Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Facile folding of insulin variants bearing a prosthetic C-peptide prepared by α-ketoacid-hydroxylamine (KAHA) ligation

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1. General methods

1.1. Regents and solvents

Solvents for flash chromatography (EtOAc, hexanes, CH_2Cl_2, MeOH) were of technical grade and distilled prior to use. Commercially available reagents and solvents were purchased from Sigma- Alrich (Buchs, Switzerland), Acros Organics (Geel, Belgium) and TCI Europe (Zwijndrecht, Belgium), Combi-Blocks (San Diego, USA), ABCR-Chemicals (Karlsruhe, Germany) Fmoc-amino acids with suitable protecting groups on the side chains, HCTU, HATU were purchased from Peptides International (Lousiville, KY, USA) and Chemimpex (Wood Dale, IL, USA). Resins were purchased form Biotage (Uppsala, Sweden).

1.2. Characterization

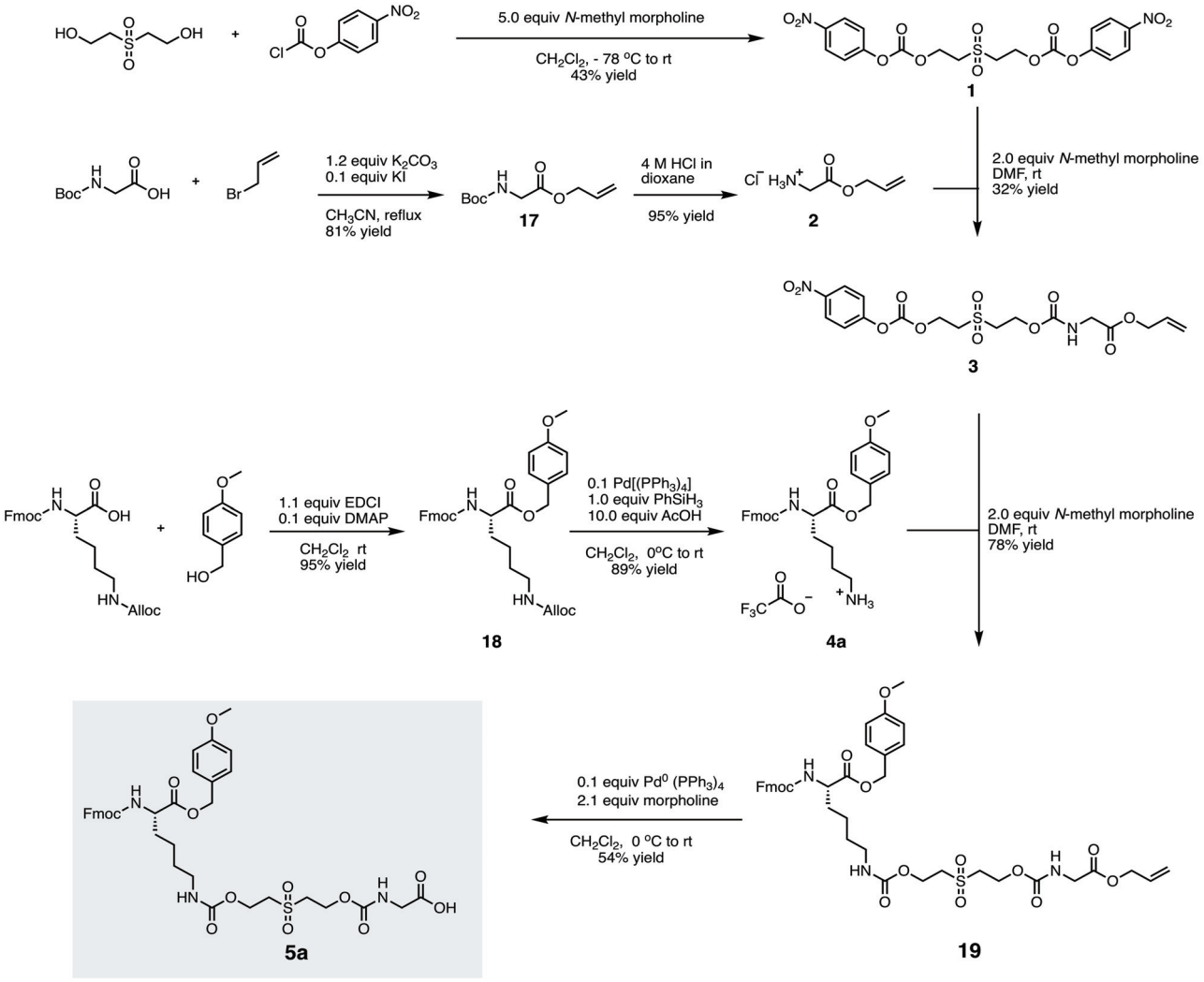
^{1}H and ^{13}C NMR spectra were recorded on Bruker AVIII400 spectrometer or Bruker AVIII600 spectrometer. Chemical shifts for ^{1}H NMR (400 and 600 MHz) and 13C NMR (101 and 150 MHz) are expressed in parts per million and are referred to residual undeuterated solvent signals. Coupling constants are reported in Hertz (Hz) and the corresponding splitting patterns are indicated as follows: s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; td, triplet of doublet; t, triplet; m, multiplet. High-resolution mass spectra were recorded by the Mass Service of the Laboratory of Organic Chemistry at ETH Zurich either with a Bruker maXis instrument (ESI-MS measurements) equipped with an ESI source and a Qq-TOF detector or with a Bruker solariX instrument (MALDI-FTICR-MS) using 4-hydroxy-α-cyanocinnamic acid as matrix. Infrared spectra were recorded on Perkin Elmer Spectrum Two FT-IR spectrometer and reported as wavenumber (cm^{-1}). CD spectra JASCO J-715 spectropolarimeter at wavelength of 300–190 nm. Peptides were analyzed by reversed phase high performance liquid chromatography (RP-HPLC) on Jasco analytical instrument equipped with dual pumps, mixer and in-line degasser. The spectra were recorded simultaneously at 220 nm, 254 nm and 301 nm. The characterization was performed on heated (Alltech column heater) Shiseido Capcell Pak C18 analytical column, Phenomenex Jupiter C4 or Vydac 214TP54 C4 analytical column. The mobile phase were CH_3CN containing 0.1% (v/v) TFA and Millipore H_2O containing 0.1% (v/v) TFA (1 mL /min flow rate).

1.3. Reactions and purification

All reactions were performed using standard techniques under an atmosphere of N_2. Reactions and fractions from flash chromatography were monitored by thin layer chromatography using precoated alumina plates (Merck, silica 60 F254) and visualized by staining with basic $KMnO_4$ solution. Flash chromatography was performed on Sigma-Aldrich SiO_2 9385 (230-400 mesh) using a forced flow of air at 0.5-1.0 bar. Peptides were purified by reversed phase high performance liquid chromatography (RP-HPLC) on Jasco preparative instrument equipped with dual pumps, mixer and in-line degasser or on Gilson preparative instrument. The spectra were recorded simultaneously at 220 nm, 254 nm and 301 nm. The purification was performed on heated (water bath) Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column, Shiseido Capcell Pak C18 Type MG II (20 mm x 250 mm, 5 μm) preparative column, Shiseido Capcell Pak C18 Type UG80 (50 mm x 250 mm, 5 μm) preparative column, Shiseido Proteonavi (50 mm x 250 mm, 5 μm) preparative column, Vydac 214MS1022 C4 preparative column (22 x 250 mm), Phenomenex

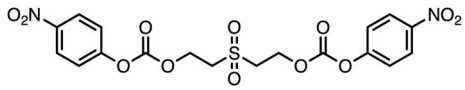
Jupiter (10 mm x 250 mm) semi-preparative column or Phenomenex Jupiter (20 mm x 250 mm) preparative column. The mobile phase were CH_3CN containing 0.1% (v/v) TFA and Millipore H_2O containing 0.1% (v/v) TFA.

2. Synthesis of linker I. for M2 insulin (5a)



Synthesis of linker I. (**5a**)

2.1. Diethoxy sulfonyl bis (4-nitrophenyl carbonate) (1)



Bis(hydroxyethyl) sulfone (5.00 g, 32.4 mmol, 1.00 equiv) and nitrophenol chloroformate (16.3 g, 81.1 mmol, 2.50 equiv) were dissolved in 250 mL CH_2Cl_2 and cooled to -78 °C. *N*-methylmorpholine (17.8 mL, 162.1 mmol, 5.00 equiv) *N*-methylmorpholine was dissolved separately in 50.0 mL CH_2Cl_2 at rt. This solution was added dropwise to the reaction mixture over the curse of 1 h. The reaction was left to stir at –78 °C for 5 h and then it was allowed to warm up to rt overnight. The formed precipitate was filtered off washed with CH_2Cl_2 to give pure **1** as a white solid (6.70 g, 13.8 mmol, 43% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 – 8.27 (m, 4H), 7.61 – 7.51 (m, 4H), 4.72 – 4.60 (m, 4H), 3.75 (t, *J* = 5.6 Hz, 4H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 155.09 (2xC), 151.50 (2xCO), 145.22 (2xC), 125.42 (4xCH), 122.52 (4xCH), 61.94 (2xCH_2), 52.12 (2xCH_2).

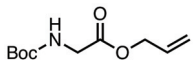
IR (cm^{-1}, neat): 1763, 1528, 1349, 1258, 1216, 1127, 1069.

HRMS (ESI): calculated for $C_{18}H_{16}N_2O_{12S}Na$ $[M+Na]^+$: 507.03162, found: 507.03132.

Rf = 0.4 (hexanes: EtOAc = 1:1)

MP: 155–156 °C

2.2. Synthesis of N-Boc Glycine allyl ester (17)



N-Boc glycine (10.0 g, 57.1 mmol, 1.00 equiv) was dissolved in 500 mL CH_3CN at rt. K_2CO_3 (9.47 g, 68.6 mmol, 1.20 equiv), KI (0.95 g, 5.71 mmol, 0.10 equiv) and allyl bromide (4.93 mL, 57.1 mmol, 1.00 equiv) were added to the mixture and heated to reflux. After 4 h CH_3CN was removed under reduced pressure, the residue was suspended in 400 mL diethyl ether and washed 400 mL water and 400 mL brine, dried over Na_2SO_4 to give pure **17** as a colourless oil (10.0 g, 46.5 mmol, 81% yield)

^{1}H NMR (300 MHz, DMSO-d_6) δ 7.23 (t, *J* = 6.2 Hz, 1H), 5.90 (m, 1H), 5.32 (m, 1H), 5.21 (m, 1H), 4.58 (m, 2H), 3.71 (d, *J* = 6.2 Hz, 2H), 1.38 (s, 9H)

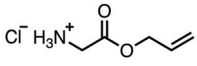
^{13}C NMR (101 MHz, DMSO-d_6) δ 170.52 (CO), 156.31 (CO), 132.90 (CH), 118.18 (CH_2), 78.70 (C), 65.09 (CH_2), 42.37 (CH_2), 28.61 (CH_3)

IR (cm^{-1}, neat): 3375, 2979, 1694, 1512, 1366, 1157, 985.

HRMS (ESI): calculated for $C_{10}H_{17}NO_4Na$ $[M+Na]^+$: 238.10498, found: 238.10492.

Rf = 0.6 (hexanes: EtOAc = 7:3)

2.3. Synthesis of glycine allyl ester hydrochloride (2)



To **17** (10.0 g, 46.5 mmol, 1.00 equiv) 100 mL 4 M HCl in dioxane was added at 0 °C and stirred under N_2 for 3 h. The mixture was diluted with 100 mL toluene and the solvents were evaporated. The residue was

dissolved in 100 mL toluene and evaporated. This procedure was repeated two times. 100 mL cold Et_2O was added to the residual colorless oil and the white precipitate was filtered off and washed with cold Et_2O to obtain pure **2** as white solid (6.70 g, 44.2 mmol, 95% yield).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.57 (s, 3H), 5.93 (m, 1H), 5.38 (m, 1H), 5.26 (m, 1H), 4.68 (m, 2H), 3.82 (s, 2H).

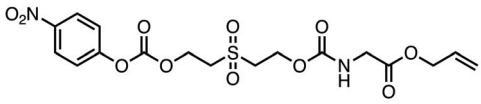
^{13}C NMR (101 MHz, DMSO-d_6) δ 167.31 (CO), 131.81 (CH), 118.46 (CH_2), 65.63 (CH_2), 39.48 (CH_2)

IR (cm^{-1}, neat): 2961, 2867, 1753, 1503, 1246, 1227, 911.

HRMS (ESI): calculated for $C_5H_{10}NO_2Na$ $[M+H]^+$: 116.0706, found: 116.0705.

MP = 80-81 °C

2.4. Synthesis of allyl diethoxy sulfonyl (4-nitrophenyl carbonate) glycinate (3)



1 (5.50 g, 11.4 mmol, 1.00 equiv) was dissolved in 110 mL *N*,*N*-dimethylformamide (DMF) in a round-bottomed flask. Separately **2** (1.72 g, 11.4 mmol, 1.00 equiv) was dissolved in 30 mL DMF and *N*-methylmorpholine (2.44 mL, 22.4 mmol, 2.00 equiv) was added to it. This solution was added drop wise to the solution of **1** and the mixture was stirred at rt. After 3 h the reaction mixture was diluted with 400 mL EtOAc and washed with 400 mL 1 M $KHSO_4$ solution, 400 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 4:1 to 1:1) to give **3** as a colorless oil (1.70 g, 5.57 mmol, 32% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.36–8.30 (m, 2H), 7.82 (t, *J* = 6.1 Hz, 1H), 7.60 – 7.54 (m, 2H), 5.90 (ddt, *J* = 17.3, 10.7, 5.4 Hz, 1H), 5.31 (dq, *J* = 17.3, 1.7 Hz, 1H), 5.21 (dt, *J* = 10.5, 1.5 Hz, 1H), 4.66–4.59 (m, 2H), 4.59 (dt, *J* = 5.4, 1.5 Hz, 2H), 4.34 (t, *J* = 5.8 Hz, 2H), 3.82 (d, *J* = 6.1 Hz, 2H), 3.71 (t, *J* = 5.7 Hz, 2H), 3.55 (t, *J* = 5.8 Hz, 2H).

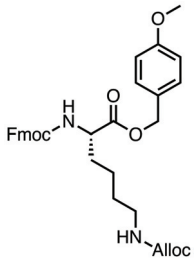
^{13}C NMR (151 MHz, DMSO-d_6) δ 169.74 (CO), 155.80 (C), 155.13 (CO), 151.53 (CO), 145.25 (C), 132.34 (CH), 125.44 (2xCH), 122.55 (2xCH), 117.89 (CH_2), 64.82 (CH_2), 62.01 (CH_2), 57.85 (CH_2), 52.74 (CH_2), 52.46 (CH_2), 42.09 (CH_2).

IR (cm^{-1}, neat): 3318, 1750, 1692, 1524, 1268, 1210.

HRMS (ESI): calculated for $C_{17}H_{21}N_2O_{11}S$ $[M+H]^+$: 461.0861, found: 461.0867.

Rf = 0.3 (hexane: EtOAc = 1:1)

2.5. Synthesis of Fmoc-Lys(NHAlloc)-OPMB (18)



Fmoc-Lys(NHAlloc)-OH (5.00 g, 11.1 mmol, 1.00 equiv) and paramethoxybenzyl alcohol (PMBOH)

(1.60 g, 11.6 mmol, 1.05 equiv) were dissolved in 110 mL CH_2Cl_2. *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) (2.33 g, 12.2 mmol, 1.10 equiv) and 4-(dimethylamino)pyridine (DMAP) 135 mg (1.10 mmol, 0.10 equiv) were added. The mixture was left to stir at rt. After 4 h the mixture was diluted with 300 mL Et_2O, washed with 200 mL 1 M HCl, 200 mL saturated aq. $NaHCO_3$ solution, 200 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 4:1 to 3:2) to give **18** as a colorless oil (6.0 g, 10.49 mmol, 95% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.89 (dt, *J* = 7.6, 0.9 Hz, 2H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.73 – 7.68 (m, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.35 – 7.29 (m, 2H), 7.29 – 7.25 (m, 2H), 7.17 (t, *J* = 5.7 Hz, 1H), 6.90 – 6.85 (m, 2H), 5.94 – 5.84 (m, 1H), 5.29 – 5.22 (m, 1H), 5.18 – 5.12 (m, 1H), 5.08 – 4.99 (m, 2H), 4.47–4.42 (m, 2H), 4.33 – 4.24 (m, 2H), 4.20 (t, *J* = 7.1 Hz, 1H), 4.04 – 3.97 (m, 1H), 3.71 (s, 3H), 2.97 – 2.90 (m, 2H), 1.71 – 1.56 (m, 2H), 1.41 – 1.32 (m, 2H), 1.32 – 1.25 (m, 2H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 172.27 (CO), 159.08(C), 156.10 (CO), 155.84 (CO), 143.79 (C), 143.69 (C), 140.38 (2xC), 133.82 (CH), 129.69 (C+2xCH), 127.59 (2xCH), 127.02 (2xCH), 125.20 (2xCH), 120.08 (2xCH), 116.79 (CH_2), 113.72 (2xCH), 65.69 (CH_2), 65.63 (CH_2), 64.07 (CH_2), 55.02 (CH_3), 53.95 (CH), 46.59 (CH), 40.03 (CH_2), 30.24 (CH_2), 28.88 (CH_2), 22.71 (CH_2).

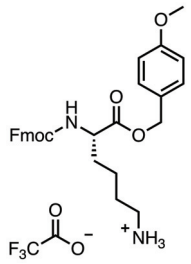
$[\alpha]^{25}_D$ (c = 0.9, $CHCl_3$): -1.8

IR (cm^{-1}, neat): 3326, 2947, 1687,1531, 1515, 1248, 736.

HRMS (ESI): calculated for $C_{33}H_{36}N_2O_7Na$ $[M+Na]^+$: 595.24147, found: 595.24142.

Rf = 0.5 (hexanes: EtOAc = 3:2)

2.6. Synthesis of Fmoc-Lys(NH_2)-OPMB TFA salt (4a)



18 (5.80 g, 10.1 mmol, 1.00 equiv) was dissolved in 100 mL dry, degassed CH_2Cl_2 at 0 °C. AcOH (5.79 mL, 101 mmol, 10.0 equiv), phenyl silane (1.25 mL, 10.1 mmol, 1.00 equiv) and tetrakis(triphenylphosphine)palladium (1.17 g, 1.01 mmol, 0.10 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt over the course of 6 h. The solvent was removed under reduced pressure and the residue was dissolved in 200 mL EtOAc, extracted 200 mL 0.2 M Na_2HPO_4 / 10 w/w % aq. citric acid solution (pH 6) buffer three times, washed with brine, dried over Na_2SO_4 and concentrated. The residue was dissolved in 100 mL CH_3CN:H_2O +0.01 v/v% TFA solution and filtered. Volatile compounds were removed by lyophilization to obtain 5.40 g (8.97 mmol, 89% yield) crude **4a**. The compound was purified by preparative HPLC for analytical purposes.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.90 (d, *J* = 7.5 Hz, 2H), 7.77 (d, *J* = 7.9 Hz, 1H), 7.74 – 7.67 (m, 2H), 7.61 (s, 3H), 7.45–7.38 (m, 2H), 7.35 – 7.29 (m, 2H), 7.29 – 7.25 (m, 2H), 6.91 – 6.82 (m, 2H), 5.09–5.01 (m, 2H), 4.38 – 4.31 (m, 1H), 4.30 – 4.22 (m, 1H), 4.20 (t, *J* = 6.9 Hz, 1H), 4.07 – 3.99 (m, 1H),

3.72 (s, 3H), 2.79 – 2.68 (m, 2H), 1.74 – 1.57 (m, 2H), 1.58 – 1.43 (m, 2H), 1.39 – 1.27 (m, 2H).

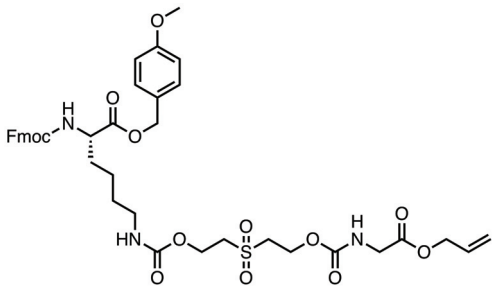
^{13}C NMR (151 MHz, DMSO-d_6) δ 172.19 (CO), 159.13 (C), 156.16 (CO), 143.81 (CH), 143.69 (C), 140.73 (2xC), 129.76 (C+2xCH), 127.77 (CH), 127.63 (CH), 127.05 (CH), 127.03 (CH), 125.18 (CH), 125.12 (CH), 120.14 (2xCH), 113.76 (2xCH, CF_3), 65.81 (CH_2), 65.63 (CH_2), 55.08 (CH_3), 53.75 (CH), 46.62 (CH), 38.57 (CH_2), 29.98 (CH_2), 26.39 (CH_2), 22.36 (CH_2)

$[\alpha]^{26}_D$ (c = 0.3, MeOH): -3.3

IR (cm^{-1}, neat): 2970, 1695, 1513, 1139, 1134, 1056, 761.

HRMS (ESI): calculated for $C_{29}H_{32}N_2O_5Na$ $[M+Na]^+$: 511.22034, found: 511.22023.

2.7. Synthesis of linker I.allyl ester (19)



3 (2.80 g, 6.08 mmol, 1.00 equiv) and *N*-methylmorpholine (1.34 mL, 12.16 mmol, 2.00 equiv) were dissolved in 50 mL DMF in a round-bottomed flask. Separately **4a** (3.68 g, 6.08 mmol, 1.00 equiv) was dissolved in 50 mL DMF. This solution was added drop wise to the solution of **3** and the mixture was stirred at rt. After 3 h the reaction mixture was diluted with 200 mL EtOAc and was washed with 200 mL 1 M $KHSO_4$ solution two times and with 400 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 4:1 to 1:1) to give **XX** as a colorless oil (3.90 g, 4.82 mmol, 78% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.89 (d, *J* = 7.6 Hz, 2H), 7.79 – 7.73 (m, 2H), 7.70 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.4 Hz, 2H), 7.35 – 7.28 (m, 2H), 7.28 – 7.21 (m, 3H), 6.87 (d, *J* = 8.6 Hz, 2H), 5.94 – 5.85 (m, 1H), 5.34 – 5.27 (m, 1H), 5.24 – 5.18 (m, 1H), 5.08 – 4.98 (m, 2H), 4.60 – 4.56 (m, 2H), 4.34 – 4.24 (m, 6H), 4.20 (t, *J* = 7.1 Hz, 1H), 4.03 – 3.97 (m, 1H), 3.81 (d, *J* = 6.1 Hz, 2H), 3.71 (s, 3H), 3.53 – 3.47 (m, 4H), 2.99 – 2.90 (m, 2H), 1.72 – 1.56 (m, 2H), 1.43 – 1.31 (m, 2H), 1.33 – 1.22 (m, 2H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 172.29 (CO), 169.70 (CO), 159.10 (C), 156.13 (CO), 155.85 (CO), 155.40 (CO), 143.81 (C), 143.72 (C), 140.70 (2xC), 132.33 (CH), 129.72 (2xCH), 127.81 (2xCH), 127.62 (2xCH), 127.05 (C), 125.19 (2xCH), 120.10 (2xCH), 117.86 (2xCH), 113.74 (CH_2), 65.72 (CH_2), 65.65 (CH_2), 64.79 (CH_2), 57.94 (CH_2), 57.32 (CH_2), 55.05 (CH_3), 53.96 (CH), 53.19 (CH_2), 53.00 (CH_2), 46.61 (CH), 42.09 (CH_2), 40.06 (CH_2), 30.26 (CH_2), 28.83 (CH_2), 22.76 (CH_2).

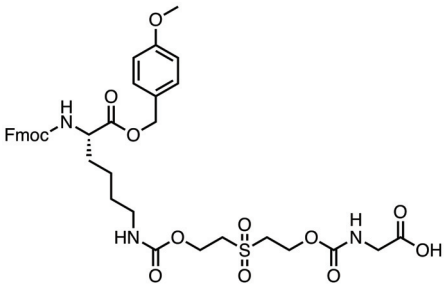
$[\alpha]^{25}_D$ (c = 0.4, $CHCl_3$): -1.1

IR (cm^{-1}, neat): 3325, 2944, 1989, 1534, 1253, 1186, 1122.

HRMS (ESI): calculated for $C_{40}H_{47}N_3O_{13}SNa$ $[M+Na]^+$: 832.27218, found: 832.27178.

Rf = 0.8 (hexanes: EtOAc = 1:2)

2.8. Synthesis of linker I. (5a)



19 (3.90 g, 4.82 mmol, 1.00 equiv) was dissolved in 50 mL dry, degassed CH_2Cl_2, cooled to 0 °C. Morpholine (0.78 mL, 10.10 mmol, 2.10 equiv) and Tetrakis(triphenylphosphine)palladium (0.56 g, 0.48 mmol, 0.10 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt in the course of 2 h.

The reaction mixture was diluted with 100 mL EtOAc, washed with 100 mL 10 w/w % aq. citric acid solution, 100 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (CH_2Cl_2:MeOH = 99:1 to 96:4 + 1% AcOH) to give **5a** as a colorless oil (2.0 g, 2.60 mmol, 54% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.92 – 7.87 (m, 2H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.73 – 7.68 (m, 2H), 7.60 (t, *J* = 6.2 Hz, 1H), 7.45 – 7.38 (m, 2H), 7.36 – 7.29 (m, 2H), 7.29 – 7.23 (m, 3H), 6.90 – 6.84 (m, 2H), 5.08 – 4.99 (m, 2H), 4.33 – 4.24 (m, 6H), 4.20 (t, *J* = 7.1 Hz, 1H), 4.08 – 3.97 (m, 1H), 3.71 (s, 3H), 3.67 (d, *J* = 6.1 Hz, 2H), 3.52 – 3.46 (m, 4H), 3.00 – 2.90 (m, 2H), 1.71 – 1.56 (m, 2H), 1.43 – 1.32 (m, 2H), 1.32 – 1.23 (m, 2H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 172.30 (CO), 171.37 (CO), 159.10 (C), 156.14 (CO), 155.81 (CO), 155.42 (CO), 143.81 (C), 143.73 (C), 140.71 (2xC), 129.72 (2xCH), 127.82 (C), 127.62 (2xCH), 127.06 (2xCH), 125.20 (2xCH), 120.10 (2xCH), 113.75 (2xCH), 65.73 (CH_2), 65.66 (CH_2), 57.84 (CH_2), 57.32 (CH_2), 55.05 (CH_3), 53.97 (CH), 53.20 (CH_2), 53.03 (CH_2), 46.62 (CH), 42.05 (CH_2), 40.06 (CH_2), 30.27 (CH_2), 28.84 (CH_2), 22.76 (CH_2)

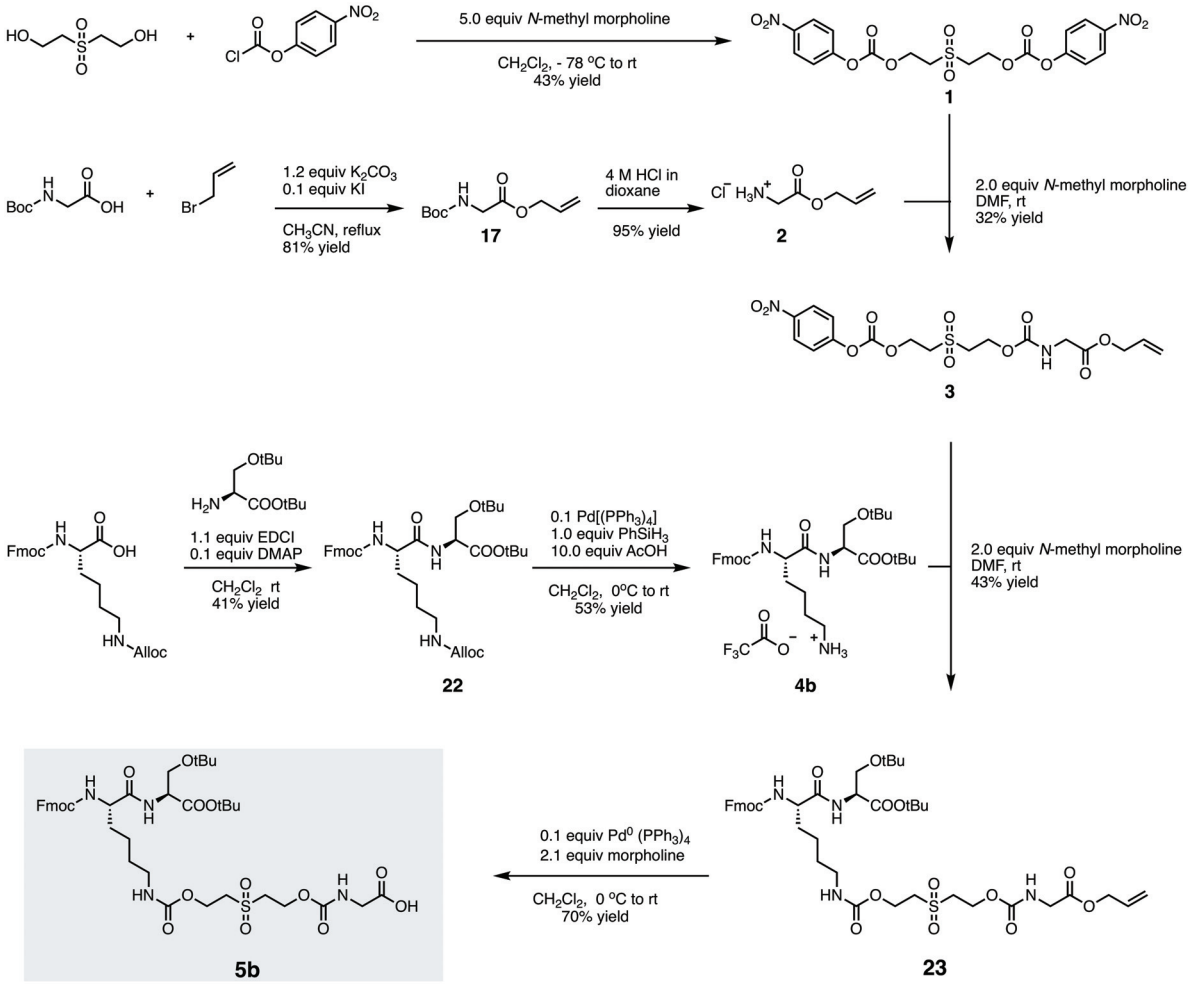
$[\alpha]^{26}_D$ (c = 1.1, $CHCl_3$): -13.7

IR (cm^{-1}, neat): 3343, 2943, 1654, 1516, 1245, 1172, 1122.

HRMS (ESI): calculated for $C_{37}H_{43}N_3O_{13}SNa$ $[M+Na]^+$: 792.24088, found: 729.24046.

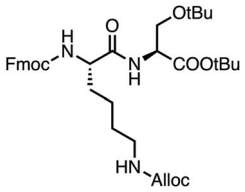
Rf = 0.3 (CH_2Cl_2:MeOH = 92:8)

3. Synthesis of linker II. for mouse insulin (5b)



Synthesis of linker II. for mouse insulin (**5b**)

3.1. Synthesis of Fmoc-Lys(NHAlloc)-Ser(tBu)-OtBu (20)



Fmoc-Lys(NHAlloc)-OH (5.50 g, 12.2 mmol, 1.00 equiv) and H-Ser(tBu)-OtBu hydrochloride (3.08 g, 12.1 mmol, 1.00 equiv) were dissolved in 120 mL CH_2Cl_2. EDCI (2.33 g, 12.15 mmol, 1.0 equiv), *N*,*N*-diisopropylethylamine (2.12 mL, 12.2 mmol, 1.00 equiv) and 148 mg (0.23 mmol, 0.10 equiv) DMAP were added. The mixture was left to stir at rt. After 3 h the mixture was diluted with 200 mL Et_2O, washed with 150 mL 1 M HCl, 300 mL saturated aq. $NaHCO_3$ solution, 150 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 65:35) to give **20** as a colorless oil (3.20 g, 4.91 mmol, 41% yield).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.92 (d, *J* = 8.0 Hz, 1H), 7.91 – 7.86 (m, 2H), 7.72 (dd, *J* = 7.6, 4.2 Hz, 2H), 7.52 (t, *J* = 9.2 Hz, 1H), 7.41 (td, *J* = 7.5, 1.1 Hz, 2H), 7.32 (td, *J* = 7.5, 1.1 Hz, 2H), 7.17 (t, *J* = 5.6 Hz, 1H), 5.95 – 5.83 (m, 1H), 5.26 (dq, *J* = 17.3, 1.8 Hz, 1H), 5.15 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.51 – 4.41 (m, 2H), 4.36 – 4.18 (m, 4H), 4.16 – 4.03 (m, 1H), 3.65 – 3.56 (m, 1H), 3.46 – 3.38 (m, 1H), 3.02 – 2.91 (m, 2H), 1.71 – 1.47 (m, 2H), 1.45 – 1.21 (m, 13H), 1.12 – 1.01 (m, 9H).

^{13}C NMR (126 MHz, DMSO) δ 172.06 (CO), 169.21 (CO), 155.95 (CO), 155.86 (CO), 143.84 (2xC), 140.68 (2xC), 133.85 (CH), 127.62 (2xCH), 127.05 (2xCH), 125.27 (2xCH), 120.09 (2xCH), 116.81 (CH), 80.55 (C), 72.67 (C), 65.62 (CH_2), 64.09 (CH_2), 61.71 (CH_2), 54.37 (CH), 53.16 (CH), 46.65 (CH), 40.06 (CH_2), 31.60 (CH_2), 29.16 (CH_2), 27.63 (3xCH_3), 27.08 (3xCH_3), 22.85 (CH_2).

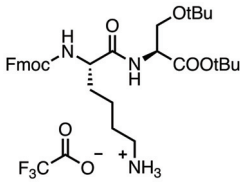
$[\alpha]^{24}_D$ (c = 1.15, $CHCl_3$): 9.2

IR (u/cm^{-1}, neat): 3313, 2974, 1702, 1525, 1246, 740

HRMS (ESI): calculated for $C_{36}H_{49}N_3O_8Na_1$ $[M+Na]^+$: 674.3412, found: 674.3403.

Rf = 0.4 (hexanes: EtOAc = 3:2)

3.2. Synthesis of Fmoc-Lys(NH_2)-Ser(tBu)-OtBu TFA salt (4b)



20 (3.20 g, 4.91 mmol, 1.00 equiv) was dissolved in 50 mL dry, degassed CH_2Cl_2 at 0 °C. AcOH (2.81 mL, 49.1 mmol, 10.0 equiv), phenyl silane (0.61 mL, 4.91 mmol, 1.00 equiv) and tetrakis(triphenylphosphine)palladium (0.57 g, 0.49 mmol, 0.1 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt in the course of 4 h. The solvent was removed under reduced pressure and the residue was dissolved in 100 mL EtOAc, extracted 100 mL 0.2 M Na_2HPO_4 / 10 w/w % aq. citric acid solution (pH 6) buffer three times, washed with brine, dried over Na_2SO_4 and concentrated. The residue was dissolved in 100 mL CH_3CN:H_2O + 0.01 v/v% TFA solution and filtered. Volatile compounds were removed by lyophilization to obtain crude **4b** (1.78 g, 2.61 mmol, 53% yield). The compound was purified by

preparative HPLC for analytical purposes.

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.95 (d, *J* = 8.0 Hz, 1H), 7.92 – 7.86 (m, 2H), 7.81 (t, *J* = 5.8 Hz, 3H), 7.72 (t, *J* = 7.5 Hz, 2H), 7.54 (d, *J* = 8.5 Hz, 1H), 7.42 (t, *J* = 7.5, 1.0 Hz, 2H), 7.32 (t, *J* = 7.5, 1.2 Hz, 2H), 4.37 – 4.30 (m, 1H), 4.28 (d, *J* = 6.4 Hz, 2H), 4.22 (t, *J* = 7.1 Hz, 1H), 4.14 – 4.05 (m, 1H), 3.66 – 3.59 (m, 1H), 3.46 – 3.40 (m, 1H), 2.82 – 2.71 (m, 2H), 1.73 – 1.62 (m, 1H), 1.59 – 1.49 (m, 3H), 1.39 (s, 12H), 1.10 (s, 9H).

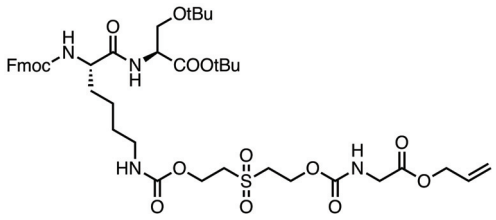
^{13}C NMR (126 MHz, DMSO) δ 171.98 (CO), 169.21 (2xCO), 158.65 (CF_3), 158.40 (CF_3), 158.14 (CF_3), 157.88 (CF_3), 156.00 (CO), 143.85 (C), 143.76 (C), 140.73 (2xC), 127.64 (2xCH), 127.07 (2xCH), 125.27 (2xCH), 120.13 (2xCH), 80.61 (C), 72.70 (C), 65.63 (CH_2), 61.73 (CH_2), 54.16 (CH), 53.18 (CH), 46.67 (CH), 38.70 (CH_2), 31.29 (CH_2), 27.64 (3xCH_3), 27.09 (3xCH_3), 26.62 (CH_2), 22.48 (CH_2).

$[\alpha]^{24}_D$ (c = 1.05, MeOH): -3.8019

IR (u/cm^{-1}, neat): 1650, 1527, 1248, 1136, 739, 722

HRMS (ESI): calculated for $C_{32}H_{46}N_3O_6$ $[M+H]^+$: 568.3381, found: 568.3379.

3.3. Synthesis of linker II. allyl ester (21)



3 (1.20 g, 2.61 mmol, 1.00 equiv) and *N*-methylmorpholine (0.57 mL, 5.21 mmol, 2.00 equiv) were dissolved in 50 mL DMF in a round-bottomed flask. Separately **4b** (1.78 g, 2.61 mmol, 1.00 equiv) was dissolved in 20 mL DMF. This solution was added dropwise to the solution of **3** and the mixture was stirred at rt. After 3 h the reaction mixture was diluted with 150 mL EtOAc and was washed with 2x150 mL 1 M $KHSO_4$ solution, 150 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 7:3 to 2:3) to give **21** as a colorless oil (1.0 g, 1.12 mmol, 43% yield).

The compound was repurified by preparative HPLC for analytical purposes.

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.92 (d, *J* = 8.0 Hz, 1H), 7.90 – 7.86 (m, 2H), 7.76 (t, *J* = 6.1 Hz, 1H), 7.74 – 7.69 (m, 2H), 7.53 (t, *J* = 9.3 Hz, 1H), 7.41 (td, *J* = 7.5, 1.0 Hz, 2H), 7.32 (td, *J* = 7.5, 1.1 Hz, 2H), 7.27 (t, *J* = 5.5 Hz, 1H), 5.96 – 5.84 (m, 1H), 5.35 – 5.18 (m, 2H), 4.58 (dt, *J* = 5.4, 1.6 Hz, 2H), 4.38 – 4.18 (m, 8H), 4.14 – 4.03 (m, 1H), 3.81 (d, *J* = 6.1 Hz, 2H), 3.65 – 3.56 (m, 1H), 3.50 (q, *J* = 6.3 Hz, 4H), 3.46 – 3.38 (m, 1H), 2.99 – 2.95 (m, 2H), 1.70 – 1.60 (m, 1H), 1.59 – 1.47 (m, 1H), 1.39 (d, *J* = 1.3 Hz, 13H), 1.09 (d, *J* = 14.1 Hz, 9H).

^{13}C NMR (126 MHz, DMSO) δ 172.07 (CO), 169.71 (CO), 169.22 (CO), 155.97 (CO), 155.87 (CO), 155.41 (CO), 143.85 (C), 143.78 (C), 140.69 (2xC), 132.33 (CH), 127.61 (2xCH), 127.06 (2xCH), 125.28 (2xCH), 120.08 (2xCH), 117.87 (CH_2), 80.57 (C), 72.68 (C), 65.63 (CH_2), 64.80 (CH_2), 61.72 (CH_2), 57.96 (CH_2), 57.34 (CH_2), 54.38 (CH), 53.21 (CH), 53.04 (2xCH_2), 46.66 (CH), 42.11 (CH_2), 40.21 (CH_2), 31.61 (CH_2), 29.12 (CH_2), 27.63 (3xCH_3), 27.09 (3xCH_3), 22.90 (CH_2).

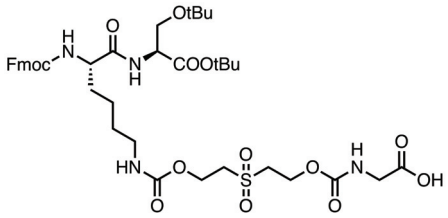
$[\alpha]^{24}_D$ (c = 0.95, $CHCl_3$): 9.2

IR (u/cm^{-1}, neat): 3332, 2974, 1710, 1525, 1159, 740

HRMS (ESI): calculated for $C_{43}H_{60}N_4Na_1O_{14}S$ $[M+Na]^+$: 911.3719, found: 911.3712.

Rf = 0.3 (hexanes: EtOAc)= 2:3)

3.4. Synthesis of linker II. (5b)



21 (1.00 g, 1.12 mmol, 1.0 equiv) was dissolved in 10 mL dry, degassed CH_2Cl_2, cooled to 0 °C. Morpholine 0.10 mL (1.24 mmol, 1.1 equiv) morpholine and tetrakis(triphenylphosphine)palladium (0.13 g, 0.11 mmol, 0.10 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt over the course of 2 h. The reaction mixture was diluted with 50 mL EtOAc, washed with 50 mL 10 w/w % aq. citric acid solution, 50 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 3:1 to 1:1 + 1% HCOOH + 1% MeOH) to give **5b** as a colorless oil (0.67 g, 0.78 mmol, 70% yield).

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.92 (d, *J* = 7.9 Hz, 1H), 7.90 – 7.86 (m, 2H), 7.76 – 7.69 (m, 2H), 7.61 (t, *J* = 6.1 Hz, 1H), 7.52 (t, *J* = 9.3 Hz, 1H), 7.45 – 7.36 (m, 2H), 7.36 – 7.30 (m, 2H), 7.30 – 7.24 (m, 1H), 4.39 – 4.17 (m, 8H), 4.13 – 4.03 (m, 1H), 3.67 (d, *J* = 6.1 Hz, 2H), 3.65 – 3.58 (m, 1H), 3.56 – 3.46 (m, 4H), 3.46 – 3.38 (m, 1H), 3.03 – 2.92 (m, 2H), 1.70 – 1.58 (m, 1H), 1.58 – 1.48 (m, 1H), 1.47 – 1.21 (m, 13H), 1.13 – 1.01 (m, 9H).

^{13}C NMR (126 MHz, DMSO) δ 172.10 (CO), 171.40 (CO), 169.24 (CO), 155.99 (CO), 155.84 (CO), 155.43 (CO), 143.86 (C), 143.80 (C), 140.72 (2xC), 127.65 (2xCH), 127.08 (2xCH), 125.30 (2xCH), 120.10 (2xCH), 80.59 (C), 72.70 (C), 65.64 (CH_2), 61.73 (CH_2), 57.87 (CH_2), 57.35 (CH_2), 54.40 (CH), 53.24 (CH), 53.18 (CH_2), 53.07 (CH_2), 46.67 (CH), 42.07 (CH_2), 40.22 (CH_2), 31.62 (CH_2), 29.14 (CH_2), 27.65 (3xCH_3), 27.10 (3xCH_3), 22.92 (CH_2).

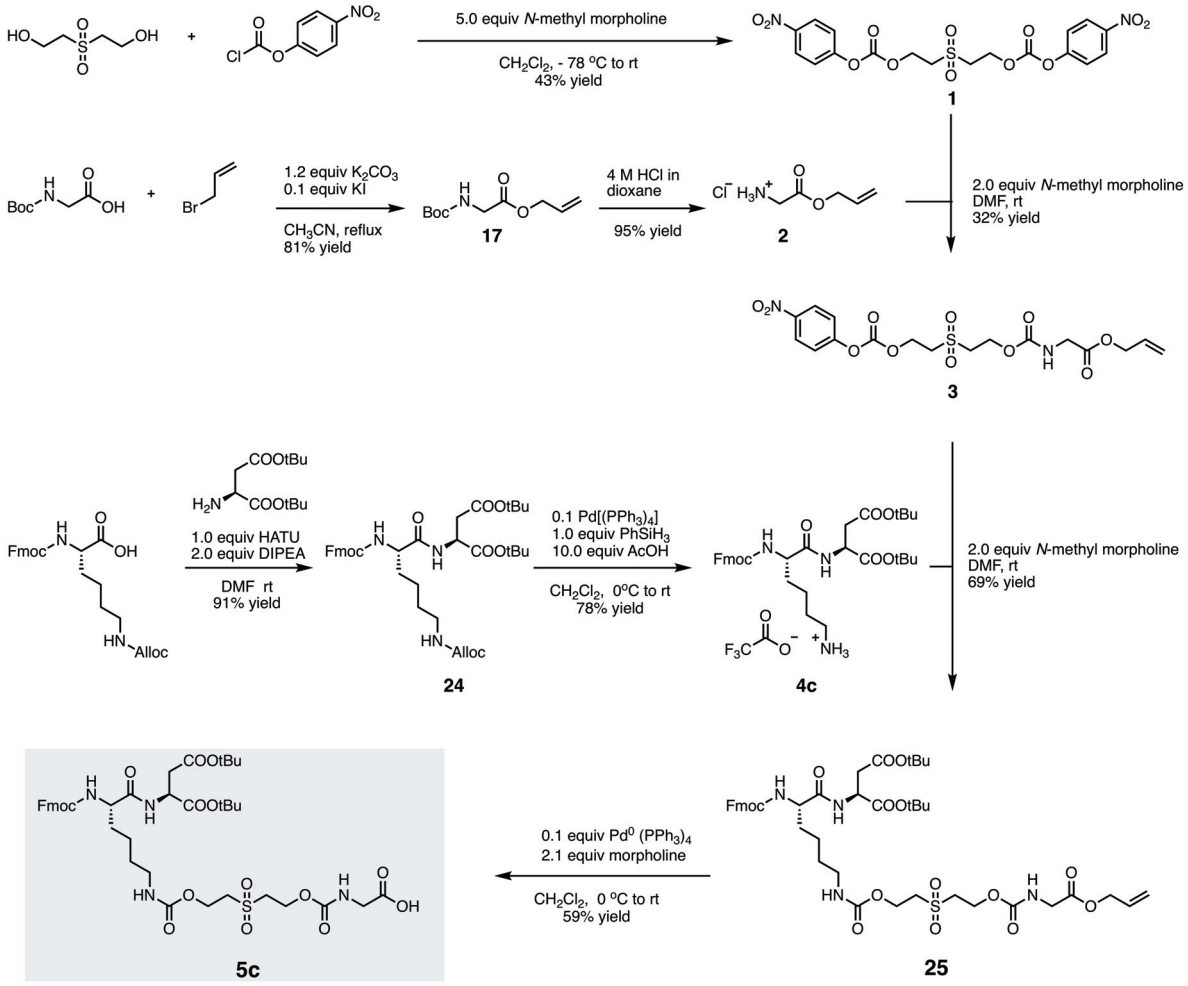
$[\alpha]^{24}_D$ (c = 0.95, $CHCl_3$): 7.9

IR (u/cm^{-1}, neat): 3676, 2973, 1706, 1527, 1250, 1124

HRMS (ESI): calculated for $C_{40}H_{56}N_4Na_1O_{14}S$ $[M+Na]^+$: 871.3406, found: 871.3407.

