Supporting information for:

One-pot N-glycosylation remodeling of IgG with non-natural sialylglycopeptides enables glycosite-specific and dual-payload antibody-drug conjugates

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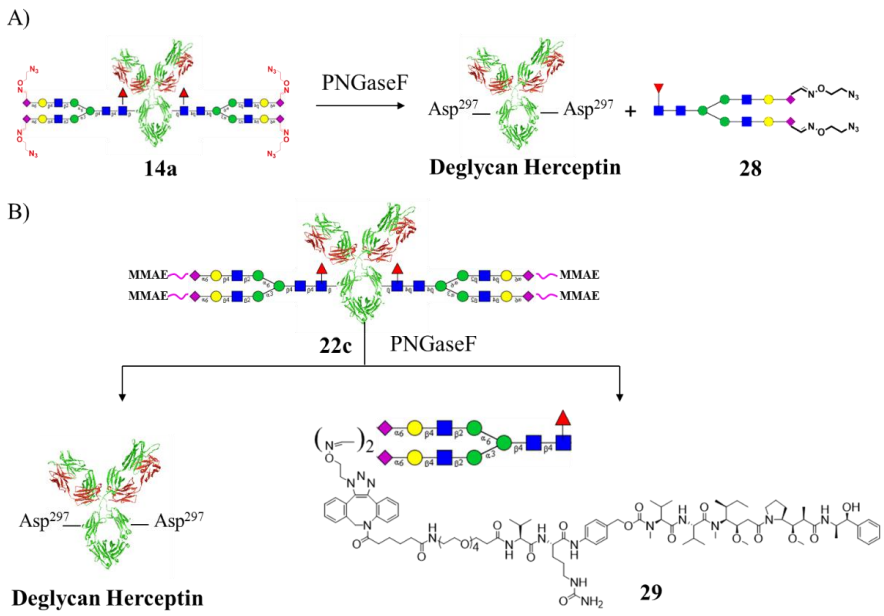
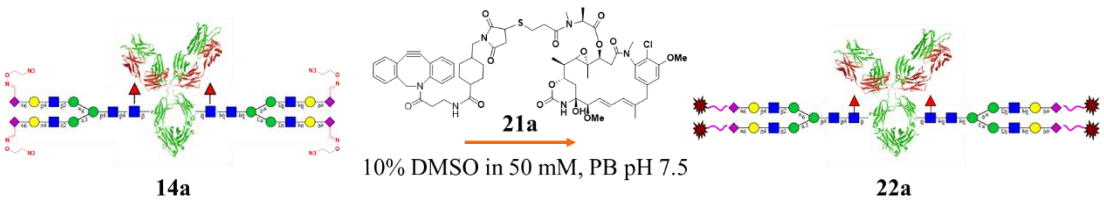
1. Supplementary schemes and figures

Scheme S1. PNGase-F digestion of **14a** and **22c**.

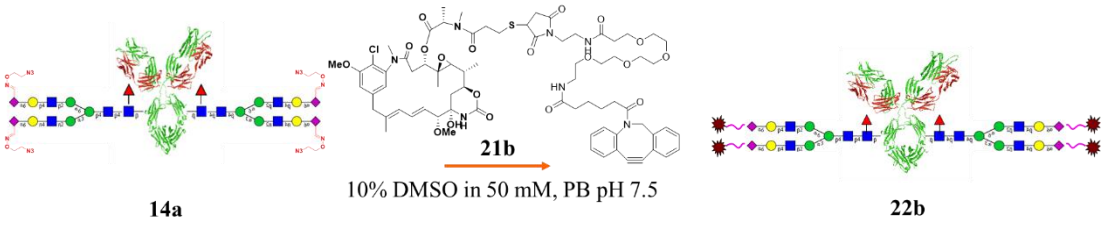


Scheme S2. Synthesis of **22a-c**

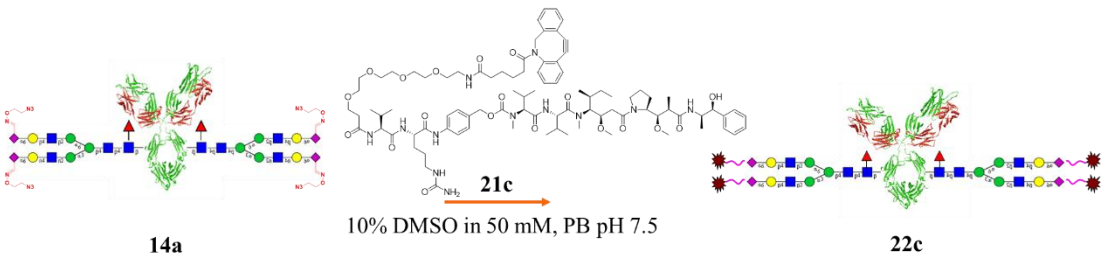
A) Synthesis of 22a



B) Synthesis of 22b

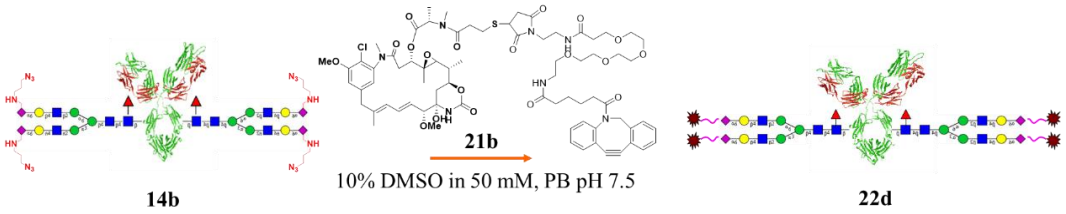


C) Synthesis of 22c

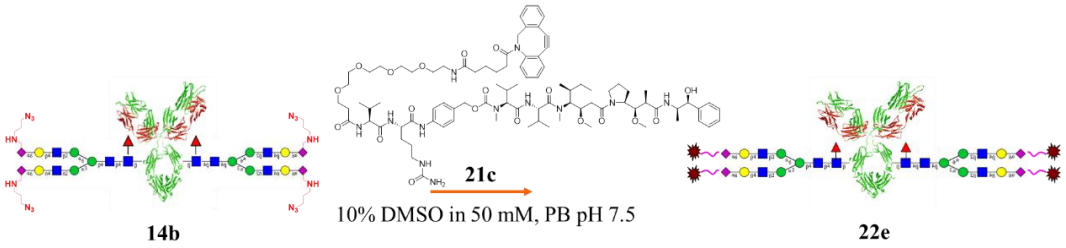


Scheme S3. Synthesis of **22d-f**

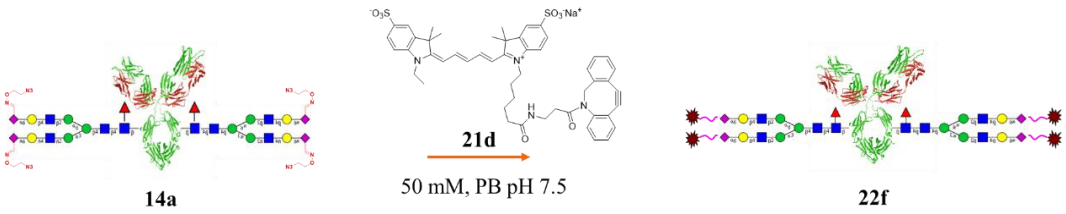
A) Synthesis of 22d



B) Synthesis of 22e

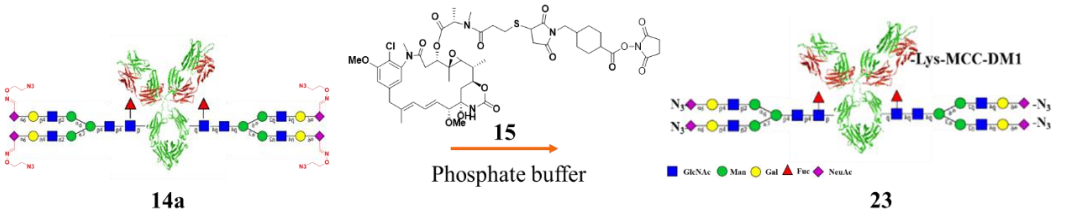


C) Synthesis of 22f

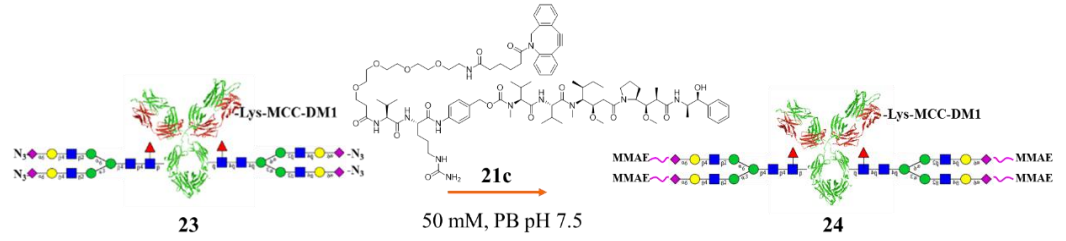


Scheme S4. Synthesis of **23-25**

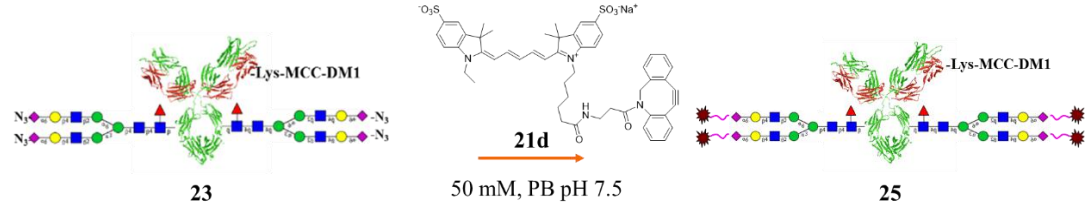
A) Synthesis of **23**



B) Synthesis of **24**



C) Synthesis of **25**



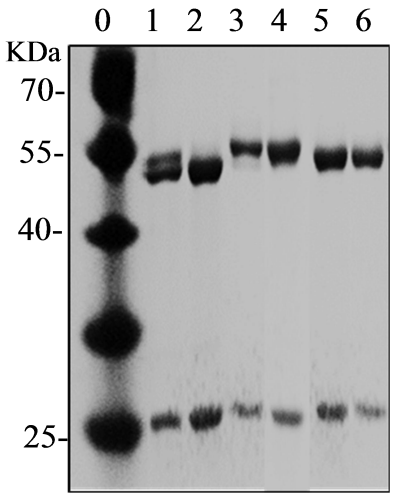


Figure S1. SDS-PAGE of glycoengineered Herceptin **7a-d**. Lane 0: Marker, Lane 1: Mixture of commercial Herceptin and Herceptin-Fucα1,6GlcNAc (**6**), Lane 2: Herceptin-Fucα1,6GlcNAc (**6**), Lane 3: S2G2F-Herceptin (**7a**), Lane 4: G2F-Herceptin (**7b**), Lane 5: G0F-Herceptin (**7c**), Lane 6: M3F-Herceptin (**7d**).

