Supporting Information for

Original article

One-step synthesis of site-specific antibody–drug conjugates by reprograming IgG glycoengineering with LacNAc-based substrates

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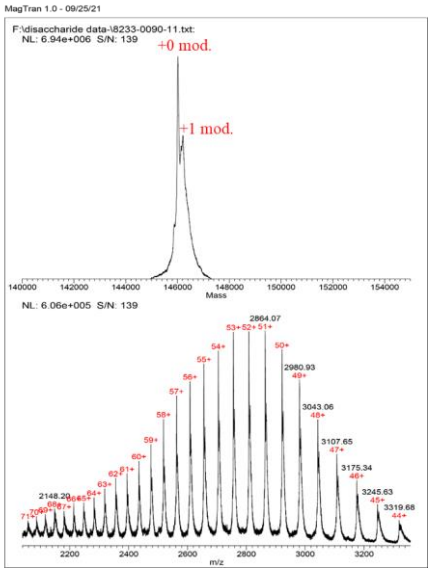
1. Supplementary scheme and figures

A. +Endo-S



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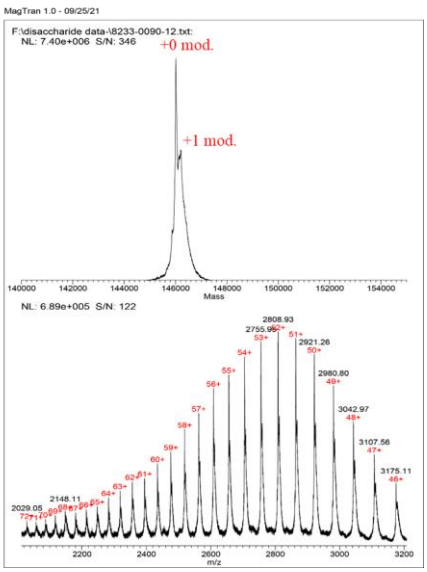

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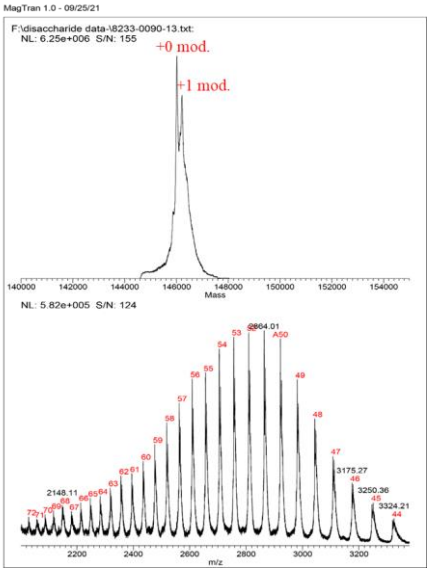

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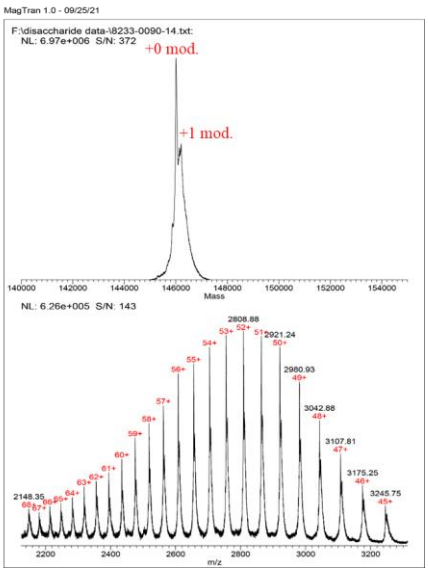

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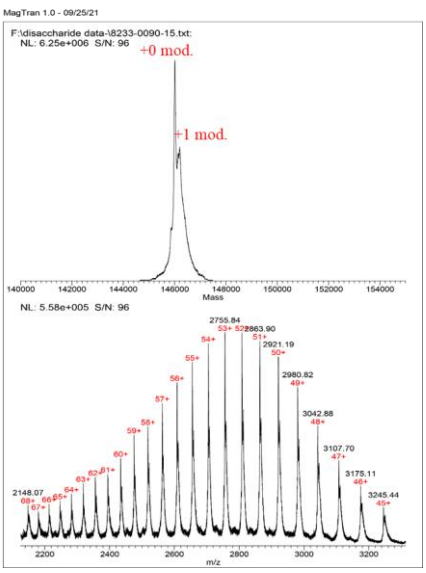

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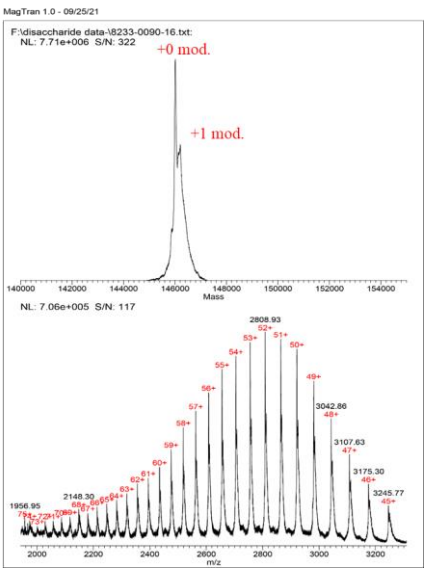
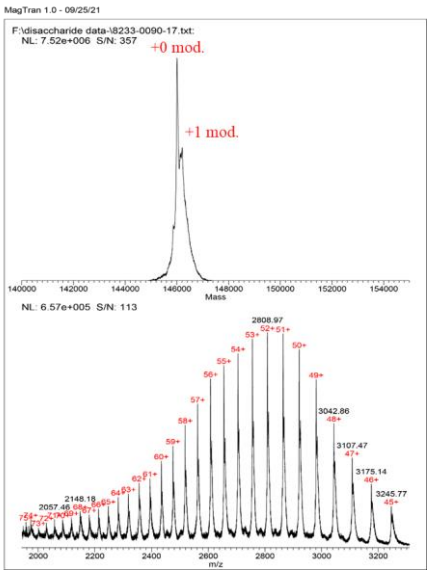


G. +Endo-D



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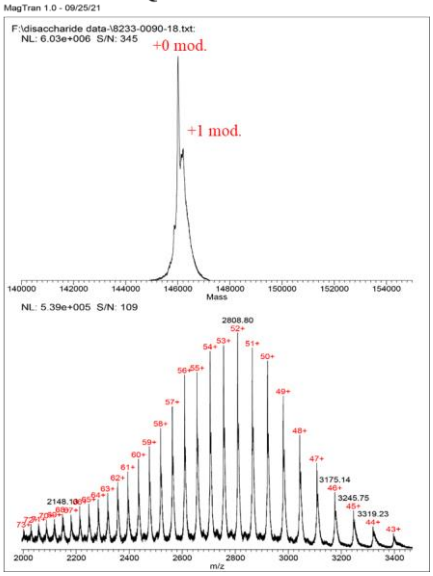
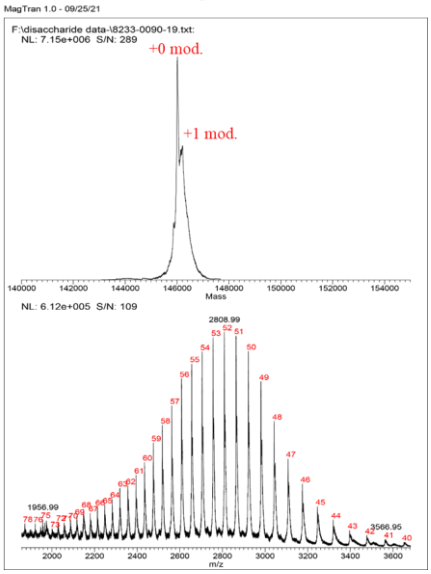


I. +Endo-D N322Q



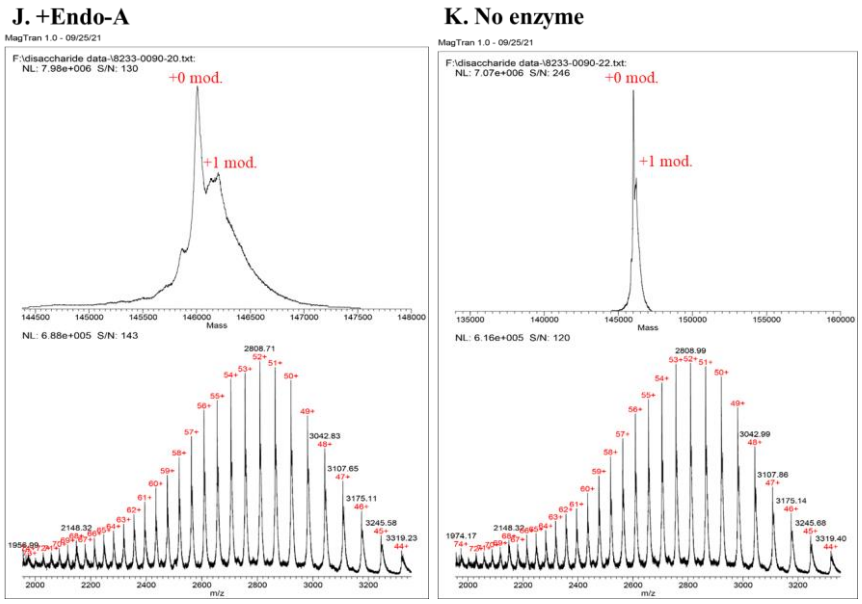
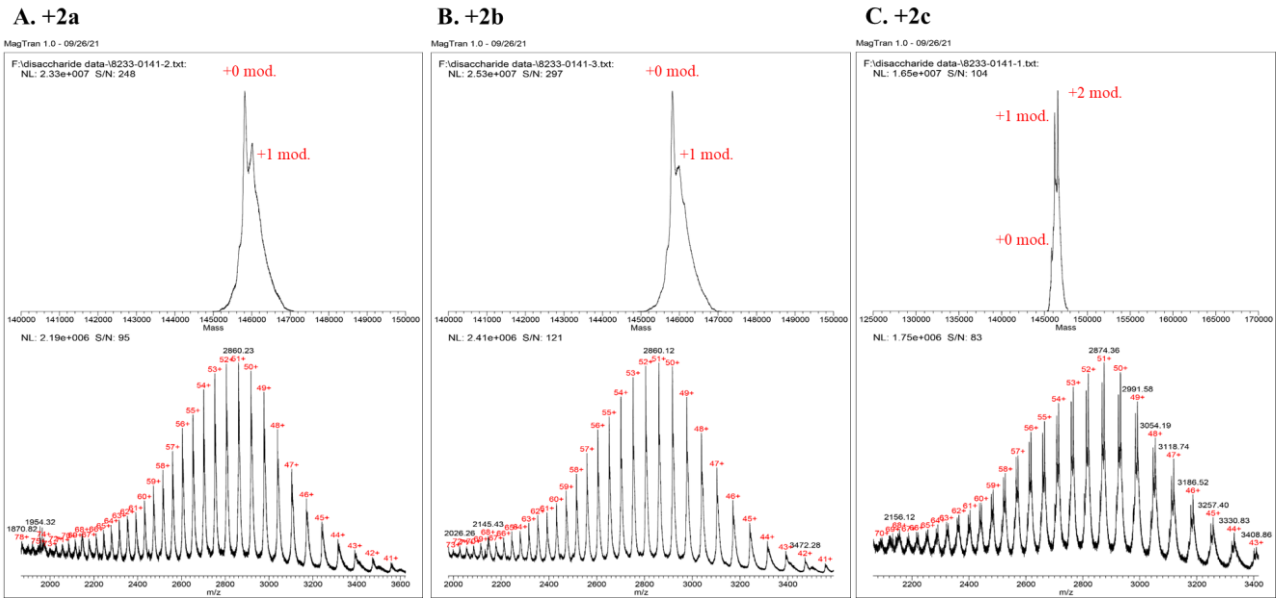


Figure S1 LC–MS profiles of transglycosylation of GlcNAc(Fuc)-trastuzumab with GlcNAc-ox and various ENGases. In each LC–MS spectrum, the deconvolution data (full mass) are shown in upper section and the multiple-charge data (*m*/*z*) are shown in lower section.



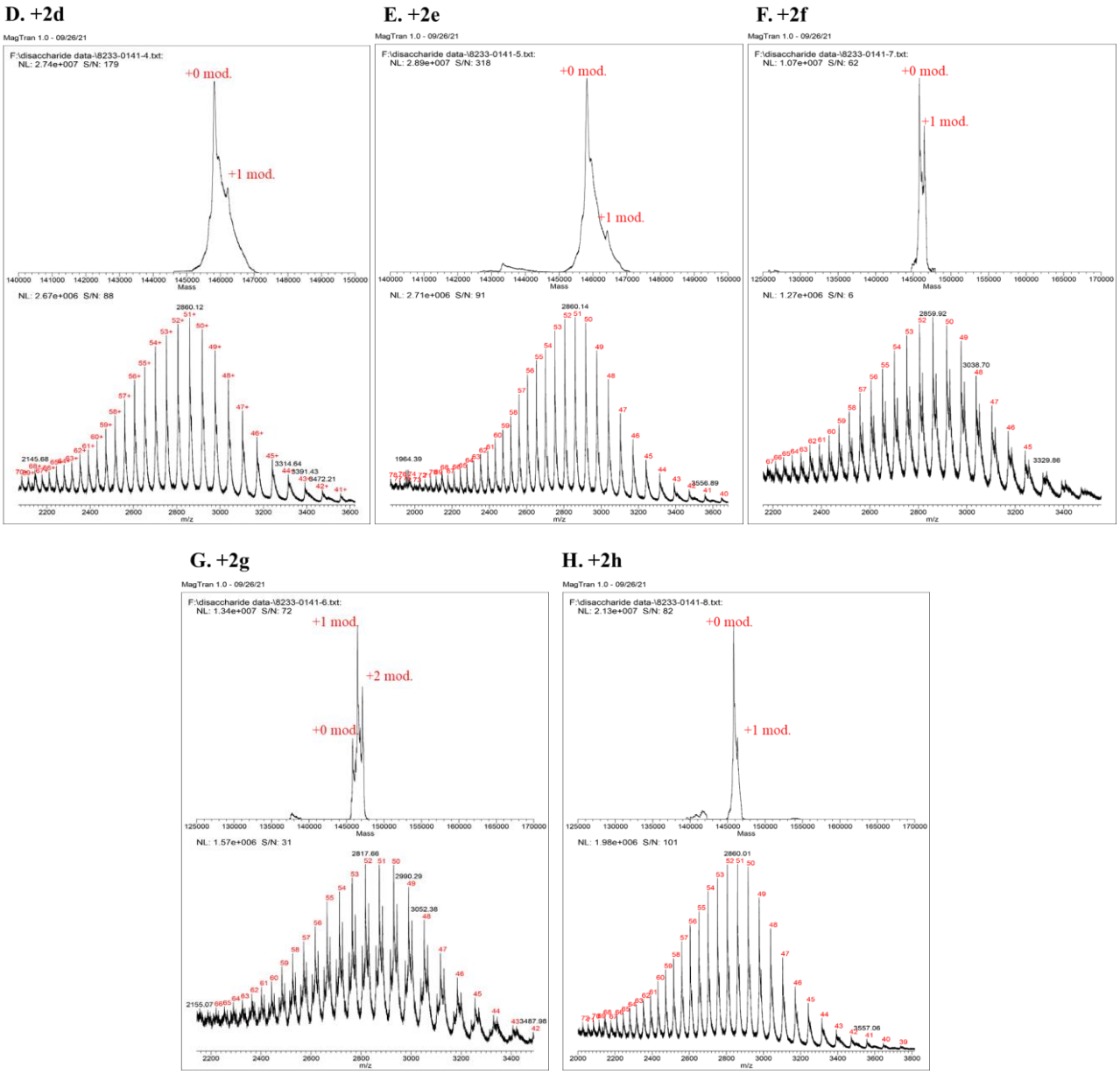


Figure S2 LC–MS profiles of Endo-S2 catalyzed transglycosylation of GlcNAc(Fuc)-trastuzumab with various glycan oxazoline substrates. In each LC–MS spectrum, the deconvolution data (full mass) are shown in upper section and the multiple-charge data (*m*/*z*) are shown in lower section.

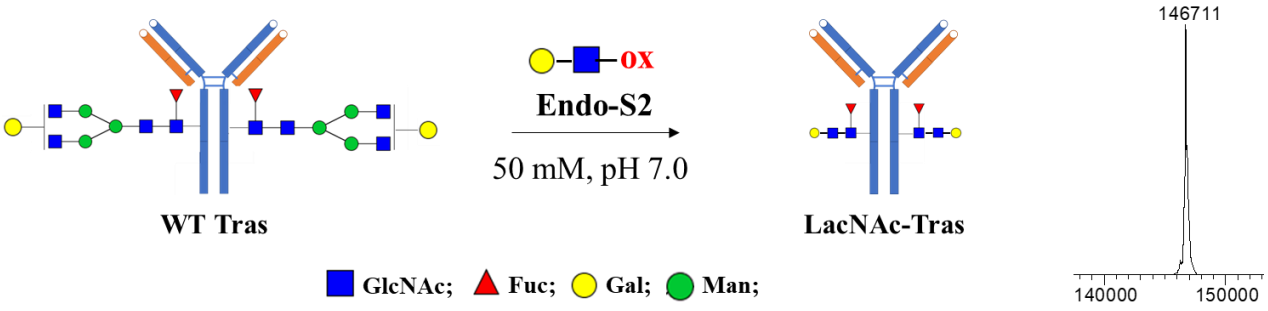
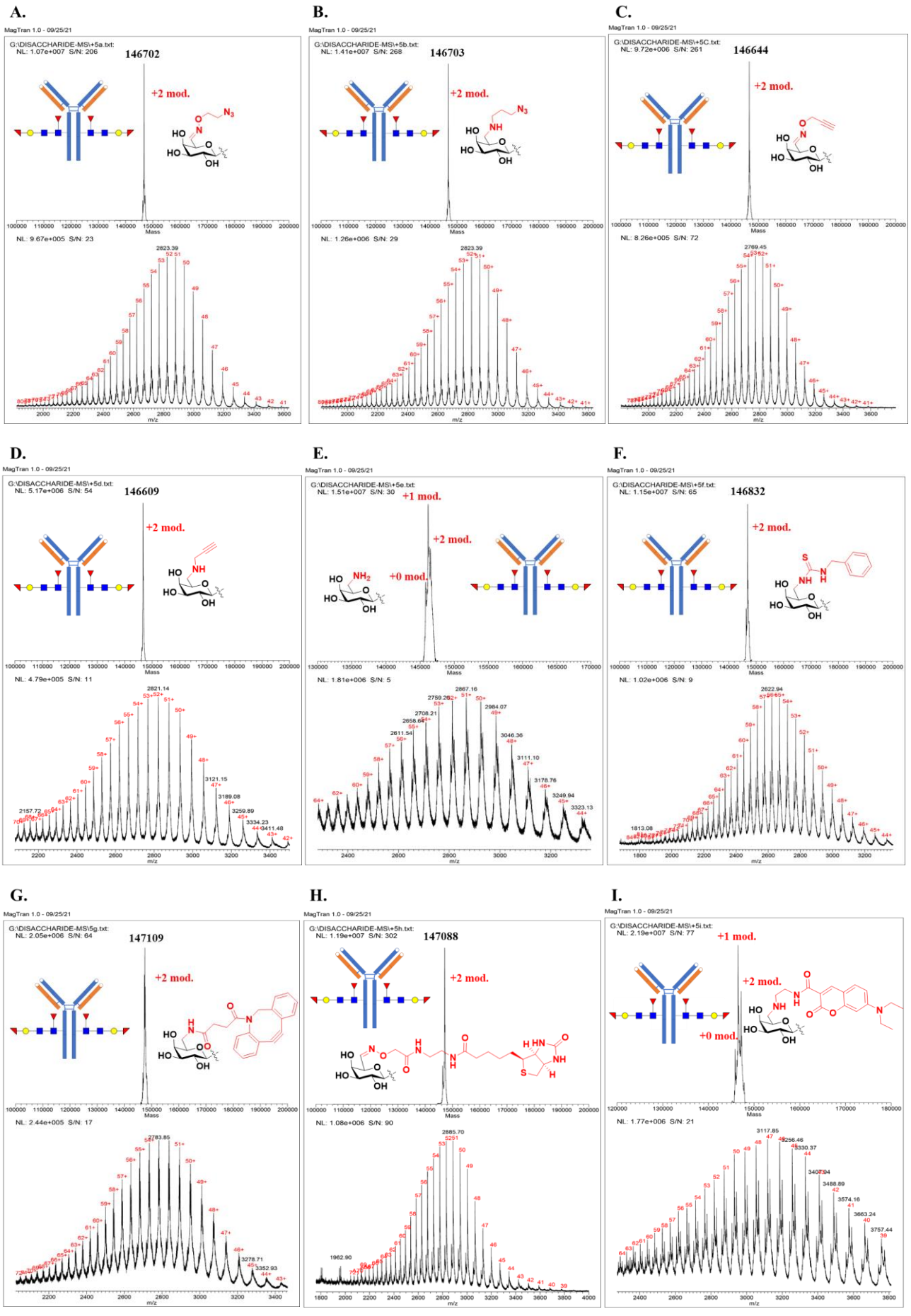


Figure S3 Endo-S2 enables efficient one-step glycoengineering of wild type trastuzumab with LacNAc oxazoline.


A.
B.
C.
D.
E.
F.
G.
H.
I.
146702
146703
146644
146609
146832
147109
147088
+2 mod.
+1 mod.
+0 mod.
Mass
m/z

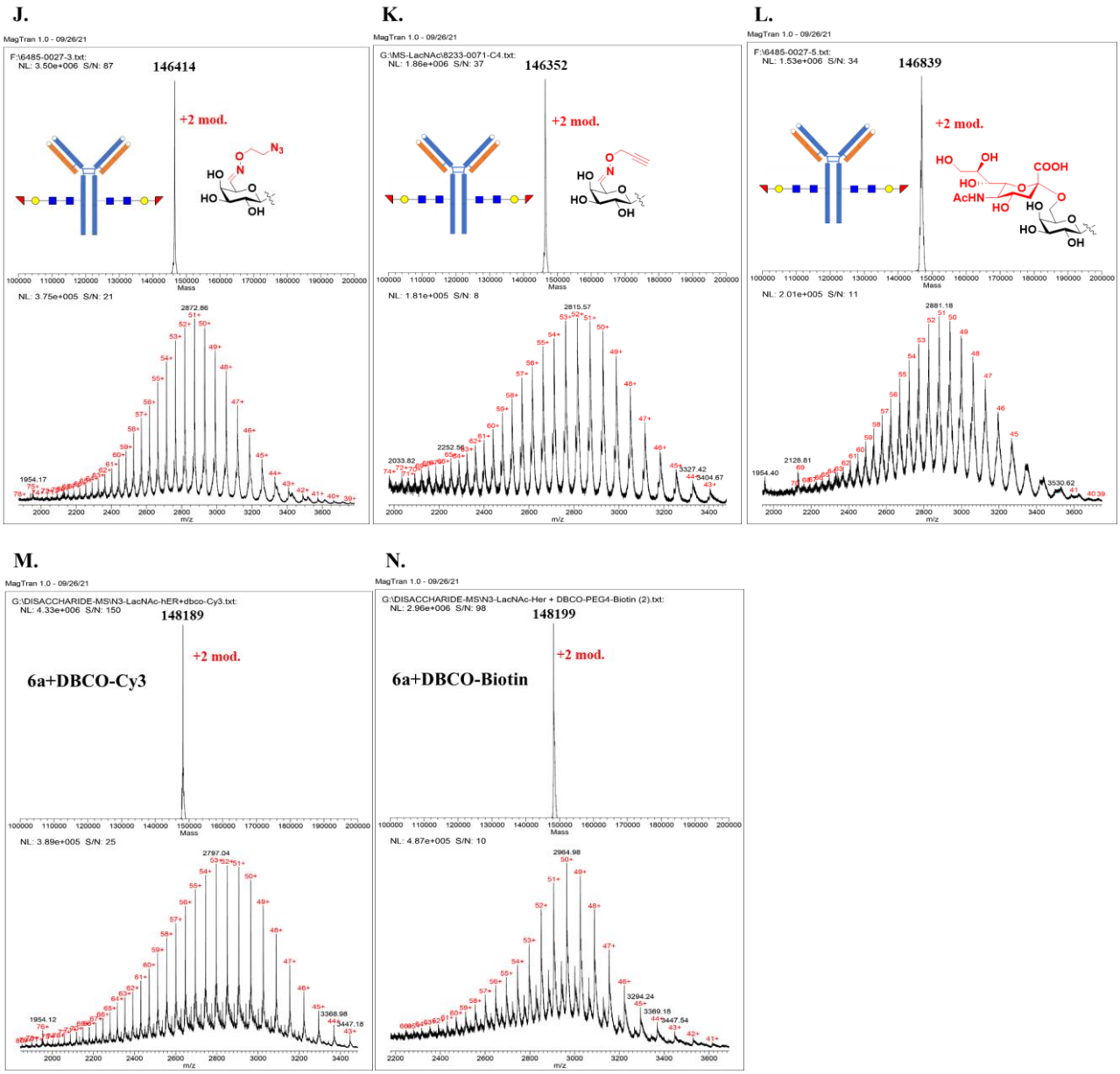


Figure S4 LC–MS profiles of Endo-S2 catalyzed transglycosylation of trastuzumab with various LacNAc derivatives. In each LC–MS spectrum, the deconvolution data (full mass) are shown in upper section and the multiple-charge data (*m*/*z*) are shown in lower section.

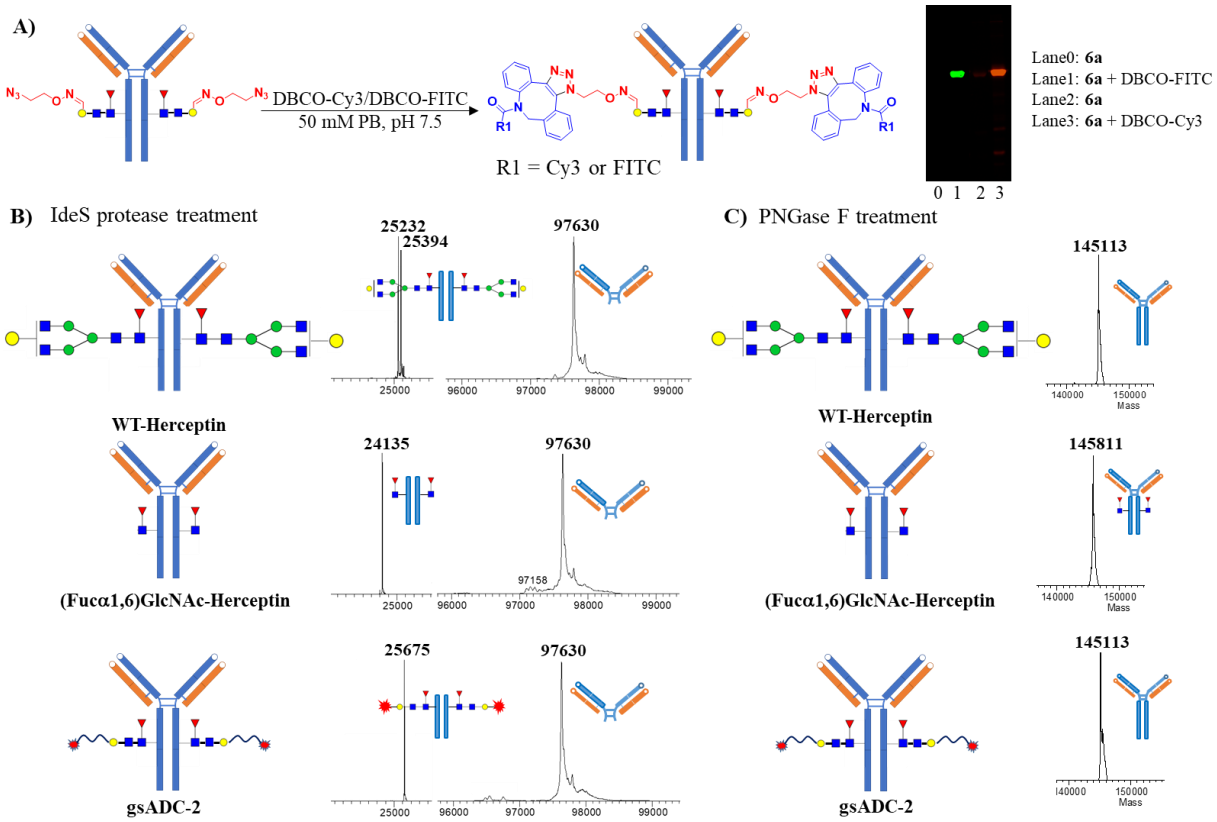


Figure S5 Digestion of Trastuzumab and gsADC-2 with PNGase F or IdeS protease to identify the conjugation site. (A) Cy3 and FITC conjugation with Az-LacNAc-Tras indicated the fluorescence of heavy chains. (B) IdeS treatment demonstrated the conjugation of drugs on the Fc domain. (C) PNGase F treatment proved the site-specific conjugation on *N*-glycosylation site of trastuzumab.