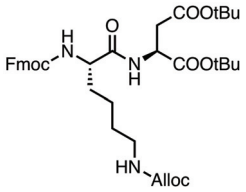
Rf = 0.3 (CH_2Cl_2:MeOH = 92:8)

4. Synthesis of linker III. for guinea pig insulin (5c)



Synthesis of linker III. for mouse insulin (**5c**)

4.1. Synthesis of Fmoc-Lys(NHAlloc)-Asp(OtBu)-OtBu (24)



Fmoc-Lys(NHAlloc)-OH (5.00 g, 11.1 mmol, 1.00 equiv) and H-Thr(tBu)-OtBu HCl (3.46 g, 12.2 mmol, 1.10 equiv) were dissolved in 45 mL DMF. HATU (4.20 g, 11.05 mmol, 1.0 equiv) and DIPEA (5.77 mL mg, 33.2 mmol, 3.00 equiv) were added. The mixture was left to stir at rt. After 2 h the mixture was diluted with 400 mL EtOAc, washed with 200 mL 1 M HCl, 200 mL saturated aq. $NaHCO_3$ solution, 200 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 3:7) to give **24** as a colorless oil (6.82 g, 10.03 mmol, 91% yield).

^{1}H NMR (600 MHz, DMSO-d6) δ 8.22 (d, J = 8.1 Hz, 1H), 7.91 – 7.86 (m, 2H), 7.72 (t, J = 7.2 Hz, 2H), 7.48 (d, J = 8.3 Hz, 1H), 7.44 – 7.38 (m, 2H), 7.36 – 7.29 (m, 2H), 7.17 (q, J = 7.8, 5.7 Hz, 1H), 6.00 – 5.81 (m, 1H), 5.29 – 5.08 (m, 2H), 4.51 – 4.42 (m, 3H), 4.28 – 4.18 (m, 3H), 4.02 – 3.95 (m, 1H), 3.02 – 2.90 (m, J = 6.5 Hz, 2H), 2.64 (dd, J = 16.3, 6.1 Hz, 1H), 2.58 – 2.51 (m, 1H), 1.67 – 1.47 (m, 2H), 1.44 – 1.21 (m, 22H).

^{13}C NMR (151 MHz, DMSO) δ 171.83 (CO), 169.62 (CO), 169.11 (CO), 155.89 (CO), 155.86 (CO), 143.87 (C), 143.74 (C), 140.68 (2xC), 133.84 (CH), 127.61 (2xCH), 127.04 (2xCH), 125.27 (2xCH), 120.09 (2xCH), 116.82 (CH_2), 80.86 (C), 80.40 (C), 65.62 (CH_2), 64.09 (CH_2), 54.38 (CH), 49.20 (CH), 46.64 (CH), 40.07 (CH_2), 37.05 (CH_2), 31.62 (CH_2), 29.14 (CH_2), 27.63 (3xCH_3), 27.50 (3xCH_3), 22.78(CH_2).

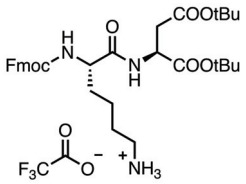
$[\alpha]^{24}_D$ (c = 0.9, CHCl3): 9.9

IR (u/cm–1, neat): 3294, 1728, 1690, 1650, 1533, 1150.

HRMS (ESI): calculated for $C_{37}H_{49}N_3Na_1O_9$ [M+Na]+: 702.3361, found: 702.3361.

Rf = 0.3 (hexanes: EtOAc = 4:1)

4.2. Synthesis of Fmoc-Lys(NH_2)-Asp(OtBu)-OtBu TFA salt (4c)



24 (6.80 g, 10.0 mmol, 1.00 equiv) was dissolved in 100 mL dry, degassed CH_2Cl_2 at 0 °C. AcOH (5.72 mL, 100.30 mmol, 10.0 equiv), phenyl silane (1.23 mL, 10.0 mmol, 1.00 equiv) and tetrakis(triphenylphosphine)palladium (1.16 g, 1.00 mmol, 0.10 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt over the course of 4 h. The solvent was removed under reduced pressure and the residue was dissolved in 300 mL EtOAc, extracted 3x300 mL 0.2 M Na_2HPO_4 / 10 w/w % aq. citric acid solution (pH 6) buffer, washed with brine, dried over Na_2SO_4 and concentrated. The residue was dissolved in 100 mL CH_3CN:H_2O +0.01 v/v% TFA solution and filtered. Volatile compounds were removed by lyophilization to obtain crude **4c** (5.50 g, 7.75 mmol, 78% yield). The compound was purified by preparative HPLC for analytical purposes.

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.25 (d, *J* = 8.1 Hz, 1H), 7.92 – 7.87 (m, 2H), 7.77 (t, *J* = 5.6 Hz, 3H), 7.74 – 7.68 (m, 2H), 7.51 (d, *J* = 8.4 Hz, 1H), 7.45 – 7.39 (m, 2H), 7.37 – 7.29 (m, 2H), 4.51 – 4.44 (m, 1H), 4.26 (d, *J* = 7.9 Hz, 2H), 4.22 (d, *J* = 6.9 Hz, 1H), 4.05 – 3.97 (m, 1H), 2.81 – 2.71 (m, 2H), 2.69 – 2.61 (m, 1H), 2.54 (dd, *J* = 16.3, 6.9 Hz, 1H), 1.69 – 1.45 (m, 4H), 1.37 (d, *J* = 4.3 Hz, 20H).

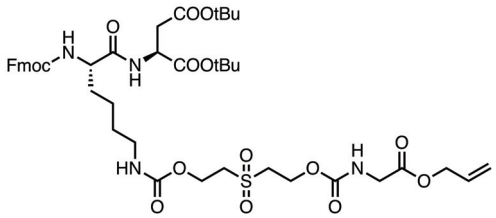
^{13}C NMR (151 MHz, DMSO) δ 171.76 (CO), 169.62 (CO), 169.15 (2xCO), 158.54 (CF_3), 158.33 (CF_3), 158.11 (CF_3), 157.89 (CF_3), 155.95 (CO), 143.88 (C), 143.73 (C), 140.72 (2xC), 127.64 (2xCH), 127.04 (2xCH), 125.30 (CH), 125.24 (CH), 120.13 (2xCH), 80.92 (C), 80.45 (C), 65.62 (CH_2), 54.19 (CH), 49.23 (CH), 46.66 (CH), 38.69 (CH_2), 37.05 (CH_2), 31.32 (CH_2), 27.64 (3xCH_3), 27.51 (3xCH_3), 26.62 (CH_2), 22.43 (CH_2).

$[\alpha]^{24}_D$ (c =0.95, MeOH): -6.2

IR (u/cm^{-1}, neat): 1666, 1520, 1201, 1143, 739, 758.

HRMS (ESI): calculated for $C_{33}H_{45}N_3Na_1O_7$ $[M+Na]^+$: 618.3150, found: 618.3151.

4.3. Synthesis of linker IV. allyl ester (25)



3 (3.57 g, 7.75 mmol, 1.00 equiv) and 1.67 mL (15.5 mmol, 2.00 equiv) *N*-methylmorpholine were dissolved in 100 mL DMF in a round-bottomed flask. Separately **4c** (5.50 g, 7.75 mmol, 1.00 equiv) was dissolved in 50 mL DMF. This solution was added dropwise to the solution of **3** and the mixture was stirred at rt. After 3 h the reaction mixture was diluted with 300 mL EtOAc and was washed with 2x300 mL 1 M $KHSO_4$ solution and with 300 mL brine, dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 4:1 to 3:7) to give **25** as a colorless oil (4.90 g, 5.34 mmol, 70% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.22 (d, *J* = 8.1 Hz, 1H), 7.89 (d, *J* = 7.6, 1.0 Hz, 2H), 7.76 (t, *J* = 6.1 Hz, 1H), 7.72 (t, *J* = 7.1 Hz, 2H), 7.48 (d, *J* = 8.3 Hz, 1H), 7.41 (t, *J* = 7.5, 1.1 Hz, 2H), 7.33 (t, *J* = 7.5, 1.4 Hz, 2H), 7.27 (t, *J* = 5.7 Hz, 1H), 5.95 – 5.85 (m, 1H), 5.34 – 5.18 (m, 2H), 4.59 (dt, *J* = 5.4, 1.5 Hz, 2H), 4.51 – 4.44 (m, 1H), 4.35 – 4.15 (m, 7H), 4.03 – 3.96 (m, 1H), 3.81 (d, *J* = 6.1 Hz, 2H), 3.58 – 3.39 (m, 4H), 3.02 – 2.92 (m, 2H), 2.68 – 2.60 (m, 1H), 2.58 – 2.51 (m, 1H), 1.68 – 1.49 (m, 2H), 1.46 – 1.22 (m, 22H).

^{13}C NMR (151 MHz, DMSO) δ 171.84 (CO), 169.71(CO), 169.63(CO), 169.12 (CO), 155.91 (CO), 155.87 (CO), 155.41 (CO), 143.88 (C), 143.75 (C), 140.69 (2xC), 132.33 (CH), 127.63 (2xCH), 127.06 (2xCH), 125.28 (2xCH), 120.10 (2xCH), 117.87 (CH_2), 80.88 (C), 80.42 (C), 65.63 (CH_2), 64.81 (CH_2), 57.96 (CH_2), 57.34 (CH_2), 54.39 (CH), 53.21 (CH_2), 53.03 (CH_2), 49.21 (CH), 46.65 (CH), 42.10 (CH_2), 40.17 (CH_2), 37.06 (CH_2), 31.63 (CH_2), 29.09 (CH_2), 27.64 (3xCH_3), 27.51 (3xCH_3), 22.83(CH_2).

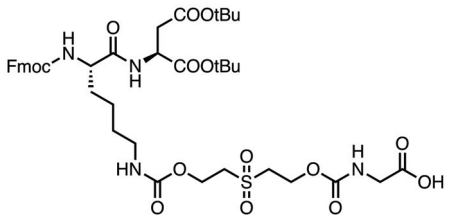
$[\alpha]^{24}_D$ (c =0.95, $CHCl_3$): 6.3

IR (u/cm^{-1}, neat): 2974, 2902, 1708, 1524, 1249, 1066, 1057.

HRMS (ESI): calculated for $C_{44}H_{60}N_4Na_1O_{15}S_1$ $[M+Na]^+$: 939.3668, found: 939.3668.

Rf = 0.3 (hexanes: EtOAc = 3:7)

4.4. Synthesis of cleavable linker IV. (5c)



25 (4.60 g, 5.02 mmol, 1.00 equiv) was dissolved in 50 mL dry, degassed CH_2Cl_2, cooled to 0 °C. Morpholine (0.44 mL, 5.52 mmol, 1.10 equiv) and tetrakis(triphenylphosphine)palladium (0.58 g, 0.50 mmol, 0.10 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt in the course of 2 h. The reaction mixture was diluted with 100 mL EtOAc, washed with 100 mL 10 w/w % aq. citric acid solution, 100 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (CH_2Cl_2:MeOH:HCOOH = 95:5:0.01) to give **5c** as a colorless oil (2.60 g, 2.96 mmol, 59% yield). The compound was repurified by preparative HPLC for analytical purposes.

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.22 (d, *J* = 8.1 Hz, 1H), 7.89 (d, *J* = 7.6, 1.0 Hz, 2H), 7.72 (t, *J* = 7.0 Hz, 2H), 7.61 (t, *J* = 6.2 Hz, 1H), 7.48 (d, *J* = 8.3 Hz, 1H), 7.41 (q, *J* = 7.5, 1.1 Hz, 2H), 7.33 (t, *J* = 7.3, 1.3 Hz, 2H), 7.27 (t, *J* = 5.8 Hz, 1H), 4.51 – 4.44 (m, 1H), 4.39 – 4.15 (m, 7H), 3.99 (s, 1H), 3.67 (d, *J* = 6.1 Hz, 2H), 3.53 – 3.46 (m, 4H), 3.03 – 2.91 (m, 2H), 2.68 – 2.52 (m, 2H), 1.68 – 1.47 (m, 2H), 1.47 – 1.21 (m, 22H).

^{13}C NMR (151 MHz, DMSO) δ 171.86 (CO), 171.40 (CO), 169.65 (CO), 169.14 (CO), 155.93 (CO), 155.84 (CO), 155.43 (CO), 143.89 (C), 143.77 (C), 140.71 (2xC), 127.65 (2xCH), 127.07 (2xCH), 125.30 (2xCH), 120.10 (2xCH), 80.90 (C), 80.44 (C), 65.64 (CH_2), 57.88 (CH_2), 57.36 (CH_2), 54.41 (CH), 53.24 (CH_2), 53.07 (CH_2), 49.22 (CH), 46.67 (CH), 42.07 (CH_2), 40.19 (CH_2), 37.07 (CH_2), 31.64 (CH_2), 29.11 (CH_2), 27.65 (3xCH_3), 27.53 (3xCH_3), 22.85 (CH_2).

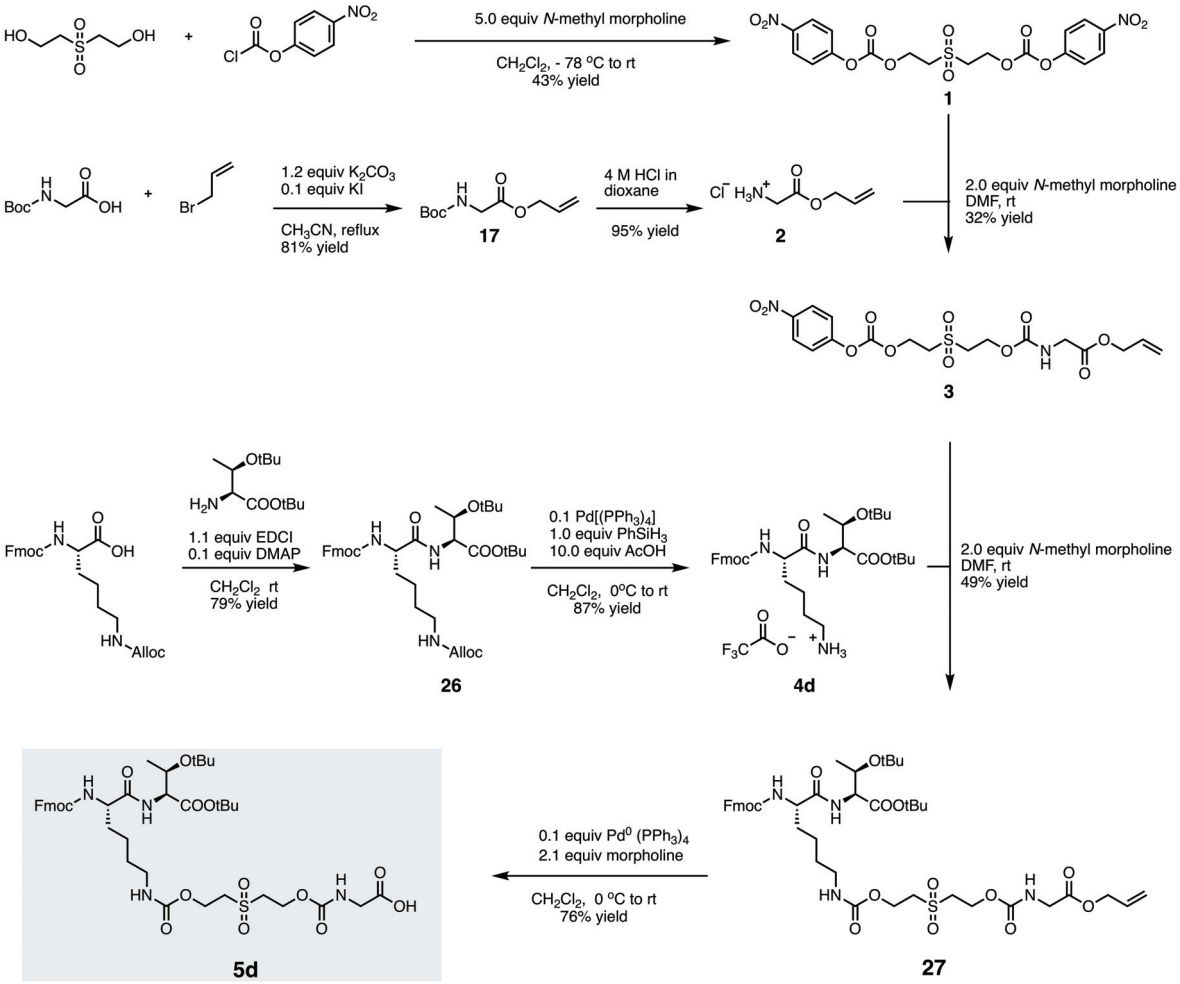
$[\alpha]^{24}_D$ (c =2.2, $CHCl_3$): 6.9

IR (u/cm^{-1}, neat): 1706, 1523, 1248, 1150, 1123, 740.

HRMS (ESI): calculated for $C_{41}H_{56}N_4Na_1O_{15}S_1$ $[M+Na]^+$: 899.3355, found: 899.3351.

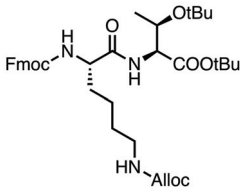
Rf = 0.3 (CH_2Cl_2:MeOH:HCOOH = 95:5:0.01)

5. Synthesis of linker IV. for human insulin (5d)



Synthesis of linker IV. (**5d**)

5.1. Synthesis of Fmoc-Lys(NHAlloc)-Thr(tBu)-OtBu (26)



Fmoc-Lys(NHAlloc)-OH (10.0 g, 22.1 mmol, 1.00 equiv) and H-Thr(tBu)-OtBu (4.60 g, 19.9 mmol, 0.90 equiv) were dissolved in 200 mL CH_2Cl_2. EDCI (4.24 g, 22.1 mmol, 1.00 equiv) EDCI and DMAP (270 mg 2.21 mmol, 0.1 equiv) were added. The mixture was left to stir at rt. After 3 h the mixture was diluted with 400 mL diethyl ether, washed with 300 mL 1 M HCl, 300 mL saturated aq. $NaHCO_3$ solution, 300 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 65:35) to give **26** as a colorless oil (10.5 g, 15.8 mmol, 79% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.91 – 7.86 (m, 2H), 7.75 – 7.69 (m, 2H), 7.60 (d, *J* = 8.8 Hz, 1H), 7.56 (d, *J* = 8.3 Hz, 1H), 7.44 – 7.38 (m, 2H), 7.36 – 7.29 (m, 2H), 7.17 (t, *J* = 5.7 Hz, 1H), 5.94 – 5.84 (m, 1H), 5.29 – 5.22 (m, 1H), 5.18 – 5.12 (m, 1H), 4.44 (dt, *J* = 5.3, 1.6 Hz, 2H), 4.33 – 4.16 (m, 4H), 4.15 – 4.02 (m, 2H), 3.01 – 2.90 (m, *J* = 6.6 Hz, 2H), 1.71 – 1.47 (m, 2H), 1.43 – 1.19 (m, 13H), 1.11 (s, 9H), 1.04 (d, *J* = 6.3 Hz, 3H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 172.35 (CO), 169.33 (CO), 155.97 (CO), 155.85 (CO), 143.84 (C), 143.75 (C), 140.69 (2xC), 133.85 (CH), 127.62 (2xCH), 127.04 (2xCH), 125.25 (2xCH), 120.10 (2xCH), 116.83 (CH_2), 80.65 (C), 73.24 (C), 66.88 (CH), 65.61 (CH_2), 64.09 (CH_2), 57.91 (CH), 54.46 (CH), 46.65 (CH), 40.09 (CH_2), 31.40 (CH_2), 29.15 (CH_2), 28.39 (3xCH_3), 27.68 (3xCH_3), 22.87 (CH_2), 19.90 (CH_3).

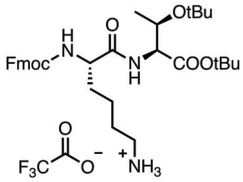
$[\alpha]^{25}_D$ (c = 0.9, $CHCl_3$): +2.3

IR (cm^{-1}, neat): 3316, 2976, 1704, 1661, 1520, 1246, 1144, 739.

HRMS (ESI): calculated for $C_{37}H_{51}N_3O_8Na$ $[M+Na]^+$: 688.35684, found: 688.35647.

Rf = 0.4 (hexanes: EtOAc = 6:4)

5.2. Synthesis of Fmoc-Lys(NH_2)-Thr(tBu)-OtBu TFA salt (4d)



26 (10.5 g, 15.77 mmol, 1.00 equiv) was dissolved in 150 mL dry, degassed CH_2Cl_2 at 0 °C. AcOH 9.02 mL, 157 mmol, 10.0 equiv), phenyl silane (1.94 mL, 15.8 mmol, 1.00 equiv) phenylsilane and tetrakis(triphenylphosphine)palladium (1.82 g, 1.58 mmol, 0.10 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt in the course of 4 h. The solvent was removed under reduced pressure and the residue was dissolved in 300 mL EtOAc, extracted 3x300 mL 0.2 M Na_2HPO_4 / 10 w/w % aq. citric acid solution (pH 6) buffer, washed with brine, dried over Na_2SO_4 and concentrated. The residue was dissolved in 100 mL CH_3CN:H_2O +0.01 v/v% TFA solution and filtered. Volatile compounds were removed by lyophilization to obtain crude **4d** (9.55 g, 13.7 mmol, 87% yield). The compound was purified by preparative HPLC for analytical purposes.

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.90 (d, *J* = 7.5 Hz, 2H), 7.71 (t, *J* = 8.1 Hz, 2H), 7.66 (s, 3H), 7.59 (d, *J* = 8.6 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 4.30 (d, *J* = 7.5 Hz, 2H), 4.26 – 4.18 (m, 2H), 4.14 (td, *J* = 9.2, 4.4 Hz, 1H), 4.11 – 4.05 (m, 1H), 2.82 – 2.71 (m, 2H), 1.73 – 1.64 (m, 2H), 1.59 – 1.50 (m, 2H), 1.41 – 1.31 (m, 11H), 1.11 (s, 9H), 1.07 – 1.02 (m, 3H).

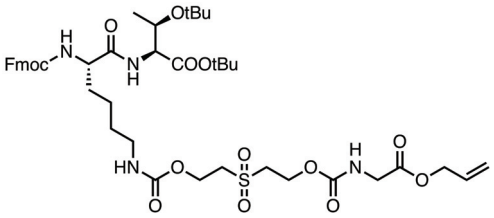
^{13}C NMR (151 MHz, DMSO-d_6) δ 172.26 (CO), 169.30 (2xCO), 157.9 (CF_3), 156.02 (CO), 143.83 (2xC), 140.71 (2xC), 127.64 (2xCH), 127.05 (2xCH), 125.22 (2xCH), 120.14 (2xCH), 80.71 (C), 73.27 (C), 66.87 (CH_2), 65.61 (CH), 57.92 (CH), 54.21 (CH), 46.66 (CH), 38.72 (CH_2), 31.05 (CH_2), 28.40 (3xCH_3), 27.69 (3xCH_3), 26.60 (CH_2), 22.48 (CH_2), 19.97 (CH_3).

IR (cm^{-1}, neat): 2977, 1668, 1520, 1138, 1080, 739.

HRMS (ESI): calculated for $C_{33}H_{47}N_3O_6Na$ $[M+Na]^+$: 604.33571, found: 604.33504.

$[\alpha]^{26}_D$ (c = 0.9, MeOH): -7.0

5.3. Synthesis of linker IV. allyl ester (27)



3 (2.30 g, 5.00 mmol, 1.00 equiv) and *N*-methylmorpholine (1.14 mL, 10.0 mmol, 2.00 equiv) were dissolved in 100 mL DMF in a round-bottomed flask. Separately **4d** (3.48 g, 5.00 mmol, 1.00 equiv) was dissolved in 50 mL DMF. This solution was added dropwise to the solution of **3** and the mixture was stirred at rt. After 3 h the reaction mixture was diluted with 300 mL EtOAc and was washed with 2x300 mL 1 M $KHSO_4$ solution and with 300 mL brine, dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 7:3 to 2:3) to give **27** as a colorless oil (2.20 g, 2.45 mmol, 49% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.91 – 7.86 (m, 2H), 7.76 (t, *J* = 6.1 Hz, 1H), 7.74 – 7.69 (m, 2H), 7.58 (dd, *J* = 14.8, 8.5 Hz, 2H), 7.44 – 7.38 (m, 2H), 7.35 – 7.29 (m, 2H), 7.27 (t, *J* = 5.8 Hz, 1H), 5.94 – 5.85 (m, 1H), 5.34 – 5.27 (m, 1H), 5.24 – 5.18 (m, 1H), 4.61 – 4.56 (m, 2H), 4.34 – 4.17 (m, 8H), 4.15 – 4.09 (m, 1H), 4.09 – 4.03 (m, 1H), 3.81 (d, *J* = 6.1 Hz, 2H), 3.53 – 3.46 (m, 4H), 3.02 – 2.90 (m, *J* = 6.7 Hz, 2H), 1.71 – 1.48 (m, 2H), 1.46 – 1.20 (m, 13H), 1.11 (s, 9H), 1.04 (d, *J* = 6.2 Hz, 3H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 172.34 (CO), 169.70 (CO), 169.33 (CO), 155.98 (CO), 155.85 (CO), 155.39 (CO), 143.84 (C), 143.75 (C), 140.69 (2xC), 132.33 (CH), 127.60 (2xCH), 127.04 (2xCH), 125.25 (2xCH), 120.10 (2xCH), 117.86 (CH_2), 80.66 (C), 73.24 (C), 66.88 (CH), 65.61 (CH_2), 64.79 (CH_2), 57.91(CH, CH_2), 57.32 (CH_2), 54.46 (CH), 53.19 (CH_2), 53.01 CH_2), 46.65 (CH), 42.09 (CH_2), 40.19 (CH_2), 31.39 (CH_2), 29.10 (CH_2), 28.39 (3xCH_3), 27.68 (3xCH_3), 22.92 (CH_2), 19.91 (CH_3)

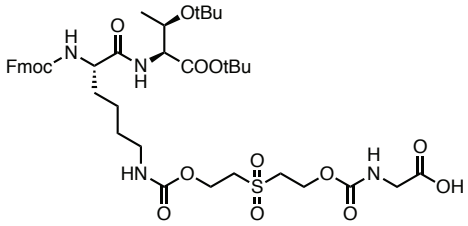
$[\alpha]^{26}_D$ (c = 0.6, $CHCl_3$): -0.5

IR (cm^{-1}, neat): 3335, 2976, 1710, 1524, 1161, 1123, 740.

HRMS (ESI): calculated for $C_{44}H_{62}N_4O_{14}SNa$ $[M+Na]^+$: 925.38754, found: 925.38662.

Rf = 0.8 (hexanes: EtOAc = 1:2)

5.4. Synthesis of cleavable linker IV. (5d)



27 (5.40 g, 5.98 mmol, 1.00 equiv) was dissolved in 50 mL dry, degassed CH_2Cl_2, cooled to 0 °C. Morpholine (0.52 mL, 6.58 mmol, 1.10 equiv) and tetrakis(triphenylphosphine)palladium (0.35 g, 0.30 mmol, 0.05 equiv) were added and the mixture was stirred under N_2 and left to warm up to rt in the course of 2 h. The reaction mixture was diluted with 100 mL EtOAc, washed with 100 mL 10 w/w % aq. citric acid solution, 100 mL brine and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by flash column chromatography (hexanes: EtOAc = 3:1 to 1:1 + 1% HCOOH + 1% MeOH) to give **5d** as a colorless oil (3.92 g, 4.55 mmol, 76% yield).

^{1}H NMR (600 MHz, DMSO-d_6) δ 7.91 – 7.86 (m, 2H), 7.74 – 7.69 (m, 2H), 7.63 – 7.54 (m, 3H), 7.45 – 7.38 (m, 2H), 7.35 – 7.30 (m, 2H), 7.27 (t, *J* = 5.7 Hz, 1H), 4.35 – 4.25 (m, 6H), 4.25 – 4.16 (m, 2H), 4.15 – 4.03 (m, 2H), 3.67 (d, *J* = 6.1 Hz, 2H), 3.50 (q, *J* = 5.5 Hz, 4H), 3.01 – 2.90 (m, *J* = 6.6 Hz, 2H), 1.71 – 1.48 (m, 2H), 1.46 – 1.19 (m, 13H), 1.11 (s, 9H), 1.04 (d, *J* = 6.2 Hz, 3H).

^{13}C NMR (151 MHz, DMSO-d_6) δ 172.35 (CO), 171.37 (CO), 169.33 (CO), 155.98 (CO), 155.81 (CO), 155.40 (CO), 143.84 (C), 143.76 (C), 140.69 (2xC), 127.63 (2xCH), 127.05 (2xCH), 125.25 (2xCH), 120.08 (2xCH), 80.66 (C), 73.25 (C), 66.88 (CH), 65.61 (CH_2), 57.92 (CH), 57.84 (CH_2), 57.32 (CH_2), 54.46 (CH), 53.20 (CH_2), 53.04 (CH_2), 46.65 (CH), 42.05 (CH_2), 40.20 (CH_2), 31.40 (CH_2), 29.11 (CH_2), 28.39 (3xCH_3), 27.68(3xCH_3), 22.92 (CH_2), 19.91 (CH_2).

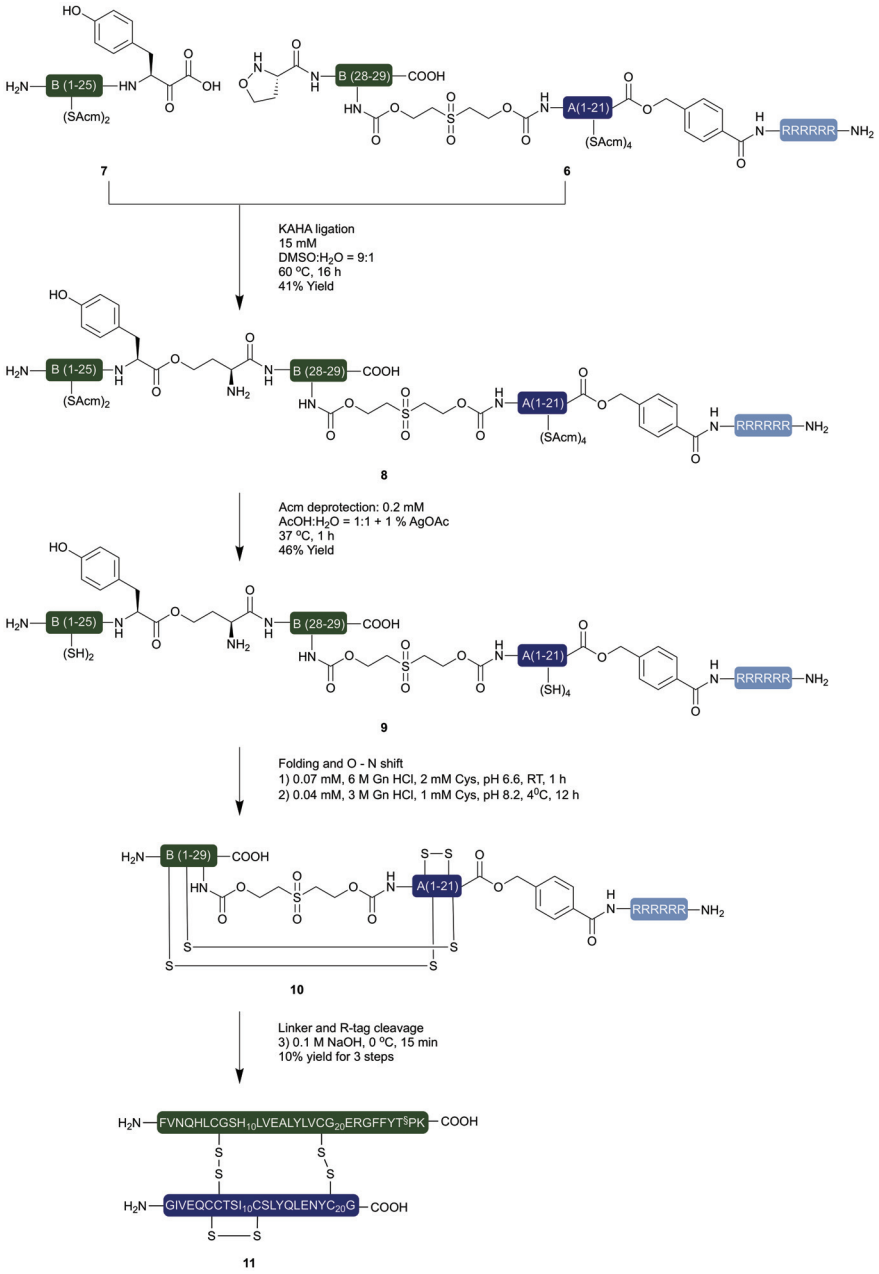
$[\alpha]^{26}_D$ (c = 0.9, $CHCl_3$): -1.8

IR (u/cm^{-1}, neat): 3333, 2977, 1706, 1525, 1160, 1123, 1077.

HRMS (ESI): calculated for $C_{41}H_{57}N_4O_{14}SNa_2$ $[M+Na]^+$: 907.33819, found: 907.33719.

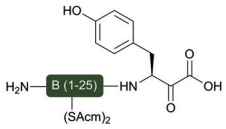
Rf = 0.3 (CH_2Cl_2:MeOH = 92:8)

6. M2 insulin



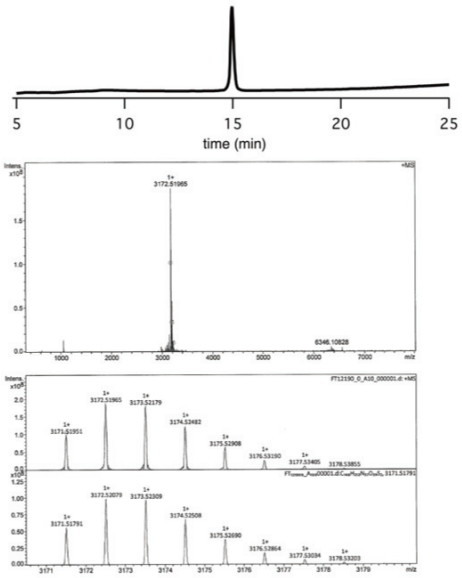
Synthesis of M2 insulin (**11**)
Mutations Thr^{B27} to Hse^{B27}

6.1. Synthesis of segment 7



α–Ketoacid segment **7** was synthesized on Rink-Amide ChemMatrix resin preloaded with Fmoc-Tyr-α-ketoacid with a loading of 0.16 mmol/g. After capping (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.80 mmol scale (5.10 g of resin) by automated Fmoc SPPS. Each amino acid (4.00 equiv) with the exception of Fmoc-Cys(Acm)-OH residues were coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.00 equiv) for 45 minutes. Fmoc-Cys(Acm)-OH (8.00 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O for 2 h at rt. 10.0 mL cleavage cocktail was used for 1.00 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged (4000 rpm for 5 min). The supernatant was discarded and the precipite was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum. Purification of crude segment **7** was performed by preparative HPLC using heated Shiseido Capcell Pak C18 column (50 x 250 mm) with a gradient of 20 to 80% CH_3CN with 0.1% TFA in 40 min. The pure product fractions were pooled and lyophilized to obtain **7** (274 mg, 86.4 μmol, 10.8% yield for peptide synthesis, resin cleavage and purification steps).

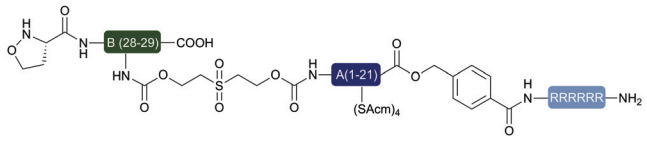
Analytical HPLC and HR-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{148}H_{212}N_{37}O_{39}S_2$ $[M+H]^+$: 3171.51791; 3171.51951 measured.



Top: **Analytical HPLC** trace of purified **7** (heated Shiseido Capcell Pak C18 analytical column,10 to 95% CH3CN with 0.1% TFA in 20 min).

Bottom: **MALDI FTMS** Measured and calculated isotopic pattern of **7**

6.2. Synthesis of segment 6

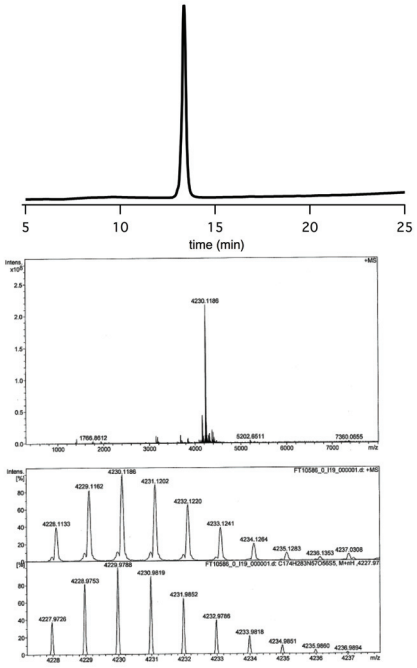


(*S*)-5-Oxaproline segment **6** was synthesized on Rink-Amide ChemMatrix resin prelaoded with Fmoc-Arg with a loading of 0.14 mmol/g. After capping (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.5 mmol scale (5.00 g of resin, 1.00 equiv) by automated Fmoc SPPS. Each aminoacid was coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.00 equiv) for 45 minutes unless otherwise specified. Fmoc-Cys(Acm)-OH (8.00 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. (4.00 equiv) 4-(Hydroxymethyl)benzoic acid was coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) for 45 minutes. Fmoc-Gly-OH was coupled with DIC (8.00 equiv), HOBt (8.00 equiv) and DMAP (0.10 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. Residues Thr^{A7} and Ser^{A8} were coupled as a pseudoproline dipeptide (3.00 equiv) HATU (3.00 equiv) *N*-methylmorpholine (6.00 equiv) for 2 h. Linker **5a** (2.00 equiv) was coupled with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h. After the linker coupling capping (acetic anhydride, *N*-methylmorpholine) was performed. For Boc-Opr-OH (2.00 equiv) double coupling was performed with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h.

The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O) for 2 h at RT. 10.0 mL cleavage cocktail was used for 1.00 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged down (4000 rpm for 5 min). The supernatant was discarded the precipitation was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum.

Purification of crude **6** was performed by preparative HPLC using Shiseido Capcell Pak Proteonavi column (50 x 250 mm) with a gradient of 20 to 80% CH_3CN with 0.1% TFA in 30 min. The fractions containing the pure product were pooled and lyophilized to obtain **6** (320 mg, 76.7 μmol, 10.8% yield for peptide synthesis, resin cleavage and purification steps).

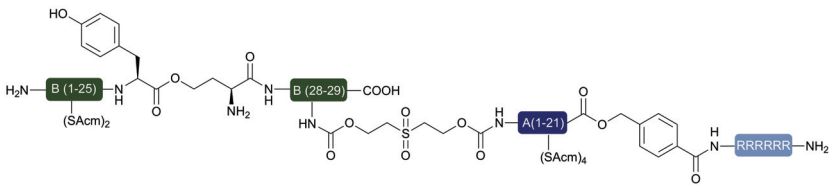
Analytical HPLC and HR-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{174}H_{283}N_{57}O_{56}S_5$ $[M+H]^+$:4227.9726; 4228.1133 measured.



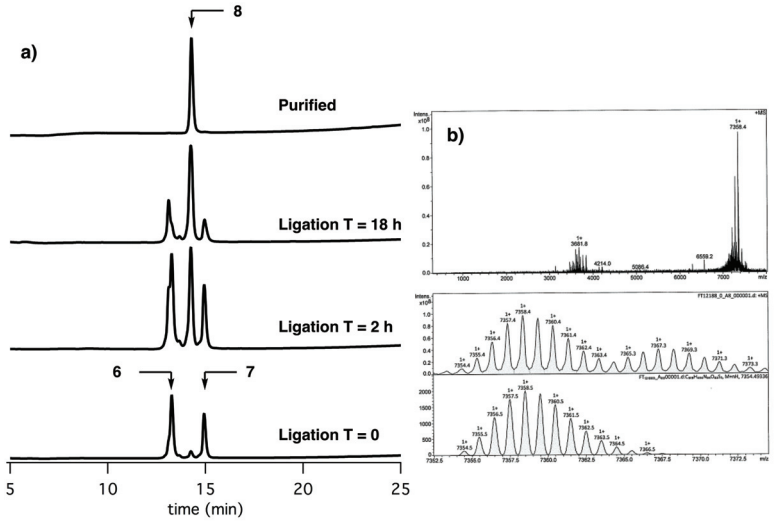
Top: **Analytical HPLC** trace of purified product **6** (heated Shiseido Capcell Pak C18 analytical column,10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **MALDI FTMS** Measured and calculated isotopic pattern of **6**

6.3. Synthesis of Acm protected linear insulin by KAHA ligation (8)



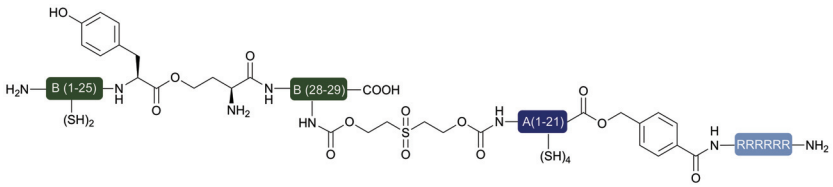
α–Ketoacid segment **7** (95.0 mg, 30.0 μmol, 0.70 equiv) and (*S*)-5-Oxaproline segment **6** (180 mg, 43.0 μmol, 1.00 equiv) were dissolved in DMSO:H_2O = 9:1, with 0.1 M oxalic acid (2.84 mL, 15 mM) and shaked at 60 ºC. The progress of the ligation was monitored by analytical HPLC using a heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min. An aliquot of the ligation mixture (0.1 μL) was taken at various time point, diluted to 12 μL with CH_3CN:H_2O = 1:1 and injected on HPLC. The reaction mixture was diluted to 10 mL with DMSO and purified by heated preparative HPLC using a Shiseido Capcell Pak C18 column (20 x 250 mm), heated at 60 ºC, with a gradient of 10 to 95% CH_3CN with 0.1% TFA in 34 min. The fractions containing the ligated product were pooled and lyophilized to give pure single chain insulin (Acm_6) **8** (95.0 mg, 12.2 μmol, 41% yield). Analytical HPLC and HR-MS confirmed the purity and identity of **8**. m/z calculated for $C_{319}H_{495}N_{94}O_{93}S_7$ measured for $[M+H]^+$:7354.5 and 7354.4 measured.



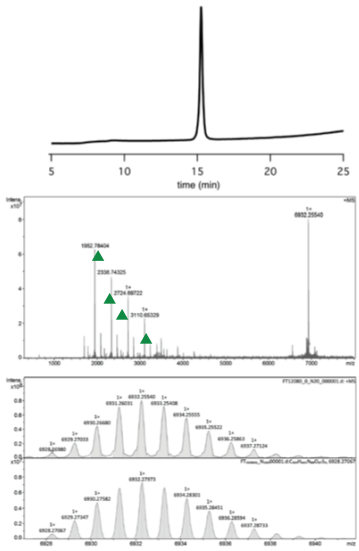
a): **Analytical HPLC** trace the ligation reaction and of the purified product **8** (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

b): **MALDI FTMS** Measured and calculated isotopic pattern of product **8**

6.4. Acm deprotection of linear insulin (9)



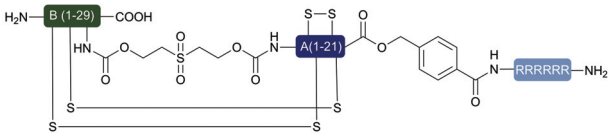
Acm protected linear insulin **8** (78.0 mg, 10.6 μmol, 1.00 equiv) was dissolved in a 50% aq. solution of AcOH (35.3 mL, 0.3 mM) containing 353 mg (2.10 mmol) 1% (m/m) AgOAc, then the mixture was agitated for 1 h at 40 °C in the dark. The reaction was quenched by the addition of DTT (489 mg, 3.20 mmol), which was dissolved in 3 mL 50% aq. solution of AcOH and added to the reaction mixture at rt; yellow precipitated formed immediately. The mixture was agitated for 15 min at rt. The formed precipitation was separated by centrifugation. The supernatant was withdrawn and the precipitate was washed with 2x5 mL 50% aq. solution of AcOH. 50.0 mg (0.18 mmol, 15.0 equiv) TCEP HCl was added to the solution and incubated for 15 min at rt. The compound was purified by preparative HPLC using a heated Phenomenex Jupiter (20 mm x 25 mm) column with a gradient of 10 to 80% CH_3CN with 0.1% TFA in 40 min. The fractions containing the reduced product were pooled and lyophilized to give pure **9** (45.0 mg, 6.49 μmol, 58% yield). Analytical HPLC and HR-MS confirmed the purity and identity of the product. m/z calculated for $C_{301}H_{464}N_{88}O_{87}S_7$ $[M+H]^+$: 6928.27067 and measured for 6928.26980.



