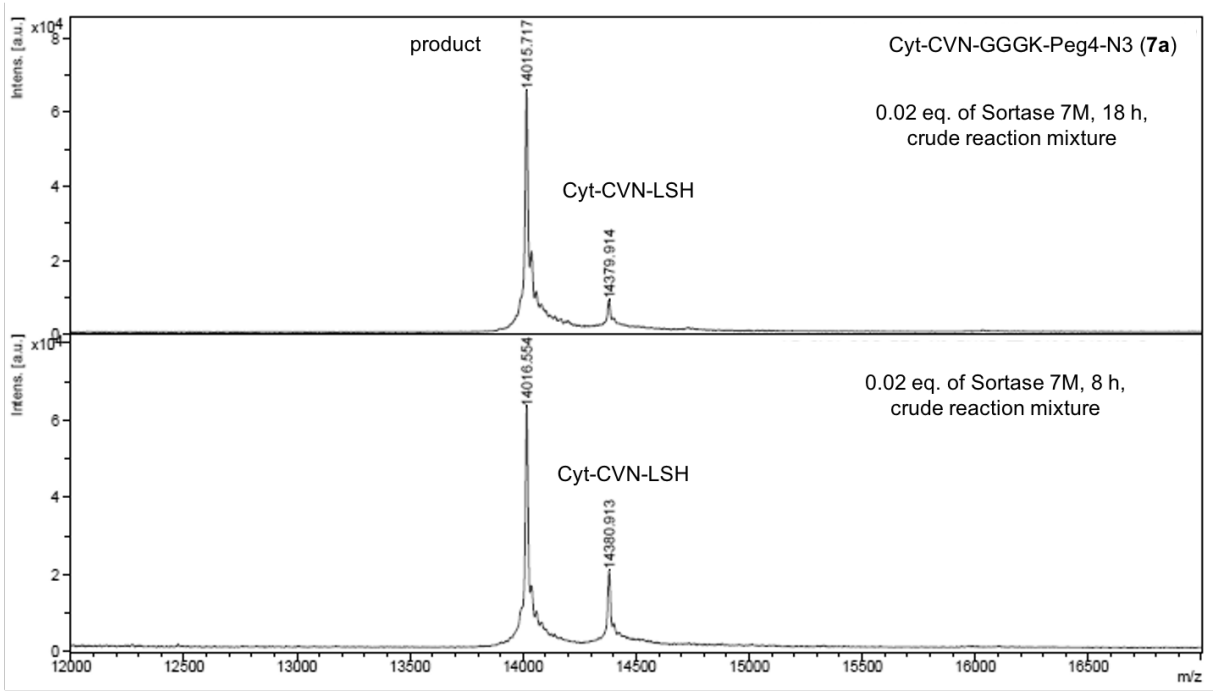


Figure S5. Consumption of starting material after 8 hours (Bottom) and 18 hours (Top)

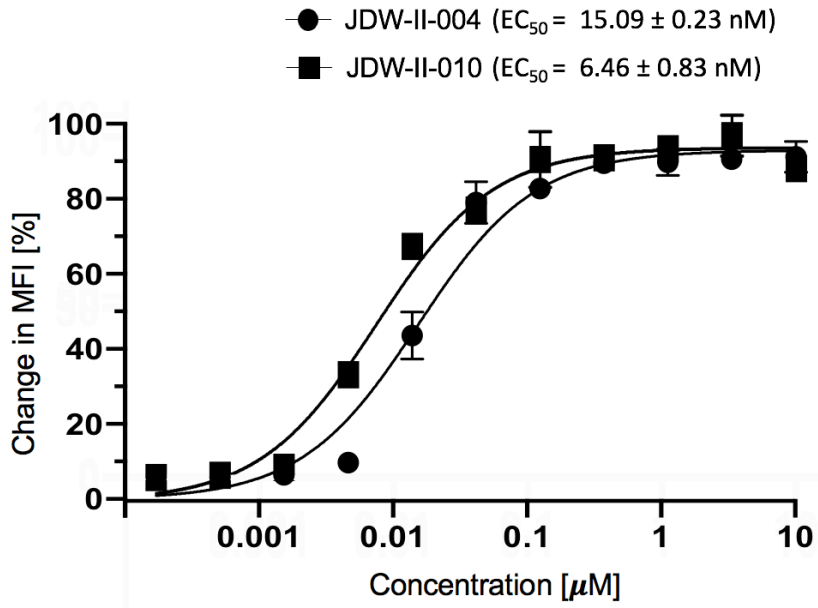


Figure S6. Activity of 11b and 11c in Jurkat cells.

Materials and Instrumentation.

All reactions were carried out in oven- or flame-dried glassware. Reagents and solvents were obtained from Fisher Chemicals, and were used as received unless otherwise mentioned. Anhydrous solvents were used directly from solvent purification system from innovative technologies. Thin layer chromatography (TLC) was carried out on aluminium-backed silica gel TLC plates w/UV254 (Sorbent Technologies), and revealed by UV irradiation (254 nm) or using CAM, 10% H_2SO_4 in EtOH or $KMnO_4$ solution followed by developing with gentle heating. Normal phase column chromatography purification of the intermediates and final compounds were performed by a Teledyne ISCO CombiFlash Rf system using appropriate silica gel pre-packed column, and preparative reverse-phase HPLC system Agilent technologies 1210 using preparative column Macherey Nagel HPLC Column Isis 21x250mm, 5 µm. All preparative runs were performed at flow rates of 20.0 ml/min. using a mixture of acetonitrile (HPLC grade, Fisher Chemicals, USA) and Milli-Q-water both acidified with 0.1% TFA. Yields mentioned refer to chromatographically and spectroscopically isolated pure compounds.

NMR spectra were recorded on a 400 MHz spectrometer (Avance III 400), a 500 MHz spectrometer (Avance III 500) with and without cryoprobe from Bruker in MeOD (^{1}H, δ = 3.31 ppm, ^{13}C, δ = 49.00 ppm), D_2O (^{1}H, δ = 4.79 ppm) or $CDCl_3$ (^{1}H, δ = 7.26 ppm, ^{13}C, δ = 77.16 ppm) obtained from Cambridge Isotopes Ltd. Chemical shifts (δ) are given in ppm and for the analysis of spectra, the software MESTRENOVA 12.0.1 was used for analysis. The signals were quoted as follows: s = singlet, bs = broad singlet, d = doublet, t = triplet, quin. = quintet, dd = doublet of doublet, ddd = doublet of doublet of doublet, td = triplet of doublet, dt = doublet of triplet and m = multiplet. High-Resolution Mass

Spectrometry (HRMS) analysis was performed using an electrospray ion source (ESI) either in positive mode or negative mode and with a time-of-flight (TOF) analyzer on a Waters LCT Premier ^{TM} mass spectrometer and are given in *m/z*. Optical rotation values were measured on a Rudolf Research analytical, Autopol III polarimeter using the sodium D line (589 nm). Protein mass were determined using Bruker Autoflex maX LRF MALDI-TOF mass spectrometer (Bruker Daltonics, Bellerica MA). Infrared (IR) spectra were recorded on a Thermo Scientific™ Nicolet™ iS™5 FT-IR Spectrometer; data are reported in frequency of absorption (cm^{-1}); only the most significant adsorption bands are reported; samples are analyzed as thin films deposited on Zn/Se crystal from solvent evaporation.

Protein expression and Purification.

Cyt-CVN-LSH

Cloning

A gene sequence of:

(GTGAAGAAAGCCAGCCTGCTGTTAAAGATTGCACTTGGCTTTATTTTCACGTTATTCGTCAGTTTTAA
CATTGTCGTCGATTCGGCAATGGCCACGGGGCAGTTCTCAAAGACGTGTGAGGACATCACGTTGGA
CGGGTCTACTTTGTCGGCGTTCTGCCAAAAGGCTGACGGATACACTCTGAATGAGACGTCCATCAAT
TTGGATGAGGAAATTGGGAATTTGGACGGGACTTTGTCTTGGGGAGACCATAACTTCTCTCTGACTT
GTGATAGCATTGGCTTAGCGCAGTCGTTGTTTACCCGCACCTACGTGCTGGCAGCCGAATGCGAAC
GCCGTGATGGATACACATATATCCCGACAGAGATTGAGTTGGACGAGCACATTGCCAATATCGACGG
TACTCTGACGTATGAGGGCGGCGGTGGAAGTGGAGGCGGTGGCTCAGGGGGTGGGGGCAGTTTAC
CGGAGACGGGCGGTCATCATCACCACCATCACTGA**)** Sig-CVNH-LSH was synthesized by IDT.

The CVNH-LSH construct was amplified by PCR with the following forward and reverse primers using the synthesized gene above:

(5'-AAAAATTGGCCATGACGGGGCAGTTCTCAAAGA-3')

(5'-TATTTCTCGAGTTACTCATACGTCAGAGTACCGTCGAT-3')

The PCR fragment was cloned into pET26, resulting the pET26-CNVH-LSH construct. The Sanger sequencing method confirmed the DNA sequence.

pET26-CNVH-LSH amino acid sequence is shown below:

MKYLLPTAAAGLLLLAAQPAMA/MTGQFSKTCEDITLDGSTLSAFCQKADGYTLNETSINLDEEI
GNLDGTLSWGDHNFSLTCDSIGLAQSLFTRTYVLAAECERRDGYTYIPTEIELDEHIANIDGTLT
YEGGGGSGGGGSGGGGSLPETGGHHHHHH

Cyt-CVNH-LSH Expression and Purification

A single colony of *E. coli* expression cells Tuner (DE3) (Novagen) containing harboring the plasmid pET15b-His-IpaD was inoculated in 50 mL conical tubes containing 10 mL LB (lysogeny broth) media + 40 ug/mL kanamycin 34 ug/ml of and chloramphenicol and grew overnight at 37˚C with shaking at 250 rpm. The overnight culture was transferred to

a shaker flask containing 2 liters of TB (Terrific Broth) media + 100 ug/mL ampicillin and shook at 200 rpm at 37 ˚C until OD 600 nm reached about 0.7. The culture was cooled to 17˚C protein was induced with 0.4 mM IPTG at 17˚C overnight. Cells were pelleted at 3500 relative centrifugal force (rcf) for 10 min and resuspended in Buffer A containing 50 mM Tris-HCl (pH 8.0) and 500 mM NaCl. Resuspended cells were frozen at -80˚C. Frozen cells were thawed at room temperature and sonicated until resuspension was no longer viscous using the following cycle parameters: amplitude: 50%, pulse length: 30 sec, number of pulses: 12, while incubated on ice for >1 min between pulses. A soluble fraction was obtained by centrifuging the cell lysate at 45450 rcf for 30 minutes at 4˚C. The soluble lysate was loaded to a 5 ml HisTrap™ High Performance pre-packed column from GE HealthCare on an AKTA Pure chromatography system. Protein was eluted with a gradient of Buffer B (Buffer A+ 500 mM imidazole) from 10% to 100%. Two elution peak fractions were pooled separately and concentrated by centrifugal membrane filtration (MWCO=3kDa) to < 5 ml for the next size exclusion chromatography purification. These two fractions had the same protein that were confirmed by SDS-PAGE and Mass Spectrometry. The concentrated protein purified from Ni affinity chromatography was applied to a Superdex S75 Increase10/300 GL column equilibrated with 20mM Tris (pH 7.4), 500mM NaCl, 5mM CaCl2 on an AKTA Pure. The inject volume was 1-2 ml each time. Protein concentration was determined by either Bradford assay or using the extinction coefficient of 0.95 g/L for each unit of absorbance at 280 nm. The yield of 10 to 20 mg of protein was obtained for each protein preparation. The purified protein was concentrated to 1.0 to 1.5 mg/mL, flash frozen in liquid nitrogen, and stored in a -80˚C freezer.

Sortase 7M

pet30b-7M SrtA was a gift from Hidde Ploegh (Addgene plasmid # 51141; http://n2t.net/addgene:51141; RRID:Addgene_51141). Sortase 7M was expressed as previously described.[2]

Ligation and conjugation procedures.

General Procedure Sortase-mediated ligation

A mixture of Cyt-CVNH-LSH (**3**, 1 mg, 1 equiv.) and appropriate peptide **6a or 6b** (100-200 equiv.) was treated with Sortase A (7M) (0.02 equiv.) in 1 ml of 50 mM Tris (pH 7.5, 150 mM NaCl). The resulting mixture was incubated at 25 ^{o} C for 24 hrs. The pure protein was isolated using a two-step purification protocol: (i) His tag purification using Ni-NTA. Ni-NTA beads were added to reaction mixture and resulting suspension was incubated for 1 hour on a mechanical rocker to removed unreacted His tagged Cyt-CVNH-LSH and 7M SrtA. The unbound desired protein conjugate was isolated from Ni-NTA beads (400 μl of slurry). For the maximum recovery of desired protein, the beads were washed with PBS buffer (5 x 2 ml). The combined buffer solution containing protein conjugate and unreacted small molecule was then transferred to 10KDa Amicon® Ultra-15 centrifugal filter unit and concentrated (as per suppliers' protocol). At this point majority of small molecules can be removed by repeated washing with PBS buffer (400 μl x 5-10 times). Finally, concentrated protein was recovered and then transferred to appropriate size 10 KDa Slide-A-Lyzer™ Dialysis cassette and dialyzed against PBS buffer (pH 7.4, 4 l). The buffer was exchanged every 2 h for 6 h, and then dialysis was continued for 18 h at 4 °C. The concentration of final protein and reaction yield were determined using Nano-Drop™, and purity was assessed using MALDI and SDS-PAGE. Under optimized conditions yields of >75% were observed.

General Procedure for fluorescent labelling of the protein with SPAAC.

A solution of Cyt-CVNH-GGGK-PEG_4-N_3 (1 mg, 1 equiv.) was mixed with the appropriate DBCO-Fluorophore (3 equiv., MB 488-DBCO, AF594-DBCO or AF647-DBCO) in 1ml of PBS (pH 7.4) buffer for 24 hrs. The desired fluorophore labelled CVNH protein was directly purified by dialysis using 10 KDa Slide-A-Lyzer™ dialysis cassettes in PBS buffer (4 l). The buffer was exchanged every 2 h, and dialysis was continued for 18 h at 4 °C. The concentration of final isolated protein-fluorophore conjugate and reaction yield was determined using Nano-Drop™, and purity was assessed using MALDI and SDS-PAGE. Yields of >85% were observed in all cases.

Protein analysis.

Protein analysis by SDS PAGE

Protein samples were added to RunBlue™ LDS sample buffer and DTT Reducer. The samples were heated for minutes at 70 °C. Samples (2 μg) were loaded and electrophoresed in RunBlue™ 12% TEO-Tricine Precast gel using RunBlue™ SDS Running Buffer at 180V constant for 40 minutes. Gels were stained with InstantBlue Coomassie protein stain, and partially distained with distilled deionized water. Gels were imaged using an iBright imaging system.

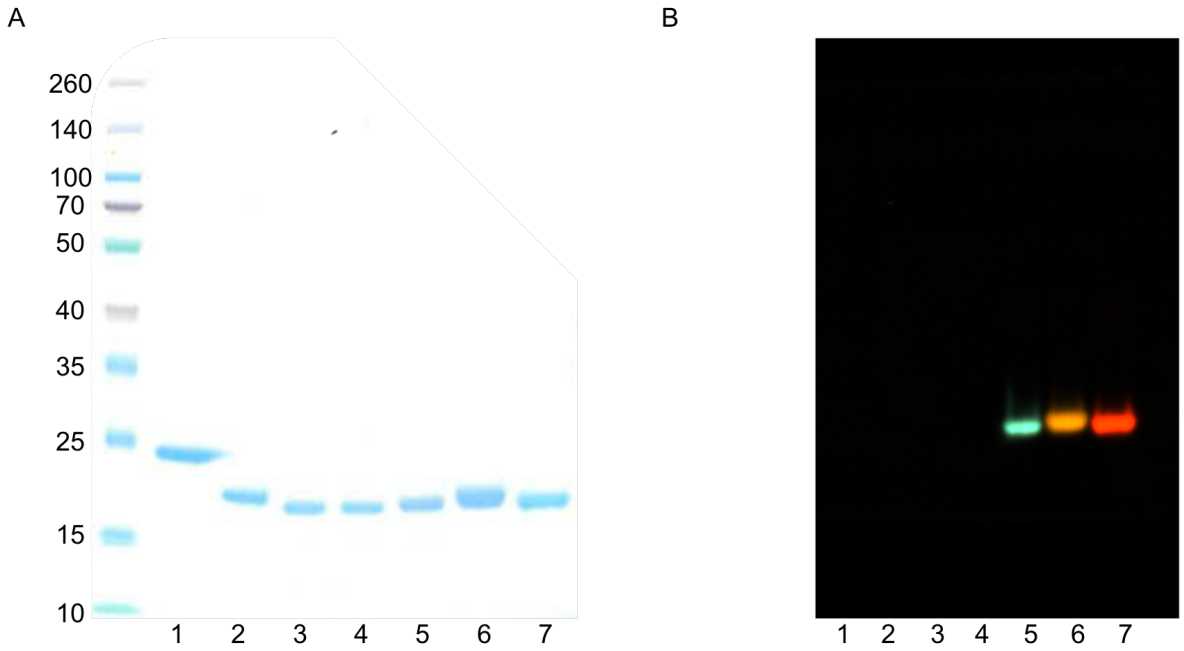


Figure S7. Protein analysis by SDS PAGE. A) Brightfield protein gel image. B) Fluorescence protein gel image. Lanes: 1. Srt A 7M; 2. Cyt-CVN-LSH (**3**); 3. Cyt-CVNH H-N_3 (**7a**); 4. Cyt-CVNH-Biotin (**7b**); 5. Cyt-CVNH-MB 488 (**9A**); 6. Cyt-CVNH-AF Dye 594 (**9B**); 7. Cyt-CVNH-AF Dye 647(**9C**).

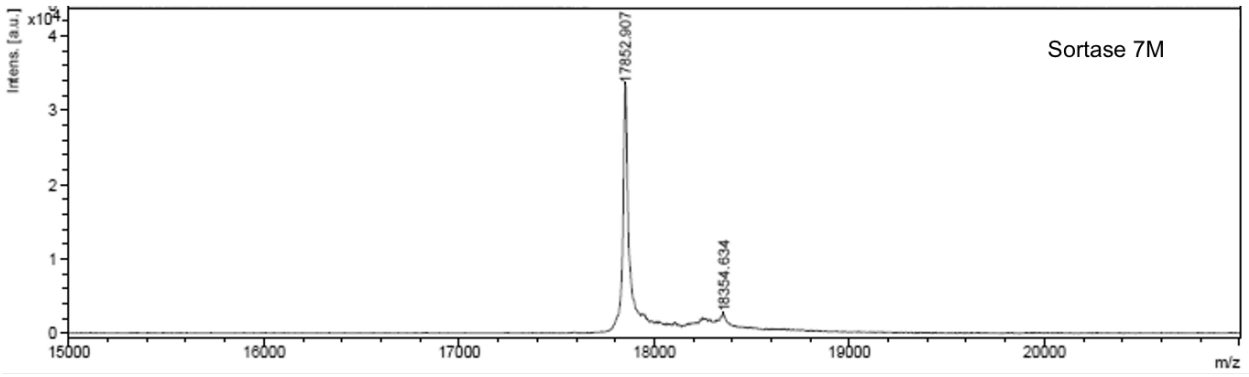
Note: The protein molecular weights do not match precisely wth the protein ladder molecular weight marker, however, the molecular weights of the proteins have been confirmed by MALDI.

MALDI Analysis Protocol:

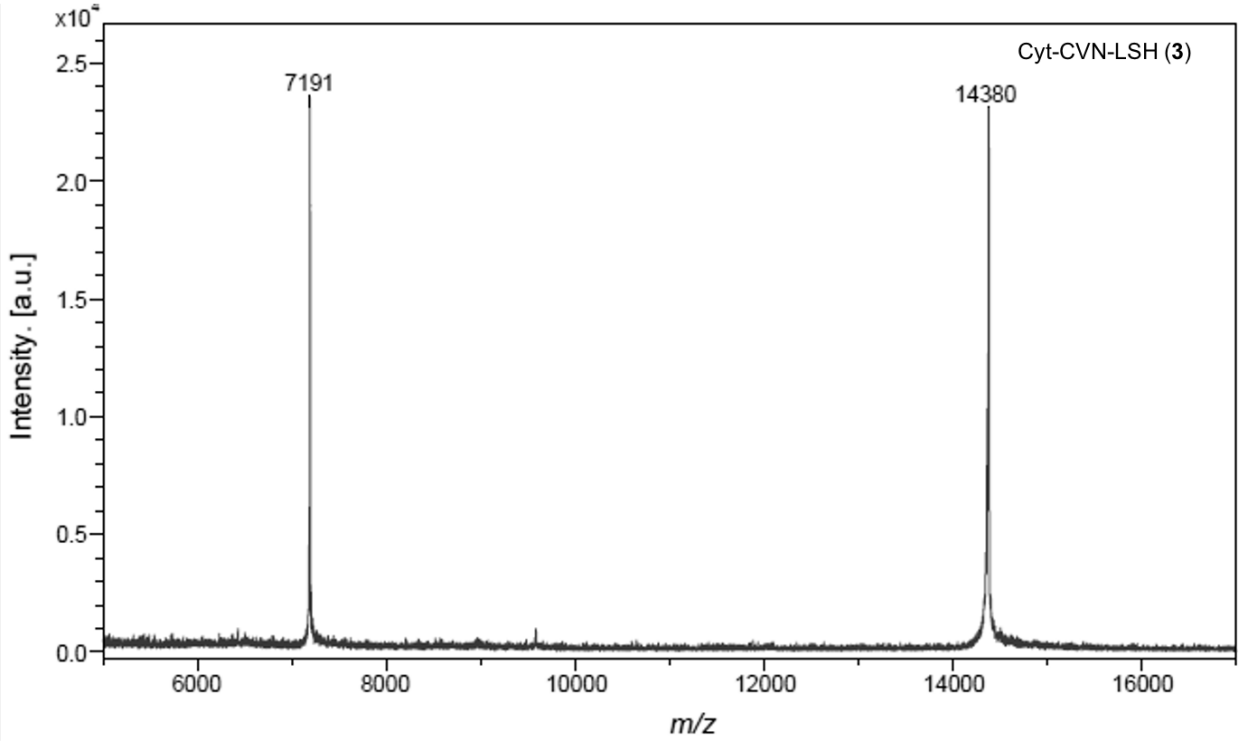
MALDI samples were prepared by combining one part of the protein, one part of 2',4',-dihydroxyacetophenone (DHAP, 15 mg/mL in ammonium citrate buffer) solution, and one part of 2% TFA. The resulting solution was mixed thoroughly. Once crystallization started, 1µL of the solution was spotted onto a stainless steel MALDI plate then dried before loading the plate into the Bruker Autoflex maX LRF MALDI-TOF mass spectrometer (Bruker Daltonics, Bellerica MA). Data were acquired using the linear-positive ion mode with the following acquisition parameters: an IS1 voltage of 19.5 kV, an IS2 voltage of 18.3 kV, a lens voltage of 7 kV, and a delay time of 350 nsec.

<u>MALDI Spectra</u>:

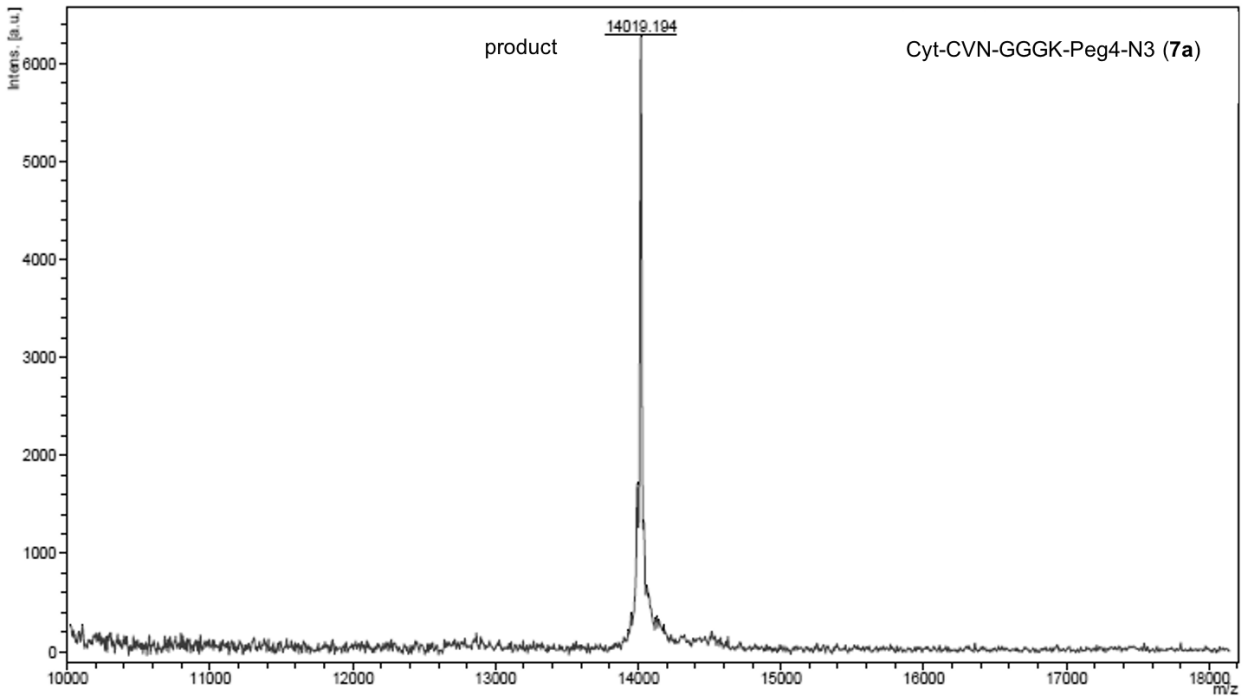
Sortase 7M



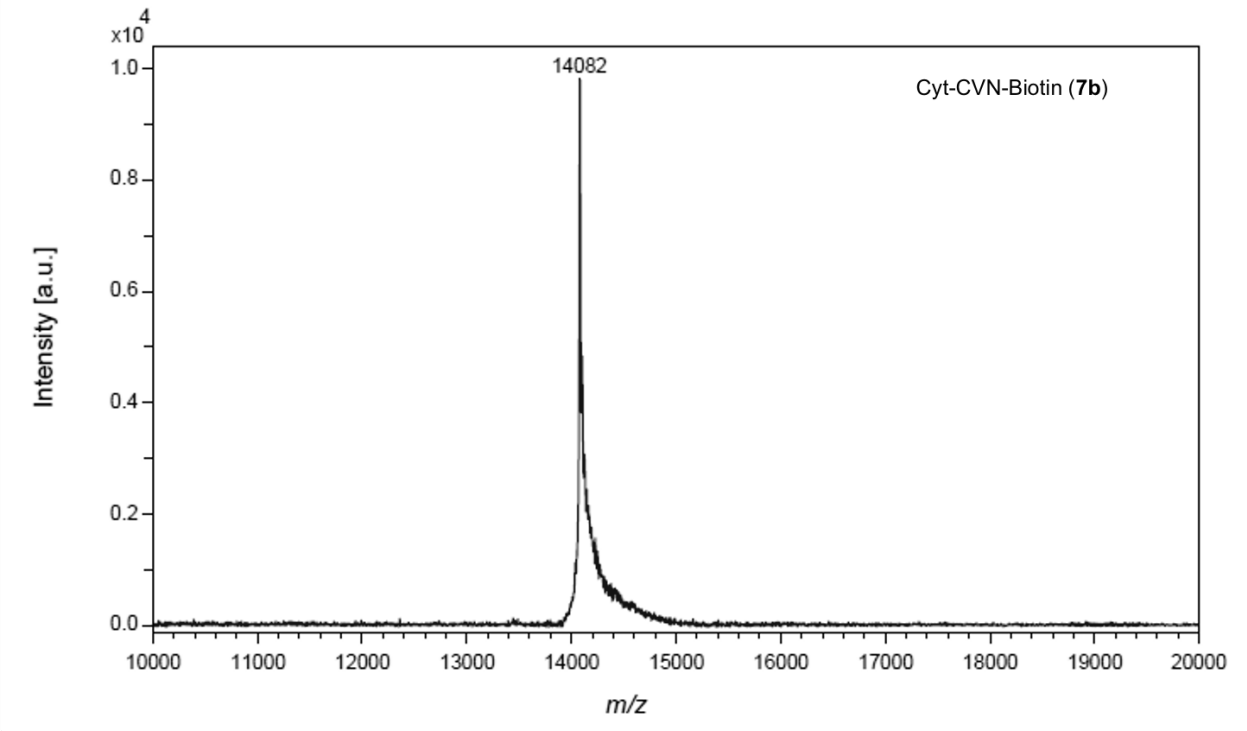
Cyt-CVNH-LSH (**3**)