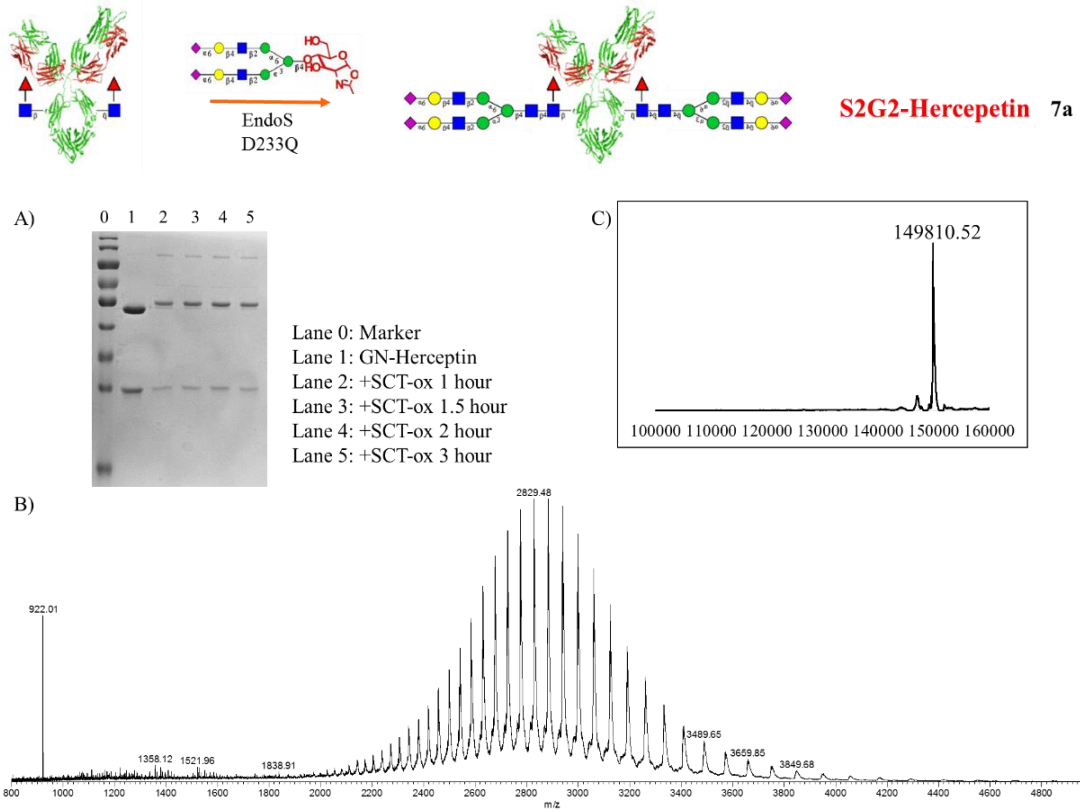


Figure S2. SDS-PAGE and LCMS profile of one-pot transglycosylation of (Fucα1,6)GlcNAc-Herceptin (**6**) with oxazoline **2**. A) SDS-PAGE of transglycosylation monitoring. B) Charged mass spectrum (*m/z*) of glycoengineered Herceptin **7a**. C) Deconvoluted mass spectrum of **7a**.

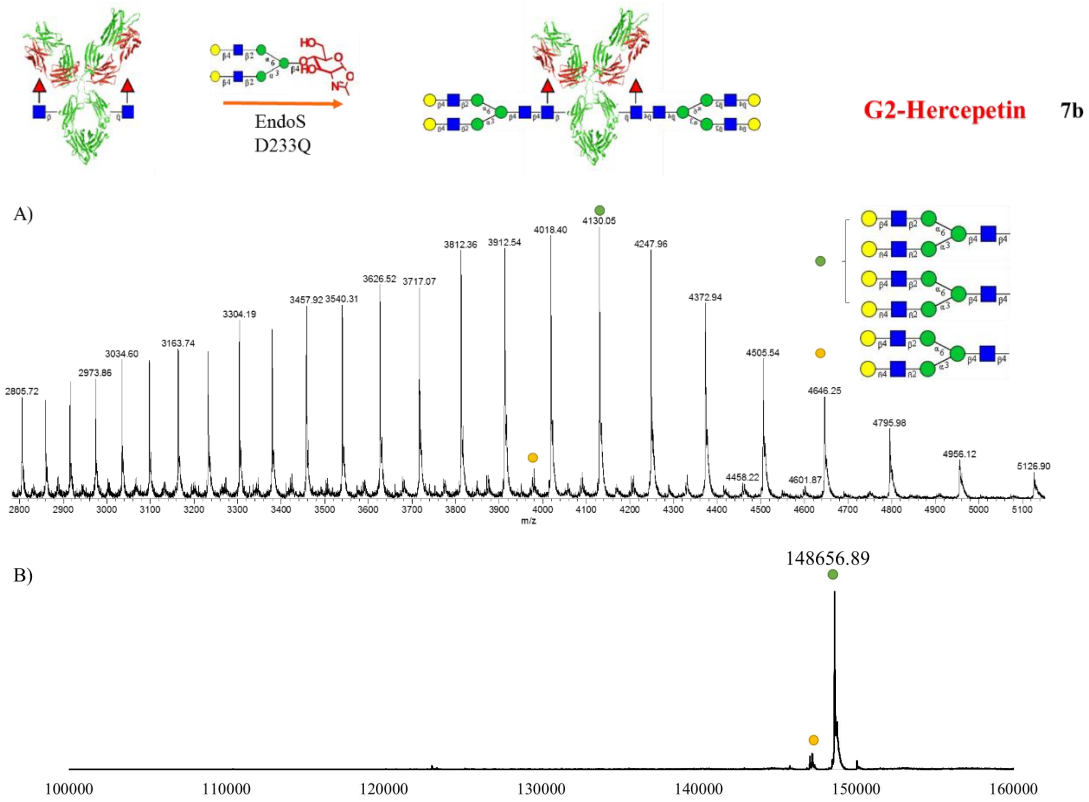


Figure S3. LCMS profile of glycoengineered Herceptin **7b**. A) The charged *m/z* spectrum of **7b**, and B) The deconvoluted spectrum of **7b**.

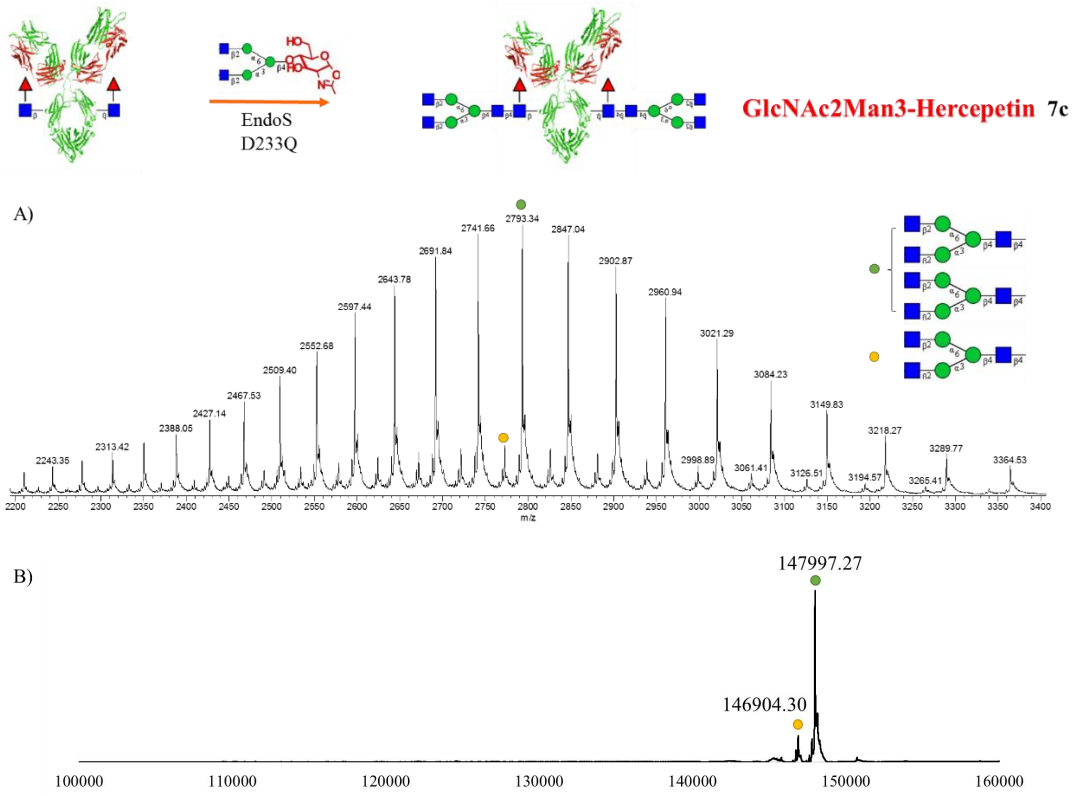


Figure S4. LCMS profile of glycoengineered Herceptin **7c**. A) The charged *m/z* spectrum of **7c**, and B) The deconvoluted spectrum of **7c**.