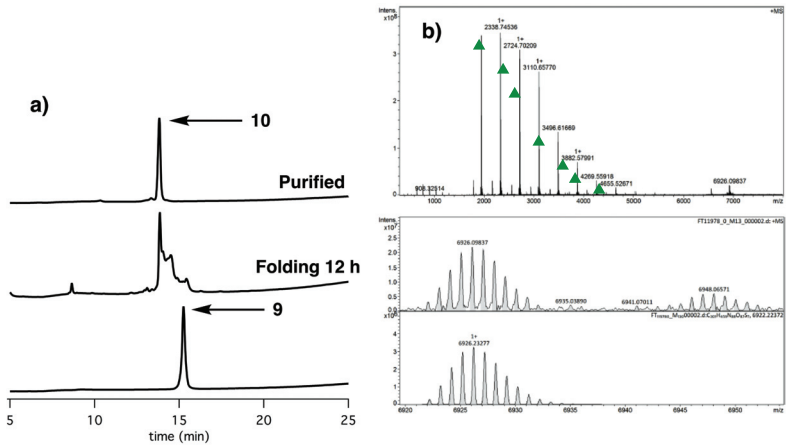
Top: **Analytical HPLC** trace of the purified product **9** (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **MALDI FTMS** Measured and calculated isotopic pattern of product **9** Green triangles highlight the calibration peaks

6.5. Folding of linear insulin (10)



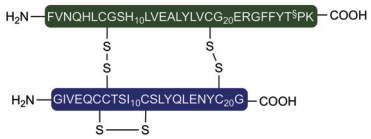
Reduced linear insulin **9** (8.0 mg, 1.2 μmol, 1.0 equiv) was dissolved in 16.0 mL freshly prepared folding buffer (6 M Gn HCl, 0.3 M Tris, 2mM Cystein hydrochloride, pH 6.6) in a 50 mL Falcon tube and was vigorously stirred open to air at rt. After 1 h 16.0 mL Millipore H_2O was added and the pH was set to 8.2 with 1 M NaOH solution. The reaction vessel was closed and kept at 4 °C overnight. After 12 h the reaction was left to warm to rt and was incubated at rt. After 4 h 3.0 mL 50% aq. solution of AcOH was added and the product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column. The fractions containing the folded product were pooled and lyophilized to give pure **10**. Analytical HPLC and HR-MS confirmed the purity and identity of the product. m/z calculated for $C_{301}H_{459}N_{88}O_{87}S_7$ $[M+H]^+$: 6926.23277 and measured for 6926.09837.



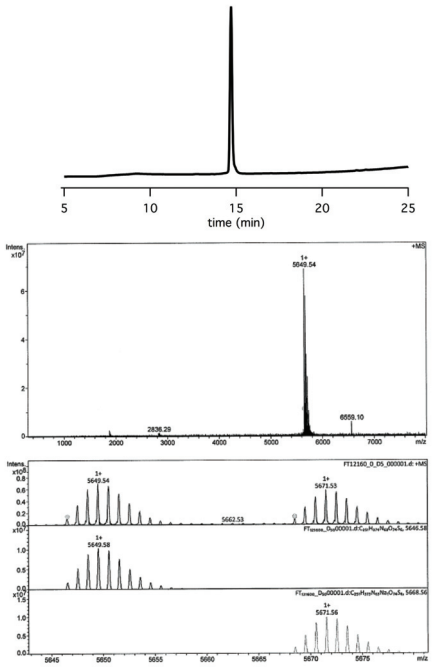
a) Analytical HPLC trace of following the folding reaction and of the purified product **10** (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

b) MALDI FTMS Measured and calculated isotopic pattern of product **10**. Green triangles highlight the calibration peaks

6.6. Cleavage of the Arg-tag and the linker (11)



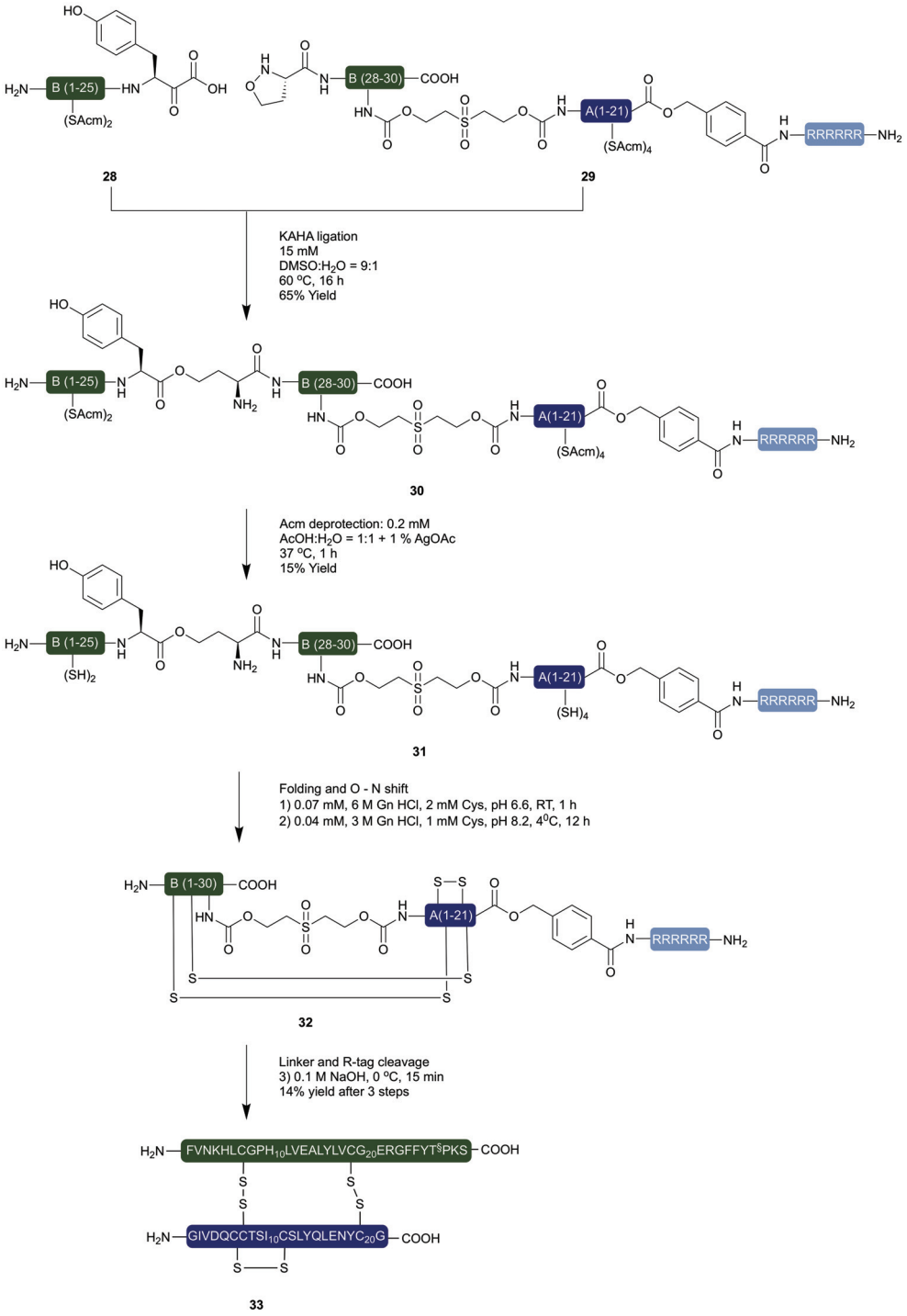
Lyophilized **10** was treated with 10 mL 0.1 M NaOH solution at 0 °C for 10 min. The reaction was quenched by the addition of 2 mL 50% aq. solution of AcOH solution and the final product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column to give pure **11** (0.7 mg, 0.12 μmol, 10% yield calculated from the folding precursor). Analytical HPLC and HR-MS were used to confirm the purity and identity of the product. m/z calculated for $C_{251}H_{374}N_{63}O_{74}S_6$ $[M+H]^+$: 5649.58 and measured for 5649.54.



Top: **Analytical HPLC** trace of the purified product **11** (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **MALDI FTMS** Measured and calculated isotopic pattern of product **11**

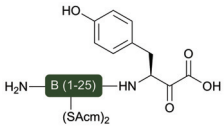
7. Mouse insulin



Synthesis of mouse insulin

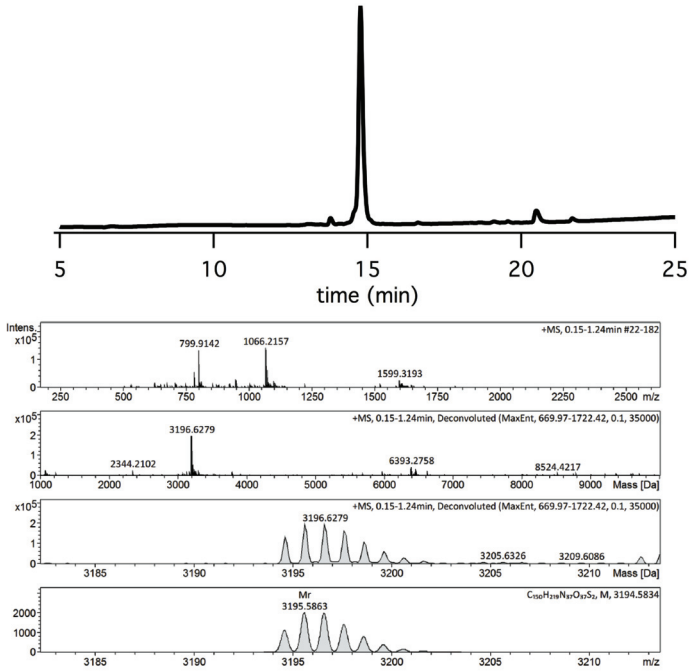
Mutations Asn^{A21} to Gly^{A21}, Thr^{B27} to Hse^{B27}

7.1. Synthesis of segment 28



α–Ketoacid segment **28** was synthesized on Rink-Amide ChemMatrix resin preloaded with Fmoc-Tyr-α-ketoacid with a loading of 0.2 mmol/g. After capping (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.40 mmol scale (2.00 g of resin) by automated Fmoc SPPS. Each amino acid (4.00 equiv) with the exception of Fmoc-Cys(Acm)-OH residues were coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.00 equiv) for 45 minutes. Fmoc-Cys(Acm)-OH (8.00 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O for 2 h at rt. 10.0 mL cleavage cocktail was used for 1.00 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged down (4000 rpm for 5 min). The supernatant was discarded the precipitation was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum. Purification of crude peptide **28** was performed by preparative HPLC using heated Shiseido Capcell Pak C18 column (50 x 250 mm) with a gradient of 20 to 80% CH_3CN with 0.1% TFA in 40 min. The pure product fractions were pooled and lyophilized to obtain **28** (235 mg, 73.5 μmol, 18 % yield for peptide synthesis, resin cleavage and purification steps).

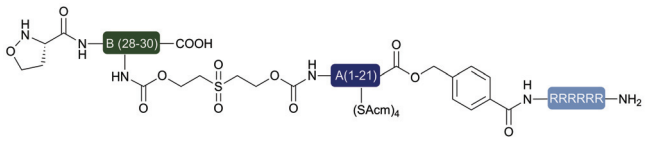
Analytical HPLC and ESI-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{150}H_{219}N_{37}O_{37}S_2$ $[M+H]^+$: 3194.5834; 3194.6277 measured.



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** trace of the purified product. Measured and calculated isotopic pattern of product **28**

7.2. Synthesis of segment 29



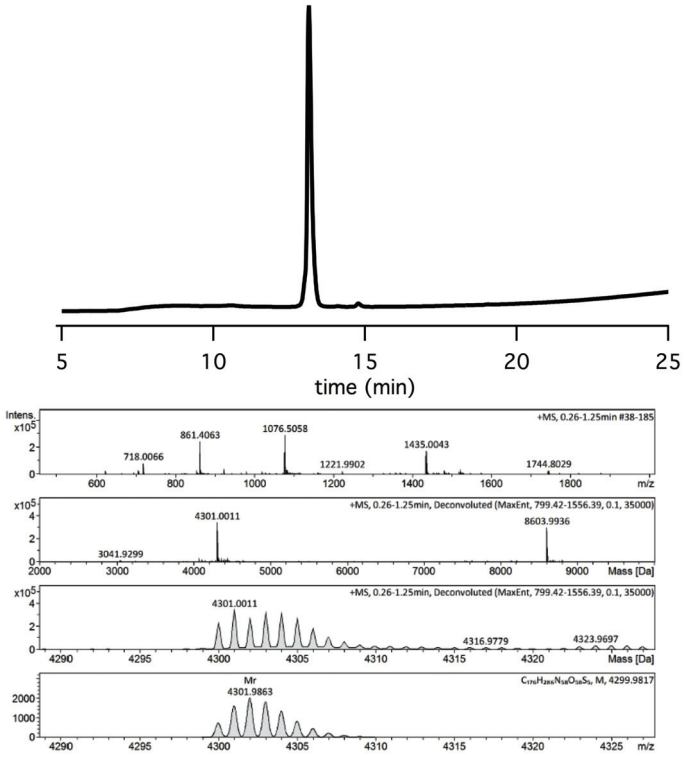
(*S*)-5-Oxaproline segment **29** was synthesized on Rink-Amide ChemMatrix resin prelaoded with Fmoc-Arg with a loading of 0.2 mmol/g. After capping (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.20 mmol scale (1.00 g of resin, 1.00 equiv) by automated Fmoc SPPS. Each aminoacid was coupled with HATU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.00 equiv) for 45 minutes unless otherwise specified. Fmoc-Cys(AcM)-OH (8.00 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. (4.0 equiv) 4-(Hydroxymethyl)benzoic acid was coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.0 equiv) for 45 minutes. Fmoc-Gly-OH was coupled with DIC (8.00 equiv), HOBt (8.00 equiv) and DMAP (0.10 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. Residues Thr^{A7} and Ser^{A8} were coupled as a pseudoproline dipeptide (3.00 equiv) HATU (3 equiv) *N*-methylmorpholine (6.00 equiv) for 2 h. Linker (**5b**) (2.00 equiv) was coupled with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h. After the linker coupling capping (Ac_2O, *N*-methylmorpholine) was performed. For Boc-Opr-OH (2.00 equiv) double coupling was performed with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h.

The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O) for 2 h at RT. 10.0 mL cleavage cocktail was used for 1.0 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was

precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged down (4000 rpm for 5 min). The supernatant was discarded the precipitation was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum.

Purification of crude **29** was performed by preparative HPLC using Shiseido Capcell Pak Proteonavi column (50 x 250 mm) with a gradient of 20 to 80% CH_3CN with 0.1% TFA in 30 min. The pure product fractions were pooled and lyophilized to obtain **29** (135 mg, 31.4 μmol) 16% yield for peptide synthesis, resin cleavage and purification steps).

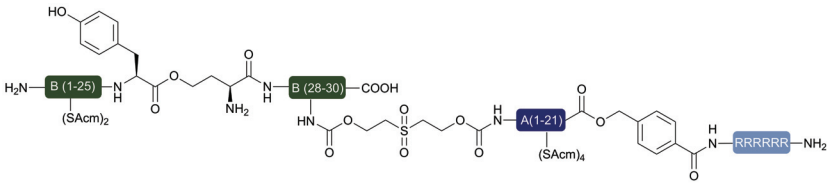
Analytical HPLC and ESI-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{176}H_{286}N_{58}O_{58}S_5$ $[M+H]^+$: 4299.9817; 4299.9997 measured.



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH3CN with 0.1% TFA in 20 min).

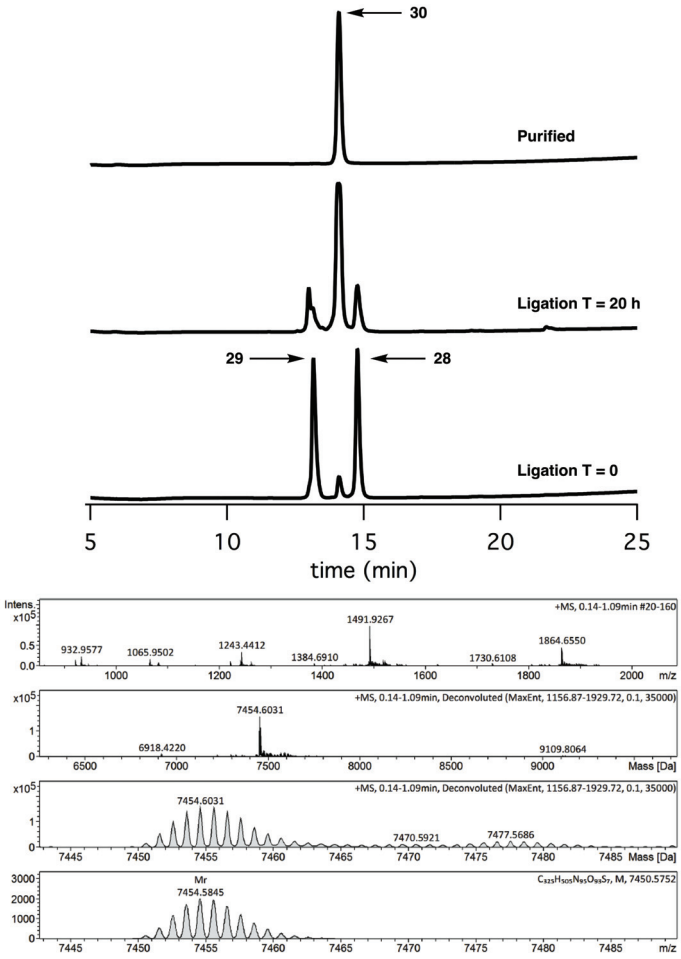
Bottom: **HR-ESI-MS** trace of the purified product. Measured and calculated isotopic pattern of product **29**

7.3. Synthesis of Acm protected linear mouse insulin by KAHA ligation (30)



α–Ketoacid segment **28** (43.5 mg, 13.6 μmol, 0.90 equiv) and (*S*)-5-Oxaproline segment **29** (65.0 mg, 15.1 μmol, 1.00 equiv) were dissolved in DMSO:H_2O = 9:1 with 0.1 M oxalic acid (1.01 mL, 15 mM) and shaked at 60 ºC. The progress of the ligation was monitored by analytical HPLC using a heated Shiseido

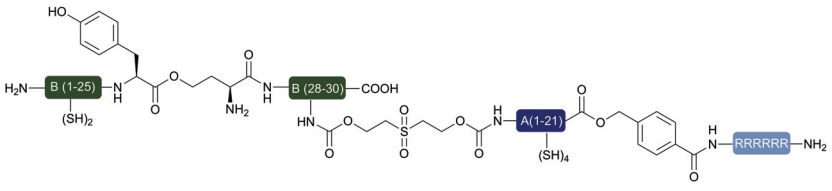
Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min. An aliquot of the ligation mixture (0.1 μL) was taken at various time points, diluted to 12 μL with CH_3CN:H_2O = 1:1 and injected on HPLC. The reaction mixture was diluted to 10 mL with DMSO and purified by heated preparative HPLC using a Shiseido Capcell Pak C18 column (50 x 250 mm, 5 μm), heated at 60 ºC, with a gradient of 10 to 95% CH_3CN with 0.1% TFA in 34 min. The fractions containing the ligated product were pooled and lyophilized to give pure **30** (66.0 mg, 8.90 μmol, 65% yield). Analytical HPLC and ESI-MS confirmed the purity and identity of **30**. m/z calculated for $C_{325}H_{505}N_{95}O_{93}S_7$ $[M+H]^+$: 7450.5752 and 7450.5902 measured.



Top: **Analytical HPLC** trace of the ligation reaction and of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **30**

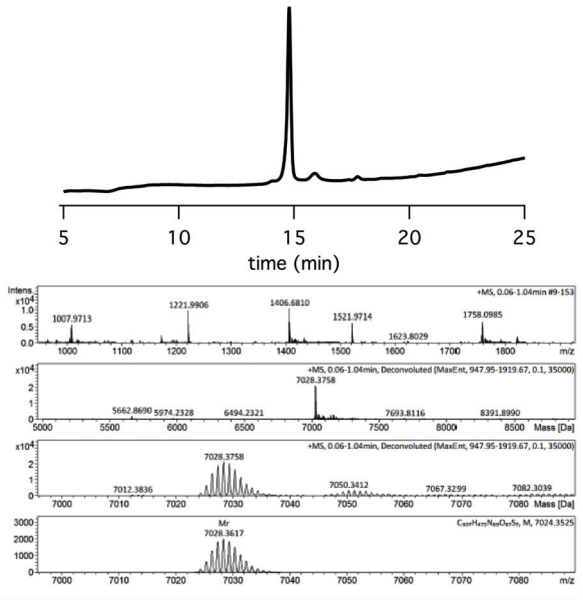
7.4. Acm deprotection of linear mouse insulin (31)



Acm protected linear insulin **30** (26.0 mg, 3.50 μmol, 1.00 equiv) was dissolved in a 50% aq. solution of AcOH (17.4 mL, 0.2 mM) containing 174 mg (1.10 mmol) 1% (m/m) AgOAc, then the mixture was agitated

for 1 h at 40 °C in the dark. The reaction was quenched by the addition of DTT (241 mg, 1.60 mmol), which was dissolved in 3 mL 50% aq. solution of AcOH and added to the reaction mixture at rt; yellow precipitated formed immediately. The mixture was agitated for 15 min at rt. The formed precipitation was separated by centrifugation. The supernatant was withdrawn and the precipitate was washed two times with 5 mL 50% aq. solution of AcOH . 15.0 mg (0.05 mmol, 15.0 equiv) TCEP HCl was added to the solution and incubated for 15 min at rt. The compound was purified by preparative HPLC using a heated Shiseido Capcell Pak C18 (20 mm x 250 mm, 5 μm) column with a gradient of 10 to 80% CH_3CN with 0.1% TFA in 40 min. The fractions containing the reduced product were pooled and lyophilized to give pure **31** (4.0 mg, 0.6 μmol, 15% yield). Analytical HPLC and ESI-MS confirmedthe purity and identity of the product. m/z calculated for $C_{307}H_{475}N_{89}O_{87}S_7$ $[M+H]^+$: 7024.3525 and measured for 7024.3576.

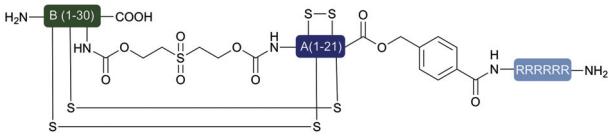
31



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

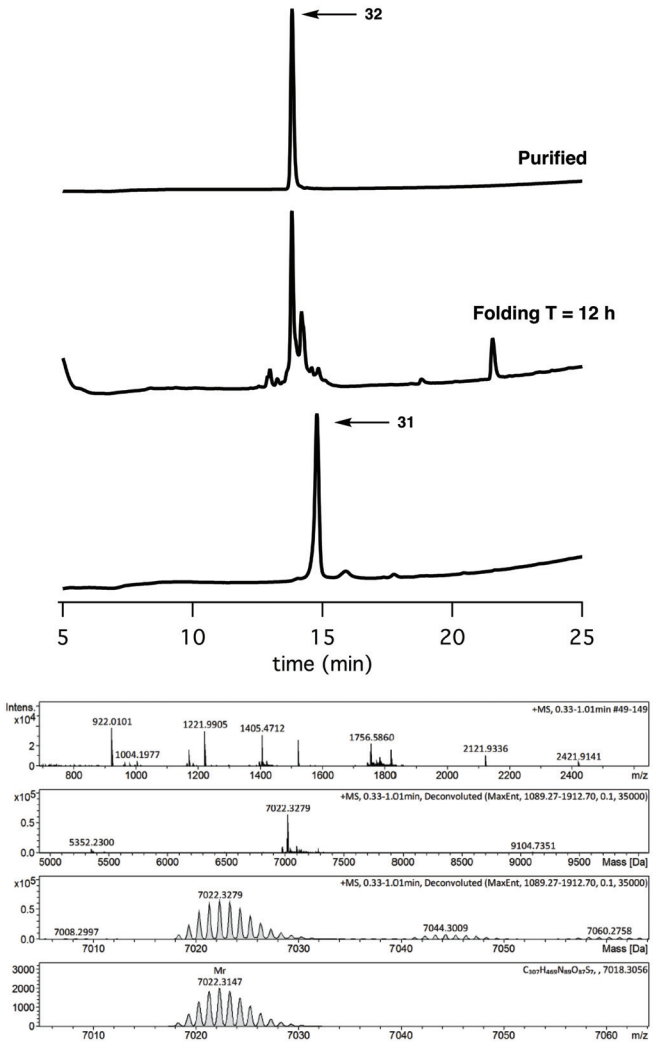
Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **31**

7.5. Folding of linear insulin (32)



Reduced linear insulin **31** (4.0 mg, 0.6 μmol, 1.0 equiv) was dissolved in 8.0 mL freshly prepared folding buffer (6 M Gn HCl, 0.3 M Tris, 2mM Cystein hydrochloride, pH 6.6) in a 50 mL Falcon tube and was vigorously stirred open to air at rt. After 1 h 8.0 mL Millipore H_2O was added and the pH was set to 8.2 with 1 M NaOH solution. The reaction vessel was closed and kept at 4 °C overnight. After 12 h the reaction was left to warm to rt and was incubated at rt. After 4 h 2.0 mL 50% aq. solution of AcOH was added and the product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column. The fractions containing the folded product were pooled,

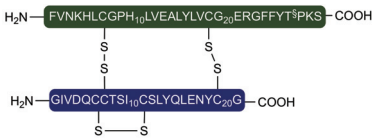
lyophilized to give pure **32**. Analytical HPLC and ESI-MS confirmed the purity and identity of the product. m/z calculated for $C_{307}H_{469}N_{89}O_{87}S_7$ $[M+H]^+$: 7018.3056 and measured for 7018.3140.



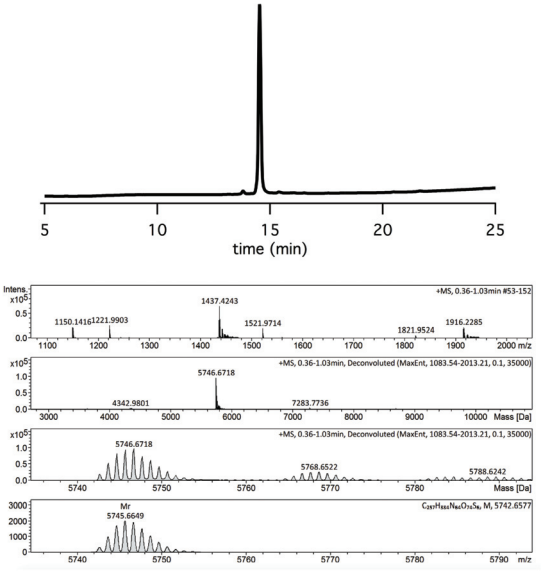
Top: **Analytical HPLC** trace of following the folding reaction and of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **32**

7.6. Cleavage of the Arg-tag and the linker (33)



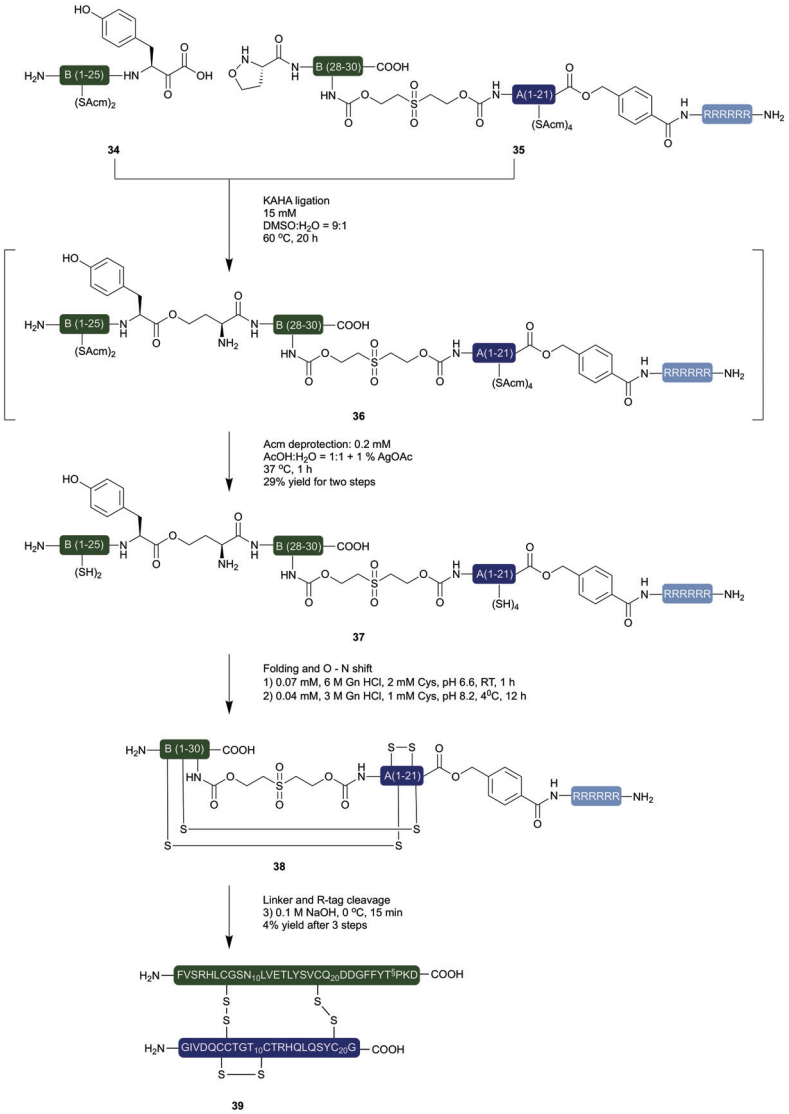
Lyophilized **32** was treated with 10 mL 0.1 M NaOH solution at 0 °C for 10 min. The reaction was quenched by the addition of 2 mL 50% aq. solution of AcOH solution and the final product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column to give pure **33** (0.5 mg, 0.1 μmol, 14% yield calculated from the folding precursor). Analytical HPLC and HR-MS were used to confirm the purity and identity of the product. m/z calculated for $C_{257}H_{384}N_{66}O_{74}S_6$ $[M+H]^+$: 5746.6577 and measured for 5746.6718.



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

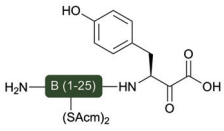
Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **33**

8. Guinea pig insulin



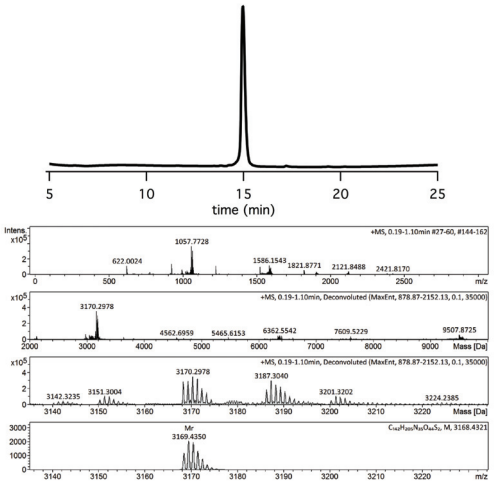
Synthesis of guinea pig
Mutations Asn^{A21} to Gly^{A21}, Ile^{B27} to Hse^{B27}

8.1. Synthesis of segment 34



α–Ketoacid segment was synthesized **34** on Rink-Amide ChemMatrix resin preloaded with Fmoc-Tyr-α-ketoacid with a substitution capacity of 0.2 mmol/g. After capping (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.4 mmol scale (2.00 g of resin) by automated Fmoc SPPS. Each amino acid (4.00 equiv) with the exception of Fmoc-Cys(Acm)-OH residues were coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.00 equiv) for 45 minutes. Fmoc-Cys(Acm)-OH (8.00 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O for 2 h at RT. 10.0 mL cleavage cocktail was used for 1.0 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged down (4000 rpm for 5 min). The supernatant was discarded the precipitation was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum. Purification of crude peptide **34** was performed by preparative HPLC using heated Shiseido Capcell Pak C18 column (50 x 250 mm) with a gradient of 20 to 60% CH_3CN with 0.1% TFA in 40 min. The pure product fractions were pooled and lyophilized to obtain **34** (230 mg, 72.6 μmol, 18 % yield for peptide synthesis, resin cleavage and purification steps).

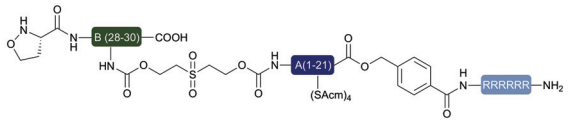
Analytical HPLC and ESI-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{142}H_{205}N_{35}O_{44}S_2$ $[M+H]^+$: 3168.4321 measured for 3168.3033.



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** trace of the purified product. Measured and calculated isotopic pattern of product **34**

8.2. Synthesis of segment 35

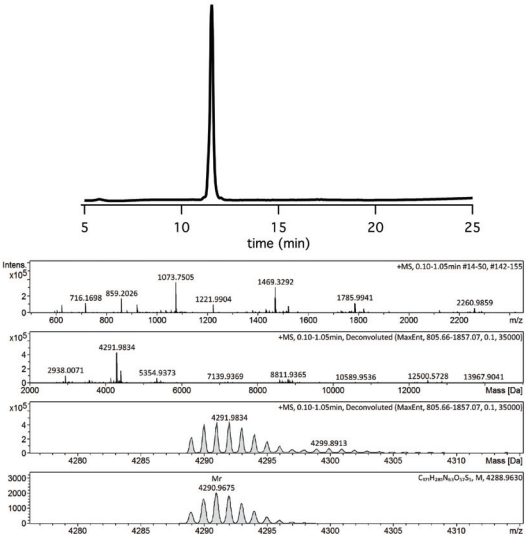


(*S*)-5-Oxaproline segment **35** was synthesized on Rink-Amide ChemMatrix resin prelaoded with Fmoc-Arg with a substitution capacity of 0.14 mmol/g. After capping (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.3 mmol scale (2.00 g of resin) by automated Fmoc SPPS. Each aminoacid was coupled with HATU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.00 equiv) for 45 minutes unless otherwise specified. Fmoc-Cys(Acm)-OH (8.00 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. (4.00 equiv) 4-(Hydroxymethyl)benzoic acid was coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) for 45 minutes. Fmoc-Gly-OH was coupled with DIC (8.00 equiv), HOBt (8.00 equiv) and DMAP (0.10 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. Residues Gly^{A9} and Thr^{A10} were coupled as a pseudoproline dipeptide (3.00 equiv) HATU (3.00 equiv) *N*-methylmorpholine (6.00 equiv) for 2 h. Linker **5c** (2.00 equiv) was coupled with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h. After the linker coupling capping (Ac_2O, *N*-methylmorpholine) was performed. For Boc-Opr-OH (2.00 equiv) double coupling was performed with HATU (2.00 equiv) N-methylmorpholine (4.00 equiv) for 2 h.

The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O) for 2 h at RT. 10.0 mL cleavage cocktail was used for 1.0 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged down (4000 rpm for 5 min). The supernatant was discarded the precipitation was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum.

Purification of crude **35** was performed by preparative HPLC using Shiseido Capcell Pak C18 column (50 x 250 mm) with a gradient of 20 to 80% CH_3CN with 0.1% TFA in 30 min. The pure product fractions were pooled and lyophilized to obtain **35** (200 mg, 46.6 μmol, 16% yield for peptide synthesis, resin cleavage and purification steps).

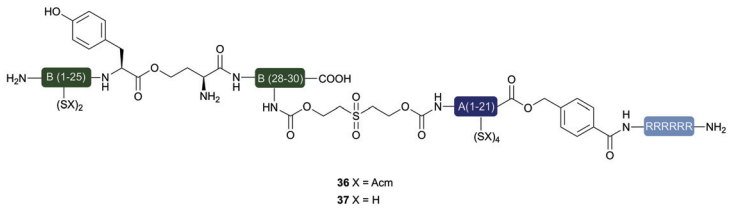
Analytical HPLC and HR-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{171}H_{281}N_{63}O_{57}S_5$ $[M+H]^+$: 4288.930 measured for 4288.9696.



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH3CN with 0.1% TFA in 20 min).

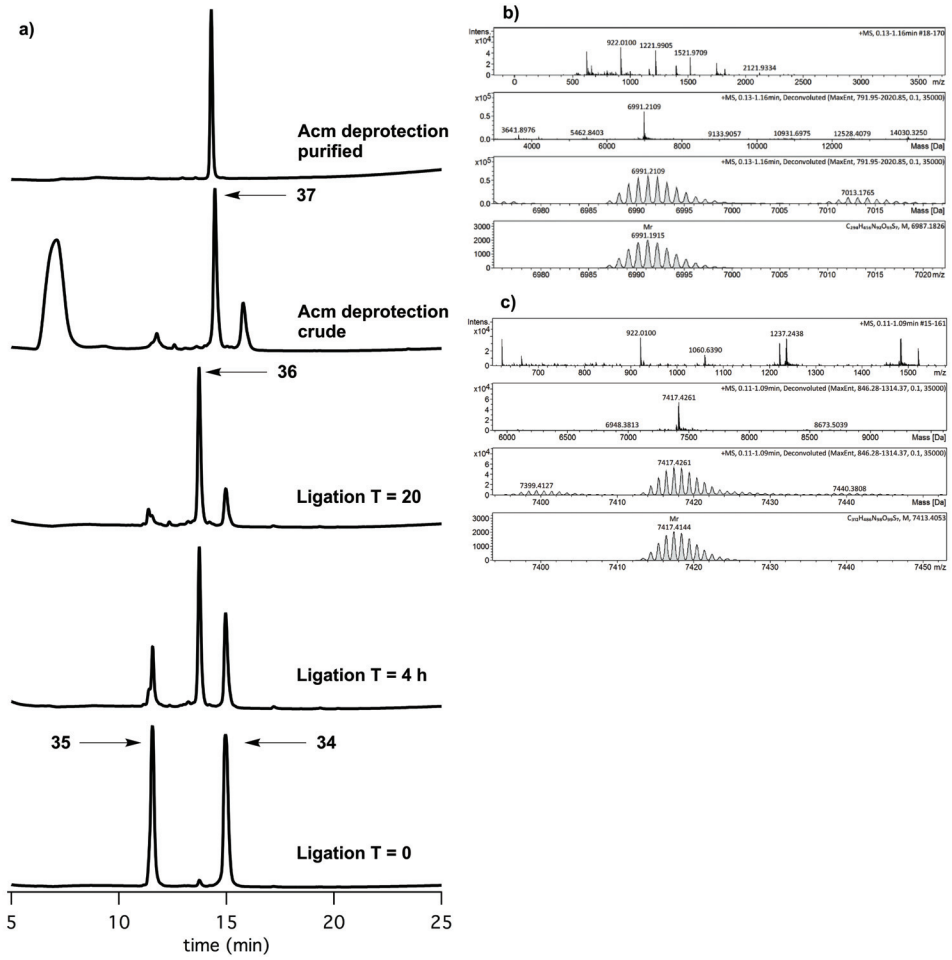
Bottom: **HR-MS** trace of the purified product. Measured and calculated isotopic pattern of product **35**

8.1. One pot synthesis of Acm protected linear guinea pig insulin by KAHA ligation (36) and Acm deprotection of the ligated product (37)



36 X = Acm
37 X = H

α–Ketoacid segment **34** (9.5 mg, 3.0 μmol, 1.00 equiv) and (*S*)-5-Oxaproline segment **35** (12.9 mg, 3.0 μmol, 1.00 equiv) were dissolved in DMSO:H_2O = 9:1 with 0.1 M oxalic acid (1.01 mL, 15 mM) and shaked at 60 ºC. The progress of the ligation was monitored by analytical HPLC using a heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min. An aliquot of the ligation mixture (0.1 μL) was taken at various time points, diluted to 12 μL with CH_3CN:H_2O = 1:1 and injected on HPLC. After 20 h the ligation was complete. The reaction mixture was diluted with 10.1 mL 50% aq. solution of AcOH and 101.10 mg (0.60 mmol, 1m/m %) AgOAc was added then the mixture was agitated for 1 h at 40 °C in the dark. The reaction was quenched by the addition of DTT (140.0 mg, 0.9 mmol), which was dissolved in 3 mL 50% aq. solution of AcOH and added to the reaction mixture at rt; yellow precipitated formed immediately. The mixture was agitated for 15 min at rt. The formed precipitation was separated by centrifugation. The supernatant was withdrawn and the precipitate was washed two times with 4 mL of the same solution. 289 mg (1.01 mmol, 500 equiv) TCEP HCl was added to the solution and incubated for 15 min at rt. The compound was purified by preparative HPLC using a heated Shiseido Capcell Pak Proteonavi column (50 x 250 mm) column with a gradient of 15 to 80% CH_3CN with 0.1% TFA in 40 min. The fractions containing the reduced product were pooled and lyophilized to give pure **37** (6.0 mg, 0.9 μmol, 29% yield for two steps). Analytical HPLC and ESI-MS confirmed the purity and identity of the products. m/z calculated for $C_{312}H_{486}N_{98}O_{99}S_7$ $[M+H]^+$: 7413.4053. and measured for 7413.3990 for **36** and m/z calculated for $C_{294}H_{456}N_{92}O_{93}S_7$ $[M+H]^+$: 6987.1826. and measured for 6987.2016 for **37**.

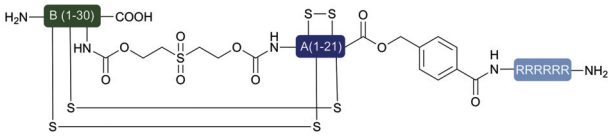


a) Curse of the ligation and the Acm deprotection followed by analytical HPLC (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH3CN with 0.1% TFA in 20 min).

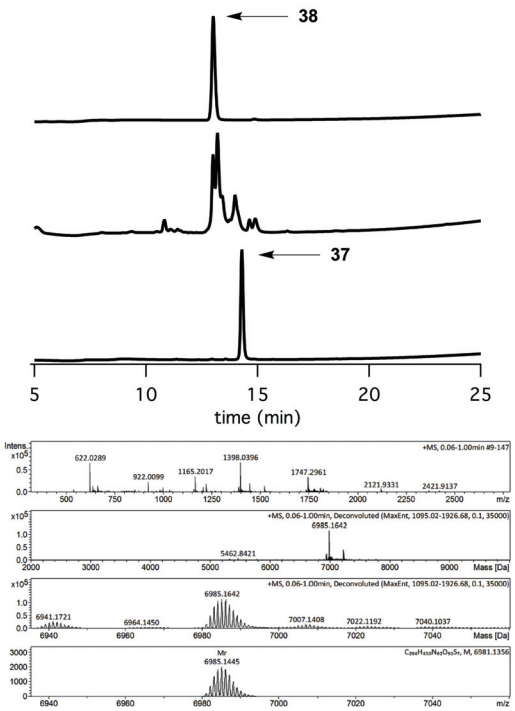
b): **HR-ESI-MS** trace of the purified Acm deptotected peptide **37**.

c) **HR-MS** trace of the ligated peptide **36**

8.2. Folding of linear guinea pig insulin (38)



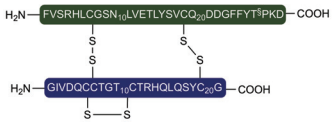
Reduced linear guinea piginsulin **37** (6.0 mg, 0.9 μmol, 1.0 equiv) was dissolved in 12.0 mL freshly prepared folding buffer (6 M Gn HCl, 0.3 M Tris, 2mM Cystein hydrochloride, pH 6.6) in a 50 mL falcon tube and was vigorously stirred open to air at rt. After 1 h 12.0 mL Millipore H_2O was added and the pH was set to 8.2 with 1 M NaOH solution. The reaction vessel was closed and kept at 4 °C overnight. After 12 h the reaction was left to warm to rt and was incubated at rt. After 4 h 2.0 mL 50% aq. solution of AcOH was added and the product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column. The fractions containing the folded product were pooled lyophilized to give pure **38**. Analytical HPLC and ESI-MS confirmed the purity and identity of the product. m/z calculated for $C_{294}H_{450}N_{92}O_{93}S_7$ $[M+H]^+$: 6981.1356 and measured for 6981.1597.