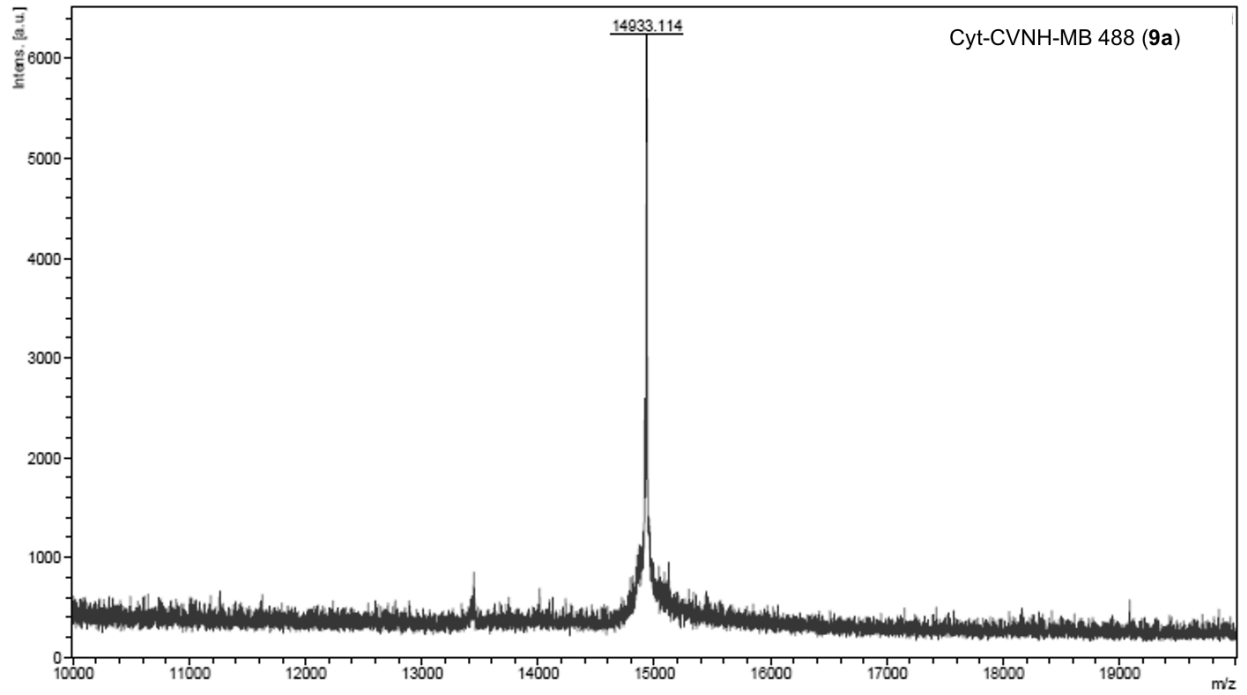
Cyt-CVNH H-N_3 (**7a**)



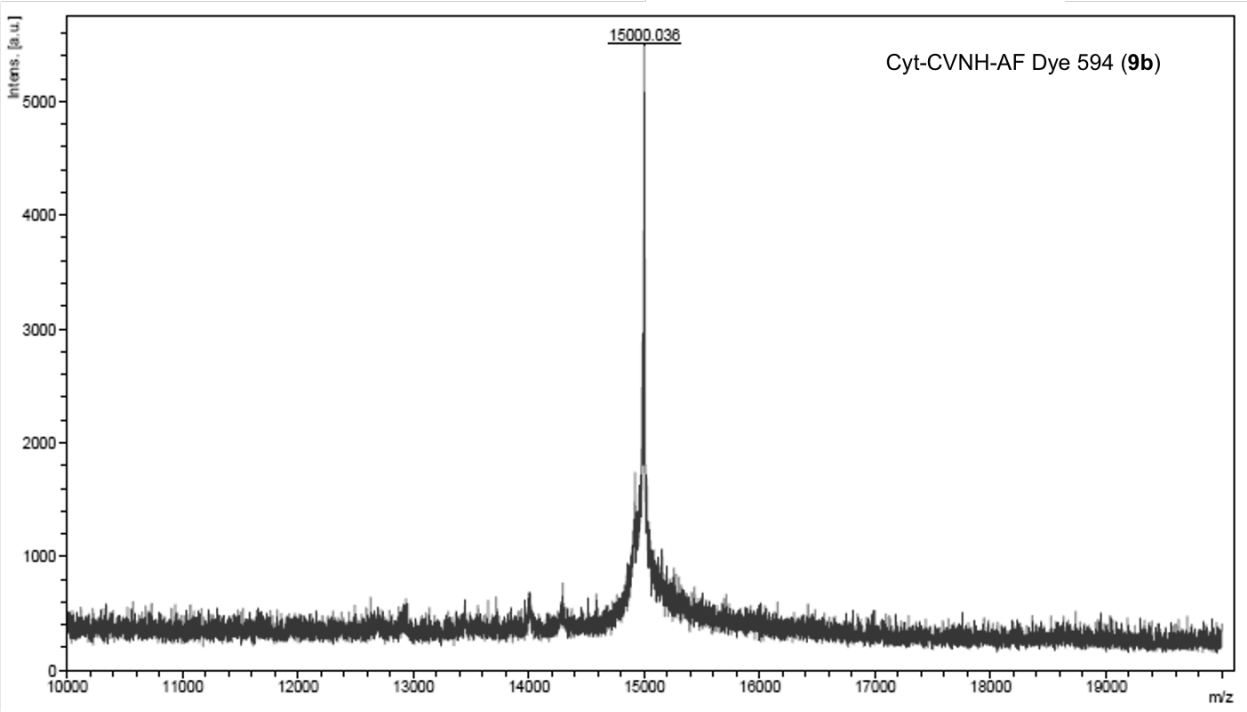
Cyt-CVNH-Biotin (**7b**)



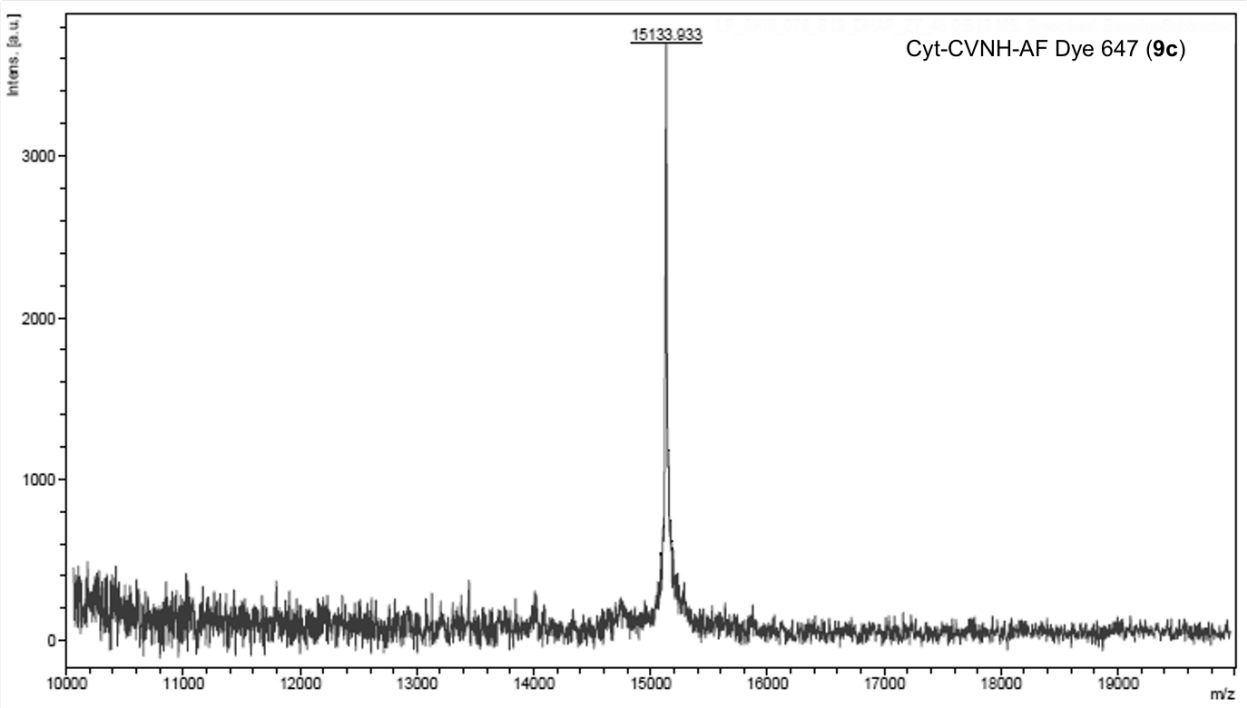
Cyt-CVNH-MB 488 (**9A**)



Cyt-CVNH-AF Dye 594 (**9B**)



Cyt-CVNH-AF Dye 647(**9C**)



Cell culture and assay procedures.

Cell culture

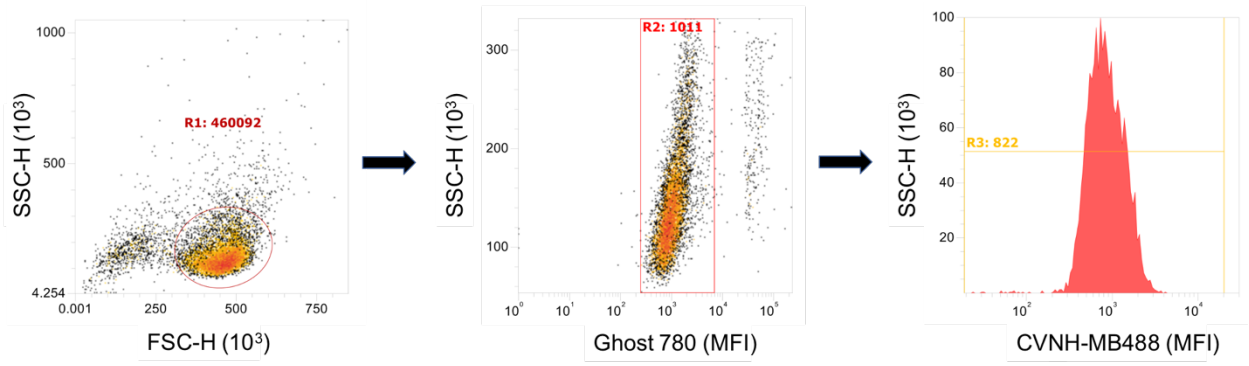
Raji, Jurkat, CHO K1, HEK 293 and MDA-MB-231 cell lines were purchased from ATCC. Raji, Jurkat and CHO K1 cells were maintained in RPMI 1640 (GIBCO) with HEPES 5 mmol/L, sodium pyruvate 1 mmol/L, 1% NEAA (GIBCO), 10% premium heat-inactivated FBS (Corning), and 100 μg/mL Normicin (Invivogen) at 37 °C in a fully humidified atmosphere of 5% CO_2. MDA-MB-231 and HEK 293 cells were maintain in DMEM (GIBCO) with 10% premium heat-inactivated FBS (Corning), and 100 μg/mL Normicin (Invivogen) at 37 °C in a fully humidified atmosphere of 5% CO_2. Cell lines were maintained in T25 or T75 flasks, and were disposed of after a maximum of 15 passage cycles.

CHO K1 and HEK 293 cell staining and analysis by flow cytometry.

Media was removed by aspiration from a T75 flask containing cells. Cells were then washed with warm (~ 37 °C) sterile PBS twice. The PBS was removed by aspiration, before 3 ml of enzyme free dissociation buffer was added. The flask was then incubated for 1-5 minutes and was gently tapped to facilitate cell dissociation. 7 ml of complete media was then added and the cells were transferred to a 15 ml conical tube. The number of cells per ml was then determined using counting beads and flow cytometry. 200,000 cells were added to each flow tube. 3-5 tubes were used per experimental condition (i.e., stained and unstained). Cells were then centrifuged for 10 minutes at 1,500 RPM, media was removed by aspiration. Cells were then washed twice with 250 μl of cold carbo free blocking buffer (CFBB Vector Laboratories) was added to each tube. Cells were then

centrifuged for 10 minutes at 1,500 RPM and CFBB was removed by aspiration. The resulting cells were stained with 100 μl of 10 μg/ml Cyt-CVNH MB 488 (**9a**) or 6.25 μg/ml of Con A AF488 for 1 hour at 4 °C. Subsequently, cells were washed twice with flow buffer (PBS containing 0.5 % BSA and 0.1% sodium azide), and stained with 7-aminoactinomycin (2.5 μg/mL, Tonbo Biosciences) or Ghost 780 (1 μl/ml Tonbo Biosciences) in flow buffer to assess viability. Cells were then analyzed by flow cytometry (Attune NxT, ThermoScientific).

Gating:



Statistical significance was calculated using a 2way ANOVA and a Dunnett's multiple comparisons test. Calculations were carried out using PRISM 8. *P < .05, **P < .01, ***P < .001, ****P < .0001)

Mannosidase inhibitor treatment, staining and analysis by flow cytometry.

Suspension cells: Raji and Jurkat cell suspension were taken directly from their respective culture flasks. The number of cells per ml was then determined using counting beads and flow cytometry. A volume of the cell suspension that equates to 500,000 cells was added to a 15 ml tube, and the tubes were then centrifuged for 10 minutes at 1,500 RPM. Media was removed by aspiration and the pellet was suspended in 10 ml of complete RPMI

media containing DMSO control or the specified concentrations of mannosidase inhibitors. The resulting suspension were transferred to 6-well plates and incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 for 48 hours. The number of cells per ml was again determined using counting beads and flow cytometry. 200,000 cells were added to flow tubes and at least 3 tubes were used per experimental condition. Cells were then centrifuged for 10 minutes at 1,500 RPM, media was removed by aspiration. Cells were then washed twice with 250 μl of cold carbo free blocking buffer (Vector Laboratories) was added to each tube. Cells were then centrifuged for 10 minutes at 1,500 RPM and CFBB was removed by aspiration. The resulting cells were stained with 100 μl of 0.25 μg/ml Cyt-CVNH MB 488 (**9a**) or 0.25 μg/ml of Con A AF488 for 1 hour at 4 °C. Subsequently, cells were washed twice with flow buffer (PBS containing 0.5 % BSA and 0.1% sodium azide), and stained with 7-aminoactinomycin (2.5 μg/mL, Tonbo Biosciences) or Ghost 780 (1 μl/ml Tonbo Biosciences) in flow buffer to assess viability. Cells were then analyzed by flow cytometry. Gating was applied as outlined above.

Adherent cells: MDA-MB-231 cells were dissociated from T75 flasks as described above using enzyme free cell dissociation buffer. The number of cells per ml was then determined using counting beads and flow cytometry. A volume of the cell suspension that equates to 300,000 cells was added to a 15 ml tube, and the tubes were then centrifuged for 10 minutes at 1,500 RPM. Media was removed by aspiration and the pellet was suspended in 10 ml of complete DMEM media containing DMSO control or the specified concentrations of mannosidase inhibitors. The resulting suspension were transferred to 6-well plates and incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 for 48 hours. The number of cells per ml was again determined using counting beads

and flow cytometry. 200,000 cells were added to flow tubes and at least 3 tubes were used per experimental condition. Cells were then centrifuged for 10 minutes at 1,500 RPM, media was removed by aspiration. Cells were then washed twice with 250 μl of cold CFBB was added to each tube. Cells were then centrifuged for 10 minutes at 1,500 RPM and CFBB was removed by aspiration. The resulting cells were stained with 100 μl of 0.25 μg/ml Cyt-CVNH MB 488 (**9a**) or 0.25 μg/ml of Con A AF488 for 1 hour at 4 °C. Subsequently, cells were washed twice with flow buffer (PBS containing 0.5 % BSA and 0.1% sodium azide), and stained with 7-aminoactinomycin (2.5 μg/mL, Tonbo Biosciences) or Ghost 780 (1 μl/ml Tonbo Biosciences) in flow buffer to assess viability. Cells were then analyzed by flow cytometry. Gating was applied as outlined above.

Fold change in MFI for Kif was calculated using the following formula:

$$\textbf{Fold increase in MFI} = \left(\frac{\textbf{MFI at concentration X}}{\textbf{MFI of DMSO treated control}} \right) - 1$$

Percentage change in MFI was calculated relative to 200 μM KIF control sample included in each experiment and was calculated using the following formula:

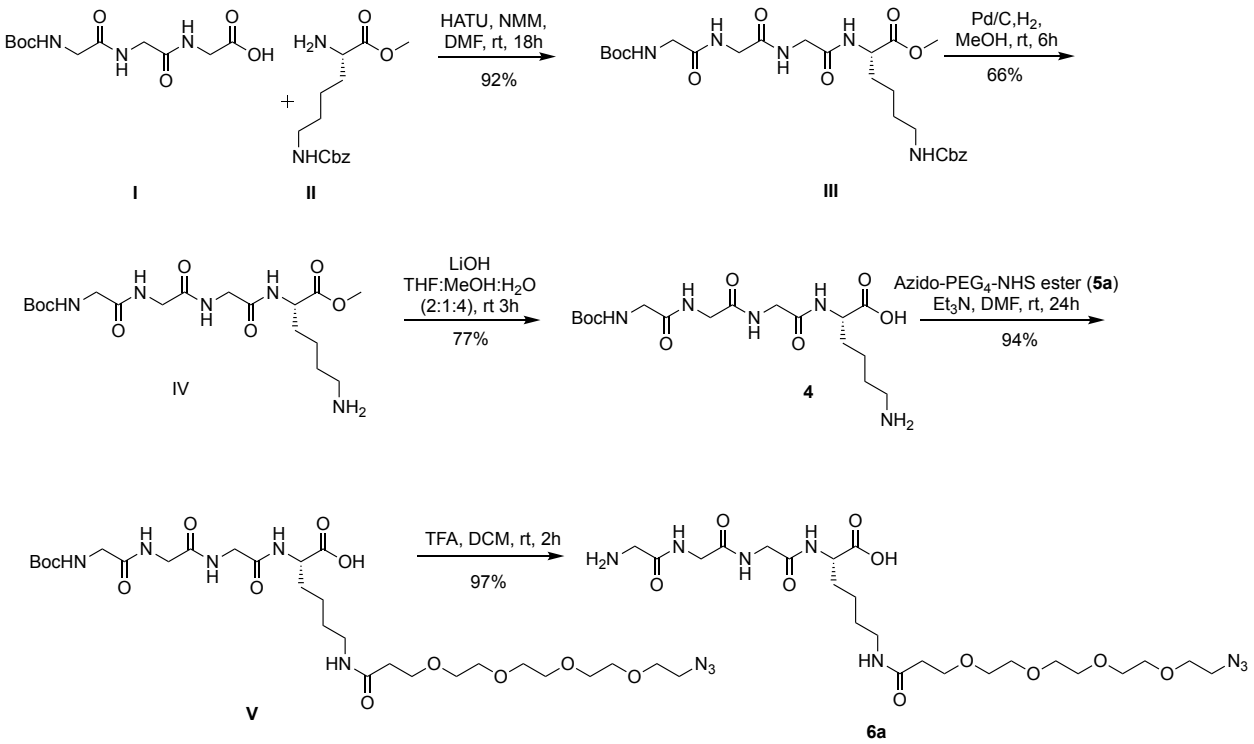
$$\textbf{\% Change in MFI} = \left(\frac{\textbf{MFI at concentration X - MFI of DMSO treated control}}{\textbf{MFI of KIF control - MFI of DMSO treated control}} \right) \textbf{X 100}$$

Values arising from these calculations were then plotted Vs concentration to calculate EC50 using PRISM 8.

Mannosidase inhibitor treatment, staining and analysis by microscopy.

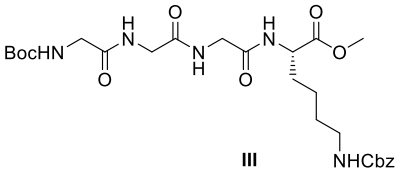
A suspension of MDA-MB-231 cells was prepared as described above. A volume of the cell suspension that equates to 50,000 cells was added to a 1.5 ml tube, and the tubes were then centrifuged for 10 minutes at 1,500 RPM. Media was removed by aspiration and the pellet was suspended in 1 ml of complete DMEM media containing DMSO control or the specified concentrations of mannosidase inhibitors. 300 μl of the resulting suspensions was then added to individual wells of an μ-Slide ibiTreat coated 8 Well slide (Ibidi). The slides were there incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 for 48 hours. Media was then removed by carful aspiration. Cells were washes twice with cold PBS, fixed with 100 μl of 4% formaldehyde (ThermoFisher Scientific) for 15 minutes, washed with cold PBS, and blocked overnight with 250 μl of carbo free blocking buffer. Samples were then stained with 10 μg/ml Cyt-CVNH MB 488 (**9a**) for 2 hours at 4 °C. Samples were then washed twice with cold PBS, and SlowFade Diamond Antifade Mountant with DAPI was applied to each sample. Samples were analyzed by confocal microscopy using an inverted Leica TCS SPE confocal laser-scanning microscope fitted with a Leica 40x oil-immersion objective was used for imaging. Cyt-CVNH MB 488 was excited with a 488 nm solid-state laser and emitted photons were collected from 500–600 nm.

Small molecule synthesis procedures.



Synthesis of Azido-Peptide 6a:

III (SK-II-039)



An ice-cold solution of Boc-Gly-Gly-OH (**I**, 1.0 g, 3.460 mmol, 1 equiv.) and *N*$^{\varepsilon}$-Z-L-lysine methyl ester (**II**, 1.02 g, 3.460 mmol, 1 equiv.) in anhydrous DMF (10 mL) was treated with HATU (1.58 g, 4.15 mmol, 1.2 equiv.) followed by NMM (0.83 g, 7.612 mmol, 2.2 equiv.). The reaction was allowed to warm to ambient temperature and was stirred for 18 hrs. Subsequently, volatiles were removed under reduced pressure, and the resulting residue was diluted with ice cold water, and extracted with ethyl acetate (3 x 25 mL). The organic phase was washed with cold brine (2×50 mL), dried over Na_2SO_4, filtered, and

concentrated under reduced pressure. The crude residue was then purified by CombiFlash using a mixture of methanol in DCM (1:9) affording pure **III** (1.80 g, 92%) as a colorless foam.

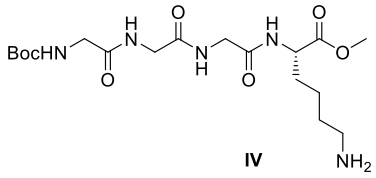
^{1}H NMR (400 MHz, CD_3OD): δ 7.35 – 7.28 (m, 5H), 5.07 (s, 2H), 4.39 (dd, *J* = 8.0 Hz, *J* = 4.0 Hz, 1H), 3.90 (s, 2H), 3.89 (s, 2H), 3.74 (s, 2H), 3.70 (s, 3H), 3.11 (t, *J* = 8.00 Hz, 2H), 1.89 – 1.70 (m, 2H), 1.57 – 1.25 (m, 4H), 1.45 (s, 9H)

^{13}C NMR (101 MHz, CD_3OD): δ 174.0, 173.4, 172.2, 171.6, 158.9, 158.7, 138.5, 129.4, 128.8, 128.9, 81.0, 67.3, 53.7, 52.7, 44.9, 43.8, 43.2, 41.4, 32.0, 30.3, 28.7, 24.0

HRMS (ESI) calcd for $[M + Na]^+$ $C_{26}H_{39}N_5O_9Na$ 588.2646, Found 588.2668

FTIR (thin film): 3321, 1660, 1522, 1454, 1249,1106 cm^{-1}.

IV (SK-II-044)



IV

Pd/C (10 wt. % loading, 80 mg) was carefully added to a solution of **III** (800 mg, 1.410 mmol) in MeOH (4 mL) under a constant flow of nitrogen, and resulting heterogeneous mixture was stirred under hydrogen atmosphere (balloon pressure) at ambient temperature for 6 hrs. Catalyst was removed by filtration over celite pad and washed with MeOH (25 mL). Combined filtrate was concentrated under reduced pressure, and the crude residue obtained was purified using CombiFlash using a mixture of methanol in DCM (2:8) affording pure **IV** (400 g, 66%) as a white solid.

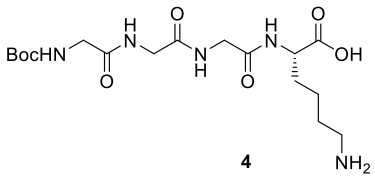
^{1}H NMR (400 MHz, CD_3OD): δ 4.48 – 4.45 (m, 1H), 3.90 (s, 4H), 3.77 (s, 2H), 3.72 (s, 3H), 2.94 (t, *J* = 8.00 Hz, 2H), 1.95 – 1.74 (m, 2H), 1.72 – 1.61 (m, 2H), 1.57 – 1.35 (m, 2H), 1.45 (s, 9H)

^{13}C NMR (101 MHz, CD_3OD): δ 173.7, 172.5, 171.7, 158.8, 81.0, 53.2, 52.8, 44.9, 3.9, 43.5, 40.5, 31.7, 28.7, 27.8, 23.7

HRMS (ESI) calcd for $[M + H]^+$ $C_{18}H_{34}N_5O_7$ 432.2458, Found 432.2427

FTIR (thin film): 3332, 1742, 1516, 1245, 1522, 1454, 1249,1024 cm^{-1}

4 (SK-III-057)



4

Lithium hydroxide monohydrate (19.4 mg, 0.463 mmol, 3 equiv.) was added to a solution of **IV** (100 mg, 0.231 mmol) in a mixture of THF/ MeOH/ H_2O (3 mL, 2:1:4 ratio), and the resulting mixture was stirred for 3 hrs. at ambient temperature. The crude reaction mixture was purified using RP HPLC followed by lyophilization to obtain **V** TFA salt (75 mg, 77%) as a white solid.