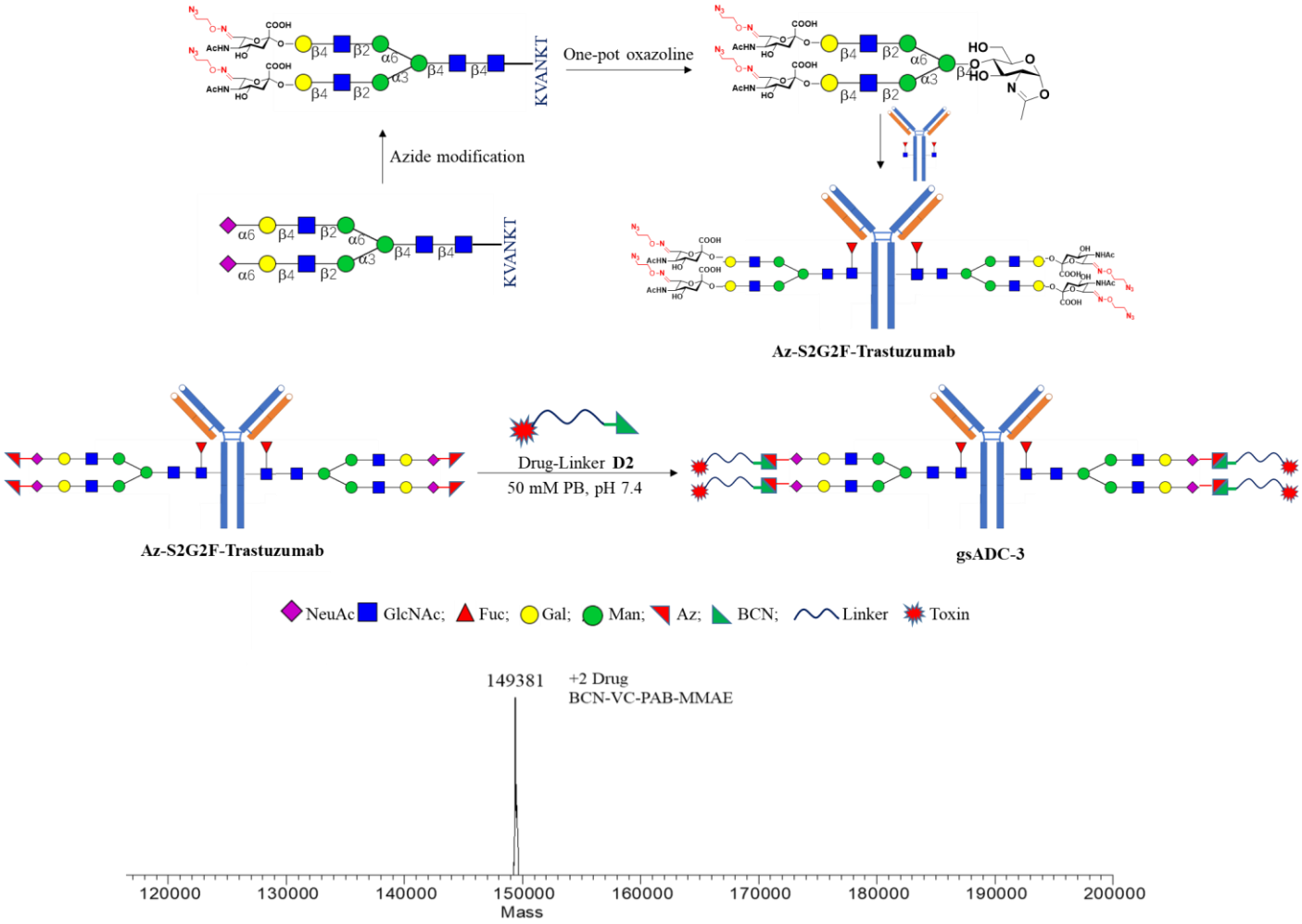
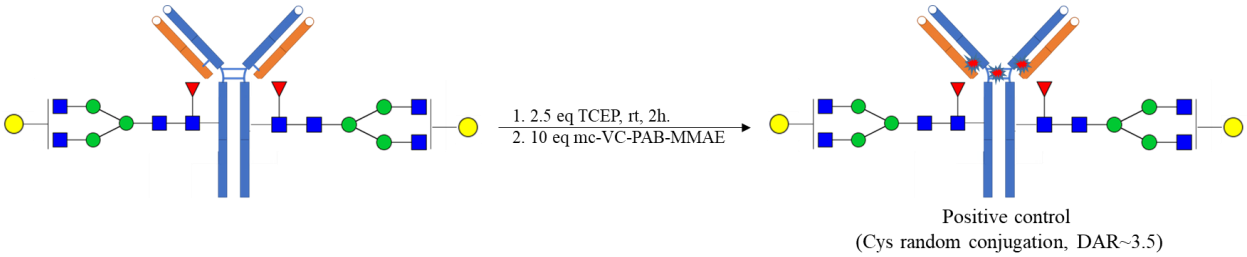


Figure S6 Preparation of gsADC-3 based on sialylated complex type glycan.



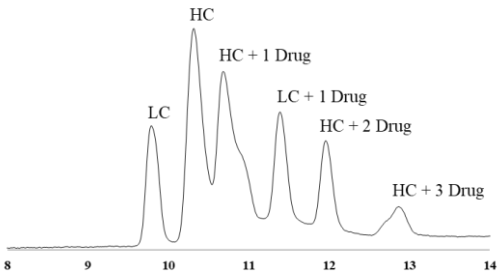
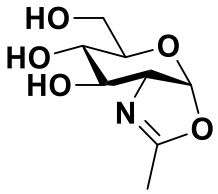


Figure S7 Preparation of one ADC based on Cys random conjugation (DAR~3.5).

2. Experimental section

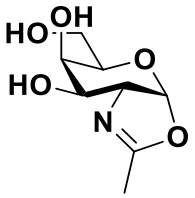
*2.1 synthesis of glycan oxazoline **2a**–**h***

GlcNAc-ox **2a**



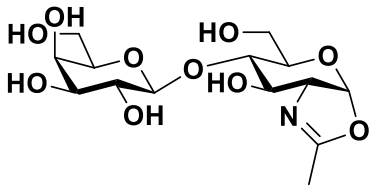
ESI-MS calcd. for $C_8H_{13}NO_5[M+H]^+$ *m/z*= 204.0872, found *m/z*= 204.0809.
^{1}H NMR (600 MHz, deuterium oxide) δ 6.01 (d, *J* = 7.3 Hz, 1H), 4.04 (ttd, *J* = 6.2, 4.4, 3.9, 2.4 Hz, 1H), 3.90 (t, *J* = 3.6 Hz, 1H), 3.74–3.70 (m, 1H), 3.60 (dd, *J* = 12.5, 6.3 Hz, 1H), 3.57–3.51 (m, 1H), 3.29 (ddd, *J* = 8.9, 6.3, 2.5 Hz, 1H), 1.97–1.95 (m, 3H).

GalNAc-ox **2b**



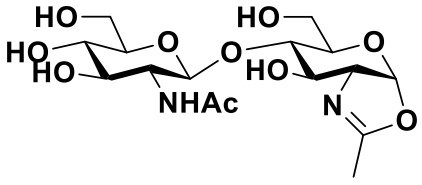
ESI-MS calcd. for $C_8H_{13}NO_5[M+H]^+$ *m/z*= 204.0872, found *m/z*= 204.0859.
^{1}H NMR (600 MHz, deuterium oxide) δ 6.00 (d, *J* = 7.2 Hz, 0.85H), 5.14 (d, *J* = 3.7 Hz, 0.15H), 4.02–3.97 (m, 1H), 3.86–3.84 (m, 1H), 3.81 (ddd, *J* = 7.0, 4.9, 1.8 Hz, 1H), 3.76 (td, *J* = 7.1, 1.3 Hz, 1H), 3.70–3.59 (m, 2H), 1.94 (d, *J* = 1.3 Hz, 3H).

LacNAc-ox **2c**



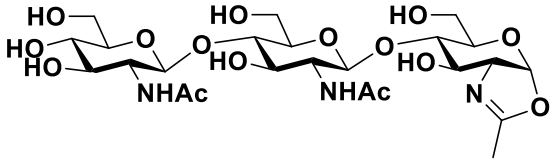
ESI-MS calcd. for $C_{14}H_{23}NO_{10}[M+H]^+$ *m/z*= 366.14, found *m/z*= 366.1421.
^{1}H NMR (600 MHz, deuterium oxide) δ 6.01 (d, *J* = 7.3 Hz, 0.84H), 4.67 (s, 0.16H), 4.34 (d, *J* = 7.9 Hz, 1H), 4.32 (dd, *J* = 3.2, 1.8 Hz, 1H), 4.11 (ddq, *J* = 6.8, 3.5, 1.7 Hz, 1H), 3.84 (dd, *J* = 3.4, 1.1 Hz, 1H), 3.76–3.52 (m, 7H), 3.42 (dd, *J* = 9.9, 7.8 Hz, 1H), 3.37 (ddd, *J* = 8.8, 6.2, 2.5 Hz, 1H), 1.98 (d, *J* = 1.8 Hz, 3H).

$(GlcNAc)_2$-ox **2d**



ESI-MS calcd. for $C_{16}H_{26}N_2O_{10}[M+H]^+$ *m/z*= 407.1665, found *m/z*= 407.1636.
^{1}H NMR (600 MHz, deuterium oxide) δ 6.00 (d, *J* = 7.3 Hz, 1H), 4.49 (d, *J* = 8.4 Hz, 1H), 4.33 (dd, *J* = 3.2, 1.6 Hz, 1H), 4.11 (ddp, *J* = 6.7, 3.5, 1.7 Hz, 1H), 3.84 (dd, *J* = 12.5, 2.1 Hz, 1H), 3.69 (dd, *J* = 12.5, 5.0 Hz, 1H), 3.63–3.54 (m, 3H), 3.53–3.45 (m, 2H), 3.42–3.34 (m, 2H), 3.20 (ddd, *J* = 8.9, 6.5, 2.4 Hz, 1H), 1.98–1.94 (m, 6H).

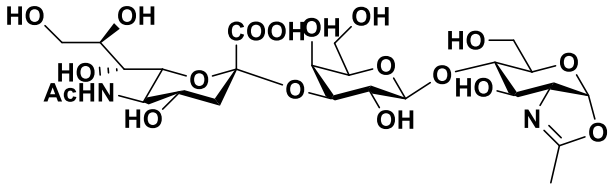
$(GlcNAc)_3$-ox **2e**



ESI-MS calcd. for $C_{24}H_{39}N_3O_{15}[M+H]^+$ *m*/*z*= 610.2459, found *m*/*z*= 610.2451.

^{1}H NMR (600 MHz, deuterium oxide) δ 5.99 (d, *J* = 7.3 Hz, 1H), 4.52–4.45 (m, 2H), 4.34–4.32 (m, 1H), 4.10 (ddq, *J* = 7.0, 3.3, 1.8 Hz, 1H), 3.85–3.20 (m, 16H), 2.00–1.90 (m, 9H).

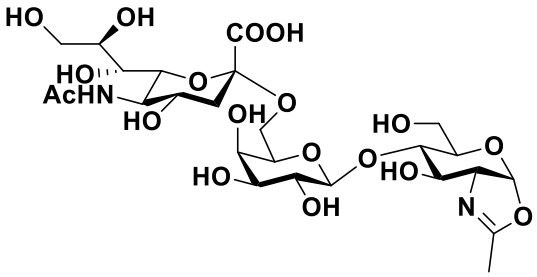
NeuNAc-α2,3-LacNAc-ox **2f**



ESI-MS calcd. for $C_{25}H_{40}N_2O_{18}[M+H]^+$ *m*/*z*= 657.2354, found *m*/*z*= 657.2351.

^{1}H NMR (600 MHz, deuterium oxide) δ 5.97 (d, *J* = 7.3 Hz, 1H), 4.39 (d, *J* = 7.8 Hz, 1H), 3.96 (dd, *J* = 9.8, 3.2 Hz, 1H), 3.83 (d, *J* = 3.2 Hz, 1H), 3.79–3.32 (m, 17H), 2.51 (1H), 1.97–1.90 (m, 6H), 1.67 (t, *J* = 12.1 Hz, 1H).

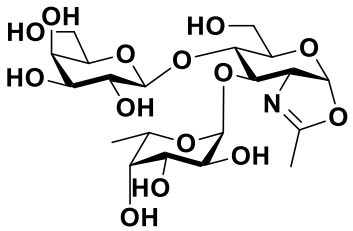
NeuNAc-α2,6-LacNAc-ox **2g**



ESI-MS calcd. for $C_{25}H_{40}N_2O_{18}[M+H]^+$ *m*/*z*= 657.2354, found *m*/*z*= 657.2343

^{1}H NMR (600 MHz, deuterium oxide) δ 5.96 (d, *J* = 7.3 Hz, 1H), 4.32–4.25 (m, 1H), 3.88–3.31 (m, 20H), 1.94 (d, *J* = 1.8 Hz, 3H), 1.91 (s, 3H), 1.56 (t, *J* = 12.2 Hz, 1H).

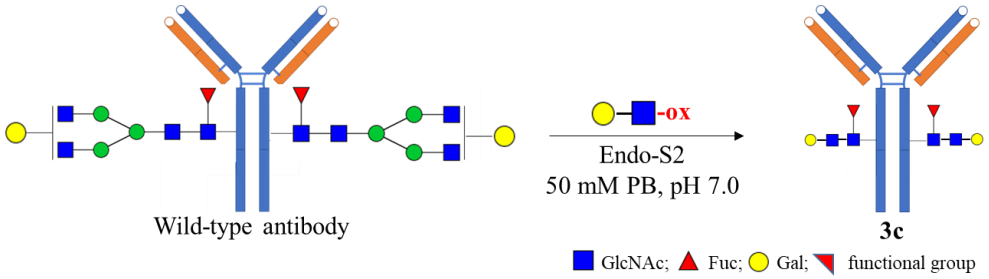
Gal-β1,4-(Fucα1,3)GlcNAc-ox **2h**



ESI-MS calcd. for $C_{20}H_{33}NO_{14}[M+H]^+$ *m*/*z*= 512.1979, found *m*/*z*= 512.1966.

^{1}H NMR (600 MHz, deuterium oxide) δ 6.01 (d, *J* = 7.3 Hz, 1H), 5.10 (d, *J* = 4.0 Hz, 1H), 4.38 (dd, *J* = 3.0, 1.2 Hz, 1H), 4.34 (d, *J* = 7.8 Hz, 1H), 4.28–4.24 (m, 1H), 4.11 (q, *J* = 6.5 Hz, 1H), 3.96 (dt, *J* = 8.7, 1.4 Hz, 1H), 3.84–3.32 (m, 12H), 1.98 (d, *J* = 1.8 Hz, 3H), 1.12 (3H).

2.2. LacNAc transglycosylation optimization via Endo-S2



A solution of wild-type trastuzumab (50 μg) and LacNAc-ox (5 nmol) in a PB buffer (50 mmol/L, pH 7.0, 10 μL) was incubated with Endo-S2 (4 μg) at 30 ℃ for 2 h. After 2–3 h, LC–MS monitoring indicated the complete reaction of wild-type trastuzumab to give the transglycosylation product carrying LacNAc **3c**.

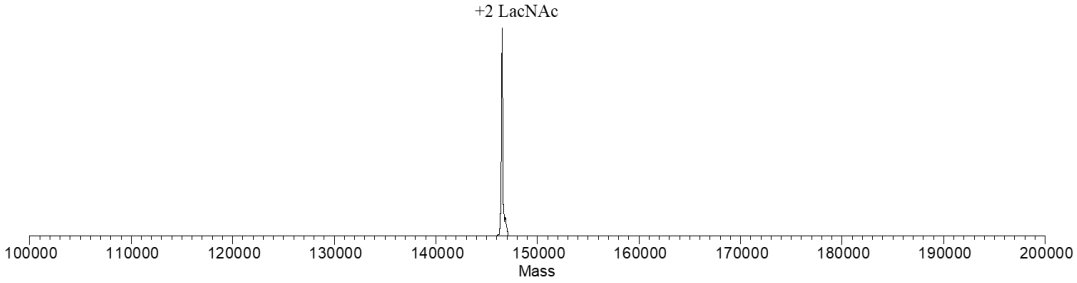


Figure S9 MS spectra of glycoengineered antibody **3c.**

2.3. Synthesis of LacNAc derivatives

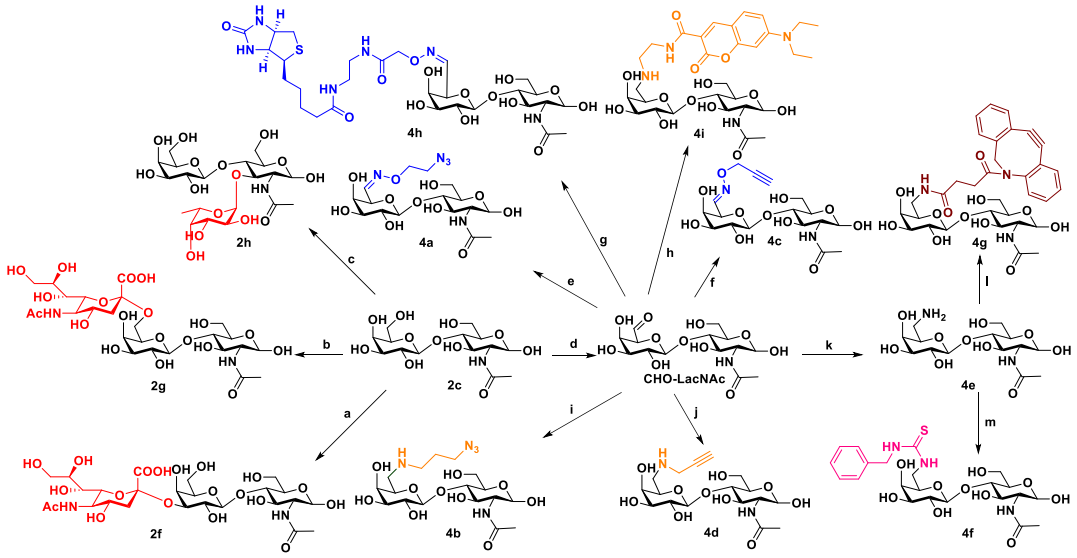


Figure S10 The synthetic route of LacNAc derivatives. a. α(2,3)-sialyltransfererase, CMP-sialic acid, cacodylate buffer, pH 7.6; b. α(2,6)-sialyltransferase (Pd2,6ST), CMP-sialic acid, 100 mmol/L Tris, pH 8.0; c. α(1,3)-fucosyltransferase; d. GOase, HRP, catalase, O_2, 50 mmol/L PB, pH 7.0, 30 ℃; e. *O*-(2-Azidoethyl)-hydroxylamine hydrochloride, pH 7.2, rt; f. *O*-(2-Propynylethyl)-hydroxylamine hydrochloride; g. Biotin-ONH_2, pH 7.2, rt; h. Coumarin-NH_2, $NaCNBH_3$, pH 6.0, 0 ℃; i. 3-azido-1-propanamine, $NaCNBH_3$, pH 6.0, 0 ℃; j. 2-Propynylamine, $NaCNBH_3$, pH 6.0, 0 ℃; k. $NH_2OH{\cdot}HCl$, Na_2CO_3, CH_3OH/H_2O, rt; $NaBH_4$, $NiCl_2{\cdot}6H_2O$, 0 ℃; l. DBCO-CONHS, pH 7.4, rt; m. *N*-Benzylthiourea, pH 8.0, rt.

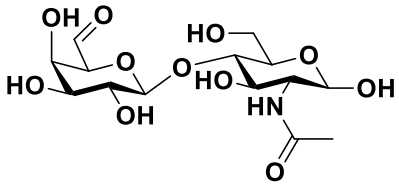
Synthesis of Gal-β1,4-(Fucα1,3)GlcNAc ***2h***. To a solution of LacNAc (20 mmol/L), GDP-Fuc (30 mmol/L, 1.5 eq), $MnCl_2$ (5 mmol/L) in Tris-HCl buffer (100 mmol/L, pH 7.5) was added Hp α1,3FT (40 μg/mL), and the mixture was incubated at 37 ℃ for overnight. TLC analysis (ethyl acetate/methanol/water/acetic acid 5:2:2:0.5) showed the conversion of LacNAc into a major product. The reaction mixture was centrifuged, and the resulting supernatant was loaded on Bio-Gel P2 column (eluent: 50 mmol/L NH_4HCO_3). Product containing fractions were combined and lyophilized to give α1,3-Fuc-LacNAc as a white

amorphous solid.

Synthesis of NeuNAc-α2,6-LacNAc ***2g.*** To a solution of LacNAc (20 mmol/L), CMP-Neu5Ac (30 mmol/L, 1.5 eq) in Tris-HCl buffer (100 mmol/L, pH 8.0) was added Pd2,6ST (20 μg/mL), and the mixture was incubated at 37 ℃ for 6 h. TLC analysis (ethyl acetate/methanol/water/acetic acid 5:2:2:0.5) showed the conversion of LacNAc into a major product. The reaction mixture was centrifuged, and the resulting supernatant was loaded on Bio-Gel P2 column (eluent: 50 mmol/L NH_4HCO_3). Product containing fractions were combined and lyophilized to give α2,6-Neu5Ac-LacNAc as a white amorphous solid.

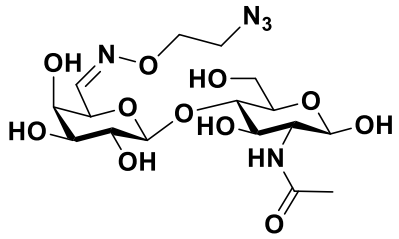
Synthesis of NeuNAc-α2,3-LacNAc ***2f.*** To a solution of LacNAc (20 mmol/L), CMP-Neu5Ac (30 mmol/L, 1.5 eq) in Tris-HCl buffer (100 mmol/L, pH 8.0) was added PmST1 (5 μg/mL), and the mixture was incubated at 37 ℃ for 30 min. TLC analysis (ethyl acetate/methanol/water/acetic acid 5:2:2:0.5) showed the conversion of LacNAc into a major product. The reaction mixture was centrifuged, and the resulting supernatant was loaded on Bio-Gel P2 column (eluent: 50 mmol/L NH_4HCO_3). Product containing fractions were combined and lyophilized to give α2,3-Neu5Ac-LacNAc as a white amorphous solid.

Synthesis of CHO-LacNAc[1]



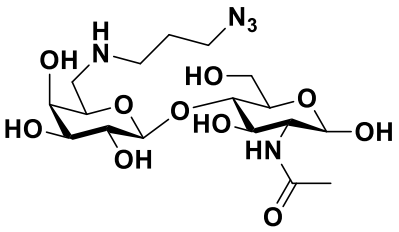
To a stirred solution of LacNAc **1c** (20 mg) in 50 mmol/L phosphate buffer (pH 7.0, 1 mL) was blowed oxygen for 10 min and galactose oxidase (47.6 U), HRP (480 U) and catalase (9.52 kU) was added respectively. The reaction mixture was stirred for 3 h and monitored by LC–MS. After that, the solution was subjected to a Bio-gel P2 column, the corresponding product CHO-LacNAc was combined and lyophilized to give a white powder. ESI-MS calcd. for $C_{14}H_{23}NO_{11}$ $[M+H]^+$ *m*/*z*= 382.1349, found *m*/*z*=382.1331. ^{1}H NMR (500 MHz, deuterium oxide) *δ* 5.15 (q, *J* = 1.4 Hz, 1H), 5.08 (ddd, *J* = 7.4, 3.2, 1.4 Hz, 1H), 4.43 (dt, *J* = 7.7, 1.7 Hz, 1H), 4.04 (dt, *J* = 3.1, 1.5 Hz, 1H), 3.96–3.89 (m, 1H), 3.88–3.74 (m, 3H), 3.70–3.52 (m, 3H), 3.41 (dq, *J* = 7.5, 1.5 Hz, 1H), 1.99 (d, *J* = 1.2 Hz, 3H).

Synthesis of N_3-ON=CH-LacNAc ***4a***



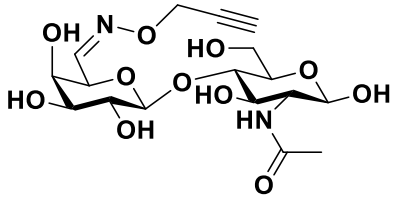
To a stirred solution of CHO-LacNAc (18 mg) in 50 mmol/L phosphate buffer (pH 7.4, 200 μL) was added *O*-(2-azidoethyl)-hydroxylamine hydrochloride (5.3 mg), the reaction mixture was stirred at room temperature for 2 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **4a** was combined and lyophilized to get a white powder. ESI-MS calc. for $C_{16}H_{27}N_5O_{11}$ [M+H]+ *m*/*z*= 466.1785, found *m*/*z*= 466.1732. ^{1}H NMR (500 MHz, deuterium oxide) *δ* 7.57 (dd, *J* = 4.1, 1.7 Hz, 0.7H), 6.91 (dd, *J* = 4.6, 3.1 Hz, 0.3H), 5.16 (q, *J* = 1.5 Hz, 0.7H), 4.80 (ddd, *J* = 4.7, 3.5, 1.2 Hz, 0.3H), 4.67–4.65 (m, 0.3H), 4.49 (d, *J* = 7.9 Hz, 0.65H), 4.46 (dd, *J* = 7.9, 1.6 Hz, 0.35H), 4.42 (ddd, *J* = 4.1, 2.8, 1.2 Hz, 0.7H), 4.29–4.19 (m, 2H), 4.04 (dt, *J* = 2.8, 1.3 Hz, 1H), 3.97–3.89 (m, 1H), 3.88–3.78 (m, 3H), 3.73–3.60 (m, 3H), 3.54 (m, 3H), 2.00 (d, *J* = 1.8 Hz, 3H).

Synthesis of N_3-NH-LacNAc ***4b***



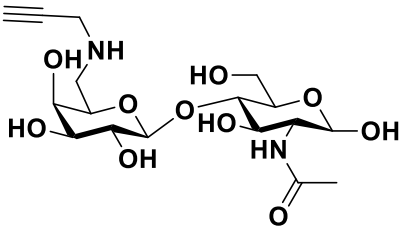
To a stirred solution of CHO-LacNAc (18 mg) in 0.2 mol/L phosphate buffer (pH 6.0, 400 μL) was added 3-azido-1-propanamine (23.6 mg) and $NaCNBH_3$ (59.5 mg) respectively, the reaction mixture was stirred at 0 ℃ for 4 h and monitored by LC–MS. The solution was subjected to a Bio-gel P2 column, the corresponding product N_3-NH-LacNAc **4b** was combined and lyophilized to get a white powder (16 mg, yield 72.8%). ESI-MS calcd. for $C_{17}H_{31}N_5O_{10}$ [M+H]+ *m*/*z* = 466.2149, found *m*/*z* = 466.2132. ^{1}H NMR (500 MHz, deuterium oxide) *δ* 5.14 (d, *J* = 3.3 Hz, 1H), 4.47 (dd, *J* = 7.8, 2.3 Hz, 1H), 3.96–3.70 (m, 7H), 3.70–3.60 (m, 1.65H), 3.51 (m, 1.35H), 3.45 (td, *J* = 6.4, 1.5 Hz, 2H), 3.35 (dd, *J* = 13.4, 9.3 Hz, 1H), 3.28 (t, *J* = 3.0 Hz, 0.65H), 3.26 (t, *J* = 3.0 Hz, 0.35H), 3.21–3.09 (m, 2H), 1.99 (s, 3H), 1.93 (dq, *J* = 14.5, 7.5, 6.9 Hz, 2H). ^{13}C NMR (126 MHz, D2O) *δ* 174.77, 174.50, 102.24, 94.96, 90.73, 76.59, 76.25, 74.97, 72.33, 72.18, 70.76, 70.54, 70.29, 70.25, 69.18, 69.09, 59.75, 59.68, 56.39, 53.85, 48.27, 48.19, 45.86, 25.00, 24.97, 22.20, 21.93.