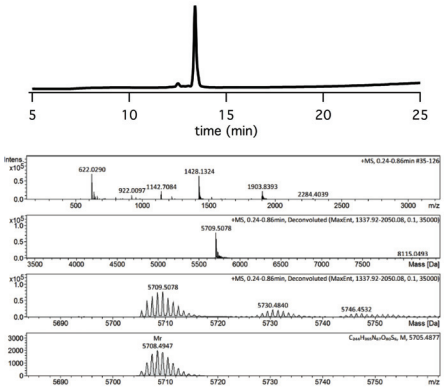
Top: **Analytical HPLC** trace following the folding and of the purified product **38** (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **38**

8.3. Cleavage of the Arg-tag and the linker (39)



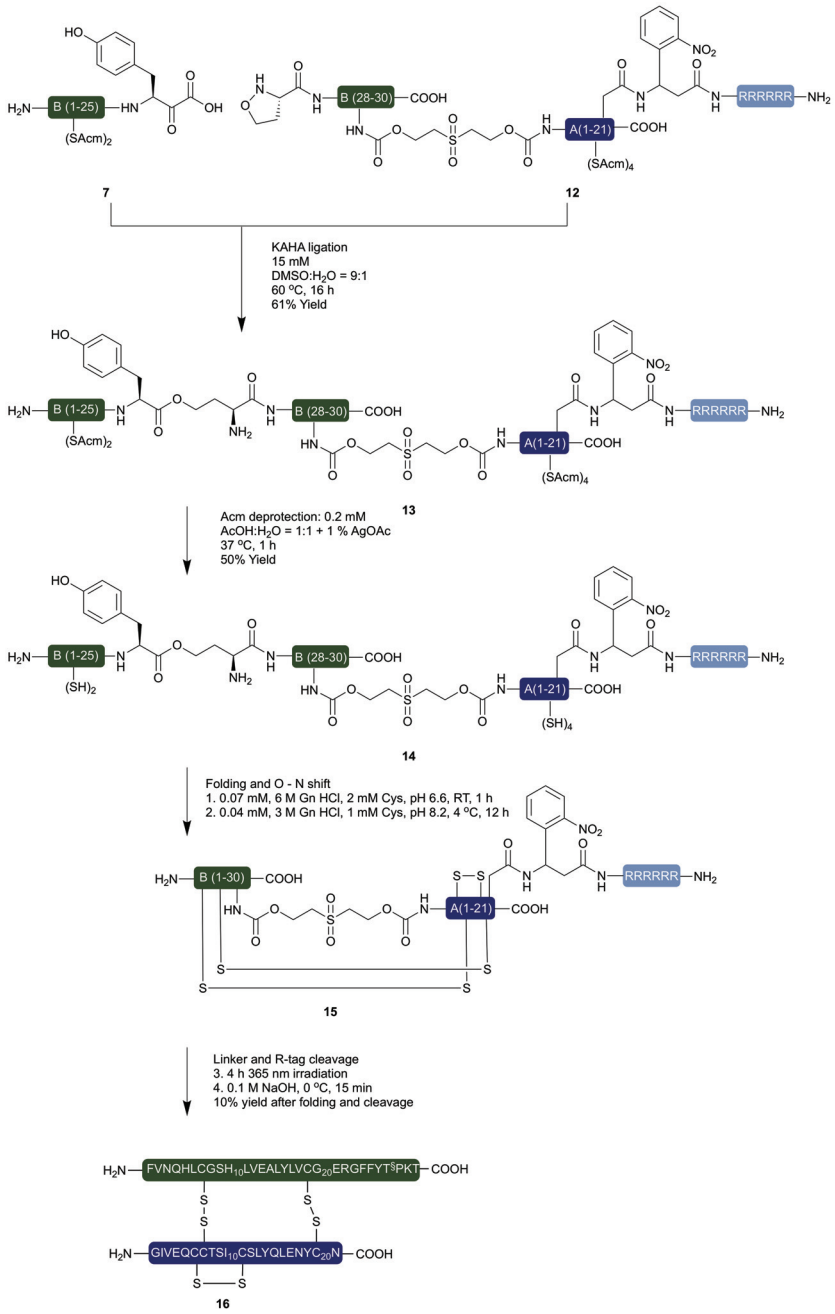
Lyophilized **38** was treated with 5 mL 0.1 M NaOH solution at 0 °C for 10 min. The reaction was quenched by the addition of 2 mL 50% aq. solution of AcOH solution and the final product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column to give pure **39** (0.2 mg, 0.04 μmol, 4% yield for two steps). Analytical HPLC and HR-MS confirmed the purity and identity of the product. m/z calculated for $C_{244}H_{365}N_{67}O_{80}S_6$ $[M+H]^+$: 5705.4877 and measured for 5705.5009.



Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

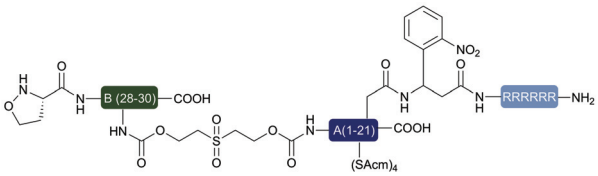
Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product (**39**)

9. Human insulin



Synthesis of human insulin **16**
Mutations Thr^{B27} to Hse^{B27}

9.1. Synthesis of segment 12

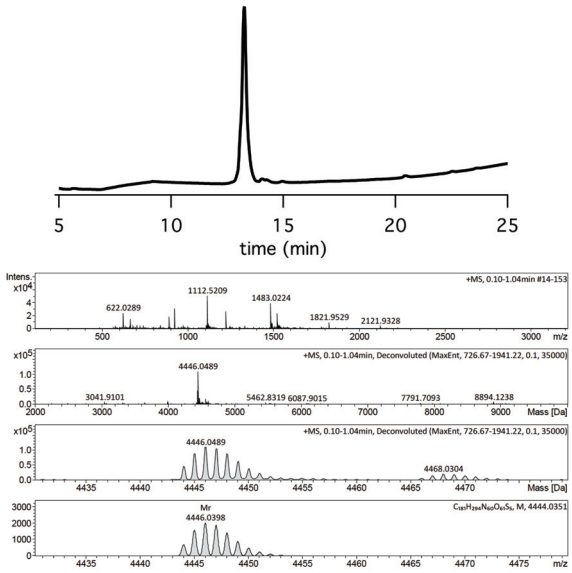


(*S*)-5-Oxaproline segment **12** was synthesized on Rink-Amide ChemMatrix resin prelaoded with Fmoc-Arg with a substitution capacity of 0.2 mmol/g. After capping with (Ac_2O, *N*-methylmorpholine), the synthesis was performed on 0.4 mmol scale (2.00 g of resin, 1.00 equiv) by automated Fmoc SPPS. Each amino acid were coupled with HCTU (3.98 equiv) *N*-methylmorpholine (8.00 equiv) Fmoc protected amino acid (4.0 equiv) for 45 minutes unless otherwise specified. Fmoc-Cys(Acm)-OH (8.0 equiv) residues were coupled with DIC (8.00 equiv) and HOBt (8.00 equiv) for 2 h after 10 min preactivation of the amino acid and the reagents. Fmoc-amino-3-(2-nitrophenyl)propionic acid (4.00 equiv) was coupled with HATU (3.98 equiv) *N*-methylmorpholine (8.00 equiv). Residues Thr^{A7} and Ser^{A8} were coupled as a pseudoproline dipeptide (3.00 equiv) HATU (3.00 equiv) *N*-methylmorpholine (6.00 equiv) for 2 h. Linker **5d** (2.00 equiv) was coupled with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h. After the linker coupling capping (Ac_2O, *N*-methylmorpholine) was performed. For Boc-Opr-OH (2.00 equiv) double coupling was performed with HATU (2.00 equiv) *N*-methylmorpholine (4.00 equiv) for 2 h.

The peptide was cleaved from resin with the following cleavage cocktail: 95 v/v% TFA; 2.5 v/v% DODT; 2.5 v/v% H_2O) for 2 h at rt. 10.0 mL cleavage cocktail was used for 1.0 g of peptidic resin. After 2 h the resin was filtered off and the volatile compounds were removed under reduced pressure. The peptide was precipitated by the addition of Et_2O. The mixture was sonicated for 30 sec and the precipitate was centrifuged down (4000 rpm for 5 min). The supernatant was discarded the precipitation was suspended in Et_2O, sonicated and centrifuged down as described above two times. The crude peptide was dried briefly under high vacuum.

Purification of crude **12** was performed by preparative HPLC using Shiseido Capcell Pak Proteonavi column (50 x 250 mm) with a gradient of 20 to 80% CH_3CN with 0.1% TFA in 30 min. The pure product fractions were pooled and lyophilized to obtain **12** (591 mg, 0.133 mmol) 33.3% yield for peptide synthesis, resin cleavage and purification steps).

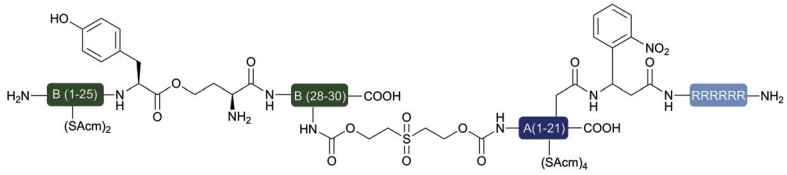
Analytical HPLC and HR-MS confirmed the purity and exact mass of the product. m/z calculated for $C_{181}H_{294}N_{60}O_{61}S_5$ $[M+H]^+$:4444.0351, 4444.0413 measured.



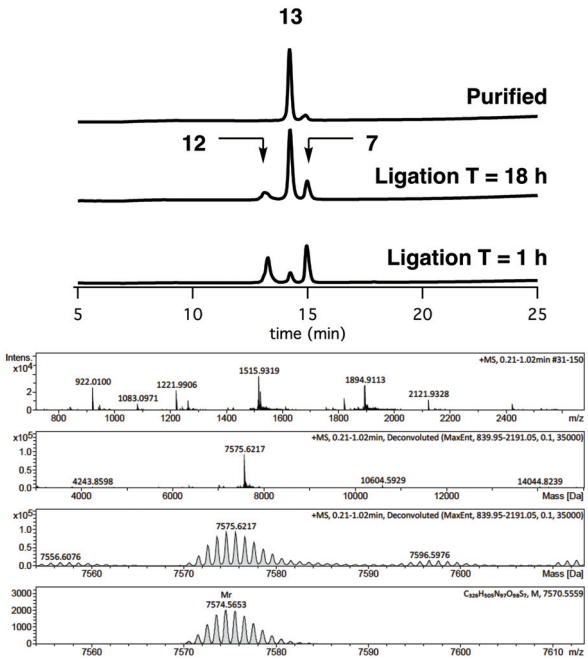
Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **12**.

9.1. Synthesis of Acm protected linear insulin by KAHA ligation 13



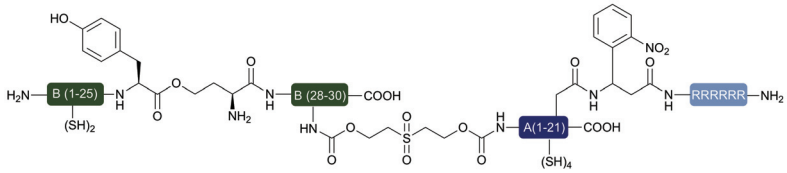
α–Ketoacid segment **7** (62.8 mg, 19.8 μmol, 0.80 equiv) and (*S*)-5-Oxaproline segment **12** (110 mg, 24.7 μmol, 1.00 equiv) were dissolved in DMSO:H_2O = 9:1 with 0.1 M oxalic acid (1.65 mL, 15 mM) and shaked at 60 ºC. The progress of the ligation was monitored by analytical HPLC using a heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min. An aliquot of the ligation mixture (0.1 μL) was taken at various time points, diluted to 12 μL with CH_3CN:H_2O = 1:1 and injected on HPLC. The reaction mixture was diluted to 10 mL with DMSO and purified by heated preparative HPLC using a Shiseido Capcell Pak C18 column (20 x 250 mm), heated at 60 ºC, with a gradient of 10 to 95% CH_3CN with 0.1% TFA in 34 min. The fractions containing the ligated product were pooled and lyophilized to give pure **13** (97.0 mg, 12.2 μmol) 61% yield for ligation. Analytical HPLC and HR-MS confirmed the purity and identity of **13**. m/z calculated for $C_{326}H_{505}N_{97}O_{98}S_7$ measured for $[M+H]^+$: 7570.5559 and 7570.6099 measured.



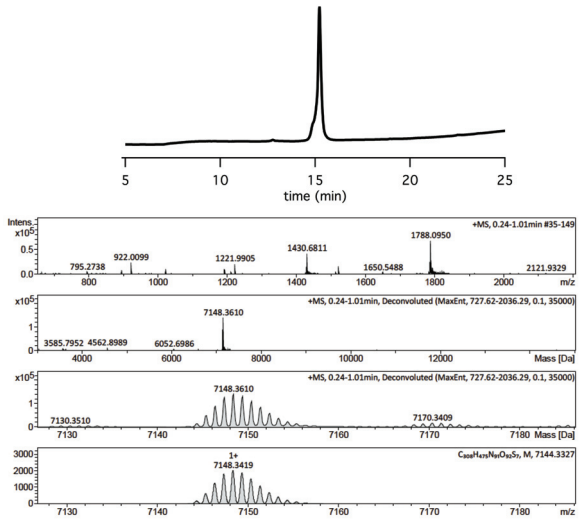
Top: **Analytical HPLC** trace the ligation reaction and of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **13**.

9.1. Acm deprotection of linear insulin 14



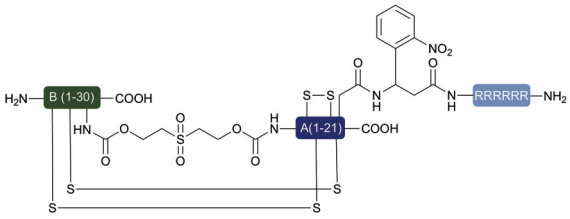
Acm protected linear insulin **13** (43.0 mg, 5.80 μmol, 1.00 equiv) was dissolved in a 50% aq. solution of AcOH (29.2 mL, 0.2 mM) containing 292 mg (1.6 mmol) 1% (m/m) AgOAc, then the mixture was agitated for 1 h at 40 °C in the dark. The reaction was quenched by the addition of DTT (404 mg, 2.60 mmol), which was dissolved in 3 mL 50% aq. solution of AcOH and added to the reaction mixture at rt; yellow precipitated formed immediately. The mixture was agitated for 15 min at rt. The formed precipitation was separated by centrifugation. The supernatant was withdrawn and the precipitate was washed two times with 5 mL 50% aq. solution of AcOH. 25.0 mg (0.09 mmol, 15.0 equiv) TCEP HCl was added to the solution and incubated for 15 min at rt. The compound was purified by preparative HPLC using a heated Phenomenex Jupiter (20 mm x 25 mm) column with a gradient of 10 to 80% CH_3CN with 0.1% TFA in 40 min. The fractions containing the reduced product were pooled and lyophilized to give pure **14** (21.0 mg, 2.94 μmol, 51% yield). Analytical HPLC and HR-MS were used to confirm the purity and identity of the product. m/z calculated for $C_{308}H_{475}N_{91}O_{92}S_7$ $[M+H]^+$: 7144.3333 and measured for 7144.3591.



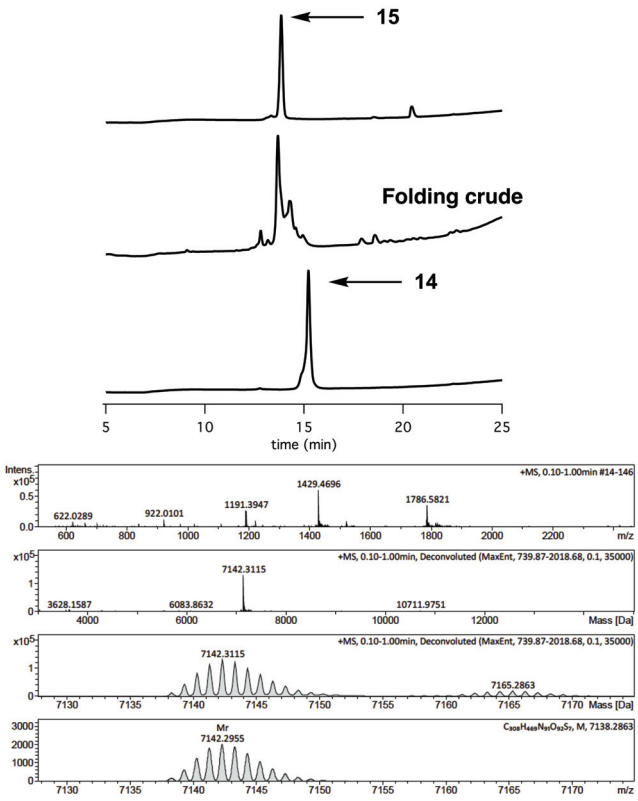
Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **HR-ESI-MS** Measured and calculated isotopic pattern of product **14**.

9.1. Folding of linear insulin 15



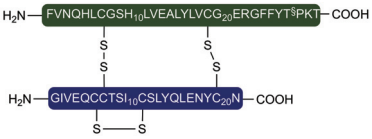
Reduced linear insulin **14** (8.5 mg, 0.6 μmol, 1.0 equiv) was dissolved in 17.0 mL freshly prepared folding buffer (6 M Gn HCl, 0.3 M Tris, 2mM Cystein hydrochloride, pH 6.6) in a 50 mL falcon tube and was vigorously stirred open to air at rt. After 1 h 17.0 mL Millipore H_2O was added and the pH was set to 8.2 with 1 M NaOH solution. The reaction vessel was closed and kept at 4 °C overnight. After 12 h the reaction was left to warm to rt and was incubated at rt. After 4 h 2.0 mL 50% aq. solution of AcOH was added and the product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column. The fractions containing the folded product were pooled to give pure **15**. Analytical HPLC and HR-MS confirmed the purity and identity of the product. m/z calculated for $C_{308}H_{469}N_{91}O_{92}S_7$ $[M+H]^+$: 7138.2863 and measured for 7138.3026.



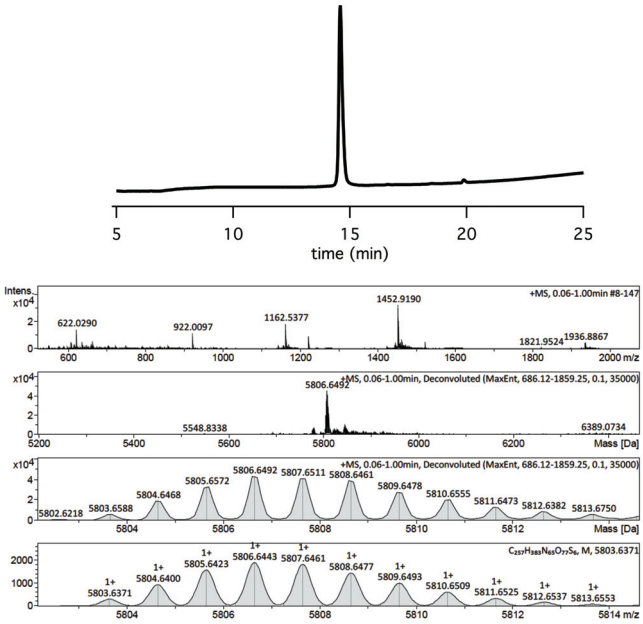
Top: **Analytical HPLC** trace the folding reaction and of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

Bottom: **ESI-MS** Measured and calculated isotopic pattern of product **15**

9.2. Cleavage of the R-tag and the linker 16



The pooled preparative HPLC fractions of **15** were irradiated for 1 h at 365 nm with a hand held UV lamp at rt. After irradiation the solvents were removed by lyophilization. The crude lyophilized peptide was treated with 8 mL 0.1 M NaOH solution at 0 °C for 10 min. The reaction was quenched by the addition of 2 mL 50% aq. solution of AcOH solution and the final product was isolated by preparative HPLC using a heated Shiseido Capcell Pak C18 Type MG II (10 mm x 250 mm, 5 μm) semi-preparative column to give pure **16** (0.7 mg, 0.12 μmol, 10% yield for three steps). Analytical HPLC and HR-MS confirmed the purity and identity of the product. m/z calculated for $C_{257}H_{383}N_{65}O_{77}S_6$ $[M+H]^+$: 5803.6376 and measured for 5803.6571.

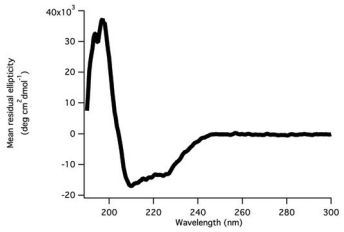


Top: **Analytical HPLC** trace of the purified product (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH3CN with 0.1% TFA in 20 min).

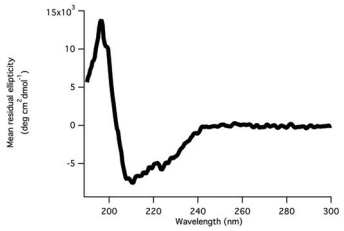
Bottom: **HR-MS** trace of the purified product. Measured and calculated isotopic pattern of product **16**

10. Circular Dichroism measurments

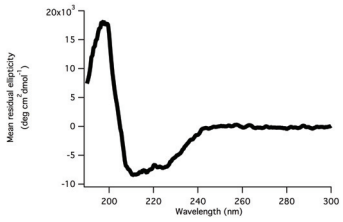
Peptide samples recombinant Insulin (Human, Aldrich), synthetic M2 (**11**) and synthetic human (**16**) were dissolved in 10 mM aq. potassium phosphate buffer (pH 7.0) at a concentration of 21 μM. Data were collected through 10 scans in a 1 mm path length cuvette at a scan speed of 100 scan/min. The previously recorded background was subtracted from the recorded spectra. The measured millidegree values were converted to mean residual ellipticity by the following equation: mean residue ellipticity = millidegrees/(pathlength in mm x concentration of sample in mol/liter x number of residues).



CD spectrum of authentic human recombinant insulin

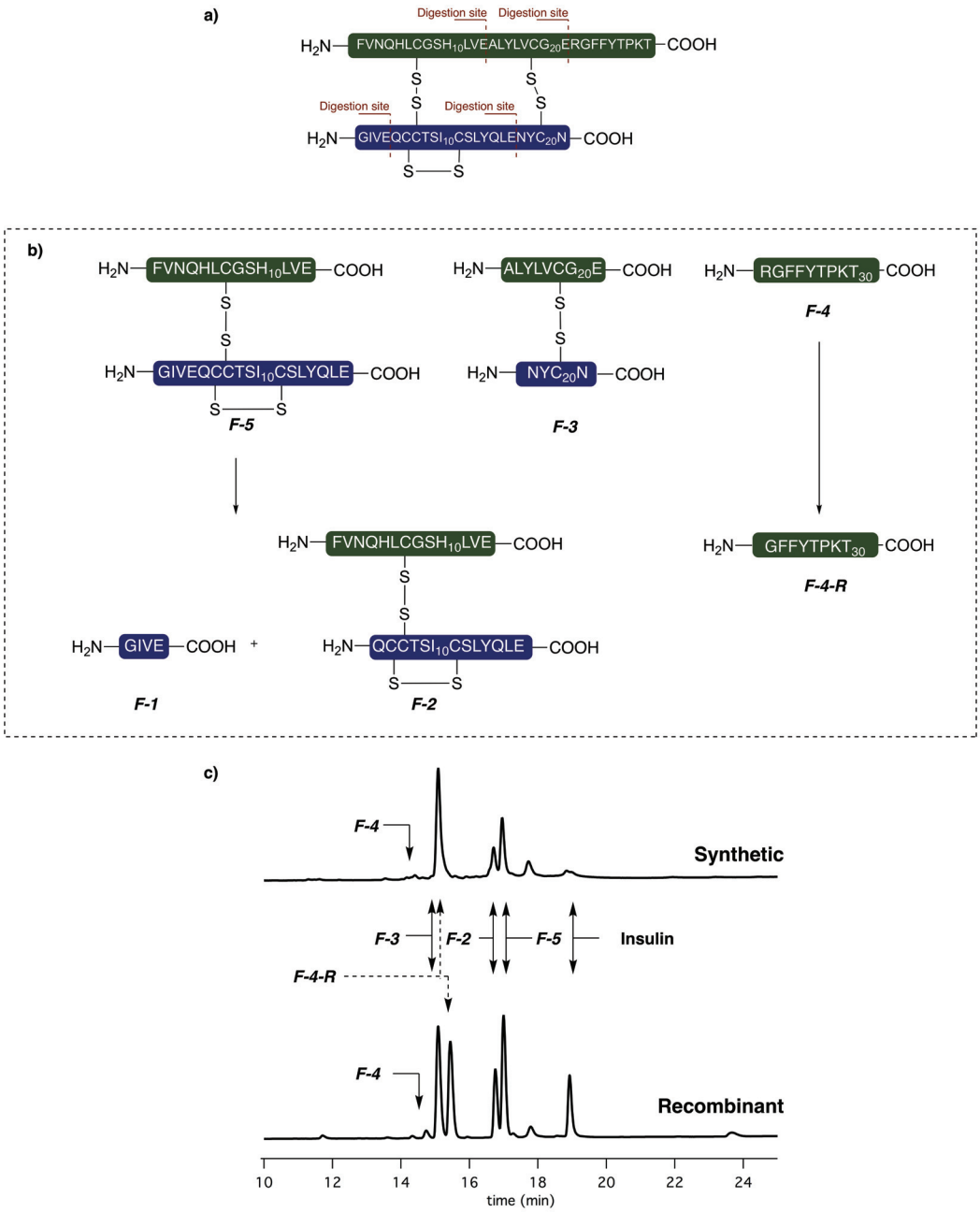


CD spectrum of synthetic human insulin (**11**)



CD spectrum of synthetic M2 insulin (**16**)

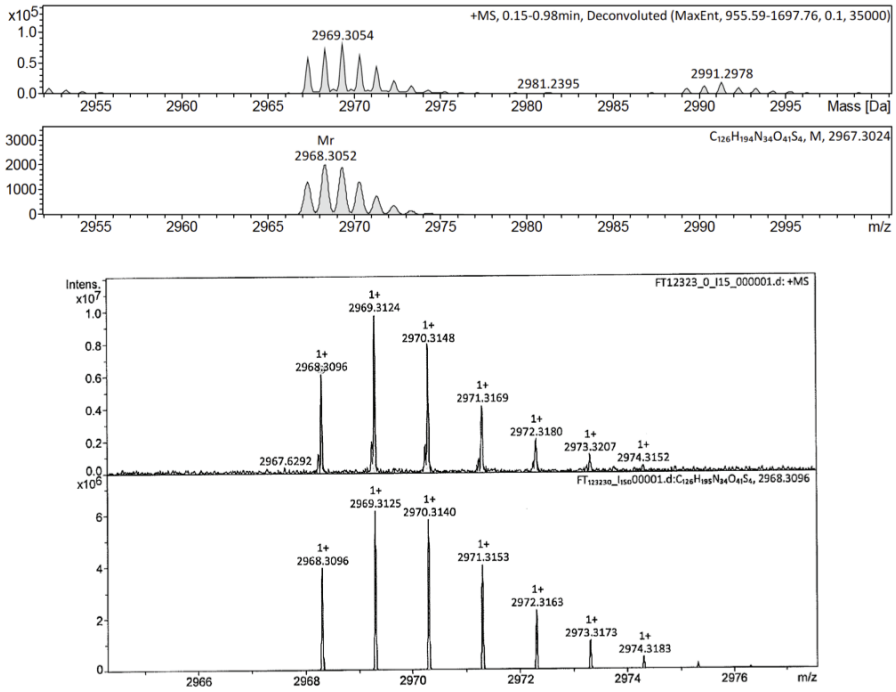
11. Digestion study



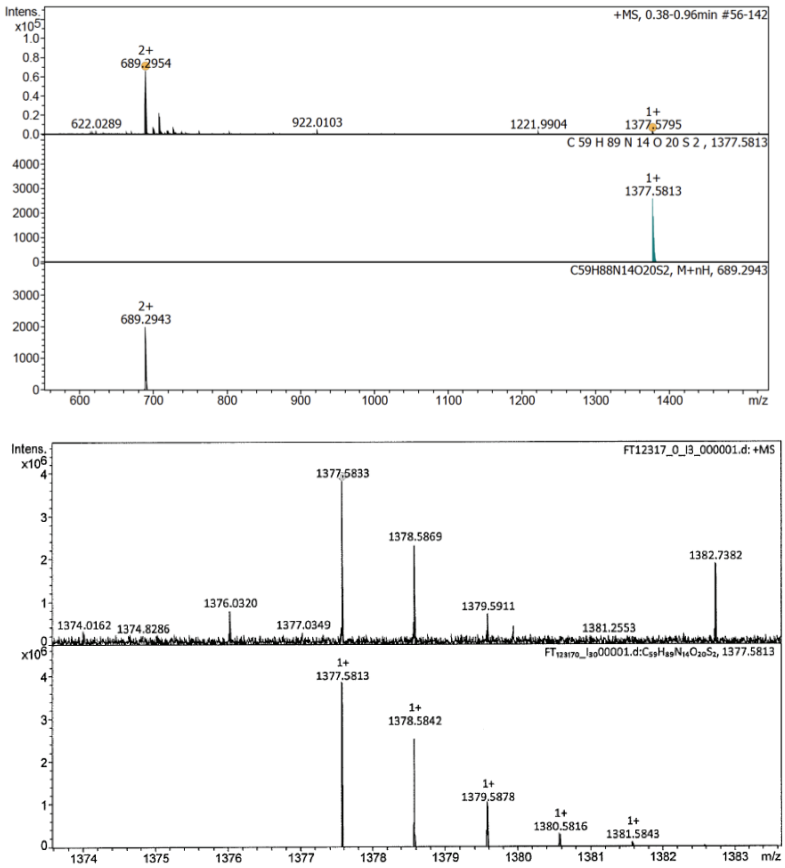
a) Digestion sites of insulin by endoproteinase Glu C (in synthetic human insulin Thr^{B27} is mutated to Hse^{B27} **b)** fragments obtained by digestion **c)** Top: Analytical HPLC trace the of the digested synthetic insulin Bottom: analytical HPLC trace the of the digestion reaction of recombinant insulin (heated Shiseido Capcell Pak C18 analytical column, 10 to 95% CH_3CN with 0.1% TFA in 20 min).

For digestion experiments Endoproteinase GluC was used from New England BioLabs Inc. 0.10 mg (0.02 μmol) insulin placed into a 1000 mmL eppendorf. 0.24 mL GluC buffer was added. The mixture was diluted with 0.24 mL Millipore-H_2O. 50.0 ng GluC endoproteinase was reconstituted in 0.2 mL Millipore-H_2O. 0.02 mL GluC endoproteinase solution was added to the buffer containing the peptide sample and was incubated at 37 °C for 3 h. The reaction was analyzed by analytical HPLC using a heated Shiseido Capcell Pak C18 analytical column, 5 to 60% CH_3CN with 0.1% TFA in 20 min.

Synthetic human insulin variant (Hse^{B27}) and recombinant human insulin were submitted to digestion. The reaction mixtures were analyzed by HPLC and HR MS. In both experiments **F-2**, **F-3**, **F-4**, **F-4-R**, **F-5** fragments were observed by HPLC and identified by mass spectrometry respectively. **F-1** fragment was not observed in any of the experiments. Under the digestion conditions **F-4** further hydrolyzed to give **F-4-R** fragment in both experiments. **F-2** fragment showed earlier elution time in the case of synthetic insulin compared to **F-2** fragment obtained from recombinant insulin. The difference in the retention time is attributed to the Thr to Hse mutation.



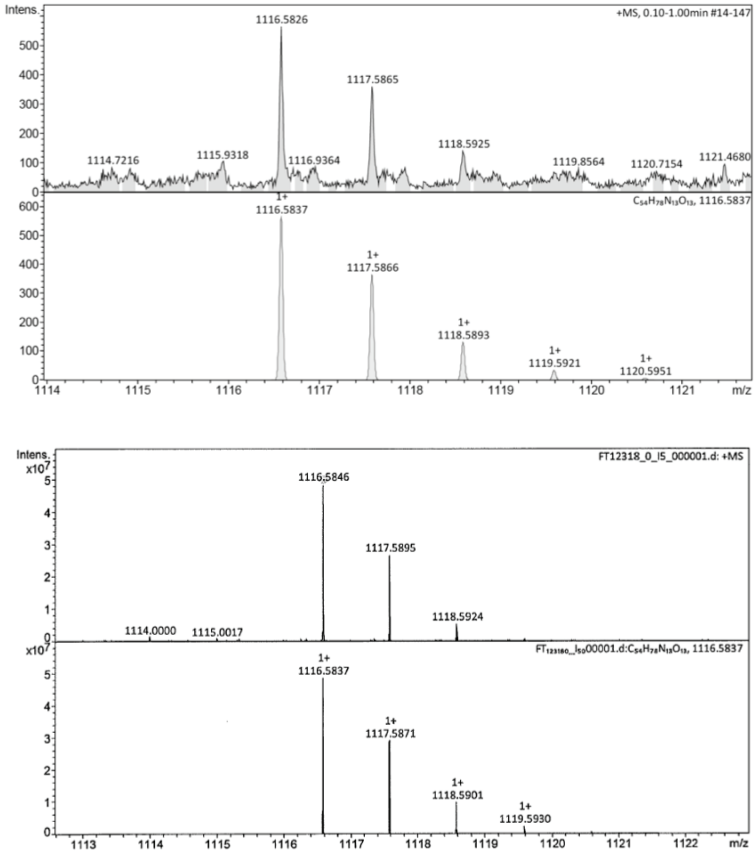
Measured and calculated isotopic pattern of digested fragment **F-2**
Top: Recombinant insulin
Bottom: Synthetic insulin



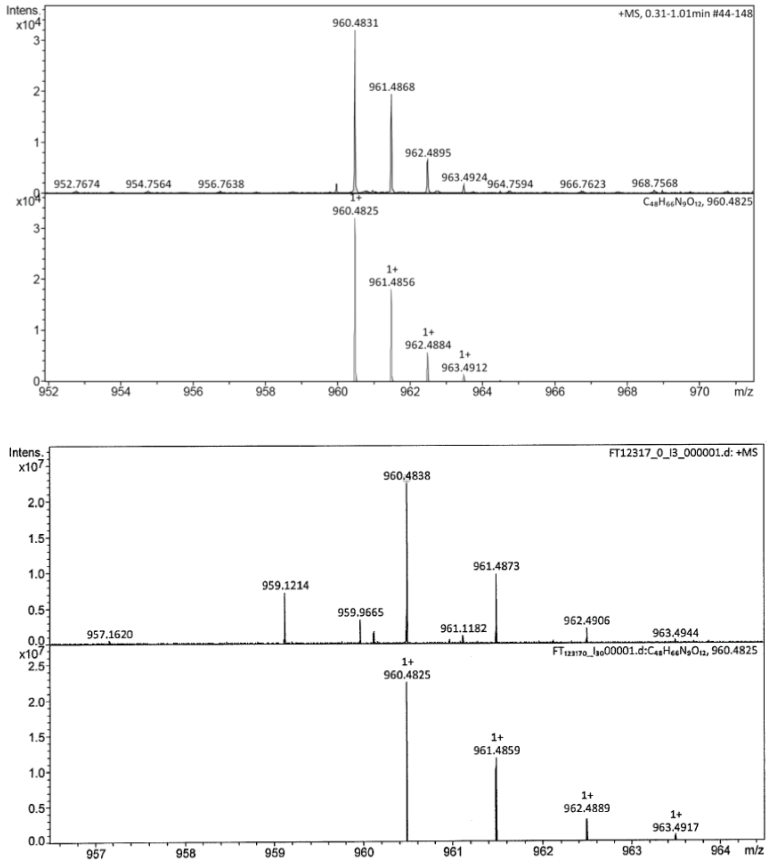
Measured and calculated isotopic pattern of digested fragment **F-3**

Top: Recombinant insulin

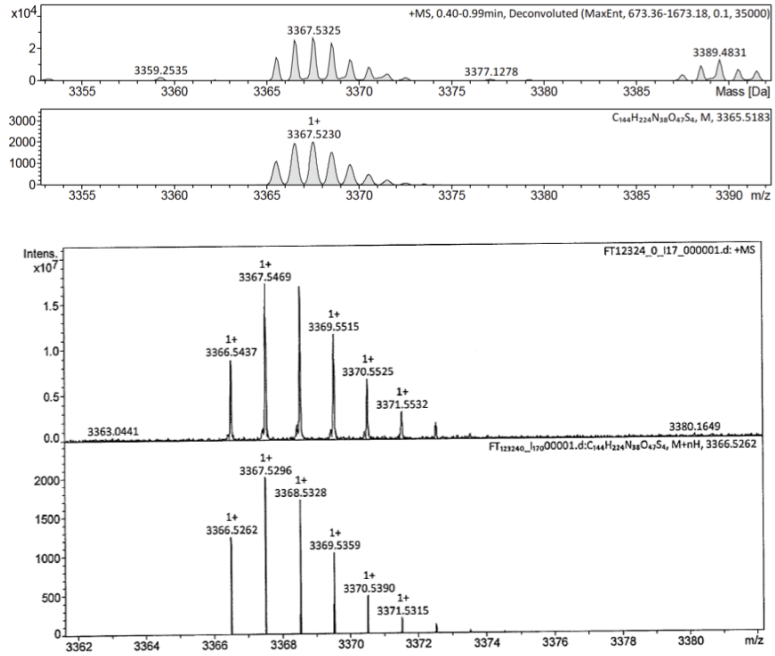
Bottom: Synthetic insulin



Measured and calculated isotopic pattern of digested fragment **F-4**
Top: Recombinant insulin
Bottom: Synthetic insulin (Thr^{27} is mutated to Homoserine)



Measured and calculated isotopic pattern of digested fragment **F-4R**
Top: Recombinant insulin
Bottom: Synthetic insulin (Thr^{27} is mutated to Homoserine)

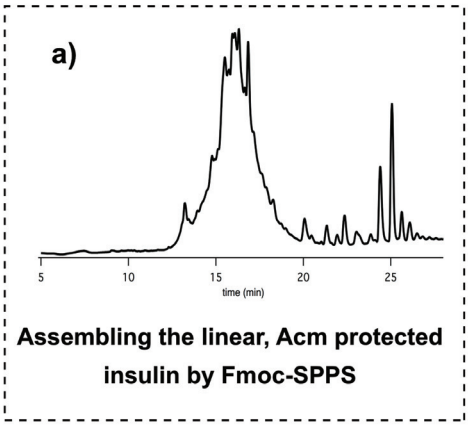


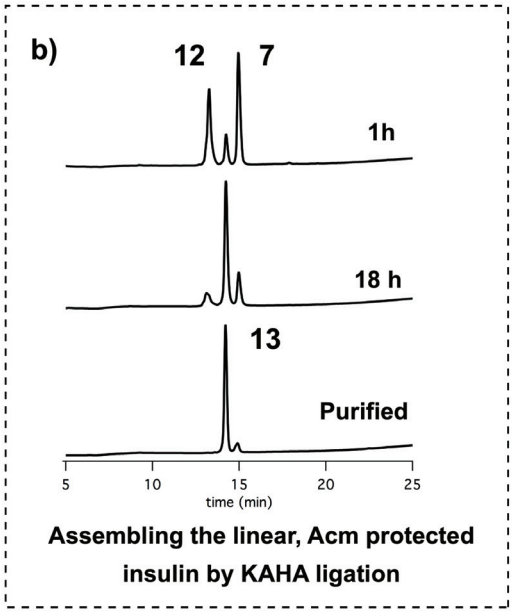
Measured and calculated isotopic pattern of digested fragment **F-5**

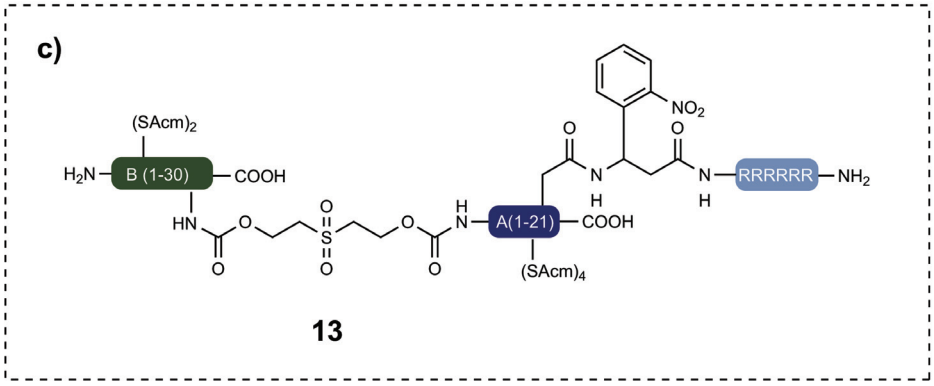
Top: Recombinant insulin

Bottom: Synthetic insulin

12. Attempt for the preparation of Acm protected linear insulin by Fmoc-SPPS







a) Crude HPLC of the attempted synthesis of Acm protected linear insulin by Fmoc-SPPS. b) HPLC monitoring of the assembling of the Acm protected linear insulin by KAHA ligation. c) Structure of Acm protected linear insulin.

