Strategic Design of GalNAc-Helical Peptide Ligands for Efficient Liver Targeting

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Materials

Reagents and solvents were purchased from Sigma-Aldrich Co. LLC, Watanabe Chemical Industries., Tokyo Chemical Industry Co. Ltd., Fujifilm Wako Pure Chemicals Co. Inc., Kanto Chemicals Co. Inc., Kishida Chemical Co. Ltd., Combi-blocks Inc., and CEM corporation. Biotinylated Tri-GalNAc was purchased from Sussex Research Laboratories Inc. Alexafluor-647-labeled Streptavidin was purchased from Jackson ImmunoResearch Laboratories Inc. Maleimide-functionalized Tri-GalNAc was purchased from Peptide Institute.

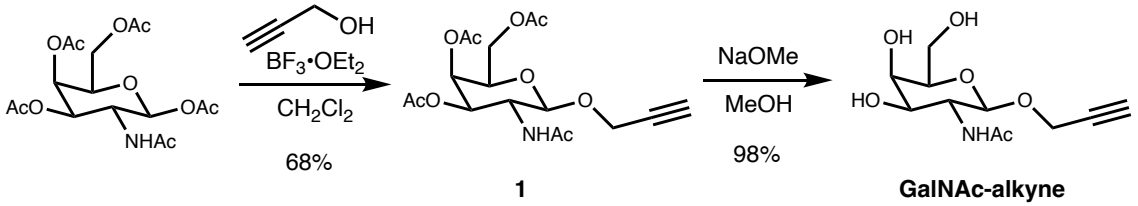
Instruments

High resolution mass spectrometry was carried on using SHIMADZU LCMS-IT-TOF or LCMS-9050 equipped with electro spray ionization source. Analytical HPLC was carried on using JASCO analytical HPLC system. ^{1}H NMR and ^{13}C NMR spectra were recorded on JEOL ECZ-600 NMR spectrometer. Spectra were reported in ppm and are referenced to the residual solvent signal.

Analytical HPLC condition

Column: J-Pak Core C18 (4.6 mm*100 mm, 5 μm) (JASCO, Japan)
Solvent A: 0.1% TFA/water
Solvent B: 0.1% TFA/MeCN
Flow rate: 1.2 mL/min
Gradient: 10%~90% B over A in 10 min.
Detection: 220 nm or 254 nm
Temperature: 40 °C

Synthesis of GalNAc-alkyne



Penta-acetyl galactosamine (389 mg, 1.00 mmol) was dissolved in CH_2Cl_2 and propargyl alcohol (162 μL, 1.50 mmol) and molecular sieves 4A were added. After stirring at 0°C for 30 min, BF_3•OEt_2 (230 μL, 1.80 mmol) was added and bring to room temperature. After stirring for 17 h, K_2CO_3 (100 mg) was added and stirred for 30 min. Reaction mixture was filtered through celite pad, filtrate was washed with water and Brine. Organic phase was dried over Na_2SO_4 and concentrated under vacuum. Following procedures were done one more time. Residue was purified by silica gel column chromatography (*n*-Hexane : EtOAc = 1 : 9 to 0 : 10) to obtain compound **1** (260 mg, 68%) as a white foam.

Compound **1** (260 mg, 0.675 mmol) was dissolved in MeOH (7 mL) and NaOMe (15 mg, 0.270 mmol) was added. After stirring for 3 h, acidic ion exchange resin was added until the solution was neutralized. Reaction mixture was filtered and concentrated under vacuum to obtain GalNAc-alkyne (172 mg, 98%) as a white solid.

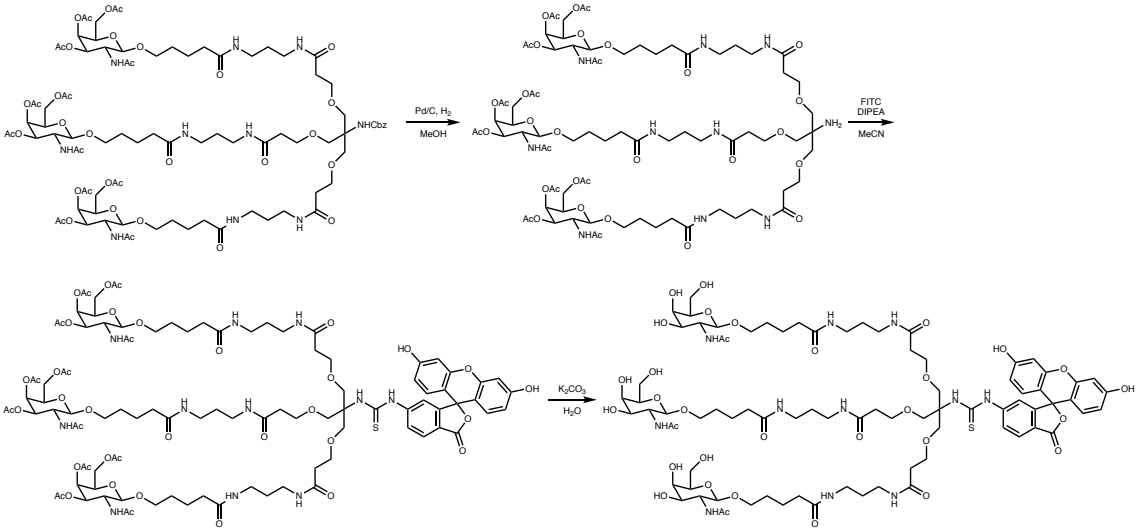
^{1}H NMR (600 MHz,CD_3OD) δ 4.56 (d, *J* = 8.4 Hz, 1H), 4.36 (t, *J* = 2.7 Hz, 2H), 3.91 (dd, *J* = 10.6, 8.5 Hz, 1H), 3.82 (d, *J* = 3.1 Hz, 1H), 3.79-3.70 (m, 2H), 3.62 (dd, *J* = 10.7, 3.3 Hz, 1H), 3.50-3.48 (m, 1H), 2.83 (t, *J* = 2.4 Hz, 1H), 1.98 (s, 3H)

^{13}C NMR (151 MHz, CD_3OD) δ 174.2, 100.8, 80.1, 76.9, 76.0, 73.1, 69.6, 62.5, 56.3, 54.0, 23.0.

HRMS-ESI (m/z): $[M + Na]^+$ calcd for $C_{11}H_{19}NO_6Na^+$ 282.0954, found 282.0928

The data was similar to previous report.[1]

Synthesis of Tri-GalNAc-FITC



Cbz-Tri-GalNAc was synthesized following the previous report.[2] Cbz-Tri-GalNAc (100 mg, 51.9 μmol) was dissolved in MeOH (1 mL), 10% Pd/C (10 mg) was added. Degassed the reaction mixture and substituted with H_2 atmosphere. After stirring for 18 h, the solution was filtered through celite pad and concentrated under vacuum. The residue was dissolved in MeCN (500 μL), FITC (40 mg, 104 μmol) and DIPEA (9.0 μL, 51.9 μmol) were added. After stirring for 27 h, the reaction mixture was purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 45%~75% in 40 min). K_2CO_3 (100 mg) was added to the HPLC fraction (30mL). After stirring for 18 h, the reaction mixture was concentrated under vacuum and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 45%~75% in 40 min) (Waters, MA). Fractions containing desired products were lyophilized to obtain (38.2 mg, 41% in 3 steps) as a yellow solid. The product was identified by LC-MS and checked purity by RP-HPLC equipped with J-Pak Core C18 (4.6 mm*100 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 1.2 mL/min, gradient: 10%~90% A over B in 10 min).

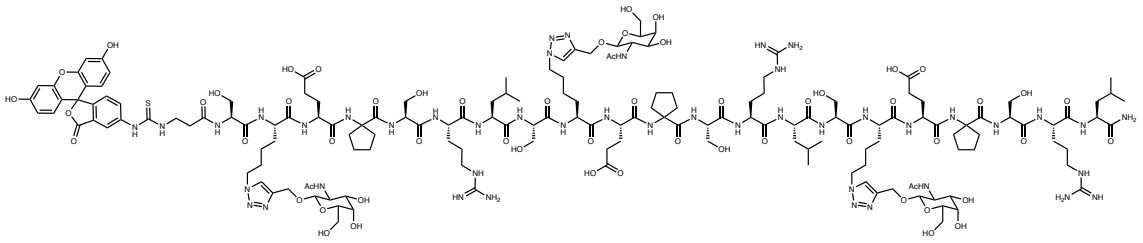
HRMS-ESI (m/z): $[M + 2H]^+$ calcd for $C_{82}H_{123}N_{11}O_{32}S_2^+$ 902.9023, found 902.9020

Purity: 98.8%, Rt = 3.98 min

General synthetic procedure of 5S-FITC and LD-FITC

Peptides were synthesized by microwave-assisted solid phase method (Liberty Blue; CEM, NC) using DIC and Oxyma pure as a coupling agent, 20% piperidine in DMF for a deprotection reagent. Before synthesizing peptides, Protide LL Rink-amide resin (50 μmol) was soaked in CH_2Cl_2 : DMF = 1 : 1 solution. FITC (2 eq) and DIPEA (2 eq) were added to the DMF suspension (2 mL) of synthesized resin-bounded peptides. After agitating for 2 h, resin was washed with DMF and CH_2Cl_2. The Synthesized peptides were cleaved with cleavage cocktail (TFA : H_2O : TIPS = 95 : 2.5 : 2.5) for 45 min at 40 °C. Cleavage cocktail was removed by N_2 gas stream and ether was poured to yield solid residues. Ether suspensions were centrifuged, and supernatants were removed. The final products were identified by LC-MS and checked purity by RP-HPLC.