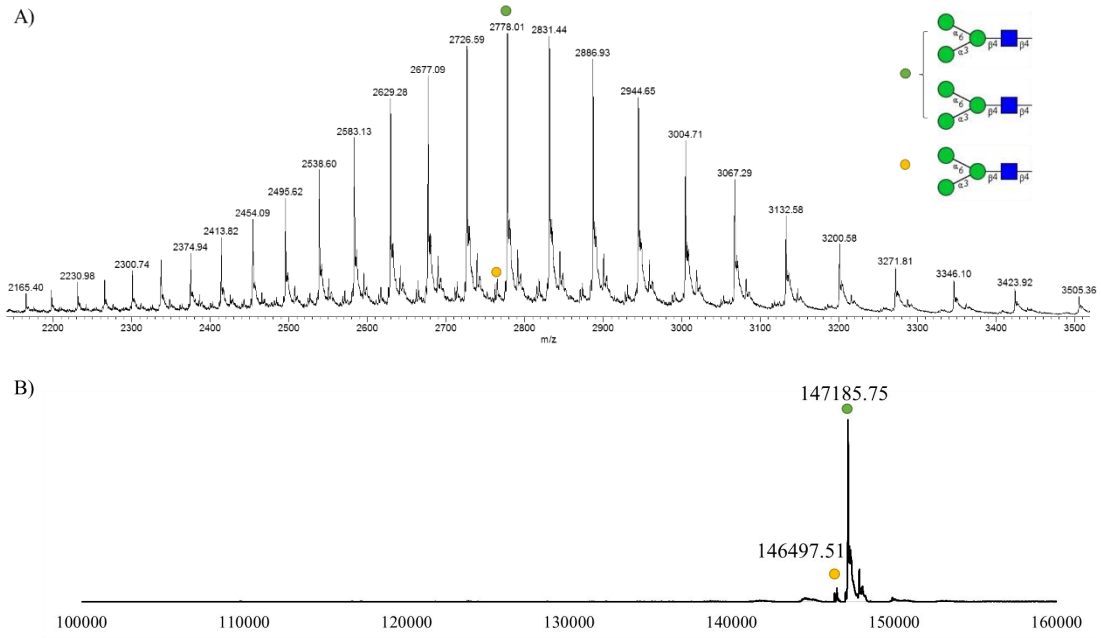


Figure S5. LCMS profile of glycoengineered Herceptin **7d**. A) The charged *m/z* spectrum of **7d**, and B) The deconvoluted spectrum of **7d**.

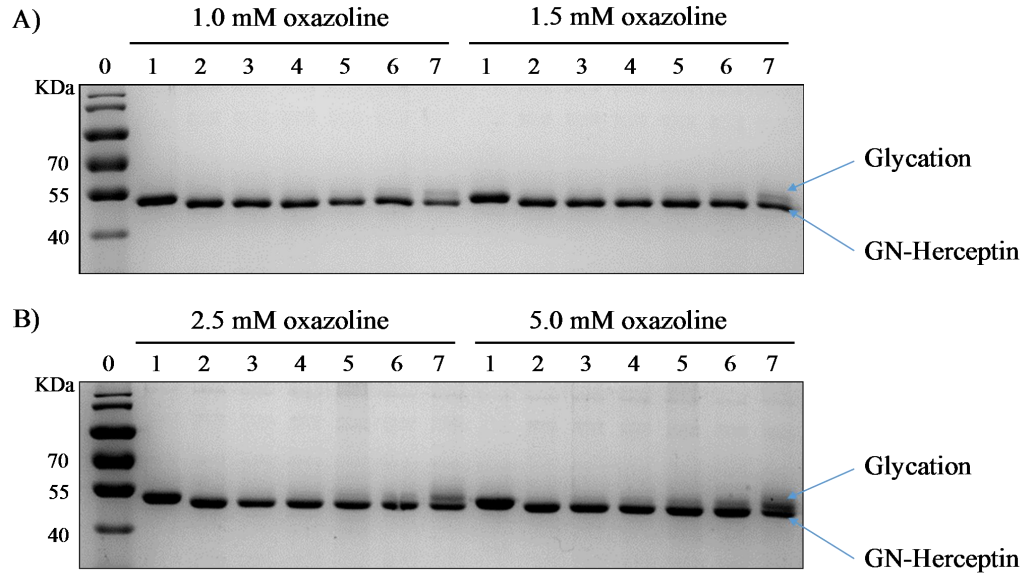


Figure S6. The SDS-PAGE analysis of non-enzymatic reaction of Herceptin-Fucα1,6GlcNAc (**6**) and oxazoline **2**.

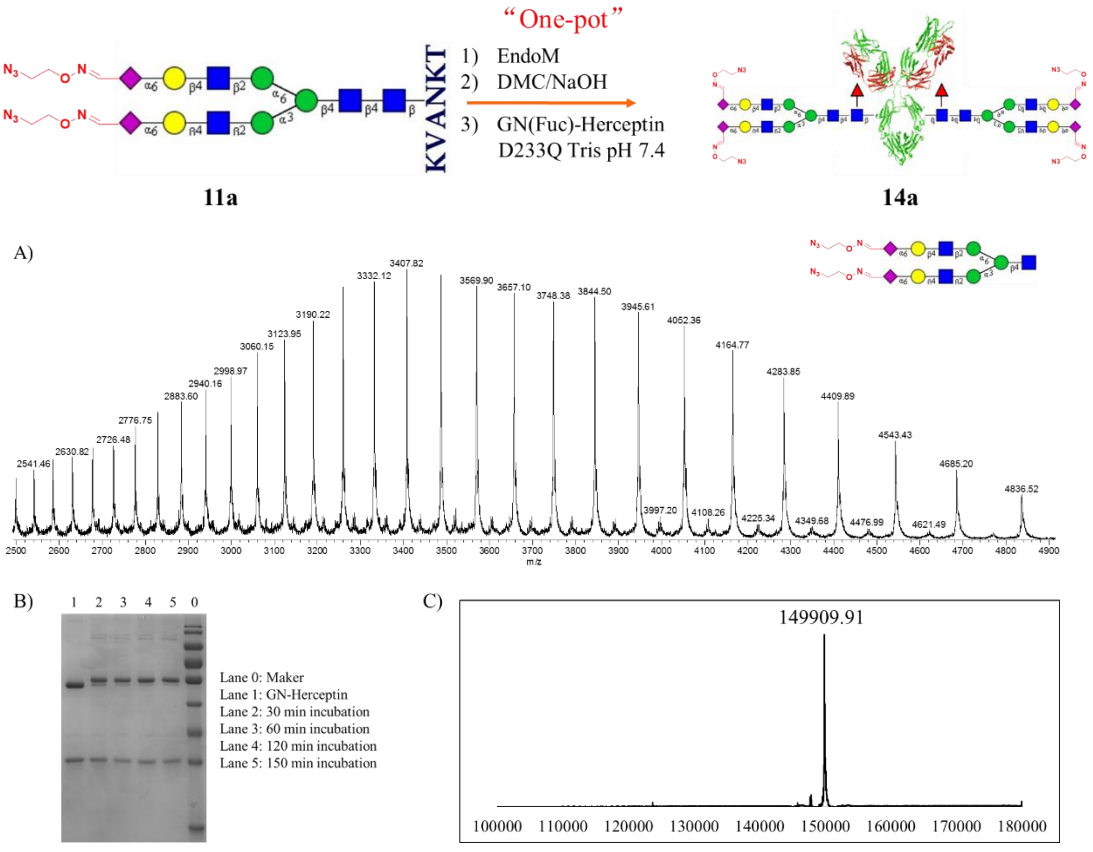


Figure S7. LCMS and SDS-PAGE profile of glycoengineered Herceptin **14a**. A) The charged *m/z* spectrum of **14a**, B) SDS-PAGE monitoring on one-pot glycosylation of **11a** and **6**, C) The deconvoluted spectrum of **14a**.