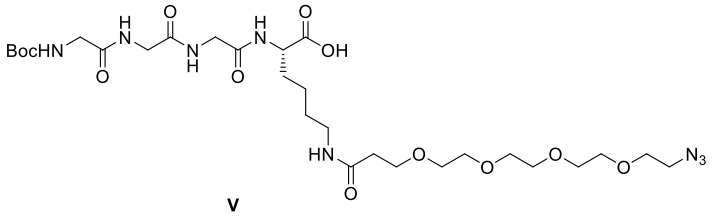
^{1}H NMR (400 MHz, D_2O): δ 4.42 (dd, *J* = 9.4, *J* = 4.7 Hz, 1H), 4.05 (s, 1H), 4.03 – 3.93 (m, 3H), 3.91 (s, 1H), 3.83 (s, 1H), 3.00 (t, *J* = 7.6 Hz, 2H), 2.03 – 1.89 (m, 2H), 1.86 – 1.76 (m, 2H), 1.70 (quin., 2H), 1.43 (s, 9H)

^{13}CNMR (101 MHz, CD_3OD): δ 175.6, 171.8, 171.2, 163.1, 81.8, 52.5, 42.3, 42.1, 40.4, 39.2, 30.0, 29.6, 27.6, 26.1, 22.0

HRMS (ESI) calcd for $[M + H]^+$ $C_{17}H_{32}N_5O_7$ 418.2302, Found 418.2316

FTIR (thin film): 3277, 3073, 1607, 1532 cm^{-1}.

VI (SK-III-066)



V

To a solution of **V** (58 mg, 0.139 mmol, 1 equiv.) in an anhydrous DMF (1.5 mL) was added commercially available Azido-PEG_4-NHS ester (54 mg, 0.139 mmol, 1 equiv.) and Et_3N (38.7 µL, 0.278 mmol, 2 equiv.) at ambient temperature. The resulting mixture was stirred for 24 hrs before volatiles were removed under reduced pressure and the crude residue was purified using RP HPLC to obtain **VI** as a white solid (91 mg, 94%) after lyophilization.

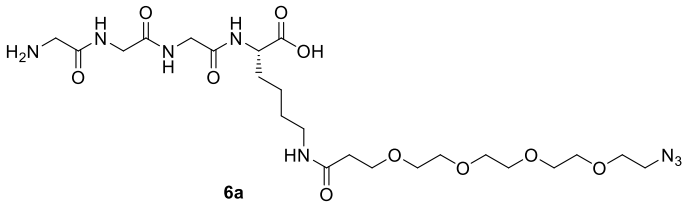
^{1}H NMR (500 MHz, CD_3OD): δ 4.39 (dd, *J* = 9.20 Hz, *J* = 4.70 Hz, 1H), 3.91 (d, *J* = 6.20 Hz, 4H), 3.76 (s, 2H), 3.72 (t, *J* = 6.10 Hz, 2H), 3.69 – 3.59 (m, 14H), 3.38 (t, *J* = 5.00 Hz, 2H), 3.19 (t, *J* = 10.00 Hz, 2H), 2.44 (t, *J* = 5.00 Hz, 2H), 1.94 – 1.83 (m, 1H), 1.81 – 1.72 (m, 1H), 1.57 – 1.47 (m, 2H), 1.45 (s, 9H), 1.48 – 1.36 (m, 2H).

^{13}CNMR (101 MHz, CD_3OD): δ 175.2, 173.9, 173.5, 173.2, 171.5, 158.7, 80.1, 71.6, 71.5, 71.5, 71.3, 71.1, 53.5, 51.8, 44.9, 43.7, 43.2, 40.0, 37.7, 32.2, 29.8, 28.7, 24.2

HRMS (ESI) calcd for $[M + Na]^+$ $C_{28}H_{50}N_8O_{12}Na$ 713.3446, Found 713.3452

FTIR (thin film): 3315, 2111, 1668, 1515, 1246, 1011 cm^{-1}.

6a (SK-III-067)



6a

VI (90 mg, 0.130 mmol, 1 equiv.) was stirred in solution of DCM:TFA (1:1 ratio, 200 mL) at ambient temperature for 2 hrs. Volatiles were removed under reduced pressure and the resulting mixture was purified using RP HPLC to afford **6a** TFA salt (75 mg, 97%) as a highly hygroscopic white foam after lyophilization.

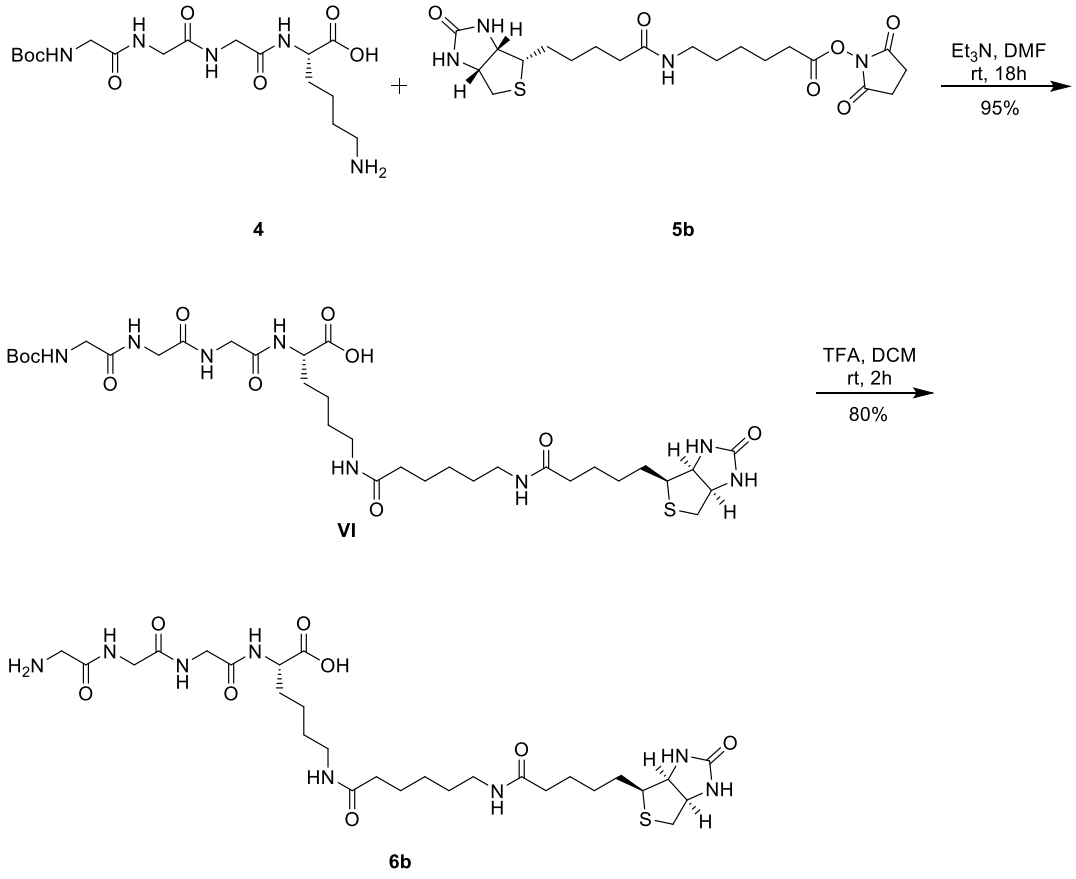
^{1}H NMR (500 MHz, D_2O): δ 4.38 (dd, *J* = 10.00 Hz, *J* = 5 Hz, 1H), 4.05 (s, 2H), 3.99 (s, 2H), 3.91 (s, 2H), 3.78 (t, *J* = 5.00 Hz, 2H), 3.74 – 3.64 (m, 14H), 3.49 (t, *J* = 5.00 Hz, 2H), 3.20 (t, *J* = 5.00 Hz, 2H), 2.51 (t, *J* = 5.00 Hz, 2H), 1.97 – 1.86 (m, 1H), 1.83 – 1.73 (m, 1H), 1.61 – 1.49 (m, 2H), 1.47 – 1.32 (m, 2H)

^{13}C NMR (125.7 MHz, D_2O): δ 178.2, 176.4, 174.2, 173.7, 170.4, 72.1, 72.1, 72.0, 72.0, 72.0, 71.7, 69.3, 55.2, 52.7, 44.8, 44.6, 42.9, 41.5, 38.6, 32.6, 30.3, 24.8

HRMS (ESI) calcd for $[M + H]^+$ $C_{23}H_{43}N_8O_{10}$ = 591.3102, Found 591.3129

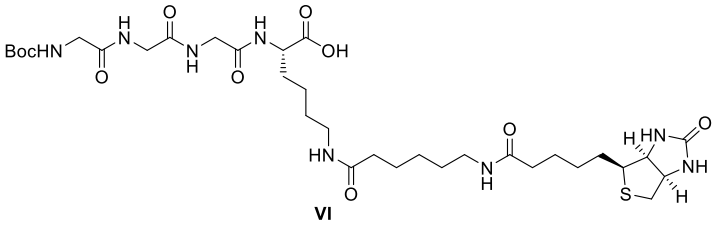
FTIR (thin film): 3330, 2116, 1670, 1525, 1230, 1020 cm^{-1}.

Synthesis of Biotin-Peptide 6b:



4 5b VI 6b

VI (SK-III-055)



VI

To a solution of **4** (100 mg, 0.239 mmol, 1 equiv.) in an anhydrous DMF (2 mL) was added commercially available Biotinamidohexanoic acid N-hydroxysuccinimide ester (**5b**, 126 mg, 0.286 mmol, 1.2 equiv.) and Et_3N (33 µL, 0.239 mmol, 1 equiv.) at ambient temperature and the resulting reaction mixture was stirred for 18 hrs. Volatiles were

removed under reduced pressure and crude residue was recrystallized using DCM to yield **VI** as a white solid (166 mg, 95%)

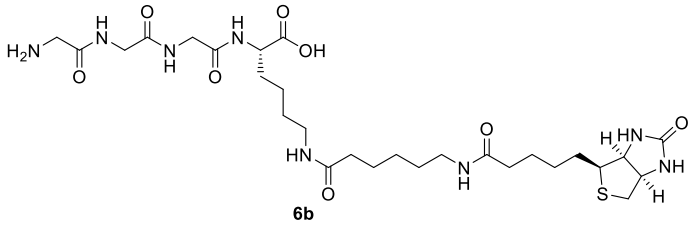
^{1}H NMR (500 MHz, CD_3OD): δ 4.50 (dd, *J* = 7.90 Hz, *J* = 4.90 Hz, 1H), 4.39 (dd, *J* = 9.10 Hz, *J* = 4.80 Hz, 1H), 4.31 (dd, *J* = 7.90 Hz, *J* = 4.40 Hz, 1H), 3.92 (s, 2H) 3.91 (s, 2H), 3.76 (d, *J* = 1.60 Hz, 2H), 3.25 – 3.19 (m, 1H), 3.17 (td, *J* = 7.00 Hz, *J* = 6.30 Hz, *J* = 2.90 Hz, 4H), 2.93 (dd, *J* = 12.80 Hz, *J* = 5.00 Hz, 1H), 2.71 (d, *J* = 12.70 Hz, 1H), 2.19 (td, *J* = 7.40 Hz, *J* = 3.20 Hz, 4H), 1.95 – 1.84 (m, 1H), 1.81 – 1.69 (m, 2H), 1.68 – 1.56 (m, 5H), 1.56 – 1.48 (m, 4H), 1.45 (s, 9H), 1.48 – 1.30 (m, 6H)

^{13}C NMR (125.7 MHz, CD_3OD): δ 176.1, 176.0, 175.2, 173.5, 172.2, 171.5, 166.1, 158.7, 81.0, 63.4, 61.7, 57.0, 53.5, 45.0, 43.8, 43.3, 41.0, 40.2, 40.0, 37.0, 36.8, 32.2, 30.1, 29.9, 29.8, 29.5, 28.7, 27.6, 26.9, 26.7, 24.2

HRMS (ESI) calcd for $[M + Na]^+$ $C_{33}H_{56}N_8O_{10}SNa$ 779.3738, Found 779.3767

FTIR (thin film): 3330, 2937, 1664, 1528, 1250, 1161 cm^{-1}.

6b (SK-III-056)



VI (70 mg, 0.0924 mmol, 1 equiv.) was stirred in solution of 20% TFA in DCM (1.50 mL) at room temperature for 2 hrs. The volatiles were removed under reduced pressure and purified using RP HPLC to afford **6b** TFA salt (48 mg, 80%) as a colorless white solid after lyophilization.

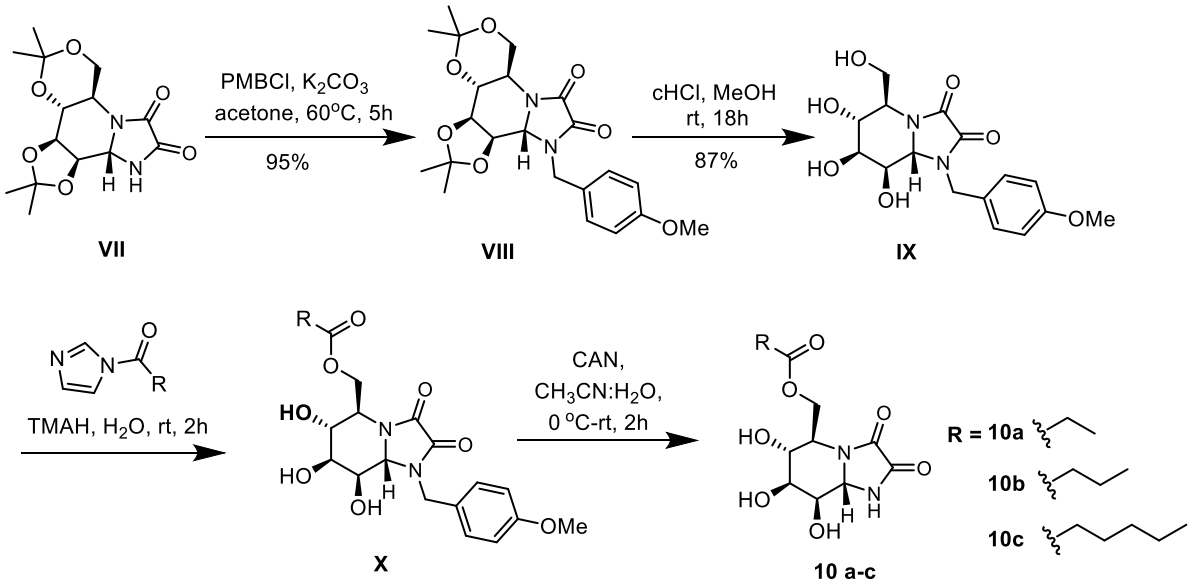
^{1}H NMR (500 MHz, D_2O): δ 4.58 (dd, *J* = 8.0 Hz, *J* = 4.90 Hz, 1H), 4.39 (dd, *J* = 8.0 Hz, *J* = 4.50 Hz, 1H), 4.35 (dd, *J* = 9.00 Hz, *J* = 5.20 Hz, 1H), 4.02 (s, 2H), 3.96 (s, 2H), 3.88 (s, 2H), 3.30 (dt, *J* = 9.70 Hz, *J* = 4.40 Hz, 1H), 3.15 (t, *J* = 6.80 Hz, 4H), 2.96 (dd, *J* = 13.00 Hz, *J* = 5.00 Hz, 1H), 2.75 (d, *J* = 13.00 Hz, 1H), 2.21 (td, *J* = 6.90 Hz, *J* = 3.50 Hz, 4H), 1.93 – 1.81 (m, 1H), 1.81 –1.21 (m, 17H)

^{13}C NMR (125.7 MHz, D_2O): δ 176.7, 176.5, 175.6, 171.6, 171.2, 167.8, 165.3, 62.1, 60.3, 55.4, 52.7, 42.4, 42.1, 40.4, 39.7, 39.1, 39.0, 35.7, 35.5, 30.1, 28.0, 27.9, 27.8, 27.7, 25.5, 25.25, 25.1, 22.4

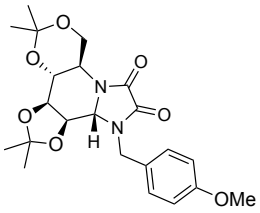
HRMS (ESI) calcd for $[M + Na]^+$ $C_{28}H_{48}N_8O_8SNa$ 679.3214, Found 679.3237

FTIR (thin film): 3323, 1742, 1724, 1516, 1245, 1024 cm^{-1}.

Synthesis of Kifunensine analogues 10a-c.



VIII (SK-II-131)



To a solution of 2,3:4,6-di-*O*-isopropylidene kifunensine[3] (**VII**, 800 mg, 2.561 mmol, 1 equiv.) in anhydrous acetone (20 mL), powdered K_2CO_3 (700 mg, 5.102 mmol, 1.5 equiv.), tetrabutylammonium iodide (39 mg, 0.256, 0.1 equiv.) and PMBCl (510 µL, 3.823 mmol, 1.5 equiv.) were added. The reaction mixture was heated to 60 °C for 5 hours and then cooled to room temperature, inorganics were removed by filtration and the organic layer was concentrated under reduced pressure. The resulting residue was diluted with cold water (20 mL) and was extracted with EtOAc (3 x 25 mL). The organic phase was washed with brine (25 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was then purified by CombiFlash using EtOAc in Hexanes (1:1) to yield pure **VIII** (1.05 g, 95%) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.30 (d, *J* = 8.00 Hz, 2H), 6.88 (d, *J* = 8.00 Hz, 2H), 5.23 (dd, *J* = 14.50 Hz, *J* = 2.40 Hz, 1H), 4.66 (ddd, *J* = 10.90 Hz, *J* = 4.80 Hz, *J* = 1.80 Hz, 1H), 4.57 (d, *J* = 8.50 Hz, 1H), 4.41 – 4.29 (m, 2H), 4.16 (td, *J* = 8.30, *J* = 2.00 Hz, 1H), 4.08 (dd, *J* = 11.2, *J* = 8.30 Hz, 1H), 3.81 (s, 3H), 3.69 (t, *J* = 10.60 Hz, 1H), 3.57 (td, *J* = 10.7, *J* = 4.70 Hz, 1H), 1.61 (s, 3H), 1.51 (s, 3H), 1.47 (s, 3H), 1.41 (s, 3H)

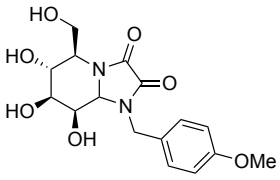
^{13}C NMR (101 MHz, $CDCl_3$): δ 159.7, 159.6, 130.6, 127.4, 114.2, 112.1, 100.1, 78.3, 75.9, 70.6, 66.4, 61.6, 55.4, 47.8, 44.7, 29.1, 26.6, 24.2, 19.1

HRMS (ESI) calcd for $[M + H]^+$ $C_{22}H_{29}N_2O_7$ 433.1975, Found 433.1973

FTIR (thin film): 1754, 1721, 1514, 1244, 1038 cm^{-1}

$[\alpha]_D^{25}$ = +87 ° (*c* = 0.0023 g/mL, MeOH)

IX (SK-II-139)



VIII (1.0 g, 2.312 mmol, 1 equiv.) was dissolved in MeOH (5 mL) and cooled to approximately 5 ° C. Concentrated HCl (2 mL) was added dropwise over 5 minutes and the mixture was stirred at ambient temperature for 18 hrs. Volatiles were removed by evaporation under reduced pressure, and the crude residue was then purified by CombiFlash using MeOH in DCM (9:1) to yield pure **IX** (710 mg, 87%) as an off-white solid.

^{1}H NMR (400 MHz, CD_3OD): δ 7.33 (d, *J* = 8.00 Hz, 2H), 6.89 (d, *J* = 8.00 Hz, 2H), 5.13 (d, *J* = 14.50 Hz, 1H), 4.87 (d, *J* = 8.90 Hz, 1H), 4.78 (d, *J* = 14.60 Hz, 1H), 4.40 (dd, *J* =

8.80 Hz, *J* = 4.70 Hz, 1H), 4.01 (dd, *J* = 3.70 Hz, 1.20 Hz, 1H), 3.92 (t, *J* = 3.30 Hz, 1H), 3.86 (dd, *J* = 11.90 Hz, *J* = 8.80 Hz, 1H), 3.78 (s, 3H), 3.75 (d, *J* = 3.10 Hz, 1H), 3.67 (dd, *J* = 12.00 Hz, *J* = 4.80 Hz, 1H)

^{13}C NMR (101 MHz, CD_3OD): δ 160.8, 160.4, 160.2, 131.0, 129.3, 115.1, 73.6, 73.5, 70.2, 66.9, 61.7, 60.0, 55.7, 46.5

HRMS (ESI) calcd for $[M + H]^+$ $C_{16}H_{21}N_2O_7$ 353.1349, Found 353.1342

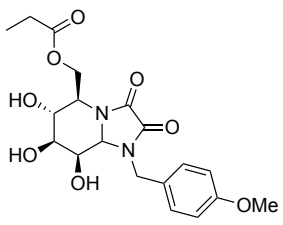
FTIR (thin film): 3315, 1667,1515,1275,1024

$[\alpha]_D^{25}$ = +291 ° (*c* = 0.0010 g/mL, MeOH)

General Procedure for Regioselective acetylation of N-PMB-Kifunensine:

N-PMB-kifunensine (**IX,** 1 equiv.) was dissolved in water (1 mL/15 mg of **IX**) and dissolved by heating at 60 °C, and then allowed to cool room temperature. Tetramethyl-ammonium hydroxide (TMAH) (25% aq. 1.3 equiv.) was added, followed by the desired *N*-acyl imidazole derivatives[4] (3.0 equiv.) in two portions. The resulting reaction mixture was stirred at room temperature for another 2-3 hours. Reaction mixture was diluted with acetonitrile, and directly purified by reverse phase HPLC to afford acetylated products

Xa (SK-IV-044)



Yield: 43%

^{1}H NMR (500 MHz, MeOD) δ 7.31 (d, *J* = 8.70 Hz, 2H), 6.92 (d, *J* = 8.60 Hz, 2H), 5.15 (d, *J* = 14.70 Hz, 1H), 4.99 (d, *J* = 9.0 Hz, 1H), 4.82 – 4.70 (m, 2H), 4.54 (dd, *J* = 10.70 Hz, *J* = 3.70 Hz, 1H), 4.14 (dd, *J* = 11.80 Hz, *J* = 3.80 Hz, 1H), 4.03 (dd, *J* = 3.70, *J* = 1.20 Hz, 1H), 3.96 (t, *J* = 3.40 Hz, 1H), 3.81 (s, 3H), 3.79 (d, merged in singlet, *J* = 3.0 Hz, 1H), 2.27 (q, *J* = 7.60 Hz, 2H), 1.05 (t, *J* = 7.50 Hz, 3H)

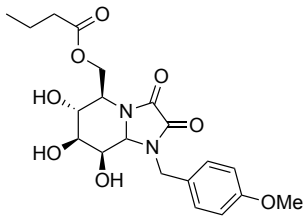
^{13}C NMR (126 MHz, MeOD) δ 174.3, 159.5, 158.7, 158.6, 129.3, 127.8, 113.7, 72.1, 72.0, 68.6, 65.3, 61.4, 55.9, 54.3, 45.3, 26.7, 7.9

HRMS (ESI) calcd for $[M + Na]^+$ $C_{19}H_{24}N_2O_8Na$ 431.1430, found 431.1423

FTIR (thin film): 3360, 1734, 1514,1248

$[\alpha]_D^{25}$ = +82 ° (*c* = 0.002 g/mL, MeOH)

Xb (SK-IV-046)



Yield: 38%

^{1}H NMR (400 MHz, MeOD) δ 7.31 (d, *J* = 8.60 Hz, 2H), 6.92 (d, *J* = 8.70 Hz, 2H), 5.15 (d, *J* = 14.60 Hz, 1H), 4.99 (d, *J* = 9.0 Hz, 1H), 4.82 – 4.75 (m, 2H), 4.55 (dd, *J* = 10.70 Hz, *J* = 3.60 Hz, 1H), 4.10 (dd, *J* = 11.80 Hz, *J* = 3.70 Hz, 1H), 4.03 (dd, *J* = 3.80 Hz, *J* = 1.30 Hz, 1H), 3.96 (t, *J* = 3.30 Hz, 1H), 3.81 (s, 3H), 3.79 (dd, merged in singlet, *J* = 3.0 Hz, 1H), 2.23 (t, *J* = 7.30 Hz, 2H), 1.54 (h, *J* = 7.40 Hz, 2H), 0.87 (t, *J* = 7.40 Hz, 3H)

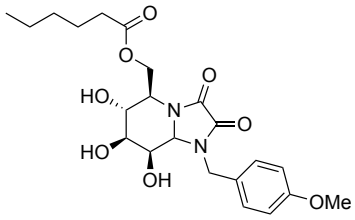
^{13}C NMR (101 MHz, MeOD) δ 174.9, 160.9, 160.2, 160.1, 130.8, 129.2, 115.1, 73.6, 73.5, 70.1, 66.7, 62.7, 57.3, 55.7, 46.7, 36.7, 19.3, 14.0

HRMS (ESI) calcd for $[M + Na]^+$ $C_{20}H_{26}N_2O_8Na$ 445.1587, found 445.1575

FTIR (thin film): 3374, 1728, 1642, 1513, 1216, 1057

$[\alpha]_D^{25}$ = +41 ° (*c* = 0.001 g/mL, MeOH)

Xc (SK-IV-052)



Yield: 35%

^{1}H NMR (400 MHz, MeOD) δ 6.82 (d, *J* = 8.30 Hz, 2H), 6.40 (d, *J* = 8.30 Hz, 2H), 4.65 (d, *J* = 14.70 Hz, 1H), 4.50 (d, *J* = 8.90 Hz, 1H), 4.35 – 4.25 (m, 2H), 4.05 (dd, *J* = 10.70 Hz, *J* = 3.70 Hz, 1H), 3.60 (dd, *J* = 11.90 Hz, *J* = 3.70 Hz, 1H), 3.53 (d, *J* = 3.60 Hz, 1H), 3.46 (t, *J* = 3.40 Hz, 1H), 3.31 (s, 3H), 3.27 – 3.30 (m, merged in singlet, 1H), 1.75 (t, *J* = 7.40 Hz, 2H), 1.03 (p, *J* = 7.40 Hz, 2H), 0.89 – 0.69 (m, 4H), 0.41 (t, *J* = 7.0 Hz, 3H)

^{13}C NMR (101 MHz, MeOD) δ 175.0, 160.9, 160.0, 160.1, 130.7, 129.2, 115.1, 73.6, 73.5, 70.1, 66.7, 62.7, 57.3, 55.7, 46.7, 34.7, 32.3, 25.5, 23.4, 14.2

HRMS (ESI) calcd for $[M + Na]^+$ $C_{22}H_{30}N_2O_8Na$ 473.1900, found 473.1909

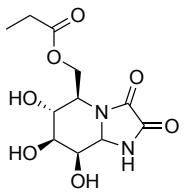
FTIR (thin film): 3368, 1728, 1612, 1513, 1216, 1098

$[\alpha]_D^{25}$ = +65 ° (*c* = 0.001 g/mL, MeOH)

General Procedure of PMB deprotection:

CAN (3 equiv.) in water was added dropwise to an ice-cold solution of **Xa-c** (1 equiv.) in acetonitrile (1:1 ratio of acetonitrile to water was used) over a period of 15 minutes. Then, the reaction mixture was warmed to ambient temperature, and stirred for another 1.5 hrs. The reaction mixture was concentrated under reduced pressure to remove acetonitrile and the reaction solution was extracted with EtOAc (3 x), washed with brine and dried over Na_2SO_4, filtered and the resulting solution was concentrated under reduced pressure. The crude residue was purified by reverse phase HPLC followed by lyophilization to obtain desired 6-O-acylated kifunensine analogs **10a-c**.