Synthesis of 5S-FITC

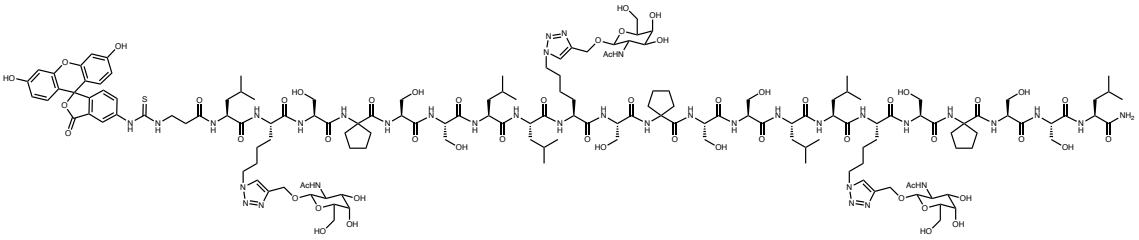


Following general synthesis procedure. Then, precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 10~50% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (14.2 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 10~50% in 40 min). Fractions containing desired products were lyophilized to yield 5S-FITC (3.6 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{162}H_{251}N_{45}O_{57}S^+$ 942.6957, found 959.6957

Purity: 99.6%, Rt = 6.58 min

Synthesis of 5-FITC

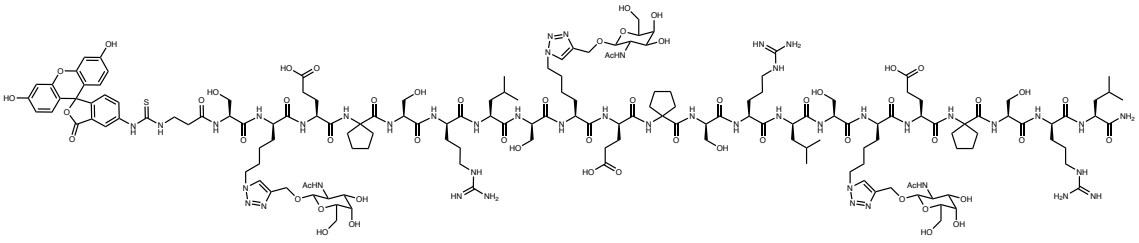


Peptide was synthesized by microwave-assisted solid phase method (Liberty Blue; CEM, NC). Synthesized peptide were cleaved with cleavage cocktail (TFA : H_2O : TIPS = 95 : 2.5 : 2.5) for 30 min at 40 °C. Cleavage cocktail was removed by N_2 gas stream and ether was poured to yield solid residues. The precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 60~80% in 10 min, 80~100% in 30 min). Fractions containing desired products were lyophilized. Yielded peptide (7.4 mg) and GalNAc-alkyne (3 eq per N_3 functional group) were dissolved in 200 μL of DMSO, then added 200 μL of the mixture of $CuSO_4$•$5H_2O$ (2 eq) and Sodium ascorbate (2 eq) in water. The mixture was stirred for 23 h. Then, FITC (3 eq) and DIPEA (10 μL) was added and stirred for additional 20 h. Reaction mixture was purified by RP-HPLC (B over A 40~80% in 40 min). Fractions containing desired products were lyophilized to yield 5-FITC (0.5 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{156}H_{242}N_{36}O_{54}S^+$ 878.9249, found 878.8245

Purity: >99%, Rt = 9.86 min

Synthesis of LD-FITC



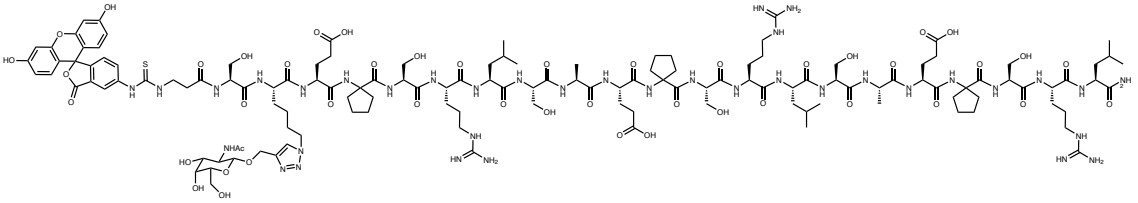
Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 20~60% in 40 min). Fractions containing desired products were lyophilized. Yielded

peptide (11.2 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 20~60% in 40 min). Fractions containing desired products were lyophilized to yield LD-FITC (2.0 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{162}H_{251}N_{45}O_{57}S^+$ 942.6957, found 959.6962

Purity: 97.6%, Rt = 6.77 min

Synthesis of 5S-1-FITC

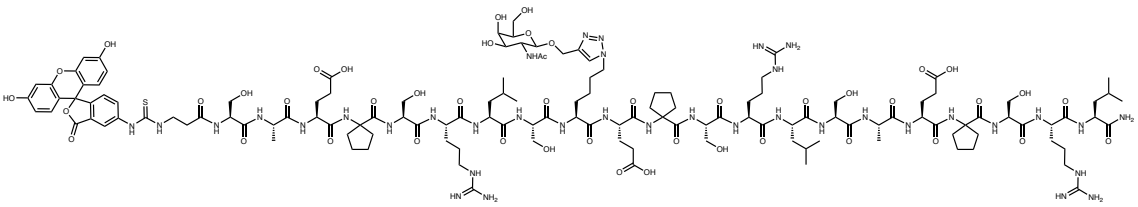


Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 20~60% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (16.0 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 20%~60% in 40 min). Fractions containing desired products were lyophilized to yield 5S-1-FITC (6.0 mg).

HRMS-ESI (m/z): $[M + 3H]^+$ calcd for $C_{134}H_{206}N_{37}O_{45}S^+$ 1028.4891, found 1028.4840

Purity: 99.8%, Rt = 6.77 min

Synthesis of 5S-1'-FITC

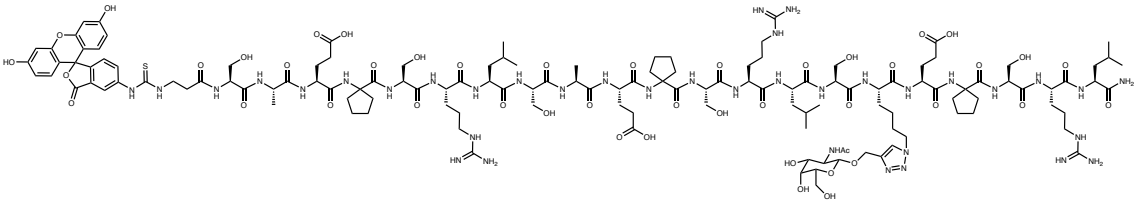


Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 35~70% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (14 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 35~70% in 40 min). Fractions containing desired products were lyophilized to yield 5S-1'-FITC (5.4 mg).

HRMS-ESI (m/z): $[M + 3H]^+$ calcd for $C_{134}H_{206}N_{37}O_{45}S^+$ 1028.4891, found 1028.4841

Purity: 99.8%, Rt = 6.83 min

Synthesis of 5S-1''-FITC

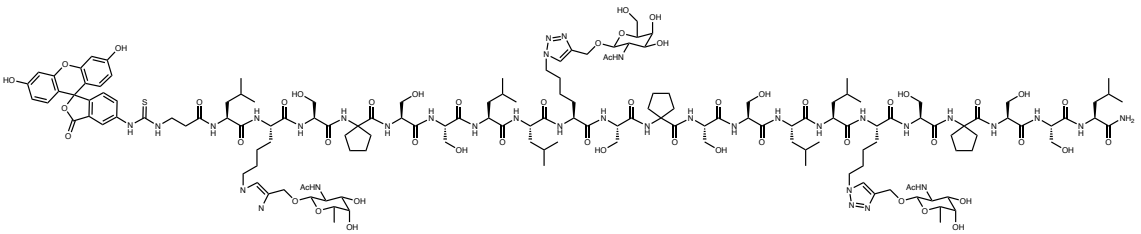


Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 35~70% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (9.5 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 35~70% in 40 min). Fractions containing desired products were lyophilized to yield 5S-1''-FITC (1.3 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{134}H_{207}N_{37}O_{45}S^+$ 771.6186, found 771.6161

Purity: 97.9%, Rt = 6.83 min

Synthesis of 5S-2-FITC

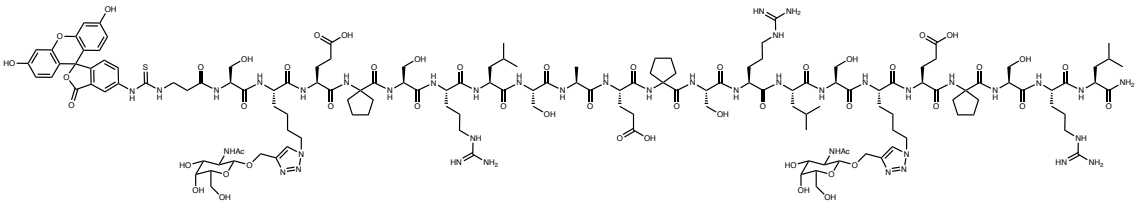


Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 20~60% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (16.0 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 20%~60% in 40 min). Fractions containing desired products were lyophilized to yield 5S-2-FITC (3.4 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{148}H_{229}N_{41}O_{51}S^+$ 857.1571, found 857.1558

Purity: 96.5%, Rt = 6.64 min

Synthesis of 5S-2'-FITC



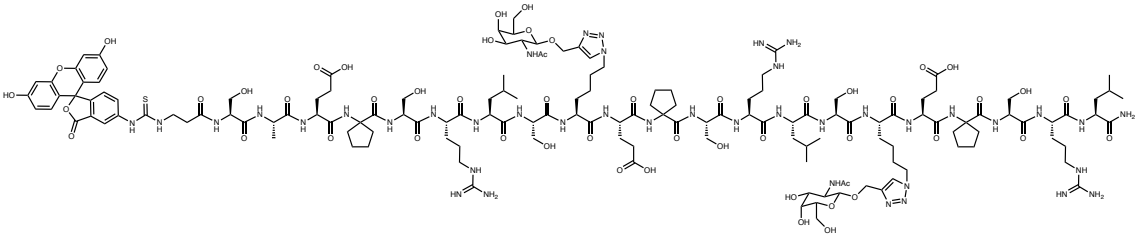
Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 30~65% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (9.0 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water.