*Synthesis of Alkyne-ON=CH-LacNAc **4c***



To a stirred solution of CHO-LacNAc (18 mg) in 50 m mol/L phosphate buffer (pH 7.4, 200 μL) was added *O*-(2-propynylethyl)-hydroxylamine hydrochloride (5.6 mg), the reaction mixture was stirred at room temperature for 2 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **4c** was combined and lyophilized to get a white powder. ESI-MS calc. for $C_{17}H_{26}N_2O_{11}$ [M+H]+ *m*/*z*= 435.1615, found *m*/*z*= 435.1610. ^{1}H NMR (500 MHz, deuterium oxide) *δ* 7.56 (dd, *J* = 4.4, 1.8 Hz, 0.75H), 6.94 (dd, *J* = 4.6, 2.7 Hz, 0.25H), 5.15 (d, *J* = 2.4 Hz, 0.6H), 4.77 (m, 0.3H), 4.68 (d, *J* = 2.5 Hz, 0.4H), 4.66 (dd, *J* = 2.5, 1.1 Hz, 2H), 4.49 (dd, *J* = 7.9, 1.1 Hz, 0.72H), 4.45 (dd, *J* = 7.9, 1.2 Hz, 0.28H), 4.41 (ddd, *J* = 4.3, 2.9, 1.2 Hz, 0.7H), 4.21 (dt, *J* = 2.8, 1.4 Hz, 0.22H), 4.03 (dq, *J* = 3.0, 1.9, 1.3 Hz, 0.78H), 3.96–3.87 (m, 1H), 3.87–3.78 (dd, *J* = 12.1, 4.9 Hz, 3H), 3.71 (dt, *J* = 9.9, 3.0 Hz, 0.5H), 3.66 (m, 1.5H), 3.59–3.49 (m, 1H), 2.9–2.84 (m, 1H), 1.99 (d, *J* = 1.3 Hz, 3H). ^{13}C NMR (126 MHz, D_2O) *δ* 174.66, 174.40, 150.58, 149.62, 149.60, 103.04, 102.55, 94.92, 94.86, 90.59, 90.55, 79.41, 79.29, 78.98, 78.56, 78.25, 76.23, 76.13, 74.78, 72.42, 72.36, 72.10, 72.04, 70.73, 70.64, 70.46, 70.35, 70.22, 70.10, 69.57, 69.20, 69.13, 68.83, 61.65, 61.46, 60.07, 59.93, 59.87, 56.24, 53.85, 53.74, 22.21, 21.92.

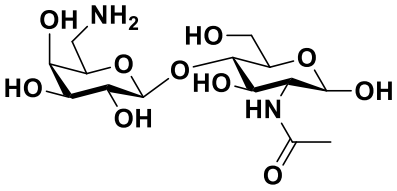
*Synthesis of Alkyne-NH-LacNAc **4d***



To a stirred solution of CHO-LacNAc (18 mg) in 0.2 mol/L phosphate buffer (pH 6.0, 400 μL) was added propargylamine (13 mg) and $NaCNBH_3$ (59.5 mg) respectively, the reaction mixture was stirred at 0 ℃ for 4 h and monitored by LC–MS. The solution was subjected to a Bio-gel P2 column, the corresponding product alkyne-NH-LacNAc **4d** was combined and lyophilized to get a white powder (16 mg, yield 72.8%). ESI-MS calcd. for $C_{17}H_{28}N_2O_{10}$ [M+H]+ *m*/*z* = 421.1822, found *m*/*z* = 421.1876. ^{1}H NMR (500 MHz, deuterium oxide) *δ* 5.15 (d, *J* = 3.2 Hz, 1H), 4.47–4.40 (m, 1H), 3.95–3.75 (m, 6H), 3.75–

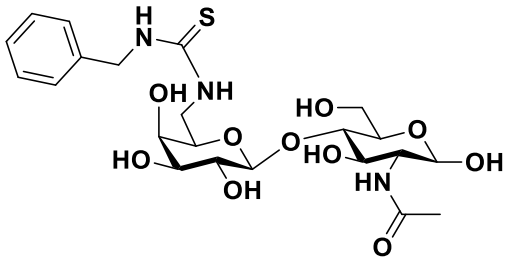
3.56 (m, 5H), 3.51 (m, 2H), 3.19–2.97 (m, 2H), 1.99 (d, J = 1.2 Hz, 3H). ^{13}C NMR (126 MHz, D_2O) δ 174.80, 174.77, 174.58, 174.50, 102.46, 102.42, 94.96, 90.66, 77.33, 77.01, 75.37, 75.02, 72.43, 72.39, 72.15, 71.00, 70.93, 70.53, 69.40, 69.15, 68.58, 61.03, 59.91, 59.82, 56.43, 53.89, 47.63, 36.81, 22.20, 21.91.

*Synthesis of NH_2-LacNAc **4e***



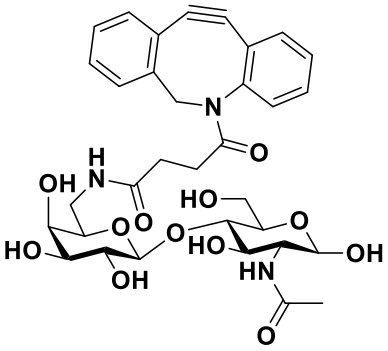
To a stirred solution of CHO-LacNAc (18 mg) in 50 mmol/L phosphate buffer (pH 7.0, 200 μL) was added hydroxyamine hydrochloride (3.6 mg), CH_3OH (180 μL) and Na_2CO_3 (2.6 mg) respectively, the reaction mixture was stirred at room temperature for 3 h. After that, $NiCl_2{\cdot}6H_2O$ (28 mg) and $NaBH_4$ (26.8 mg) were added to the mixture, the reaction was stirred at 4 ℃ overnight and monitored by LC–MS. The solution was subjected to a Bio-gel P2 column, the corresponding product NH_2-LacNAc was combined and lyophilized to get a white powder. ESI-MS calc. for $C_{14}H_{26}N_2O_{10}$ [M+H]+ m/z= 383.1665, found m/z= 383.1661. ^{1}H NMR (500 MHz, deuterium oxide) δ 5.01 (s, 0.7H), 4.34–4.19 (m, 1.3H), 3.83–3.27 (m, 11H), 3.11–2.97 (m, 1H), 1.85 (d, J = 6.2 Hz, 3H).

*Synthesis of LacNAc derivatives **4f***



To a stirred solution of CHO-LacNAc (20 mg) in 50 mmol/L phosphate buffer (pH 8.0, 200 μL) was added *N*-benzylthiourea (9.4 mg in 100 μL DMF), the reaction mixture was stirred at room temperature for 8 h and monitored by LC–MS. The solution was subjected to a semi-preparative C18 column, the corresponding product **4f** was combined and lyophilized to get a white powder (17 mg, yield 62%). ESI-MS calc. for $C_{22}H_{22}N_3O_{10}S$ [M+H]+ m/z= 532.1965, found m/z= 532.1911. ^{1}H NMR (500 MHz, deuterium oxide) δ 7.37 (td, J = 7.4, 4.5 Hz, 2H), 7.33–7.25 (m, 3H), 5.14 (d, J = 2.9 Hz, 0.65H), 4.68 (0.35H) 4.67–4.64 (m, 1H), 4.33 (s, 1H), 3.89 (m, 1H), 3.86–3.70 (m, 6H), 3.68–3.61 (m, 1H), 3.60–3.50 (m, 3H), 3.46 (dt, J = 10.0, 7.4 Hz, 2H), 1.95 (d, J = 8.6 Hz, 3H). ^{13}C NMR (126 MHz, D2O) δ 174.63, 174.33, 128.88, 127.57, 126.84, 102.97, 102.89, 94.93, 90.51, 78.99, 78.58, 74.88, 73.15, 72.39, 72.36, 70.88, 70.32, 69.09, 68.85, 68.82, 60.15, 60.01, 56.19, 53.75, 22.19, 21.88.

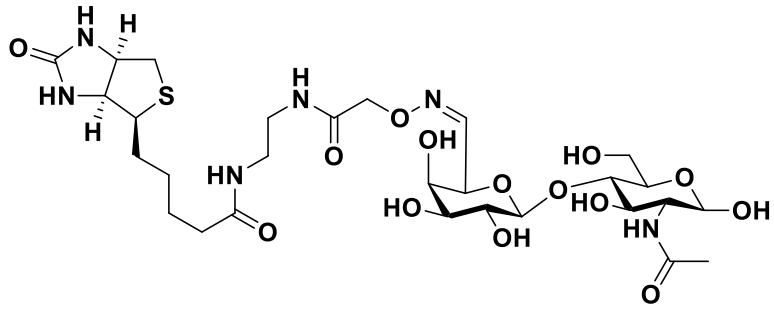
*Synthesis of DBCO-LacNAc **4g***



To a stirred solution of NH_2-LacNAc **4e** (15 mg) in 50 mmol/L phosphate buffer (pH 7.4, 200 μL) was added DBCO-CONHS

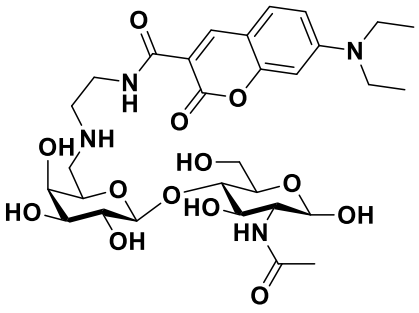
(23.7 mg in 100 μL DMF), the reaction mixture was stirred at room temperature for 4 h and monitored by LC–MS. The solution was subjected to a semi-preparative C18 column, the corresponding product DBCO-LacNAc was combined and lyophilized to get a white powder (22 mg, yield 83.7%). ESI-MS calc. for $C_{33}H_{39}N_3O_{12}$ [M+H]+ *m*/*z*= 670.2612, found *m*/*z*= 670.2661. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.77–7.54 (m, 4H), 7.41 (dt, *J* = 21.2, 7.7 Hz, 2H), 7.35–7.19 (m, 4H), 4.97 (d, *J* = 14.0 Hz, 1H), 4.90–4.80 (m, 1H), 4.39 (t, *J* = 7.9 Hz, 0.25H), 4.19–4.05 (m, 0.75H), 3.76–3.64 (m, 1H), 3.64–3.52 (m, 3H), 3.28–2.94 (m, 14H), 2.53 (dtd, *J* = 15.0, 7.5, 3.3 Hz, 2H), 2.26–2.14 (m, 2H), 2.03–1.92 (m, 1H), 1.77–1.67 (m, 3H). ^{13}C NMR (126 MHz, DMSO) δ 172.27, 172.20, 172.17, 171.61, 171.59, 169.70, 152.11, 152.09, 148.92, 148.88, 132.98, 132.90, 130.13, 130.10, 129.44, 128.60, 128.51, 128.48, 128.16, 127.26, 127.24, 125.62, 123.02, 121.88, 121.88, 114.72, 114.69, 108.65, 104.23, 104.08, 90.87, 90.82, 81.88, 81.66, 73.45, 73.42, 73.21, 71.68, 70.95, 70.93, 70.44, 68.92, 68.60, 60.99, 60.86, 60.82, 55.38, 54.39, 54.33, 30.69, 30.64, 29.93, 22.97.

Synthesis of Biotin-ON=CH-LacNAc ***4h***



To a stirred solution of CHO-LacNAc (18 mg) in 50 m mol/L phosphate buffer (pH 7.4, 200 μL) was added Biotin-ONH_2 (22.6 mg), the reaction mixture was stirred at room temperature for 8 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **4h** was combined and lyophilized to get a white powder. ESI-MS calc. for $C_{28}H_{46}N_6O_{14}S$ [M+H]+ *m*/*z*= 723.2871, found *m*/*z*= 723.2877. ^{1}H NMR (500 MHz, deuterium oxide) δ 7.64 (dd, *J* = 4.0, 2.6 Hz, 0.81H), 6.93 (dd, *J* = 4.5, 3.2 Hz, 0.19H), 5.14 (d, *J* = 3.2 Hz, 0.65H), 4.86 (td, *J* = 4.1, 3.7, 1.2 Hz, 0.25H), 4.80–4.75 (m, 2H), 4.68 (m, 0.35H) 4.58–4.50 (m, 3H), 4.47 (d, *J* = 7.9 Hz, 1H), 4.42 (ddd, *J* = 5.4, 4.1, 1.3 Hz, 0.75H), 4.39–4.35 (m, 1H), 4.27–4.24 (m, 0.2H), 4.04 (td, *J* = 3.2, 1.1 Hz, 0.8H), 3.95–3.74 (m, 4H), 3.69 (tt, *J* = 7.7, 3.2 Hz, 1H), 3.66–3.59 (m, 1H), 3.58–3.50 (m, 1H), 3.38–3.20 (m, 4H), 2.97–2.90 (m, 1H), 2.73 (d, *J* = 13.0 Hz, 1H), 2.19 (t, *J* = 7.3 Hz, 2H), 1.99 (d, *J* = 1.1 Hz, 3H), 1.73–1.47 (m, 4H), 1.35 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ 177.11, 177.08, 174.55, 174.27, 172.32, 172.29, 172.04, 165.35, 150.16, 150.13, 103.23, 103.17, 94.91, 90.59, 90.48, 79.80, 79.36, 77.26, 74.73, 72.37, 72.32, 72.19, 72.07, 70.62, 70.40, 70.14, 69.45, 69.41, 69.11, 68.81, 62.10, 60.28, 59.91, 56.19, 55.34, 53.75, 39.71, 38.51, 38.42, 38.29, 35.54, 27.91, 27.87, 27.71, 25.12, 22.21, 21.91.

Synthesis of coumarin LacNAc ***4i***



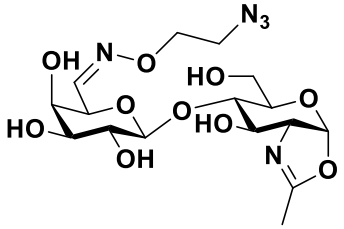
To a stirred solution of 7-(diethylamino)coumarin-3-carboxylic acid (10 mg) in DMF (200 μL) was added HATU (29 mg), mono-Fmoc ethylene diamine hydrochloride (18.3 mg) and DIPEA (19 μL) respectively, the mixture was stirred at 37 °C for 4 h and monitored by LC–MS. After 2 h, the solution was subjected to a semi-preparative column, the corresponding product NH_2-coumarin was combined and lyophilized to get a white powder (8.7 mg, yield 75%). ESI-MS calcd. for $C_{16}H_{21}N_3O_3$ [M+H]+ *m*/*z* = 304.1661, found *m*/*z* = 304.1615.

To a stirred solution of CHO-LacNAc (32.7 mg) in 0.2 mol/L phosphate buffer (pH 6.0, 400 μL) was added NH_2-coumarin (8.7 mg) and $NaCNBH_3$ (18 mg) respectively, the reaction mixture was stirred at 0 °C overnight and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product coumarin LacNAc was combined and lyophilized to get a white powder (12 mg, yield 63%). ESI-MS calcd. for $C_{30}H_{44}N_4O_{13}$ [M+H]+ *m*/*z* = 669.2983, found *m*/*z* = 669.2941.

Synthesis of derived LacNAc oxazolines

To a solution of LacNAc derivatives **4a**–**4i**, **2g** (5 μmoL) in D_2O (100 μL) was added 2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride (CDMBI, 5.4 mg, 25 μmoL) and K_3PO_4 (16 mg, 75 μmoL), The reaction mixture was incubated at 0 °C for 12 h and treated by Waters Sep-Pak Vac column before LC–MS monitoring, the mixture was directly subjected to NMR analysis.

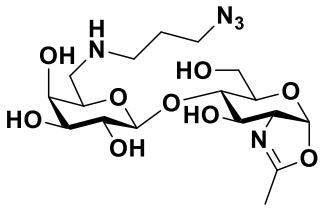
N_3-ON=CH-LacNAc-ox ***5a***



ESI-MS calc. for $C_{16}H_{25}N_5O_{10}$ [M+H]+ *m*/*z*= 448.1679, found *m*/*z*= 448.1662.

^{1}H NMR (600 MHz, deuterium oxide) δ 7.57 (d, *J* = 4.8 Hz, 0.65H), 6.91 (d, *J* = 4.6 Hz, 0.35H), 6.01 (dd, *J* = 7.3, 2.3 Hz, 1H), 4.43 (d, *J* = 7.8 Hz, 0.7H), 4.38 (d, *J* = 7.9 Hz, 0.3H), 4.34 (m, 0.3H), 4.33–4.3 (m, 1H), 4.25–4.15 (m, 3H), 4.11 (m, 1H), 3.98 (dd, *J* = 3.4, 1.1 Hz, 0.7H), 3.74 (m, 1H), 3.67–3.57 (m, 3H), 3.53–3.44 (m, 3H), 3.40–3.35 (m, 1H), 1.99 (t, *J* = 1.9 Hz, 3H).

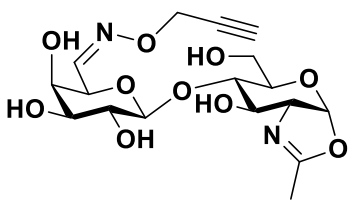
N_3-NH-LacNAc-ox ***5b***



ESI-MS calc. for $C_{17}H_{29}N_5O_9$ [M+H]+ *m*/*z*= 448.2043, found *m*/*z*= 448.2022.

^{1}H NMR (600 MHz, deuterium oxide) δ 5.97 (d, *J* = 7.3 Hz, 1H), 4.29 (d, *J* = 7.9 Hz, 1H), 4.27 (dd, *J* = 3.0, 1.6 Hz, 1H), 4.07 (m, 1H), 3.72 (d, *J* = 3.5 Hz, 1H), 3.69 (dd, *J* = 12.3, 2.5 Hz, 1H), 3.63–3.58 (m, 1H), 3.56–3.49 (m, 2H), 3.40–3.35 (m, 1H), 3.31 (m, 1H), 3.29 (t, *J* = 6.7 Hz, 3H), 2.93 (q, *J* = 7.4 Hz, 2H), 2.68 (q, *J* = 7.3 Hz, 2H), 1.95 (d, *J* = 1.7 Hz, 3H), 1.67 (p, *J* = 7.1 Hz, 2H).

Alkyne-ON=CH-LacNAc-ox ***5c***

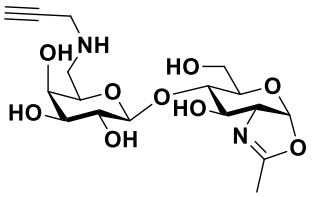


ESI-MS calc. for $C_{17}H_{24}N_2O_{10}$ [M+H]+ *m*/*z*= 417.1509, found *m*/*z*= 417.1511.

^{1}H NMR (600 MHz, deuterium oxide) δ 7.53 (d, *J* = 4.9 Hz, 0.65H), 6.95 (d, *J* = 4.8 Hz, 0.35H), 5.98 (d, *J* = 7.3 Hz, 1H), 4.61 (d, *J* = 9.9 Hz, 4H), 4.43–4.25 (m, 2H), 4.15–4.05 (m, 1.3H), 3.95 (d, *J* = 3.4 Hz, 0.7H), 3.76–3.67 (m, 1H), 3.65–3.53

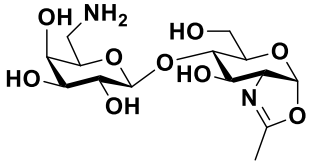
(m, 3H), 3.46 (dd, *J* = 10.1, 7.8 Hz, 1H), 3.36 (m, 1H), 1.96 (d, *J* = 1.6 Hz, 3H).

Alkyne-NH-LacNAc-ox ***5d***



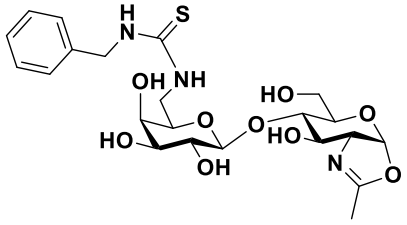
ESI-MS calc. for $C_{17}H_{26}N_2O_9$ [M+H]+ *m*/*z*= 403.1716, found *m*/*z*= 403.1741.
^{1}H NMR (600 MHz, deuterium oxide) δ 5.97 (d, J = 7.3 Hz, 1H), 4.33–4.23 (m, 2H), 4.11–4.03 (m, 1H), 3.74 (d, J = 3.6 Hz, 1H), 3.69 (dd, J = 12.3, 2.5 Hz, 1H), 3.63–3.58 (m, 1H), 3.56–3.48 (m, 2H), 3.40–3.35 (m, 1H), 2.90–2.86 (m, 3H), 2.67–2.62 (m, 2H), 1.95 (d, J = 1.8 Hz, 3H).

NH_2-LacNAc-ox ***5e***



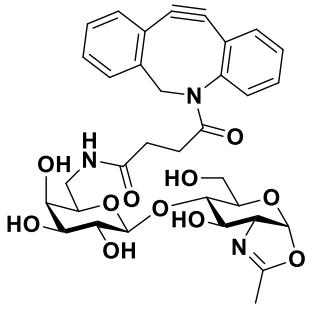
ESI-MS calc. for $C_{14}H_{24}N_2O_9$ [M+H]+ *m*/*z*= 365.156, found *m*/*z*= 365.1551.

Benzyl-LacNAc-ox ***5f***



ESI-MS calc. for $C_{22}H_{31}N_3O_9S$ [M+H]+ *m*/*z*= 514.1859, found *m*/*z*= 514.1858.
^{1}H NMR (600 MHz, deuterium oxide) δ 7.32 (t, *J* = 7.6 Hz, 2H), 7.25 (dd, *J* = 7.9, 5.7 Hz, 3H), 5.97 (d, *J* = 7.3 Hz, 1H), 4.34–4.21 (m, 2H), 4.04 (d, *J* = 7.2 Hz, 1H), 3.70 (dd, *J* = 12.3, 2.5 Hz, 3H), 3.56 (dd, *J* = 12.3, 6.4 Hz, 3H), 3.52–3.44 (m, 1H), 3.39 (dd, *J* = 9.9, 7.8 Hz, 1H), 3.34 (ddd, *J* = 8.9, 6.4, 2.4 Hz, 1H), 1.97 (d, *J* = 1.8 Hz, 3H).

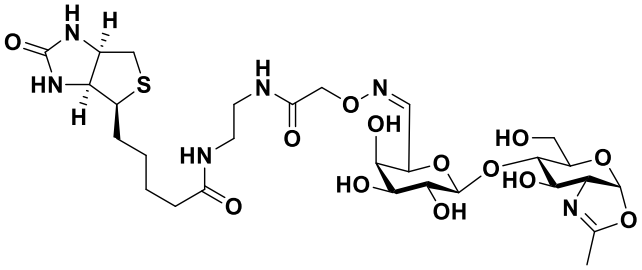
DBCO-LacNAc-ox ***5g***



ESI-MS calc. for $C_{33}H_{37}N_3O_{11}$ [M+H]+ *m*/*z*= 652.2506, found *m*/*z*= 652.2525.
^{1}H NMR (600 MHz, DMSO-d_6) δ 7.74 (dd, *J* = 6.8, 2.7 Hz, 1H), 7.68 (d, *J* = 7.4 Hz, 1H), 7.65 (q, *J* = 5.5 Hz, 1H), 7.58–7.48 (m, 2.5H), 7.47–7.33 (m, 3H), 7.29–7.01 (m, 1.5H), 5.95 (dd, *J* = 7.3, 3.0 Hz, 1H), 5.09 (d, *J* = 14.1 Hz, 1H), 4.21 (dt, *J* = 11.8, 2.1 Hz, 1H), 4.17–4.12 (m, 1H), 4.00–3.88 (m, 1H), 3.66 (m, 2H), 3.60–3.51 (m, 5H), 3.34 (m, 2H), 3.29–3.27 (m, 2H), 3.26–3.14 (m, 3H), 2.66 (dt, *J* = 16.0, 7.8 Hz, 1H), 2.34 (dt, *J* = 15.4, 7.6 Hz, 1H), 2.10 (td, *J* = 15.2, 13.6, 6.0 Hz, 1H), 2.00–

1.95 (m, 3H), 1.83 (m, 1H).

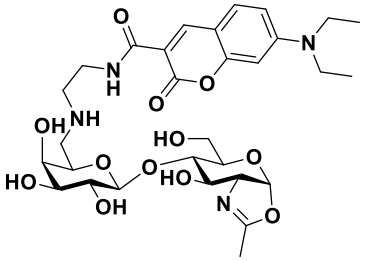
Biotin-ON=CH-LacNAc-ox ***5h***



ESI-MS calc. for $C_{28}H_{44}N_6O_{13}S$ [M+H]+ *m/z*= 705.2765, found *m/z*= 705.2711.

^{1}H NMR (600 MHz, deuterium oxide) δ 7.61 (d, J = 4.4 Hz, 0.65H), 6.75 (d, J = 4.8 Hz, 0.35H), 5.97 (t, J = 6.9 Hz, 1H), 4.47 (m, 3H), 4.39 (d, J = 7.5 Hz, 1H), 4.29 (d, J = 37.8 Hz, 3H), 4.06 (d, J = 15.6 Hz, 1H), 3.96 (s, 1H), 3.75–3.68 (m, 1H), 3.64–3.53 (m, 2H), 3.46 (m, 1H), 3.35 (m, 1H), 3.33–3.20 (m, 5H), 3.13 (d, J = 23.1 Hz, 1H), 2.85 (dd, J = 13.1, 4.9 Hz, 1H), 2.65 (d, J = 13.1 Hz, 1H), 2.15–2.06 (m, 2H), 1.96 (dd, J = 5.7, 2.0 Hz, 3H), 1.57 (s, 1H), 1.53–1.38 (m, 3H), 1.24 (s, 2H).

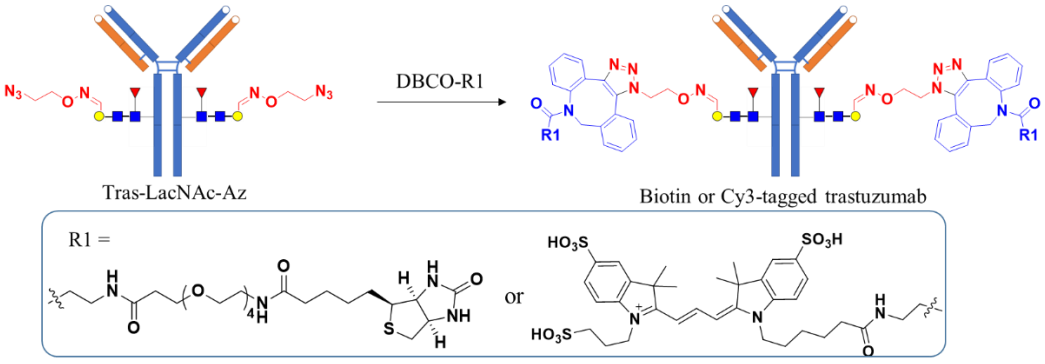
Coumarin LacNAc-ox ***5i***



ESI-MS calc. for $C_{30}H_{42}N_4O_{12}$ [M+H]+ *m/z*= 651.2877, found *m/z*= 651.2834.

2.4 The internalization trace of HER2 receptor on SK-Br-3 tumor cell

Synthesis of biotin/Cy3-tagged LacNAc-trastuzumab

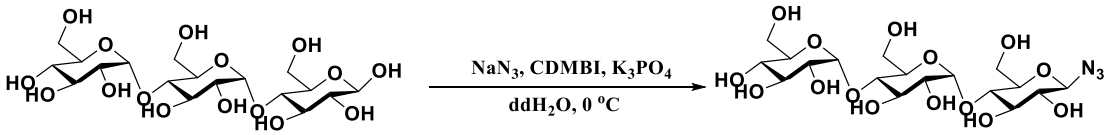


A solution of azido-LacNAc-trastuzumab (1 mg) in a phosphate buffer (50 mmol/L, pH 7.4, 200 μL) was incubated with DBCO-PEG$_4$-Biotin **8a** or DBCO-Cy3 **8b** (80 nmoL) at 37 °C for 2 h at dark. LC–MS monitoring indicated the complete reaction of azido-LacNAc-trastuzumab to give Biotin/Cy3-tagged LacNAc-trastuzumab. The reaction mixture was concentrated by centrifugal filtration (Millipore) to give the corresponding Biotin or Cy3-tagged LacNAc-trastuzumab (**9a** or **9b**).