10a (SK-IV-045)



Yield: 80%

^{1}H NMR (500 MHz, MeOD) δ 5.02 (d, *J* = 9.10 Hz, 1H), 4.77 (dd, *J* = 11.80 Hz, *J* = 10.6 Hz, 1H), 4.44 (dd, *J* = 10.60 Hz, *J* = 3.80 Hz, 1H), 4.21 (dd, *J* = 11.80, *J* = 3.80 Hz, 1H), 4.05 (dd, *J* = 3.80 Hz, *J* = 1.10 Hz, 1H), 3.98 (d, *J* = 3.30 Hz, 1H), 3.54 (dd, *J* = 9.10 Hz, *J* = 2.80 Hz, 1H), 2.34 (q, *J* = 7.50 Hz, 2H), 1.11 (t, *J* = 7.5 Hz, 3H)

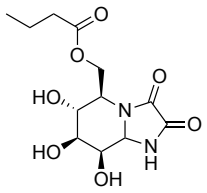
^{13}C NMR (126 MHz, MeOD) δ 175.9, 161.6, 160.2, 73.0, 73.1, 70.6, 64.6, 63.0, 57.3, 28.2, 9.3

HRMS (ESI) calcd for $[M + Na]^+$ $C_{11}H_{16}N_2O_7Na$ 311.0855, found 311.0853

FTIR (thin film): 3375, 1728, 1642, 1513, 1216, 1057

$[\alpha]_D^{25}$ = –82 ° (*c* = 0.001 g/mL, MeOH)

10b (SK-IV-047)



Yield: 75%

^{1}H NMR (500 MHz, MeOD) δ 5.06 (d, *J* = 9.0 Hz, 1H), 4.84 (t, *J* = 11.30 Hz, 1H), 4.51 (dd, *J* = 10.70 Hz, *J* = 3.80 Hz, 1H), 4.20 (dd, *J* = 11.90 Hz, 3.90 Hz, 1H), 4.07 (d, *J* = 3.80 Hz, 1H), 3.99 (t, *J* = 3.40 Hz, 1H), 3.57 (dd, *J* = 9.10, *J* = 2.80 Hz, 1H), 2.29 (t, *J* = 7.30 Hz, 2H), 1.62 (h, *J* = 7.30 Hz, 2H), 0.94 (t, *J* = 7.40 Hz, 3H)

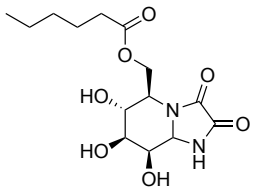
^{13}C NMR (126 MHz, MeOD) δ 175.1, 161.9, 160.5, 73.1, 73.1, 70.6, 64.5, 62.9, 57.3, 36.7, 19.2, 13.9

HRMS (ESI) calcd for $[M + Na]^+$ $C_{12}H_{18}N_2O_7Na$ 325.1012, found 325.0989

FTIR (thin film): 3374, 1730, 1642, 1513, 1057

$[\alpha]_D^{25}$ = +21° (*c* = 0.001, MeOH)

10c (SK-IV-053)



Yield: 71%

^{1}H NMR (500 MHz, MeOD) δ 5.05 (d, *J* = 9.0 Hz, 1H), 4.84 (dd, *J* = 11.9 Hz, *J* = 10.70 Hz, 1H), 4.51 (dd, *J* = 10.7 Hz, *J* = 3.80 Hz, 1H), 4.19 (dd, *J* = 11.9 Hz, *J* = 3.80 Hz, 1H), 4.07 (dd, *J* = 3.80 Hz, *J* = 1.30 Hz, 1H), 3.99 (t, *J* = 3.30 Hz, 1H), 3.57 (dd, *J* = 9.10 Hz, *J* = 2.90 Hz, 1H), 2.31 (t, *J* = 7.40 Hz, 2H), 1.59 (hept, *J* = 6.60 Hz, 2H), 1.40 – 1.24 (m, 4H), 0.93 (t, *J* = 7.10 Hz, 3H)

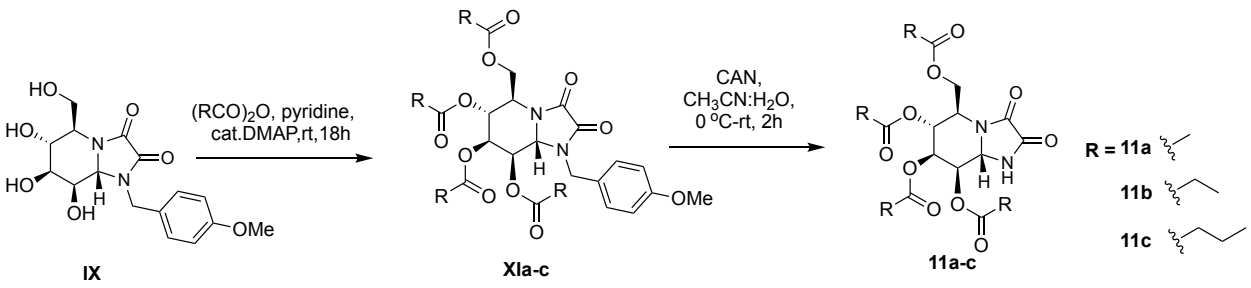
^{13}C NMR (126 MHz, MeOD) δ 175.2, 161.8, 160.5, 73.2, 73.1, 70.6, 64.5, 62.9, 57.2, 34.8, 32.3, 25.5, 23.3, 14.2

HRMS (ESI) calcd for $[M + H]^+$ $C_{14}H_{23}N_2O_7$ 331.1505, found 331.1514

FTIR (thin film): 3376, 1728, 1641, 1513, 1057

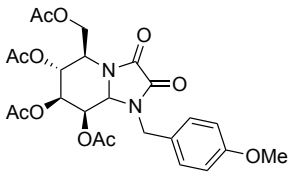
$[\alpha]_D^{25}$ = +5° (*c* = 0.001, MeOH)

Synthesis of 11a-c:



General Procedure for acylation of XI: To an ice-cold solution of **IX** (1 equiv.) in anhydrous pyridine (excess), acetic, propionic, or butanoic anhydride (10 equiv.), and DMAP (0.1 equiv.) were added. The reaction mixture was stirred for 18 hrs. Volatiles were removed by evaporation, and the resulting residue was diluted with water, extracted with EtOAc (3 times), washed with 1N HCl, brine and dried over Na_2SO_4, filtered and the solution was evaporated under reduced pressure. The crude product obtained was purified by CombiFlash using EtOAc in Hexanes to yield desired **XIa-c**.

XIa (SK-II-140)



Yield: 78%

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.12 (d, *J* = 8.00 Hz, 2H), 6.88 (d, *J* = 8.00 Hz, 2H), 5.48 – 5.40 (m, 1H), 5.11 – 5.04 (m, 2H), 4.96 – 4.88 (m, 2H), 4.77 (dd, *J* = 9.80 Hz, *J* = 5.20 Hz, 1H), 4.59 – 4.45 (m, 2H), 4.18 (dd, *J* = 11.60 Hz, *J* = 5.20 Hz, 1H), 3.80 (s, 3H), 2.13 (s, 3H), 2.11 (s, 3H), 2.03 (s, 3H), 1.92 (s, 3H)

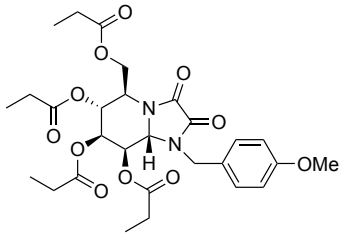
^{13}C NMR (101 MHz, $CDCl_3$): δ 170.75, 169.03, 168.64, 168.63, 159.48, 158.21, 157.46, 128.37, 126.90, 114.43, 77.36, 72.48, 67.61, 67.04, 63.51, 60.35, 55.32, 52.42, 46.44, 20.65, 20.56, 20.48

HRMS (ESI) calcd for $[M + H]^+$ $C_{24}H_{29}N_2O_{11}$ 521.1771, Found 521.1793

FTIR (thin film): 1749, 1612, 1514, 1154

$[\alpha]_D^{25}$ = +130 ° (*c* = 0.0015 g/mL, MeOH)

XIb (JDW-I-112)



Yield: 81%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12 (d, *J* = 8.60 Hz, 2H), 6.87 (d, *J* = 8.80 Hz, 2H), 5.48 – 5.45 (m, 1H), 5.09 – 5.04 (m, 2H), 5.01 (d, *J* = 15.20 Hz, 1H), 4.93 (dd, *J* = 9.3 Hz, *J* = 3.0 Hz, 1H), 4.75 (dd, *J* = 10.0 Hz, *J* = 5.10 Hz, 1H), 4.61 (dd, *J* = 11.4 Hz, *J* = 10.0 Hz, 1H), 4.40 (d, *J* = 15.10 Hz, 1H), 4.12 (dd, *J* = 11.4 Hz, *J* = 5.2 Hz, 1H), 3.80 (s, 3H), 2.37 (qd, *J* = 7.50 Hz, *J* = 2.0 Hz, 4H), 2.29 (q, *J* = 7.5 Hz, 2H), 2.23 – 2.13 (m, 2H), 1.14 (t, *J* = 7.60 Hz, 6H), 1.06 (t, *J* = 7.60 Hz, 6H)

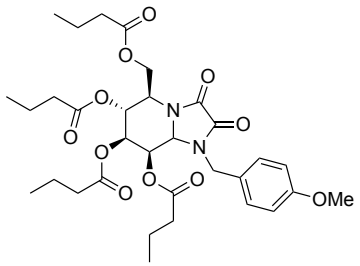
^{13}C NMR (101 MHz, $CDCl_3$): δ 174.2, 172.5, 172.2, 159.6, 158.0, 157.4, 128.7, 126.9, 114.5, 72.9, 67.1, 66.9, 63.2, 60.1, 55.4, 52.6, 46.4, 27.5, 27.4, 27.3, 27.2, 9.2, 9.0, 8.9, 8.5

HRMS (ESI) calcd for $[M +Na]^+$ $C_{28}H_{36}N_2O_{11}Na$ 599.2217, found 599.2239

FTIR (thin film): 1748, 1616, 1514, 1154

$[\alpha]_D^{25}$ = +8.8 ° (*c* = 0.003 g/mL, $CHCl_3$)

XIc JDW-I-114



Yield: 78%

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (d, *J* = 8.60 Hz, 2H), 6.87 (d, *J* = 8.70 Hz, 2H), 5.48 (t, *J* = 3.40 Hz, 1H), 5.15 – 5.02 (m, 3H), 4.95 (dd, *J* = 9.3 Hz, *J* = 3.0 Hz, 1H), 4.75 (dd, *J* = 10.0 Hz, *J* = 5.20 Hz, 1H), 4.60 (dd, *J* = 11.40 Hz, *J* = 10.0 Hz, 1H), 4.32 (d, *J* = 15.10 Hz, 1H), 4.10 (dd, *J* = 11.4 Hz, *J* = 5.20 Hz, 1H), 3.81 (s, 3H), 2.33 (t, *J* = 7.40 Hz, 4H), 2.25 (t, *J* = 7.40 Hz, 2H), 2.22 – 2.05 (m, 2H), 1.50 – 1.78 (m, 8H), 1.01 – 0.83 (m, 12H)

^{13}C NMR (101 MHz, $CDCl_3$) δ 173.3, 171.6, 171.3, 171.2, 159.6, 157.9, 157.3, 126.7, 114.4, 72.9, 67.0, 66.9, 62.9, 60.0, 55.3, 52.5, 46.2, 35.9, 35.8, 35.7, 35.5, 18.5, 18.2, 18.1, 17.7, 13.6, 13.6, 13.5

HRMS (ESI) calcd for $[M + Na]^+$ $C_{32}H_{44}N_2O_{11}Na$ 655.2843, found 655.2880

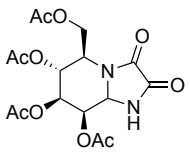
FTIR (thin film): 1749, 1616, 1514

$[\alpha]_D^{25}$ = +17.33° (*c* = 0.003 g/mL, $CHCl_3$)

General Procedure of PMB deprotection

A solution of CAN (3 equiv.) in water was added dropwise to an ice-cold solution of **XIa-c** (1 equiv.) in acetonitrile (1:1 ratio of acetonitrile-water used) over a period of 15 minutes. The resulting reaction mixture was warmed to ambient temperature and stirred for another 1.5 hours. Reaction mixture was then concentrated under reduced pressure and residue obtained was diluted with water and extracted with EtOAc (3 x), washed with brine, dried over Na_2SO_4, filtered and the resulting solution was concentrated under reduced pressure. The crude residue was purified by CombiFlash using EtOAc in Hexanes to obtain desired **11a-c**

11a (SK-II-141)



Yield: 73%

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.62 (s, 1H), 5.42 (t, *J* = 3.30 Hz, 1H), 5.21 (d, *J* = 9.30 Hz, 1H), 5.08 (d, *J* = 3.60 Hz, 1H), 5.00 (dd, *J* = 9.00 Hz, *J* = 3.00 Hz, 1H), 4.70 (dd, *J* = 9.90 Hz, *J* = 4.70 Hz, 1H), 4.61 (t, *J* = 10.70 Hz, 1H), 4.23 (dd, *J* = 11.50 Hz, *J* = 4.90 Hz, 1H), 2.19 (s, 3H), 2.11 (s, 3H), 2.10 (s, 3H), 2.05 (s, 3H)

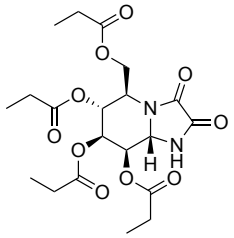
^{13}C NMR (123.7 MHz, $CDCl_3$): δ 170.9, 169.7, 169.2, 168.7, 160.8, 157.6, 71.3, 67.6, 67.5, 61.0, 60.3, 52.7, 20.8, 20.8, 20.7, 20.6

HRMS (ESI) calcd for $[M + H]^+$ $C_{16}H_{21}N_2O_{10}$ 401.1196, Found 401.1176

FTIR (thin film): 1848, 1741, 1533, 1050

$[\alpha]_D^{25}$ = +25.2 ° (*c* = 0.00115 g/mL, MeOH)

11b (JDW-II-004)



Yield: 75%

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.08 (s, 1H), 5.49 (t, *J* = 3.40 Hz, 1H), 5.24 (d, *J* = 9.50 Hz, 1H), 5.11 (d, *J* = 3.70 Hz, 1H), 4.92 (dd, *J* = 9.50 Hz, *J* = 3.0 Hz, 1H), 4.74 – 4.64 (m, 2H), 4.25 – 4.14 (m, 1H), 2.66 – 2.16 (m, 8H), 1.21 (t, *J* = 7.60 Hz, 3H), 1.16 – 0.82 (m, 9H)

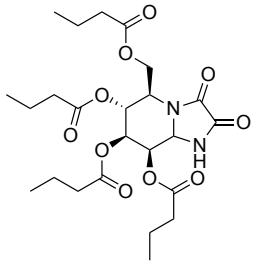
^{13}C NMR (101 MHz, $CDCl_3$) δ 174.4, 173.0, 172.6, 172.2, 160.6, 157.6, 71.5, 67.3, 61.0, 60.3, 52.8, 27.5, 27.3, 27.2, 9.1, 8.8, 8.7

HRMS (ESI) calcd for $[M + Na]^+$ $C_{20}H_{28}N_2O_{10}Na$ 479.1642, Found 479.1649

FTIR (thin film): 1738, 1462, 1151, 1093

$[\alpha]_D^{25}$ = –16 ° (*c* = 0.002 g/mL, $CHCl_3$)

11c (JDW-II-010)



Yield: 73%

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.36 (s, 1H), 5.48 (t, *J* = 3.40 Hz, 1H), 5.22 (d, *J* = 9.40 Hz, 1H), 5.09 (d, *J* = 3.70 Hz, 1H), 4.91 (dd, *J* = 9.4 Hz, *J* = 3.0 Hz, 1H), 4.74 – 4.65 (m, 2H), 4.17 (q, *J* = 6.3 Hz, 1H), 2.45 – 2.24 (m, 8H), 1.79 – 1.53 (m, 8H), 1.11 – 0.83 (m, 12H)

^{13}C NMR (126 MHz, $CDCl_3$) δ 173.6, 172.2, 171.8, 171.3, 71.7, 67.3, 67.2, 60.9, 60.1, 52.8, 36.0, 35.9, 35.8, 35.7, 18.1, 18.3, 18.3, 18.1, 13.7, 13.7, 13.6

HRMS (ESI) calcd for $[M + Na]^+$ $C_{24}H_{36}N_2O_{10}Na$ 535.2268, found 535.2251

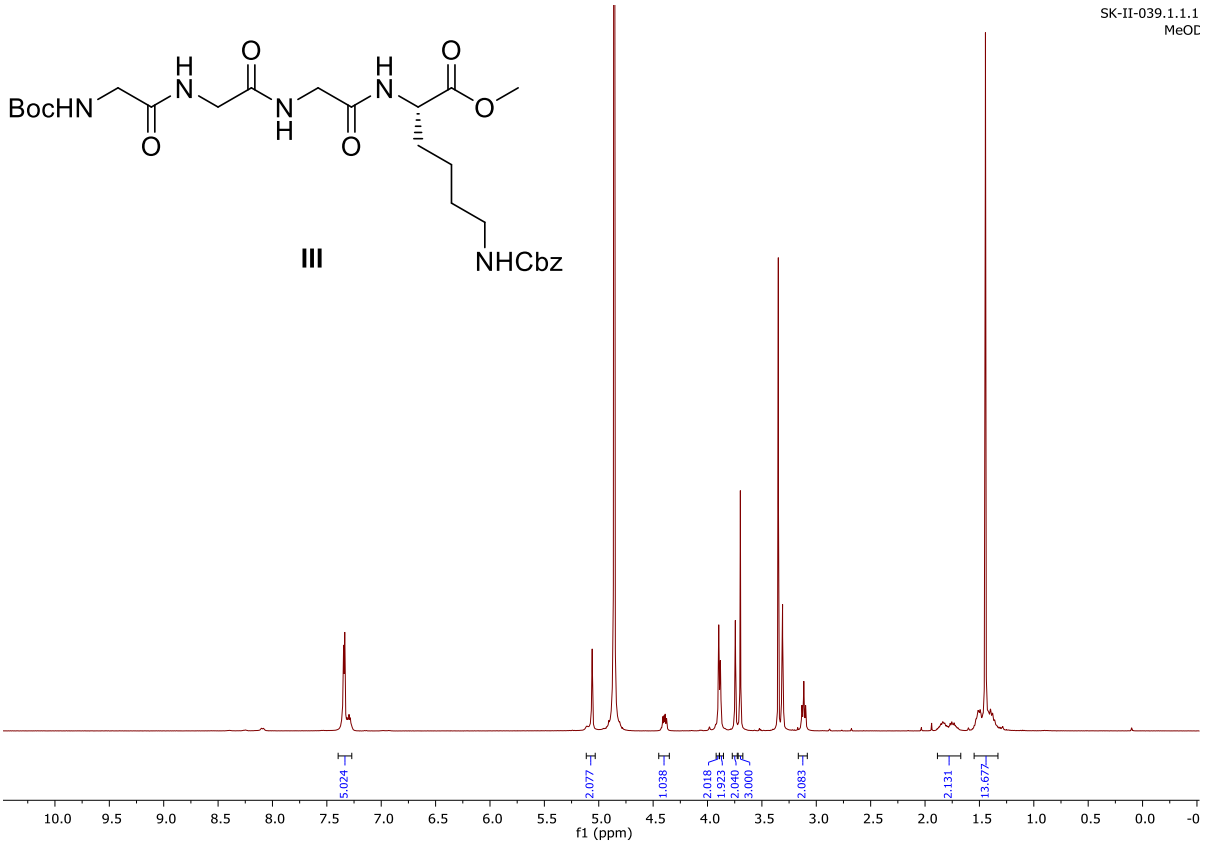
FTIR (thin film): 1747, 1418, 1153, 1050

$[\alpha]_D^{25}$ = –16.7 ° (*c* = 0.0028 g/mL, $CHCl_3$)

References:

1. E. Matei, R. Basu, W. Furey, J. Shi, C. Calnan, C. Aiken, A. M. Gronenborn, *J Biol Chem* **2016**, *291*, 18967-18976
2. Q. Wu, H. L. Ploegh, M. C. Truttmann, *ACS Chem. Biol.* **2017**, *12*,664–673
3. H. Kayakiri, C. Kasahara, T. Oku, M. Hashimoto, *Tetrahedron Lett.***1990**, *31*, 225–226
4. J. Zhuo, Y. Zhang, Z. Li, C. Li, *ACS Catal.* **2020**, *10*, 3895−3903

NMR Spectra:


SK-II-039.1.1.1
MeOD
BocHN
NHCbz
III
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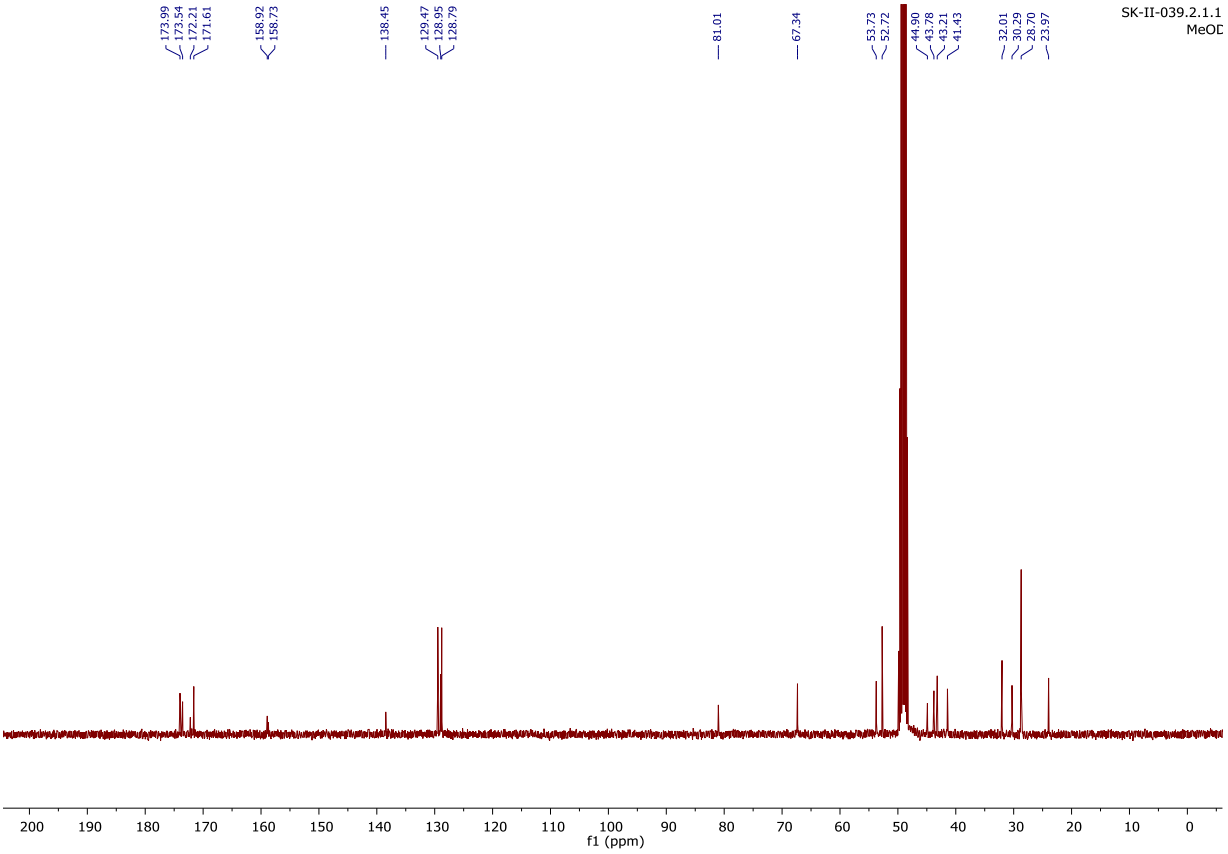
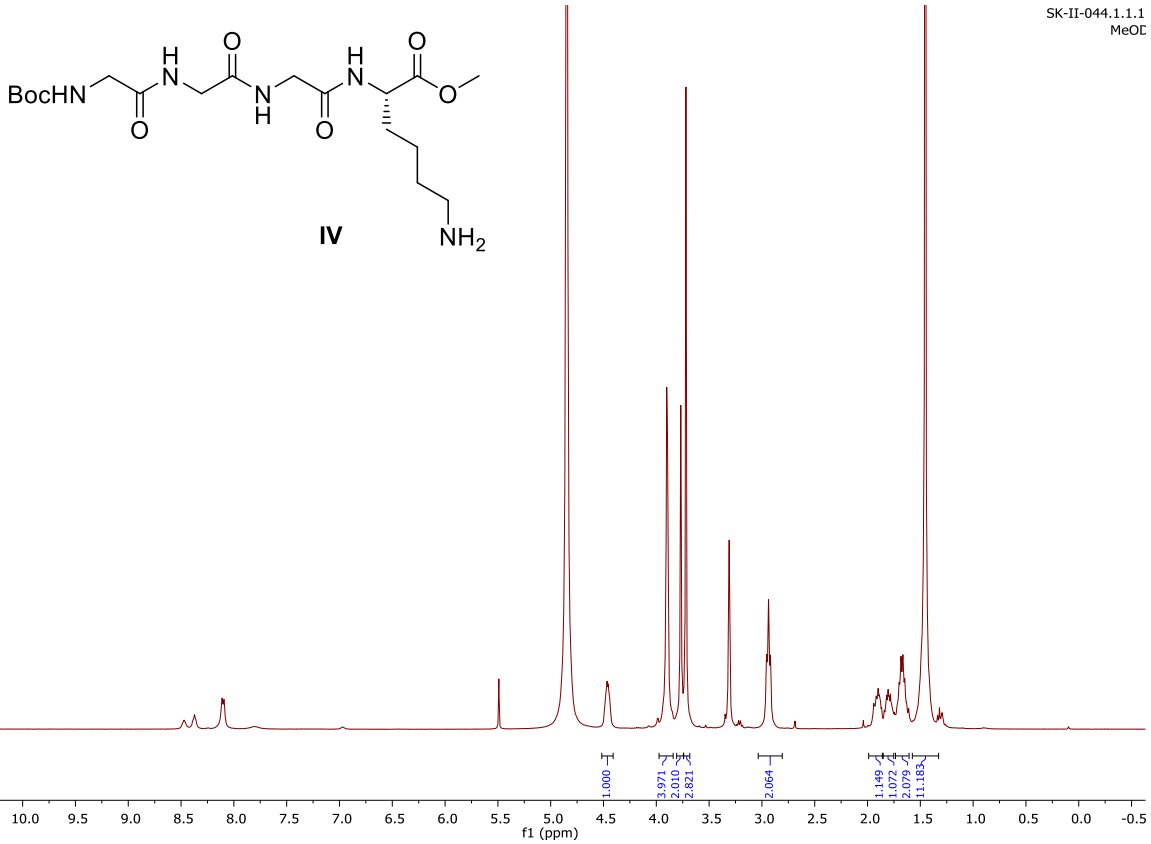
2.077
1.038
2.018
1.923
2.040
3.000
2.083
2.131
13.677
f1 (ppm)