The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 30%~65% in 40 min). Fractions containing desired products were lyophilized to yield 5S-2'-FITC (3.5 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{148}H_{229}N_{41}O_{51}S^+$ 857.1571, found 857.1530

Purity: 99.4%, Rt = 6.73 min

Synthesis of 5S-2''-FITC

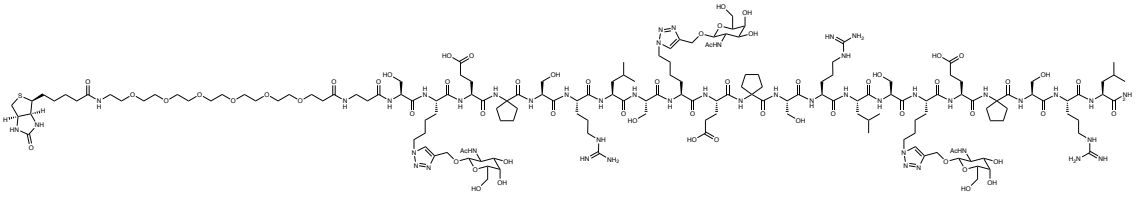


Following general synthesis procedure, then precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 30~65% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (16.0 mg) and GalNAc-alkyne (6 eq) were dissolved in 200 μL of DMSO, then added 100 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 30~65% in 40 min). Fractions containing desired products were lyophilized to yield 5S-2''-FITC (2.9 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{148}H_{229}N_{41}O_{51}S^+$ 857.1571, found 857.1533

Purity: >99%, Rt = 6.73 min

Synthesis of 5S-Bio



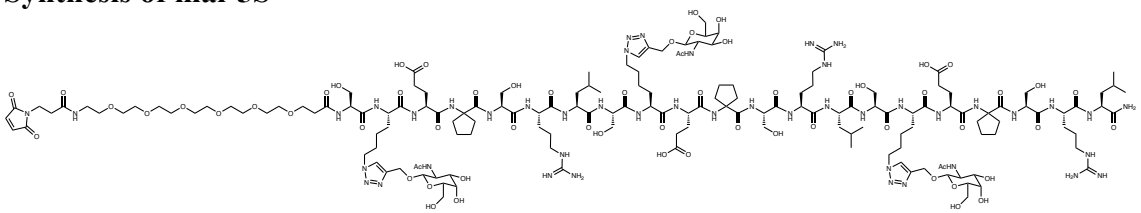
Peptide was synthesized by microwave-assisted solid phase method (Liberty Blue; CEM, NC). Biotin-PEG6-CO_2H (4 eq), COMU (4 eq) and DIPEA (8 eq) were added to the DMF suspension (2 mL) of synthesized resin-bounded peptides. After agitating for

2 h, resin was washed with DMF and CH_2Cl_2. The Synthesized peptide was cleaved with cleavage cocktail (TFA : H_2O : TIPS = 95 : 2.5 : 2.5) for 45 min at 40 °C. Cleavage cocktail was removed by N_2 gas stream and ether was poured to yield solid residues. Ether suspensions were centrifuged, and supernatants were removed. The precipitate was dissolved in 50% MeCN aq. and purified by RP-HPLC using JASCO preparative HPLC system equipped with XBridge® Prep C18 OBD column (19 mm*250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min, Gradient; B over A 20~60% in 40 min). Fractions containing desired products were lyophilized. Yielded peptide (19.1 mg) and GalNAc-alkyne (6 eq) were dissolved in 300 μL of DMSO, then added 200 μL of the mixture of $CuSO_4$•$5H_2O$ (1 eq) and Sodium ascorbate (1 eq) in water. The mixture was reacted under microwave irradiation for 1 h at 60 °C. Reaction mixture was purified by RP-HPLC (B over A 20%~60% in 40 min). Fractions containing desired products were lyophilized to yield 5S-Bio (18.6 mg).

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{166}H_{283}N_{47}O_{61}S^+$ 985.7547, found 985.7547

Purity: 96.9%, Rt = 6.27 min

Synthesis of mal-5S



GalNAc conjugated Peptide 5S without β-alanine was synthesized following the method of synthesis of 5S. Mal-PEG6-CO_2H (4 eq), HSTU (4 eq) and DIPEA (8 eq) were dissolved in DMF (40 μL) and reacted for 10 min. DMF solution was added to the solution of GalNAc conjugated Peptide 5S (18.1 mg) in 0.1 M Tris-HCl (pH 8.3) (200 μL) and stirred for 14 h at room temperature. Reaction mixture was purified by RP-HPLC (B over A 30%~70% in 40 min) to yield mal-5S (18.0 mg)

HRMS-ESI (m/z): $[M + 4H]^+$ calcd for $C_{160}H_{269}N_{45}O_{61}S^+$ 949.2327, found 949.2299

Purity: >99%, Rt = 6.59 min

In silico structural prediction

The in silico-based peptide modeling was performed on MOE 2022.07 software (CCD). Peptide conformation minimization is carried on by molecular dynamics (MD) as initial conformation set to the α-helical structure. The force fields are set to AMBER-10.

The flexibility of side-chain was determined by MD following the program below.

Time (ps)	Step	Temp (K)
0–10	Minimalization	0
10–110	Heating	10~300
110–210	Equilibration	300
210–510	Production	300

CD spectrometry

CD spectra were recorded on JASCO J-1100 CD spectrometer using a 1.0 mm path length cell. The data are expressed in terms of [θ]; i.e., total molar ellipticity (deg cm2 dmol-1). 20 mM phosphate buffer solution (pH = 7.4) or 50% MeCN aq. were used as a solvent and peptide concentration was 100 μM. The helicity was evaluated on secondary structure analysis add-in on JASCO spectra manager software based on Yang's reference CD spectra.[3]

Cellular uptake evaluation

HepG2 cells were seeded (150,000 cells per well in a 24-well plate) and incubated for overnight before experiment. Medium was removed and cells washed with D-PBS (-). Cells were incubated with 5 μM of ligands in Dulbecco's modified Eagle's medium (DMEM) low glucose with 10% FBS and 1% penicillin/streptomycin for 2 h. After incubation, the cell were collected by trypsin treatment and washed with D-PBS(-), then suspended in D-PBS(-), then supplemented with 2% FBS. Suspension was filtered before flow cytometry analysis. Data was collected by Acculi C6 Plus Flowcytometer (BD Biosciences) The value shown in the article is expressed in relative MFI defined as Tri-GalNAc as one. Significant differences were calculated by the Dunnett's test for Tri-

GalNAc-FITC using EZR software.[4]

The cellular uptake study on non-hepatocytes were conducted with the same method above using HeLa cells (125,000 cells per well in a 24-well plate) as an alternative cultured in DMEM high glucose.

Binding activity studies against ASGPR using live cells

HepG2 cells were seeded (150,000 cells per well in a 24-well plate) and incubated for overnight before experiment. Medium was removed and cells washed with D-PBS (-). Cells were incubated with 5 μM of ligands and 0.03 mM~100 mM of GalNAc in DMEM low glucose with 10% FBS and 1% penicillin/streptomycin for 2 h. After incubation, the cell were collected by trypsin treatment and washed with D-PBS(-), then suspended in D-PBS(-), then supplemented with 2% FBS. Suspension was filtered before flow cytometry analysis. Mean fluorescence intensity of each concentration of GalNAc was plotted and drew inhibition curve using Kaleida graph software (Synergy Software).

Internalization study of streptavidin

HepG2 cells were seeded (150,000 cells per well in a 24-well plate) and incubated for overnight before experiment. Medium was removed and cells washed with D-PBS (-). Cells were incubated with 250 nM of Alexa Fluor 647 labelled streptavidin and 1 μM biotinylated ligands in DMEM low glucose with 10% FBS and 1% penicillin/streptomycin for 2 h. After incubation, the cell were collected by trypsin treatment and washed with D-PBS(-), then suspended in D-PBS(-), then supplemented with 2% FBS. Suspension was filtered before flow cytometry analysis. Data was collected by Acculi C6 Plus Flowcytometer (BD Biosciences).

Synthesis method of 5S-ctx

DTPA (161 μL, 10 mM in PBS, final concentration 1 mM) and sodium tetraborate decahydrate (30.8 mg, final concentration 50 mM) to cetuximab (1372 μL, 5 mg/mL in PBS) (Merck & Co., NJ). The solution was treated with TCEP (6 eq, 10 mM in PBS) for 2 h at 37 °C. The solution was applied on MidiTrap G-25 column (preequilibrated with PBS containing 1 mM DTPA). The column was centrifuged for 2

min (1000 x g). Eluate was kept in refrigerator (4 °C), then mal-5S (10 eq, 10 mM in water) was added and incubated for 1 h at 4 °C. The solution was applied on MidiTrap G-25 column (preequilibrated with PBS). The column was centrifuged for 2 min (1000 x g). MidiTrap G-25 column was washed out with PBS by centrifuging for 2 min (1000 x g). The eluent was combined and concentrated using Amicon 10K for 15 min (7500 x g). The concentration was determined by measuring UV absorbance at 280 nm.