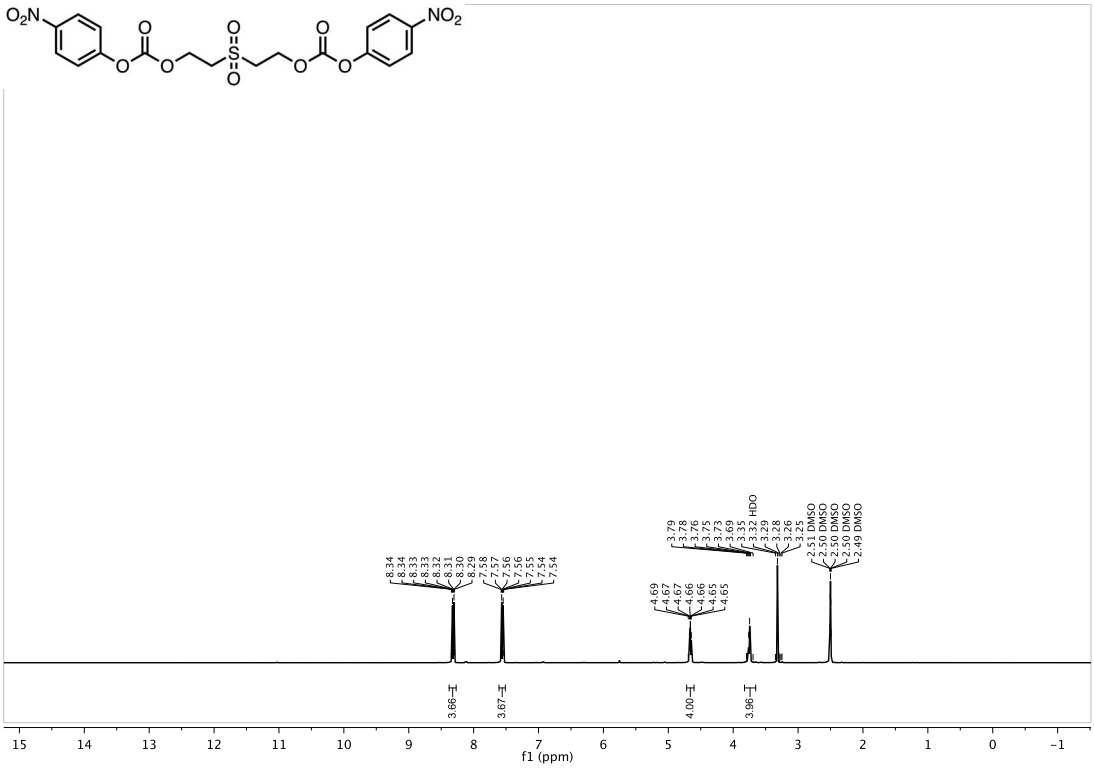
13. Insulin receptor phosphorylation assay

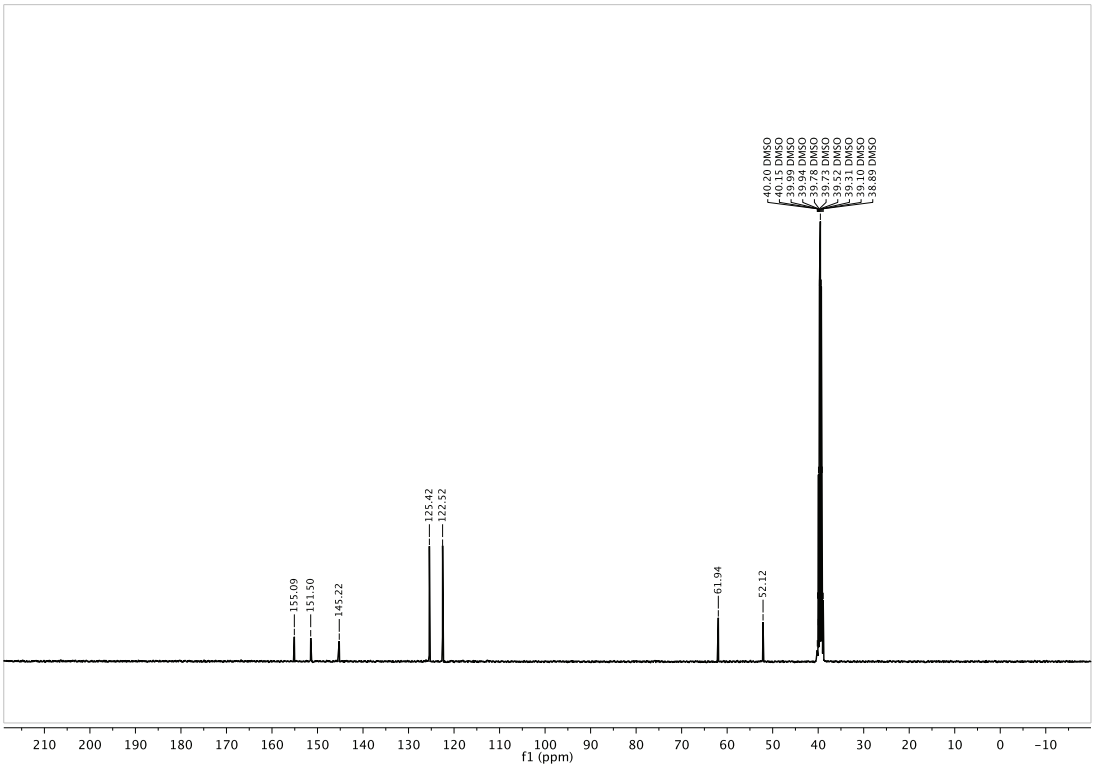
Biological activity of each prepared synthetic insulin analogues were measures by a cell-based assay as described before.[1]

[1] M. R. Sommerfeld, G. Müller, G. Tschank, G. Seipke, P. Habermann, R. Kurrle, N. Tennagels, *Plos One*, 2010, **5**, 9540

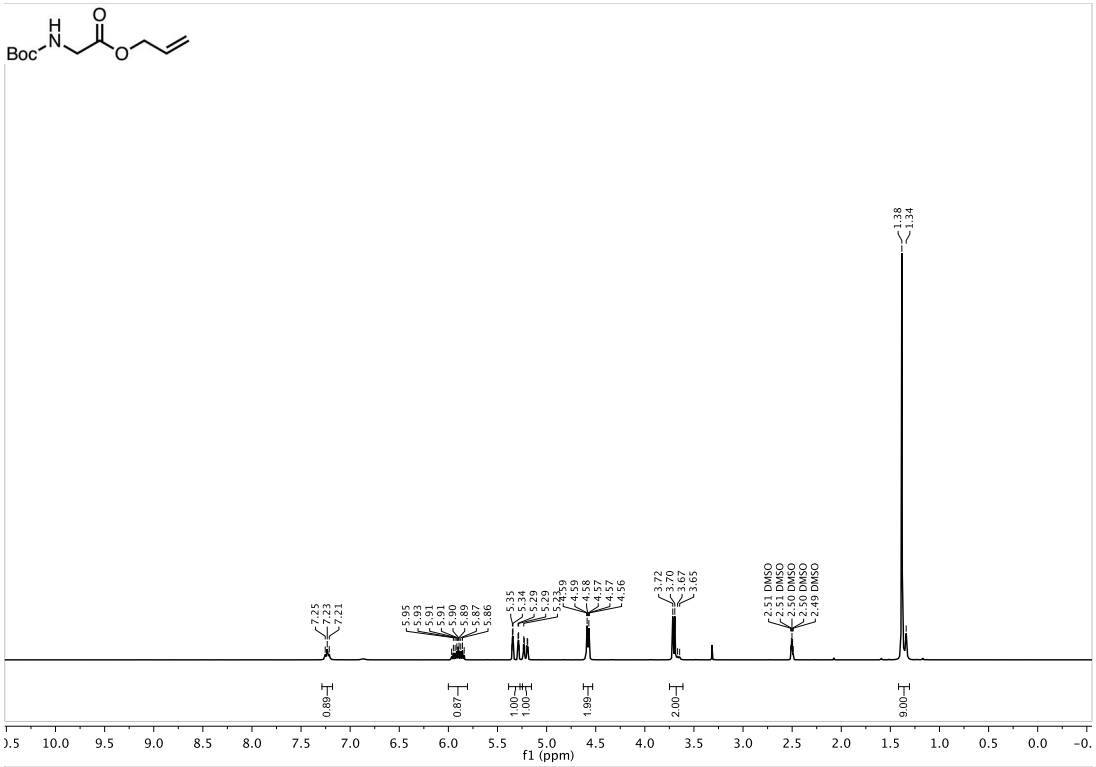
14. NMR spectra

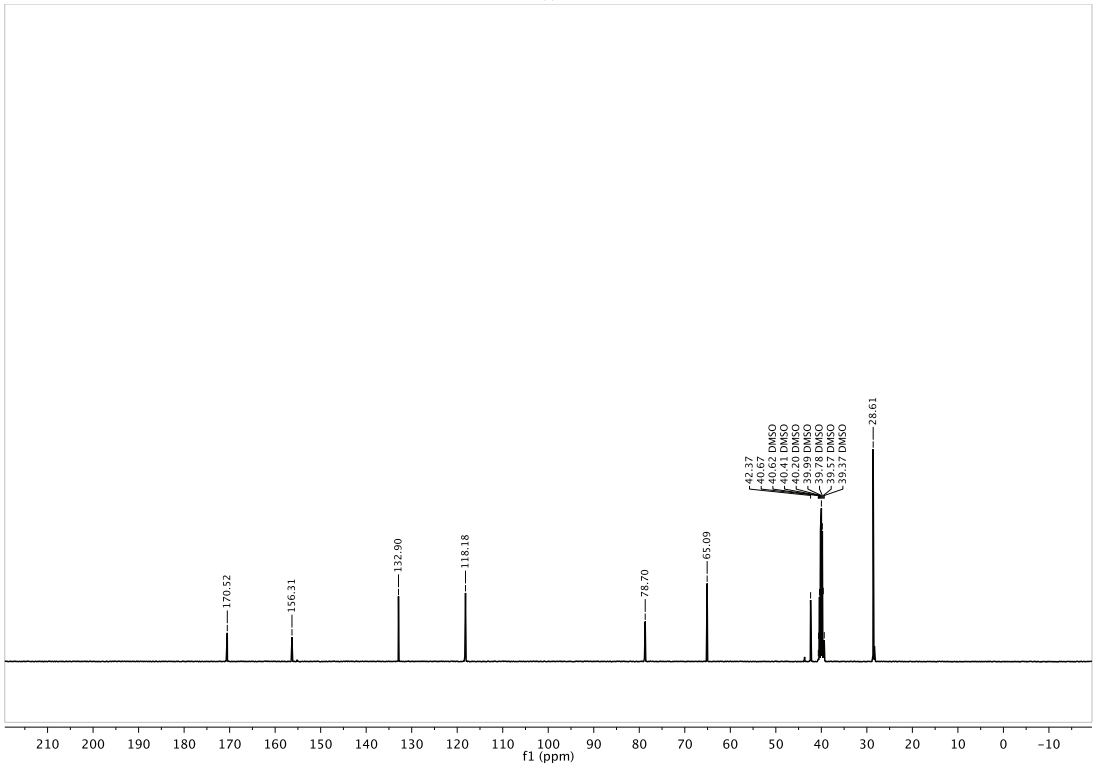
14.1. Sulfonyl bis (4-nitrophenyl carbonate) (1)



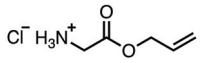


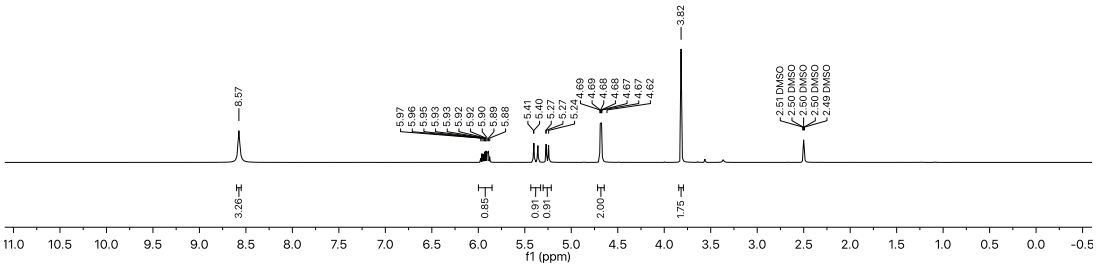
14.2. N-Boc Glycine allyl ester (17)

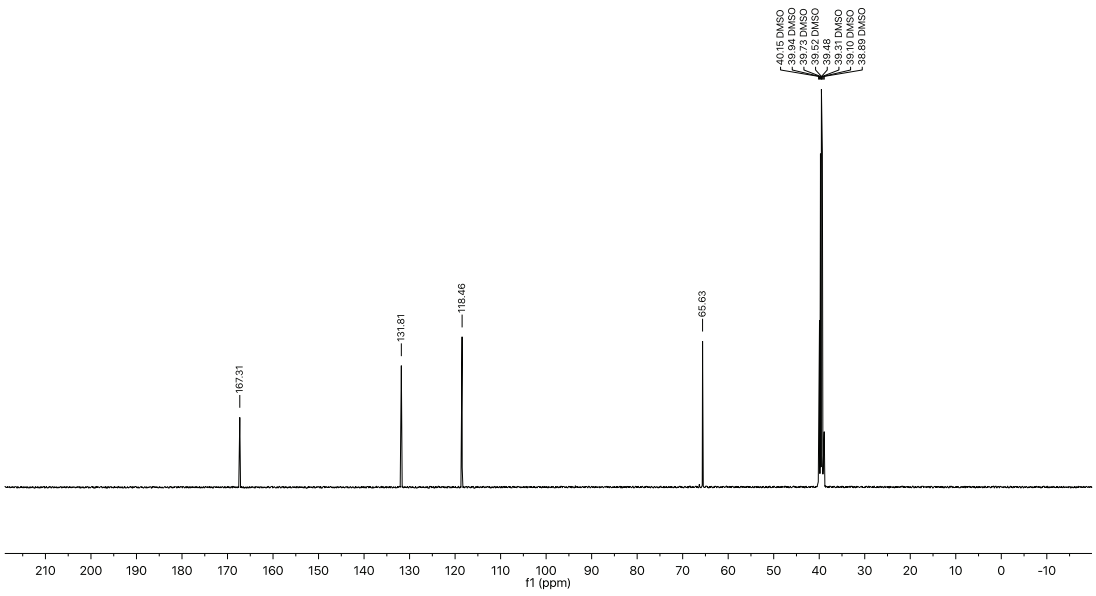




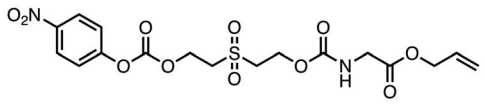
14.3. Glycine allyl ester hydrochloride (2)

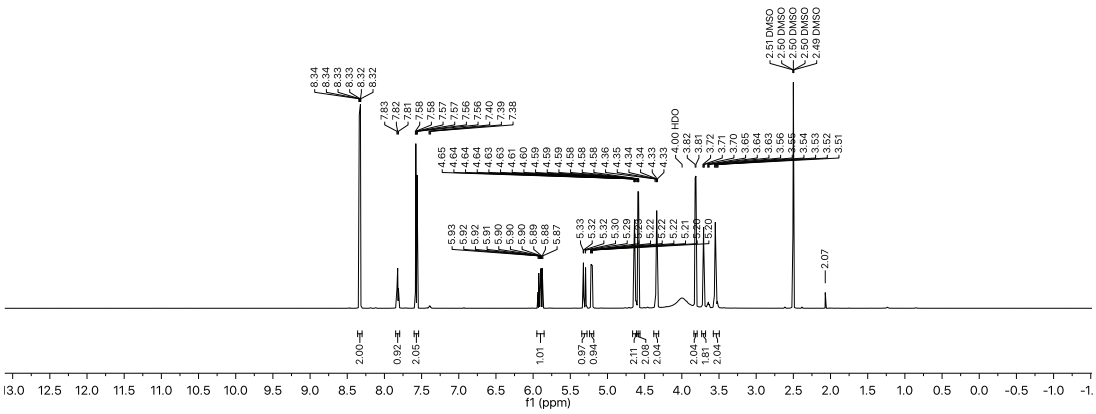


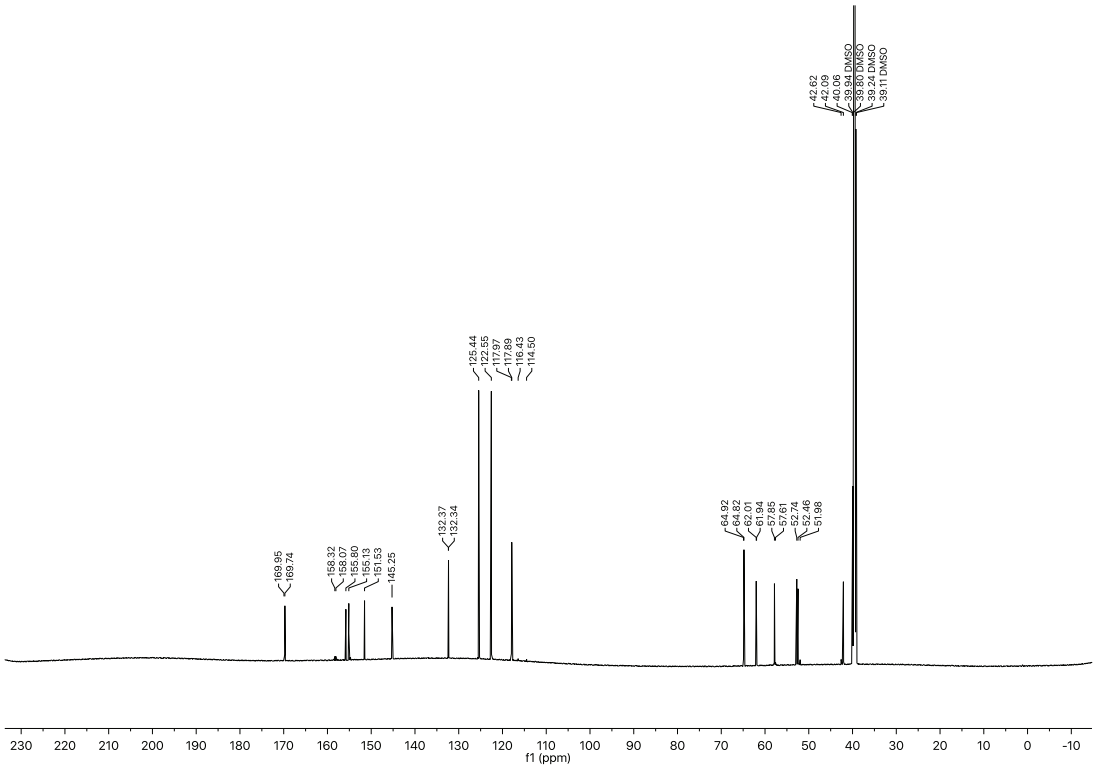




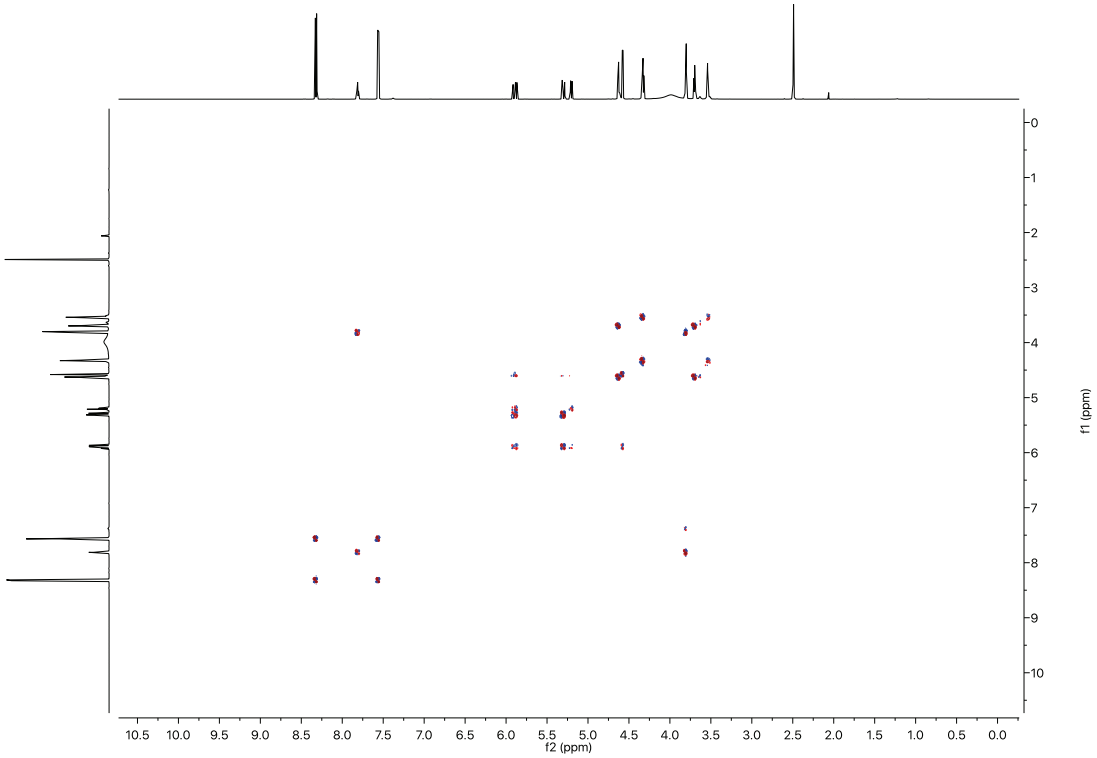
14.4. Allyl diethoxy sulfonyl (4-nitrophenyl carbonate) glycinate (3)



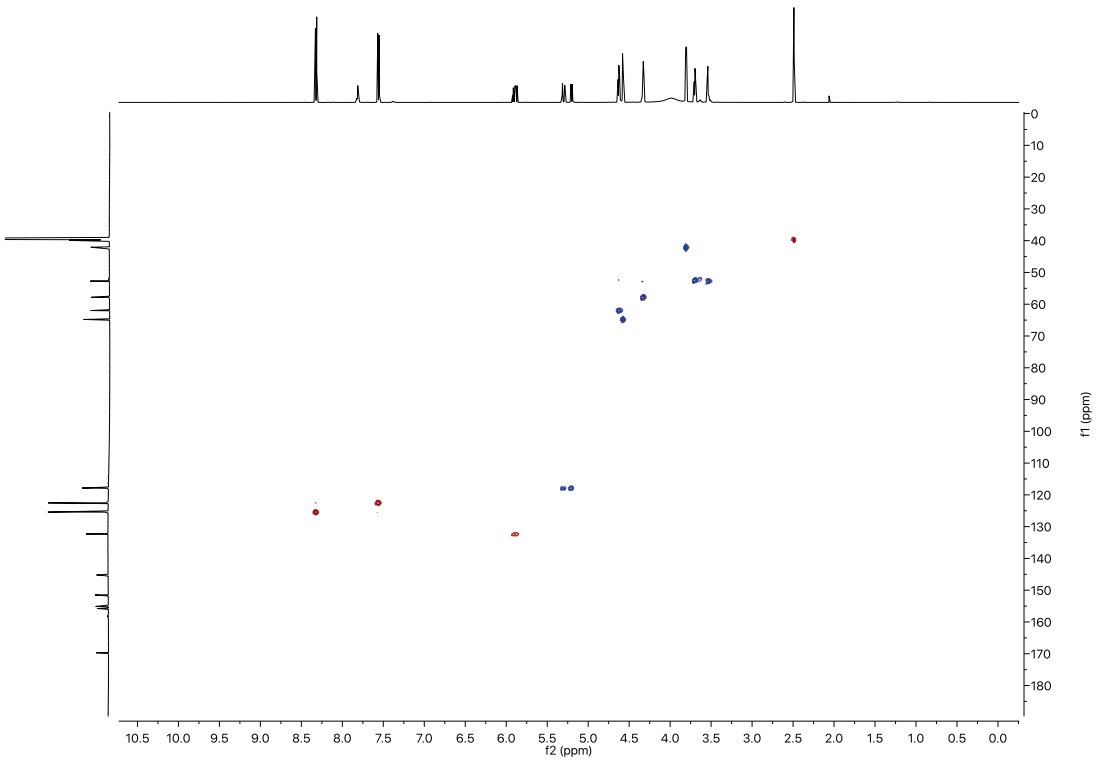




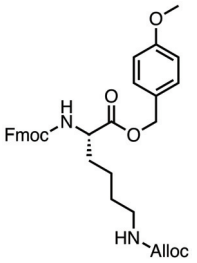
^{1}H, ^{1}H-COSY

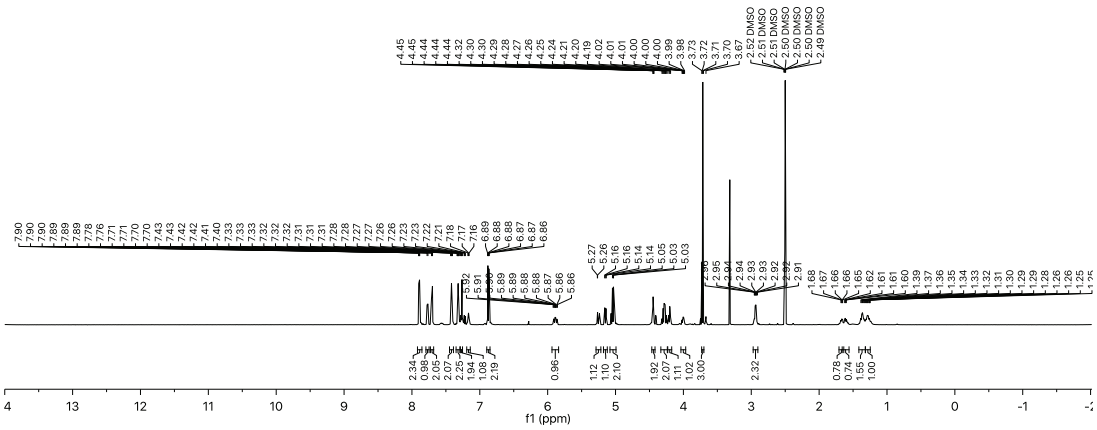


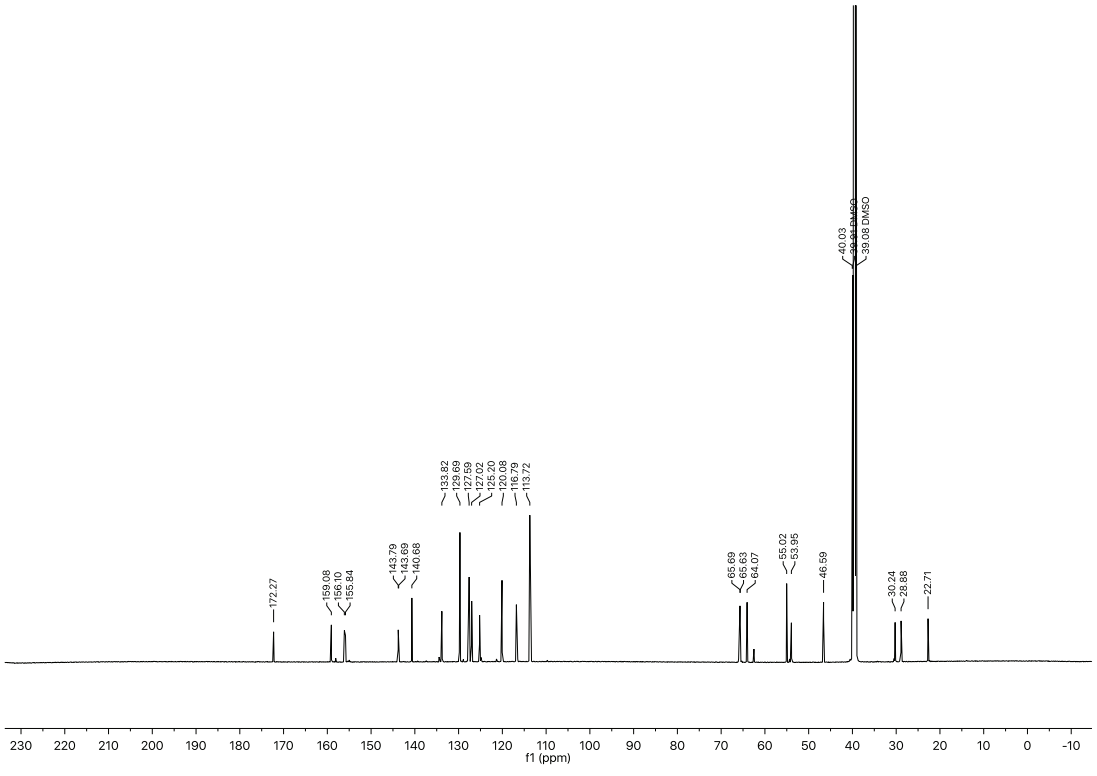
^{1}H, ^{13}C-HSQC



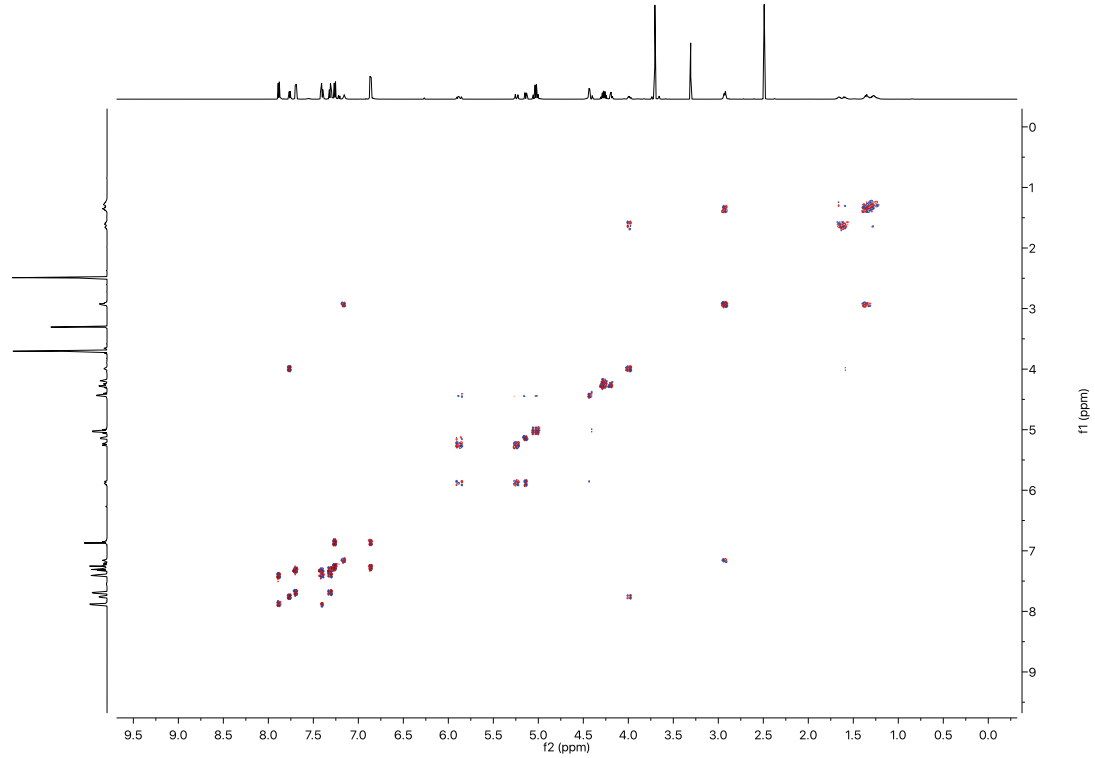
14.5. Fmoc-Lys(Alloc)-OPMB (18)