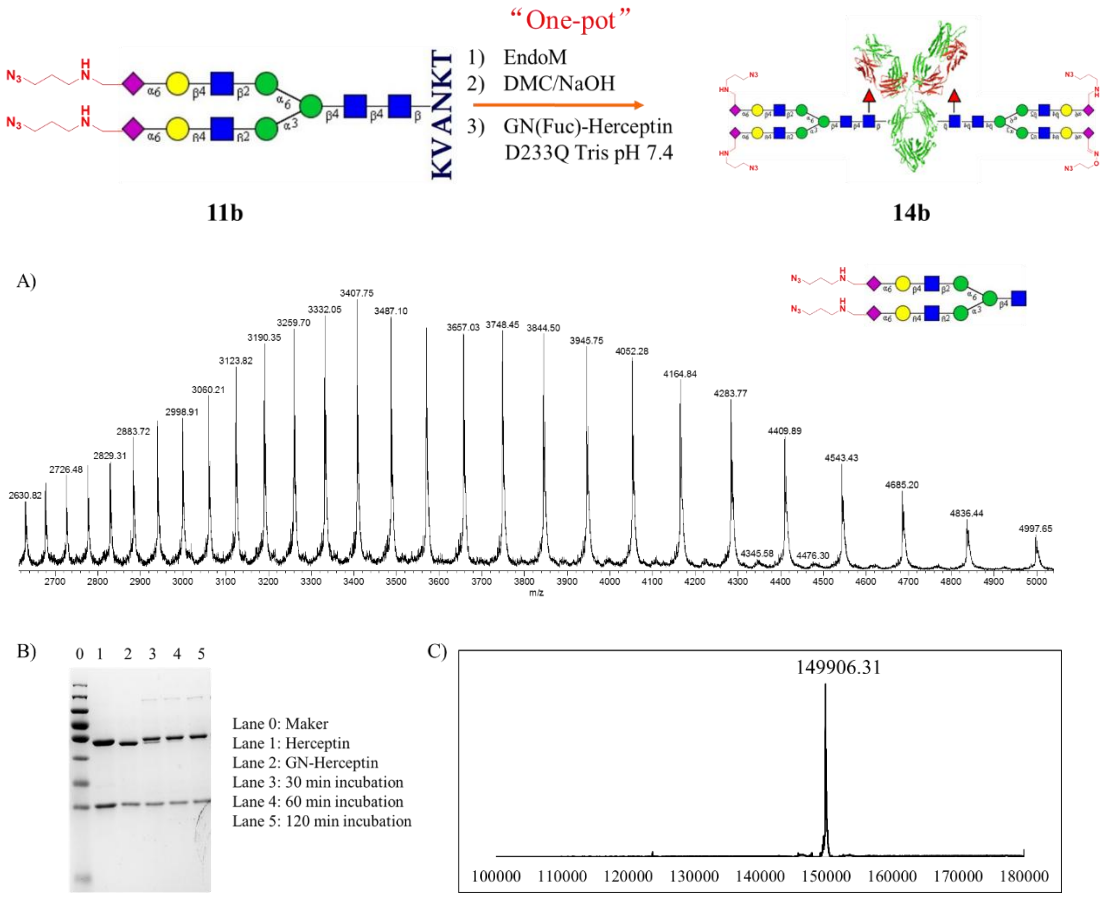
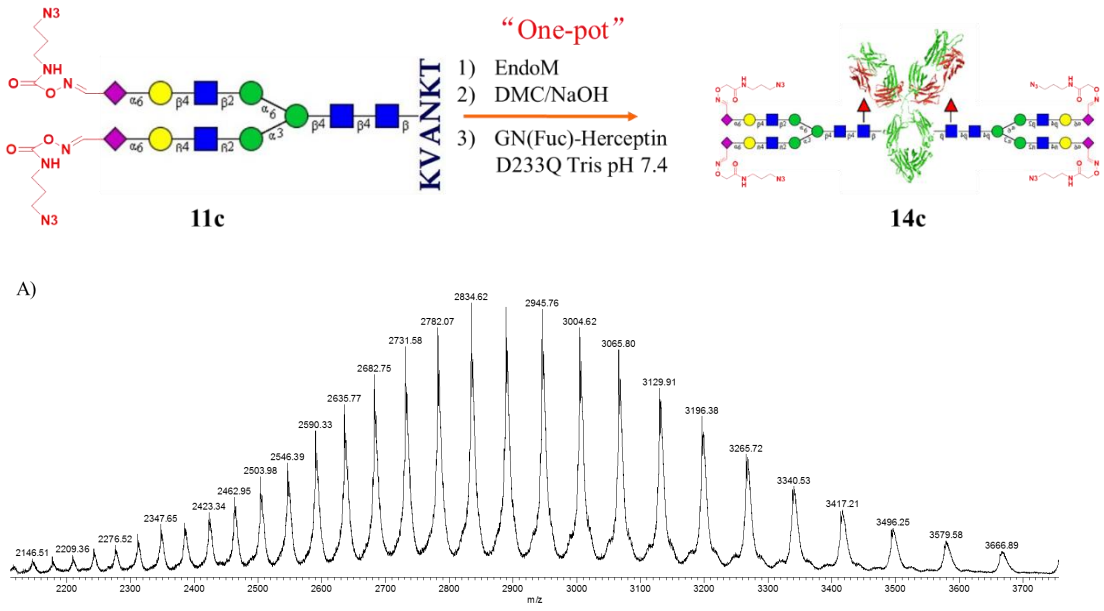


Figure S8. LCMS and SDS-PAGE profile of glycoengineered Herceptin **14b**. A) The charged *m/z* spectrum of **14b**, B) SDS-PAGE monitoring on one-pot glycosylation of **11b** and **6**, C) The deconvoluted spectrum of **14b**.



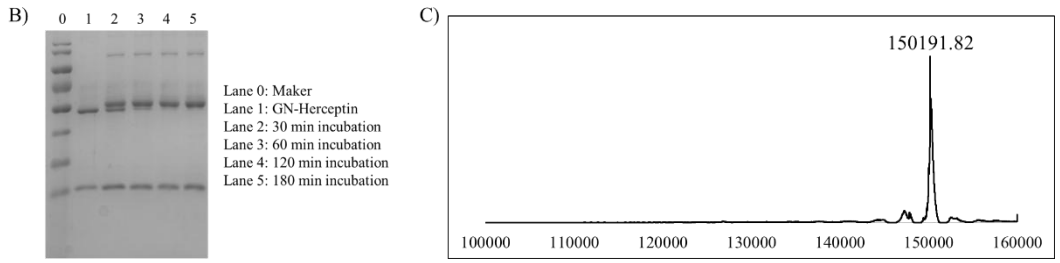


Figure S9. LCMS and SDS-PAGE profile of glycoengineered Herceptin **14c**. A) The charged *m/z* spectrum of **14c**, B) SDS-PAGE monitoring on one-pot glycosylation of **11c** and **6**, C) The deconvoluted spectrum of **14c**.

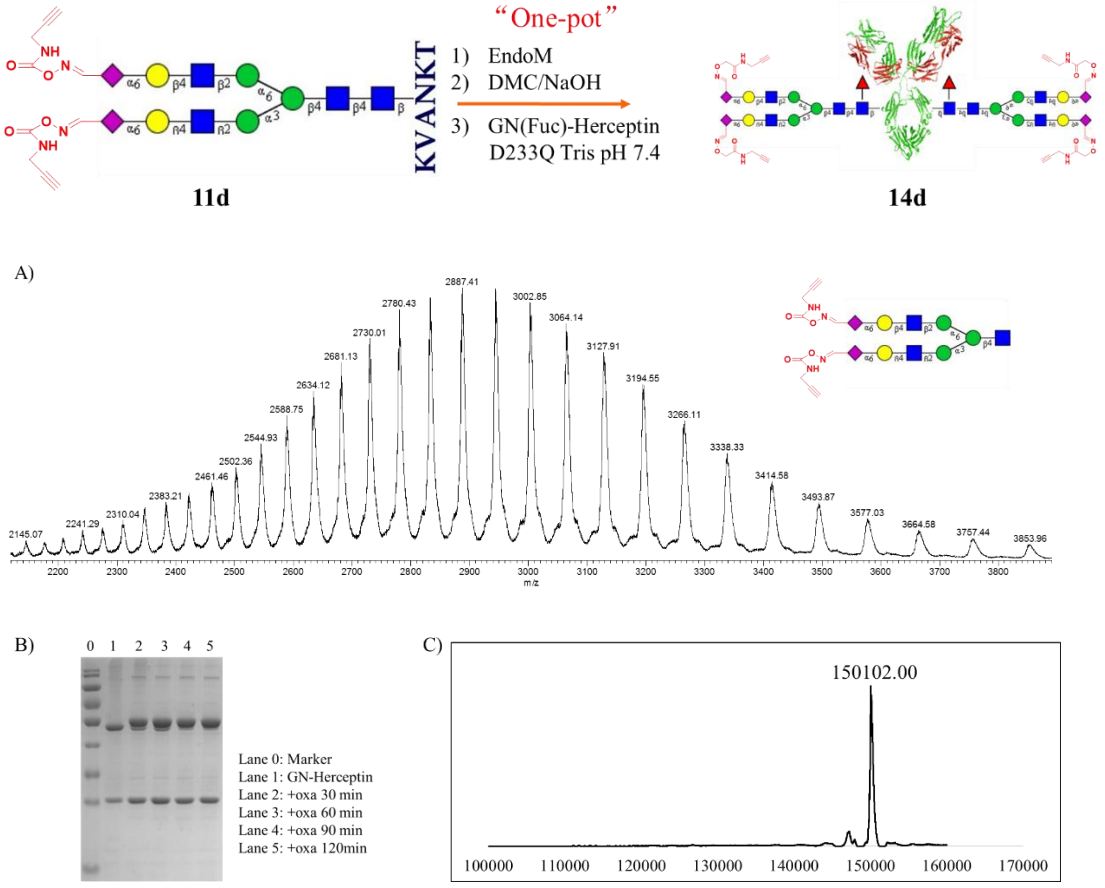


Figure S10. LCMS and SDS-PAGE profile of glycoengineered Herceptin **14d**. A) The charged *m/z* spectrum of **14d**, B) SDS-PAGE monitoring on one-pot glycosylation of **11d** and **6**, C) The deconvoluted spectrum of **14d**.

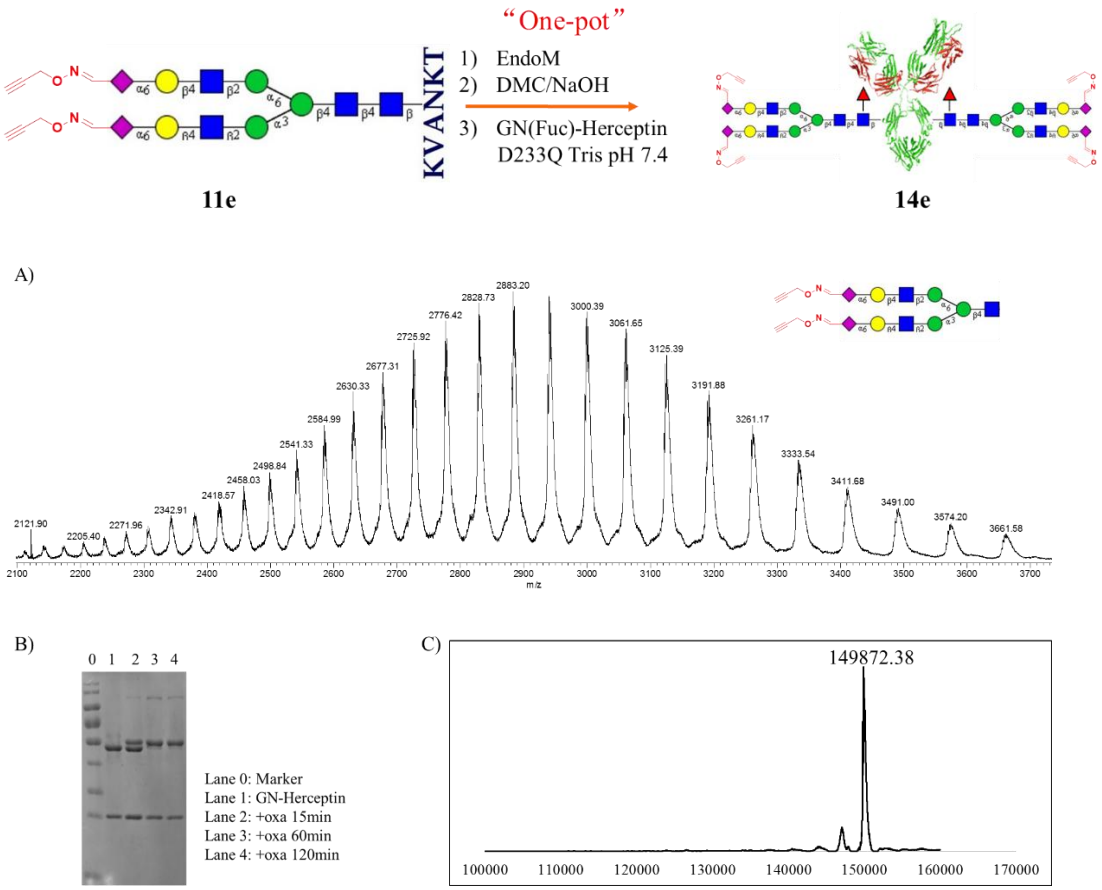


Figure S11. LCMS and SDS-PAGE profile of glycoengineered Herceptin **14e**. A) The charged *m/z* spectrum of **14e**, B) SDS-PAGE monitoring on one-pot glycosylation of **11e** and **6**, C) The deconvoluted spectrum of **14e**.