Synthesis method of Tri-ctx

DTPA (111 µL, 10 mM in PBS, final concentration 1 mM) and sodium tetraborate decahydrate (21.2 mg, final concentration 50 mM) to cetuximab (1000 µL, 5 mg/mL in PBS). The solution was treated with TCEP (6 eq, 10 mM in PBS) for 2 h at 37 °C. The solution was applied on MidiTrap G-25 column (preequilibrated with PBS containing 1 mM DTPA). The column was centrifuged for 2 min (1000 x g). Eluate was kept in refrigerator (4 °C), then mal-Tri-GalNAc (10 eq, 4.9 mM in water) was added and incubated for 1 h at 4 °C. The solution was applied on MidiTrap G-25 column (preequilibrated with PBS). The column was centrifuged for 2 min (1000 x g). The concentration was determined by measuring UV absorbance at 280 nm.

Degradation study of EGFR

HepG2 cell line (ATCC) cultured in DMEM (Merck) and supplemented with 10% heat inactivated FBS and 100 µg/mL Kanamycin (Merck). Cells were maintained in humidified incubator with 95% air/5%CO_2 at 37°C. HepG2 cells were treated with various concentrations of compounds for 24 h in 6 well plate. Cells were lysed with SDS lysis buffer (0.1M Tris-HCl, pH 8.0, 10% glycerol, 1% SDS) and immediately boiled for 10 min to obtain clear lysates. Protein concentrations were measured using Pierce™ BCA protein assay kit (Thermo Fisher Scientific). Lysates containing equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Merck, Germany) for Western blot analysis using the appropriate antibodies. Immunoreactive proteins were visualized using the Immobilon Western chemiluminescent HRP substrate (Merck) or Clarity Western ECL substrate (Bio-Rad); light emission intensity was quantified using an LAS-3000 lumino-image analyzer equipped with Image Gauge v2.3 software (Fuji).

The antibodies used in this study were: anti-EGFR antibody (1:1000, #4267, Cell signaling), β-actin antibody (1:2000, #A2228, Merck), anti-rabbit-IgG-HRP (1:5000, #NA934, GE Healthcare) and anti-mouse-IgG-HRP (1:5000, #NA931, GE Healthcare).

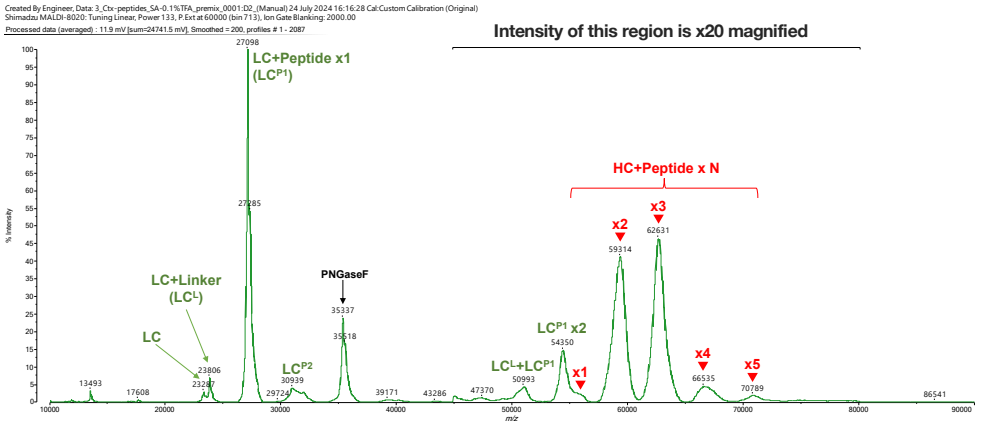
Evaluation of EGFR clearance activity of ligand-cetuximab conjugates

HepG2 cells in DMEM high glucose supplemented with GlutaMAX™ (Thermo) and 10% FBS were seed (50000 cells per well in a 24-well plate) and incubated for overnight before experiment. The cells were incubated with 1 nM ctx, Tri-ctx, and 5S-ctx for 2, 4, 8, 16, 24 h at 37ºC. The cells were washed with PBS, and collected by Accutase (Nacalai tesque) treatment. The cells were incubated with 1.5 μg/mL Alexa Fluor 488 labelled F(ab')2 Fragment Goat Anti-Human IgG, Fcγ Fragment Specific (Jackson ImmunoResearch) in FACS stain buffer (PBS with 0.5% BSA, 0.1%NaN_3, and 2 mM EDTA) for 30 min on ice. The data were collected by FACSCanto II (BD Biosciences). The EGFR internalization of ctx, Tri-ctx, and 5S-ctx are indicated as the mean fluorescence intensity (MFI) of four samples. Error bars represent the standard error of four samples.

DAR analysis of ligand-cetuximab conjugates

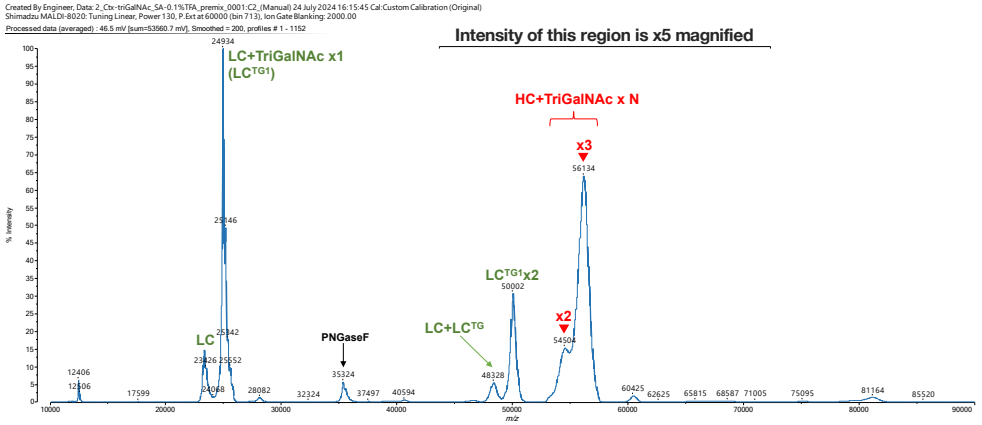
Cetuximab conjugates (100 μL) were treated with PNGaseF (5 μL) (Promega) for 25 h at 37 °C. The solution was desalted using Amicon 3K for 30 min (14000 x g). Pure water (200 μL) was added and repeated previous step for 3 times. Final concentration was determined by measuring UV absorbance at 280 nm. The sample was mixed with 20 mg/mL sinapinic acid in 50% MeCN, 0.1% TFA in a same volume and dropped to a sample plate. Mass spectra was collected by SHIMADZU MALDI-8020 and detected on positive-ion linear mode. BSA was used for external standard to calibrate.

Mass spectra of 5S-ctx and DAR calculation



	DAR	Intensity(mV)	Ratio	Average DAR of each subunit	Average DAR
LC	0	698.5	2.6%	1.01	7.20
	1	24742.5	93.6%		
	2	981.8	3.7%		
HC	1	32.8	2.8%	2.59	
	2	509.7	43.1%		
	3	573.7	48.5%		
	4	43.5	3.7%		
	5	23.4	2.0%		

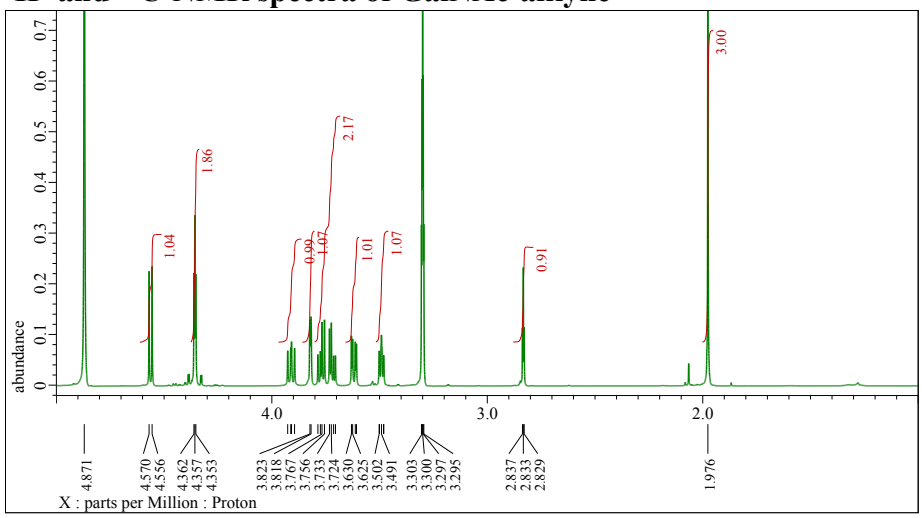
Mass spectra of Tri-ctx and DAR calculation