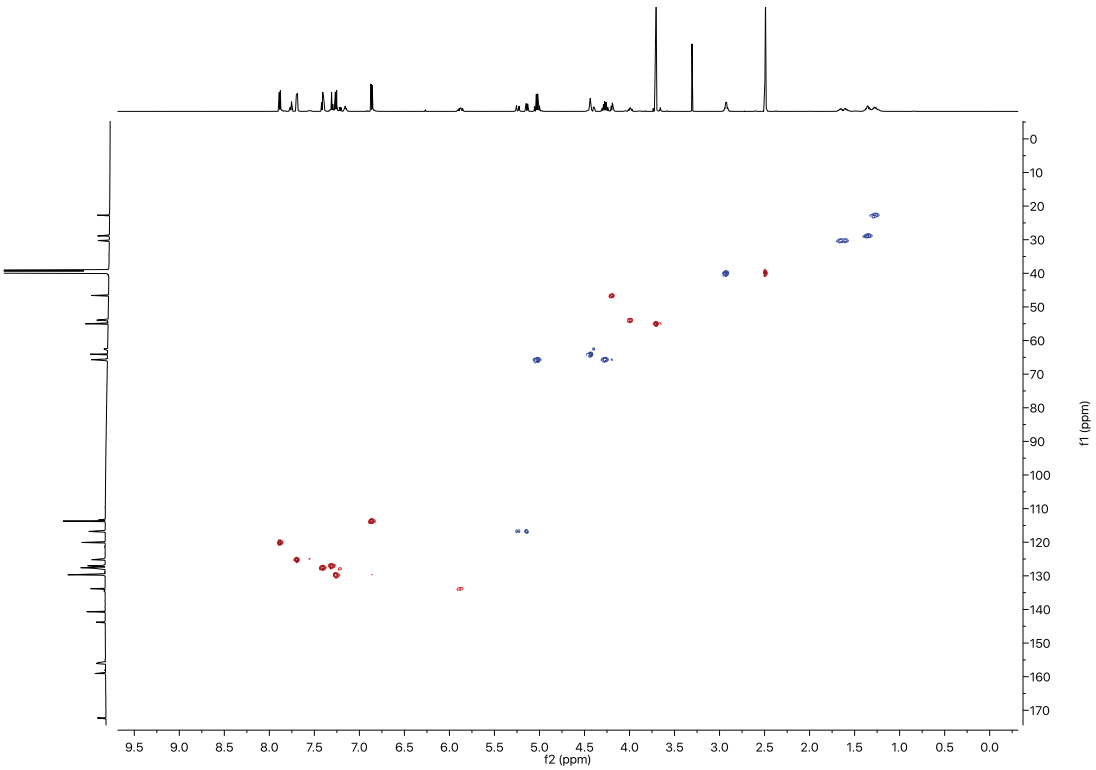




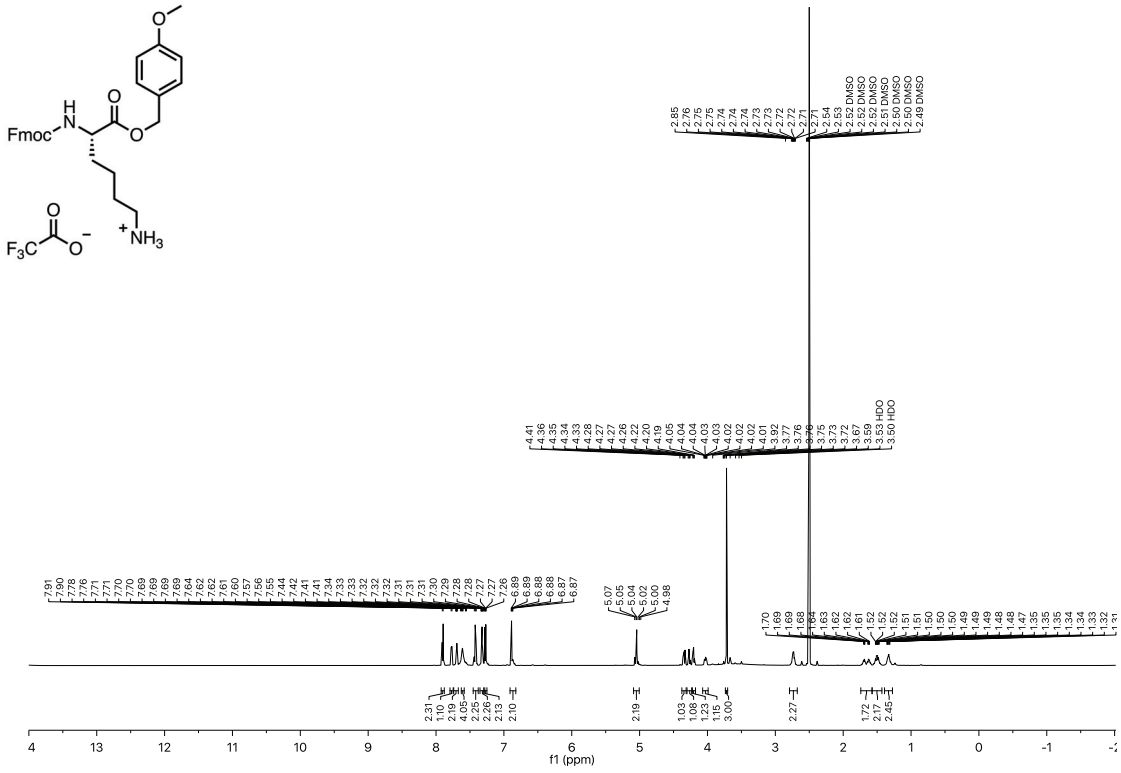
^{1}H, ^{1}H-COSY

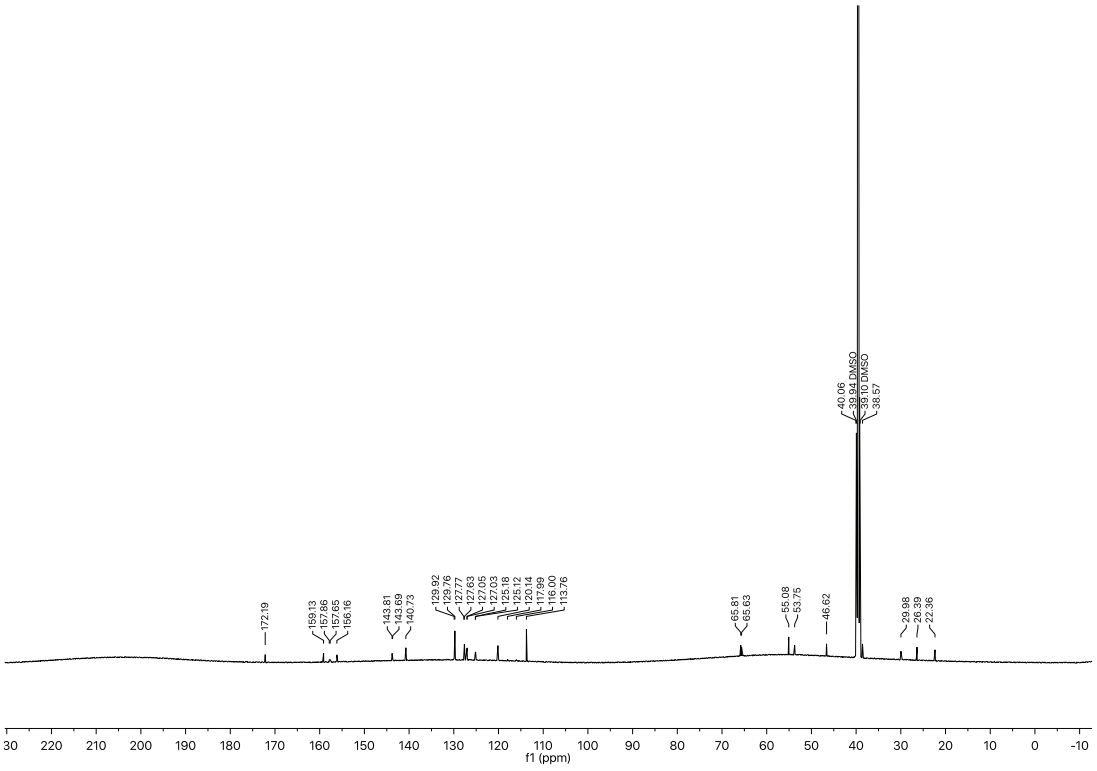


^{1}H, ^{13}C-HSQC

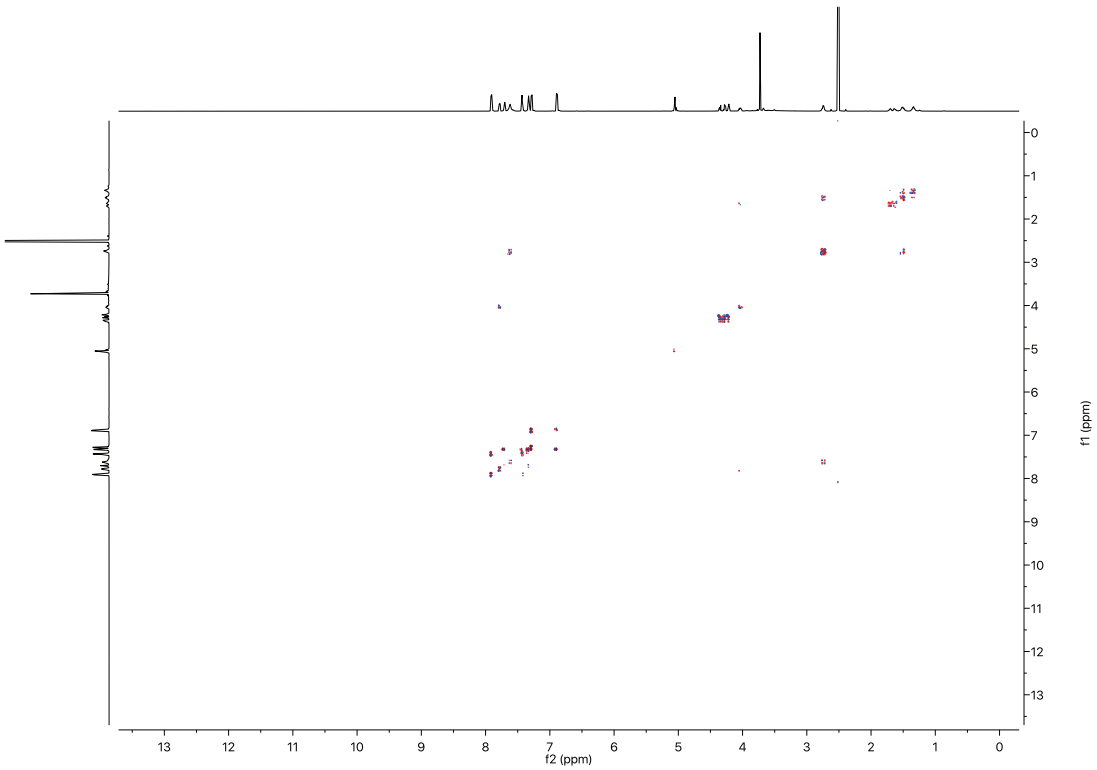


14.6. Fmoc-Lys(NH_2)-OPmb TFA salt (4a)

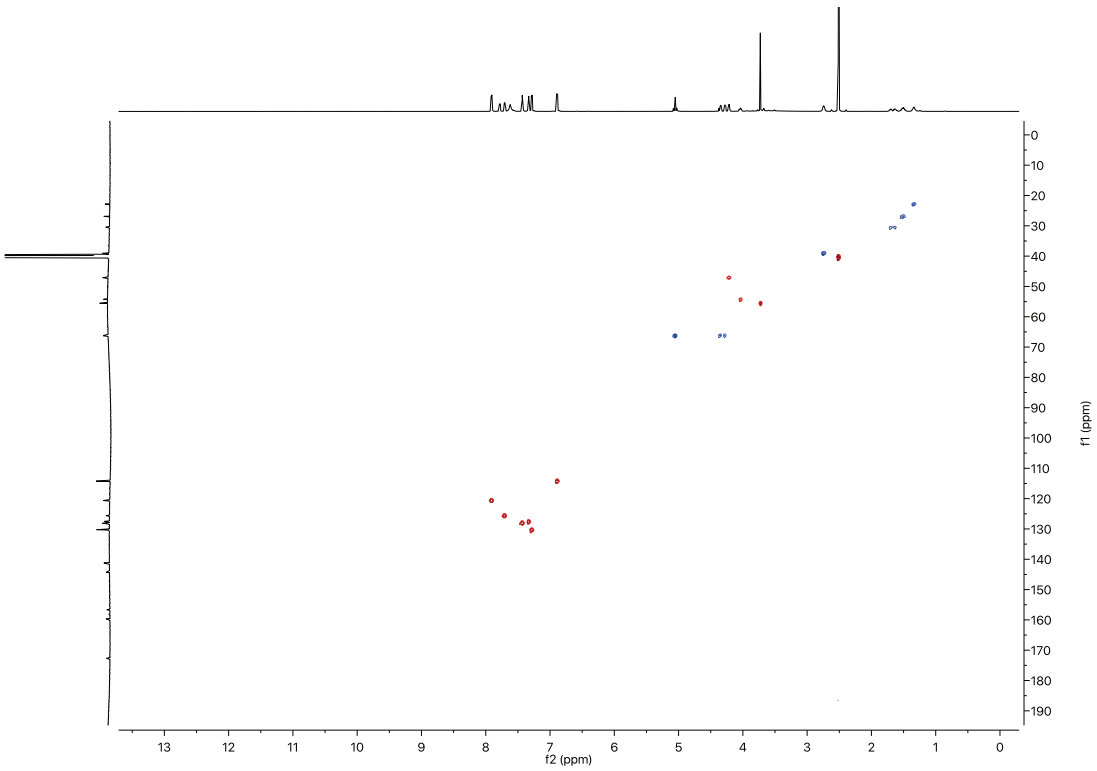




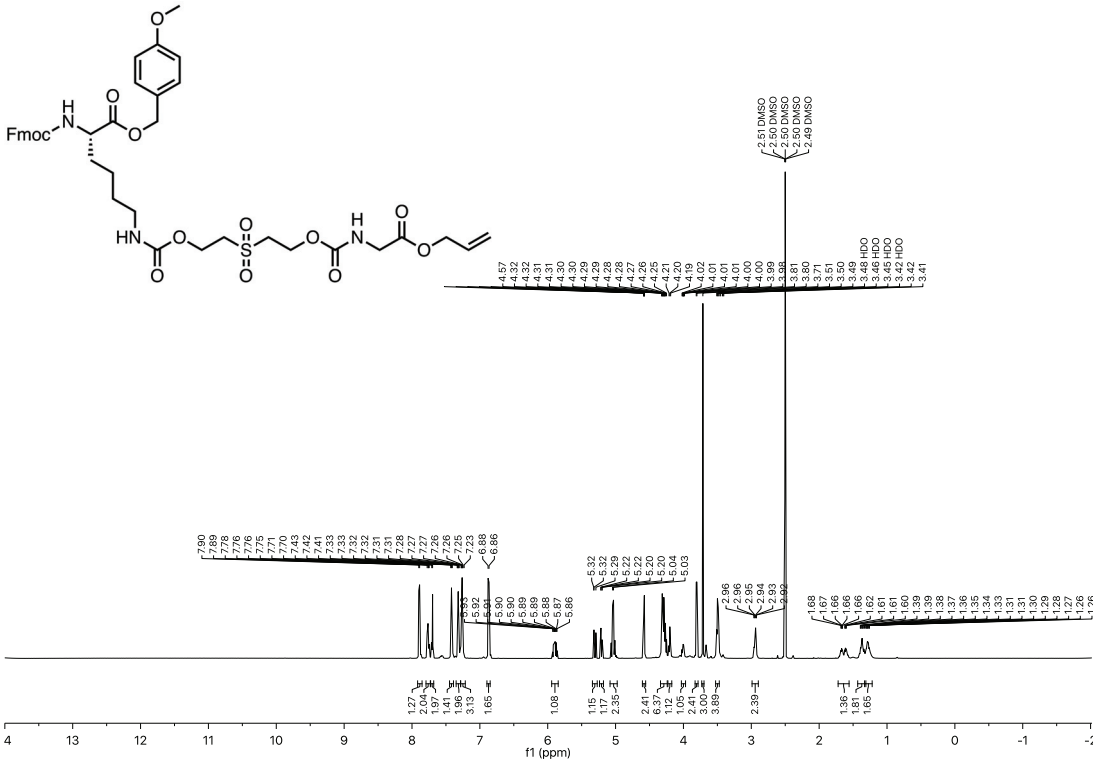
^{1}H, ^{1}H-COSY

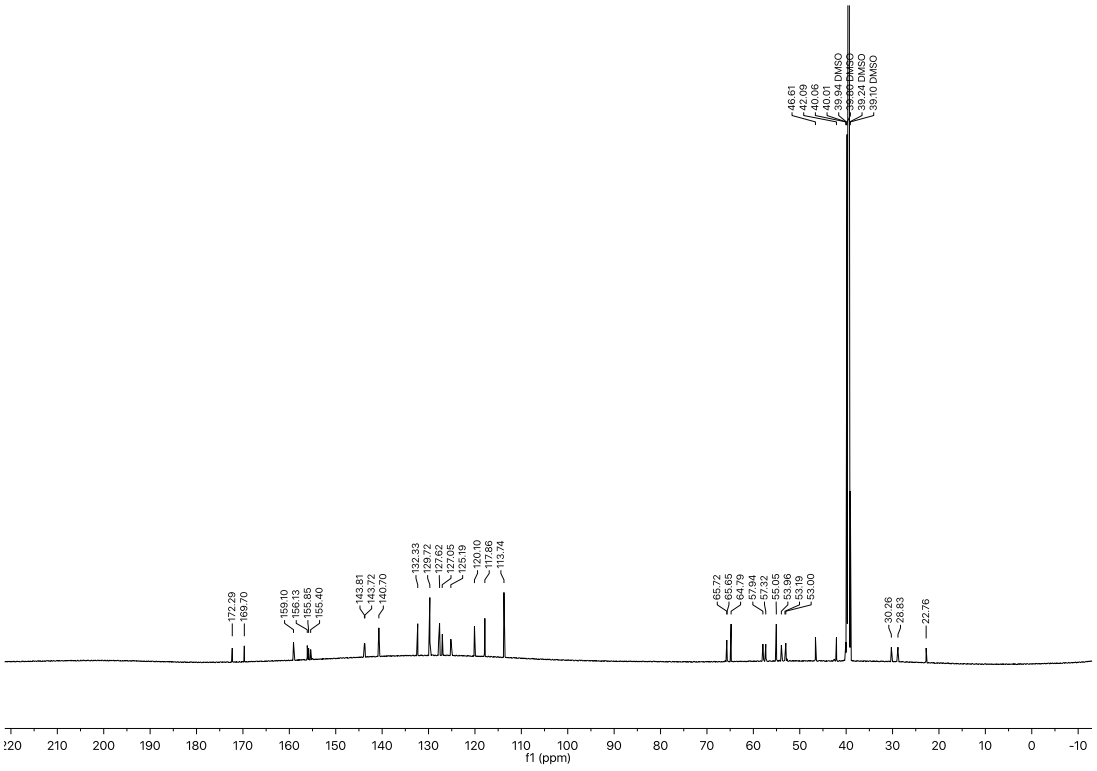


^{1}H, ^{13}C-HSQC

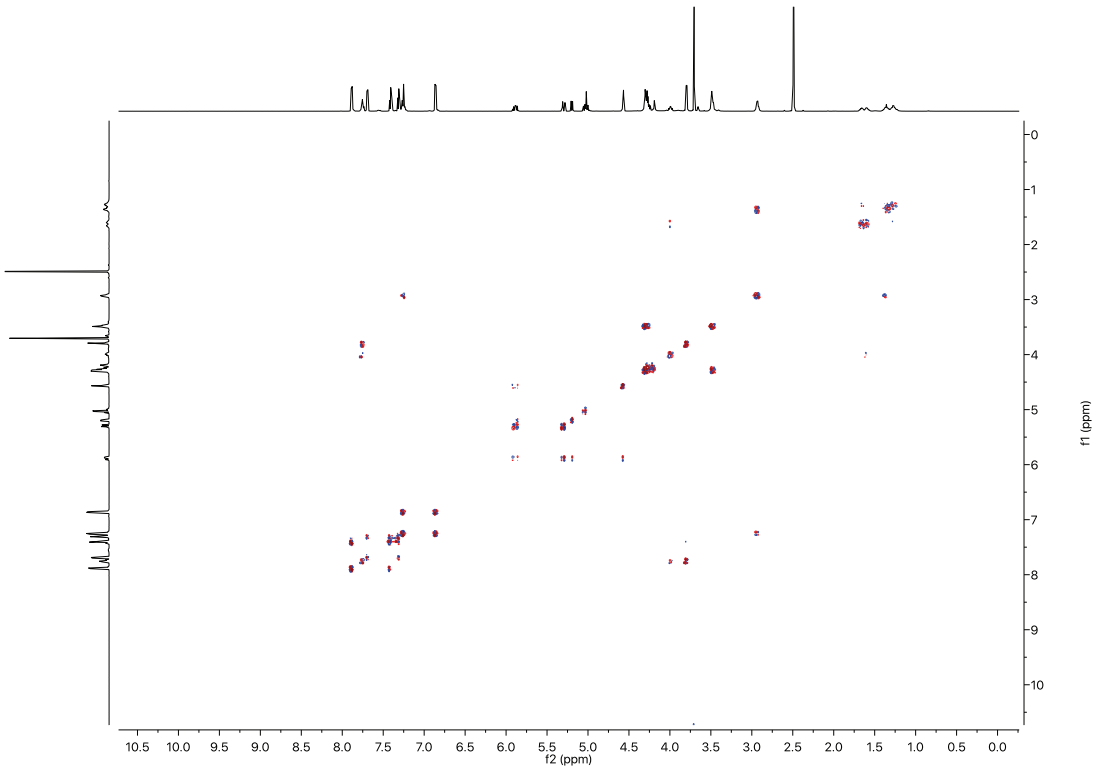


14.7. Linker l. allyl ester (19)

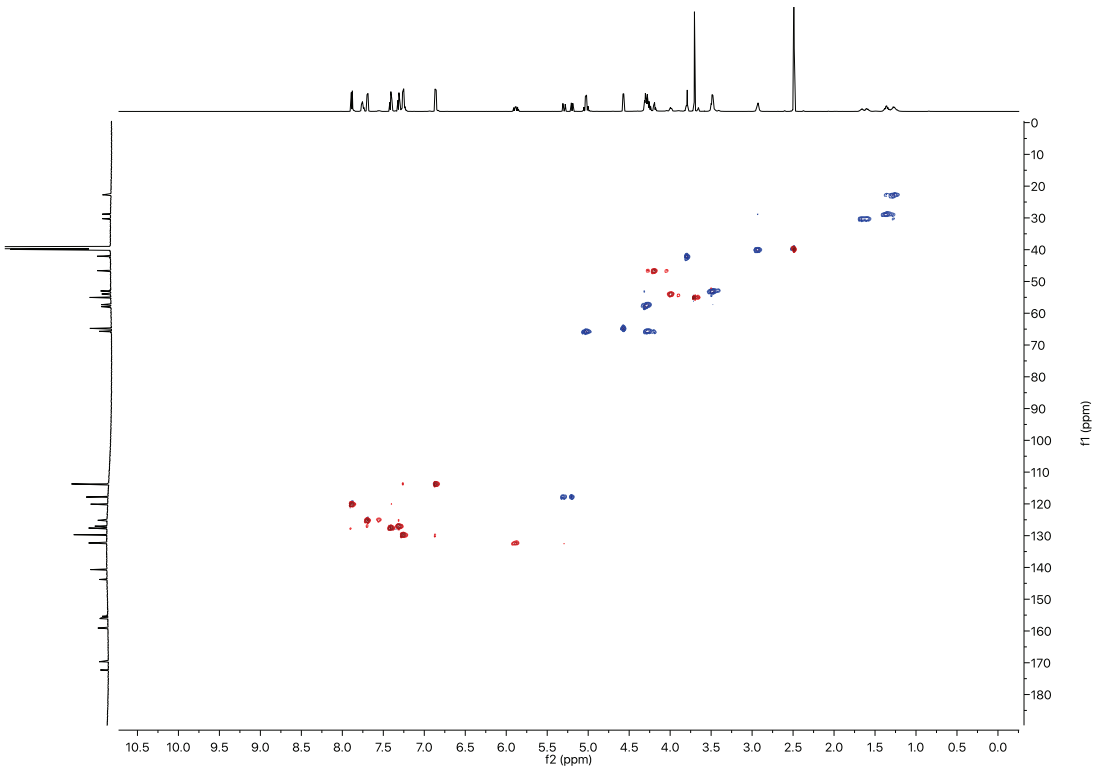




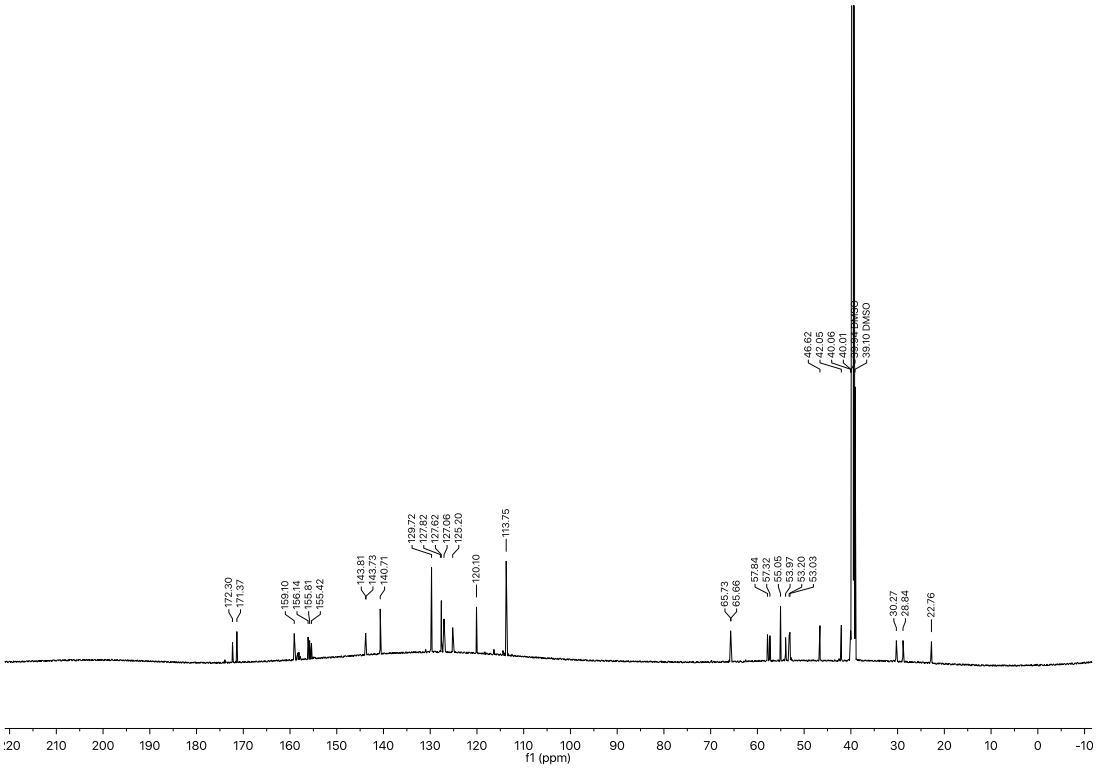
^{1}H, ^{1}H-COSY



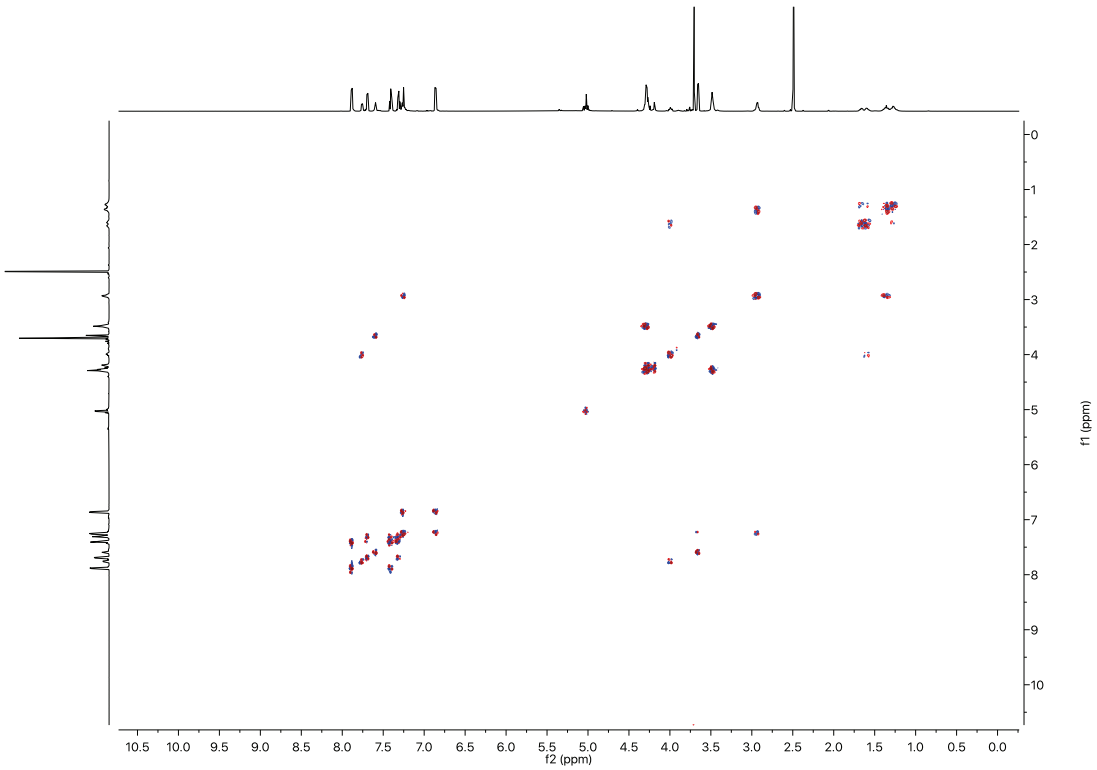
^{1}H, ^{13}C-HSQC



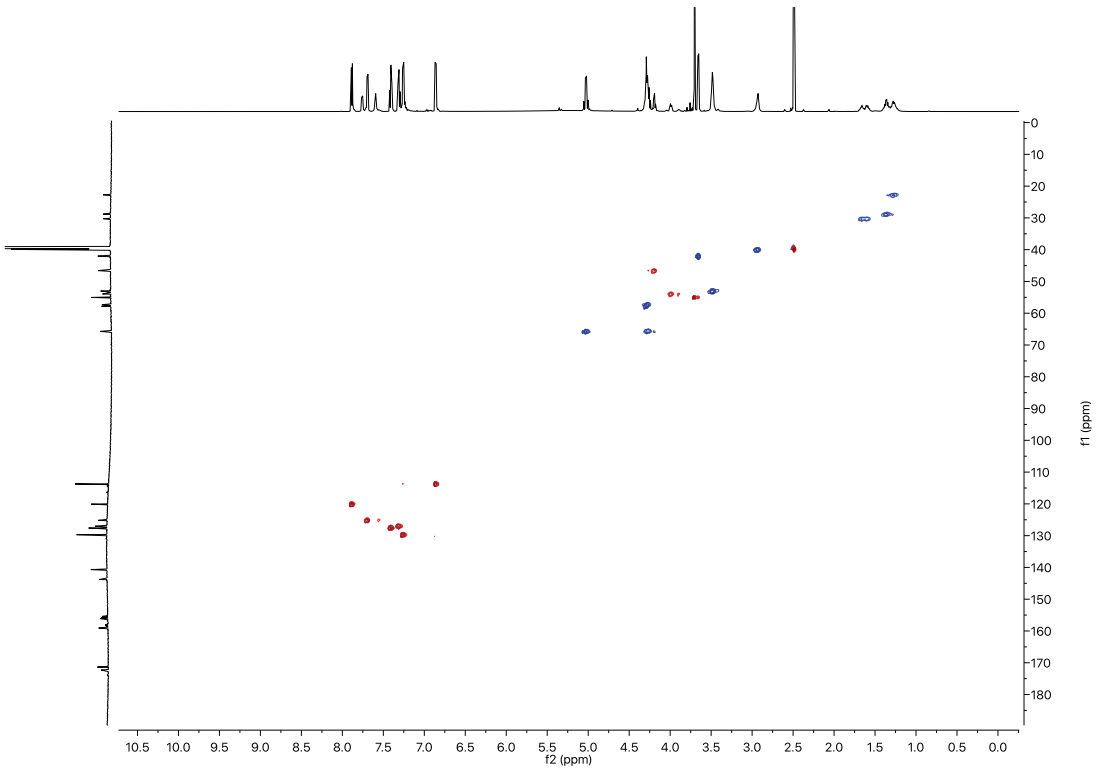
14.1. Linker I. (5a)



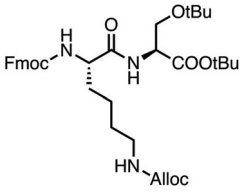
^{1}H, ^{1}H-COSY

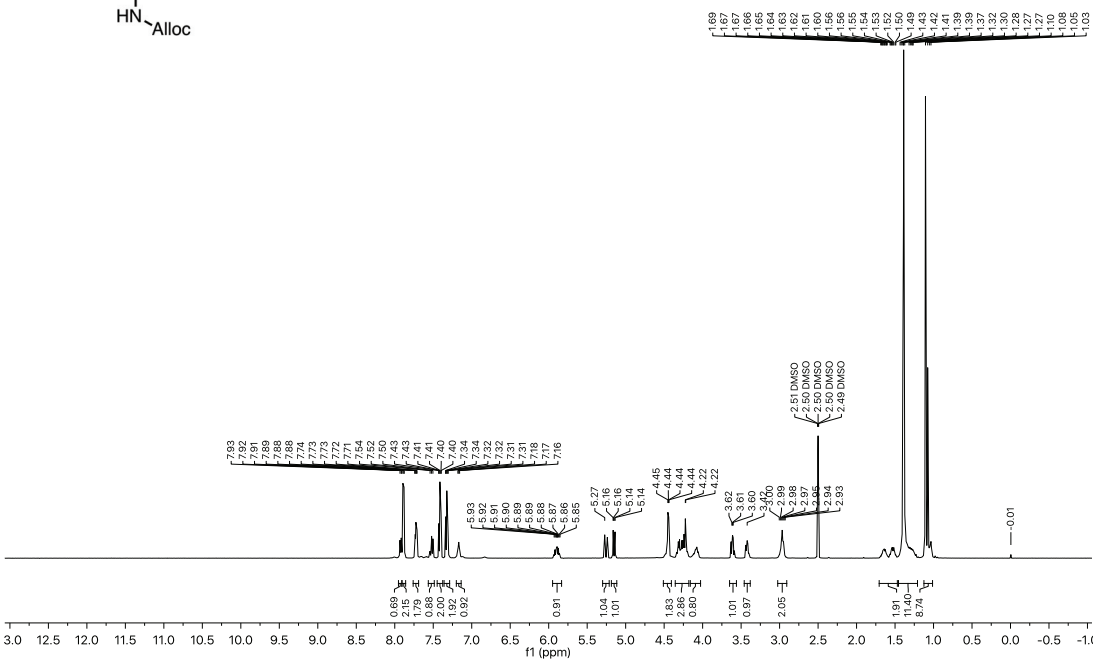


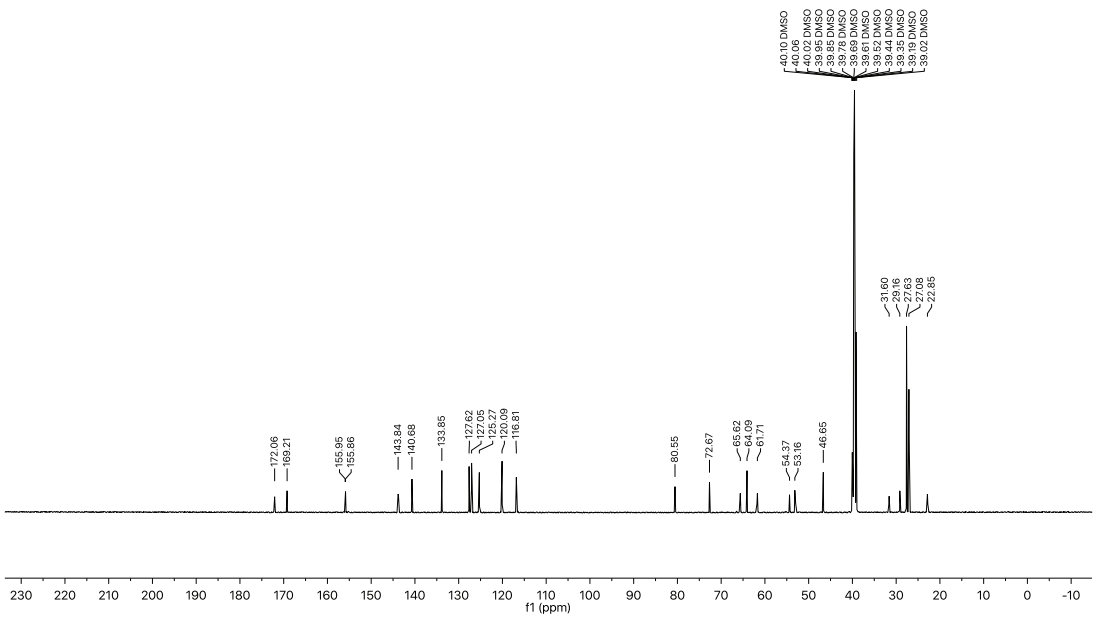
^{1}H, ^{13}C-HSQC



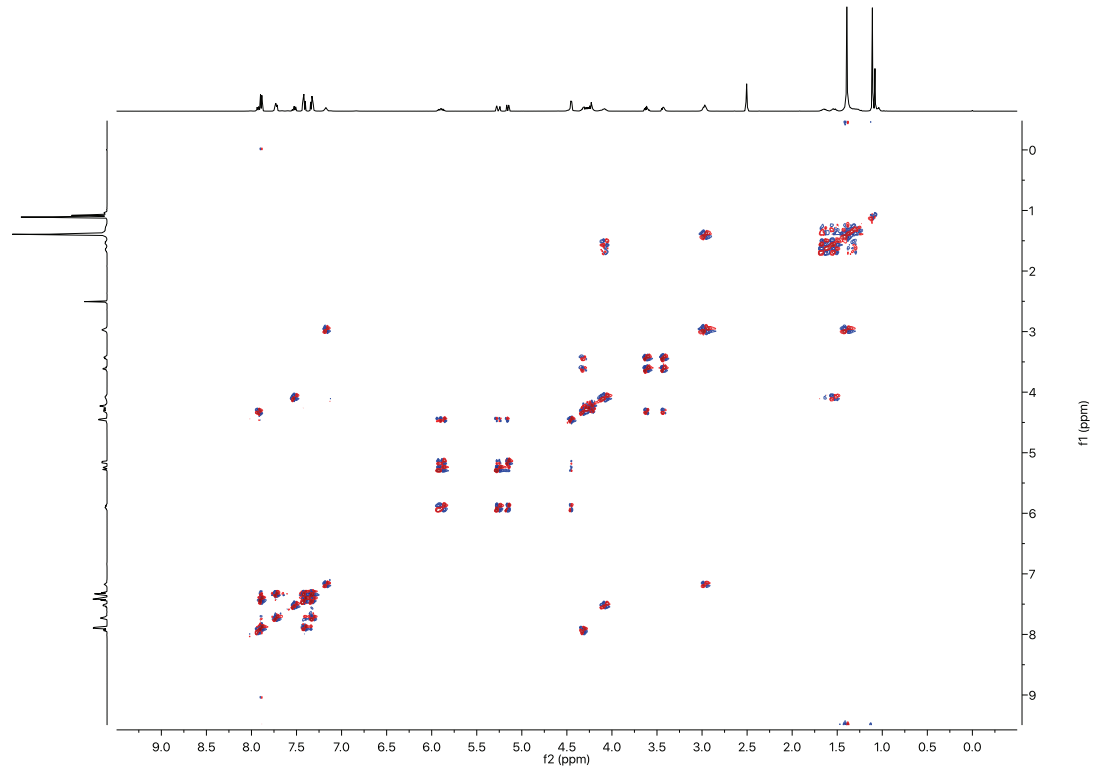
14.2. Fmoc-Lys(NHAlloc)-Ser(tBu)-OtBu (20)