SK-II-039.2.1.1
MeOD
173.99
173.54
172.21
171.61
158.92
158.73
138.45
129.47
128.95
128.79
81.01
67.34
53.73
52.72
44.90
43.78
43.21
41.43
32.01
30.29
28.70
23.97
f1 (ppm)


SK-II-044.1.1.1
MeOD
BocHN
IV
NH2
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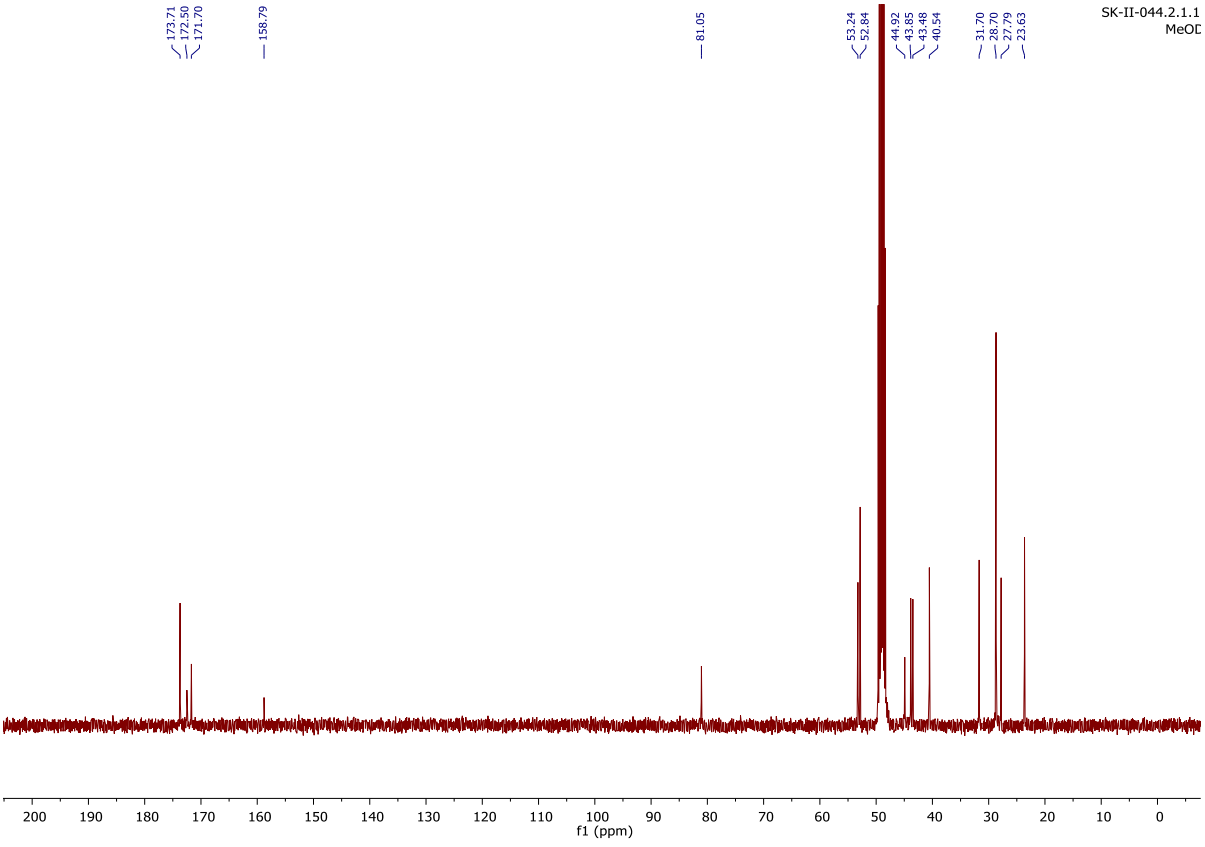
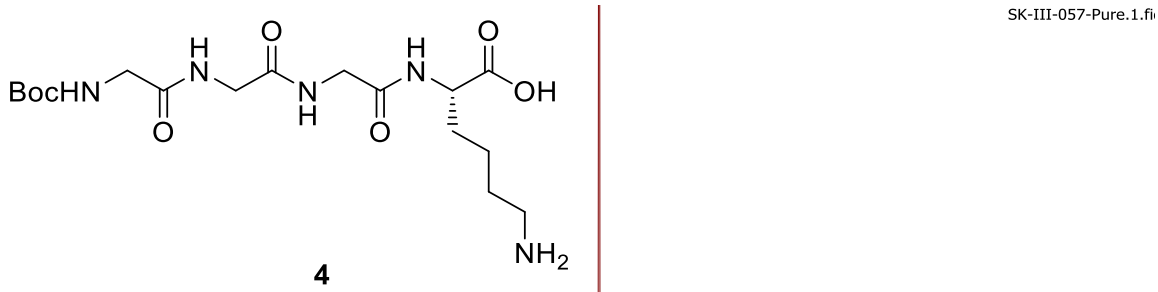
2.010
2.821
2.064
1.149
1.072
2.079
11.183
f1 (ppm)

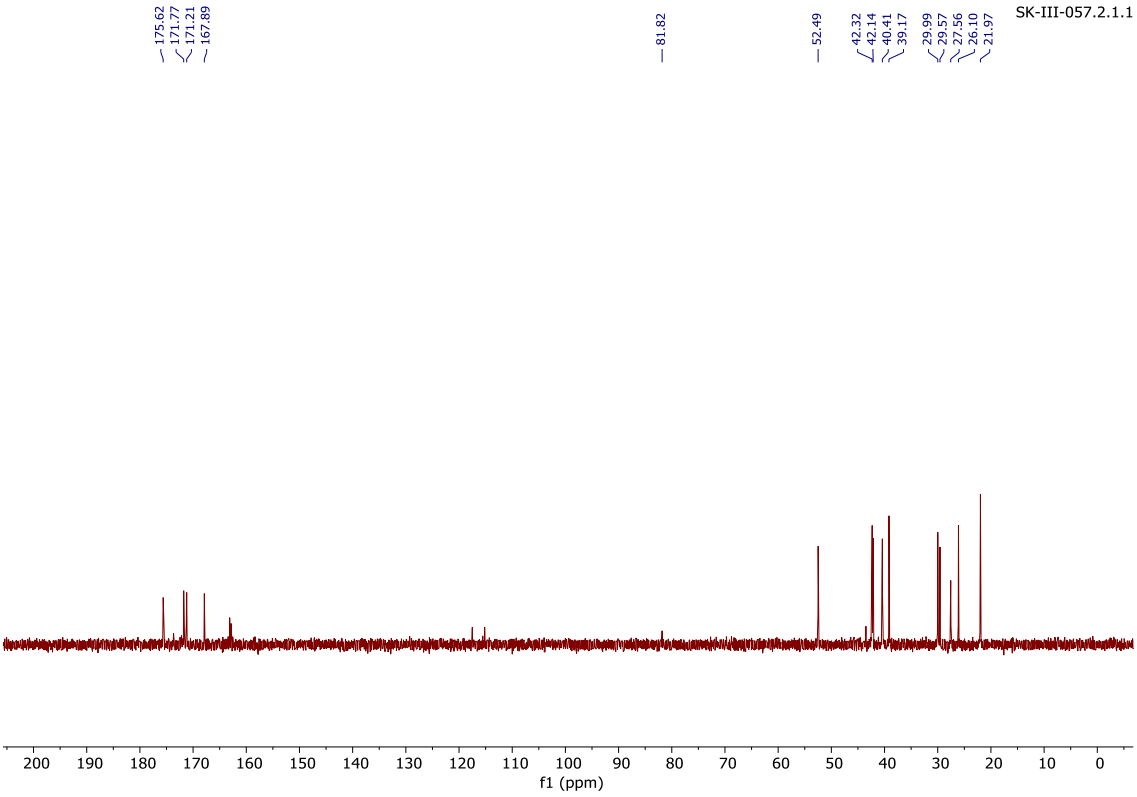

SK-II-044.2.1.1
MeOD
173.71
172.50
171.70
158.79
81.05
53.24
52.84
44.92
43.85
43.48
40.54
31.70
28.70
27.79
23.63
f1 (ppm)

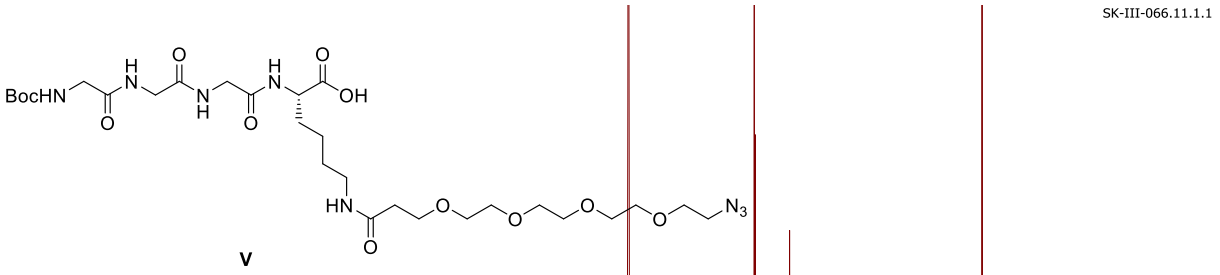


4

SK-III-057-Pure.1.fi

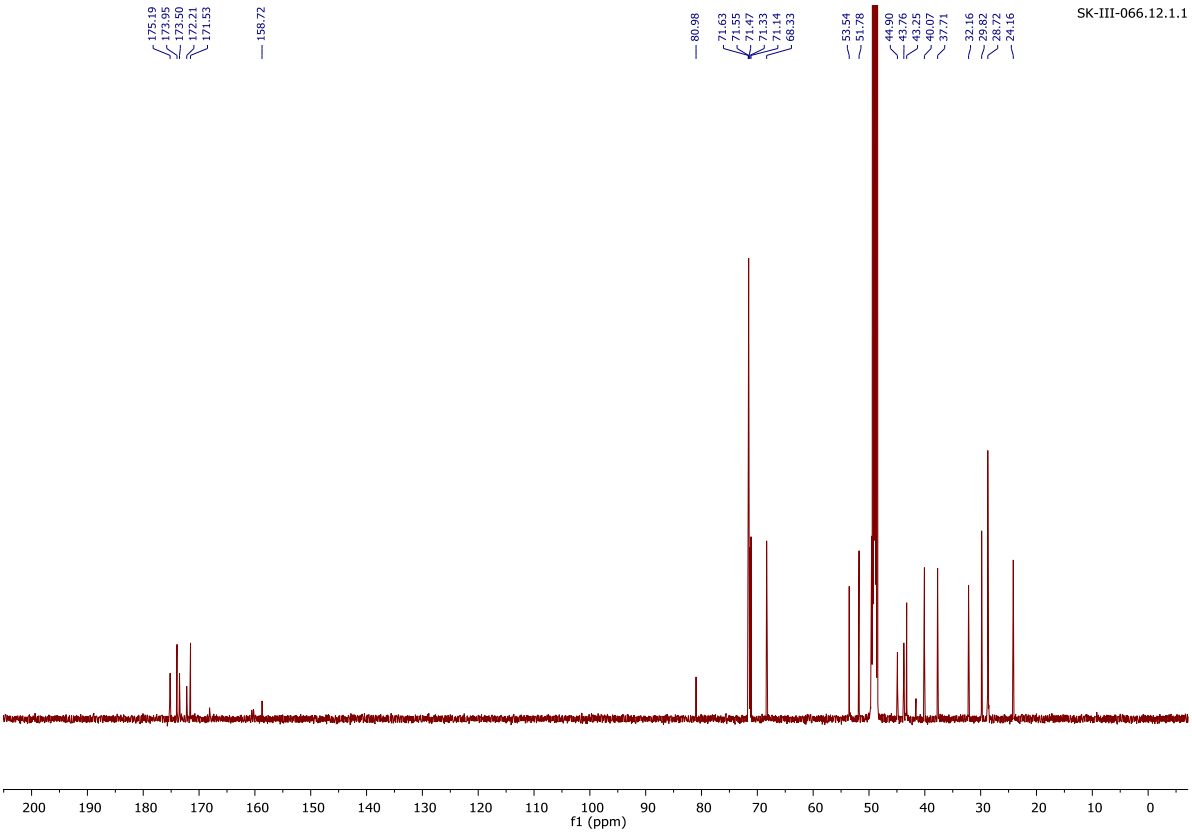
SK-III-057.2.1.1

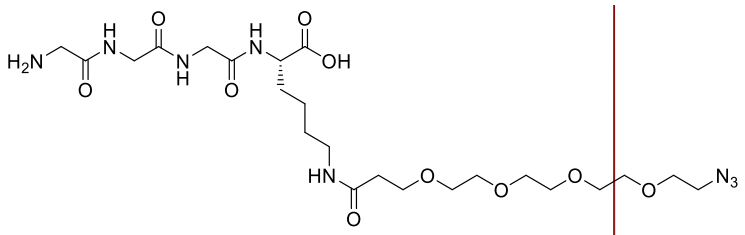



BocHN
OH
HN
N3

V

SK-III-066.11.1.1
1.025
4.132
2.113
2.107
14.213
2.255
2.290
2.037
1.138
1.045
2.254
11.222
f1 (ppm)

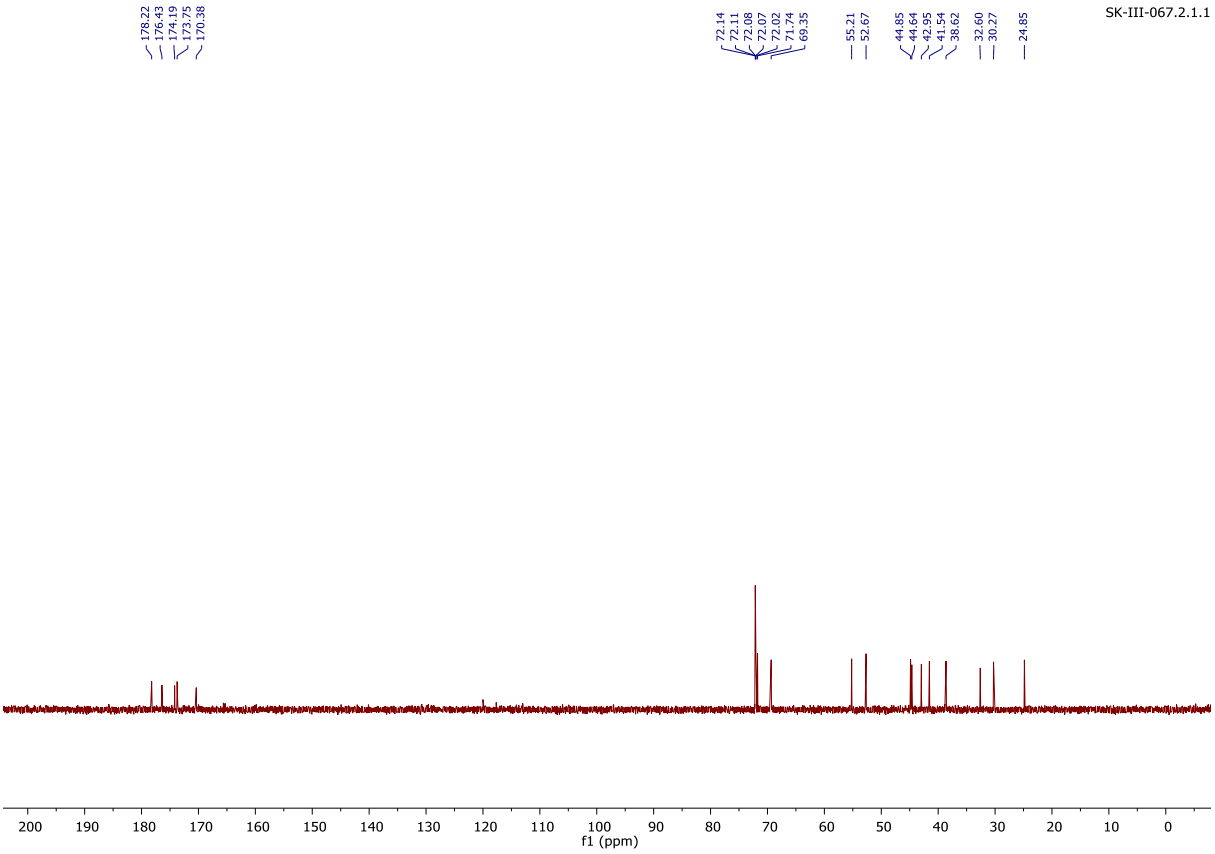

SK-III-066.12.1.1
175.19
173.95
173.50
172.21
171.53
158.72
80.98
71.63
71.55
71.47
71.33
71.14
68.33
53.54
51.78
44.90
43.76
43.25
40.07
37.71
32.16
29.82
28.72
24.16
f1 (ppm)

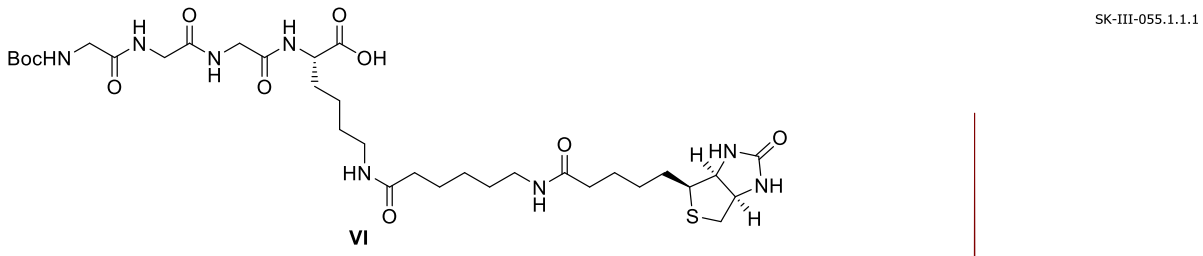


6a

SK-III-067.1.1.1

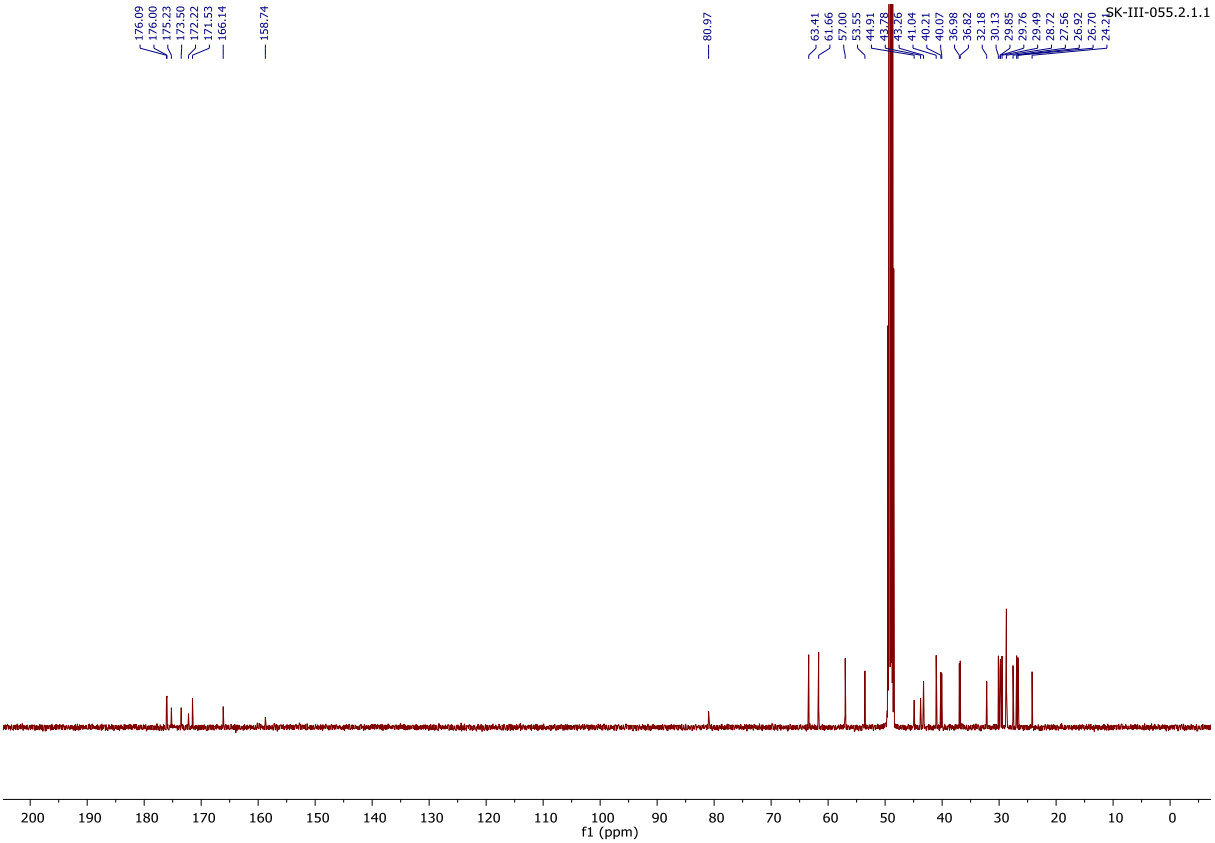
SK-III-067.2.1.1

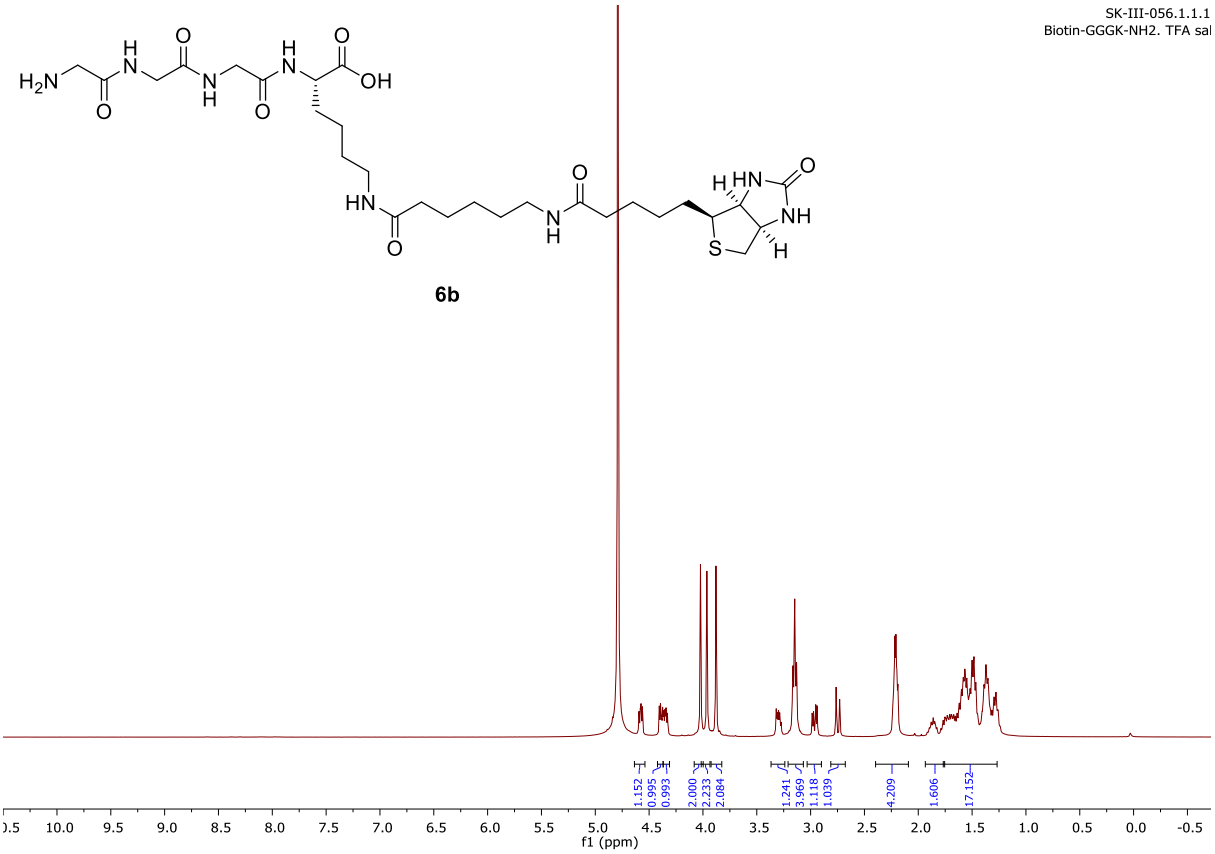


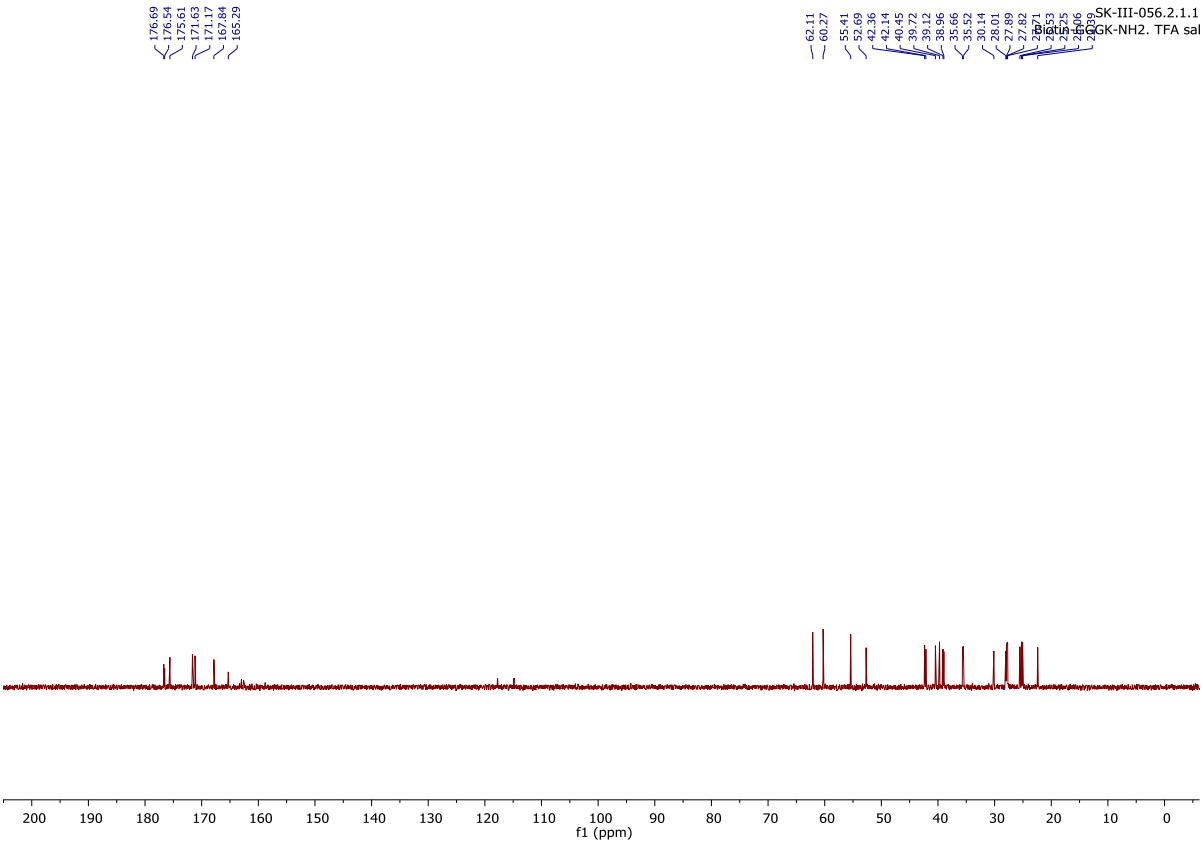


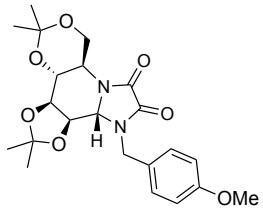
SK-III-055.1.1.1

SK-III-055.2.1.1

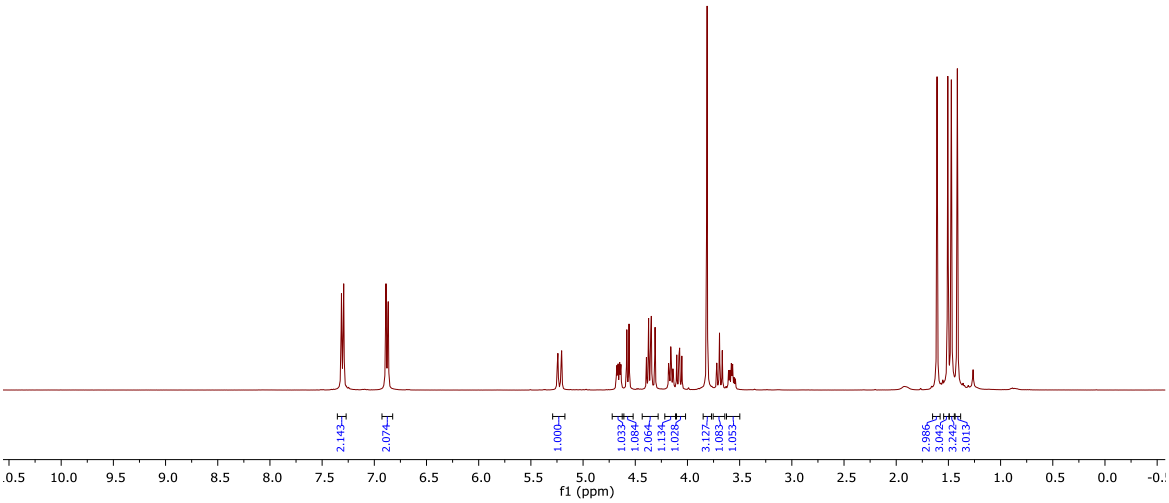



SK-III-056.1.1.1
Biotin-GGGK-NH2. TFA sal
6b
1.152
0.995
0.993
2.000
2.233
2.084
1.241
3.969
1.118
1.039
4.209
1.606
17.152
f1 (ppm)