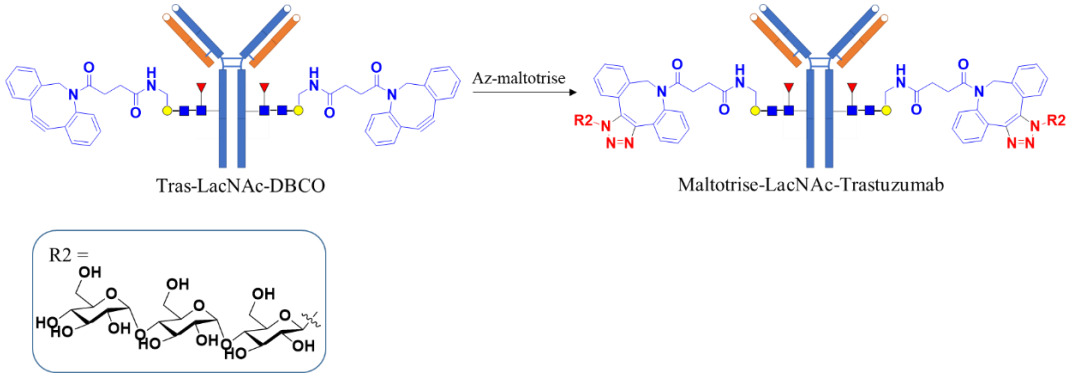
Cell culture. SK-Br-3 cells were cultured in a 10% FBS-containing RPMI 1640 medium and incubated under 5% CO_2 at 37 °C in a water-saturated cell incubator (Thermo Scientific). Cells were transferred to an 8-well chamber for confocal microscopy analysis.

Imaging of glycoengineered trastuzumab uptake and HER2 internalization. For glycoengineered trastuzumab uptake assays, SK-Br-3 cells cultured on 8-well chamber were incubated with Cy3-tagged LacNAc-trastuzumab (10 μg/mL) for 1 h on ice, then washed 3 times with 5% FBS-containing PBS buffer. After that, SK-Br-3 cells were cultured in a 10% FBS-containing RPMI 1640 medium and incubated under 5% CO_2 at 37 ºC for 0–8 h. Then the cells were washed 3 times again with 5% FBS-containing PBS and were fixed at room temperature for 15 min with immune staining fix solution (200 μL). The cell nuclei were stained with Hoechst 33258 (1:1000 dilution, 100 μL) at room temperature for 15 min. All the cells in 8-wells were washed and preserved in cell grade 1 × PBS on ice at dark. Image analysis was performed on a Leica TCS-SP8 STED instrument. Hoechst 33258 was excited at 405 nm and the nuclei was detected within the window of 415–460 nm. Cy3 was excited at 552 nm and the internalization process was detected with the window of 562-590 nm.

2.5 Antibody glycosylation remodeling with extra sugar moiety

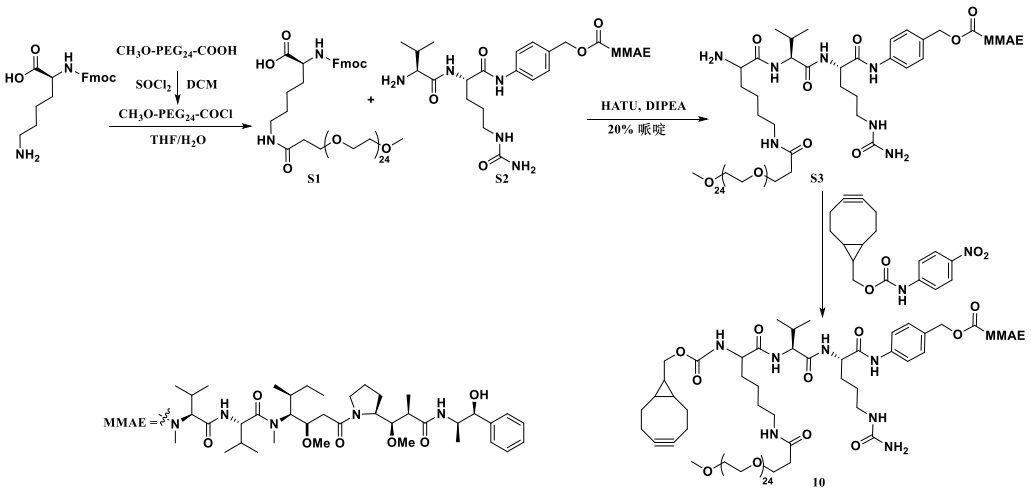


Synthesis of 1-azido-maltotriose. To a solution of maltotriose (10 mg) in ddH_2O (400 μL) was added NaN_3 (64 mg), CDMBI (21.6 mg) and K_3PO_4 (64 mg) respectively, the reaction mixture was stirred at 0 ℃ overnight and monitored by LC–MS. The solution was subjected to a Bio-gel P2 column, the corresponding product 1-azido-maltotriose was combined and lyophilized to get a white powder. ESI-MS calcd. for $C_{19}H_{33}N_3O_{14}$ $[M+H]^+$ m/z = 528.2041, found m/z = 528.2042.



Synthesis of glycoengineered trastuzumab with maltotriose by SPAAC reaction. A solution of DBCO-LacNAc-trastuzumab (50 μg) in a phosphate buffer (50 mmol/L, pH 7.4, 10 μL) was incubated with 1-azido-maltotriose (4 nmoL) at 37 ºC for 2 h. LC–MS monitoring indicated the complete reaction of DBCO-LacNAc-trastuzumab to give maltotriose-extended LacNAc-trastuzumab. The reaction mixture was concentrated by centrifugal filtration (Millipore) to give the corresponding maltotriose-extended LacNAc-trastuzumab.

2.6 Synthesis of drug-linker 10 BCN-Lys(PEG24)-VC-PAB-MMAE



*Synthesis of compound **S1***

To a stirred solution of CH_3O-PEG_{24}-COOH (50 mg) in CH_2Cl_2 (2 mL) was added $SOCl_2$ (two drops) under nitrogen protection, the reaction mixture was heated at 50 °C for 2 h. TLC analysis (CH_3OH: CH_2Cl_2=1:8) indicated that the reaction was completed. The reaction mixture was dried by rotary evaporation and redissolved in anhydrous THF to get a PEG solution. To a stirred solution of Fmoc-Lys-OH (16 mg) and $NaHCO_3$ (18 mg) in 900 μL THF and 300 μL ddH_2O was added the above PEG solution slowly, the reaction mixture was stirred at room temperature for 1 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **S1** was combined and lyophilized to get a white powder. ESI-MS calc. for $C_{73}H_{126}N_2O_{30}$ $[M+2H]^{2+}$ m/z=756.4275, found m/z= 756.4272.

*Synthesis of compound **S3***

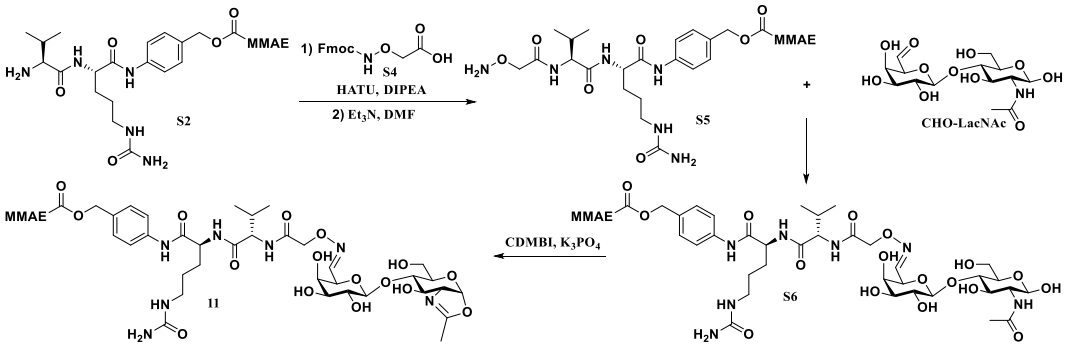
To a stirred solution of compound **S1** (10 mg) in DMF (100 μL) was added HATU (7.8 mg), **S2** (8.2 mg) and DIPEA (3.5 μL) respectively, the mixture was stirred at 37 °C for 2 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **S3** was combined and lyophilized to get a white powder (16 mg, yield 91%). ESI-MS calcd. for $C_{116}H_{208}N_{12}O_{39}$ $[M+3H]^{3+}$ m/z = 798.8299, found m/z = 798.8298. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.02 (s, 1H), 8.26 (s, 0.8H), 8.12 (d, J = 7.3 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 8.02 (s, 0.2H), 7.90 (d, J = 8.7 Hz, 1H), 7.73 (d, J = 7.7 Hz, 6H), 7.64 (d, J = 8.5 Hz, 1H), 7.58 (t, J = 5.7 Hz, 3H), 7.39–7.24 (m, 6H), 7.21–7.12 (m, 1H), 6.08 (s, 1H), 5.05 (m, 3H), 4.69 (m, 1H), 4.50 (d, J = 5.8 Hz, 1H), 4.47–4.36 (m, 2H), 4.35–4.30 (m, 1H), 4.27–4.17 (m, 2H), 4.09–3.92 (m, 1H), 3.68–3.58 (m, 3H), 3.51 (s, 89H), 3.45–3.34 (m, 3H), 3.25 (m, 9H), 3.20 (d, J = 12.2 Hz, 4H), 3.12 (s, 2H), 3.05 (t, J = 10.3 Hz, 1H), 2.97 (m, 2H), 2.87 (d, J = 18.0 Hz, 2H), 2.76 (m, 2H), 2.46–2.34 (m, 3H), 2.32–2.22 (m, 1H), 2.19–1.89 (m, 3H), 1.88–1.63 (m, 1H), 1.55 (m, 3H), 1.41–1.23 (m, 2H), 1.09–0.97 (m, 8H), 0.92–0.69 (m, 25H). ^{13}C NMR (126 MHz, DMSO) δ 172.87, 172.05, 171.39, 170.98, 170.74, 170.33, 170.27, 169.24, 159.53, 159.12, 158.85, 158.59, 158.33, 144.13, 144.09, 139.04, 138.92, 138.90, 128.63, 128.24, 128.19, 127.17, 127.11, 126.93, 126.87, 119.46, 119.31, 118.33, 115.97, 85.89, 82.12, 75.29, 75.26, 71.76, 70.26, 70.15, 70.06, 69.94, 67.25, 63.81, 61.37, 60.73, 59.14, 58.65, 58.51, 57.92, 57.61, 57.57, 54.56, 53.57, 52.68, 50.24, 49.64, 47.67, 46.70, 44.21, 43.67, 39.23, 37.63, 36.40, 35.62, 32.29, 31.97, 31.71, 31.03, 29.80, 27.17, 27.10, 25.78, 24.80, 23.56, 22.68, 19.62, 19.34, 19.22, 19.01, 18.76, 18.48, 16.32, 16.08, 15.88, 15.79, 15.71, 15.42, 10.85, 10.74.

*Synthesis of compound **10***

To a stirred solution of compound **S3** (12.8 mg) in DMF (100 μL) was added BCN-O-PNP (3.4 mg) and Et_3N (1.5 μL), the mixture was stirred at 37 °C for 3 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **10** was combined and lyophilized to get a white powder (10 mg, yield 72.7%). ESI-MS calcd. for $C_{127}H_{220}N_{12}O_{41}$ $[M+2H]^{2+}$ m/z = 1285.7825, $[M+3H]^{3+}$ m/z = 857.524, found m/z = 1285.7848, 857.5232. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.99 (s, 1H), 8.27 (s, 0.5H), 8.08 (d, J = 7.4 Hz, 1H), 8.01 (d, J = 8.5 Hz, 0.5H), 7.89 (d, J = 8.7 Hz, 1H), 7.83 (d, J = 7.9 Hz, 1H), 7.77 (t, J = 5.6 Hz, 1H), 7.63 (t, J = 7.7 Hz, 1.5H),7.57 (m, 4.5H), 7.48 (m, 3H), 7.42–7.24 (m, 10H),

7.24–7.14 (m, 1H), 6.01 (s, 1H), 5.16–4.91 (m, 3H), 4.69 (m, 1H), 4.50 (d, *J* = 5.9 Hz, 1H), 4.44 (d, *J* = 6.6 Hz, 1H), 4.37 (q, *J* = 7.5 Hz, 1H), 4.26 (dd, *J* = 13.8, 10.8 Hz, 1H), 4.17 (m, 2H), 3.99 (m, 2H), 3.78 (dd, *J* = 9.3, 2.4 Hz, 1H), 3.64–3.55 (m, 4H), 3.55–3.32 (m, 96H), 3.28–3.23 (m, 7H), 3.20 (d, *J* = 12.3 Hz, 4H), 3.12 (s, 2H), 3.08–2.92 (m, 4H), 2.86 (dd, *J* = 18.0, 4.2 Hz, 3H), 2.47–2.36 (m, 1H), 2.29 (t, *J* = 6.6 Hz, 3H), 2.22–1.87 (m, 5H), 1.87–1.62 (m, 2H), 1.63–1.40 (m, 3H), 1.39–1.12 (m, 9H), 1.08–0.97 (m, 7H), 0.93–0.72 (m, 29H). ^{13}C NMR (126 MHz, DMSO) *δ* 172.87, 172.52, 172.28, 172.16, 171.35, 170.98, 170.34, 170.27, 169.25, 168.06, 159.43, 158.86, 152.27, 148.91, 144.11, 144.09, 132.86, 130.06, 129.91, 129.36, 128.62, 128.47, 128.26, 128.20, 127.25, 127.13, 126.92, 126.87, 125.61, 122.95, 121.87, 119.44, 119.30, 114.83, 108.63, 75.29, 75.28, 71.75, 70.25, 70.14, 70.05, 69.97, 67.33, 61.37, 58.51, 57.77, 57.61, 57.58, 55.26, 53.59, 36.60, 35.26, 34.36, 31.08, 29.76, 29.27, 25.79, 25.12, 24.94, 23.56, 23.28, 19.58, 18.43, 18.40, 18.05, 15.89, 15.79, 15.75, 15.42.

*2.7 Synthesis of Drug-linker-LacNAc **11***



*Synthesis of compound **S5***

To a stirred solution of compound **S4** (5.6 mg) in DMF (100 μL) was added HATU (13.5 mg), **S2** (20 mg) and DIPEA (9.3 μL) respectively, the mixture was stirred at 37 °C for 2 h, LC–MS monitoring indicated the complete reaction, then Et_3N (100 μL) was added to the above solution. The reaction mixture was stirred at room temperature for another 1 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **S5** was combined and lyophilized to get a white powder (16.5 mg, yield 89%). ESI-MS calcd. for $C_{60}H_{97}N_{11}O_{14}$ $[M+H]^+$ *m/z* = 1196.7294, $[M+2H]^{2+}$ *m/z* = 598.8685, found *m/z* = 1196.7263, 598.8622. ^{1}H NMR (500 MHz, DMSO-d_6) *δ* 10.04 (s, 1H), 8.33 (d, *J* = 7.3 Hz, 1H), 8.20 (d, *J* = 8.2 Hz, 1H), 8.10 (m, 0.4H), 7.93 (d, *J* = 8.8 Hz, 0.6H), 7.89 (m, 0.6H), 7.73 (d, *J* = 8.5 Hz, 0.4H), 7.54 (d, *J* = 8.0 Hz, 2H), 7.28 (m, 6H), 7.13 (m, 1H), 5.19–4.91 (m, 2H), 4.77–4.58 (m, 1H), 4.53 (s, 2H), 4.5–4.25 (m, 6H), 3.72 (d, *J* = 9.4 Hz, 1H), 3.59–3.41 (m, 2H), 3.34–2.90 (m, 15H), 2.91–2.71 (m, 4H), 2.45–2.17 (m, 2H), 2.00 (m, 4H), 1.86–1.23 (m, 9H), 1.01 (dd, *J* = 13.5, 6.7 Hz, 6H), 0.79 (ddt, *J* = 43.6, 15.6, 7.3 Hz, 29H). ^{13}C NMR (126 MHz, DMSO-d_6) *δ* 173.32, 173.16, 171.27, 171.03, 170.26, 169.58, 167.78, 159.89, 159.61, 159.33, 159.05, 158.78, 156.87, 156.29, 143.84, 143.70, 138.79, 132.42, 128.73, 128.59, 128.28, 127.24, 126.94, 119.64, 119.48, 117.74, 115.41, 85.68, 82.07, 78.13, 77.32, 75.46, 71.51, 66.60, 64.15, 61.34, 60.71, 59.12, 58.77, 57.83, 57.69, 57.58, 55.77, 54.63, 54.53, 53.82, 50.13, 49.60, 47.77, 46.80, 44.21, 43.73, 39.10, 37.43, 35.51, 32.24, 32.02, 31.08, 30.40, 30.03, 29.48, 27.01, 25.67, 24.70, 23.47, 19.44, 19.32, 19.20, 19.10, 18.85, 18.63, 18.31, 16.52, 16.00, 15.69, 15.22, 10.72, 10.67, 8.96.

*Synthesis of compound **S6***

A solution of compound **S5** (10 mg) and CHO-LacNAc (3.3 mg) in DMF (100 μL) and a phosphate buffer (0.2 mol/L, pH 7.5, 100 μL) was stirred at 37 °C for 2 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **S6** was combined and lyophilized to get a white powder (10 mg, yield 74%). ESI-MS calcd. for $C_{74}H_{118}N_{12}O_{24}$ $[M+2H]^{2+}$ *m/z* = 780.4265, found *m/z* = 780.4221. ^{1}H NMR (500 MHz, DMSO-d_6) *δ* 10.00 (d, *J* = 7.4 Hz, 1H), 8.27 (d, *J* = 7.2 Hz, 1H), 8.22 (s, 0.4H), 7.98 (s, 0.6H), 7.84 (1.5H), 7.68–7.50 (m, 4.5H), 7.37–7.21 (m, 6H), 7.14 (h, *J* = 6.5 Hz, 1H), 5.13–4.93 (m, 2H), 4.91 (d, *J* = 2.8 Hz, 1H), 4.71 (d, *J* = 5.7 Hz, 1H), 4.65–4.45 (m, 4H), 4.44–4.31 (m, 3H), 4.30–4.16 (m, 3H), , 3.67–3.50 (m, 5H), 3.48–3.27 (m, 4H), 3.26–3.13 (m, 7H), 3.09 (s, 2H), 3.06–2.90 (m, 4H), 2.84 (dd, *J* = 18.6,

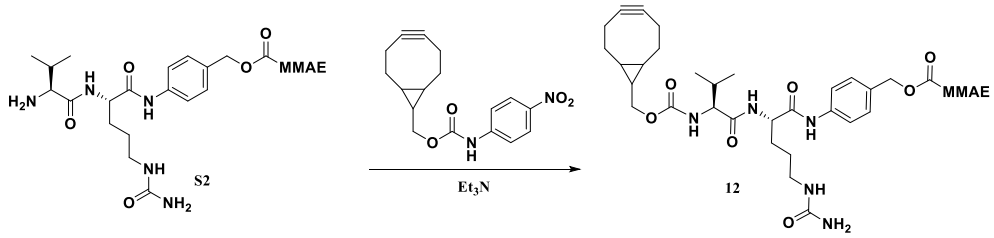
4.5 Hz, 3H), 2.38 (d, *J* = 15.9 Hz, 1H), 2.32–2.20 (m, 1H), 2.16–2.03 (m, 2H), 1.96 (m, 2H), 1.80 (d, *J* = 6.1 Hz, 3H), 1.69 (m, 1H), 1.63–1.24 (m, 4H), 0.99 (m, 6H), 0.93–0.63 (m, 28H). ^{13}C NMR (126 MHz, DMSO) δ 172.91, 171.28, 170.97, 170.33, 170.30, 169.92, 169.29, 168.86, 168.63, 159.55, 156.73, 150.11, 144.08, 144.03, 139.01, 138.89, 132.47, 132.29, 128.63, 128.26, 128.21, 127.20, 127.14, 126.93, 126.88, 119.50, 119.34, 104.09, 90.82, 85.87, 82.12, 81.42, 78.16, 77.38, 75.31, 75.28, 72.98, 72.83, 72.72, 71.14, 71.10, 70.54, 70.49, 70.41, 68.96, 66.56, 66.49, 61.37, 60.73, 60.65, 59.14, 58.67, 57.63, 57.57, 57.53, 55.54, 54.65, 54.56, 54.39, 53.69, 50.23, 49.64, 47.69, 46.72, 44.22, 43.69, 37.61, 35.60, 32.29, 31.99, 31.30, 30.47, 30.42, 30.15, 29.73, 27.16, 27.07, 25.77, 25.75, 24.79, 23.56, 23.44, 23.03, 19.59, 19.32, 19.21, 18.99, 18.79, 18.76, 18.71, 18.51, 16.35, 16.08, 15.86, 15.75, 15.39, 10.84, 10.74.

*Synthesis of compound **11***

To a solution of compound **S6** (10 mg) in ddH_2O (100 μL) was added CDMBI (6.9 mg) and K_3PO_4 (20.4 mg), the reaction mixture was stirred at 0 °C for 12 h, LC–MS monitoring indicated the oxazoline formed. The solution was subjected to a semi-preparative C18 column with base mobile phase, the corresponding product compound **11** was combined and lyophilized to get a white powder (7.2 mg, yield 72%). ESI-MS calcd. for $C_{74}H_{116}N_{12}O_{23}$ $[M+2H]^{2+}$ *m*/*z* = 771.4215, found *m*/*z* = 771.4221.

2.8 Synthesis of glycosite-specific ADCs containing branched N-glycan chains with DAR 4

*Synthesis of drug-linker **12*** (BCN-VC-PAB-MMAE)



To a stirred solution of compound **S2** (6 mg) in DMF (100 μL) was added BCN-O-PNP (3.4 mg) and Et_3N (1.5 μL), the mixture was stirred at 37 °C for 3 h and monitored by LC–MS. The solution was subjected to a semi-preparative column, the corresponding product **12** was combined and lyophilized to get a white powder (5 mg, yield 72.7%). ESI-MS calcd. for $C_{69}H_{106}N_{10}O_{14}$ $[M+2H]^{2+}$ *m*/*z* = 650.4023, found *m*/*z* = 650.4011.

2.9 Synthesis of gsADC-3

Synthesis of azido-S2G2F-trastuzumab. The azido-S2G2F Trastuzumab was prepared as described previously. Briefly, the azido group was introduced to the terminal oxidized sialic acid of sialylglycpeptide (SGP) by oxime reaction. The purified azido oxime SGP (10 mg) was incubated with Endo-M (28 μg) in a phosphate buffer (50 mmol/L, pH 6.2, 69 μL) at 30 °C and monitored by LC–MS. After the complete hydrolysis, the reaction mixture was diluted to 10 mmol/L with water, and CDMBI (5 eq.) and K_3PO_4 (15 eq.) was added. The solution was incubated on ice for 2 h. Then, the reaction mixture was further diluted into 1.95 mL Tris buffer (50 mmol/L, pH 7.1) containing (Fucα1,6)GlcNAc-trastuzumab (11.5 mg) and Endo-S D233Q (340 μg), and the mixture was incubated at 30 °C for 2 h. LC–MS monitoring indicated the complete reaction. The solution was immediately subjected to affinity chromatography via protein A resin following the above procedure to give the corresponding azido-S2G2F-Trastuzumab.

Synthesis of gsADC-3. A solution of azido-S2G2F-Trastuzumab (1 mg) and drug-linker **12** in a phosphate buffer (50 mmol/L, pH 7.4, 200 μL) was incubated at 37 °C for 4 h. LC–MS monitoring indicated the complete reaction of azido-S2G2F-Trastuzumab to gsADC-3. The reaction mixture was concentrated by centrifugal filtration (Millipore) to give the corresponding gsADC-3.

2.10 Synthesis of positive control ADC (Cys random conjugation)

A solution of wild-type Trastuzumab (1 mg) in a phosphate buffer (50 mmol/L, pH 7.4, 200 μL) was treated with TCEP (2.5 eq, 0.085 mmol/L) at rt for 2 h. Then, the Drug-Linker mc-VC-PAB-MMAE (10 eq, 0.33 mmol/L) was added to the above solution, the mixture was incubated at 37 ºC for another 2 h. Reverse-phase (RP) HPLC and MS analysis indicated the DAR value was about 3.5. The reaction mixture was concentrated by centrifugal filtration (Millipore) to give the corresponding Cys random ADC.

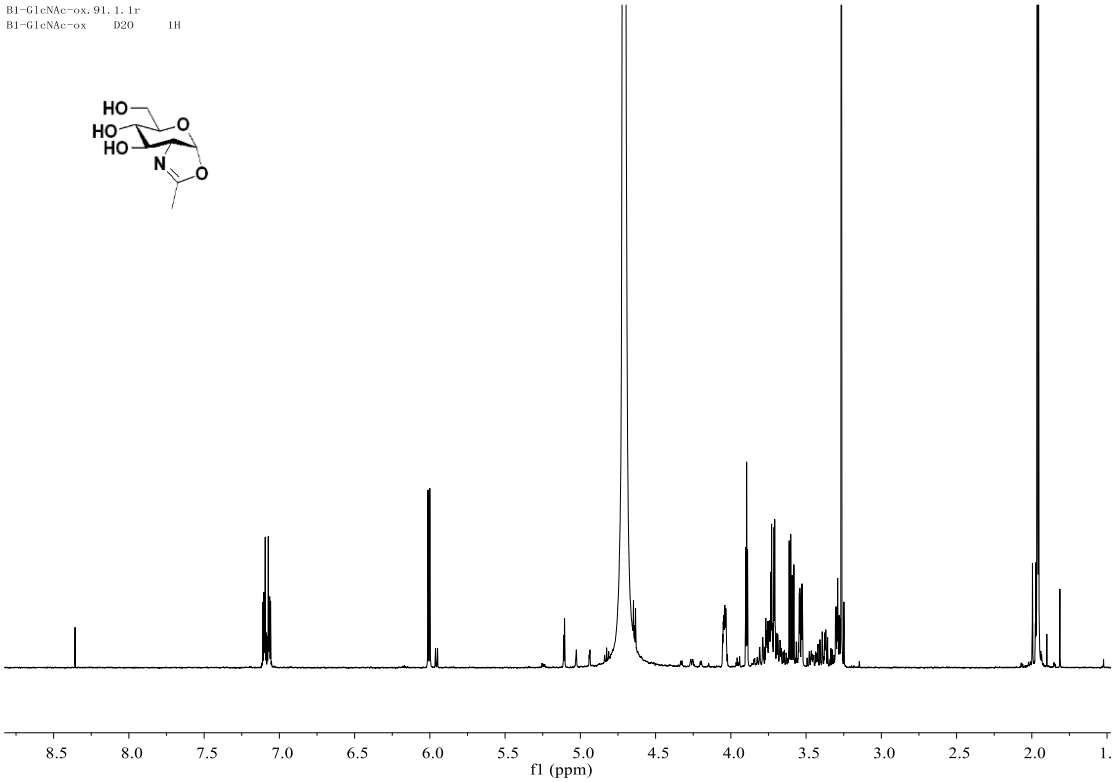
2.11 Reaction sites confirmation

Light and heavy chain analysis. A solution of **6a** (50 μg) in a phosphate buffer (50 mmol/L, pH 7.4, 10 μL) was incubated with DBCO-Cy3 or DBCO-FITC (4 nmoL) at 37 ºC for 4 h. SDS-PAGE analysis indicated that Cy3 or FITC was labeled on the heavy chain of trastuzumab.