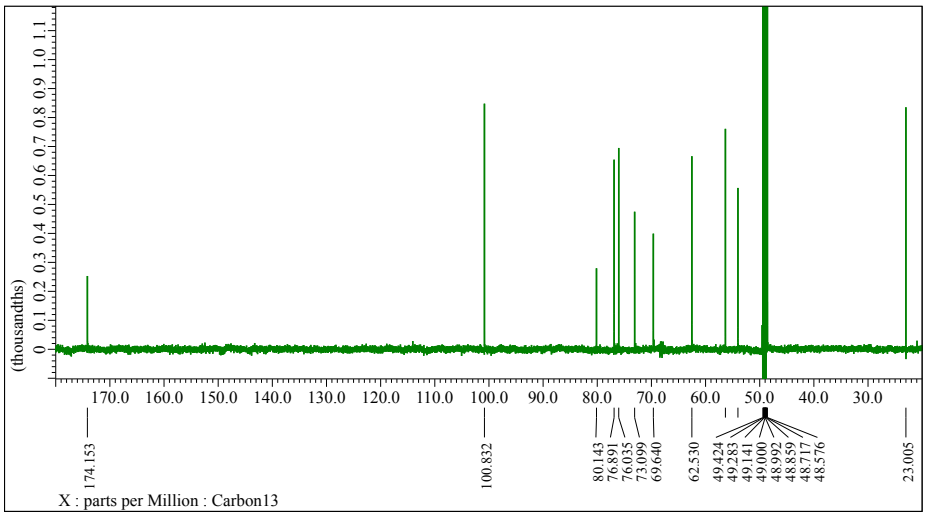


	DAR	Intensity(mV)	Ratio	Average DAR of each subunit	Average DAR

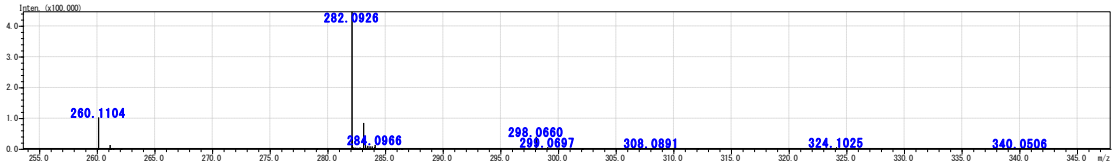
LC	0	7880.6	12.8%	0.87	7.36
	1	53560.7	87.2%		
HC	2	1642	19.4%	2.81	
	3	6841.3	80.6%		