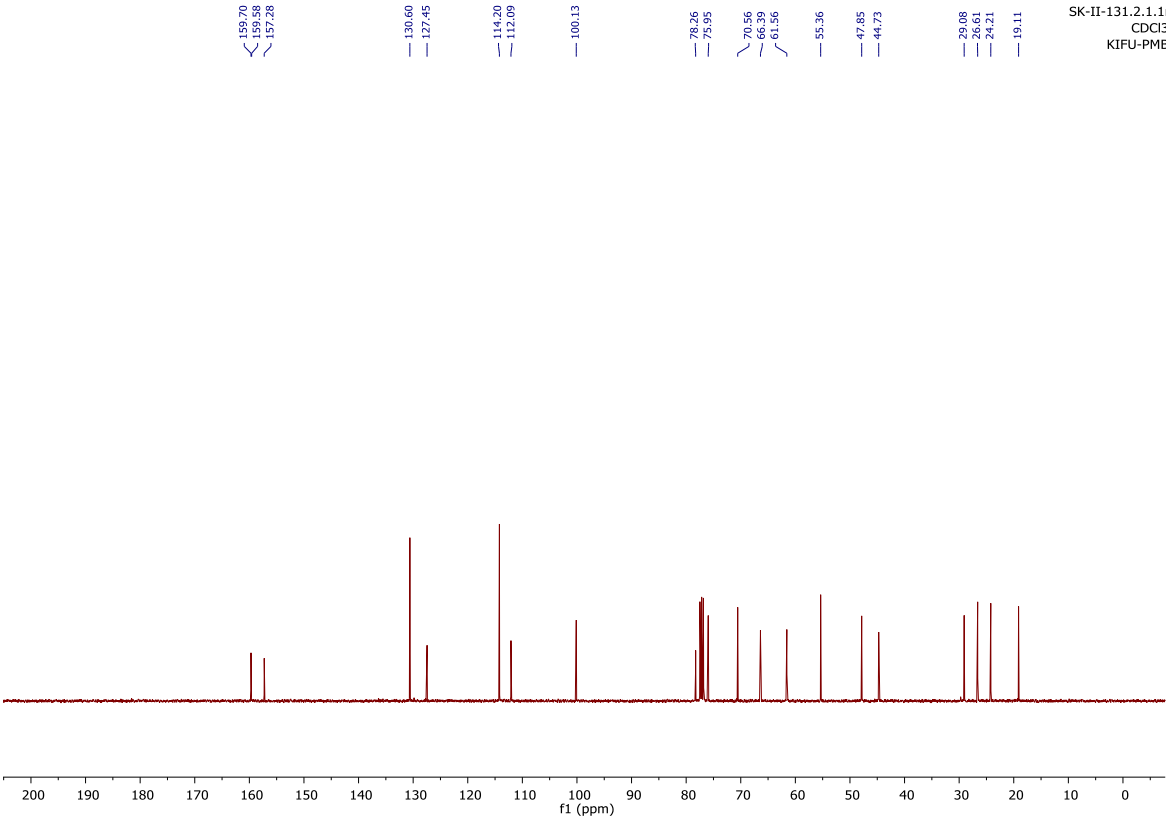

SK-III-056.2.1.1
Biotin-GGGK-NH2. TFA sal
176.69
176.54
175.61
171.63
171.17
167.84
165.29
62.11
60.27
55.41
52.69
42.36
42.14
40.45
39.72
39.12
38.96
35.66
35.52
30.14
28.01
27.89
27.82
f1 (ppm)

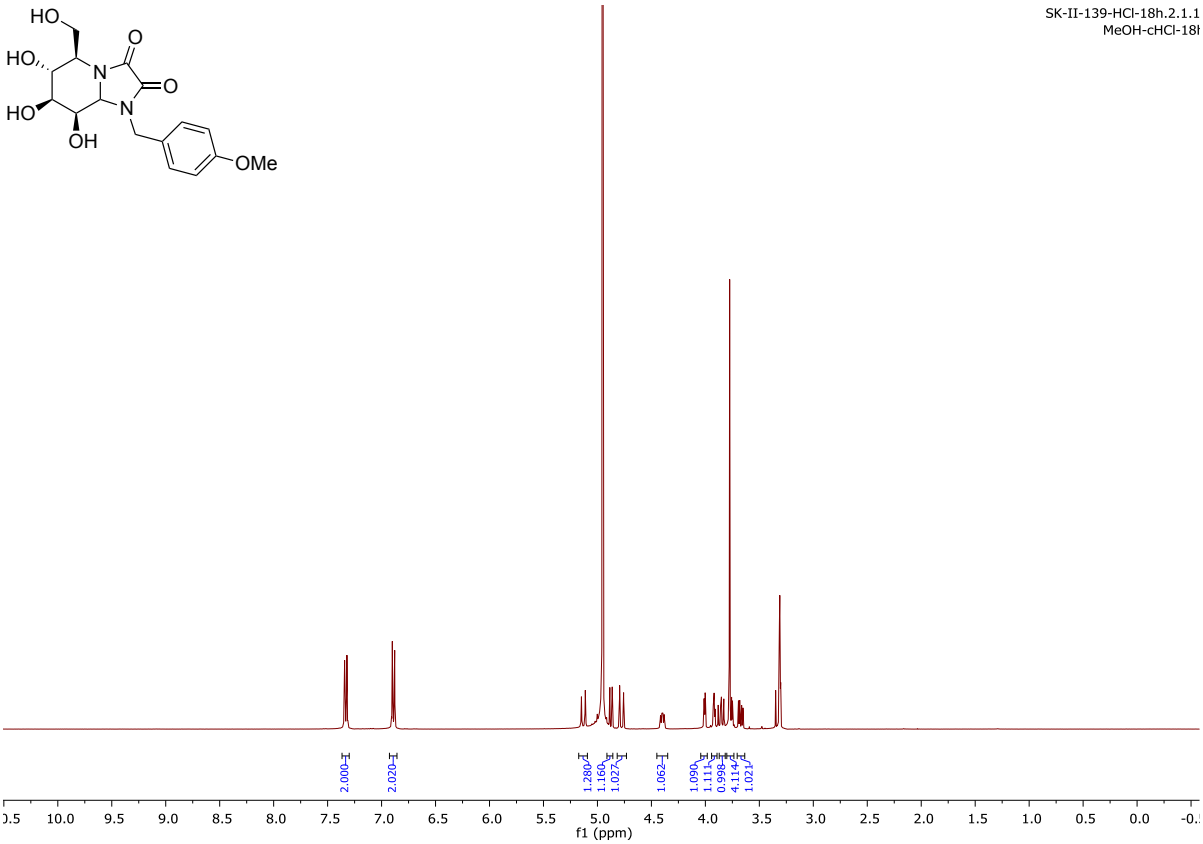


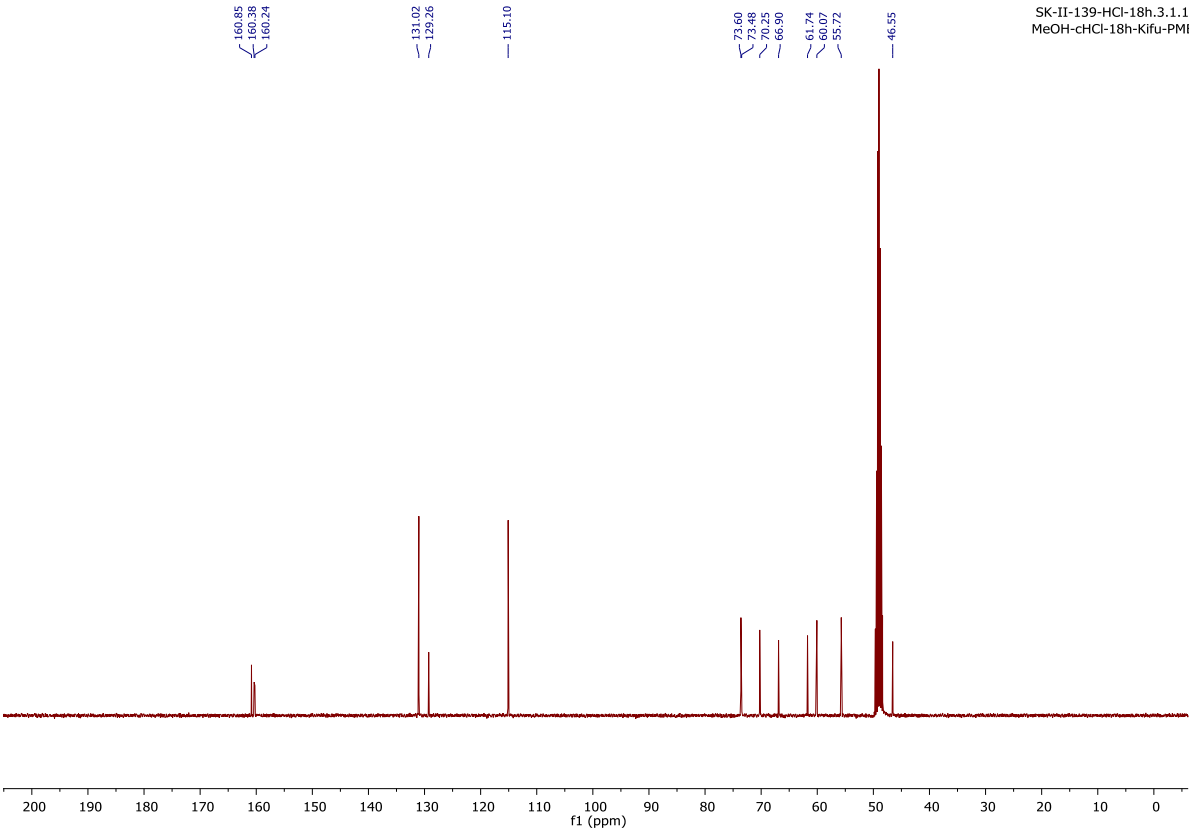
SK-II-131.1.1.1
CDCl3
KIFU-PME

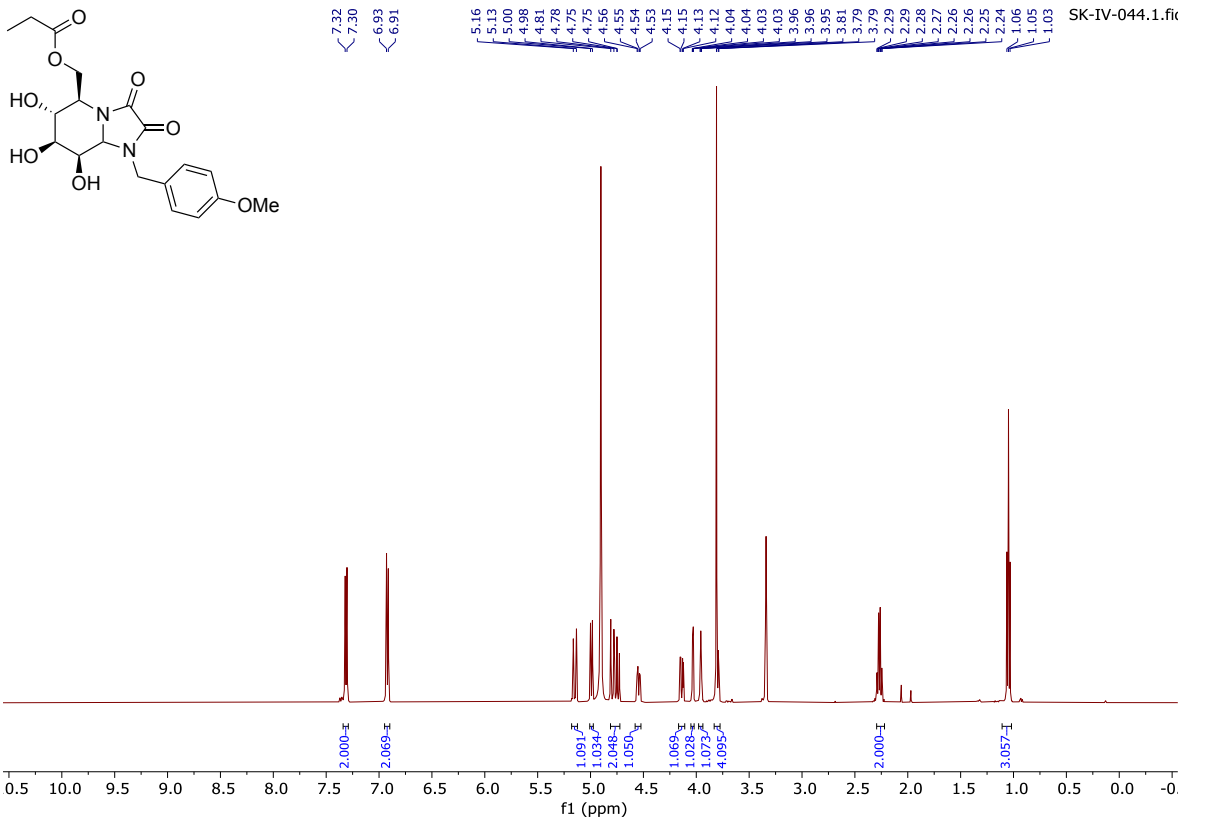


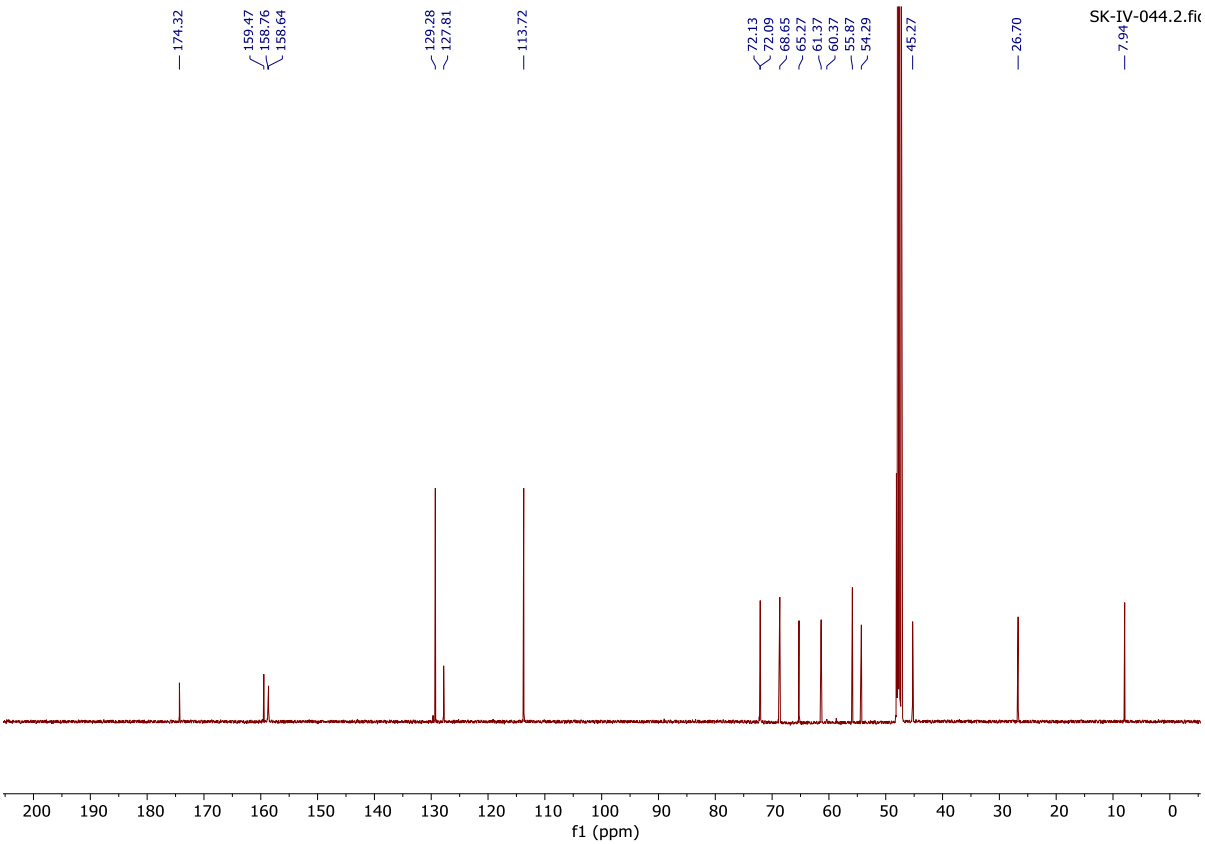
SK-II-131.2.1.1
CDCl3
KIFU-PME

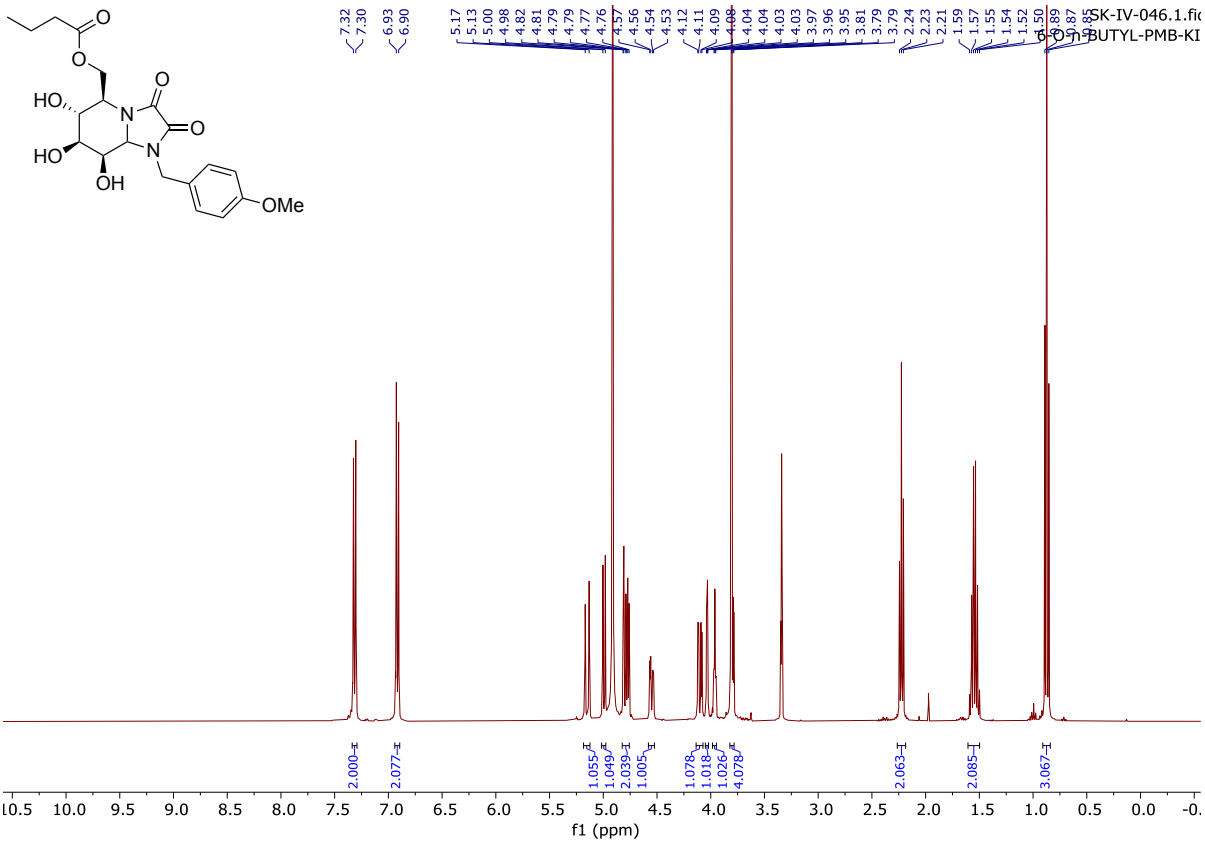


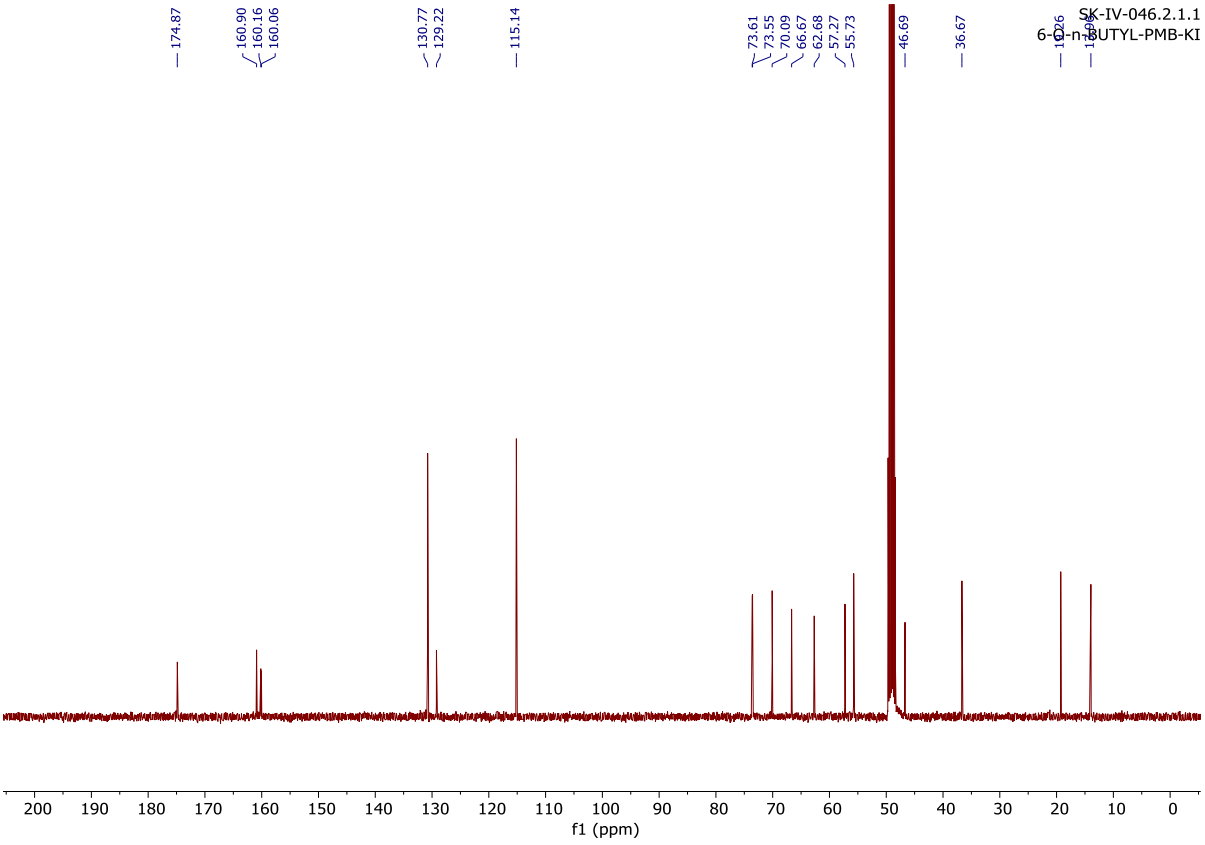

SK-II-139-HCl-18h.2.1.1
MeOH-cHCl-18h
HO
HO
HO
OH
O
O
N
N
OMe
2.000
2.020
1.280
1.166
1.027
1.062
1.090
1.111
0.998
4.414
1.021
f1 (ppm)