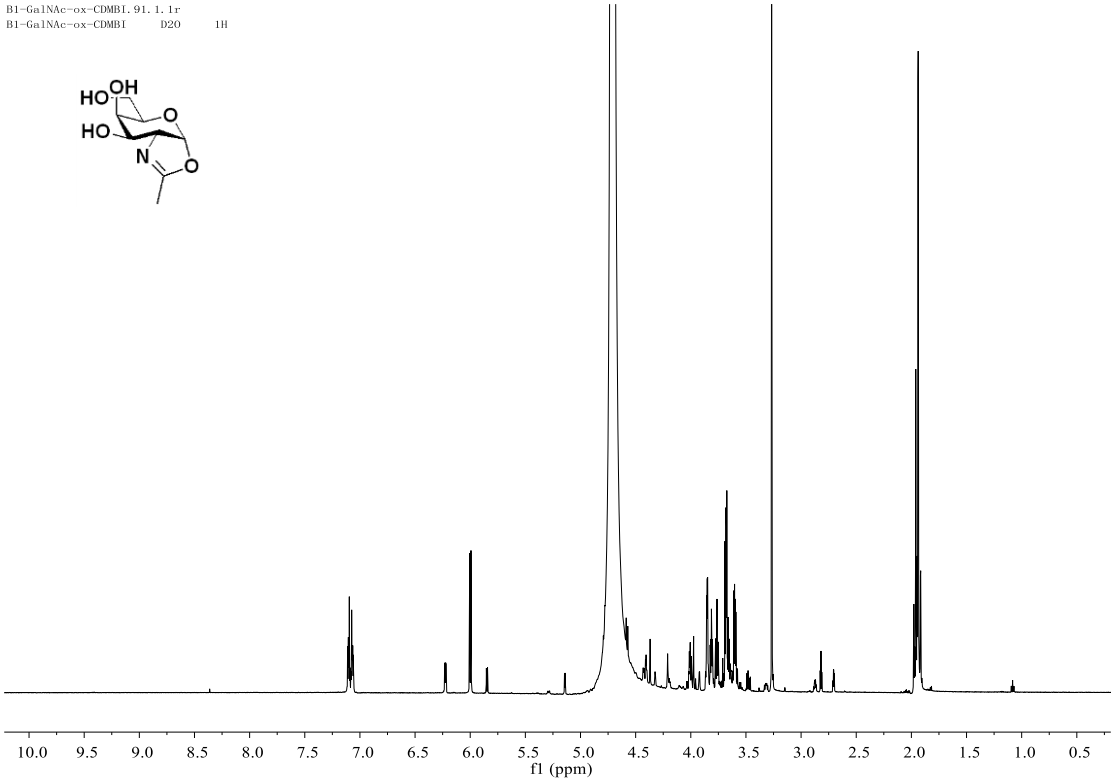
IdeS protease treatment. A solution of Wild-type trastuzumab or (Fucα1,6)GlcNAc-trastuzumab or gsADC-2 (50 μg) in a phosphate buffer (50 mmol/L, pH 7.4, 10 μL) was incubated with IdeS protease (5 U) at 37 ºC overnight. LC–MS monitoring indicated that drug-LacNAc complex were conjugated on the ScFc of trastuzumab.

PNGase F treatment. A solution of Wild-type trastuzumab or gsADC-2 or (Fucα1,6)GlcNAc-trastuzumab (50 μg) in a phosphate buffer (50 mmol/L, pH 7.4, 10 μL) was incubated with PNGase F (1 μg) at 37 ºC overnight. LC–MS monitoring indicated that azido-tagged LacNAc was conjugated on the glycosylation site of the antibody.

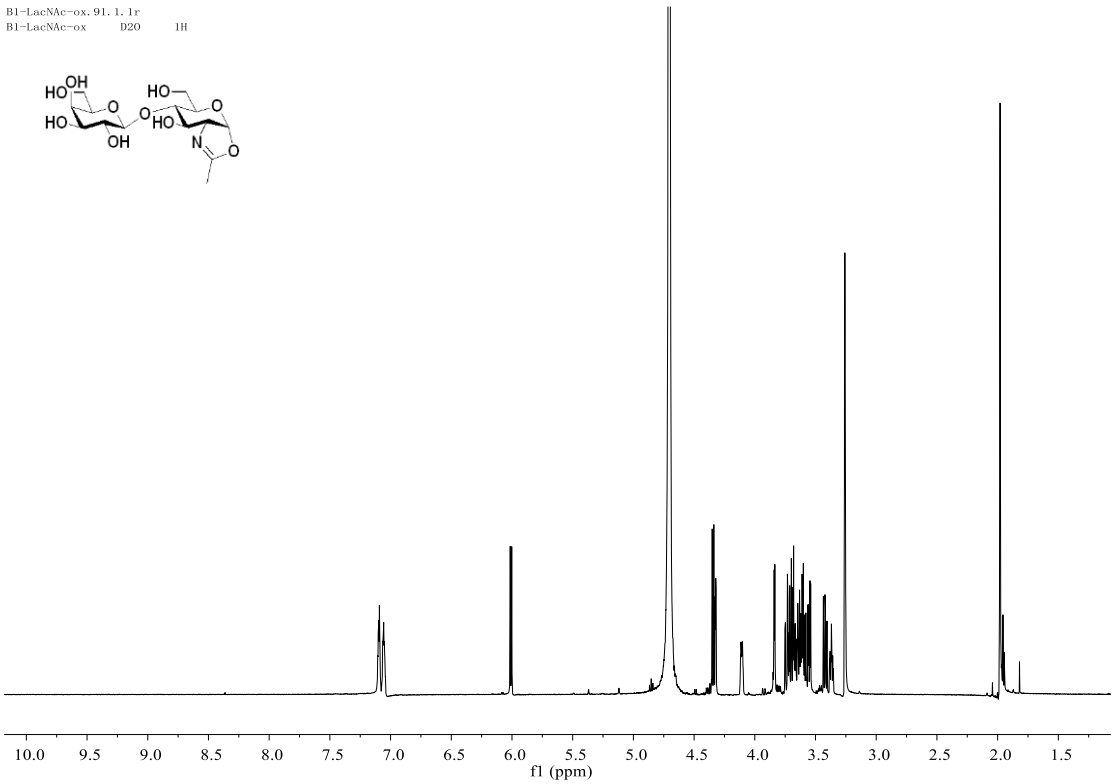
3. NMR spectra:



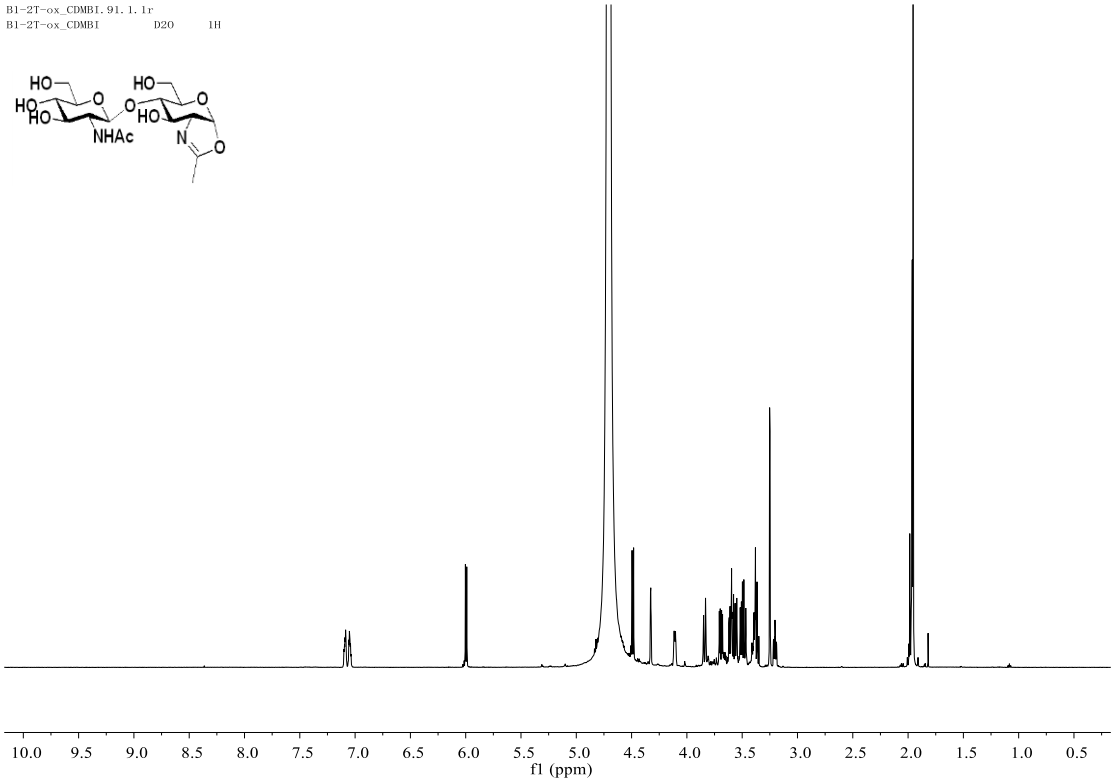
^{1}H NMR of GlcNAc-ox **2a**



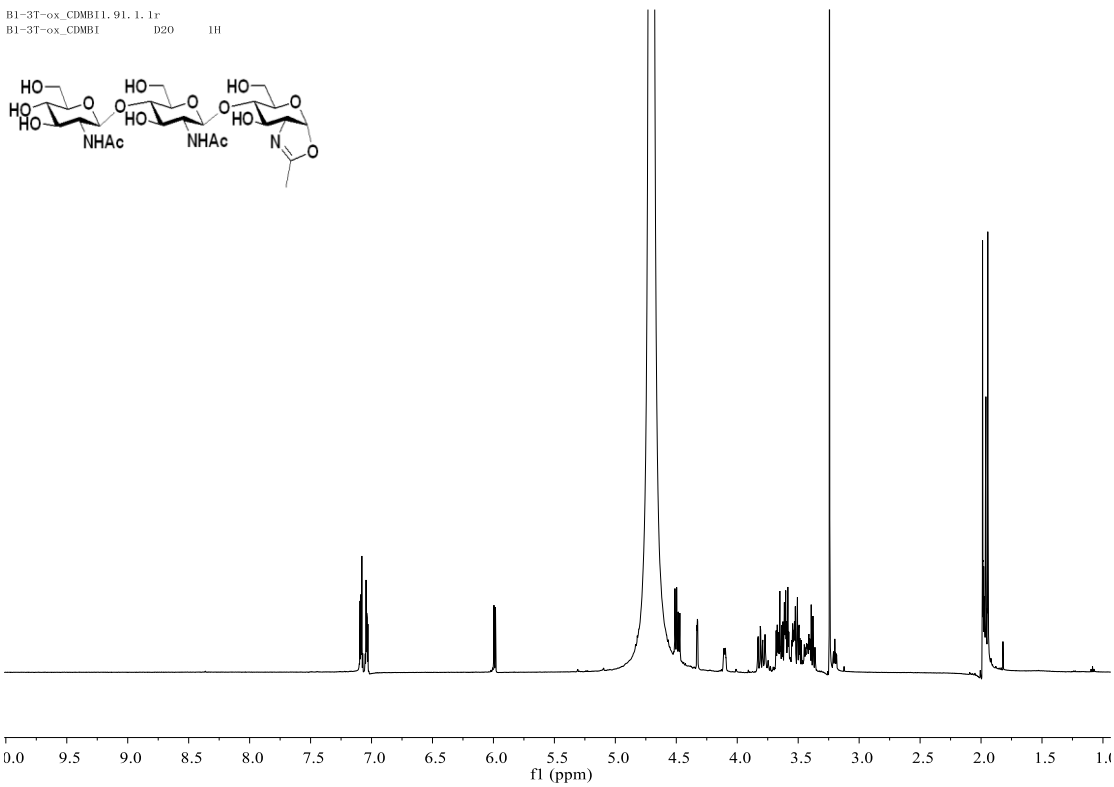
^{1}H NMR of GalNAc-ox **2b**



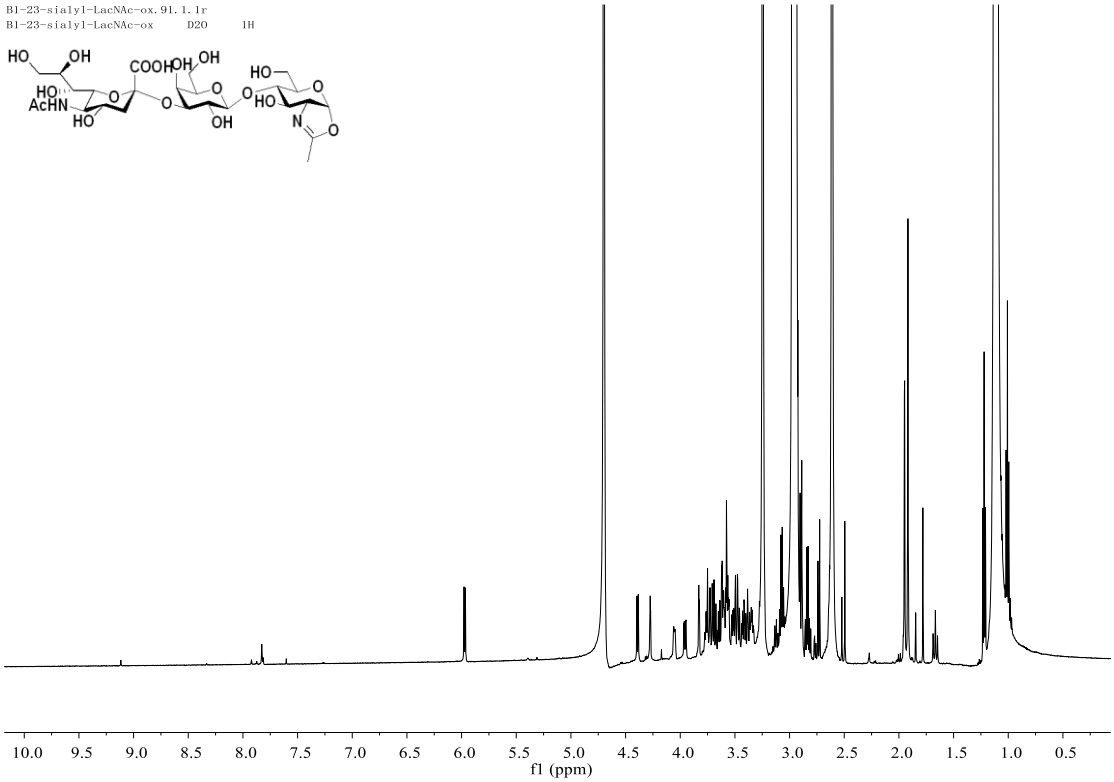
^{1}H NMR of LacNAc-ox **2c**



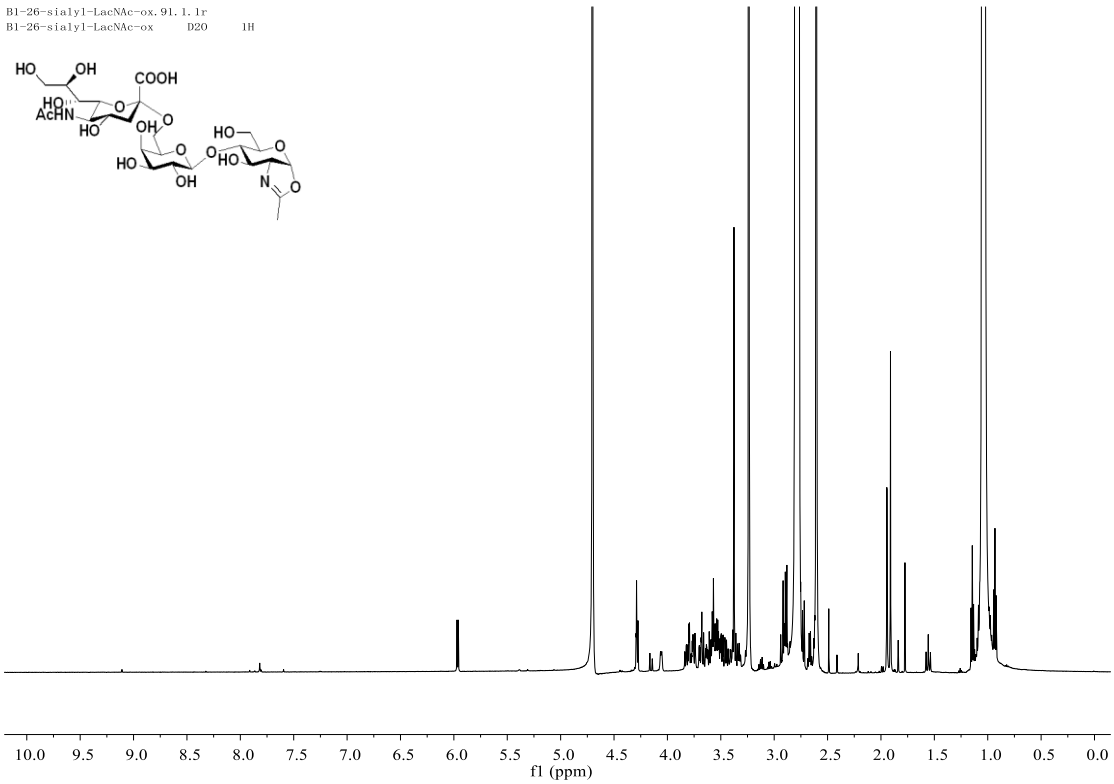
^{1}H NMR of $(GlcNAc)_2$-ox **2d**



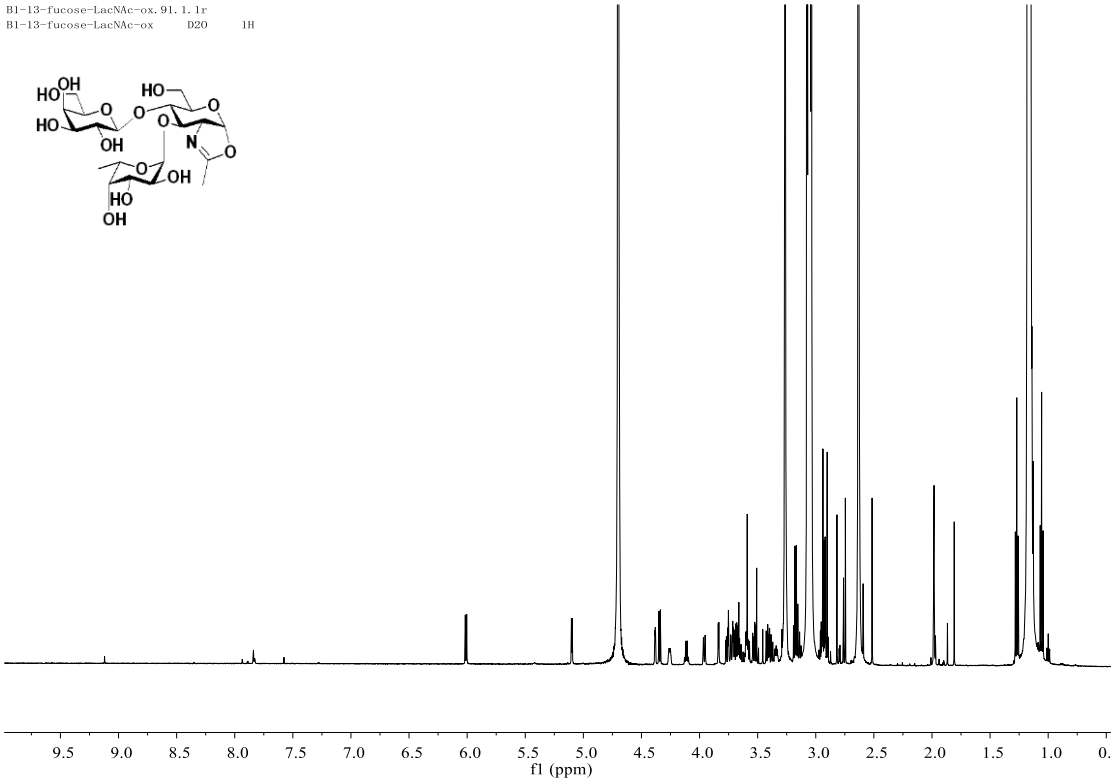
^{1}H NMR of $(GlcNAc)_3$-ox **2e**



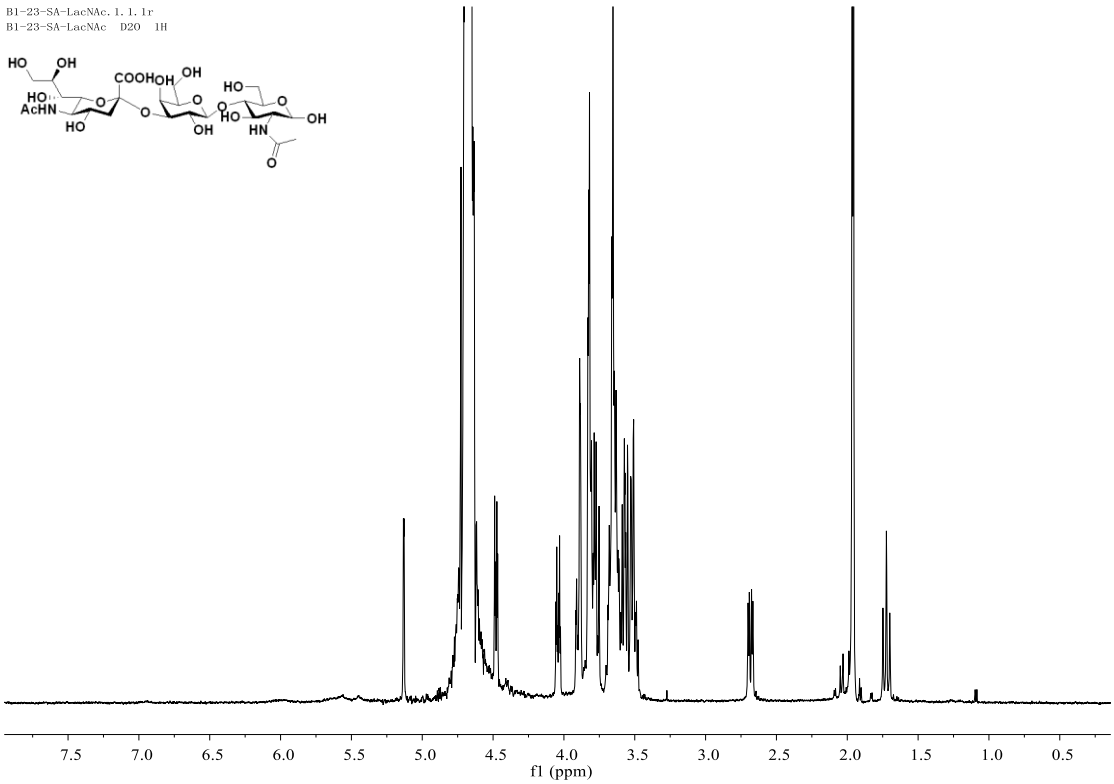
^{1}H NMR of NeuNAc-α2,3-LacNAc-ox **2f**



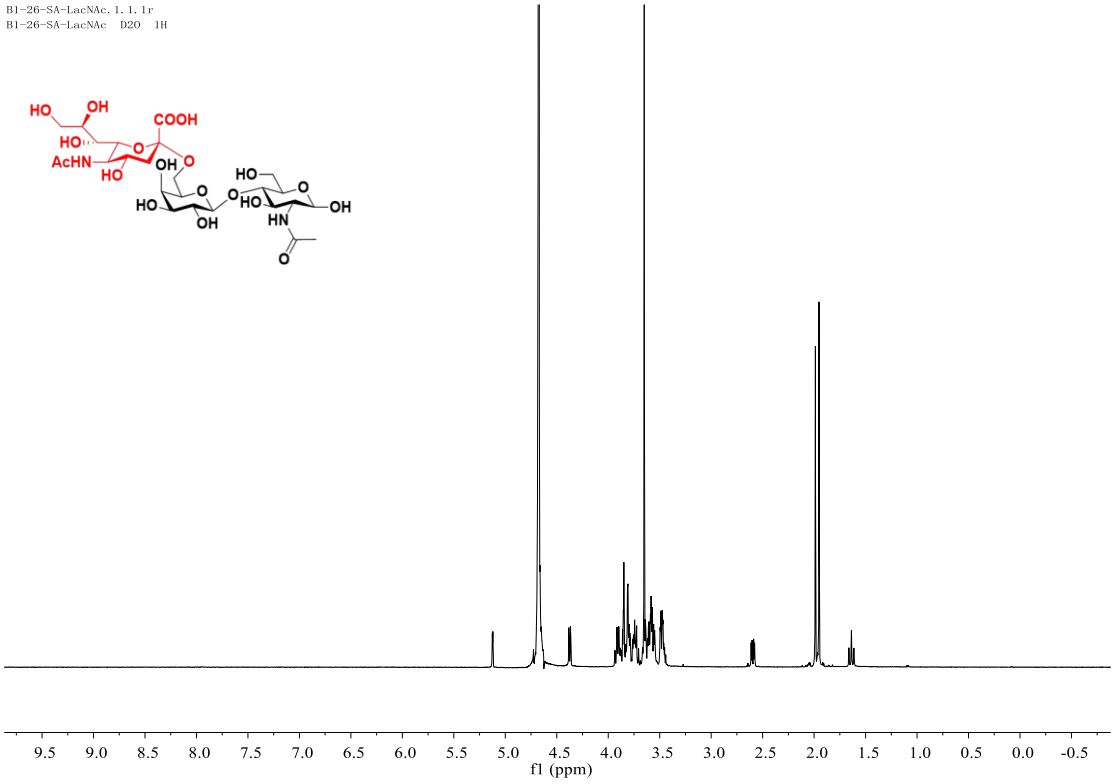
^{1}H NMR of NeuNAc-α2,6-LacNAc-ox **2g**



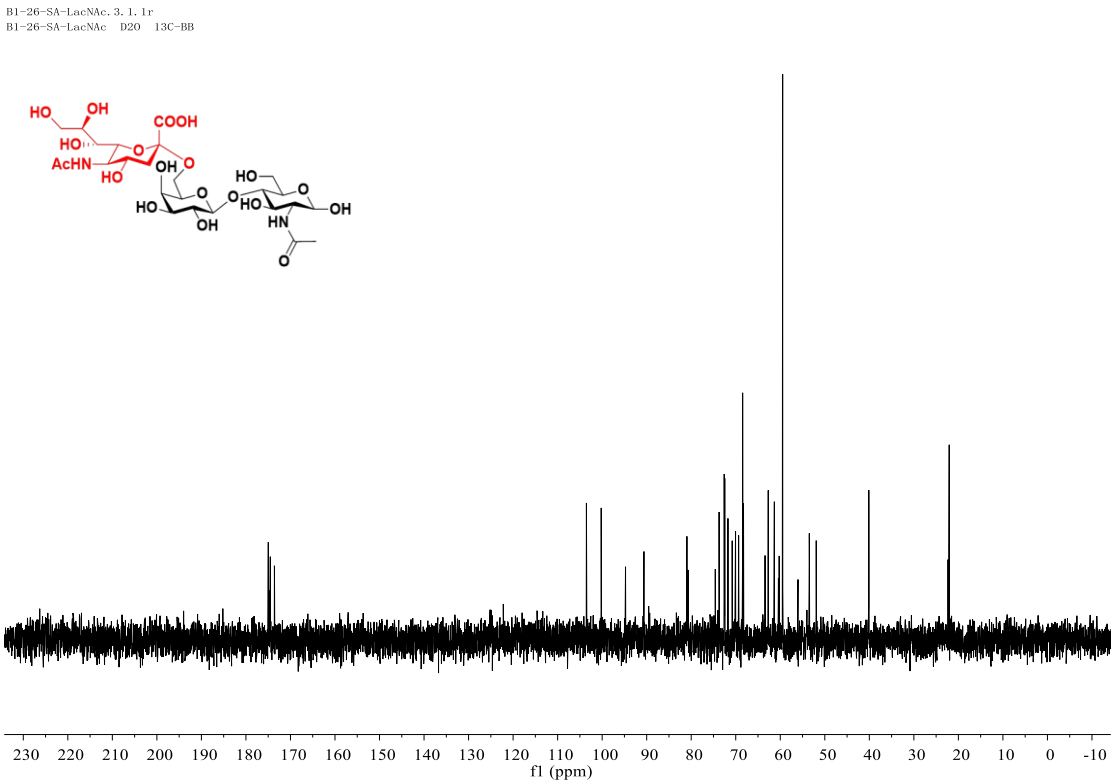
^{1}H NMR of Gal-β1,4-(Fucα1,3)GlcNAc-ox **2h**