^{1}H- and ^{13}C-NMR spectra of GalNAc-alkyne

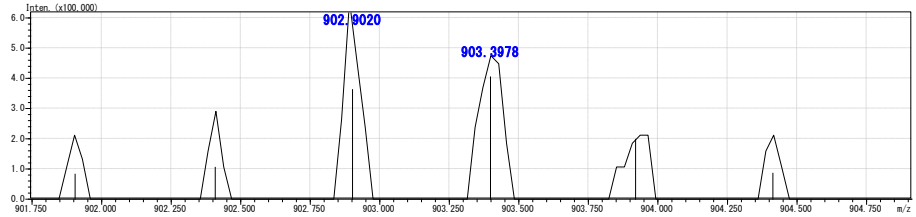




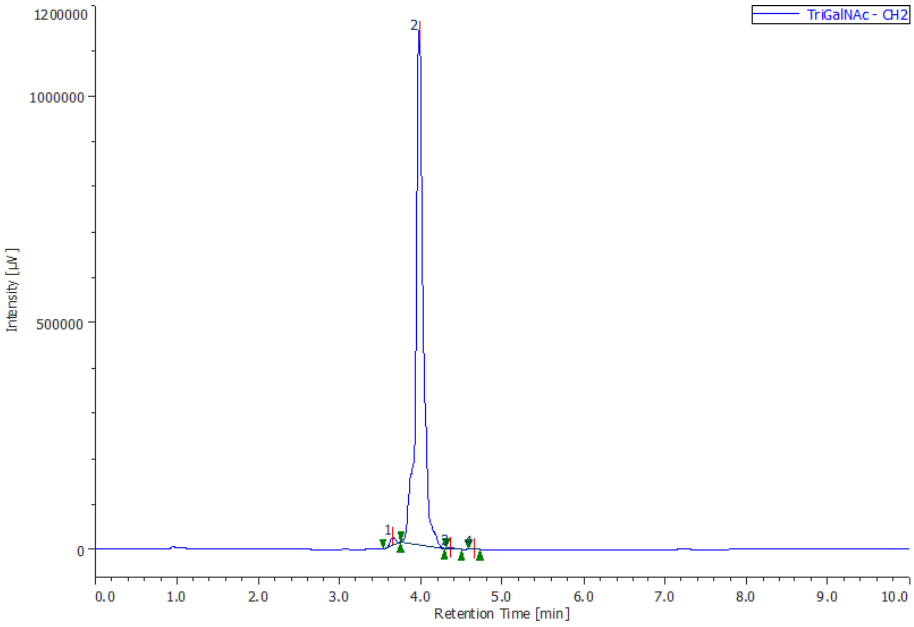
HR-MS chromatogram of GalNAc-alkyne



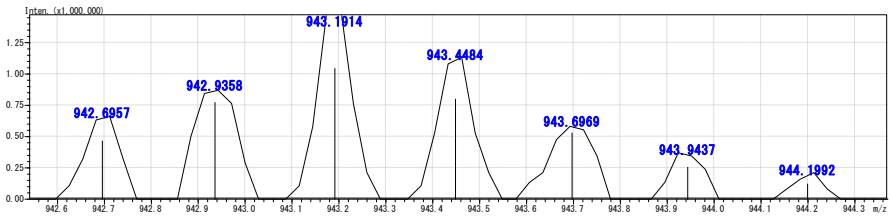
HR-MS chromatogram of Tri-GalNAc



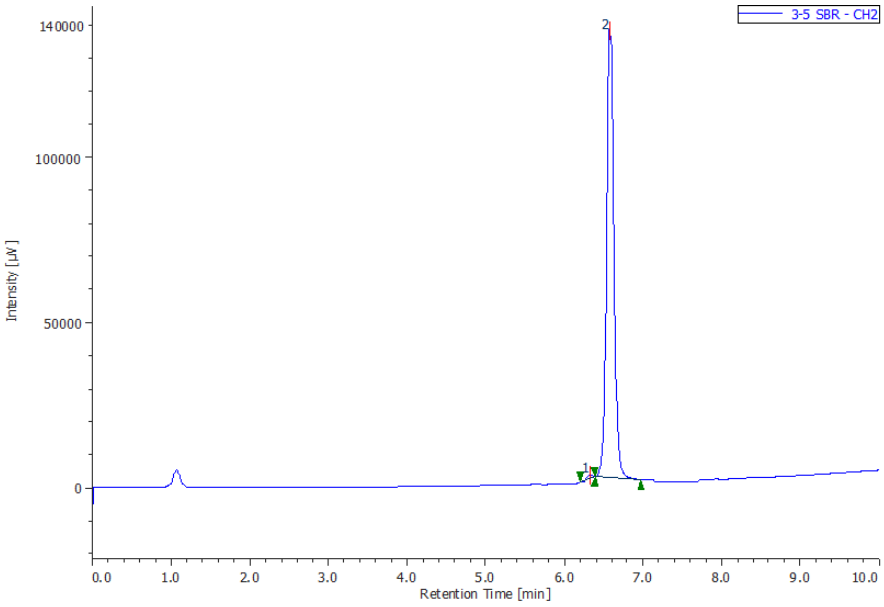
HPLC chromatogram of Tri-GalNAc



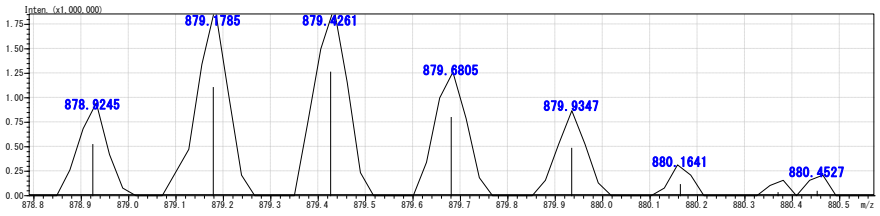
HR-MS chromatogram of 5S



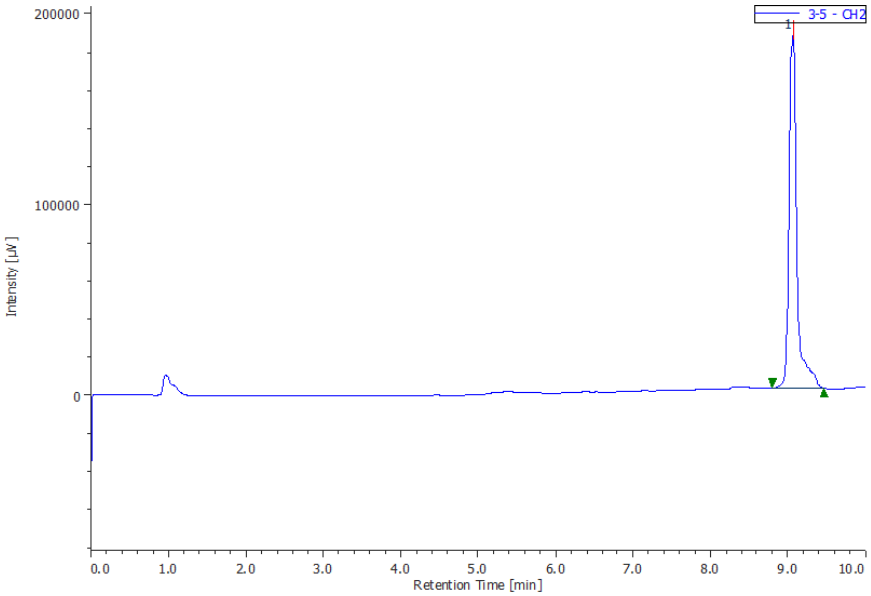
HPLC chromatogram of 5S



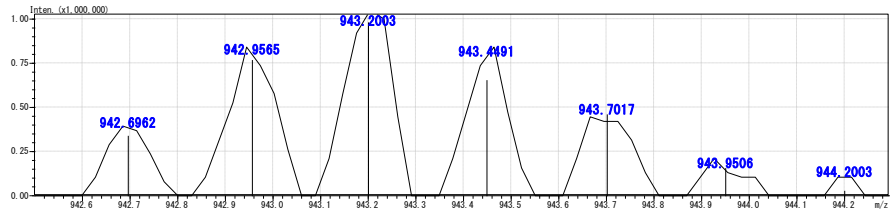
HR-MS chromatogram of 5



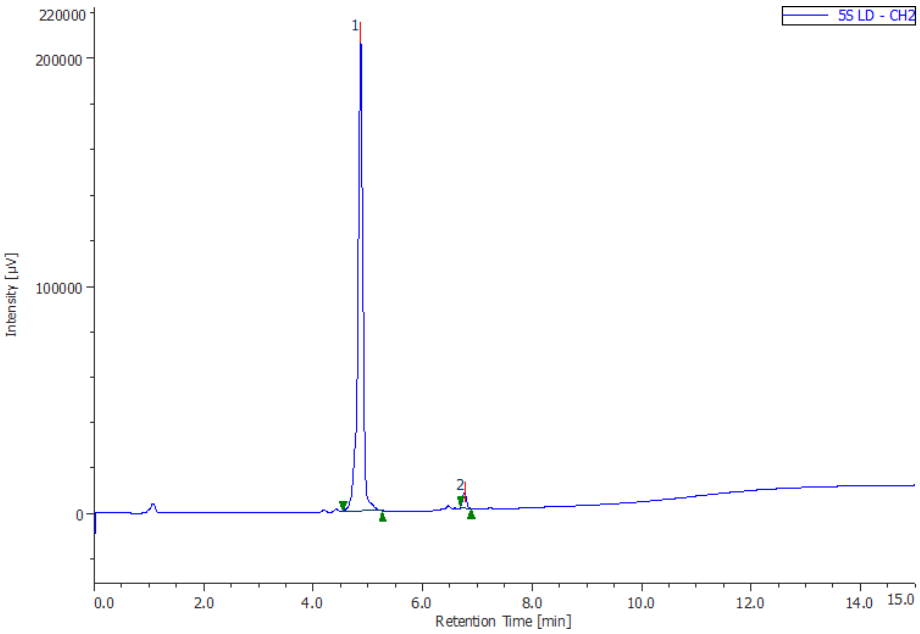
HPLC chromatogram of 5



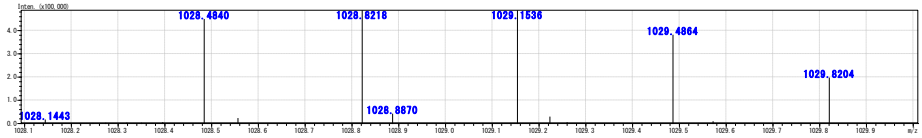
HR-MS chromatogram of LD



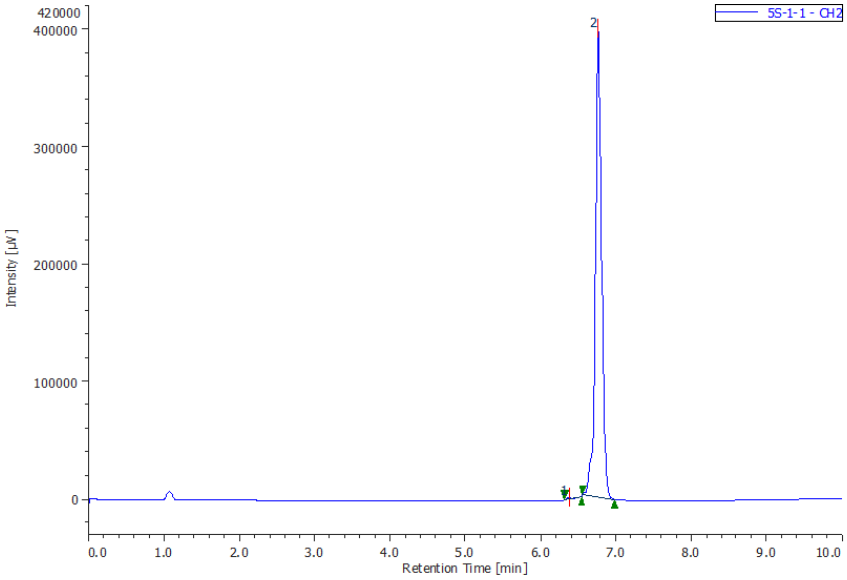
HPLC chromatogram of LD



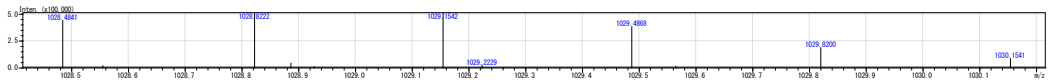
HR-MS chromatogram of 5S-1



HPLC chromatogram of 5S-1



HR-MS chromatogram of 5S-1’



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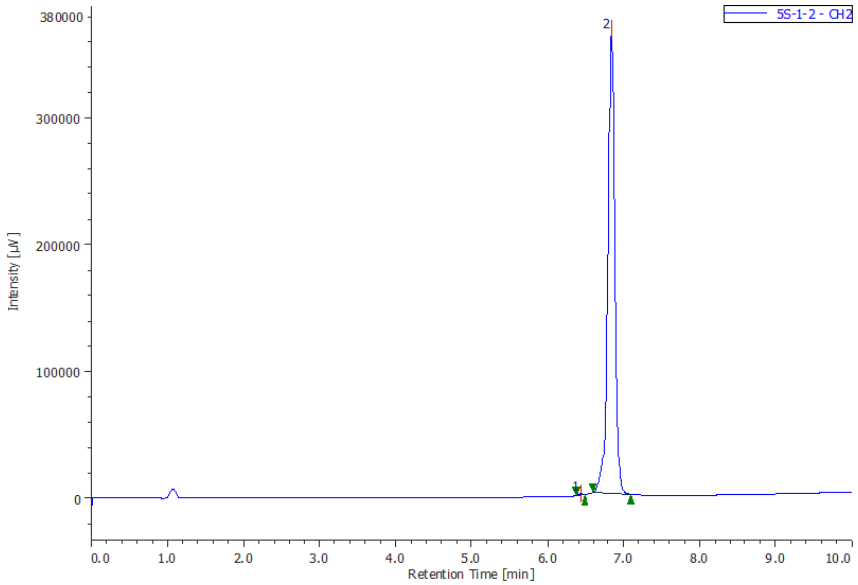
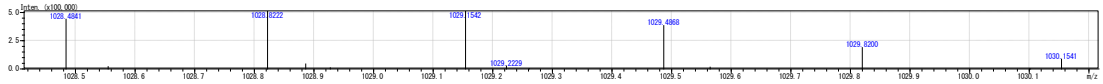


HR-MS chromatogram of 5S-1”



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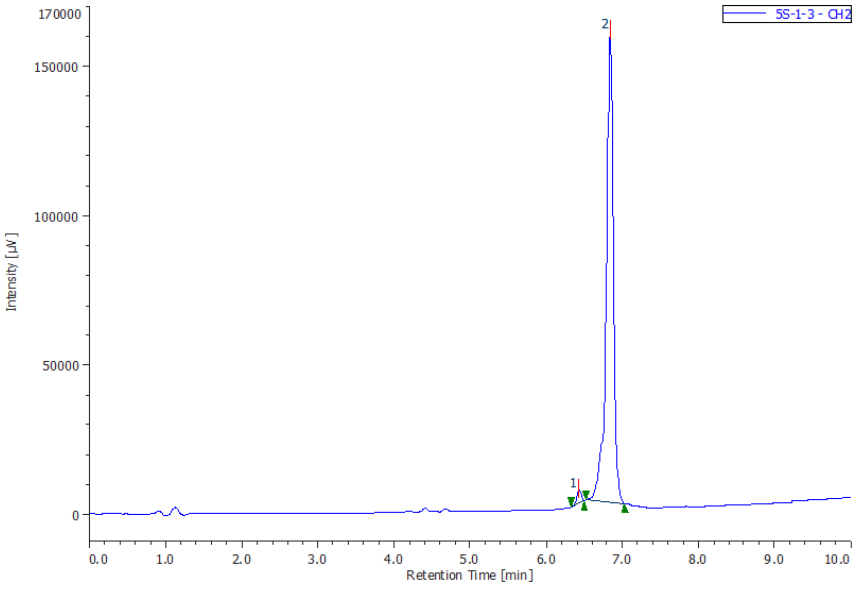
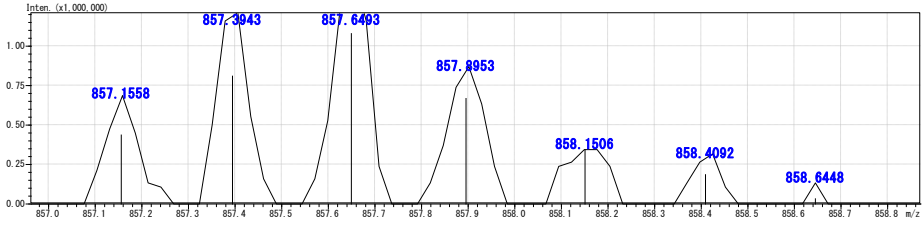
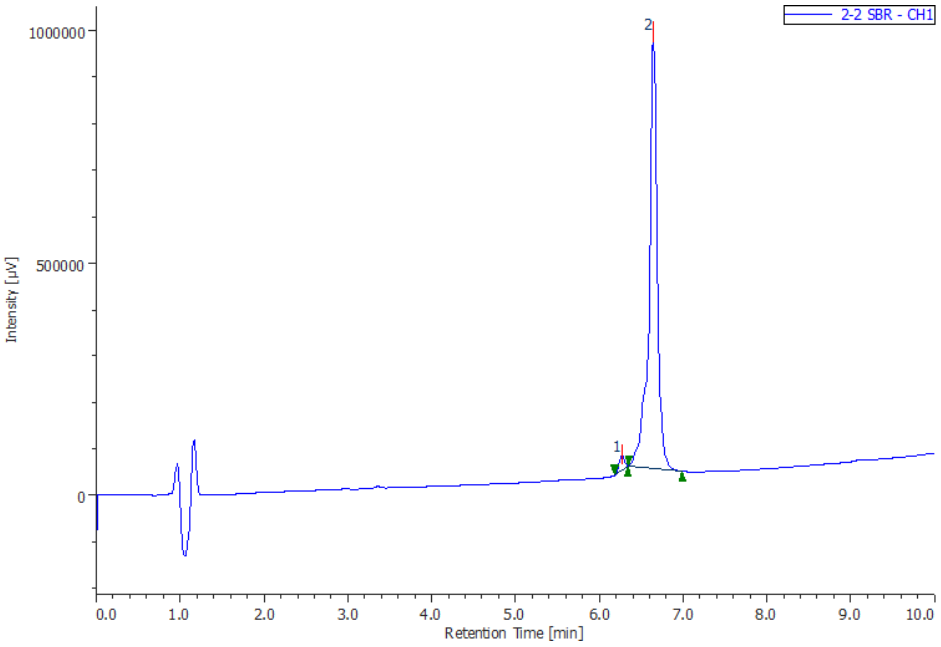