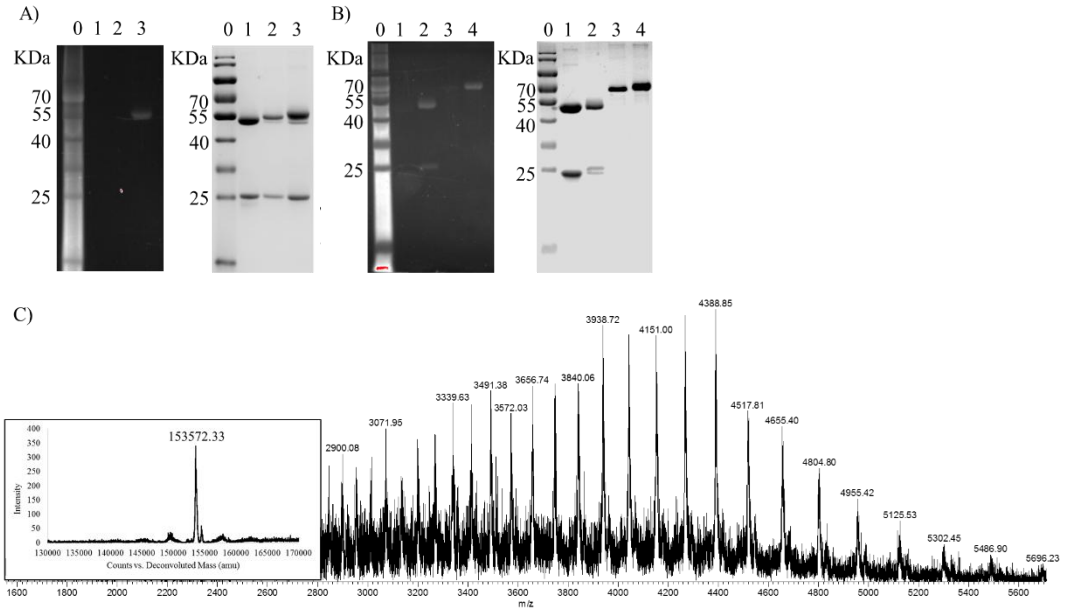


Figure S12. SDS-PAGE and MS profile of Cy5 labeling reaction with Herceptin and BSA. A) SDS-PAGE profile of glycosite-specific Cy5-labeled Herceptin **22f** stained by Fluorescence (Left) and Coomassie brilliant blue (Right). Lane 0: marker, Lane 1: Herceptin-FucαGlcNAc, Lane 2: **14a**, Lane 3: **22f**. B) SDS-PAGE profile of random labeling of Cy5 on commercial Herceptin and BSA stained by Fluorescence (Left) and Coomassie brilliant blue (Right). Lane 0: marker, Lane 1: commercial Herceptin, Lane 2: Cy5-labeled Herceptin, Lane 3: BSA, Lane 4: Cy5-labeled BSA. C) The LCMS profile of site-specific Cy5-labeled Herceptin **22f**. The insert box is the deconvoluted mass spectrum of **22f**.

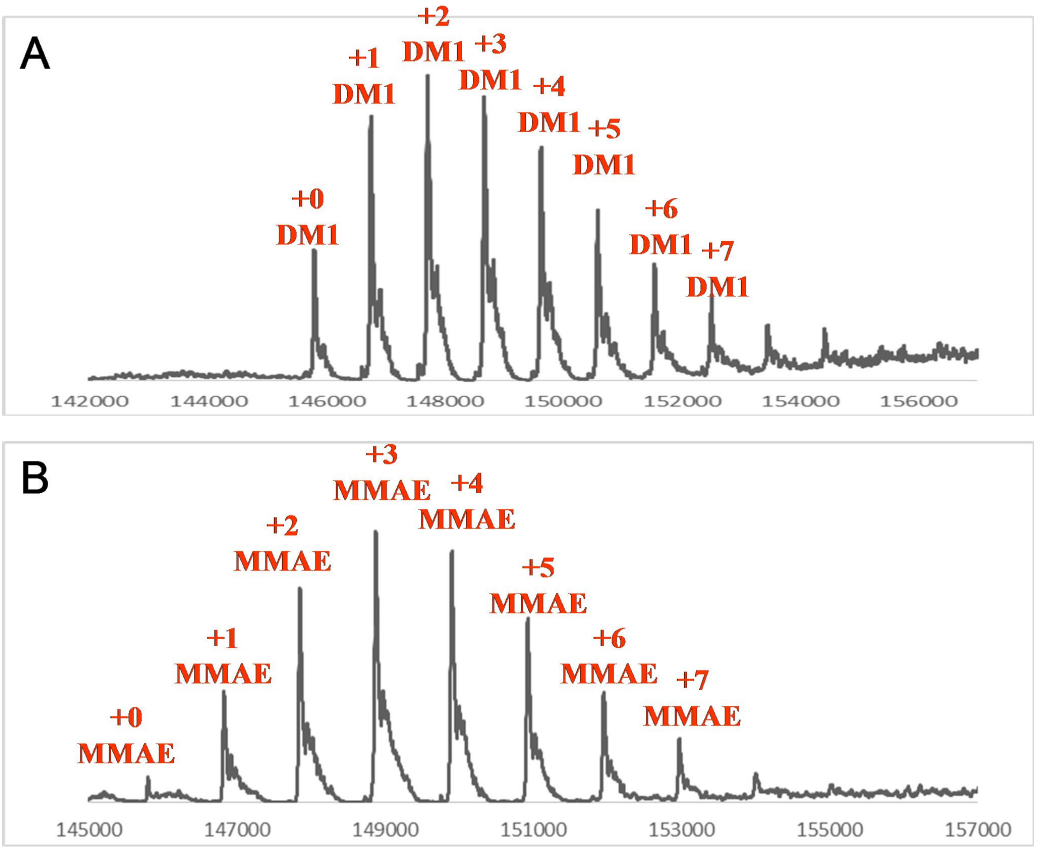
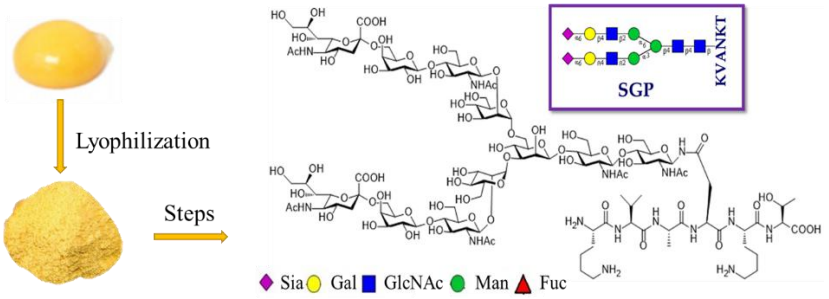


Figure S13. LCMS spectra of synthesized T-DM1 (A) and T-MMAE (B).

2. Synthesis of divergent functionalized SGPs

Extraction and purification of sialylglycopeptide (SGP, 1) from egg yolk



Isolation and purification procedure of the sialylglycopeptide (SGP) was based on our preliminary study.

Briefly, the lyophilized egg yolk powder (1.2 Kg) was washed with methyl tertiary butyl ether (4 L, twice) and 70% acetone (4 L, twice) respectively. The 40% acetone (4 L x 2) solution was combined and concentrated to a small volume before subjected to the active carbon/celite (1:1) column which had been pre-washed with 50% acetonitrile and the following pure water. The column was eluted with pure water, 5% acetonitrile solution, 10% acetonitrile solution, 20% acetonitrile and 30% acetonitrile respectively. The 20% acetonitrile part which containing the corresponding product SGP indicated by HPLC and LCMS was concentrated to a small volume before subjected to Sephadex G-25 gel column. The column was eluted with 0.1 M acetate acid solution and the fractions containing the products was combined and lyophilized to get a white powder. Finally, the white powder was further purified with preparative RP-HPLC preparative column and the corresponding product was gathered and lyophilized to get a white powder (1.1 g).

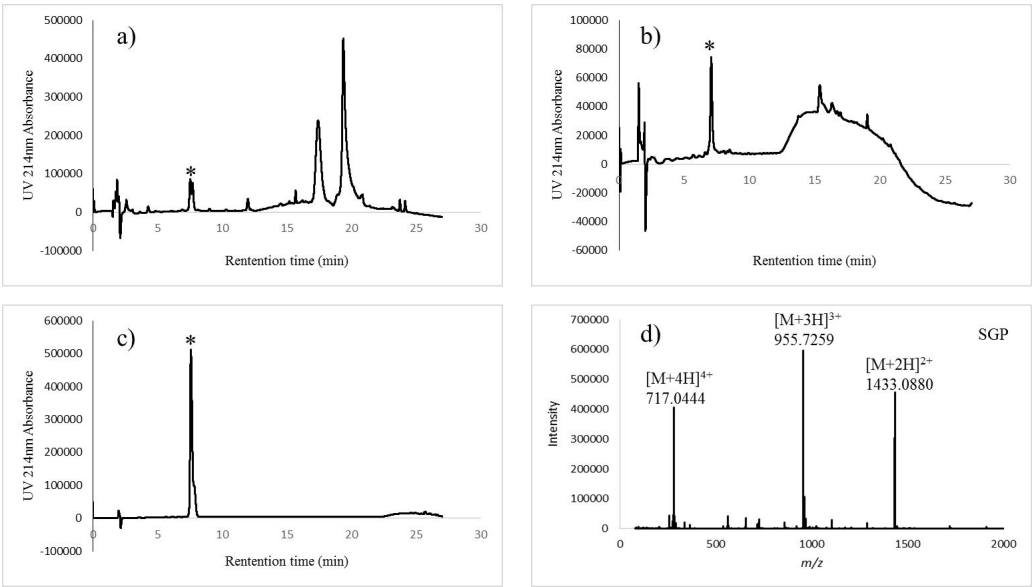


Figure S14. HPLC and HRMS profiles (HPLC method B) of SGP (marked with *) extraction and purification. (a) Crude solution after 40% acetone extraction. (b) Crude 20% acetonitrile solution

after active carbon extraction. (c) Pure SGP after Sephadex G-25 and preparative RP-HPLC column purification. (d) HRMS profile of pure SGP. The corresponding multiple charged peaks of SGP has been labeled above the peaks. Calcd for $[M+4H]^{4+}$ 717.0501, found 717.0444; $[M+3H]^{3+}$ 955.7308, found 955.7259; $[M+2H]^{2+}$ 1433.0923, found 1433.0880.