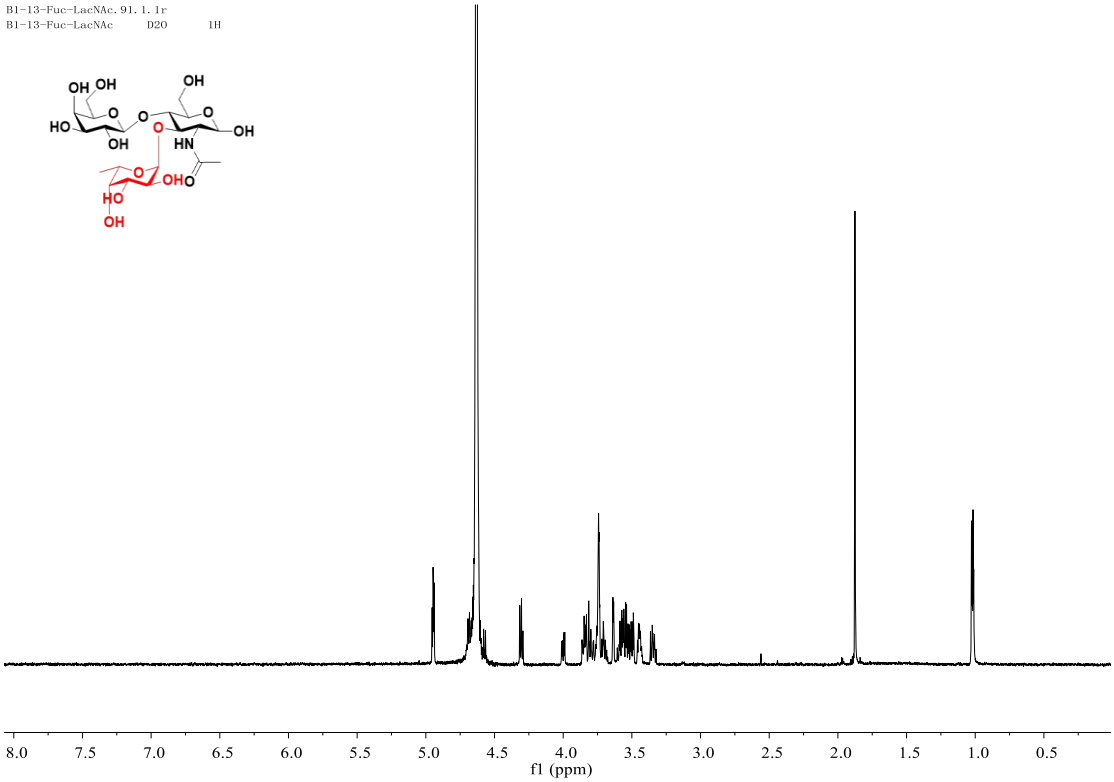
^{1}H NMR of NeuNAc-α2,3-LacNAc **4l**



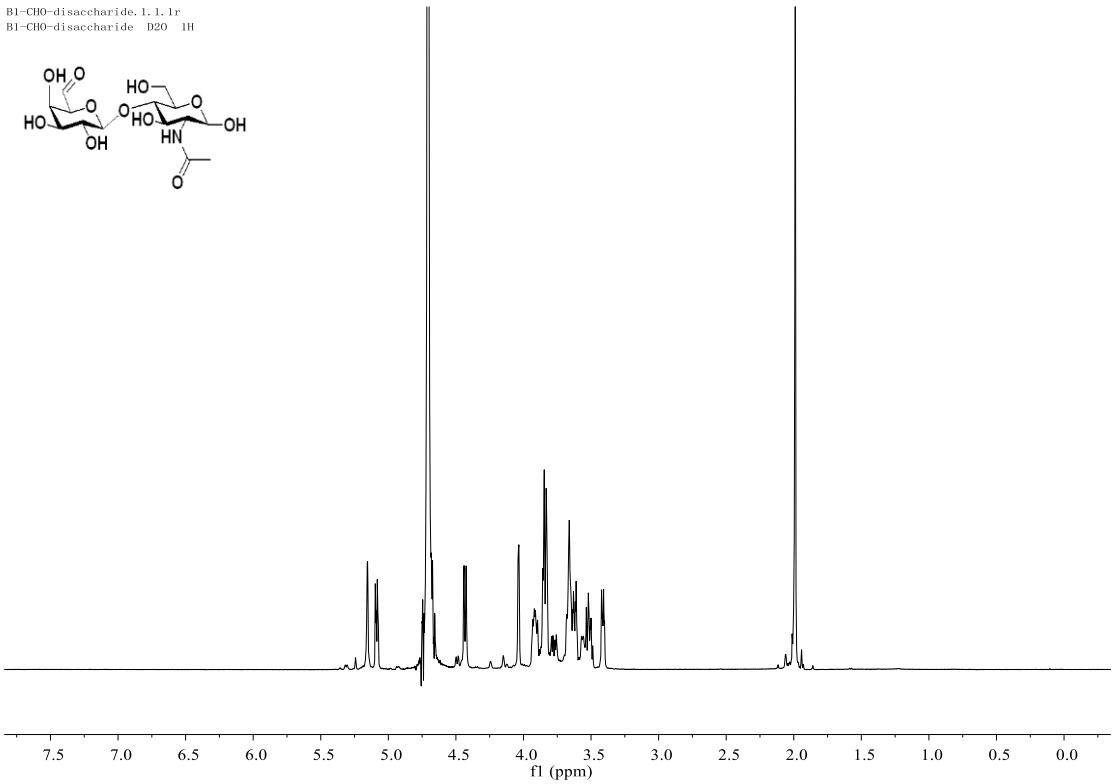
^{1}H NMR of NeuNAc-α2,3-LacNAc **4k**



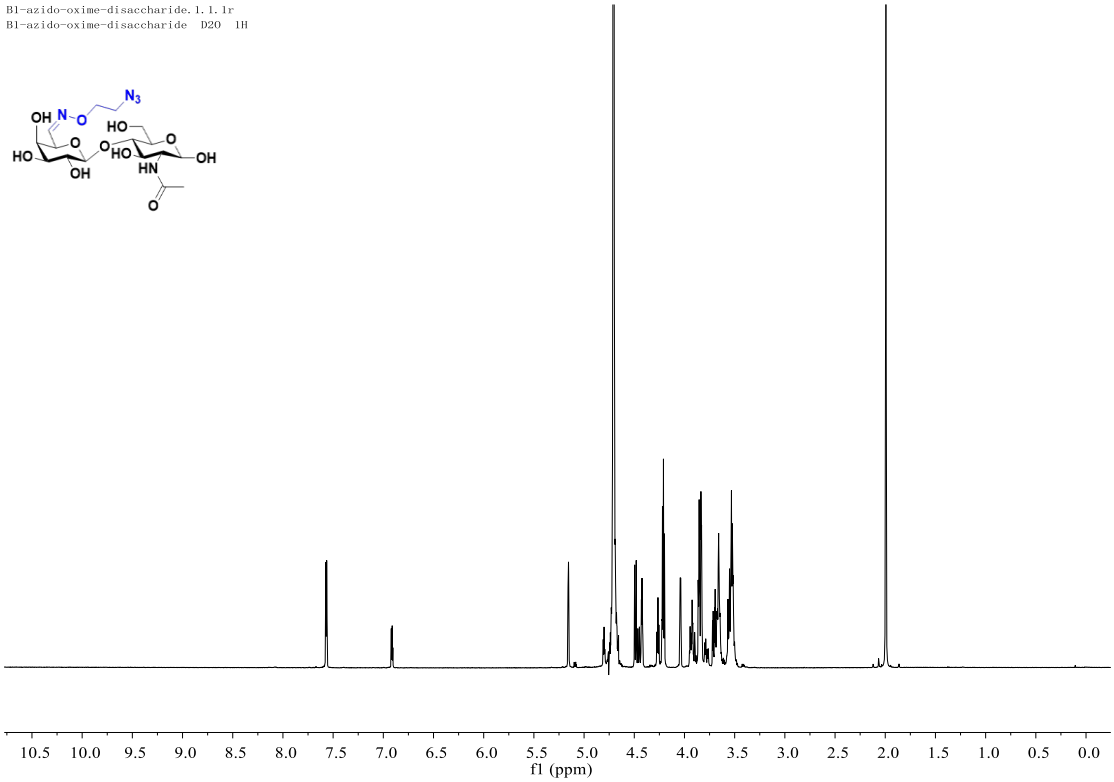
^{13}C NMR of NeuNAc-α2,3-LacNAc **4k**



^{1}H NMR of Gal-β1,4-(Fucα1,3)GlcNAc **4j**

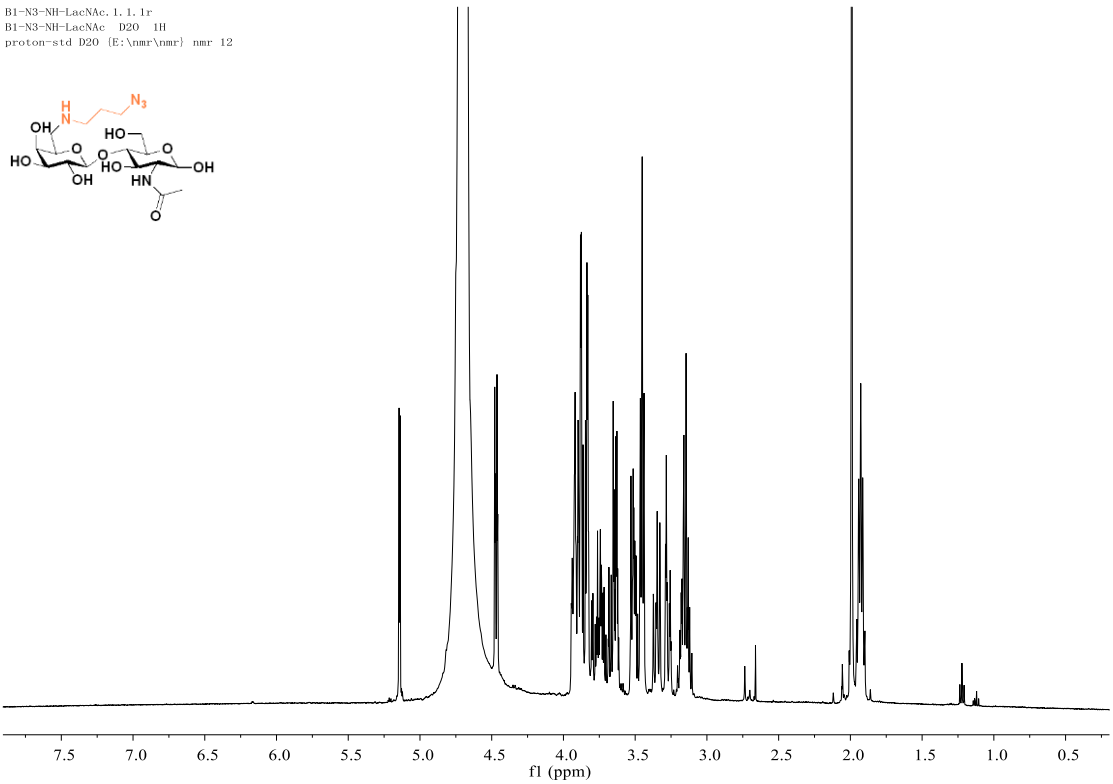


^{1}H NMR of CHO-LacNAc **4j**

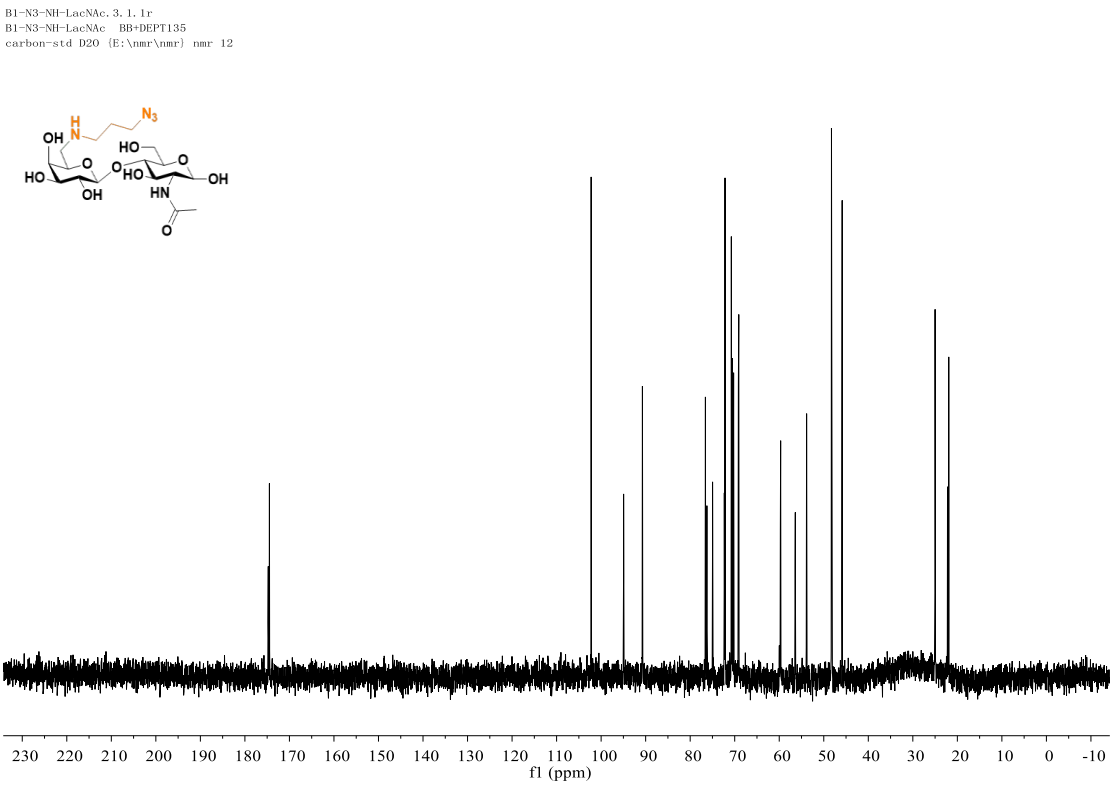


^{1}H NMR of N_3-ON=CH-LacNAc **4a**

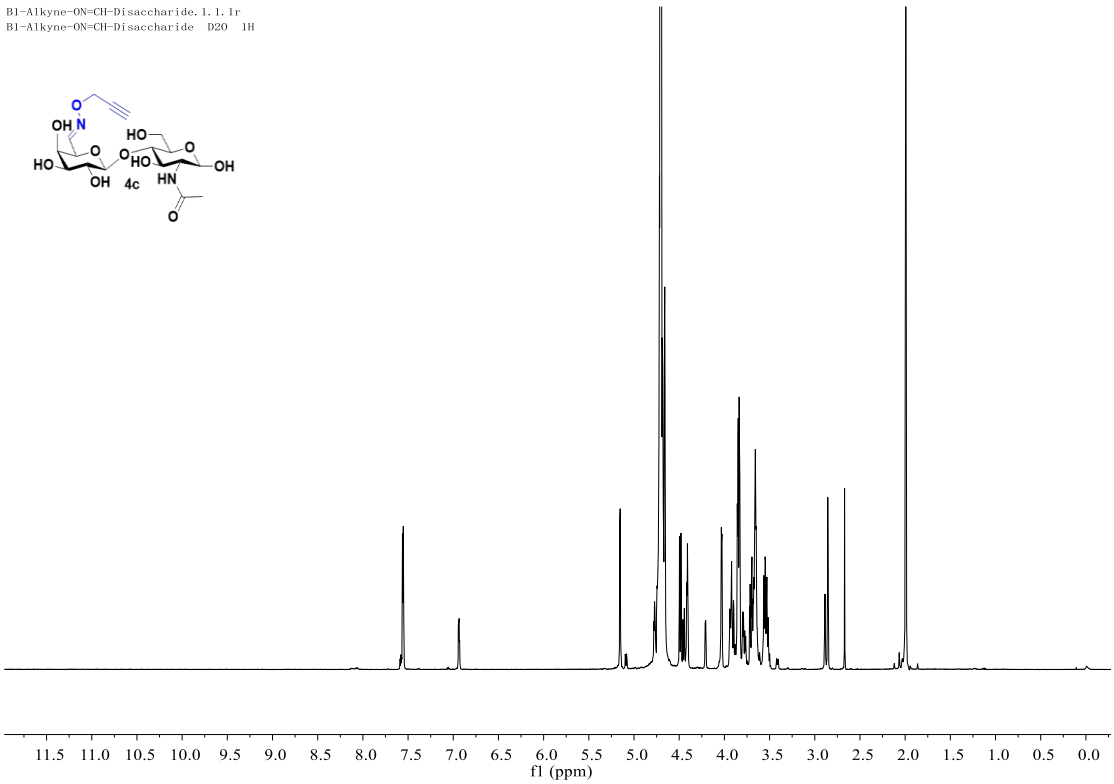
N_3-NH-LacNAc **4b**



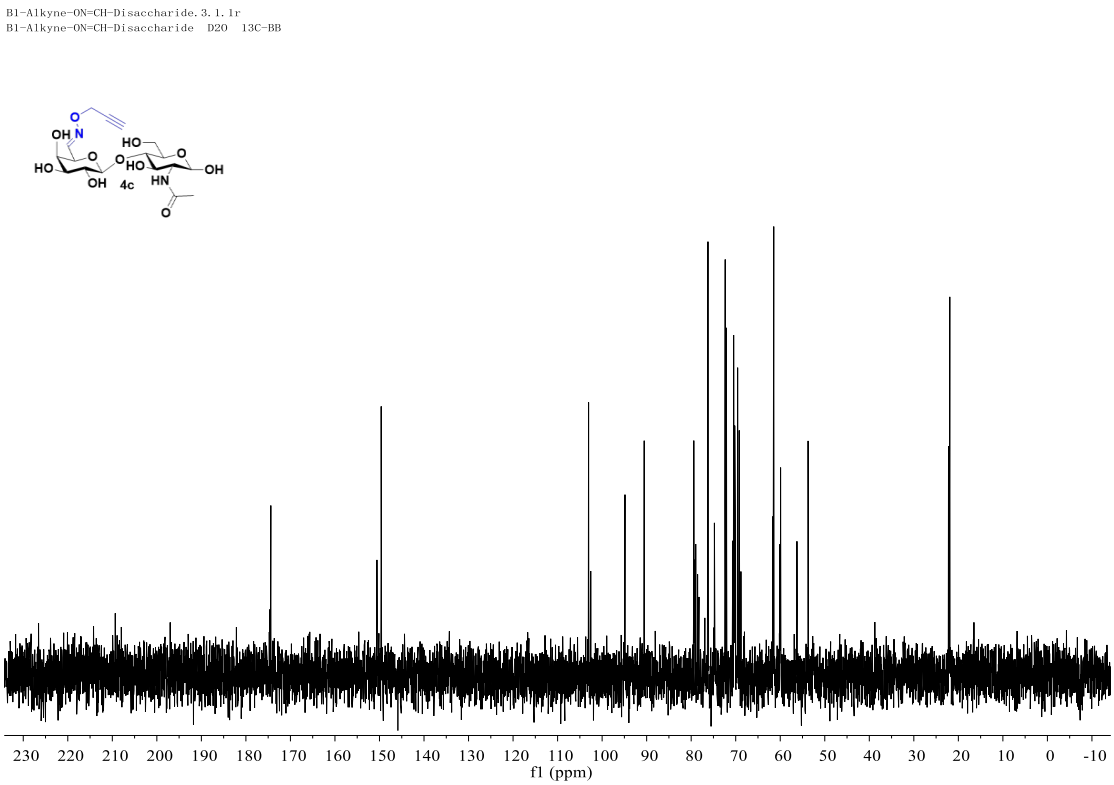
^{1}H NMR of N_3-NH-LacNAc **4b**



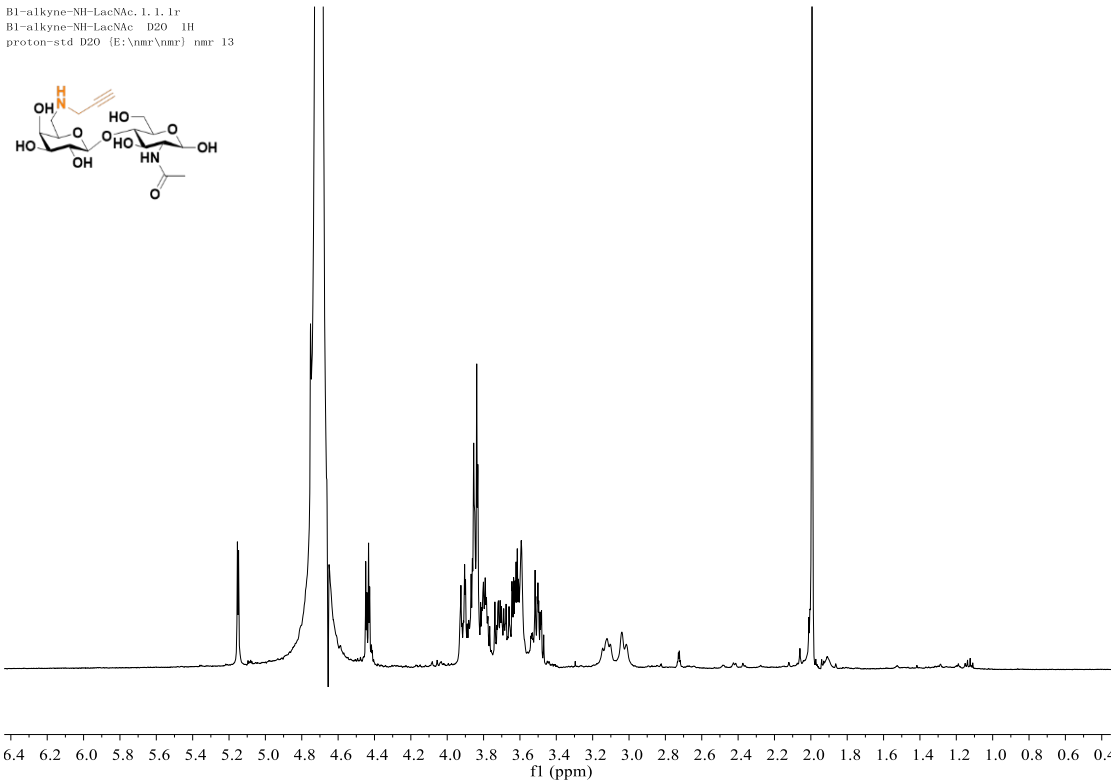
^{13}C NMR of N_3-NH-LacNAc **4b**



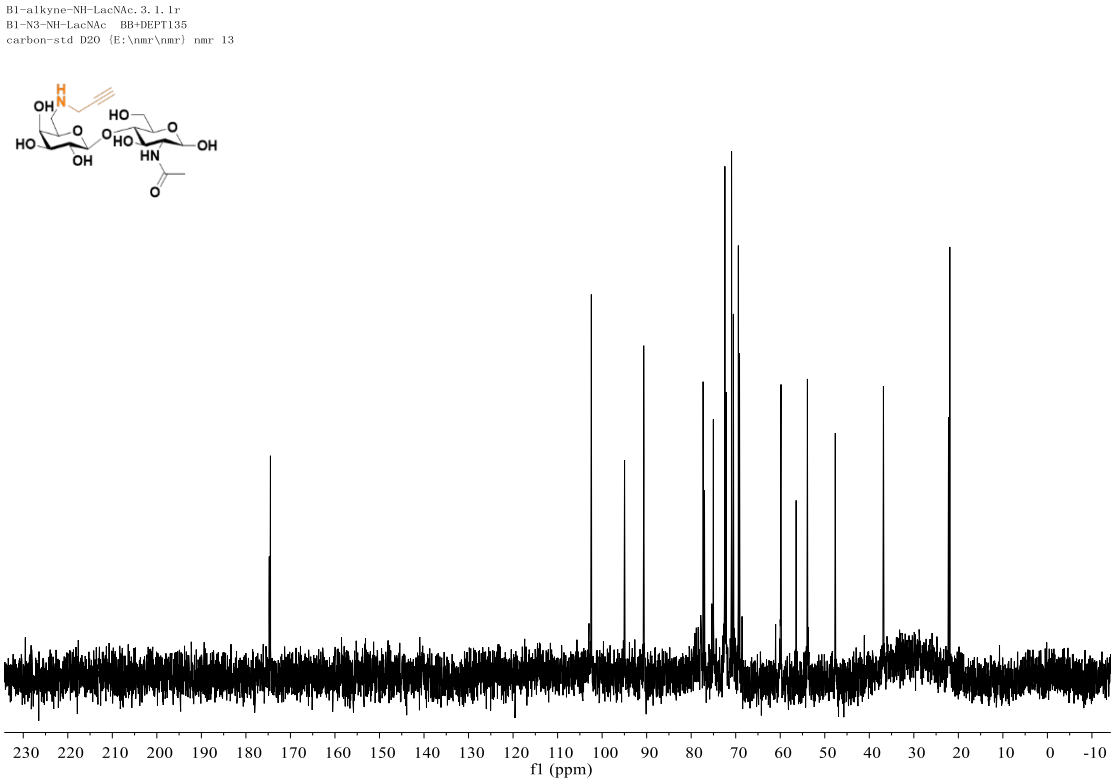
^{1}H NMR of Alkyne-ON=CH-LacNAc **4c**



^{13}C NMR of Alkyne-ON=CH-LacNAc **4c**

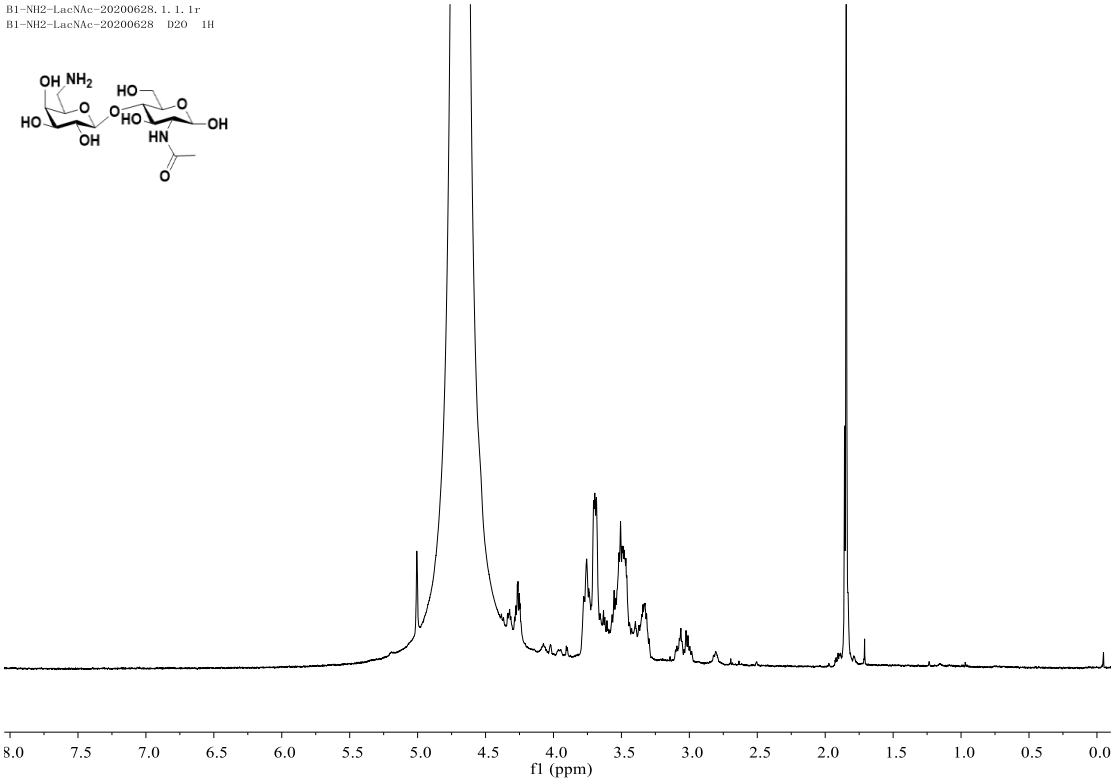


^{1}H NMR of Alkyne-NH-LacNAc **4d**

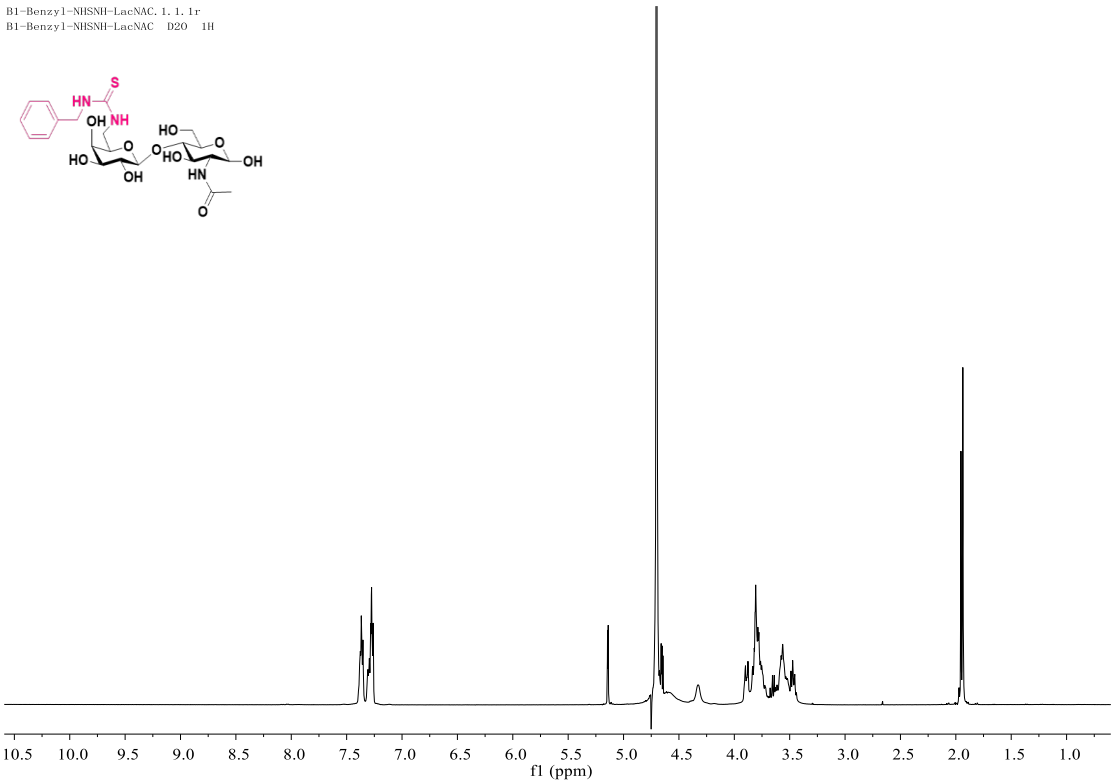