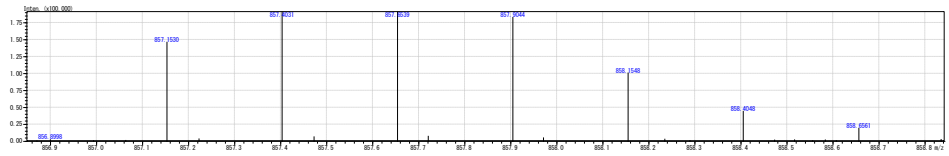
HR-MS chromatogram of 5S-2



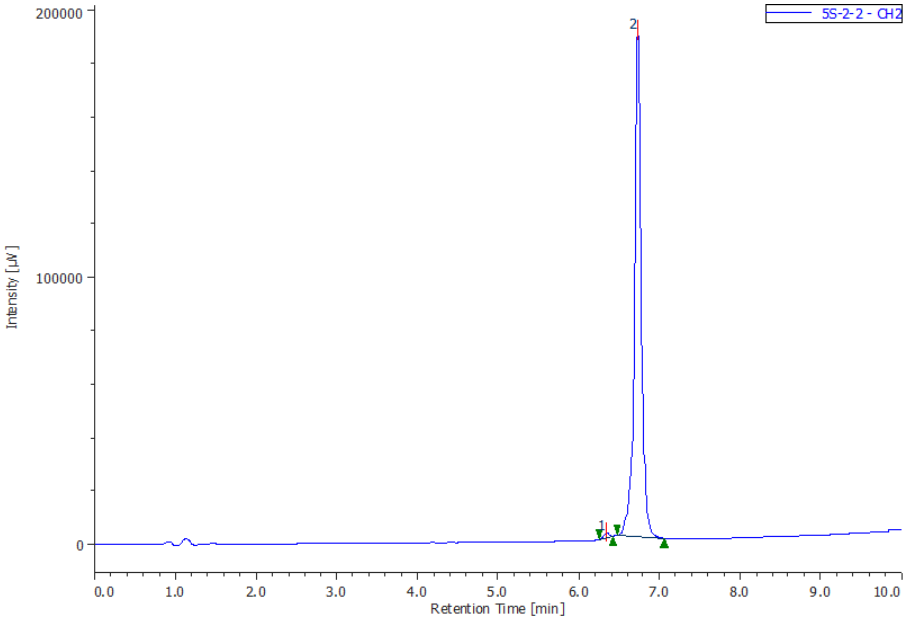
HPLC chromatogram of 5S-2



HR-MS chromatogram of 5S-2’



HPLC chromatogram of 5S-2’



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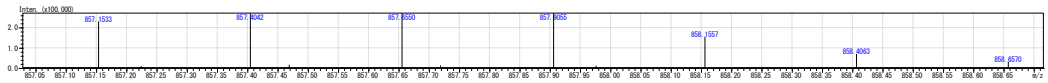
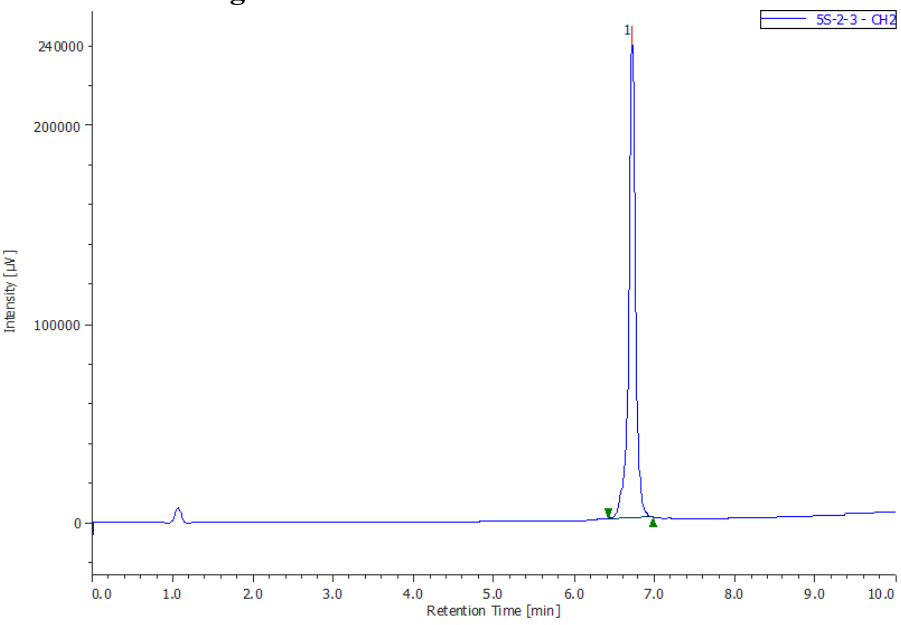
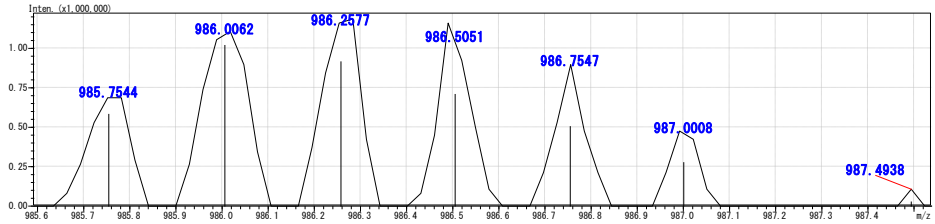


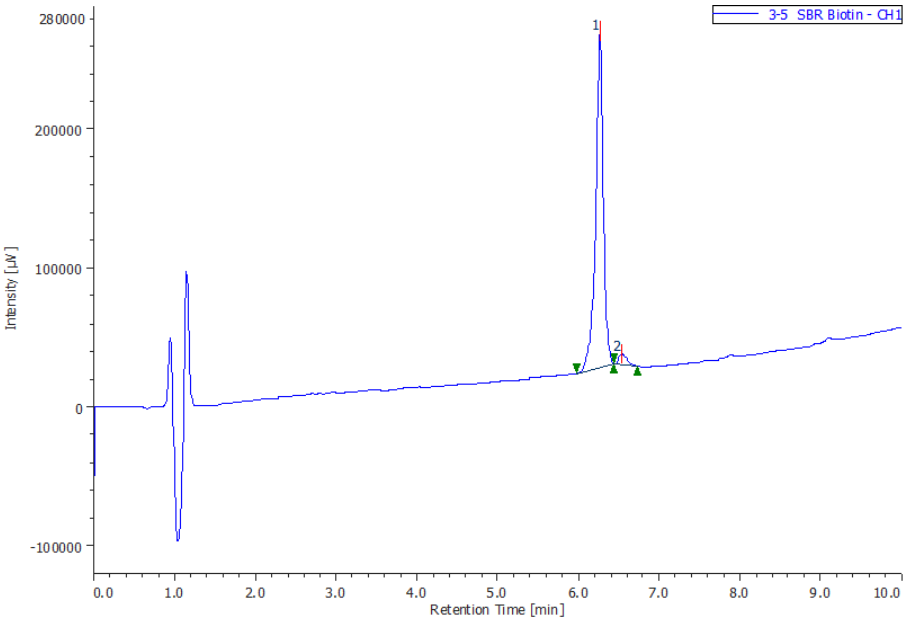
HPLC chromatogram of 5S-2”