Oxidation of SGP to give aldehyde-SGP (8)

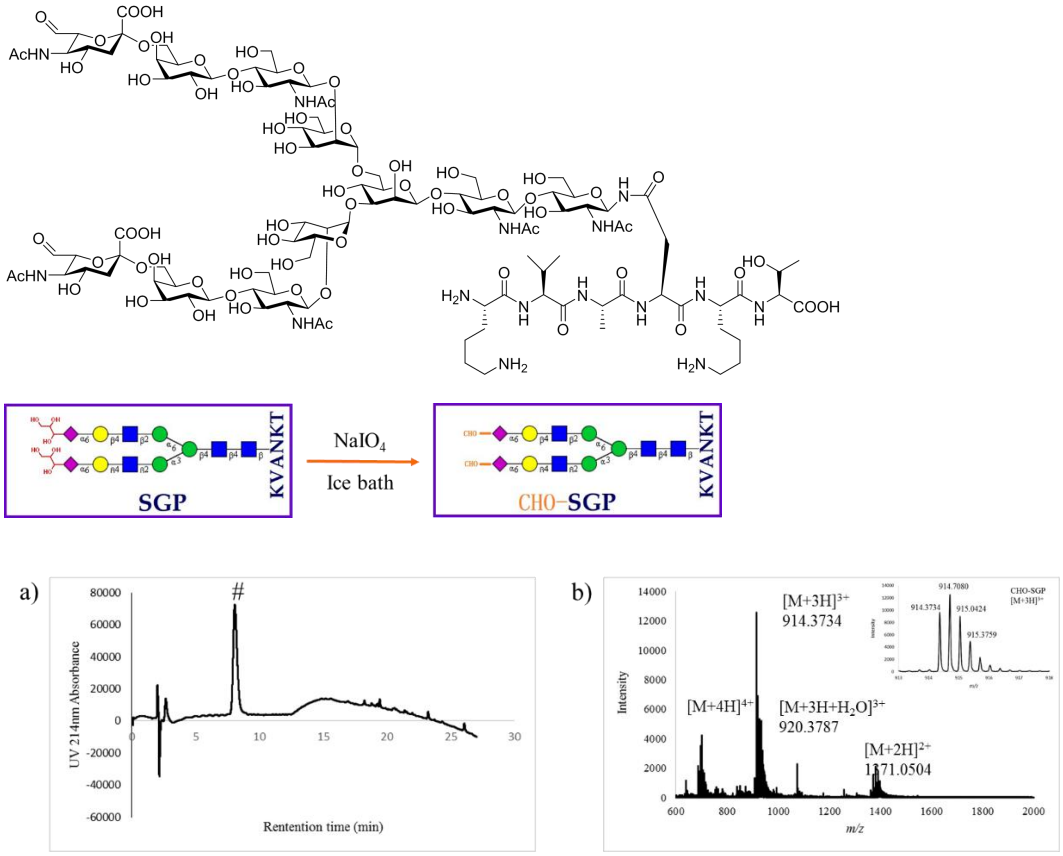


Figure S15. HPLC and HRMS profiles (HPLC method B) of the SGP oxidation reaction. a) The reaction was monitored by RP-HPLC at 15 min. The oxidative SGP was marked with # above the peak. b) The HRMS data of the corresponding peak in figure a. The multiple charged peak has been marked.

Synthesis of different functionalized SGPs with 10a-d.

Synthesis of azido-SGP with O-(2-azidoethyl) hydroxylamine hydrochloride (10a) to give 11a.

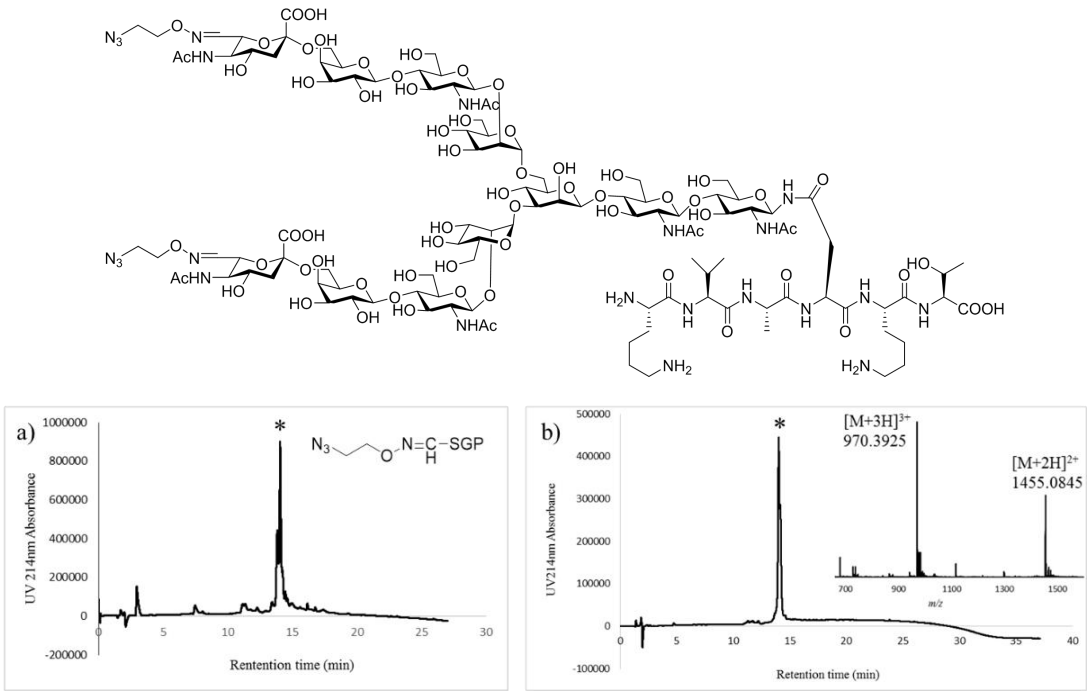


Figure S16. HPLC and LCMS profiles (HPLC method B) of CHO-SGP **8** and O-(2-propynyl)-hydroxylamine hydrochloride **10a** reaction. a) Aliquots removed from reaction mixture at 2 hour. The corresponding product was labeled with *. b) The HPLC profile of the product after preparative column purification. The insert is the HRMS profile of pure product.

Synthesis of azido-SGP with 2-Aminooxy-N-(3-azidopropyl)acetamide (10c) to give 11c

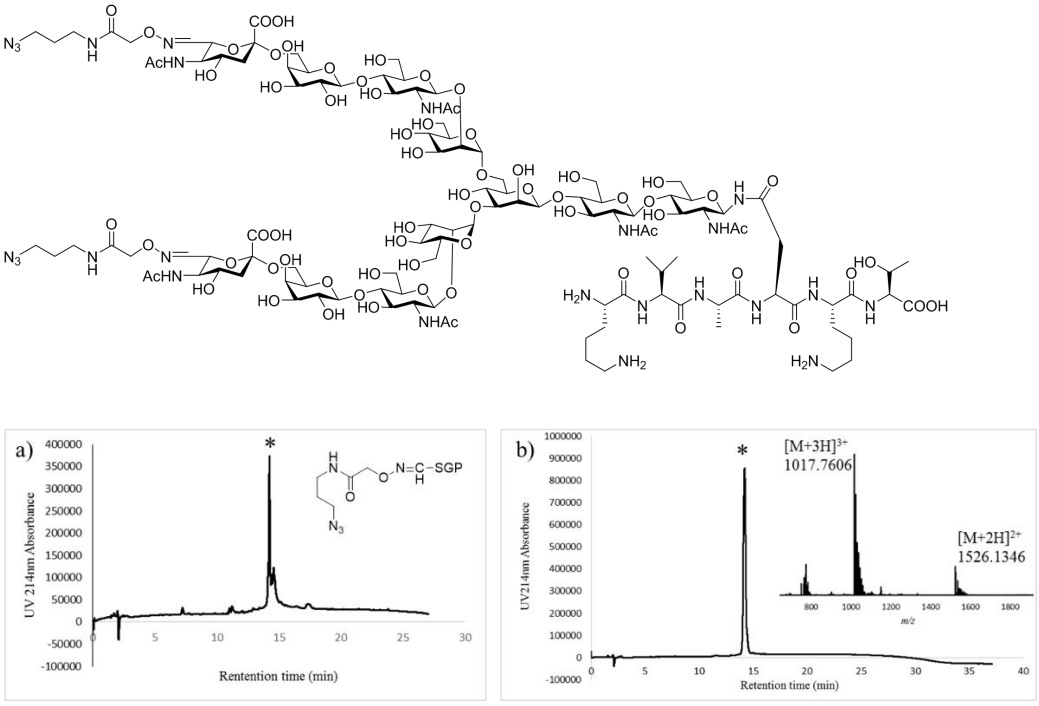


Figure S17. HPLC and LCMS profiles (HPLC method B) of CHO-SGP **8** and

O-(2-propynyl)-hydroxylamine hydrochloride **10c** reaction. a) The samples removed from reaction mixture at 2 hour. The corresponding product was labeled with *. b) The HPLC profile of the product after preparative column purification. The insert is the HRMS profile of pure product.