^{13}C NMR of Alkyne-NH-LacNAc **4d**

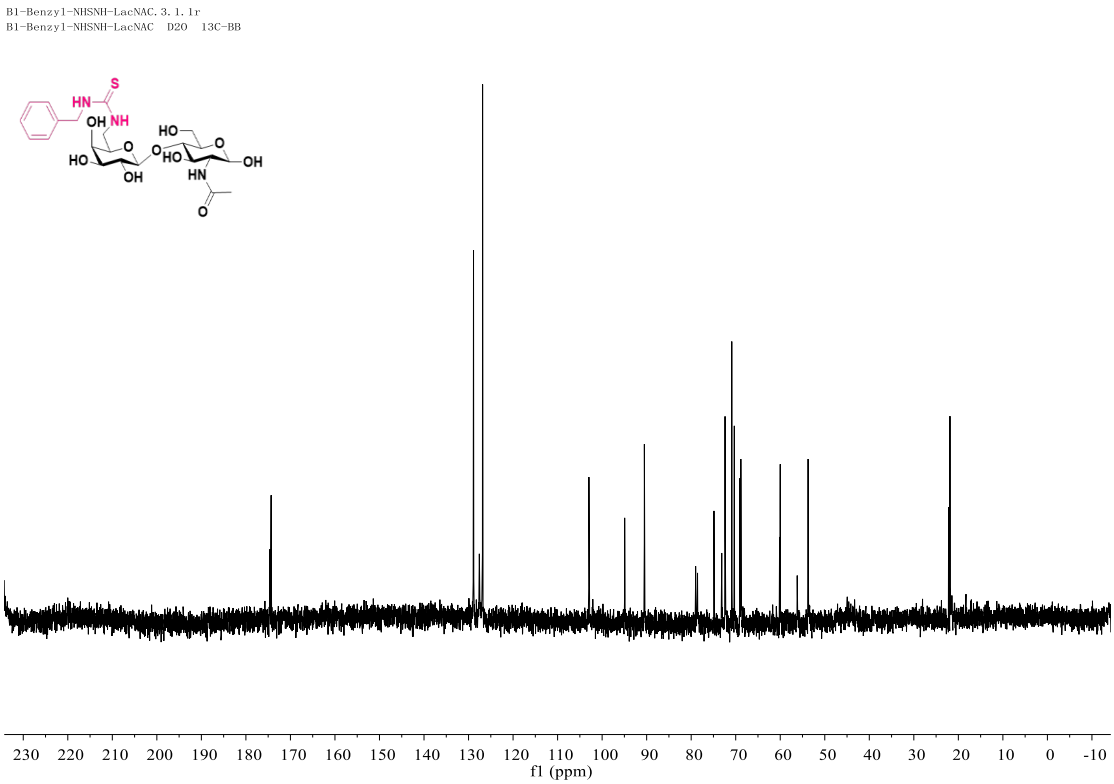
NH_2-LacNAc **4e**



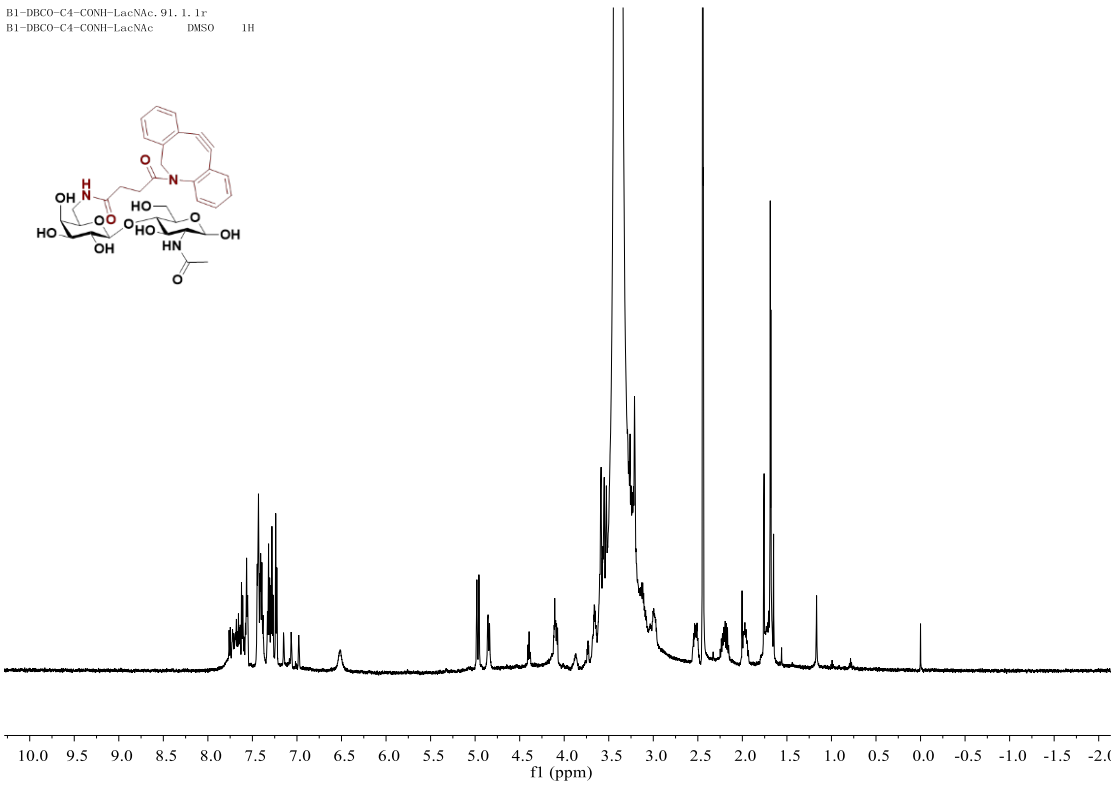
^{1}H NMR of NH_2-LacNAc **4e**



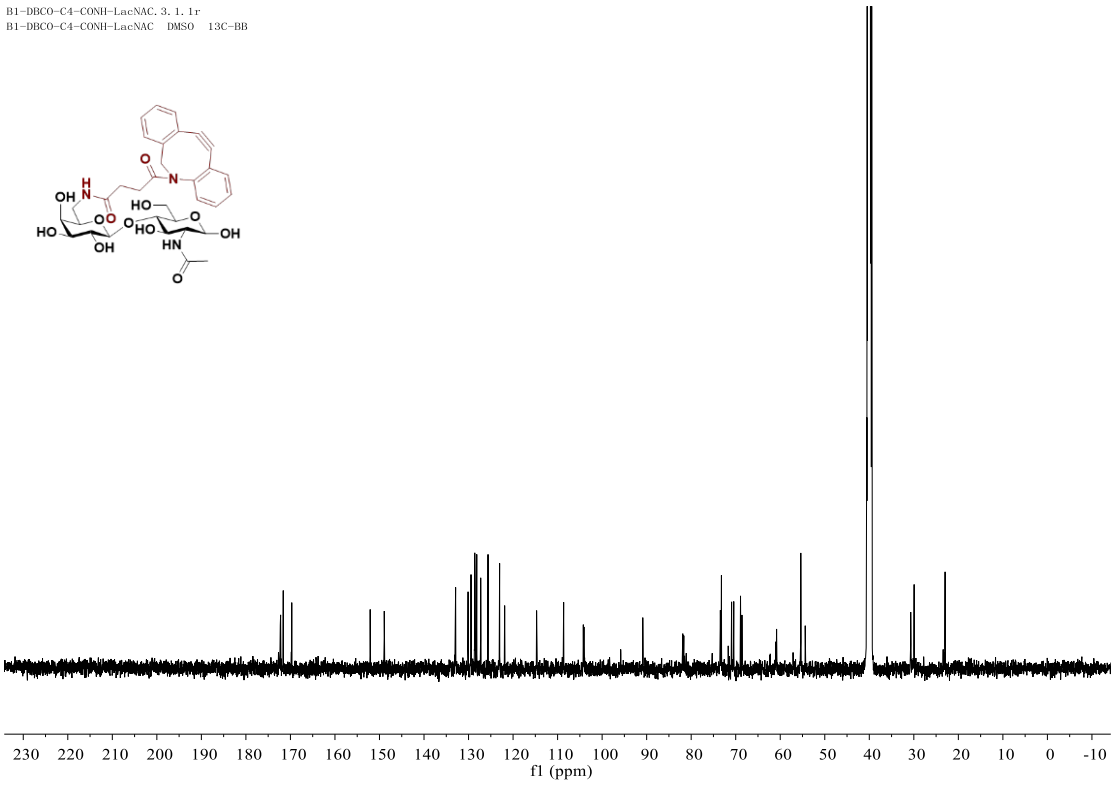
^{1}H NMR of Benzyl-LacNAc **4f**



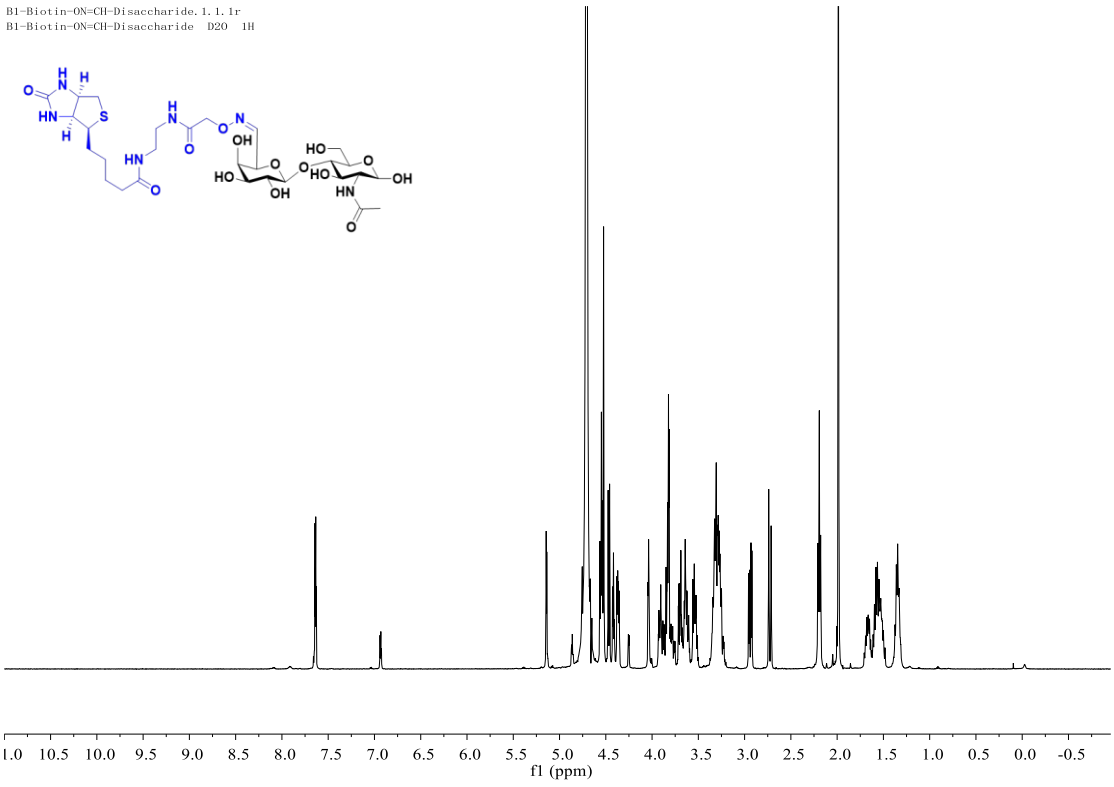
^{13}C NMR of Benzyl-LacNAc **4f**



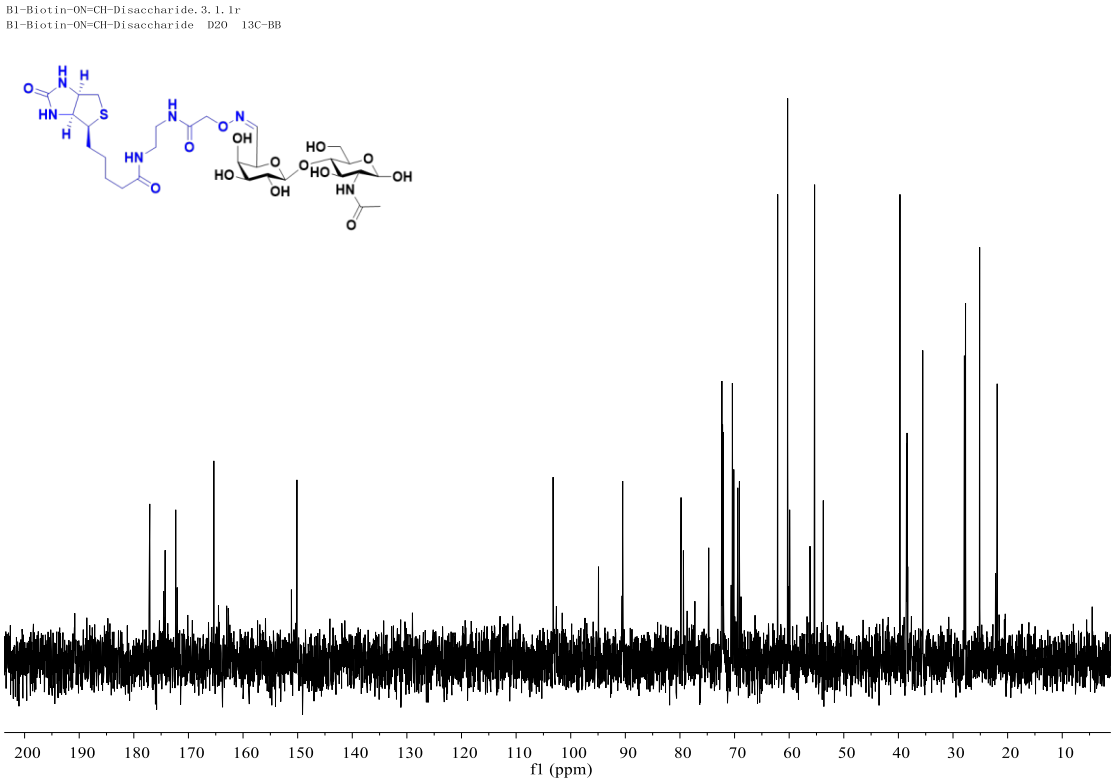
^{1}H NMR of DBCO-LacNAc **4g**



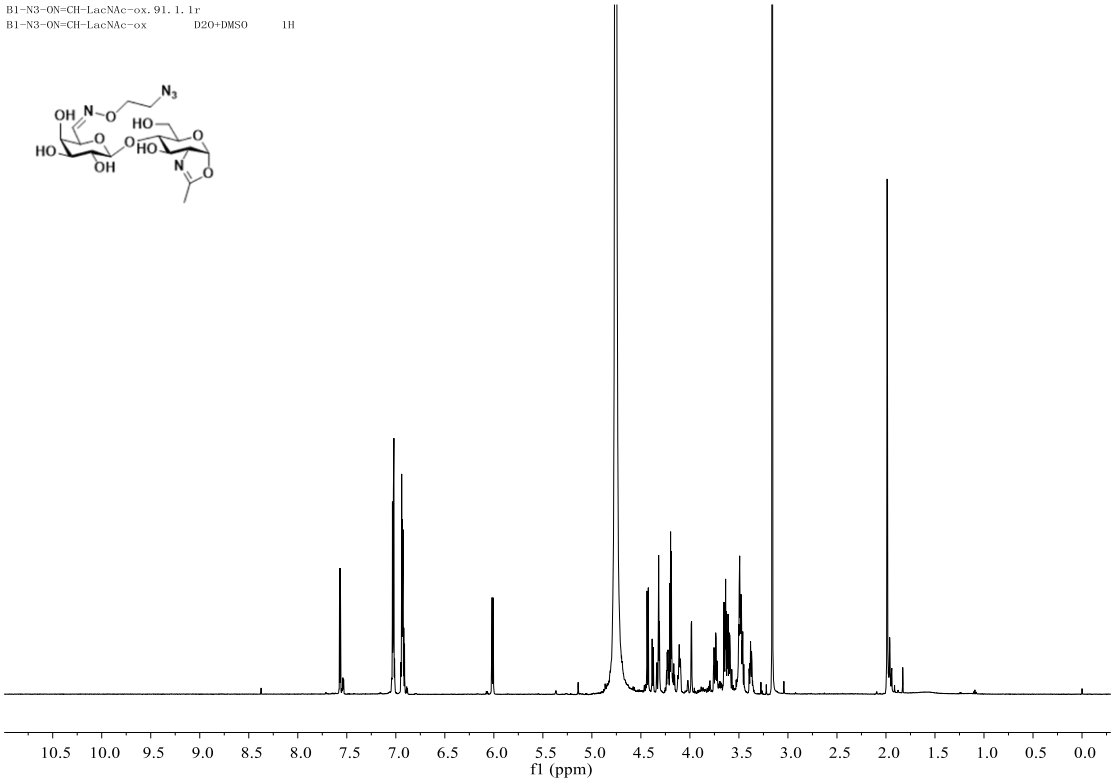
^{13}C NMR of DBCO-LacNAc **4g**



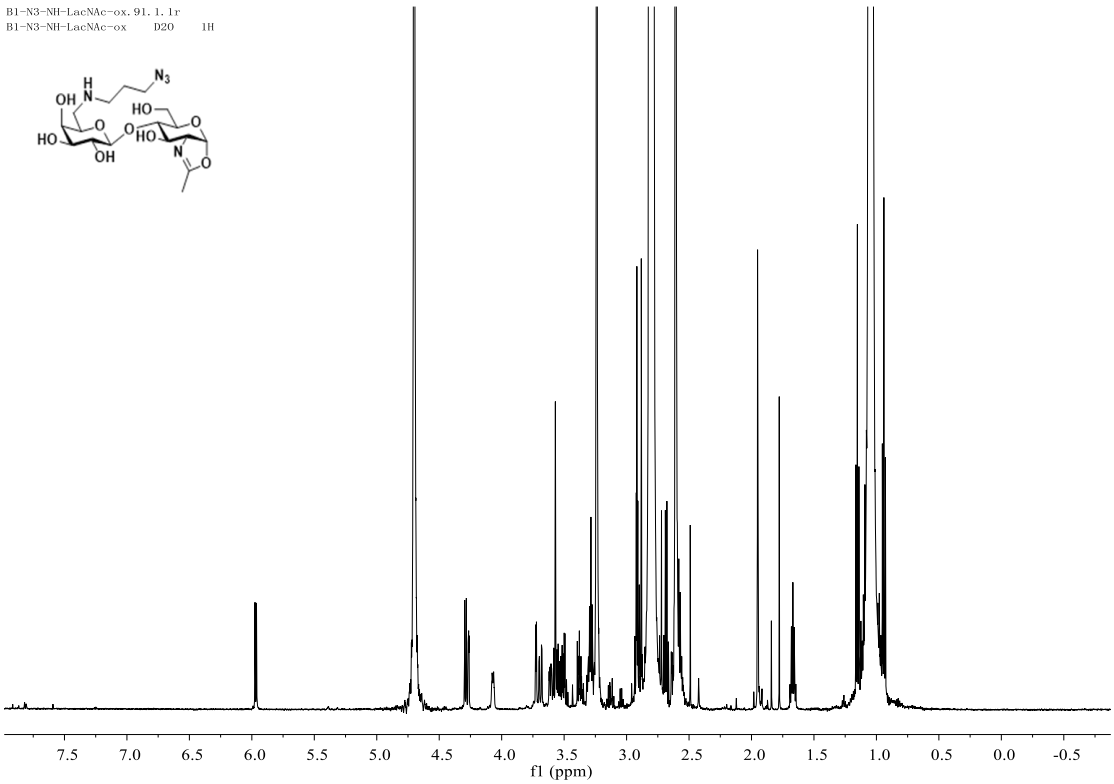
^{1}H NMR of Biotin-ON=CH-LacNAc **4h**



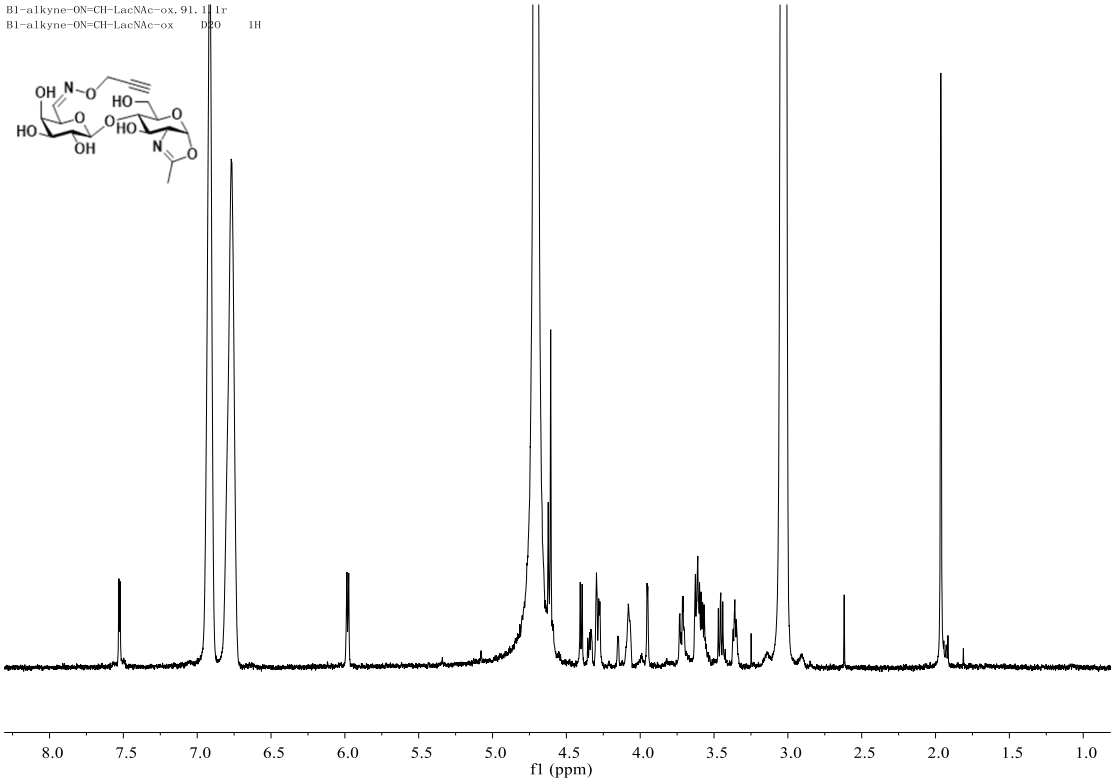
^{13}C NMR of Biotin-ON=CH-LacNAc **4h**



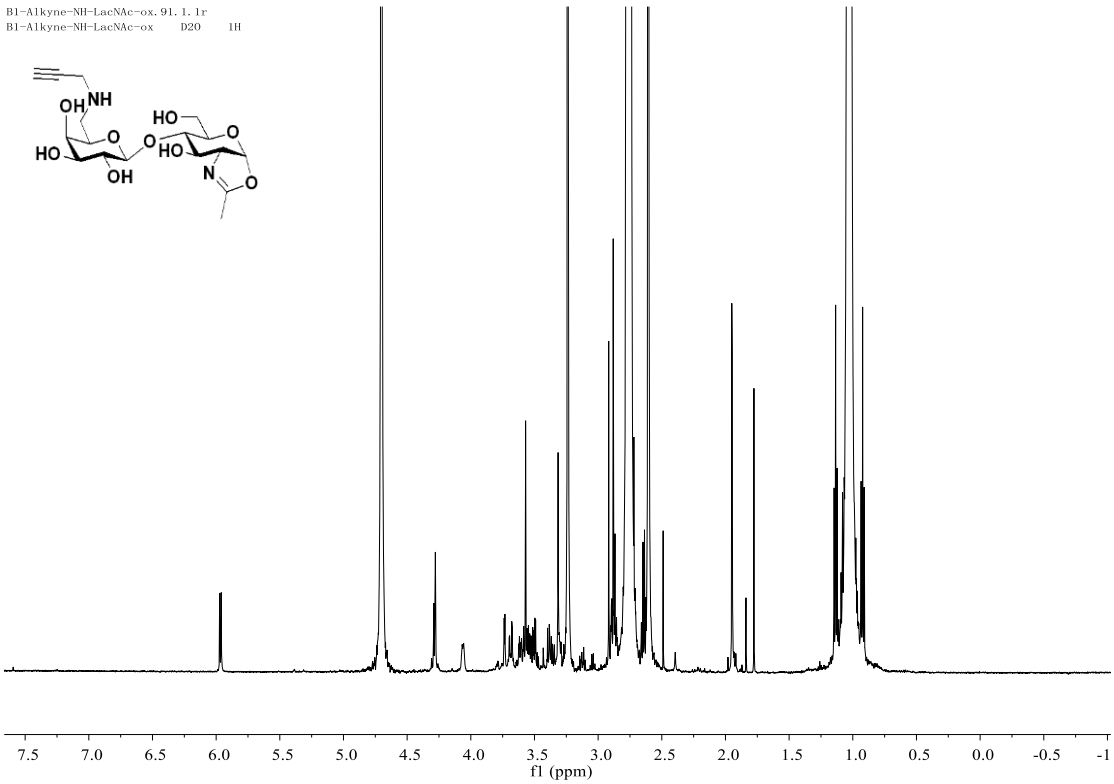
^{1}H NMR of N_3-ON=CH-LacNAc-ox **5a**



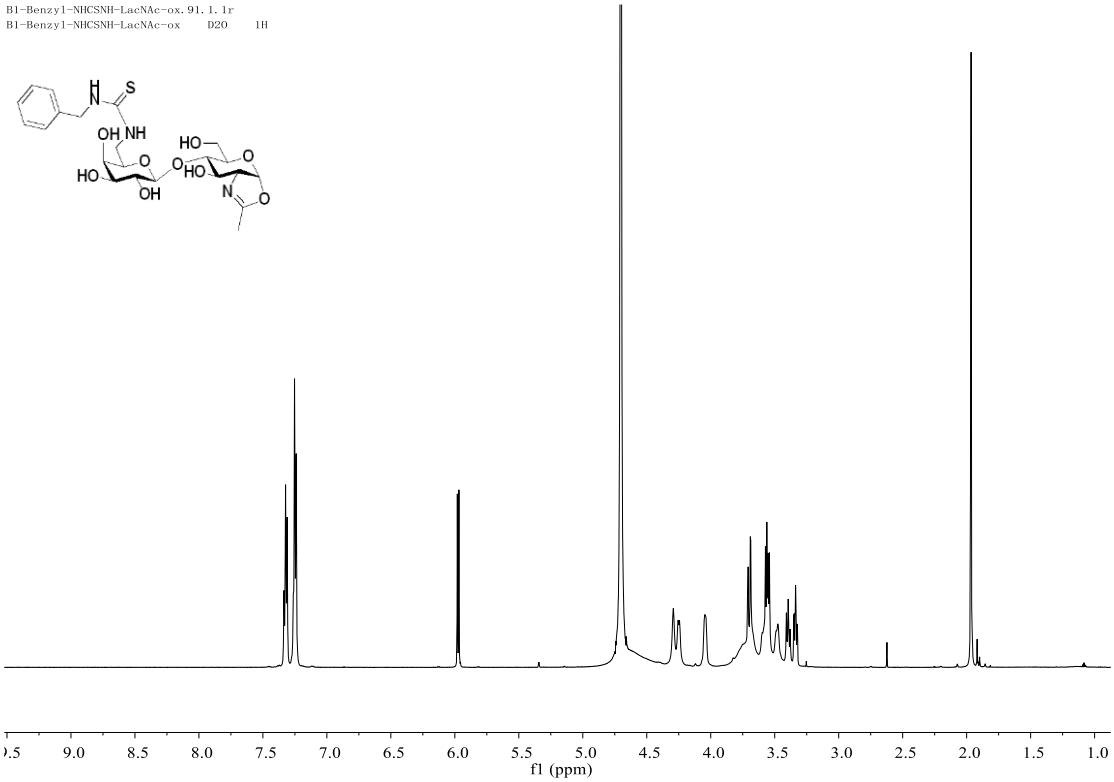
^{1}H NMR of N_3-NH-LacNAc-ox **5b**



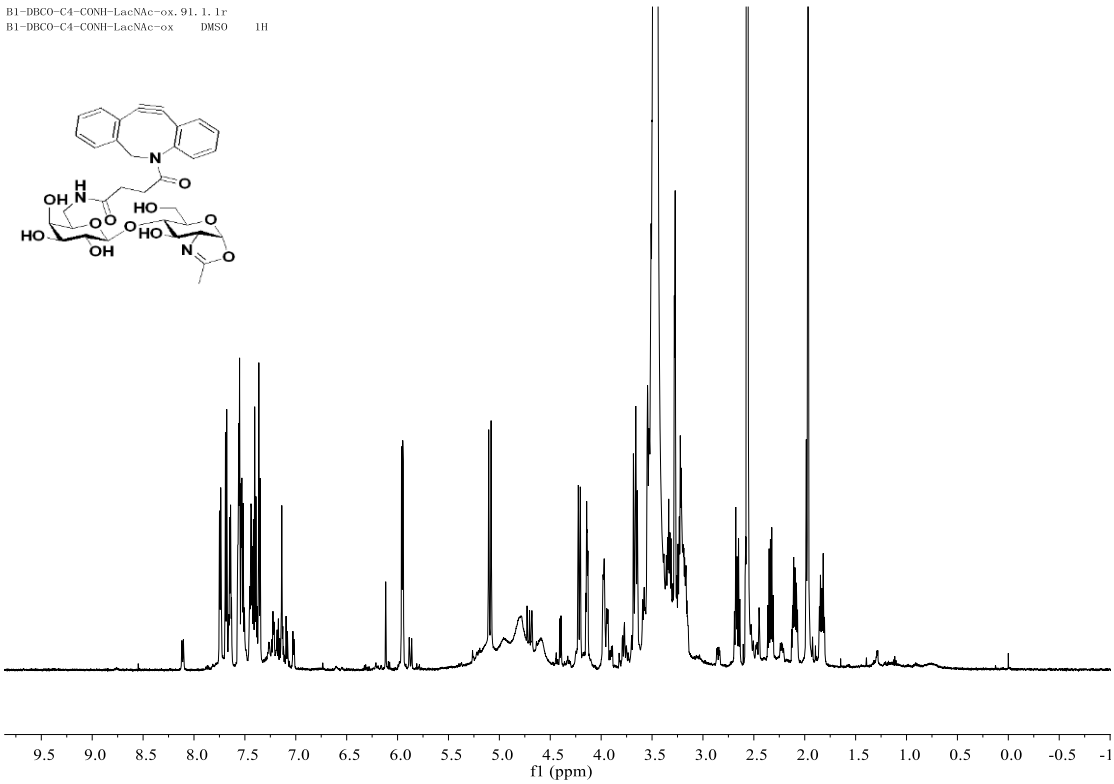
^{1}H NMR of Alkyne-ON=CH-LacNAc-ox **5c**