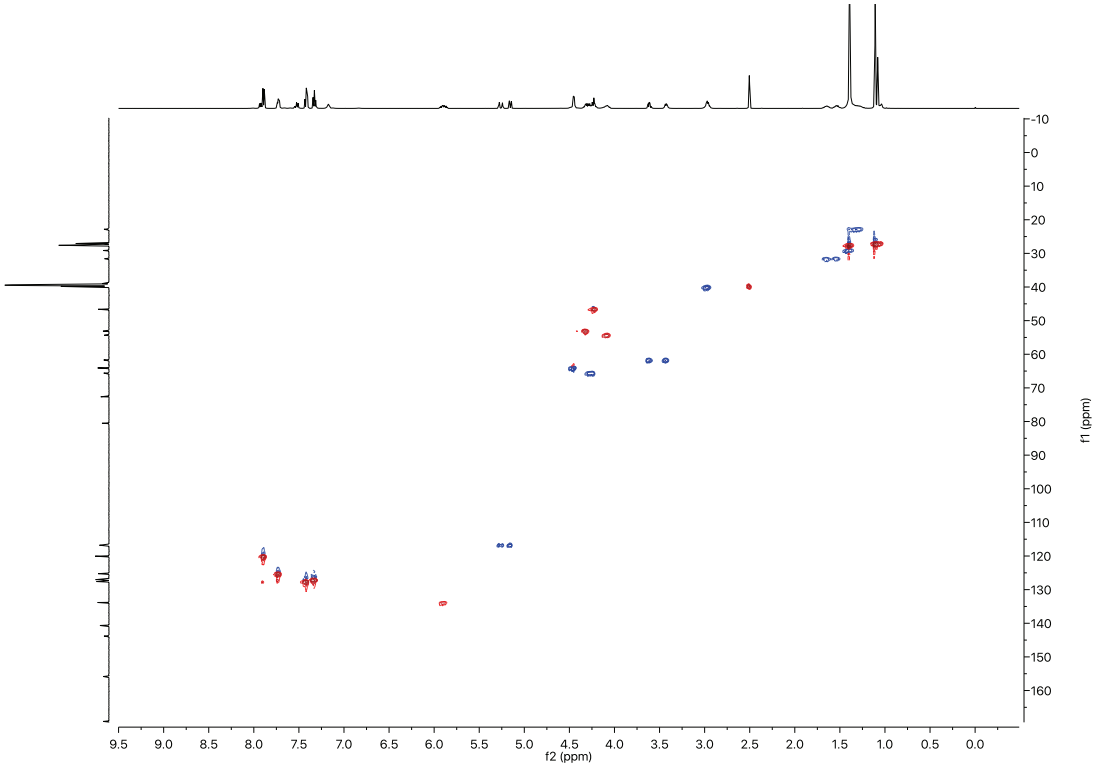




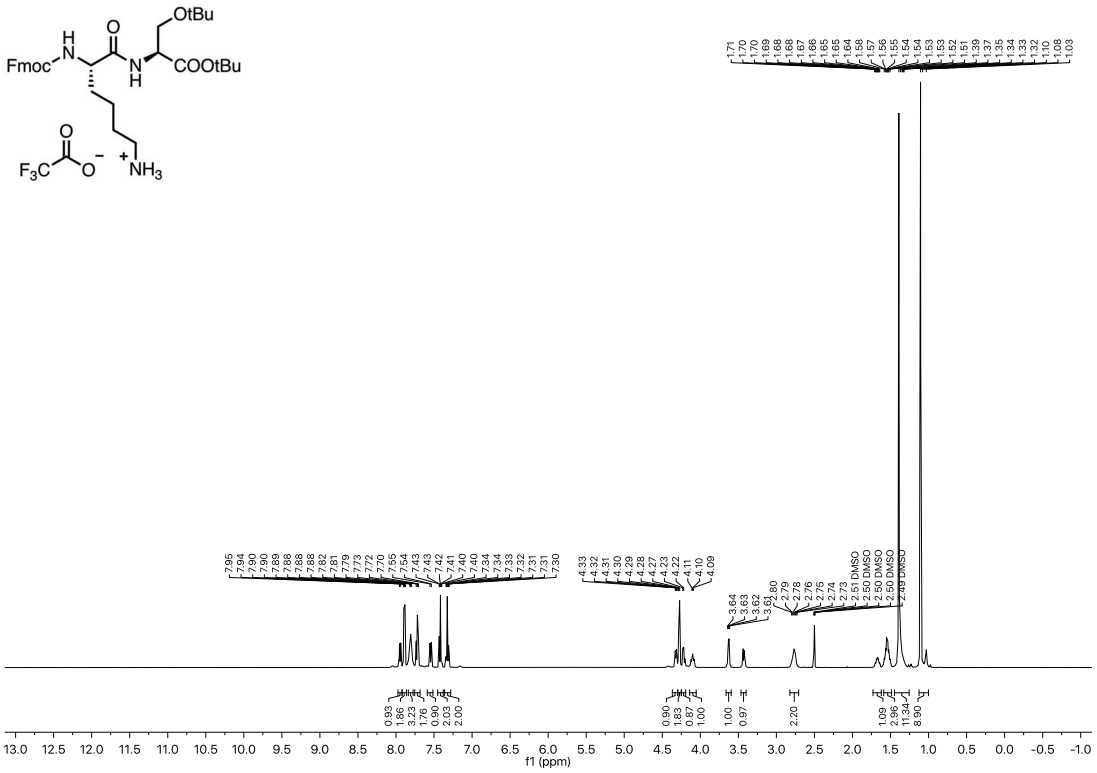
^{1}H, ^{1}H-COSY

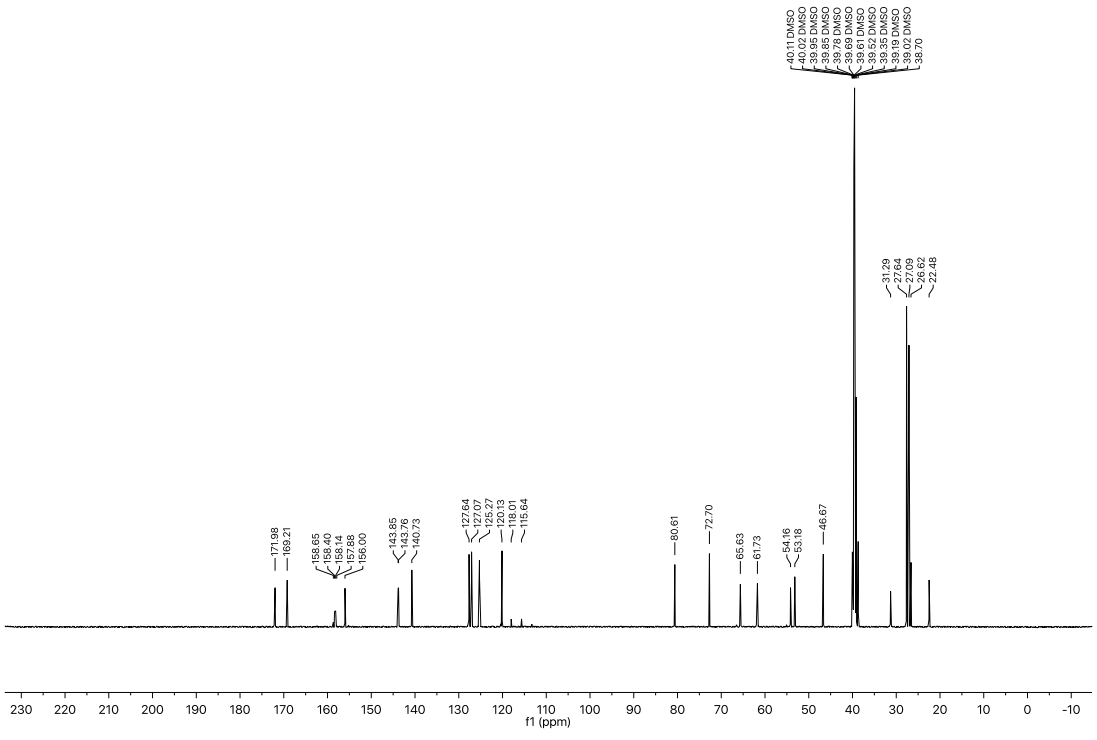


^{1}H, ^{13}C-HSQC

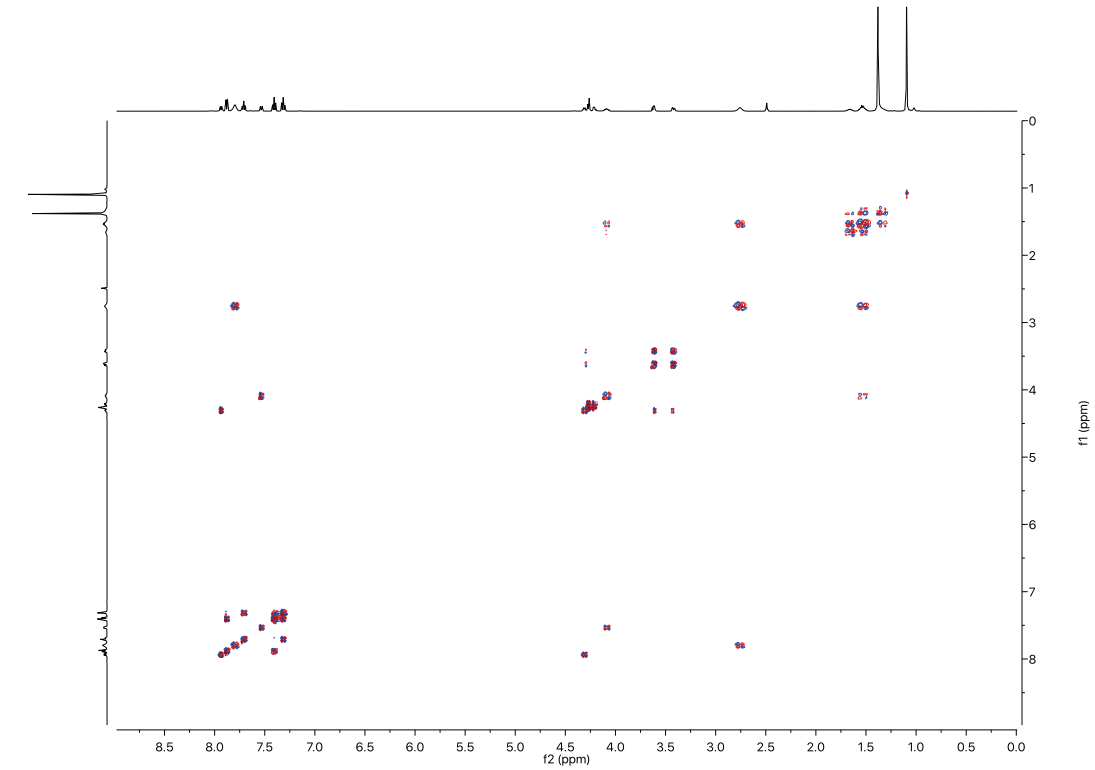


14.3. Fmoc-Lys(NH_2)-Ser(tBu)-OtBu TFA salt (4b)

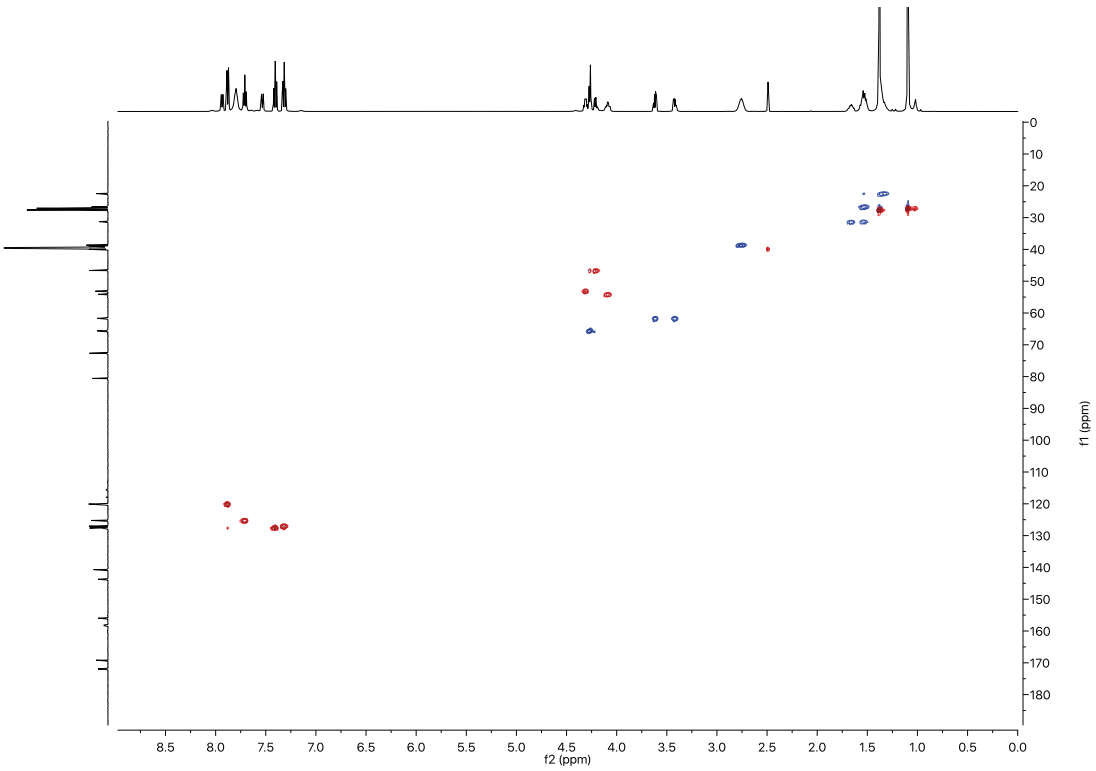




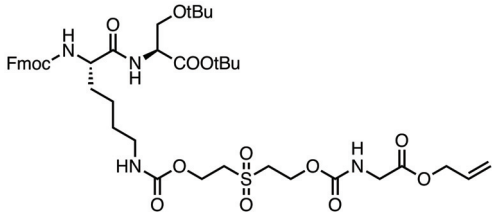
^{1}H, ^{1}H-COSY

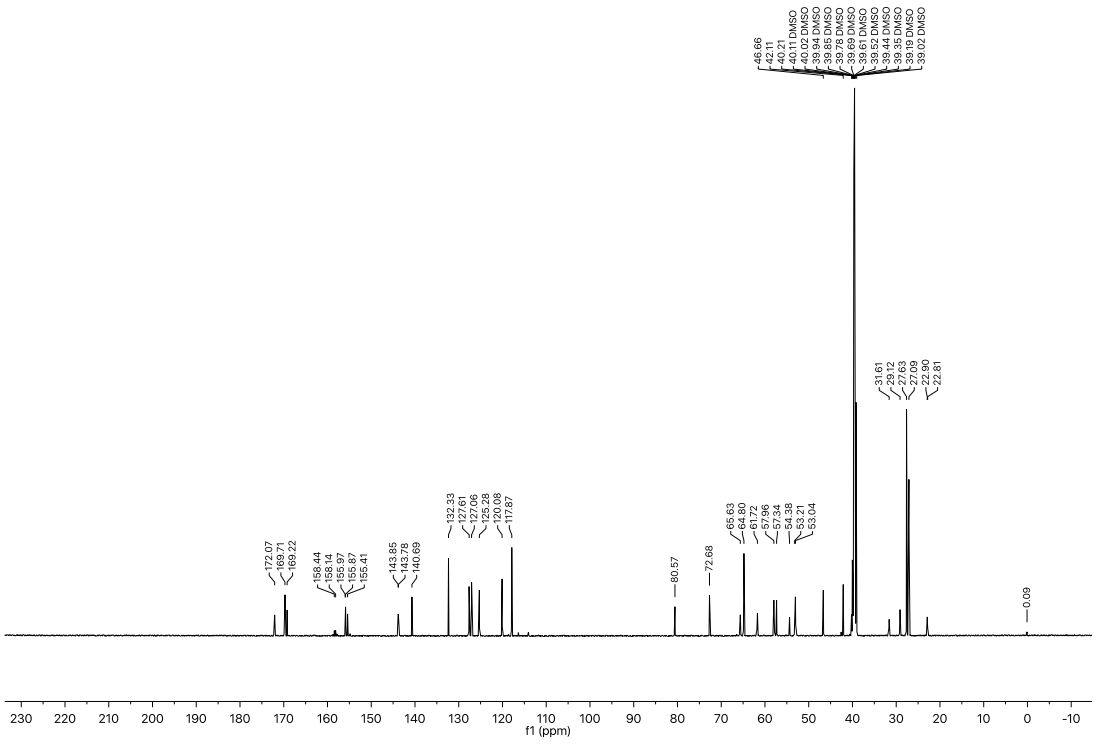


^{1}H, ^{13}C-HSQC

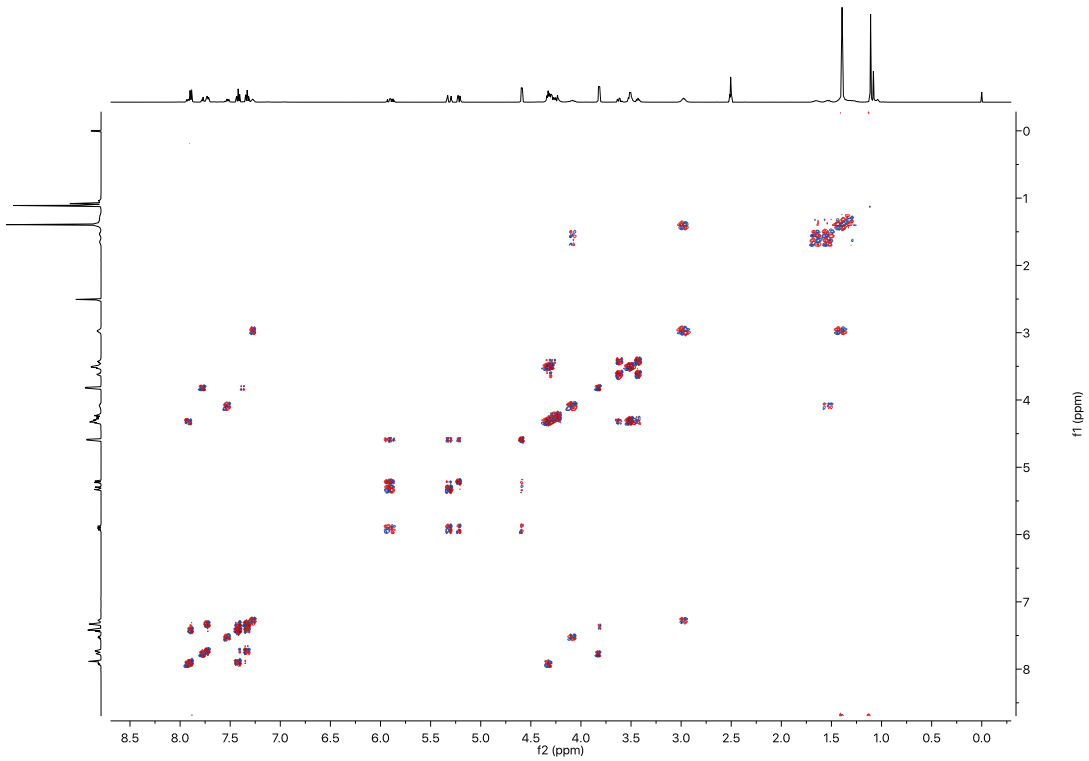


14.4. Linker II. allyl ester (21)

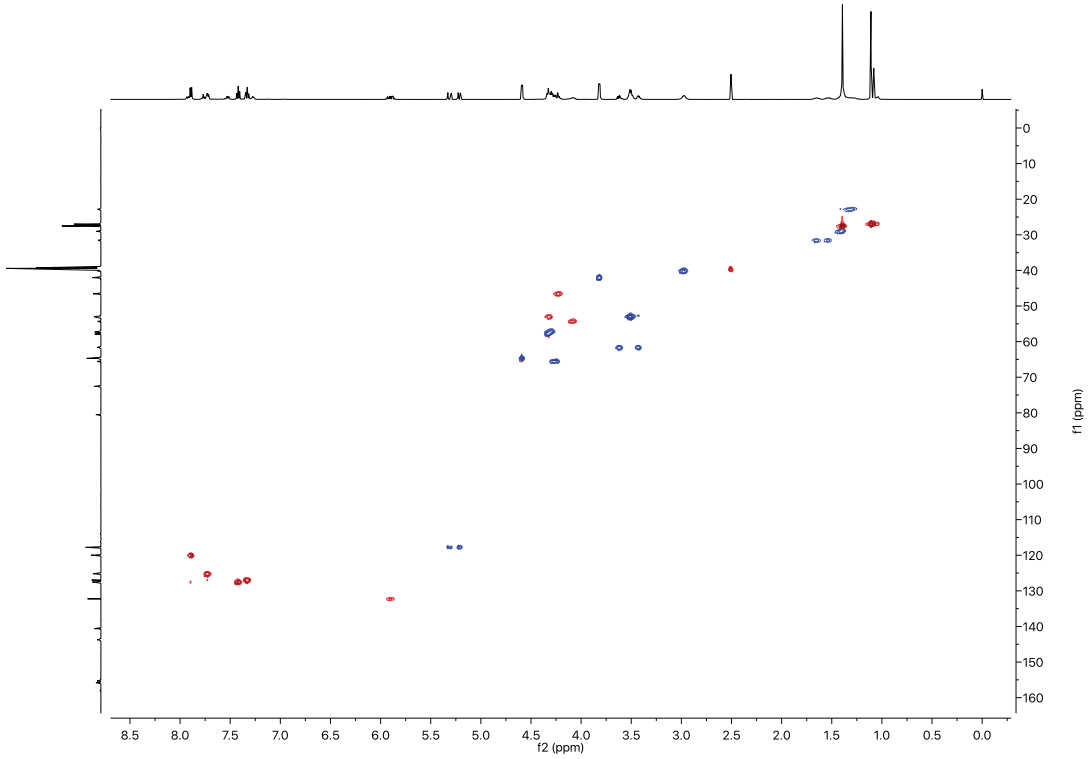




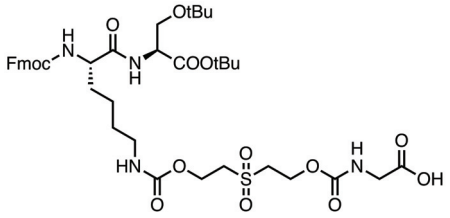
^{1}H, ^{1}H-COSY

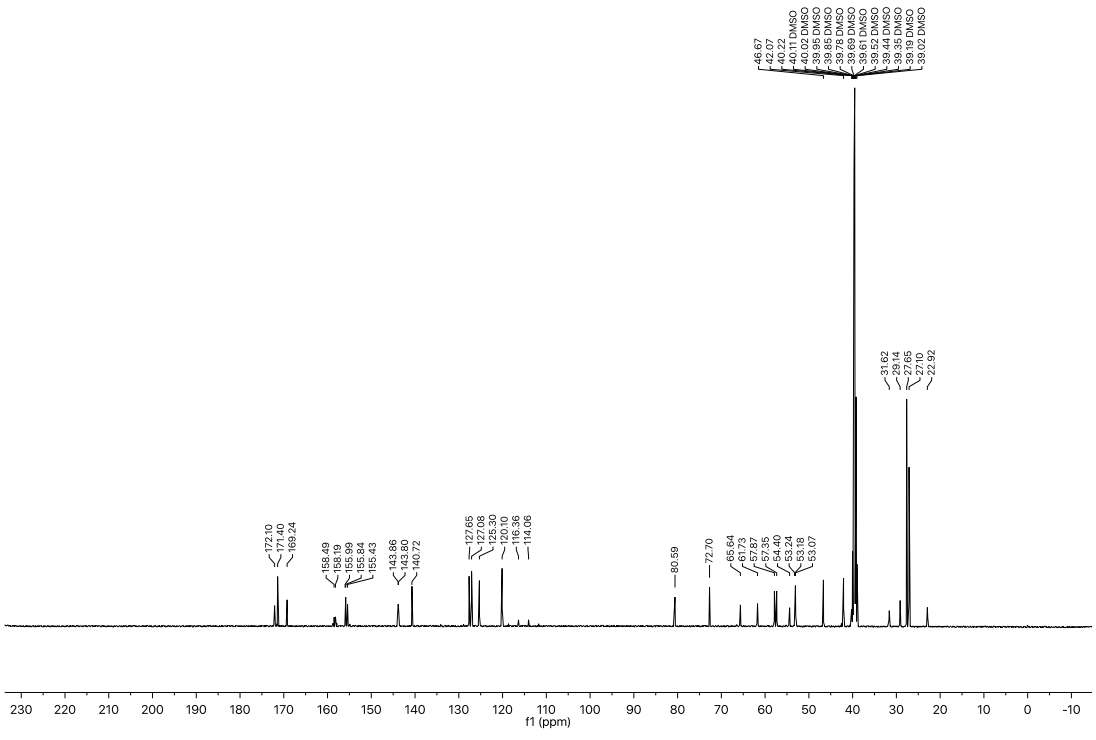


^{1}H, ^{13}C-HSQC

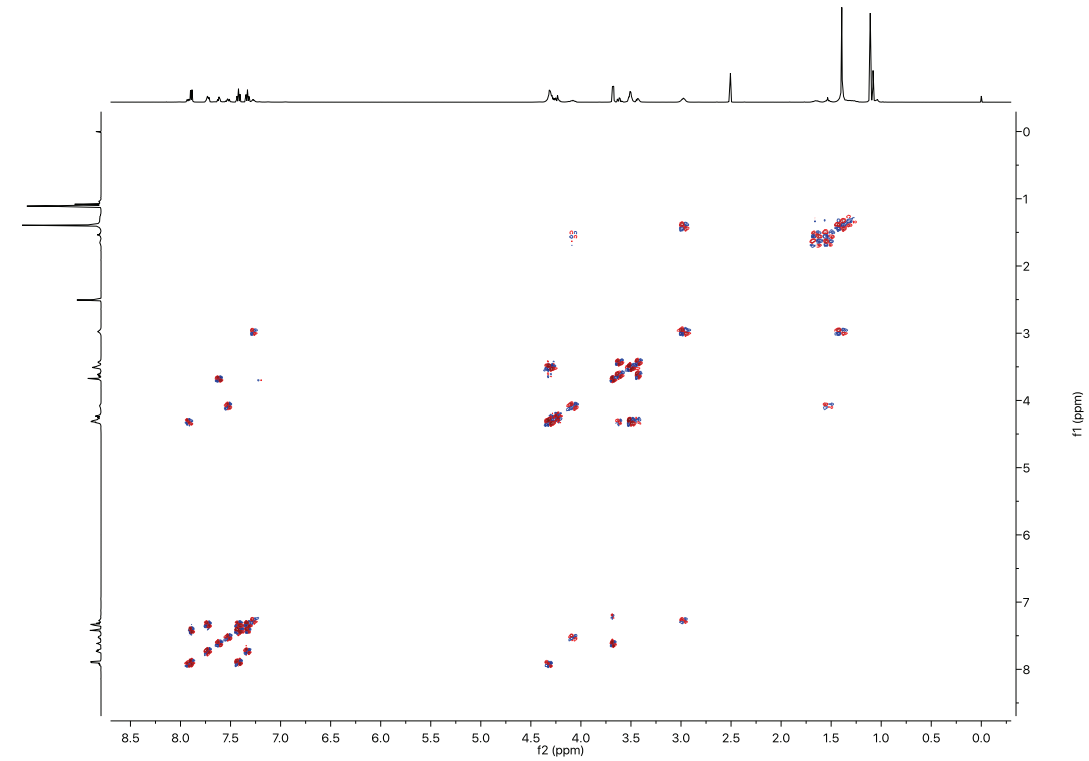


14.5. Linker II. (5b)

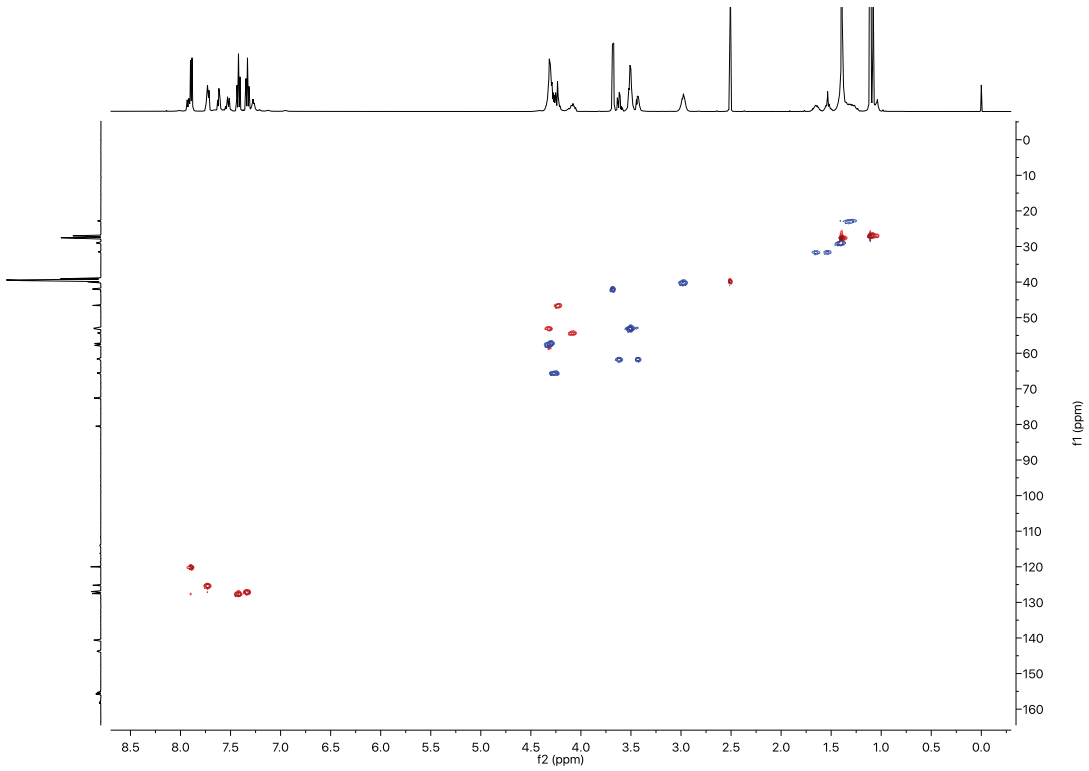




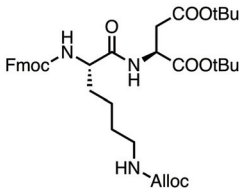
^{1}H, ^{1}H-COSY

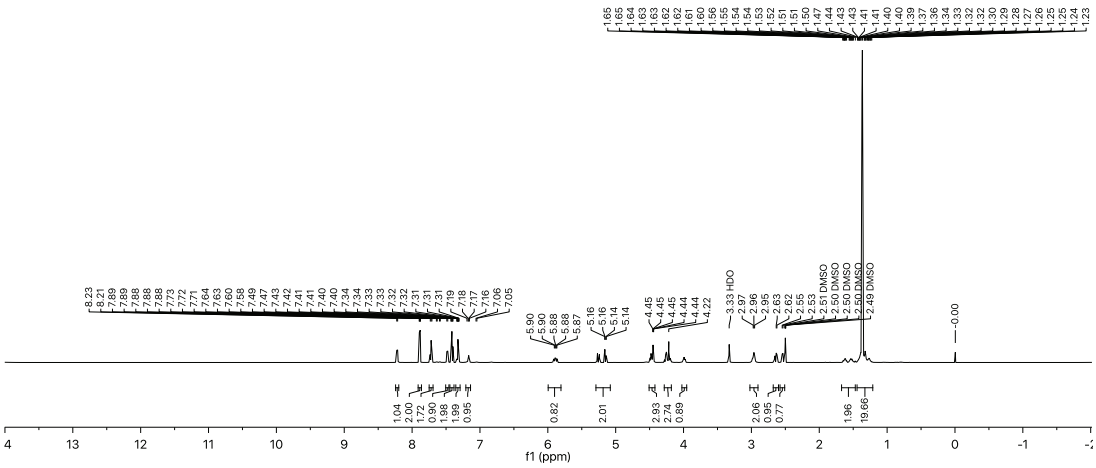


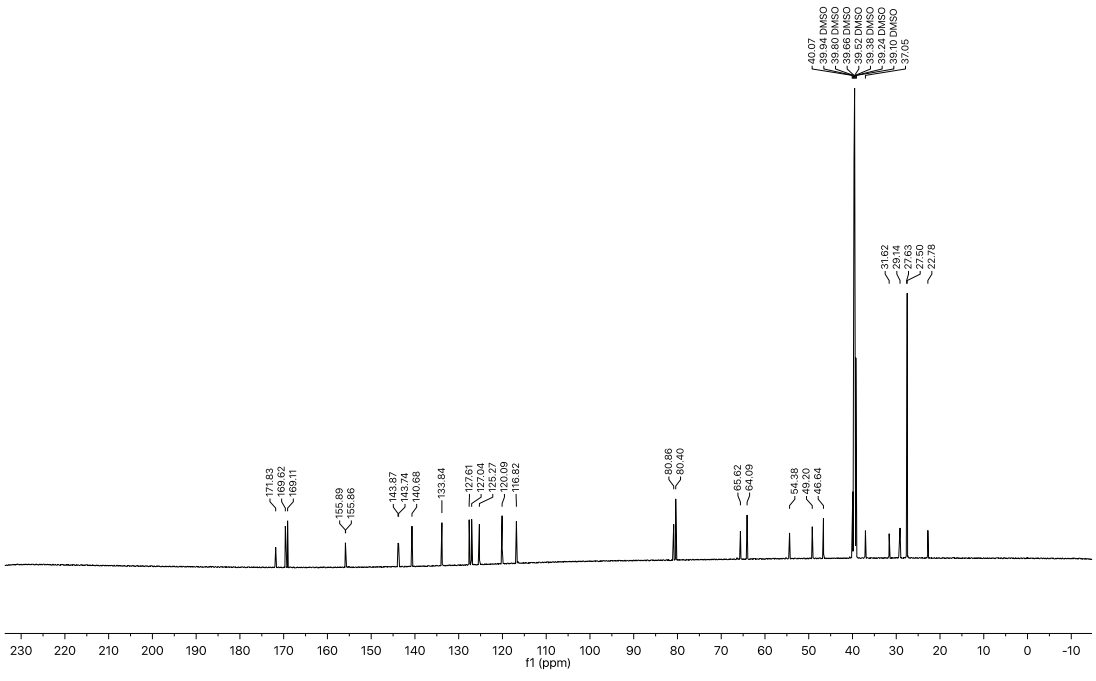
^{1}H, ^{13}C-HSQC



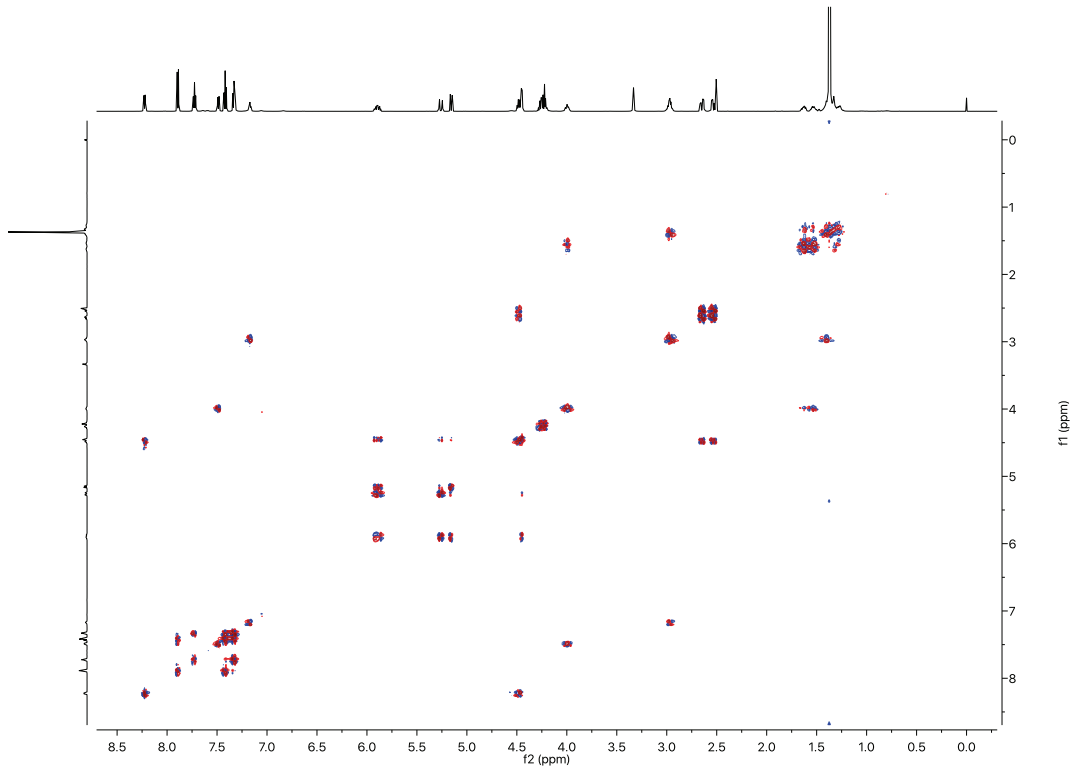
14.6. Fmoc-Lys(NHAlloc)-Asp(OtBu)-OtBu (24)



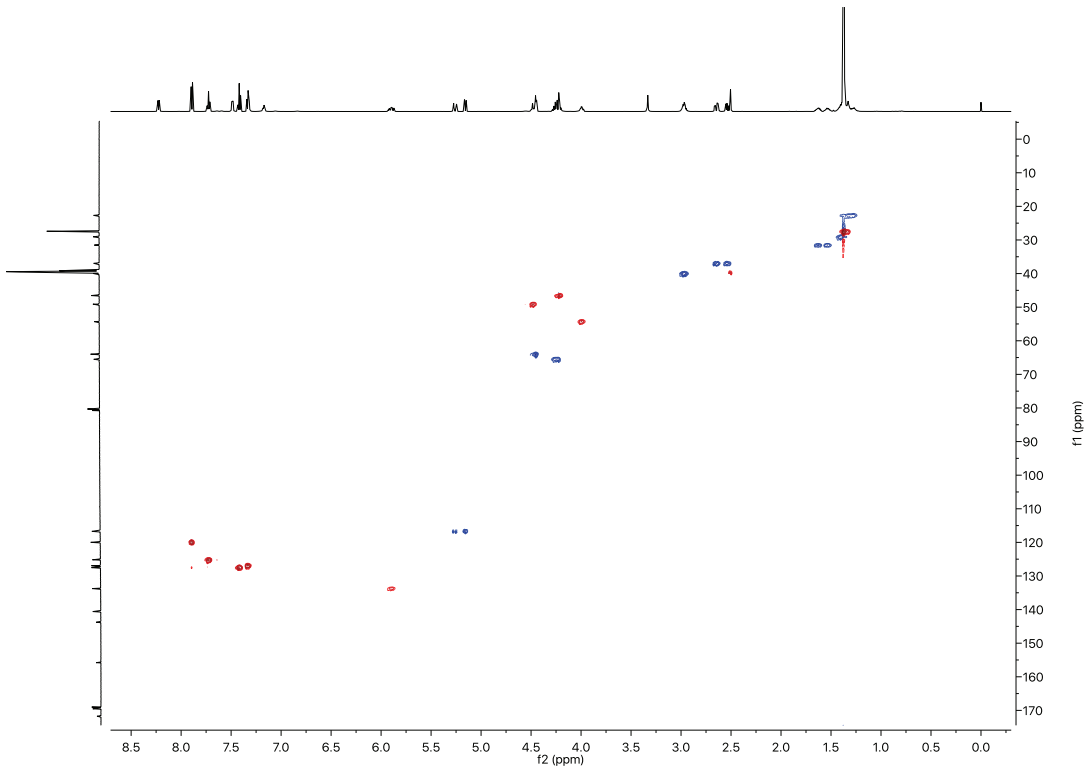




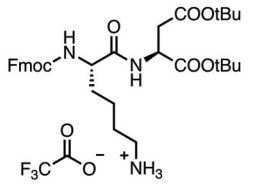
^{1}H, ^{1}H-COSY

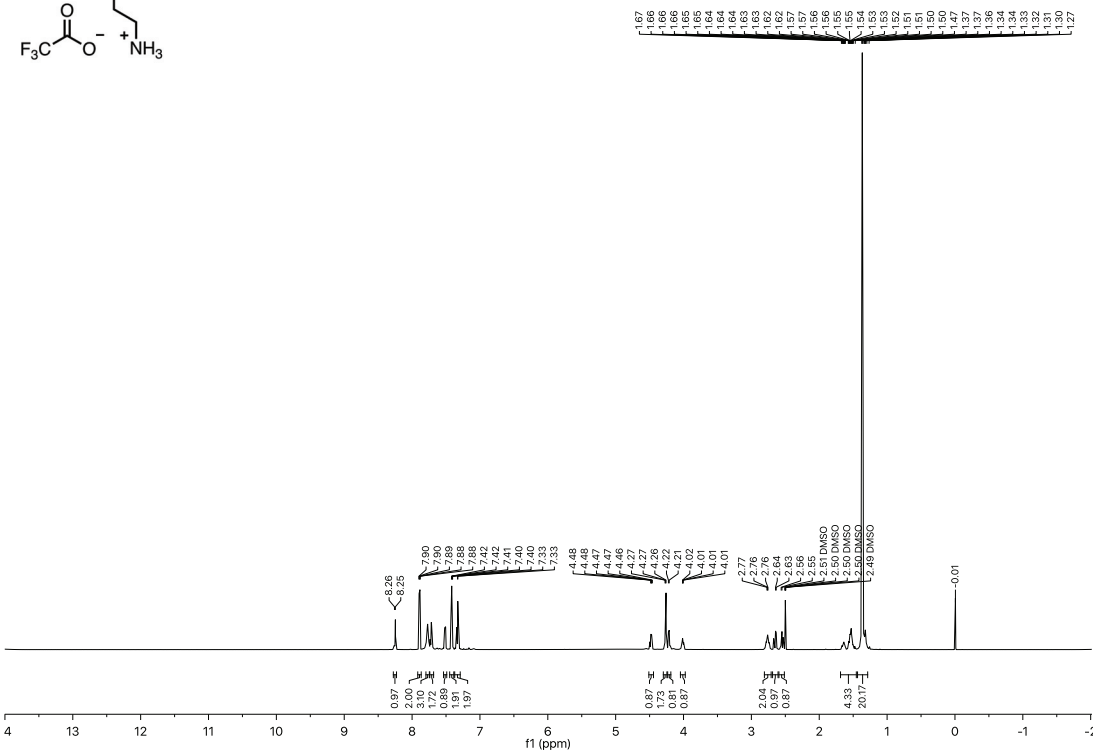


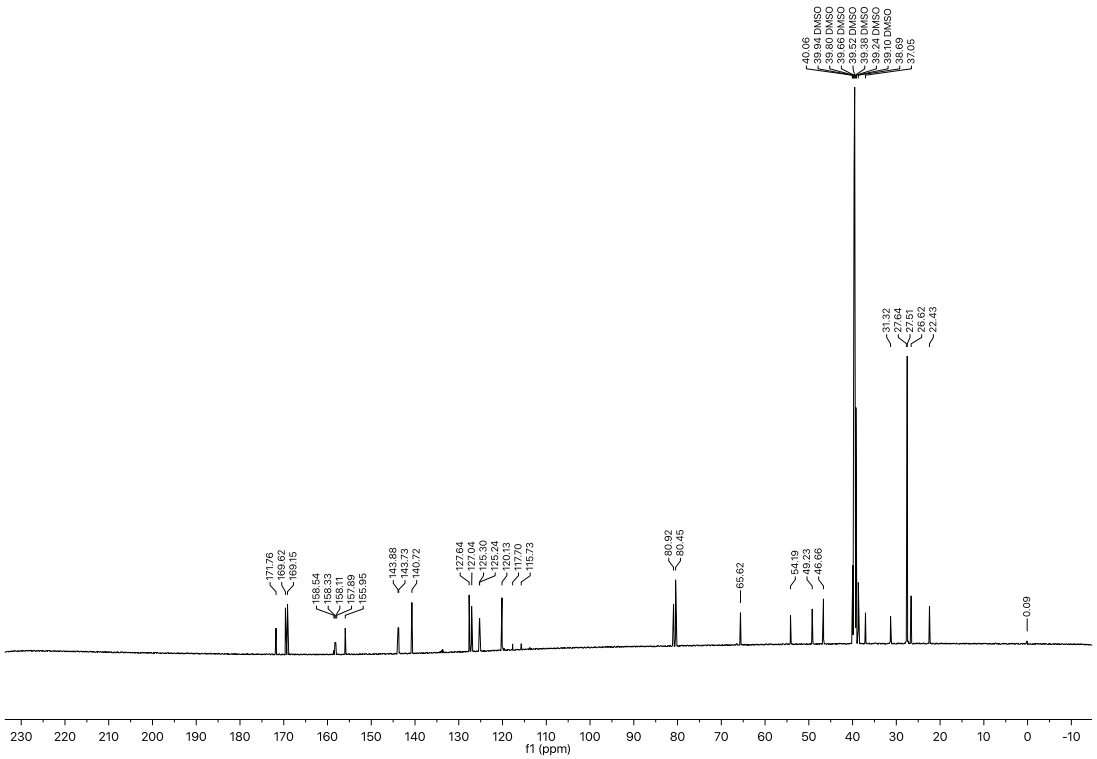
^{1}H, ^{13}C-HSQC



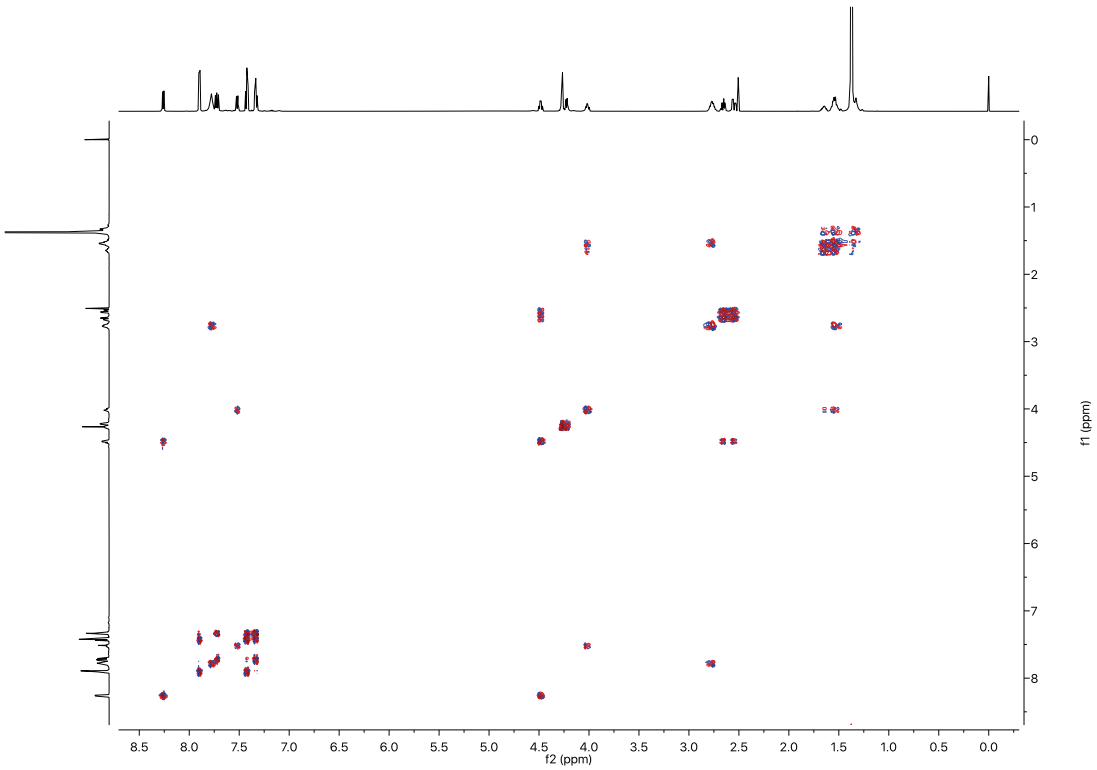
14.7. Fmoc-Lys(NH2)-Asp(OtBu)-OtBu TFA salt (4c)



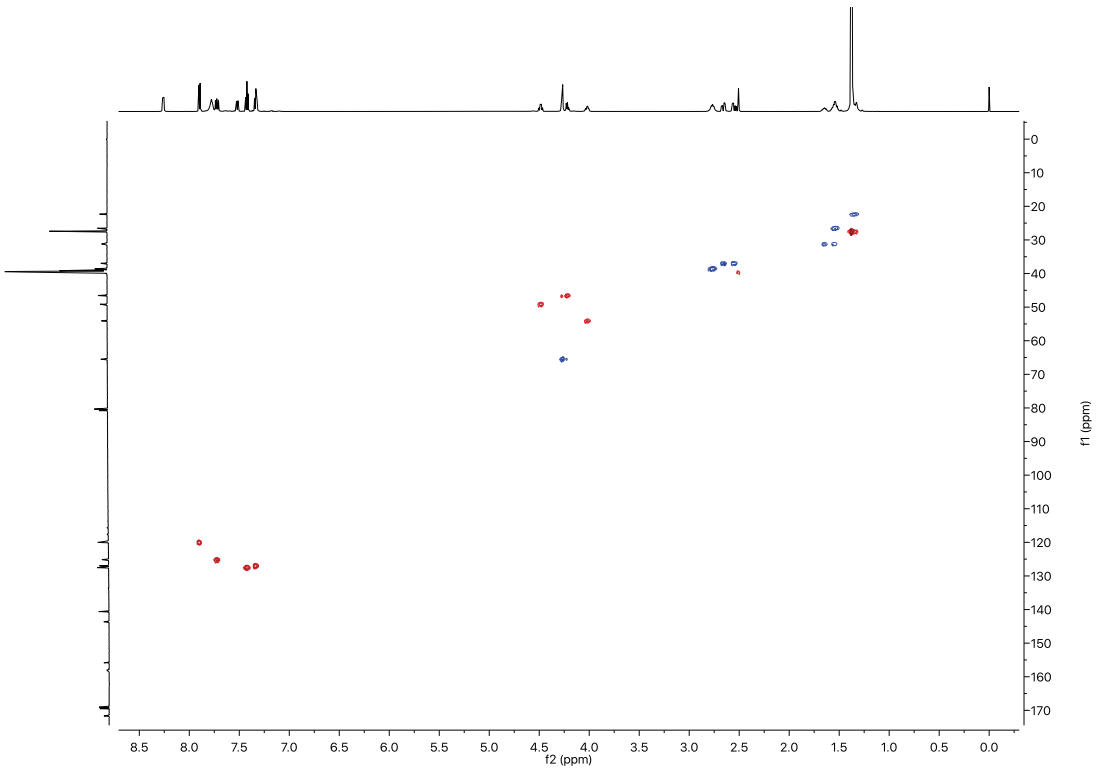




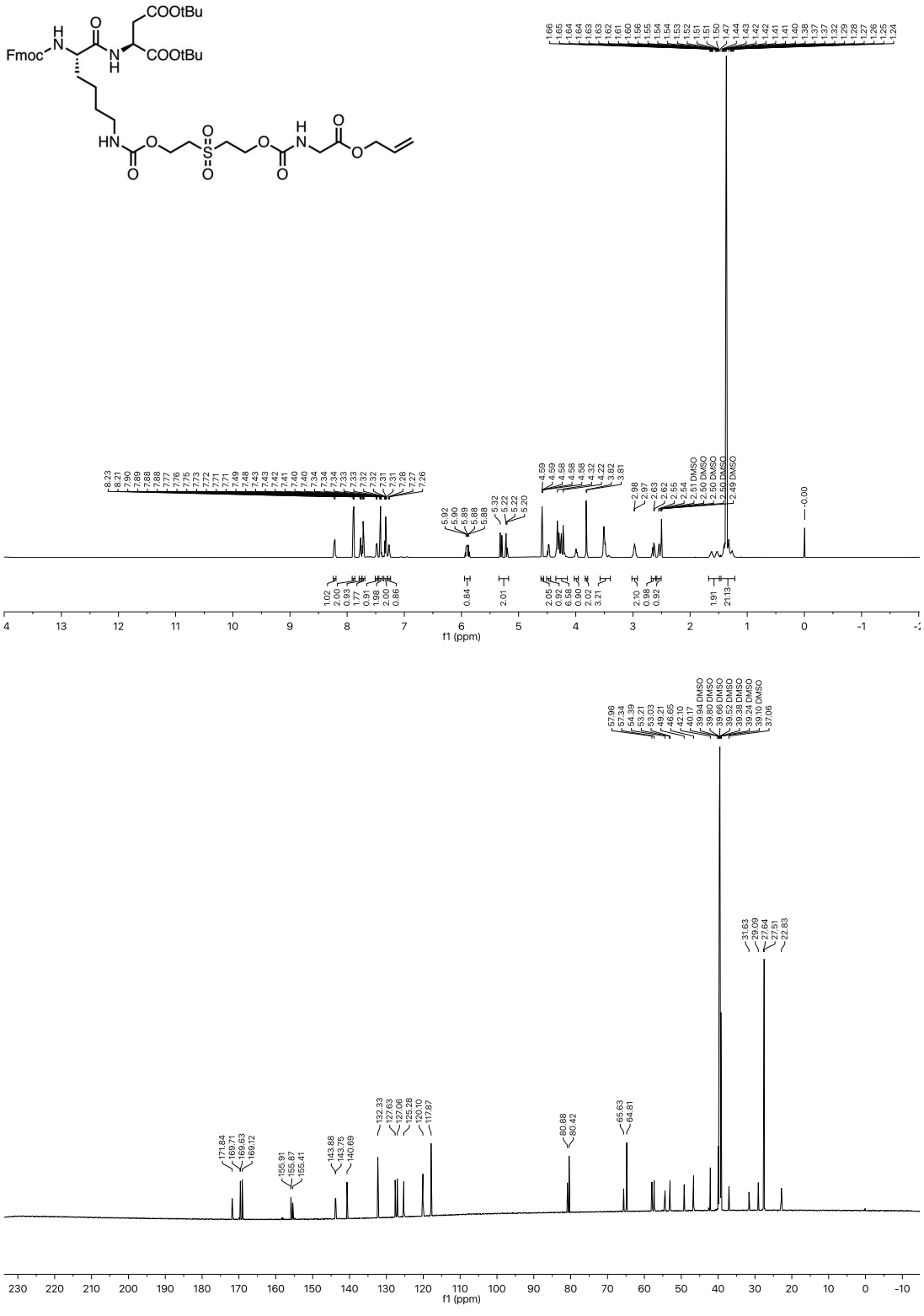
^{1}H, ^{1}H-COSY



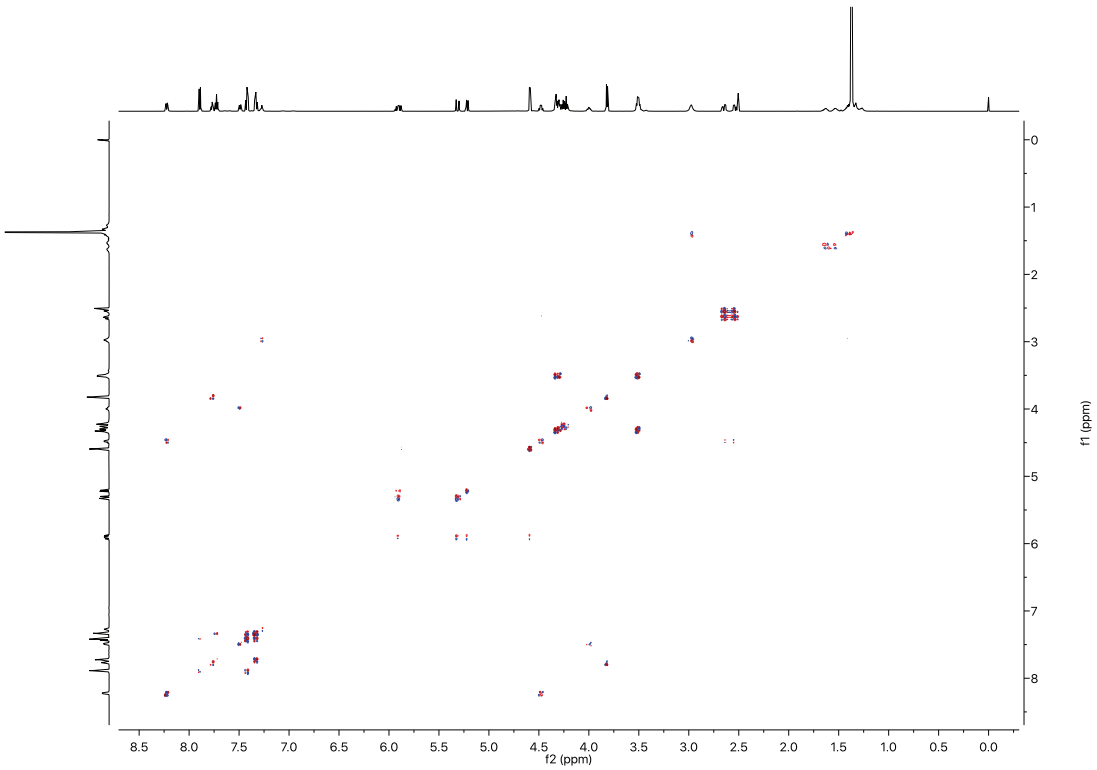
^{1}H, ^{13}C-HSQC



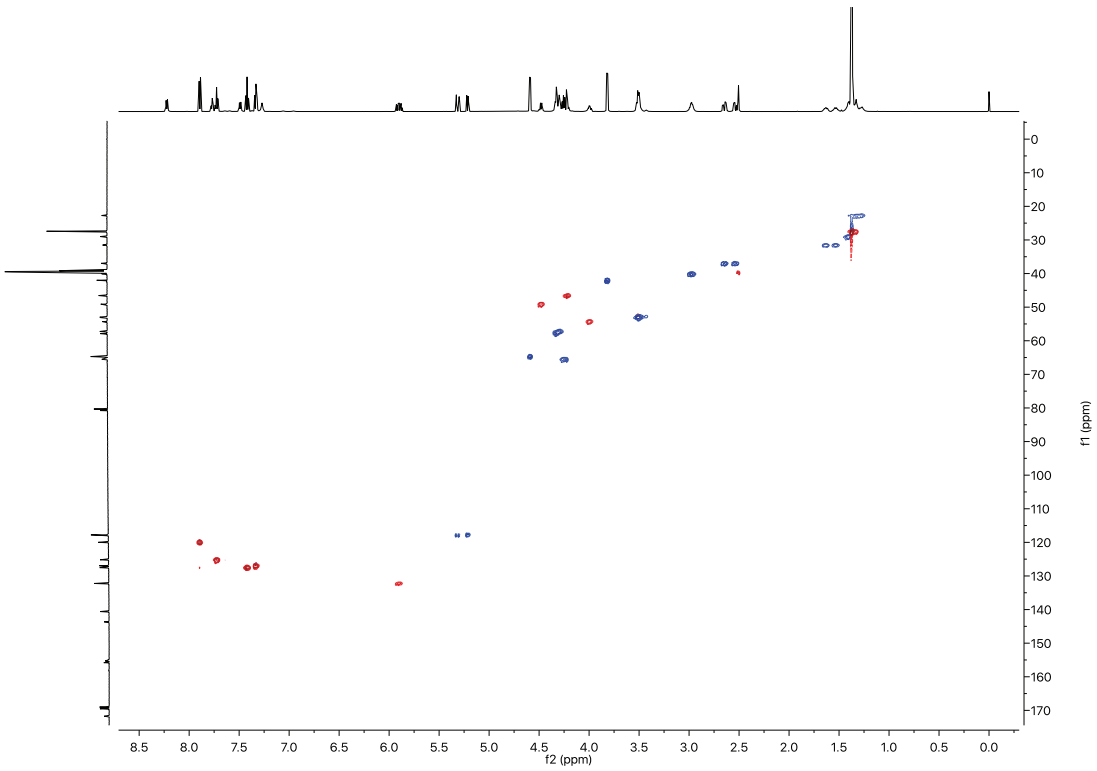
14.8. Synthesis of linker III. allyl ester (25)



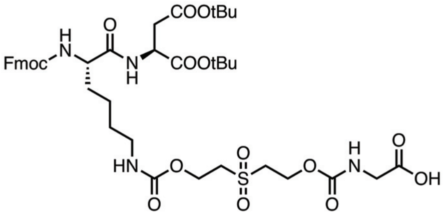
^{1}H, ^{1}H-COSY

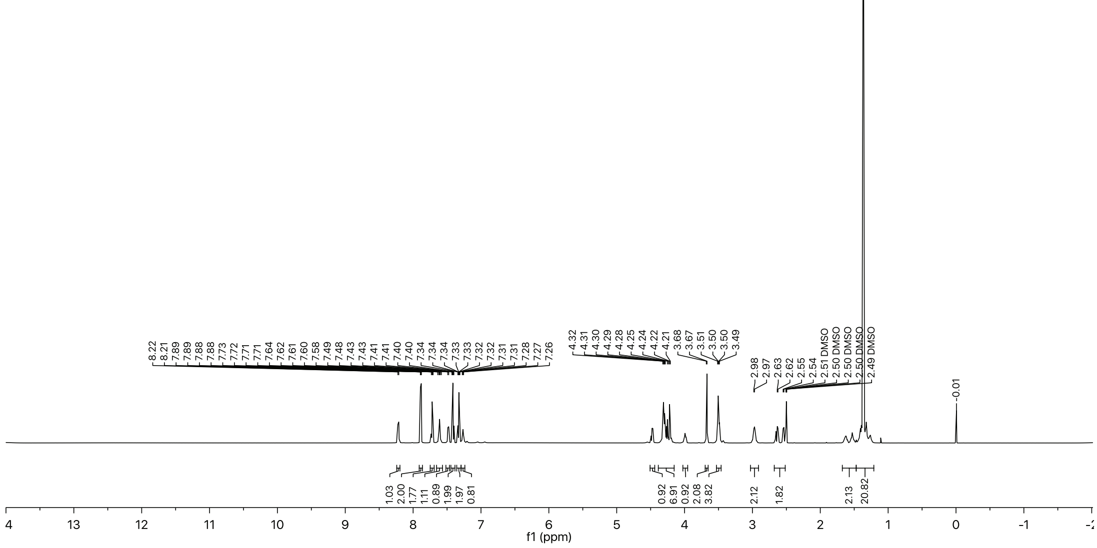


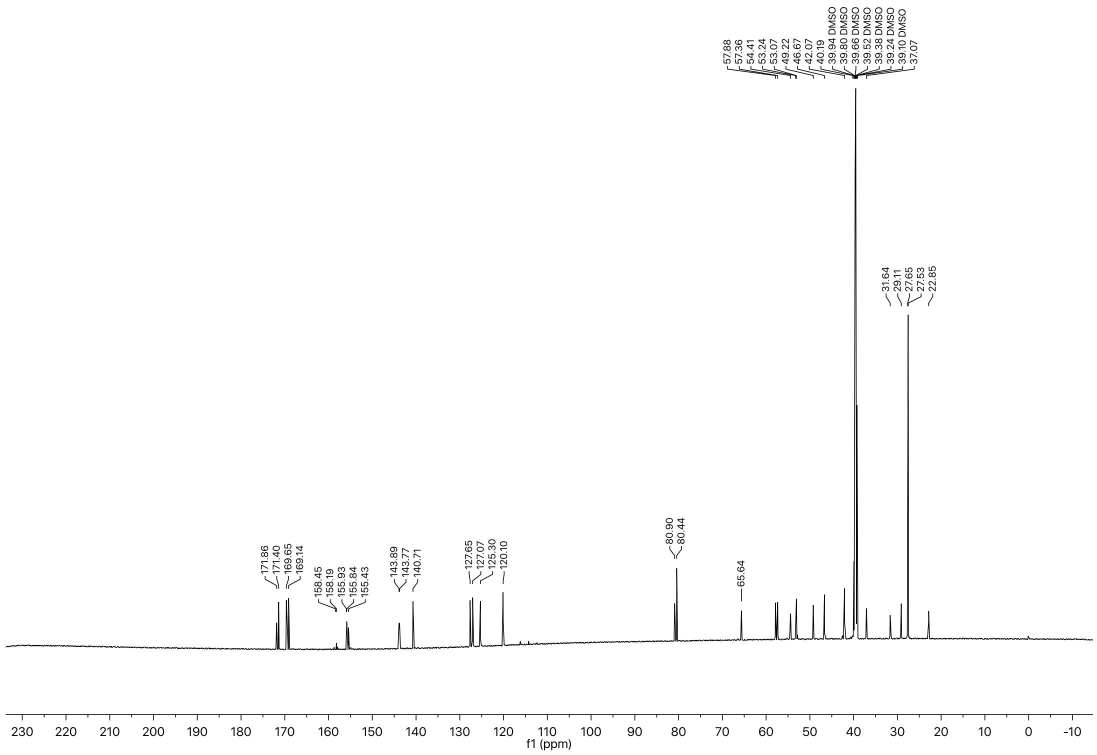
^{1}H, ^{13}C-HSQC



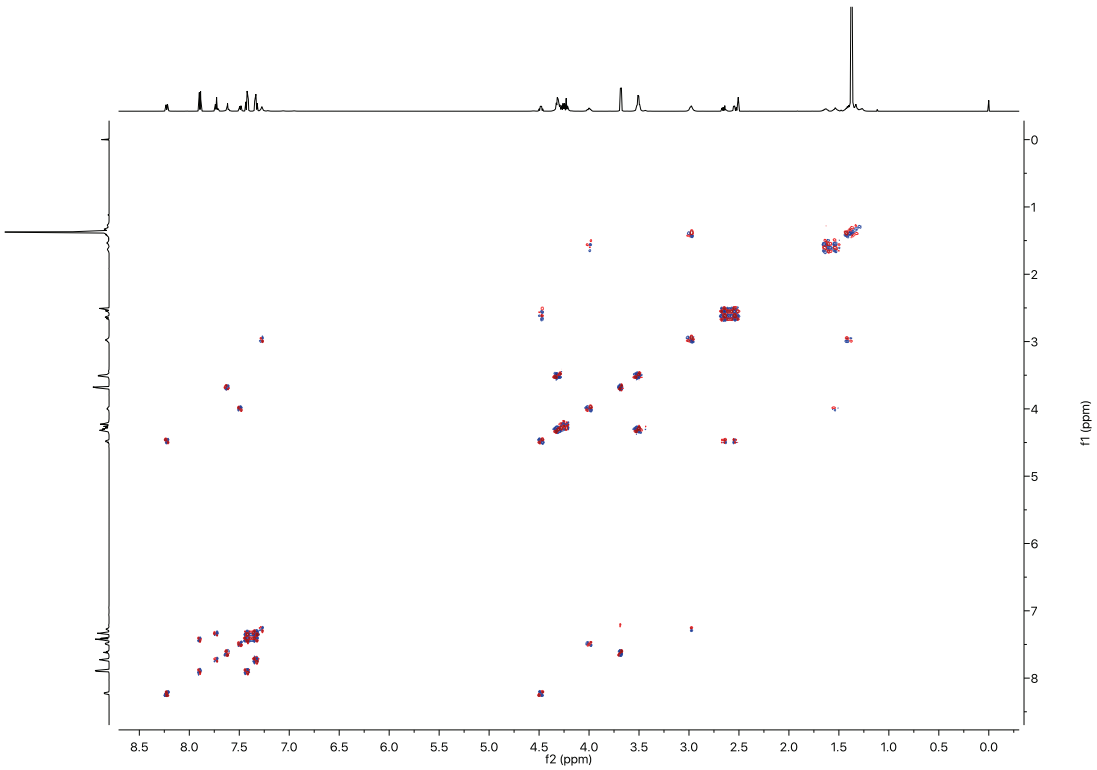
14.9. Linker III. (5c)