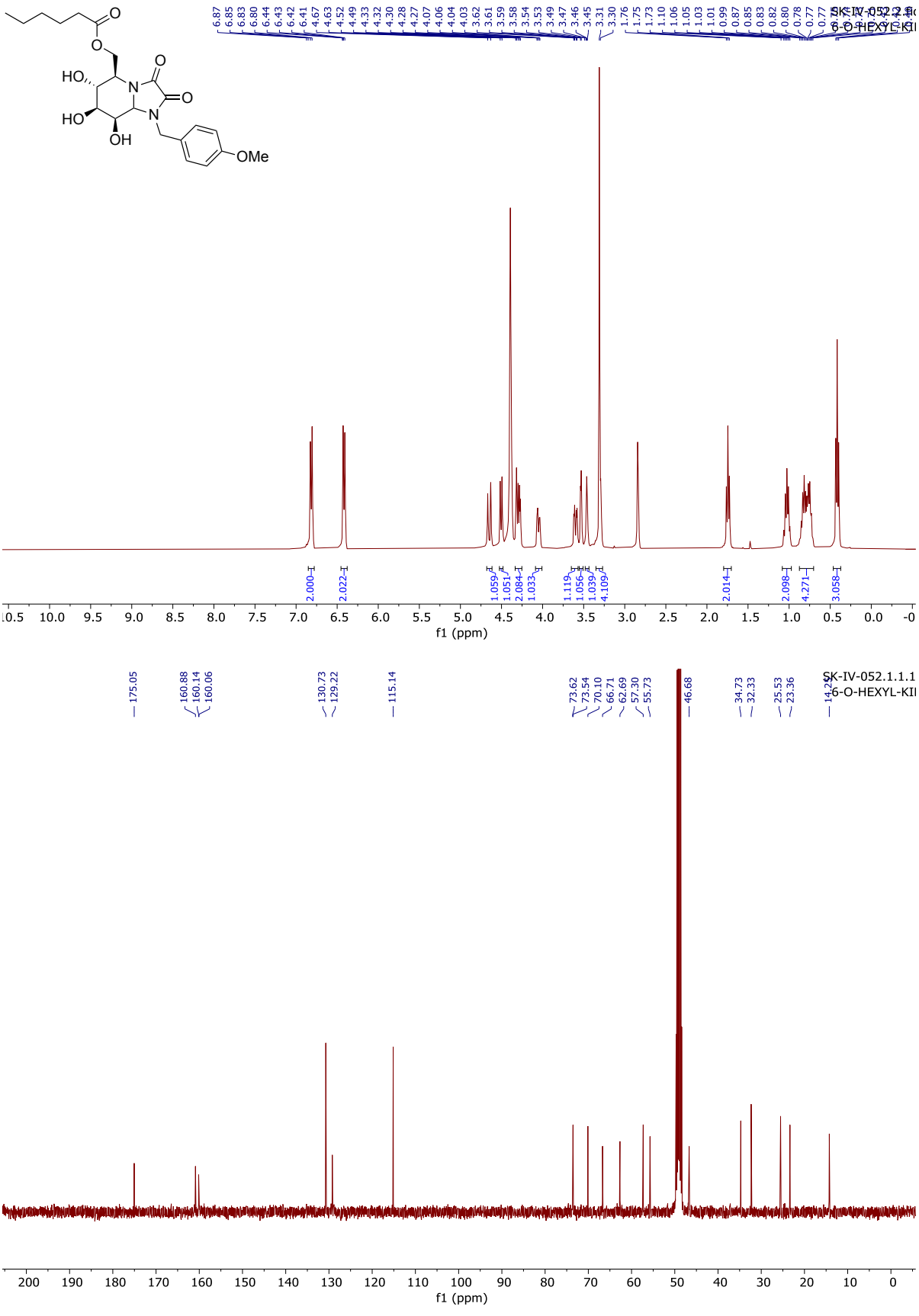

SK-II-139-HCl-18h.3.1.1
MeOH-cHCl-18h-Kifu-PMI
160.85
160.38
160.24
131.02
129.26
115.10
73.60
73.48
70.25
66.90
61.74
60.07
55.72
46.55
f1 (ppm)

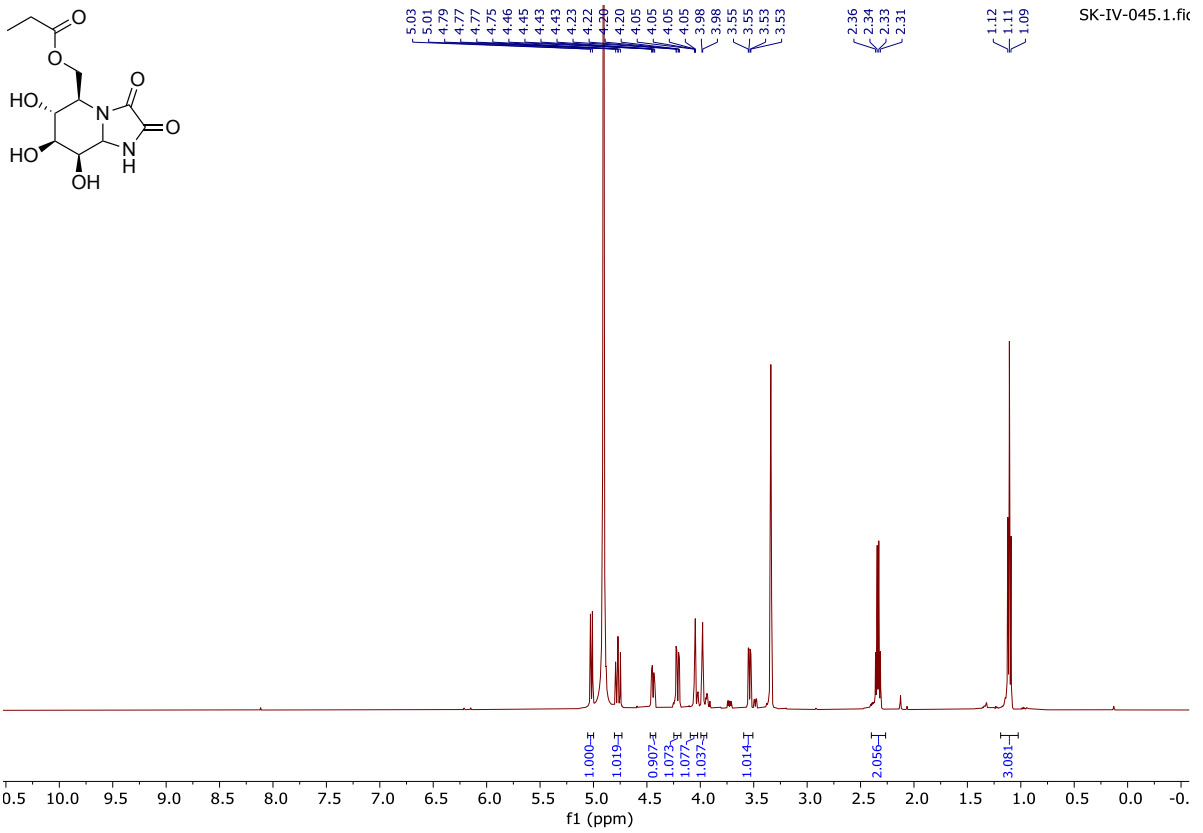

SK-IV-044.1.fi
HO
HO
OH
O
N
N
O
O
OMe
f1 (ppm)

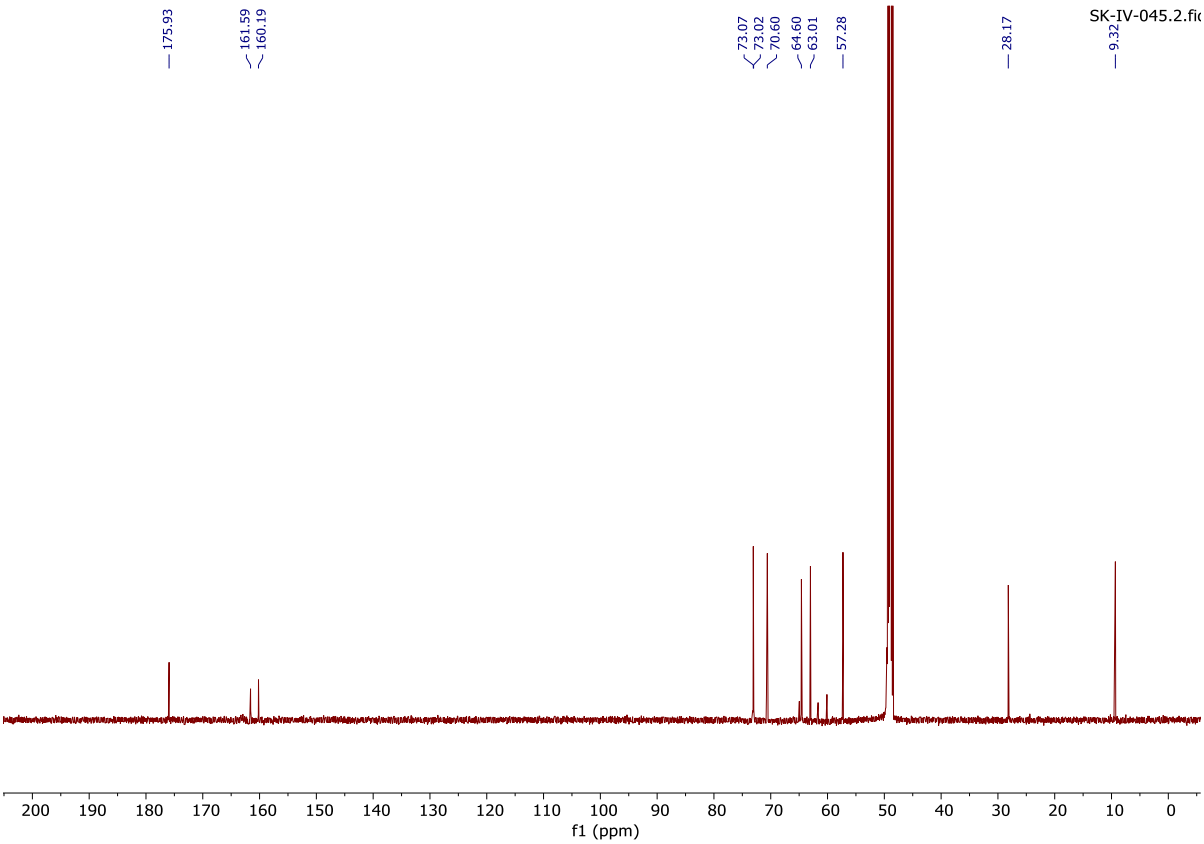

SK-IV-044.2.fi
f1 (ppm)

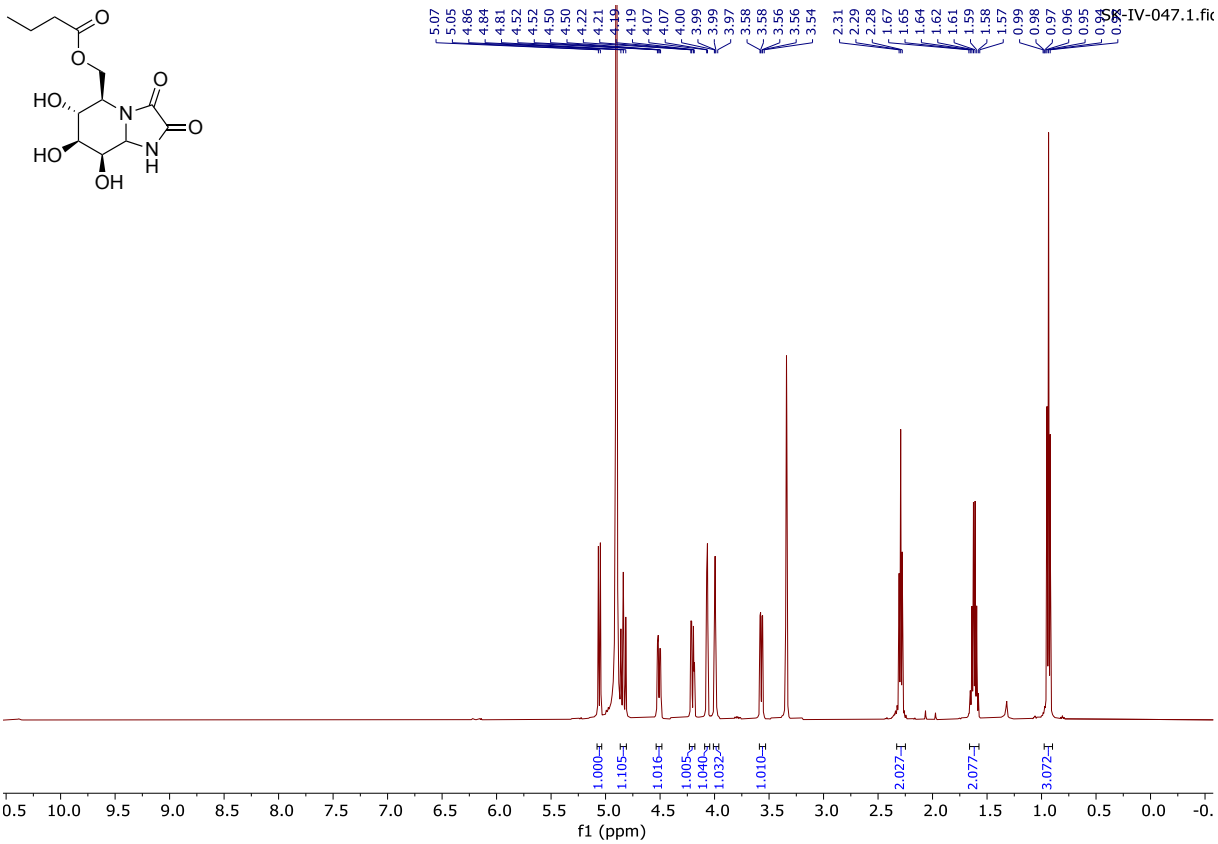

SK-IV-046.1.fi
6-O-n-BUTYL-PMB-KI
f1 (ppm)

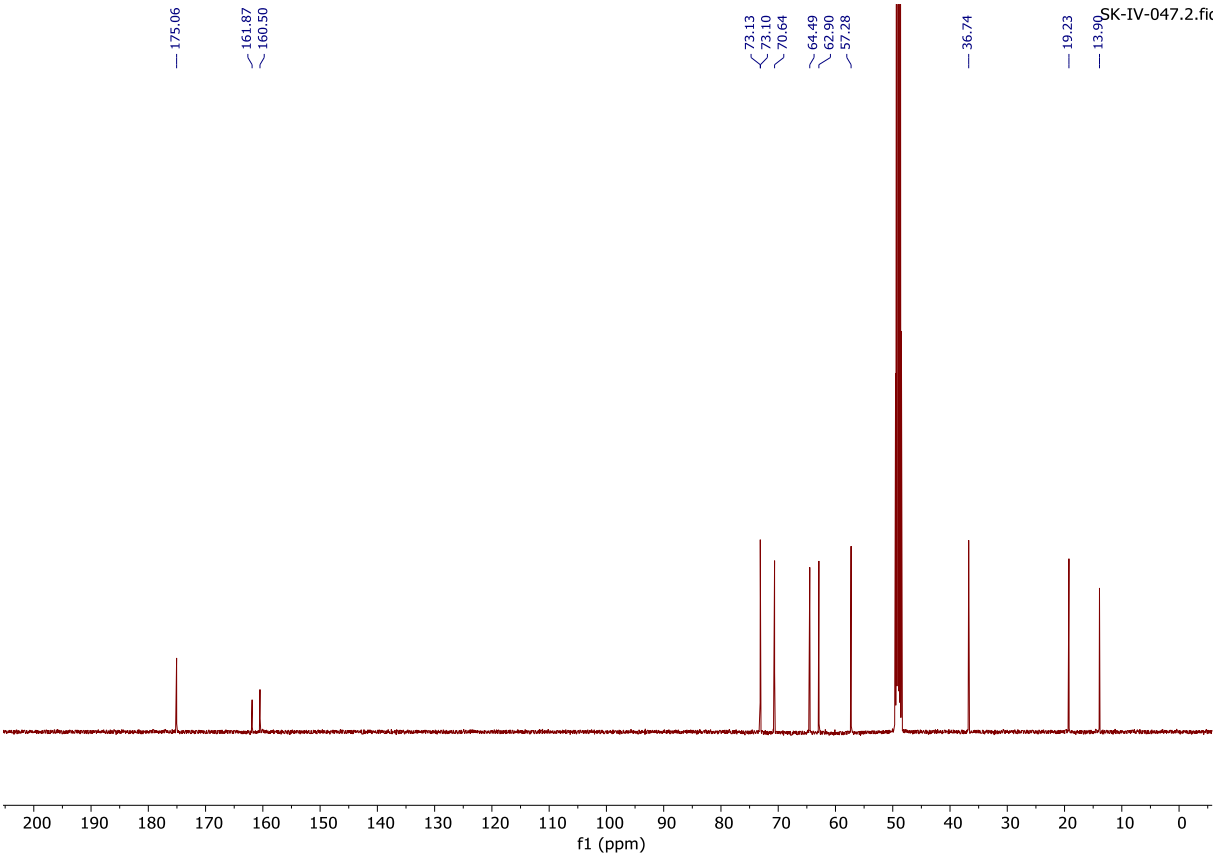

SK-IV-046.2.1.1
6-O-n-BUTYL-PMB-KI
f1 (ppm)

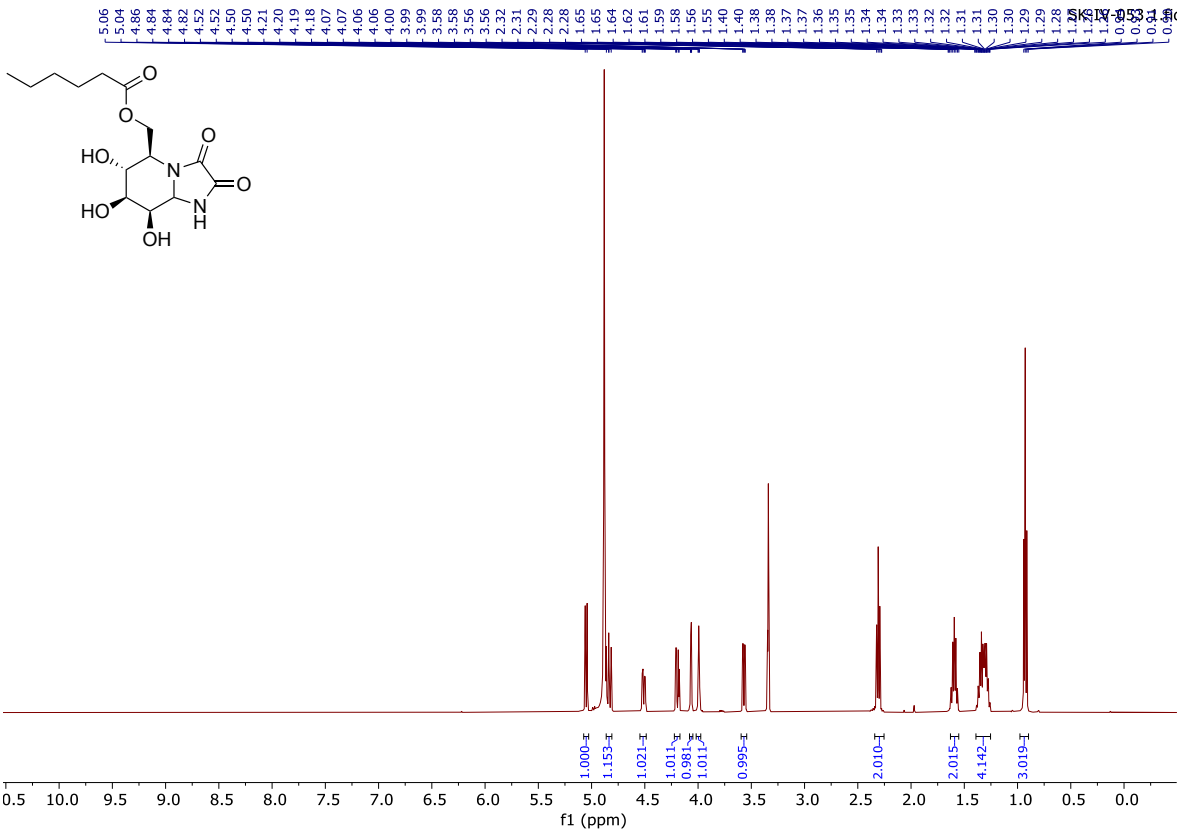

SK-IV-052.1.1.1
6-O-HEXYL-KII
f1 (ppm)
SK-IV-052.1.1.1
6-O-HEXYL-KII
f1 (ppm)

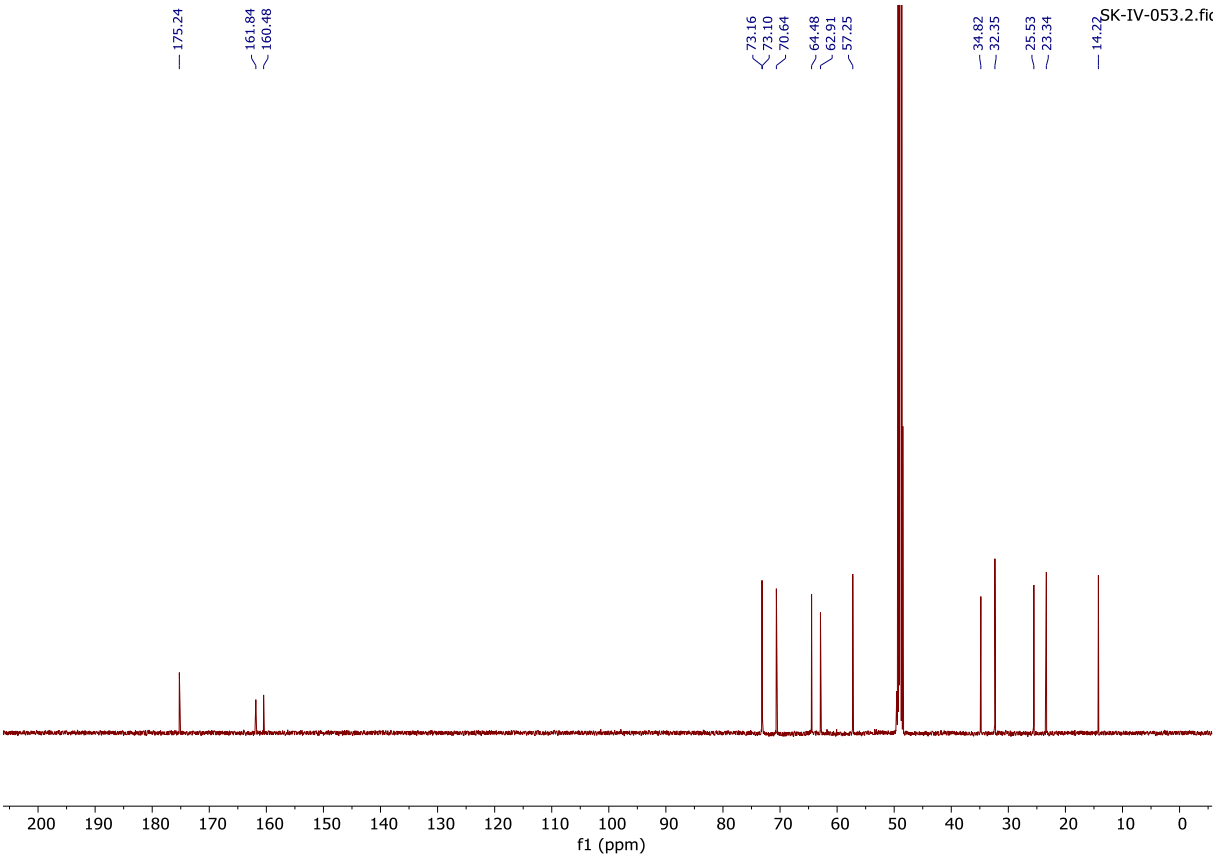

SK-IV-045.1.fi
5.03
5.01
4.79
4.77
4.77
4.75
4.46
4.45
4.43
4.43
4.23
4.22
4.20
4.20
4.05
4.05
4.05
4.05
3.98
3.98
3.55
3.55
3.53
3.53
2.36
2.34
2.33
2.31
1.12
1.11
1.09
1.000
1.019
0.907
1.073
1.077
1.037
1.014
2.056
3.081
f1 (ppm)

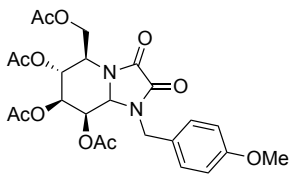

SK-IV-045.2.fi
175.93
161.59
160.19
73.07
73.02
70.60
64.60
63.01
57.28
28.17
9.32
f1 (ppm)


SK-IV-047.1.fic
5.07
5.05
4.86
4.84
4.81
4.52
4.52
4.50
4.50
4.22
4.21
4.19
4.07
4.07
4.00
3.99
3.99
3.97
3.58
3.58
3.56
3.56
3.54
2.31
2.29
2.28
1.67
1.65
1.64
1.62
1.61
1.59
1.58
1.57
0.99
0.98
0.97
0.96
0.95
1.000
1.105
1.016
1.005
1.040
1.032
1.010
2.027
2.077
3.072
f1 (ppm)

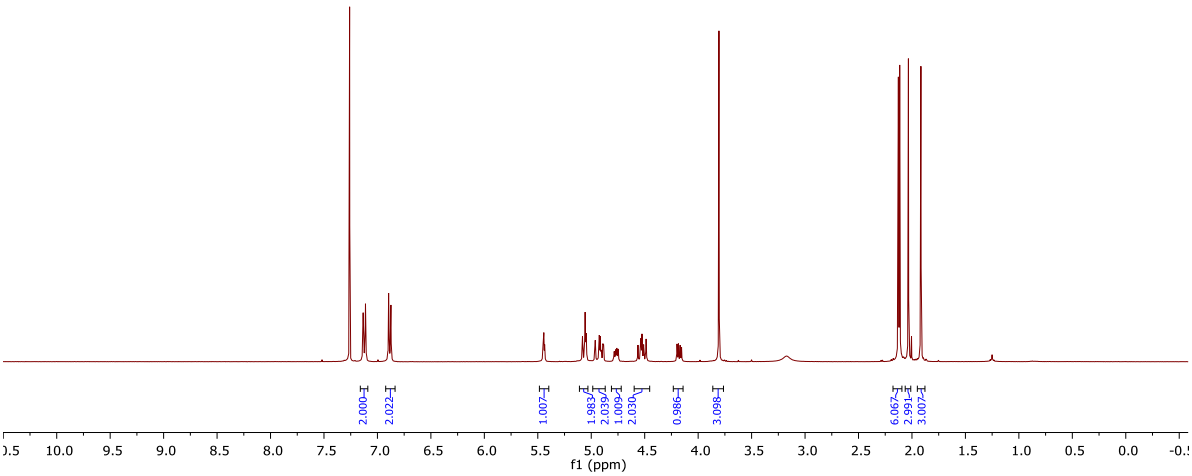

SK-IV-047.2.fic
175.06
161.87
160.50
73.13
73.10
70.64
64.49
62.90
57.28
36.74
19.23
13.90
f1 (ppm)


5.06 5.04 4.86 4.84 4.84 4.82 4.52 4.52 4.50 4.50 4.21 4.20 4.19 4.18 4.07 4.07 4.06 4.06 4.00 3.99 3.99 3.58 3.58 3.56 3.56 2.32 2.31 2.29 2.28 2.28 1.65 1.64 1.62 1.61 1.59 1.58 1.56 1.55 1.40 1.40 1.38 1.38 1.37 1.37 1.36 1.35 1.35 1.34 1.34 1.33 1.33 1.32 1.32 1.31 1.31 1.30 1.30 1.29 1.29 1.28
SK-IV-053.1.fi
O
O
HO
N
O
O
HO
N
H
OH
1.000 1.153 1.021 1.011 0.981 1.011 0.995 2.010 2.015 4.142 3.019
0.5 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 1.0 0.5 0.0
f1 (ppm)