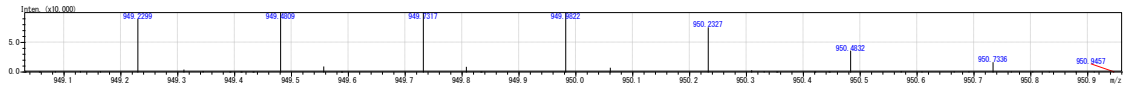
HR-MS chromatogram of 5S-Bio



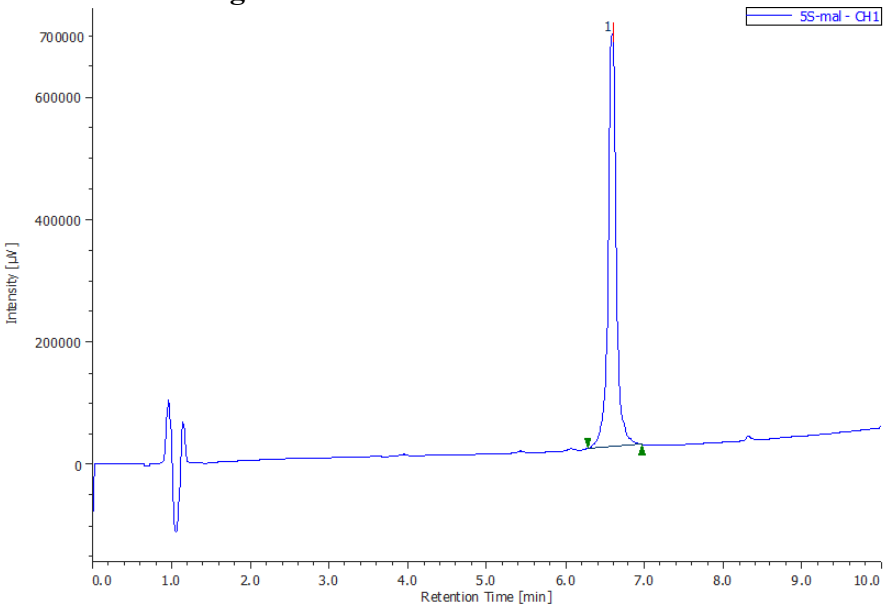
HPLC chromatogram of 5S-Bio



HR-MS chromatogram of Mal-5S



HPLC chromatogram of Mal-5S



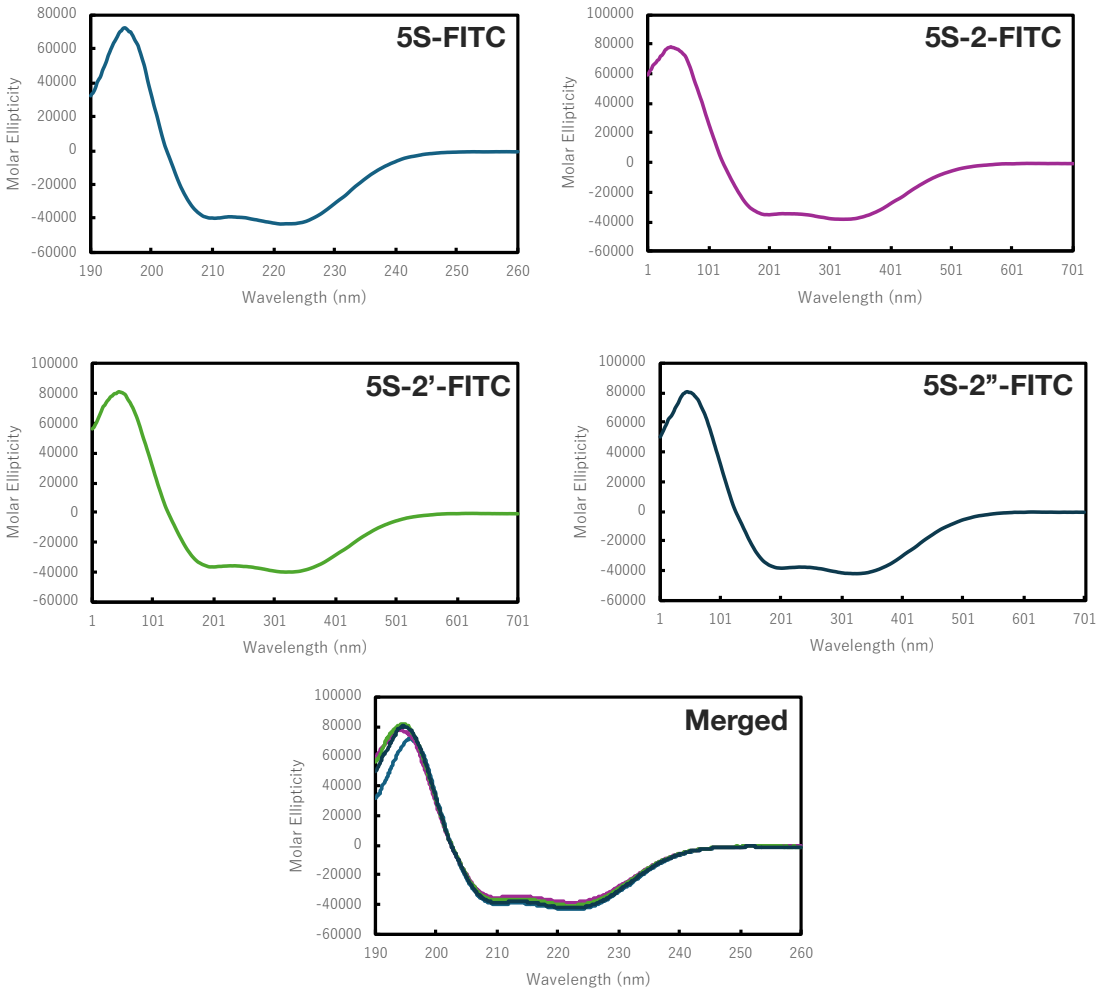
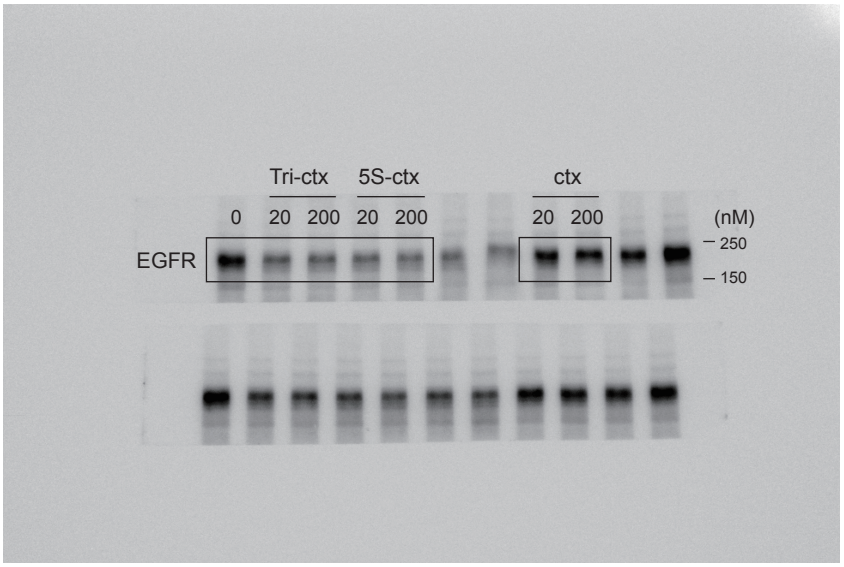
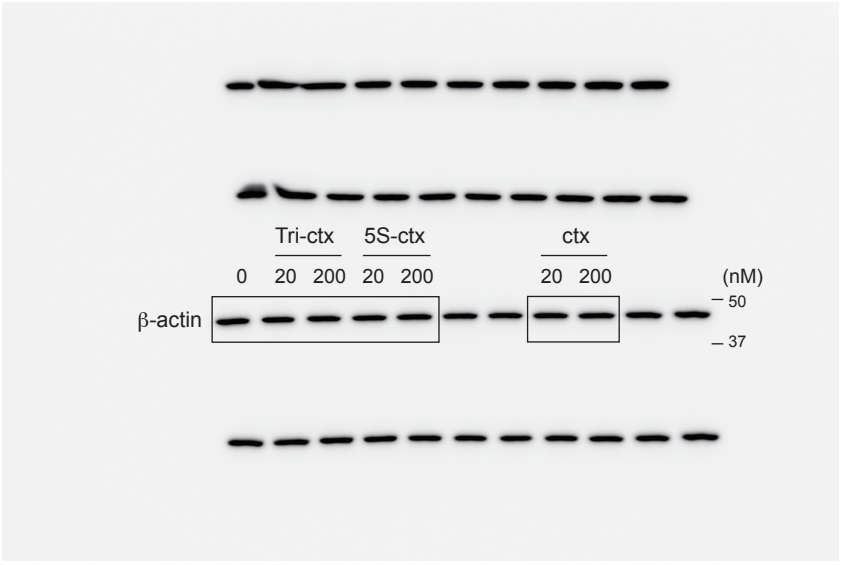


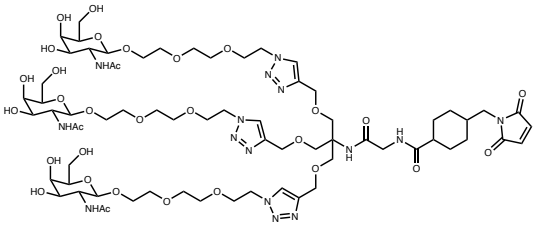
Figure S1. CD spectra of **5S-FITC**, **5S-2-FITC**, **5S-2'-FITC**, **5S-2"-FITC** and merged spectra of those four peptides. Condition: 100 μM in 20 mM phosphate buffer (pH 7.2).

Figure 8b


Tri-ctx
5S-ctx
ctx
0
20
200
20
200
20
200
(nM)
EGFR
250
150


Tri-ctx
5S-ctx
ctx
0
20
200
20
200
20
200
(nM)
β-actin
50
37

Chemical structure of mal-Tri-GalNAc



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4. Y. Kanda, Investigation of the freely available easy-touse software 'EZR' (Easy R) for medical statistics, *Bone Marrow Trasnplant*, 2013, **48**, 452-458.