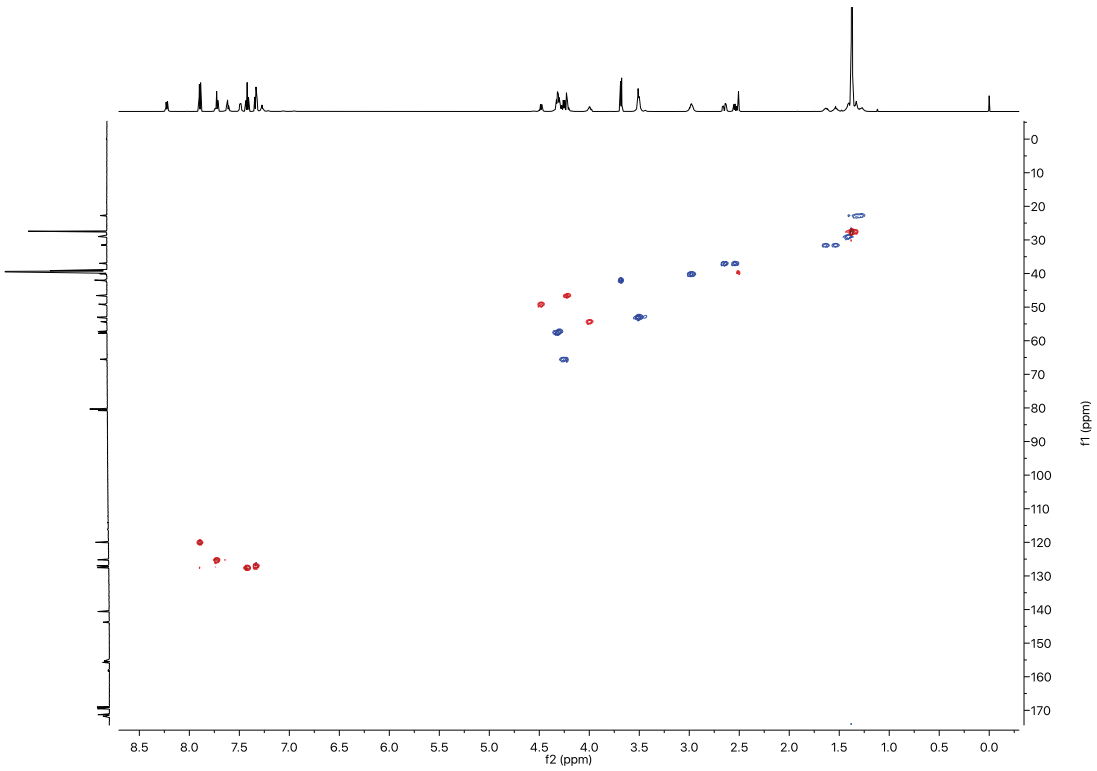




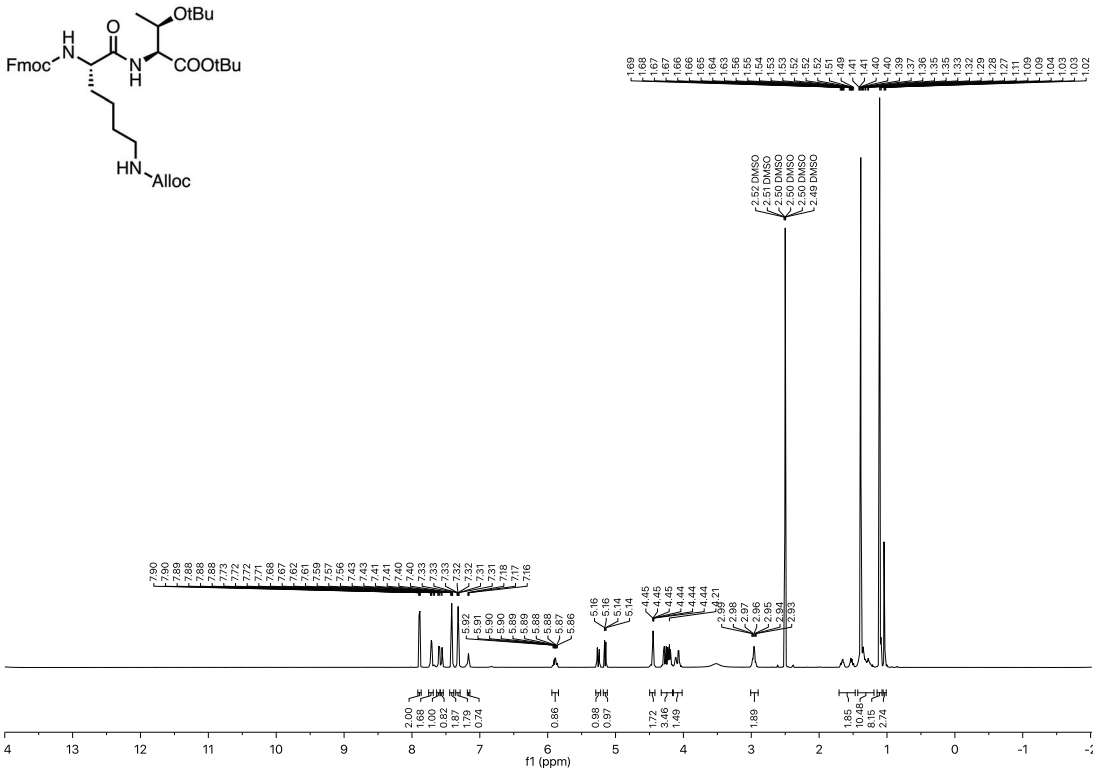
^{1}H, ^{1}H-COSY

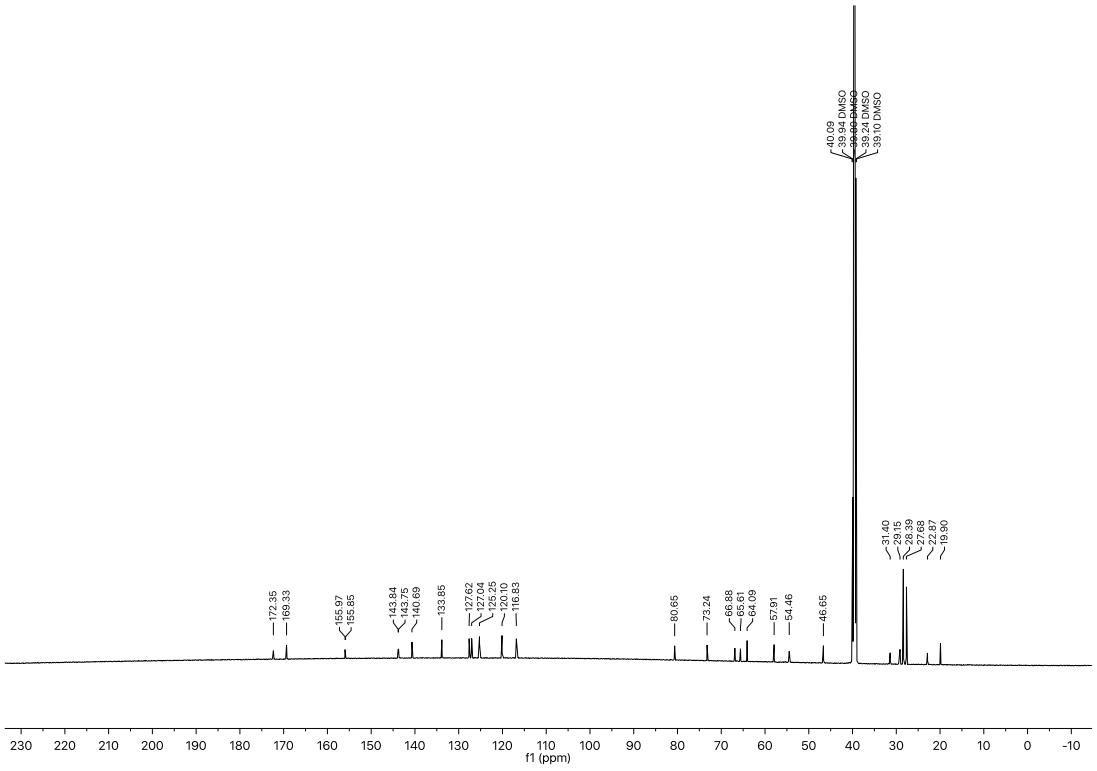


^{1}H, ^{13}C-HSQC

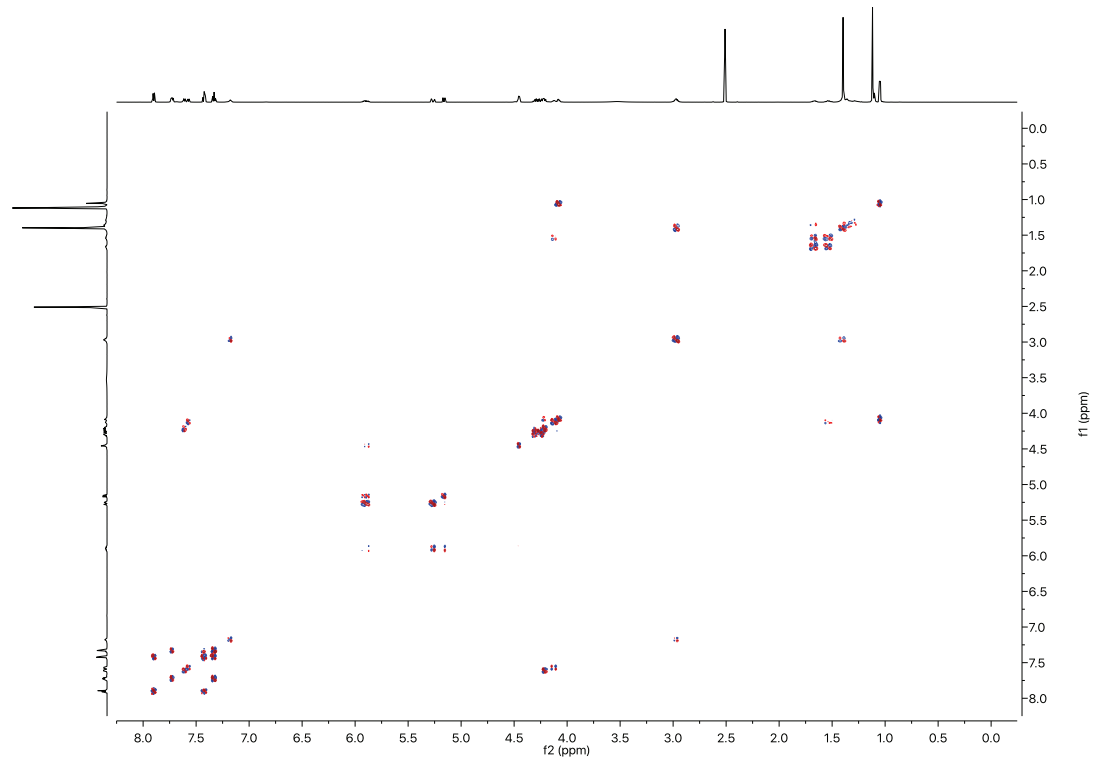


14.10. Fmoc-Lys(NHAlloc)-Thr(tBu)-OtBu (26)

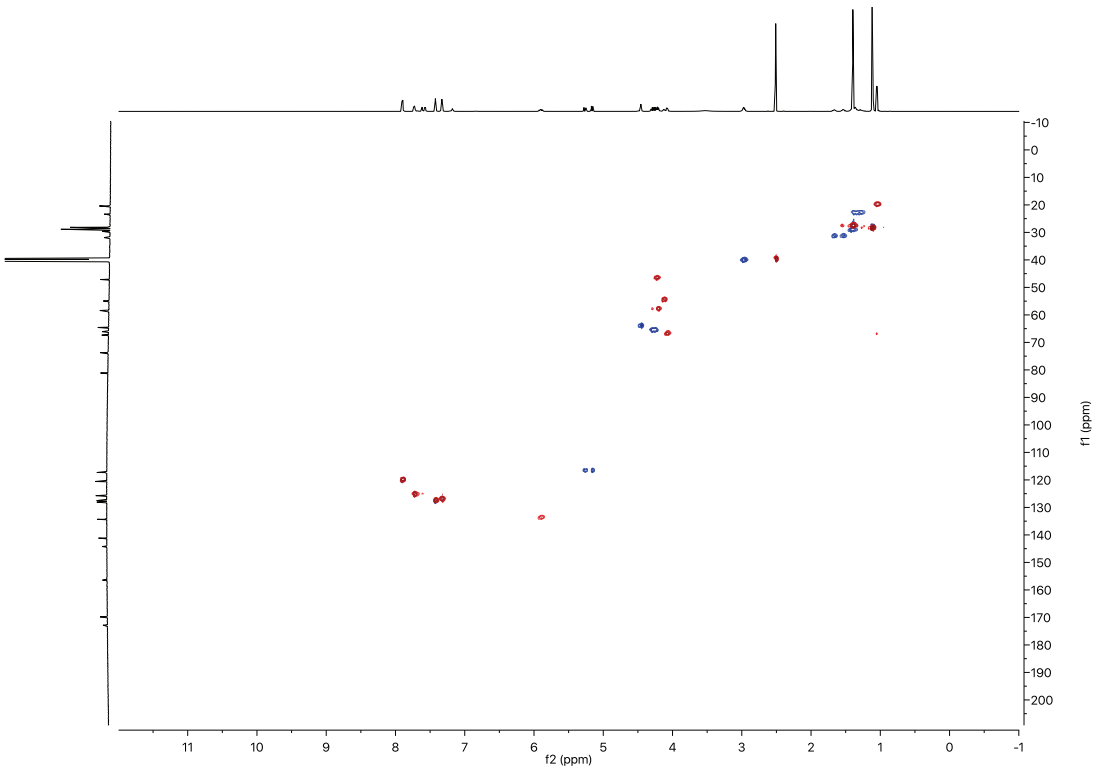




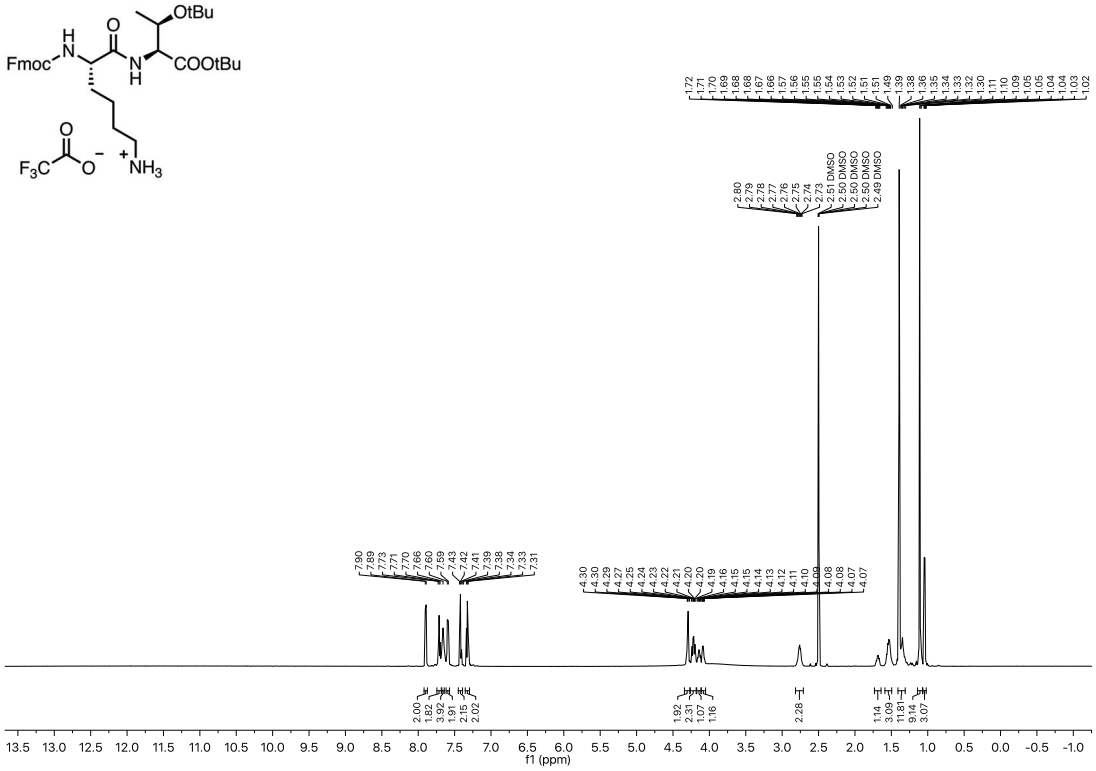
^{1}H, ^{1}H-COSY

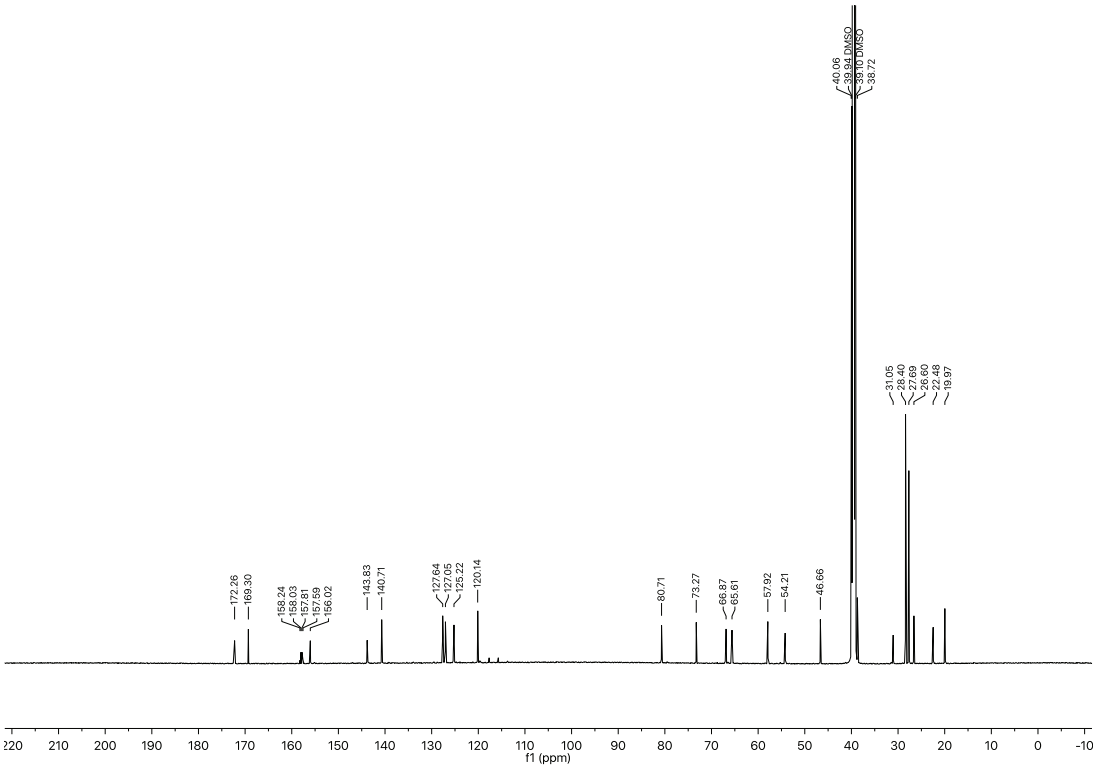


^{1}H, ^{13}C-HSQC

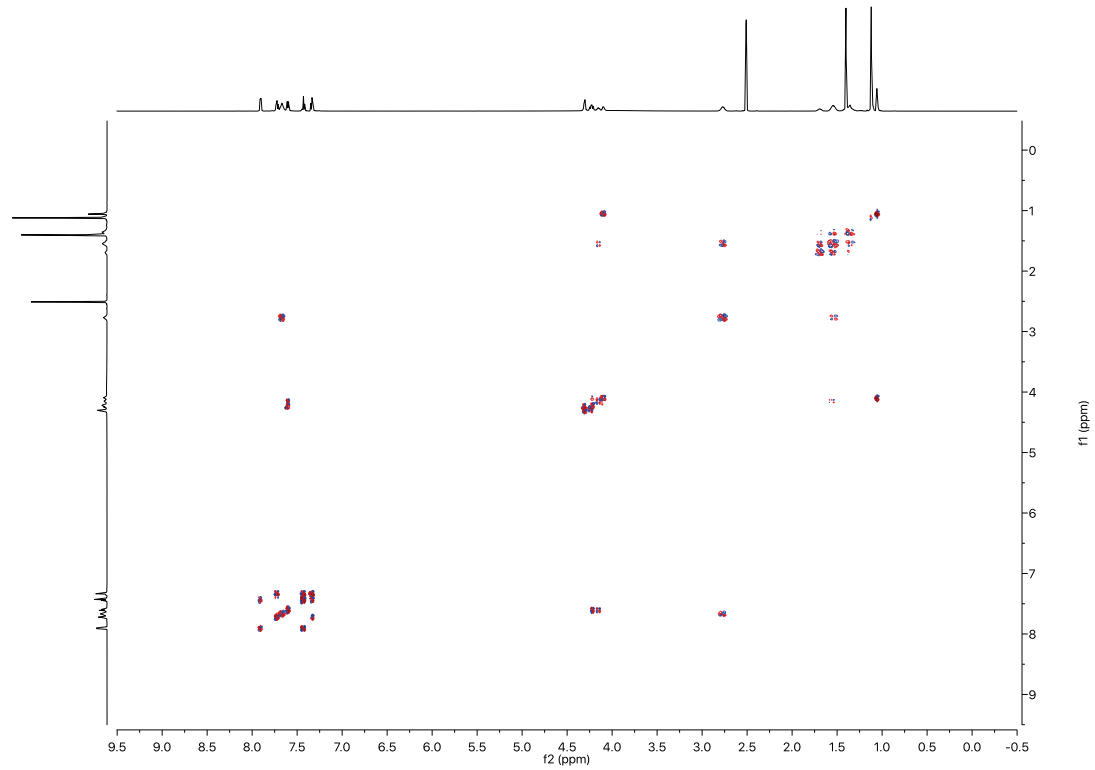


14.11. Fmoc-Lys(NH2)-Thr(tBu)-OtBu TFA salt (4d)

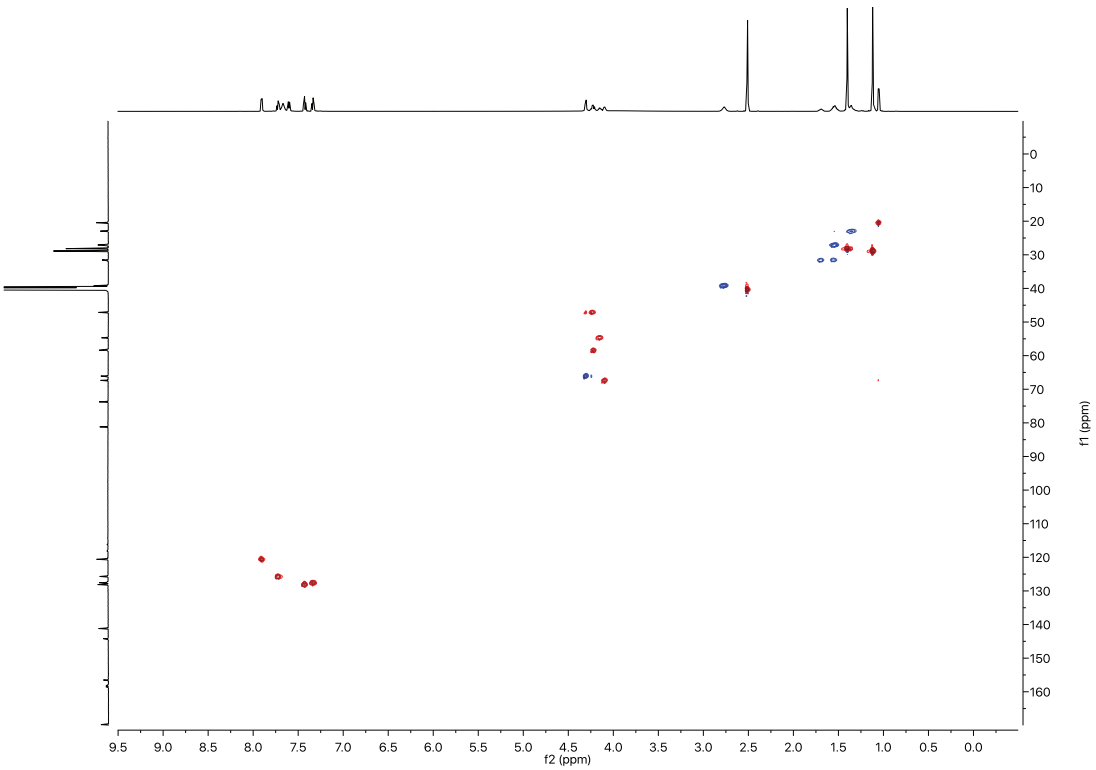




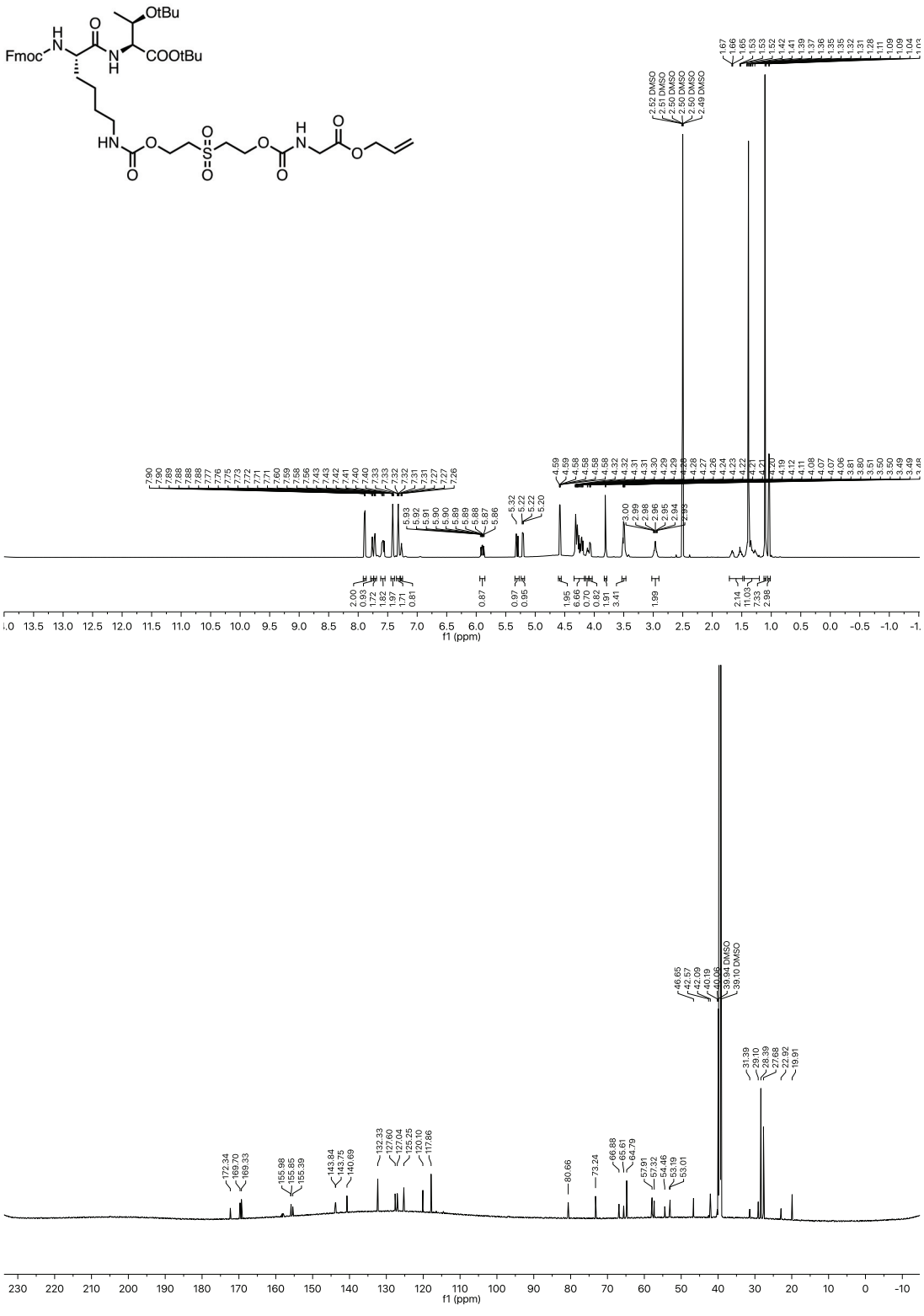
^{1}H, ^{1}H-COSY



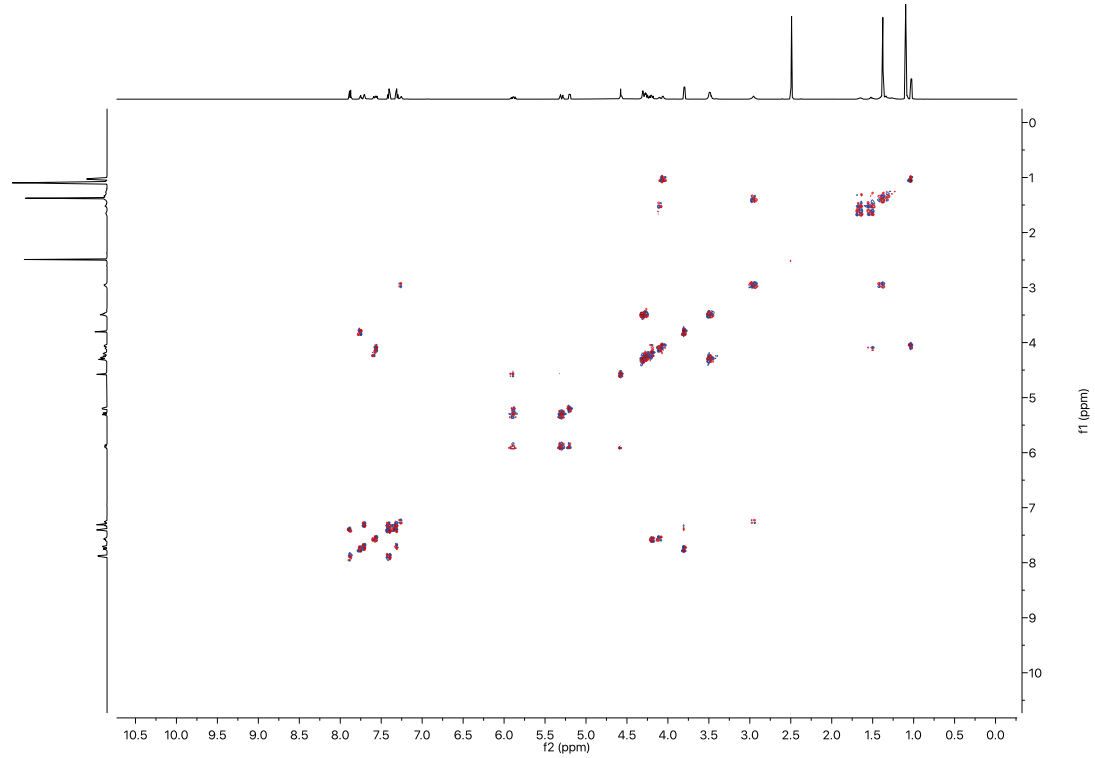
^{1}H, ^{13}C-HSQC



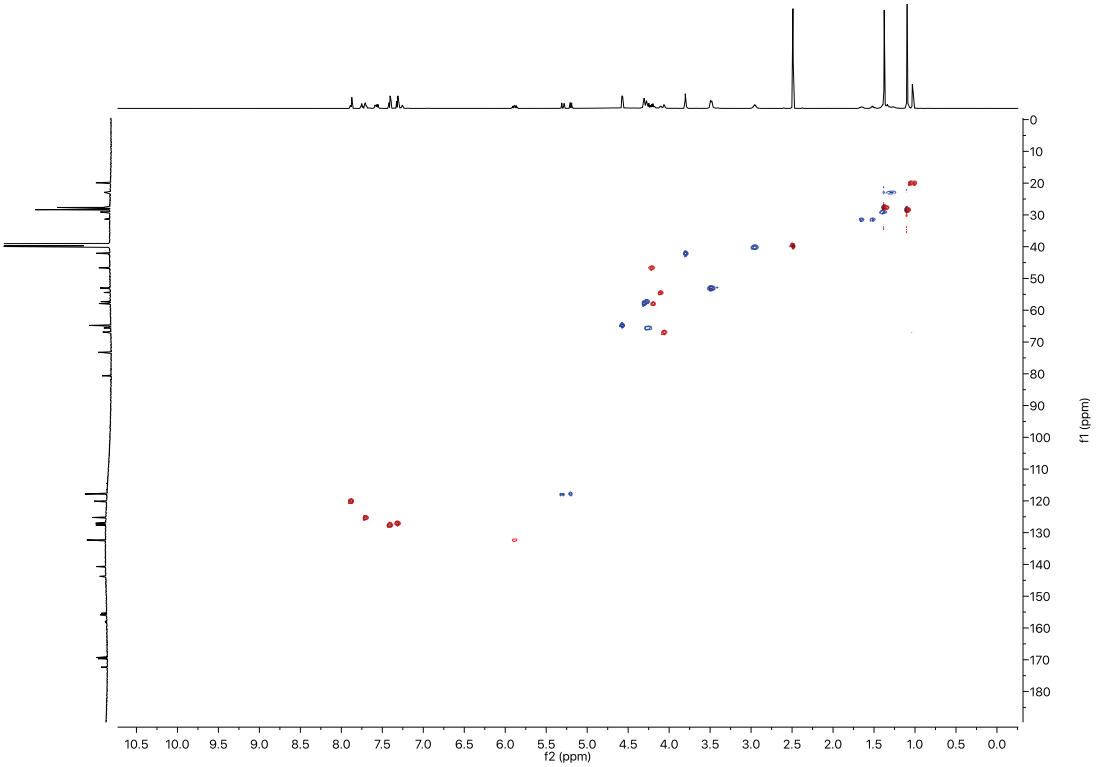
14.12. Linker IV. allyl ester (27)



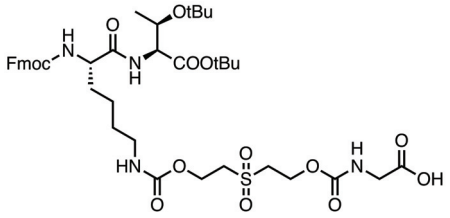
^{1}H, ^{1}H-COSY

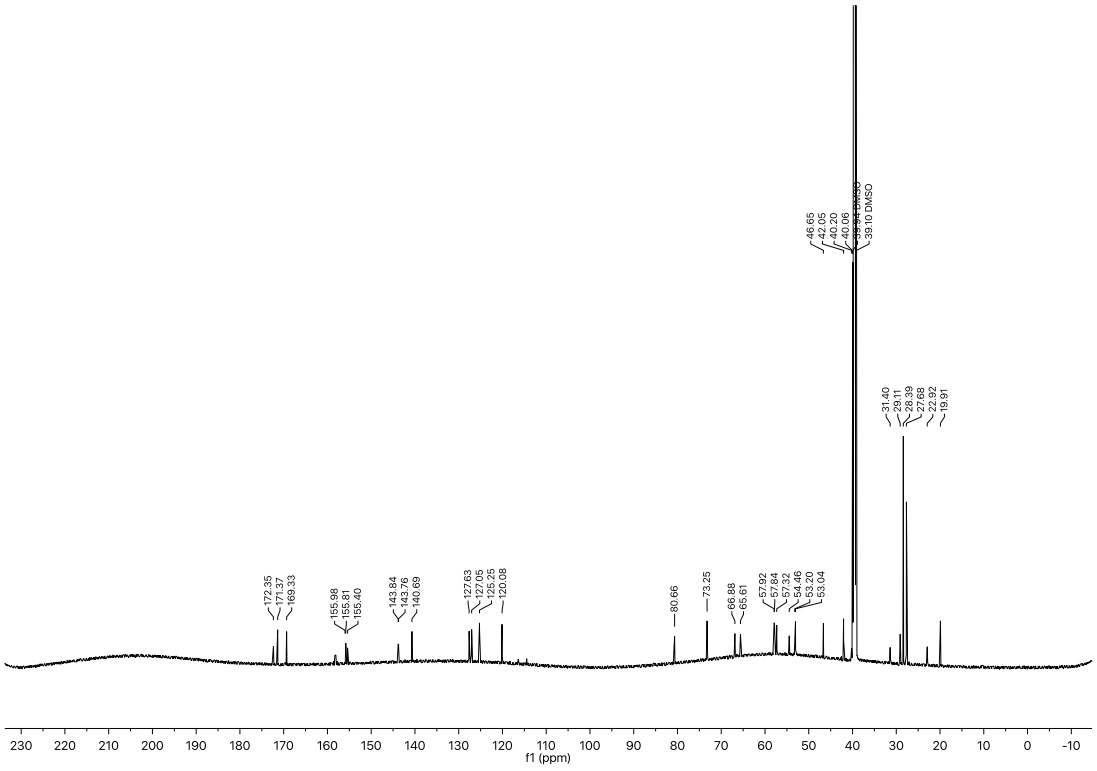


^{1}H, ^{13}C-HSQC

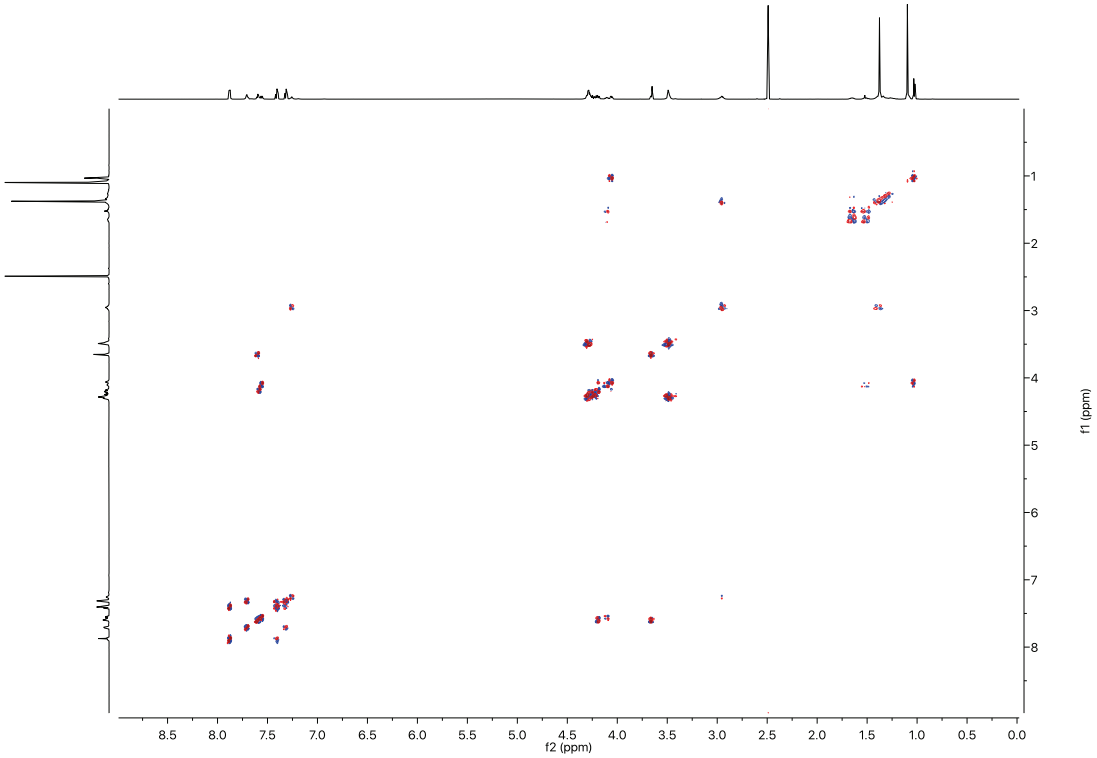


14.13. Linker IV. (5d)

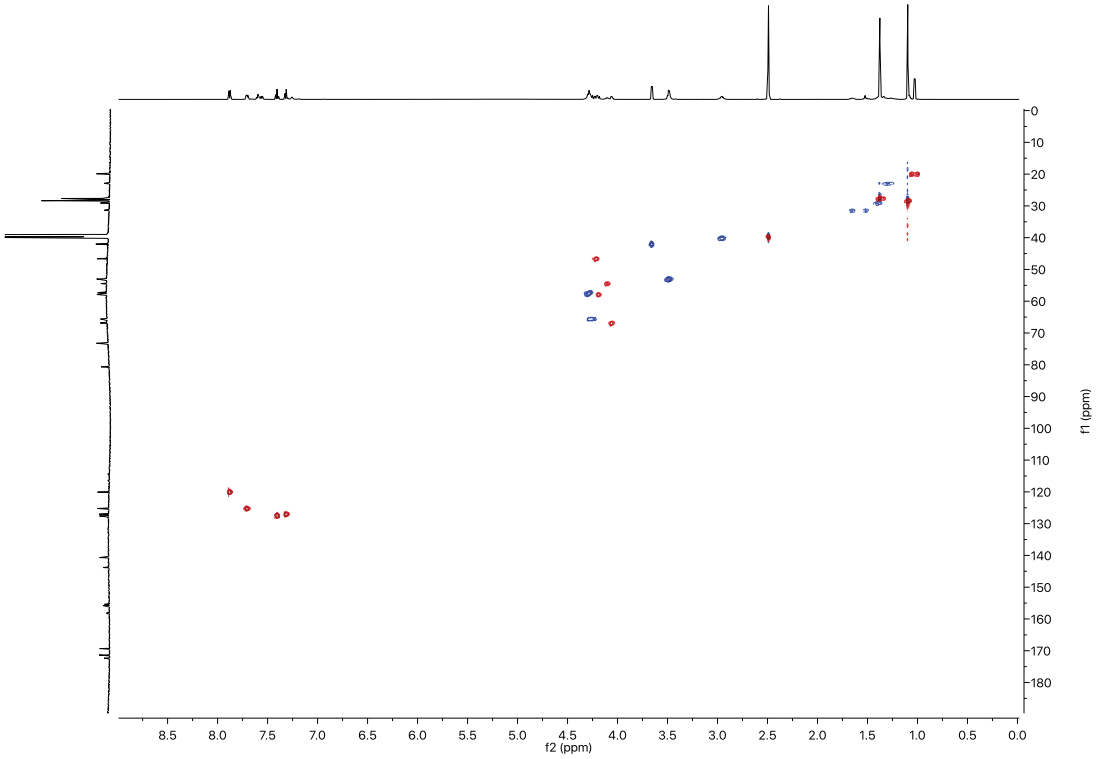




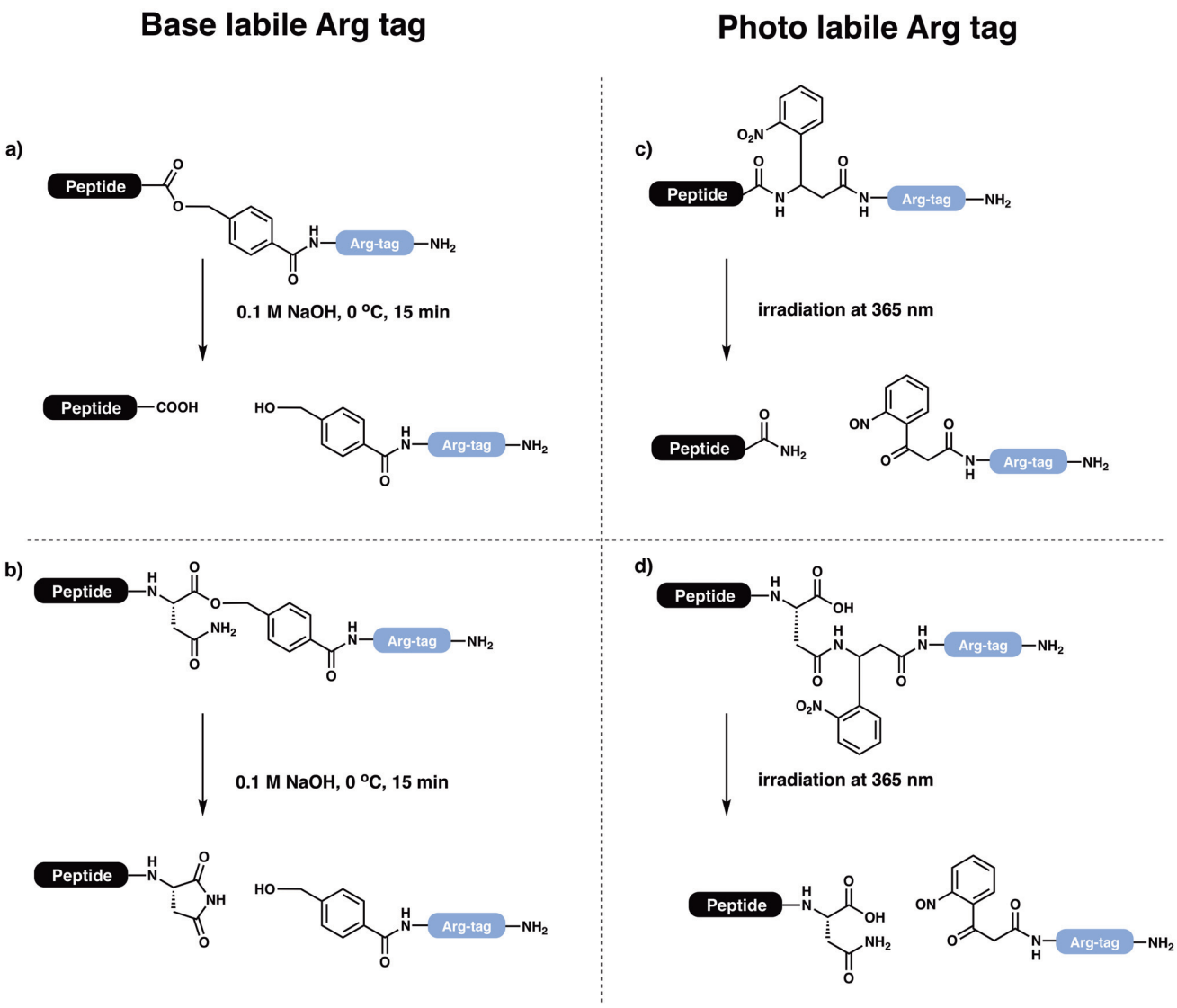
^{1}H, ^{1}H-COSY



^{1}H, ^{13}C-HSQC



15. Comparison between base labile Arg tag and Photo labile Arg tag



a) Base mediated ester hydrolysis results in C-terminal carboxylic acid. **b)** Base mediated cleavage results in aspartimide formation in the case of C terminal Asn residue. **c)** Photo-cleavage of ortho-nitrobenzyl type linker results in C-terminal carboxamide. **d)** Photo-cleavage of side chain anchored ortho-nitrobenzyl type linker results in native Asn residue on the C-terminus