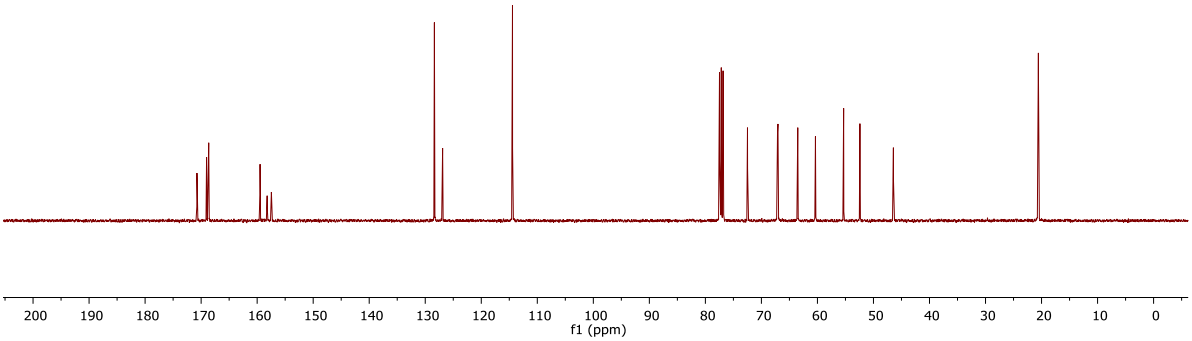

175.24
161.84
160.48
73.16
73.10
70.64
64.48
62.91
57.25
34.82
32.35
25.53
23.34
14.22
SK-IV-053.2.fi
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

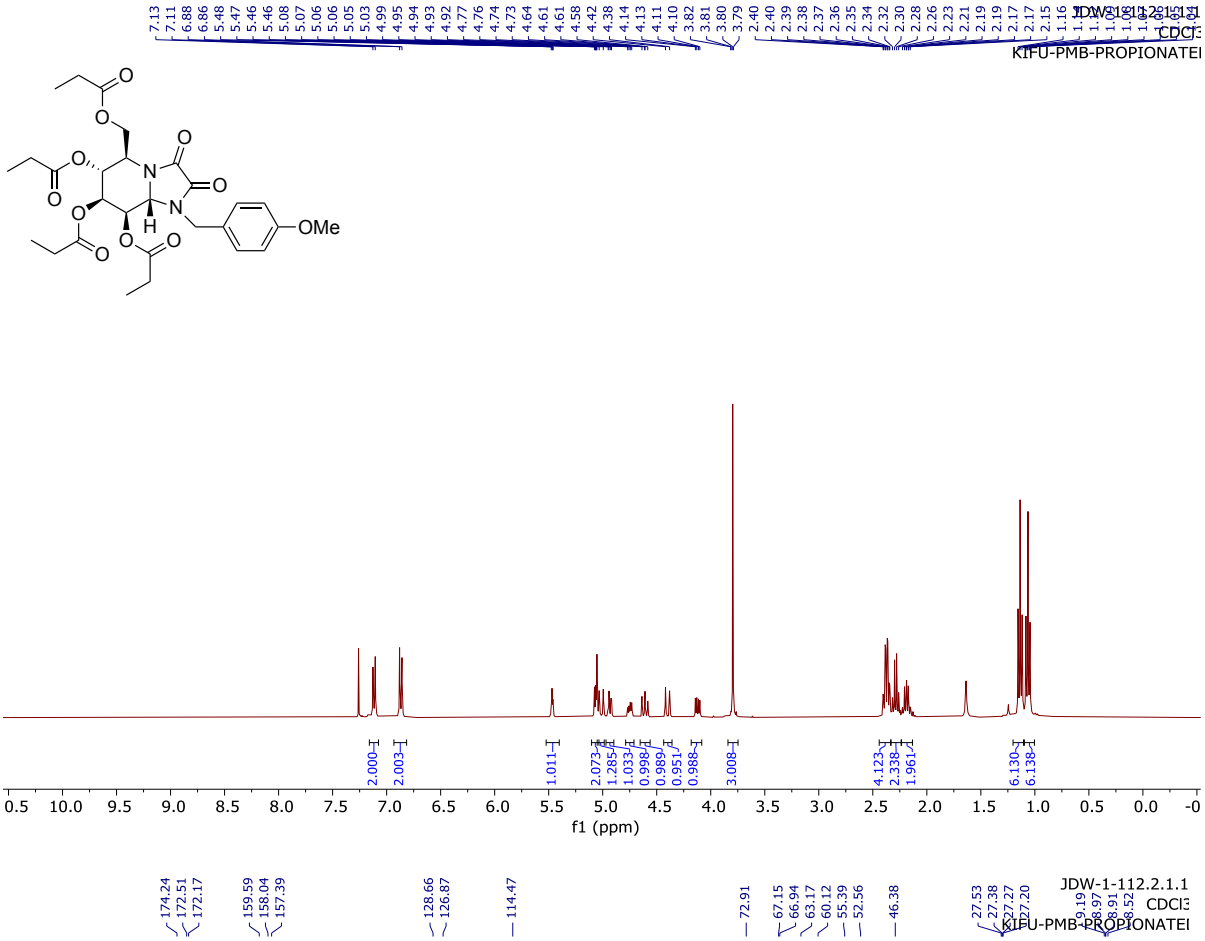


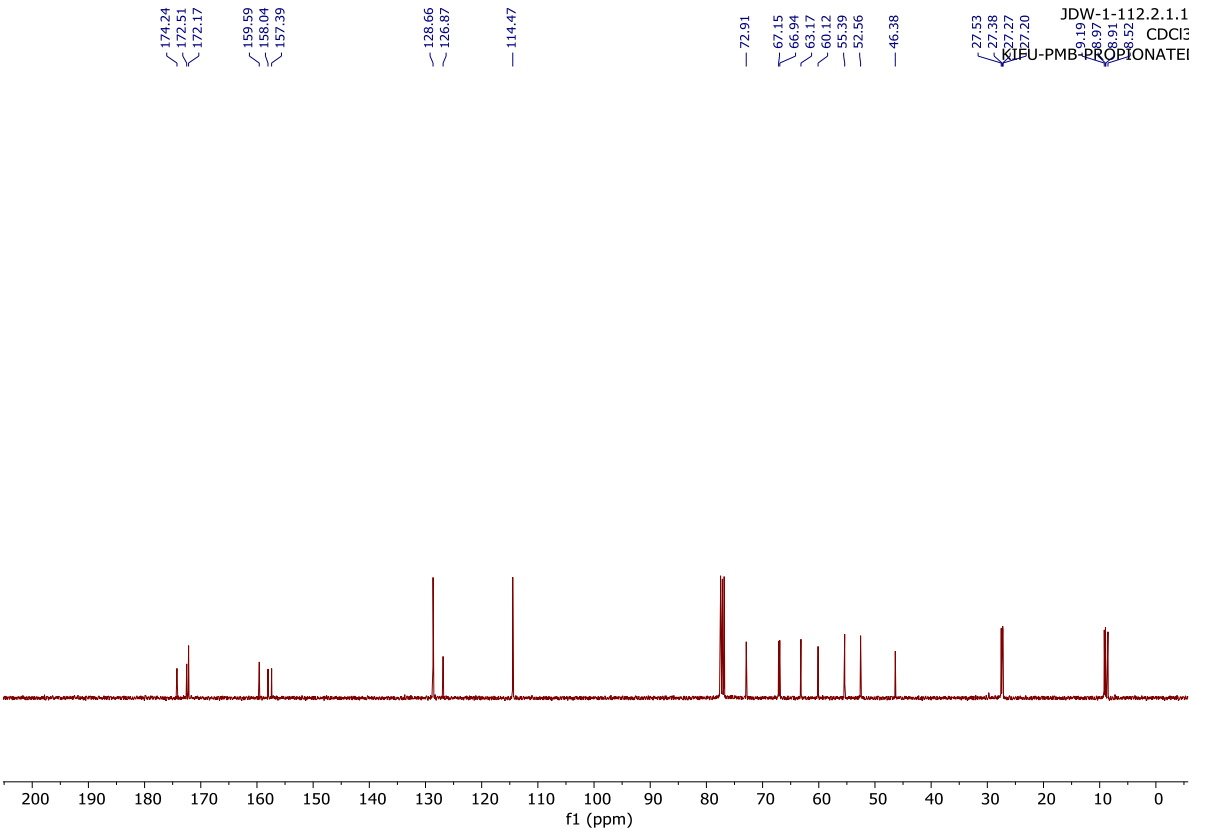
SK-II-140.1.1.1
Kifunensine-N-PMB-(OAc)

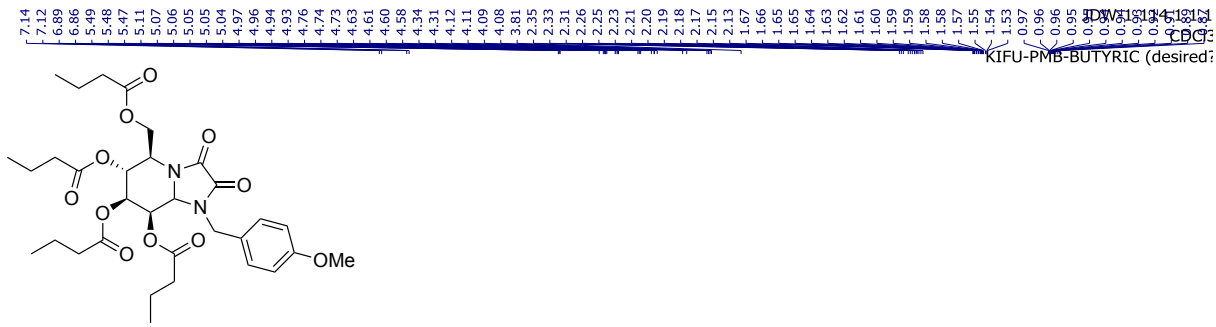


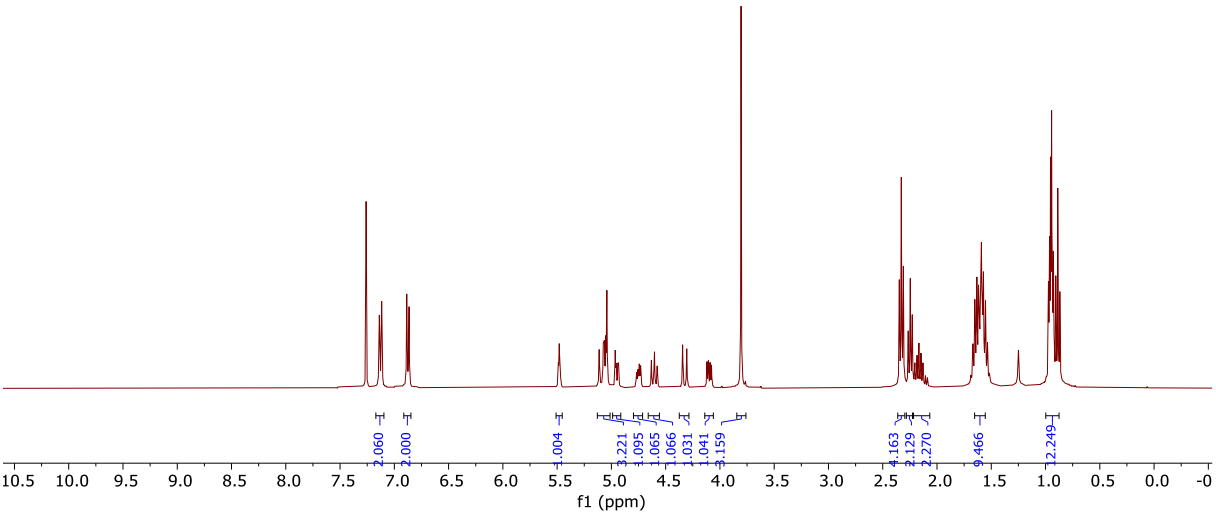
SK-II-140.2.1.1
Kifunensine-N-PMB-(OAc)

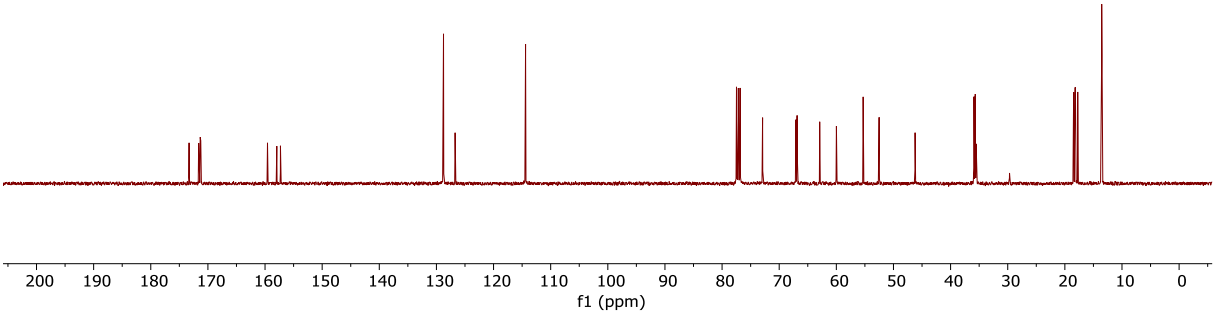


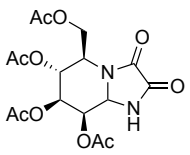

JDW-1-112.2.1.1
CDCl3
KIFU-PMB-PROPIONATEI
OMe
f1 (ppm)


JDW-1-112.2.1.1
CDCl3
KIFU-PMB-PROPIONATEI
f1 (ppm)

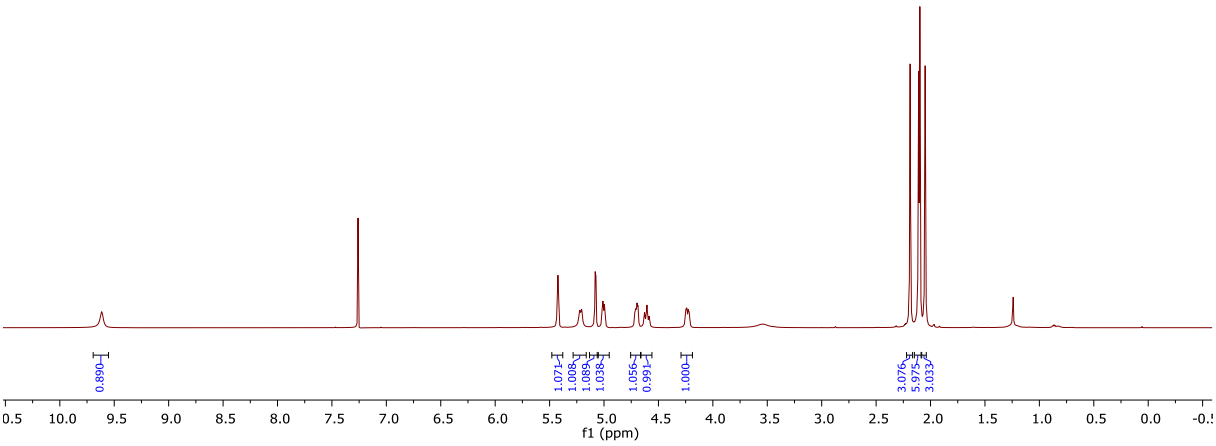

OMe


f1 (ppm)

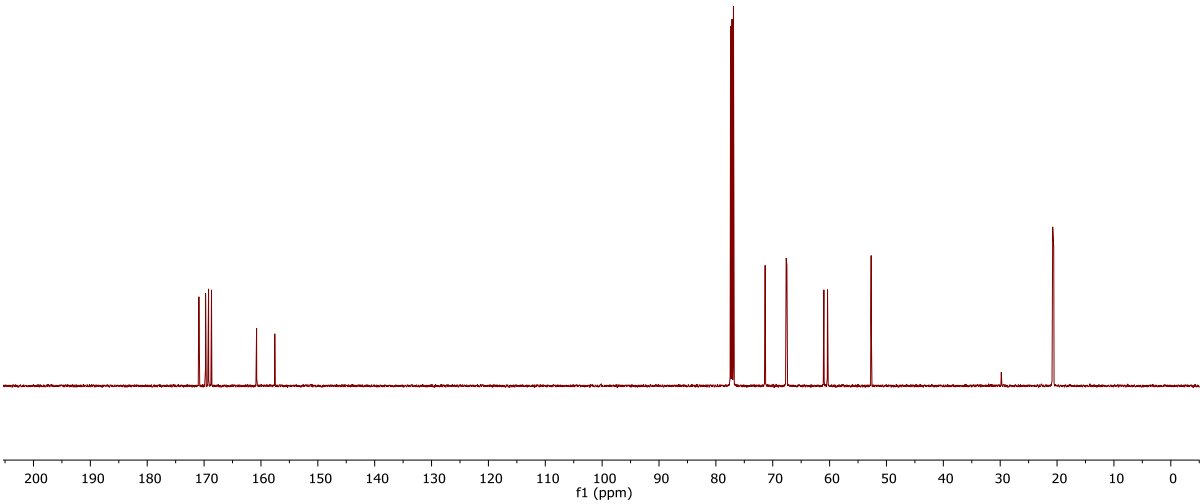

f1 (ppm)

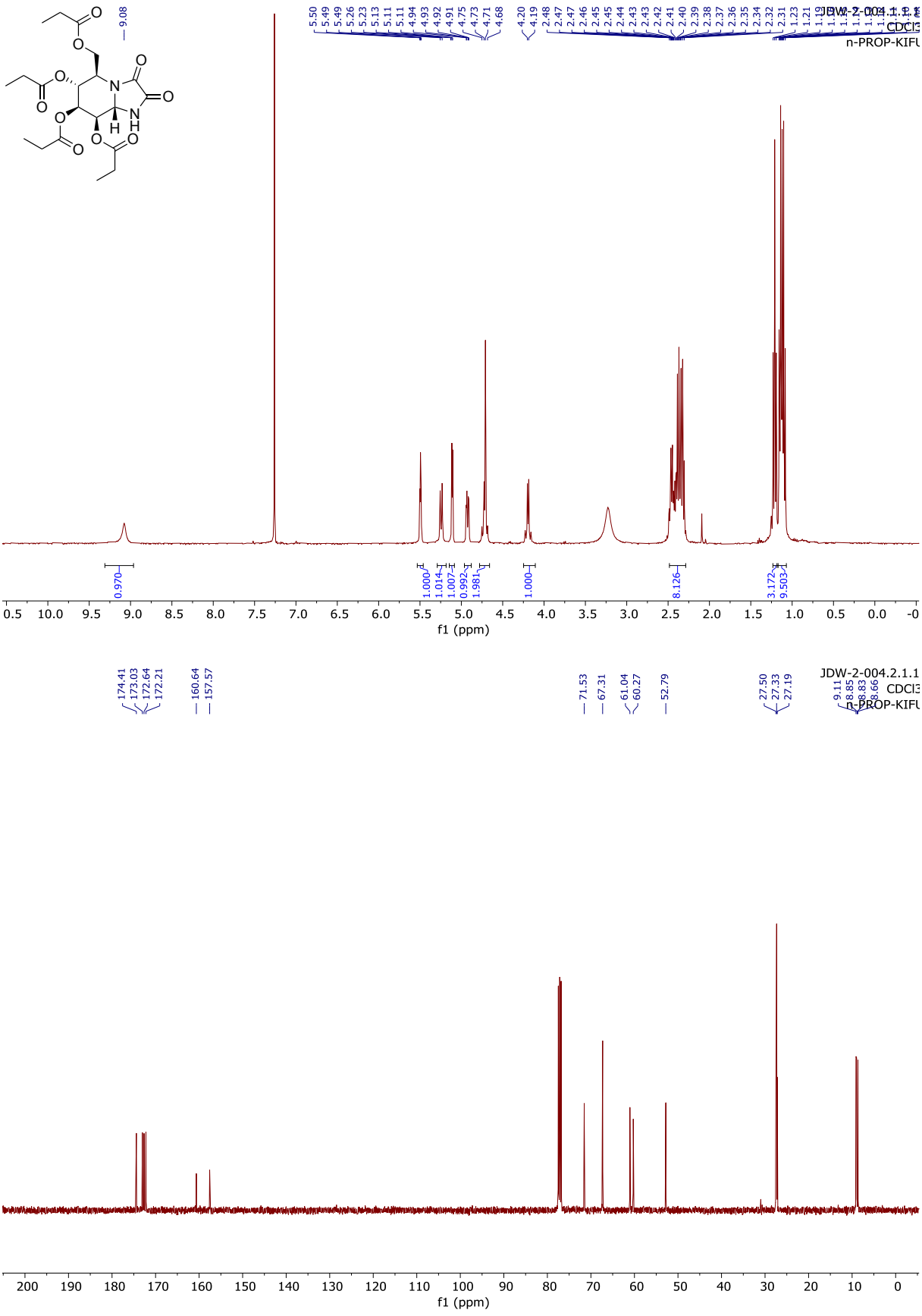


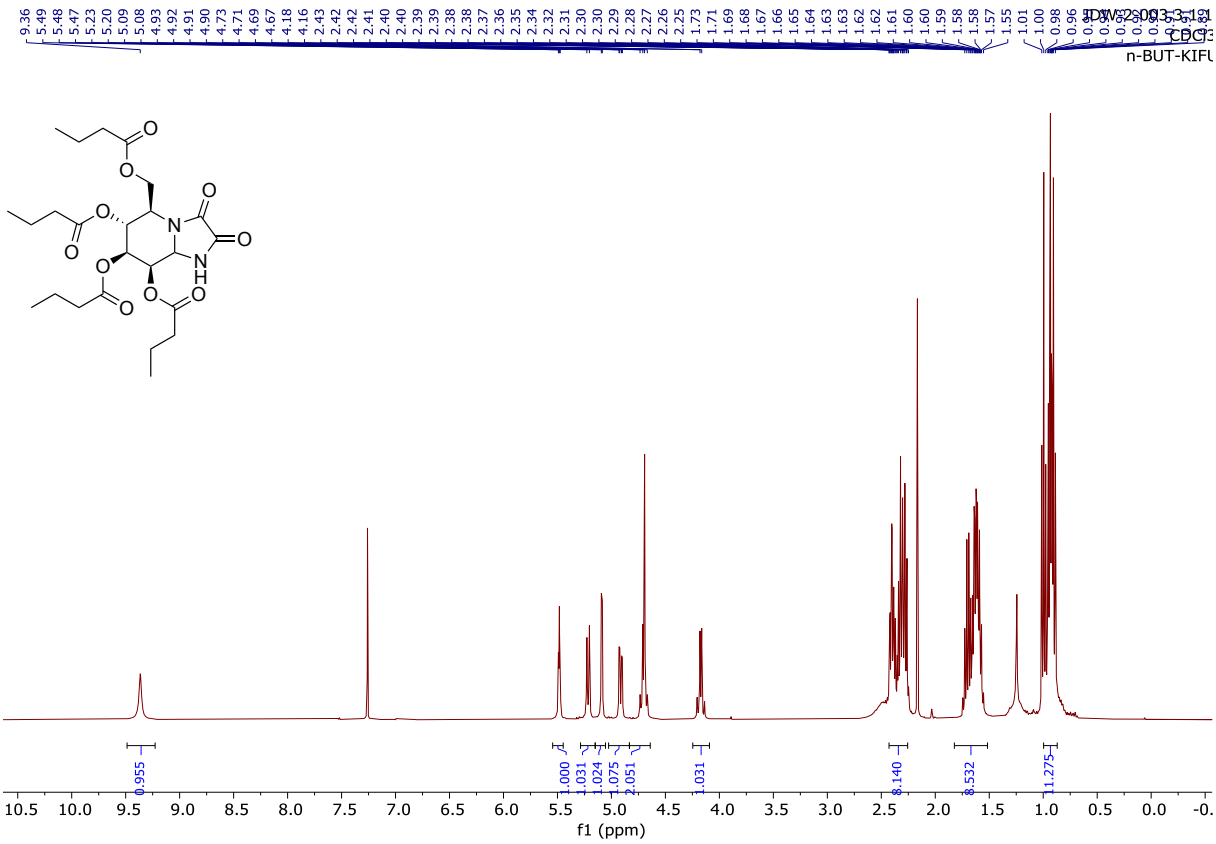
SK-II-141.1.1.1

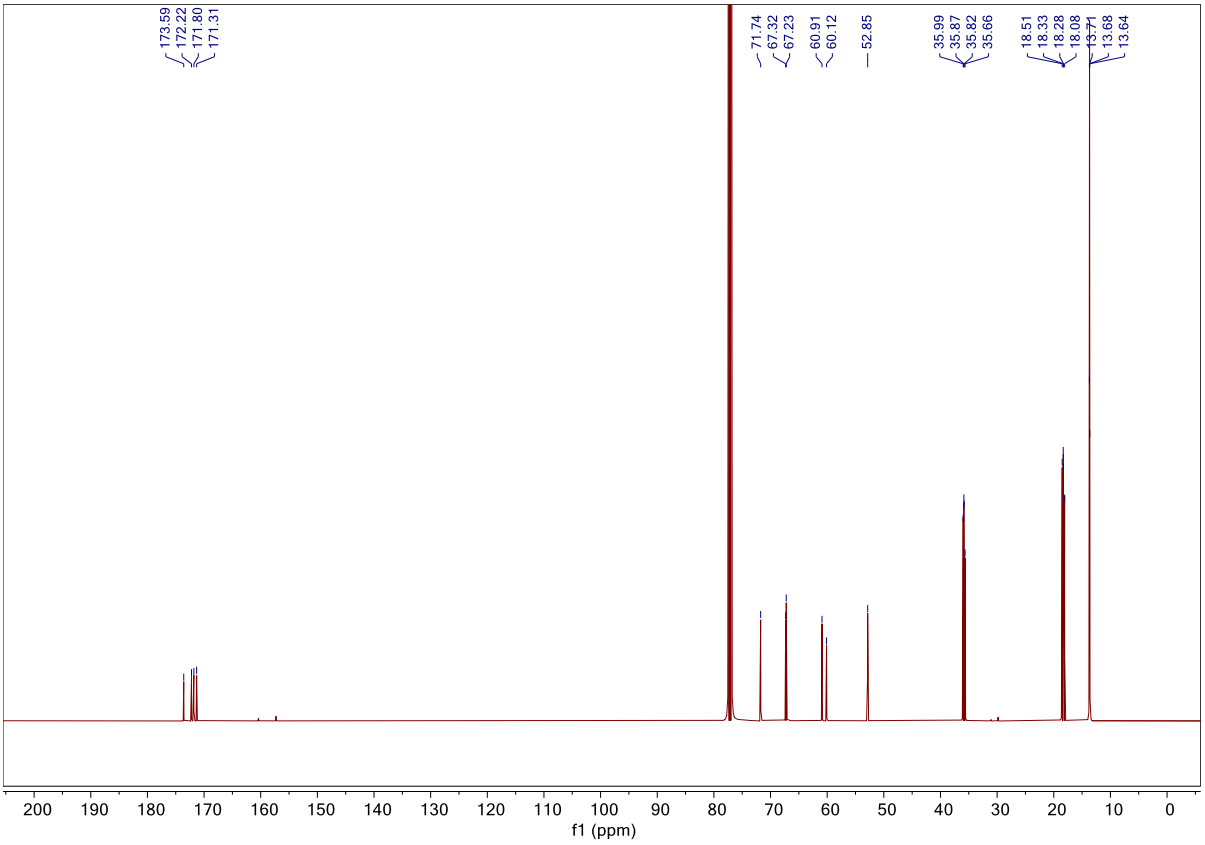


SK-II-141.2.1.1






f1 (ppm)


f1 (ppm)