Synthesis of alkyne-SGP with 2-azanyloxy-N-prop-2-ynyl-ethanamide trifluoroacetic acid salt (10d) to give 11d

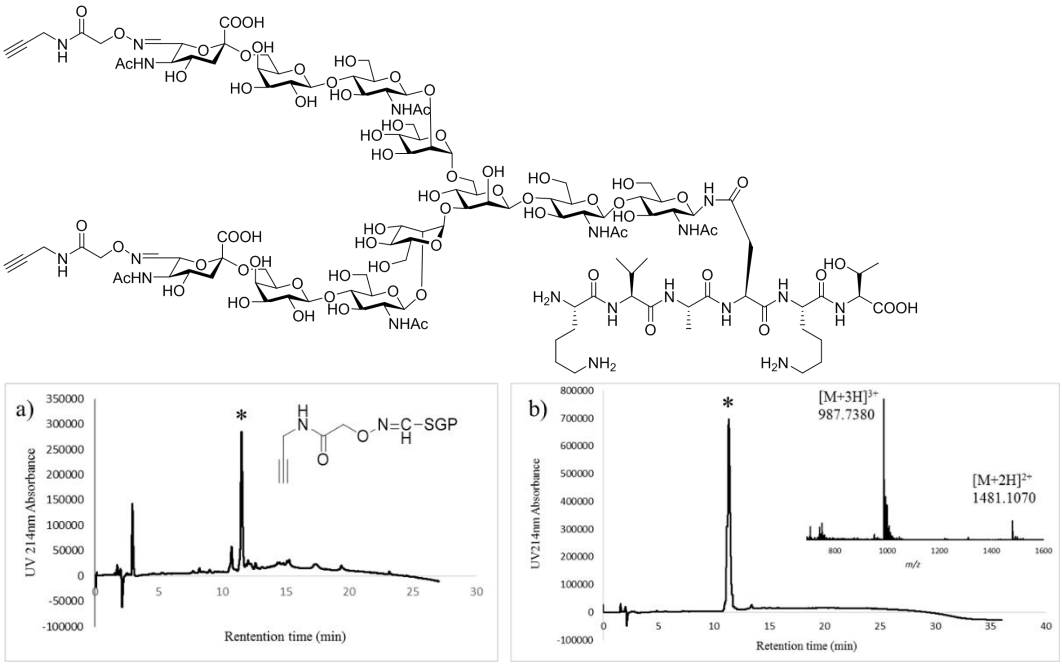
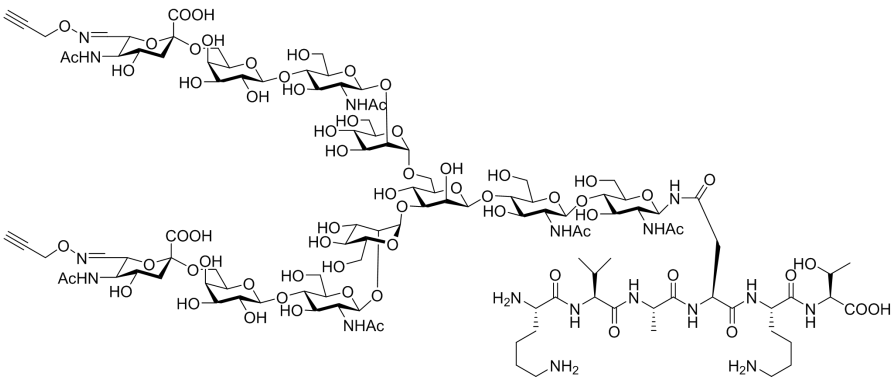


Figure S18. HPLC and LCMS profiles (HPLC method B) of CHO-SGP **7** and **M4** reaction. a) The samples removed from reaction mixture at 2 hour. The corresponding product was labeled with *. b) The HPLC profile of the product after preparative column purification. The insert is the HRMS profile of pure product.

Synthesis of alkyne-SGP with O-(2-propynyl)-hydroxylamine hydrochloride (10b) to give 11e



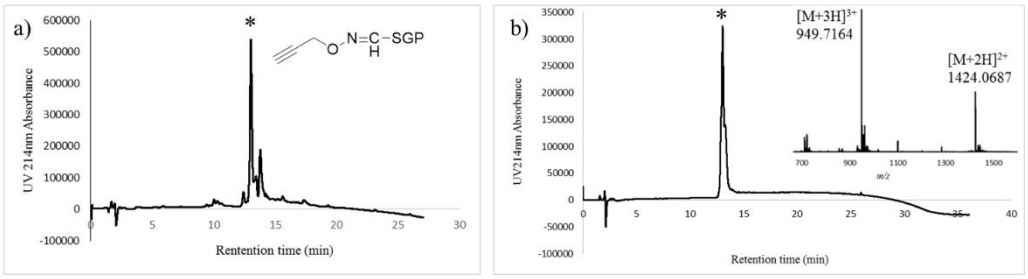


Figure S19. HPLC and LCMS profiles (HPLC method B) of CHO-SGP **8** and O-(2-propynyl)-hydroxylamine hydrochloride **10b** reaction. a) The samples removed from reaction mixture at 2 hour. The corresponding product was labeled with *. b) The HPLC profile of the product after preparative column purification. The insert is the HRMS profile of pure product.

Reductive amination of CHO-SGP (8)

Reductive amination of CHO-SGP (8) with 3-azido-1-propanamine to give 11b

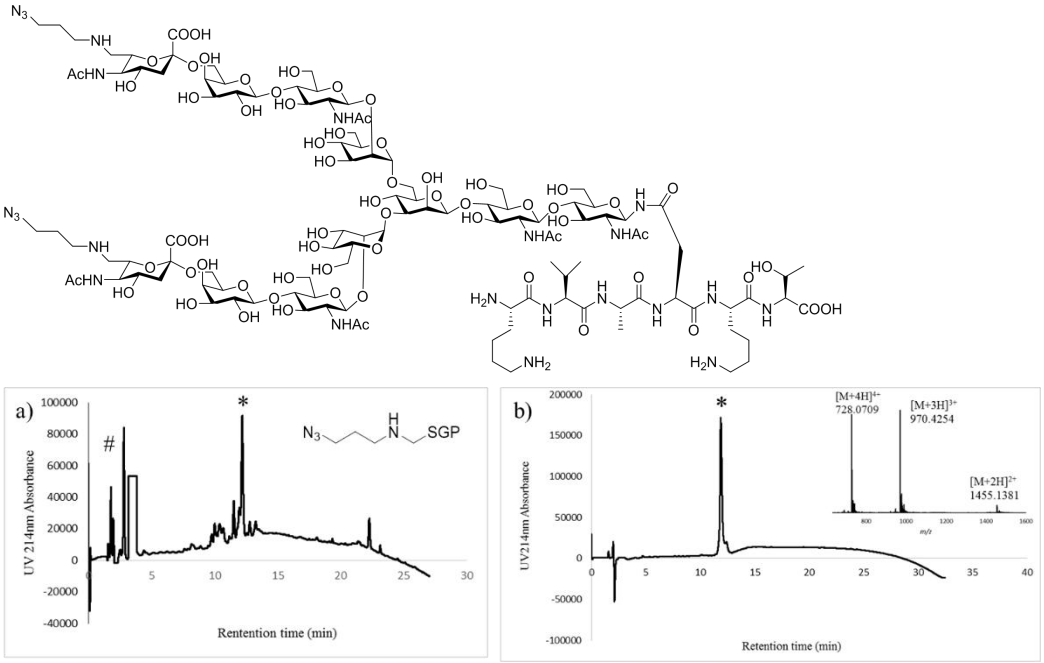


Figure S20. HPLC and LCMS profiles (HPLC method B) of reductive amination reaction with 3-azido-1-propanamine. a) Aliquots removed from reaction mixture at 3 hour. The corresponding product was labeled with *. b) The HPLC profile of the product after preparative column purification. The insert is the HRMS profile of pure product.

Reductive amination of CHO-SGP (8) with propargylamine to give 11f

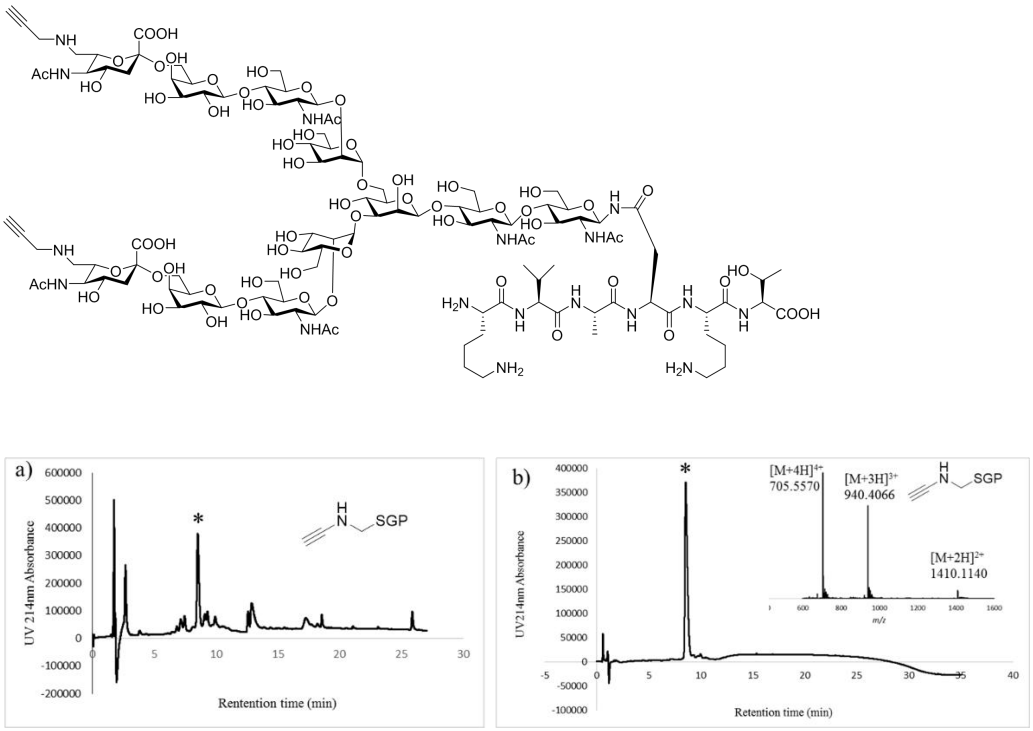
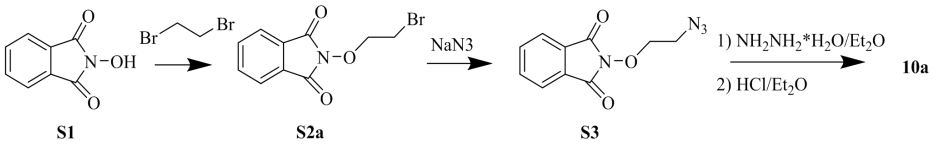


Figure S21. HPLC and LCMS profiles (HPLC method B) of reductive amination reaction with propargylamine. a) The samples removed from reaction mixture at 3 hour. The corresponding product was labeled with *. b) The HPLC profile of the product after preparative column purification. The insert is the HRMS profile of pure product.