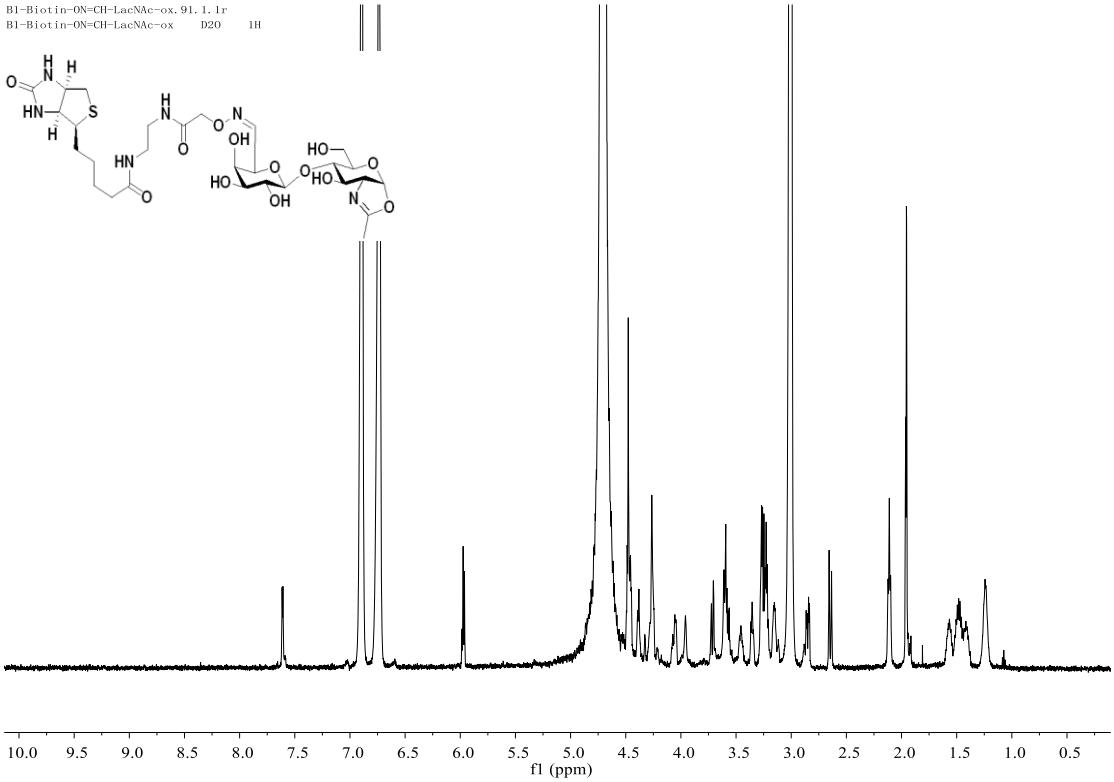
^{1}H NMR of Alkyne-NH-LacNAc-ox **5d**



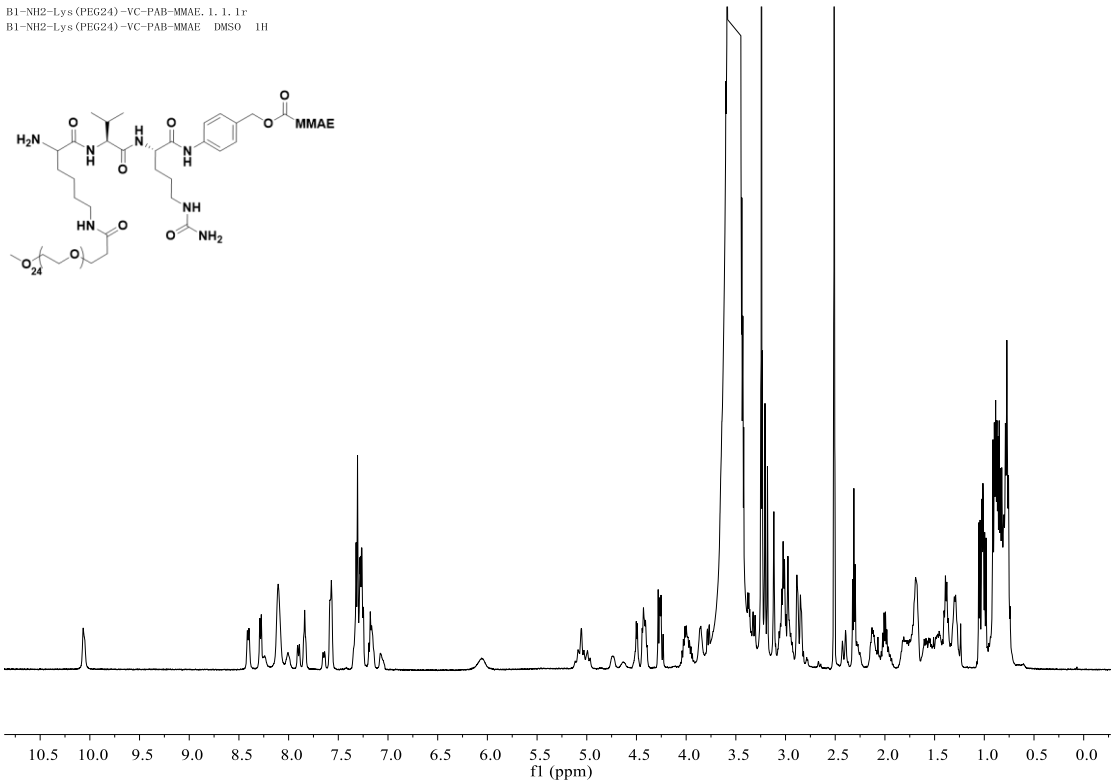
^{1}H NMR of Benzyl-LacNAc-ox **5f**



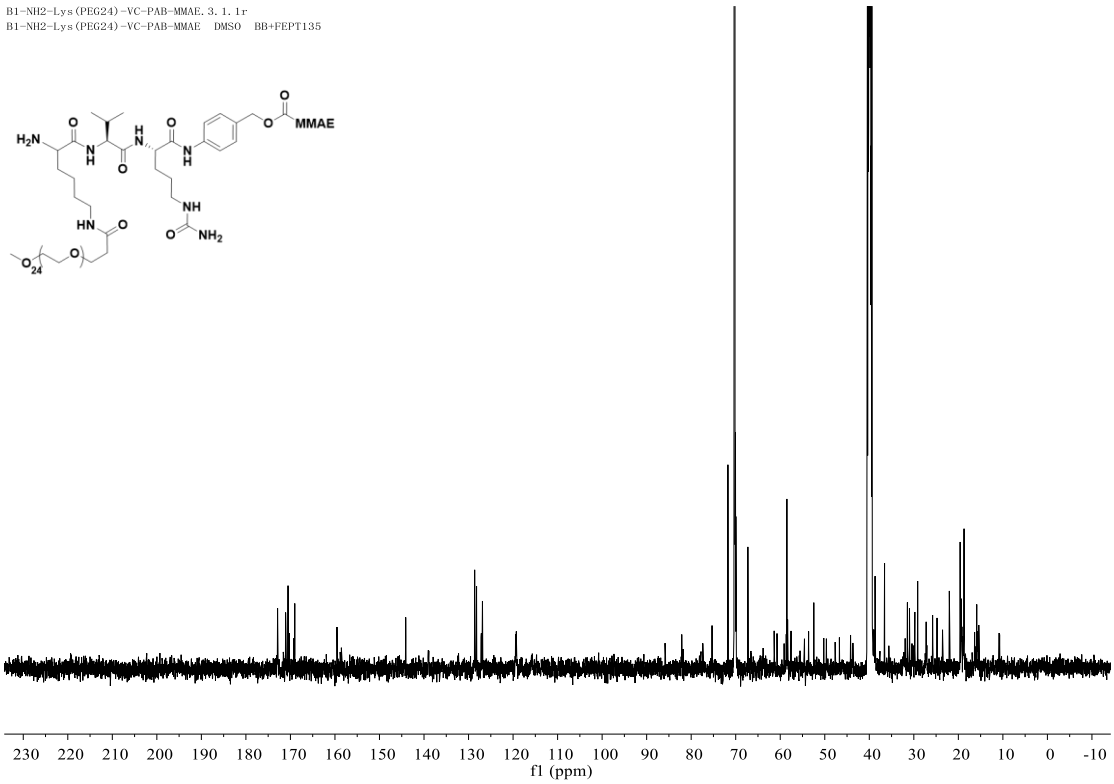
^{1}H NMR of DBCO-LacNAc-ox **5g**



^{1}H NMR of Biotin-ON=CH-LacNAc-ox **5h**

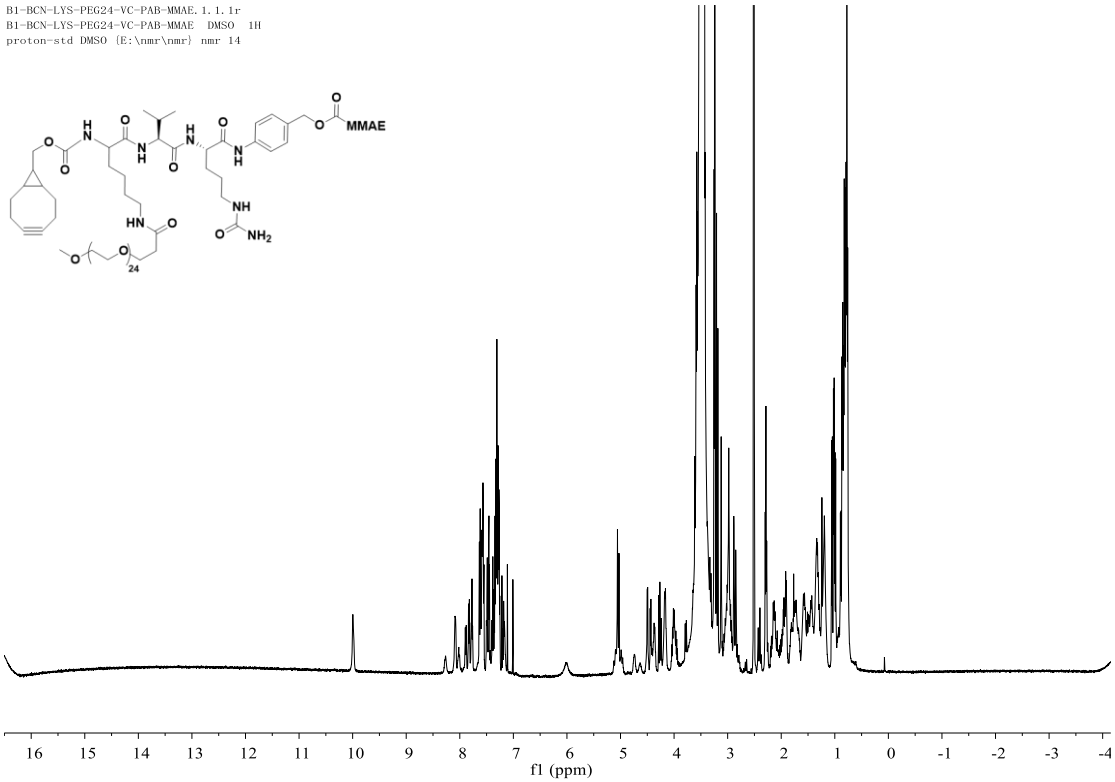


^{1}H NMR of Compound **S3**

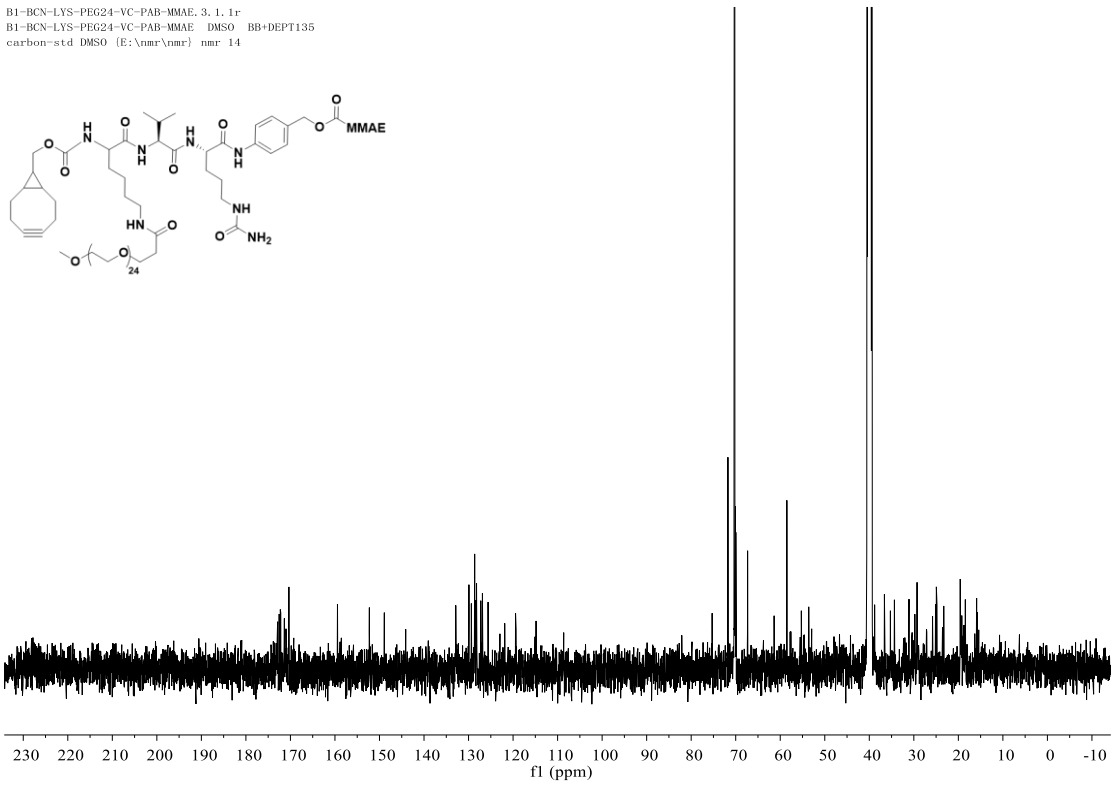


^{13}C NMR of Compound **S3**

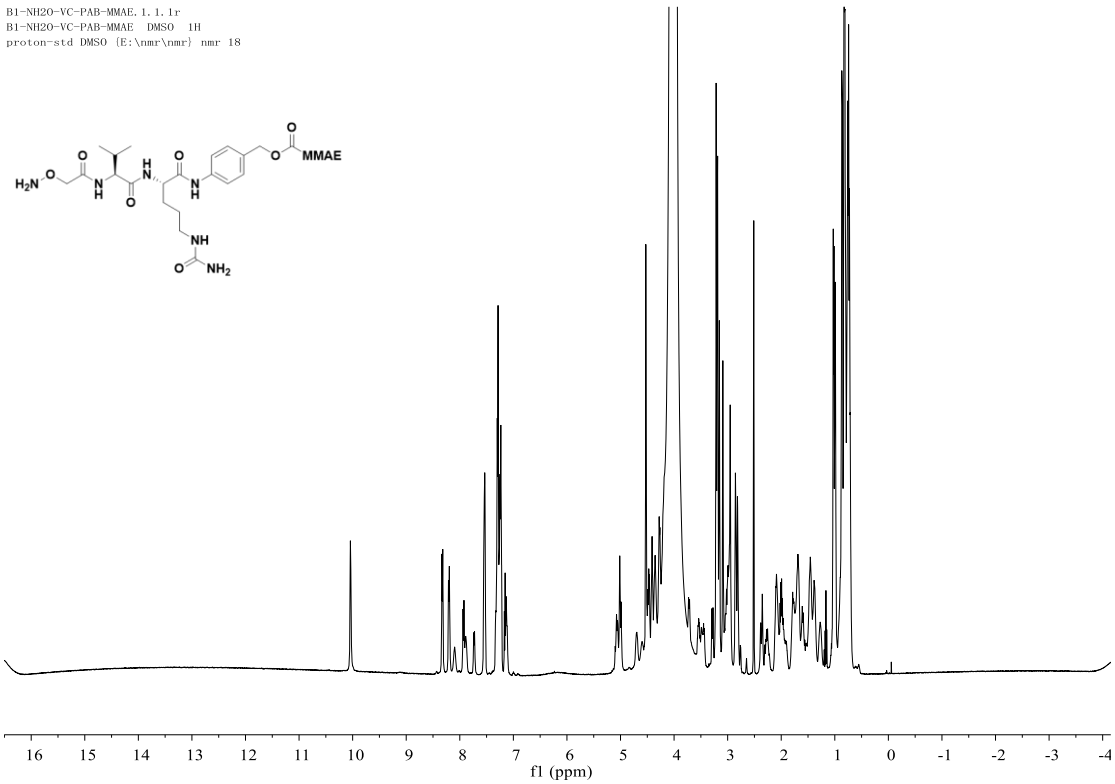
Compound **10**



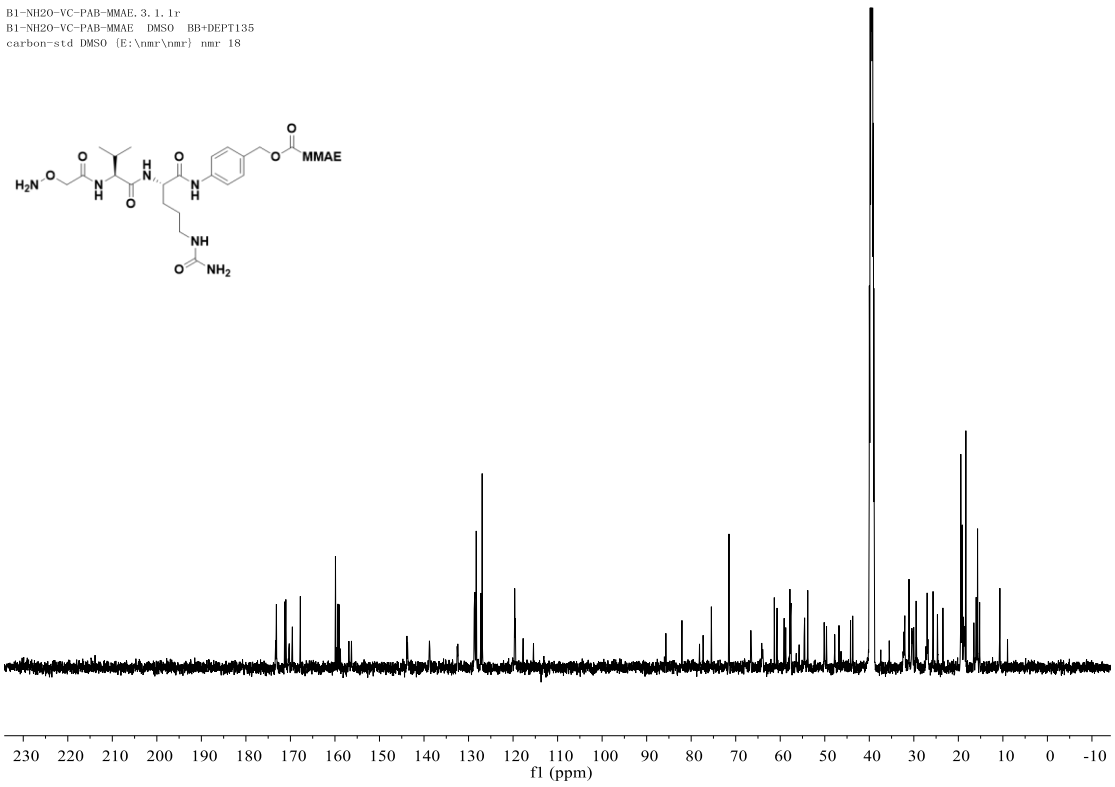
^{1}H NMR of Compound **10**



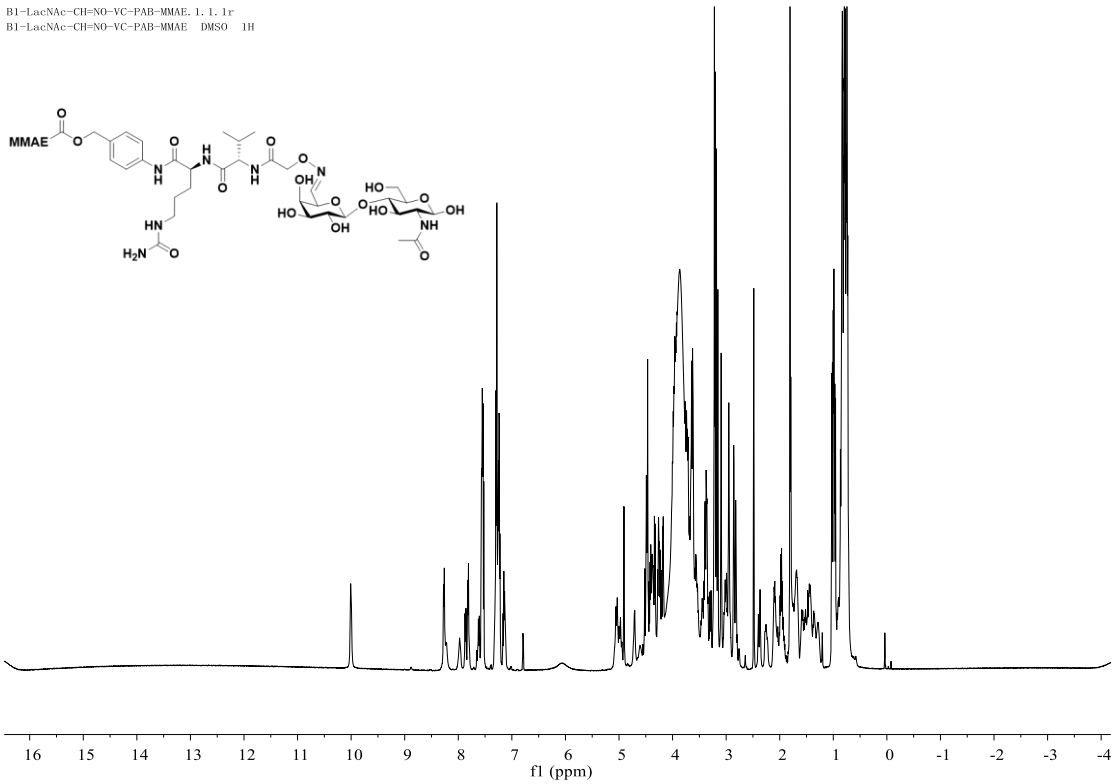
^{13}C NMR of Compound **10**



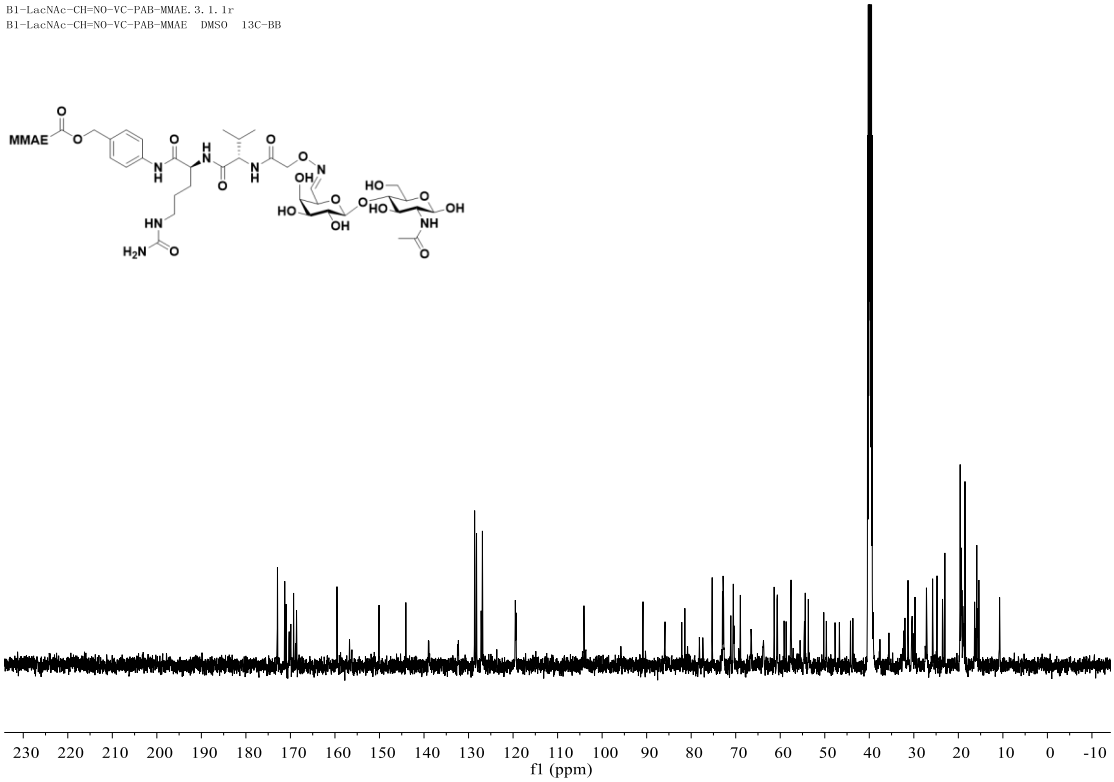
^{1}H NMR of Compound **S5**



^{13}C NMR of Compound **S5**



^{1}H NMR of Compound **S6**



^{13}C NMR of Compound **S6**

4. References

[1] K. Parikka, M. Tenkanen, *Carbohydr. Res.* **2009**, *344*, 14-20.