3. Synthesis of small molecules and drug-payloads

Synthesis of small molecule

Synthesis of O-(2-azidoethyl)-hydroxylamine hydrochloride (10a)



S1 S2a S3

Synthesis of N-(2-bromoethoxy)-phthalimide (S2a) [1]

1.14 g, 7.0 mmol N-Hydroxyphthalimide **S1** (1.0 eq) was dissolved in 8.0 mL DMF and 3.0 mL 34.8 mmol 1,2-dibromoethane (~5.0 eq) was added to the solution dropwise followed by 2.0 mL, 14 mmol triethylamine. The reaction was stirred at room temperature protecting from light and monitored by TLC (EA/PE=1:1, Rf_1=0.5 and Rf_{2a}=0.85). The solid in the reaction mixture was filtered and washed with DMF twice when the TLC indicated the complete assumption of the starting material. Most of DMF was evaporated and the residue was precipitated into excess water. The solid was gathered and wash with water twice. The precipitate was removed after that the crude product was redissolved in 5.0 mL EA. The solution was washed with 1 N HCl, water and brine respectively and dried over anhydrous Na_2SO_4. Finally the solution was concentrated to give the product **S2a** (977.4 mg, yield 52.0 %). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.89 (m, 2H ArH), 7.80 (m, 2H ArH), 4.51 (t, 2H), 3.67 (t, 2H). HRMS Calcd for $[M+Na]^+$ 291.9585, found $[M+Na]^+$ 291.9569.

Synthesis of N-(2-azidoethoxy)-phthalimide (S3) [2]

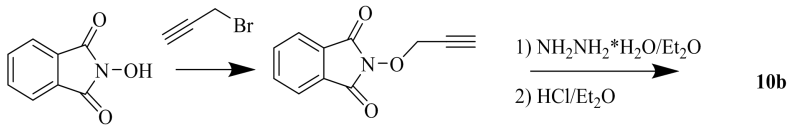
The material **S2a** (500 mg, 1.85 mmol, 1.0 eq) was dissolved in 10.0 mL acetone and to solution was added sodium azido (360 mg, 5.55 mmol, 3.0 eq) which was pre-dissolved in 2.0 mL water at an ice bath. The reaction was heated to 60 ^{o}C after stirring at the ice bath for 30 min. The reaction was monitored by TLC (EA/PE=1:2). 30.0 mL DCM was added to dilute the reaction mixture and after that the solution was washed with water and brine respectively. Then it was dried over

anhydrous Na_2SO_4 and finally concentrated under reduced pressure to afford **S3** as a white solid (184 mg, 1.46 mmol. Yield 79.0 %). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.89 (m, 2H), 7.80 (m, 2H), 4.38 (t, 2H), 3.69 (t, 2H). HRMS Calcd for $[M+Na]^+$ 255.0494, found $[M+Na]^+$ 255.0476.

Synthesis of O-(2-azidoethyl)-hydroxylamine hydrochloride (10a) [3]

To a well-stirred solution of **S3** (103 mg, 0.45 mmol, 1.0 eq) was added hydrazine hydrate (45 μL, 0.67 mmol, 1.5 eq) and the solid-hydrazine hydrate mixture was stirred at room temperature for 30 min followed by another 2 hours in 20 mL ether. The color turned from yellow solid to snow white solid in ether. The solid was gather with centrifugation and washed with ether twice. The organic part was combined and stirred with 1 N HCl in dioxane (2.0 eq) for another 2 hours. The mixture was centrifuged to get a white solid M1. ^{1}H NMR (400 MHz, DMSO-d6) δ 11.05 (s, 3H), 4.15 (q, J = 5.2, 4.4 Hz, 2H), 3.59 (t, J = 4.4 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d6) δ 73.42, 48.77. ESI-HRMS Calcd for $[M+H]^+$ 103.0614, found $[M+H]^+$ 103.0617.

Synthesis of O-(2-propynylethyl)-hydroxylamine hydrochloride (10b)



S1 **S2b**

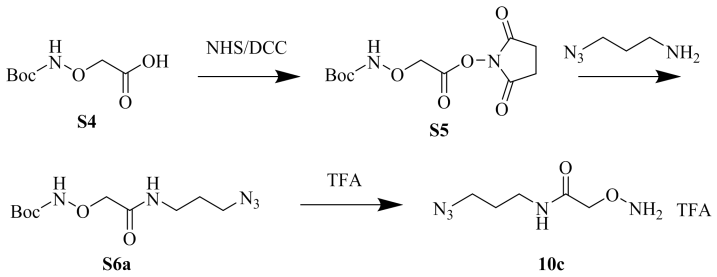
Synthesis of N-(2-propynylethoxy)-phthalimide (S2b)

The procedure was same to the synthesis of N-(2-bromoethoxy)-phthalimide (**S2a**)

Synthesis of O-(2-propynylethyl)-hydroxylamine hydrochloride (10b)

The procedure was same to the synthesis of O-(2-azidoethyl)-hydroxylamine hydrochloride (**10a**). ^{1}H NMR (400 MHz, DMSO-d6) δ 11.07 (s, 3H), 4.73 (dd, J = 5.5, 2.2 Hz, 2H), 3.86 (t, J = 2.4 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d6) δ 81.51, 76.99, 62.27. ESI-HRMS Calcd for $[M+H]^+$ 72.0444, found $[M+H]^+$ 72.0451.

Synthesis of 2-Aminooxy-N-(3-azidopropyl)acetamide (10c)



Synthesis of 2-(Boc-aminooxy)-N-hydroxysuccinimide Ester (S5) [4]

N-Boc-aminooxyacetic acid (1.0 g 5.23 mmol 1.0 eq) was dissolved in 20 mL DCM and cooled with an ice bath. To the stirred solution was added N-hydroxysuccinimide (NHS, 662.5 mg, 5.75 mmol, 1.1 eq) followed by N, N'-dicyclohexylcarbodiimide (DCC, 1.3 g, 6.28 mmol, 1.2 eq). The solution was stirred at an ice bath for 10 min and another 2 hours at room temperature. The solution was washed with saturated $NaHCO_3$ and brine respectively after that the TLC indicated the completion of the reaction. The organic part was dried with anhydrous Na_2SO_4 and concentrated under reduced pressure to afford **S5** as a white solid (1.42 g, 94%) and used without further purification. ^{1}H NMR (400 MHz, Methanol-d4) δ 4.78 (s, 2H), 2.88 (s, 4H), 1.50 (s, 9H).

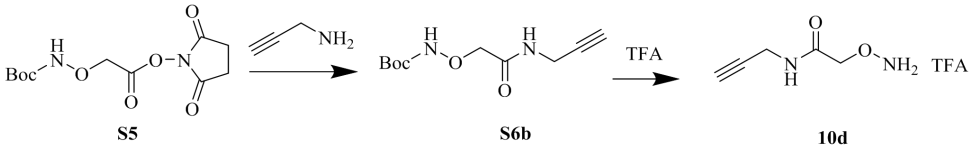
Synthesis of 2-(Boc-aminooxy)-N-(3-azidopropyl)acetamide (S6a)

To a stirred solution of **S5** (100 mg, 0.35 mmol, 1.0 eq) in 10 mL DCM was added 3-azido-1-propanamine (40.0 mg, 0.38 mmol, 1.1 eq) followed by TEA (72 μL, 2.0 eq). The reaction was stirred at room temperature for 2 hours and monitored by TLC (EA/PE=1:1). The solution was then washed with water and brine respectively, and dried over anhydrous Na_2SO_4. The organic part was gathered and concentrated under reduced pressure to afford **S6b** as a slightly yellow oil (90 mg, 95%). 1H NMR (400 MHz, Chloroform-d) δ 8.37 (s, 1H), 7.57 (s, 1H), 4.34 (s, 2H), 3.42 (p, 4H), 1.86 (p, 2H), 1.51 (s, 9H). ^{13}C NMR (126 MHz, Chloroform-d) δ 168.47, 157.47, 82.93, 48.51, 35.79, 28.19, 27.59. ESI-HRMS Calcd for $[M+Na]^+$ 296.1335, found $[M+Na]^+$ 296.1347.

2-Aminooxy-N-(3-azidopropyl)acetamide (10c)

To a stirred solution of **S6b** (90 mg) in 8.0 mL DCM was added TFA 2.0 mL (20% TFA/DCM solution) and stirred at room temperature for 1 hour at which time TLC indicated completion assumption of the starting material. The reaction was concentrated *in vacuo* and the crude yellow residue was taken up in an amount of water and washed with DCM twice. The water part was gathered and concentrated to give **10c** as a slight orange oil. HRMS Calcd for $[M+Na]^+$ 196.0805, found $[M+Na]^+$ 196.0814. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.24 (t, J = 5.6 Hz, 1H), 4.32 (s, 2H), 3.38 (t, J = 6.8 Hz, 2H), 3.18 (q, J = 6.7 Hz, 2H), 1.69 (p, J = 6.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d6) δ 167.85, 72.37, 48.76, 36.12, 28.71. ESI-HRMS Calcd for $[M+H]^+$ 174.0991, found $[M+H]^+$ 174.0982.

Synthesis of 2-Aminooxy-N-(propargyl)acetamide (10d).



S5 **S6b** **10d**

Synthesis of 2-(Boc-aminooxy)-N-propargylacetamide (S6b)

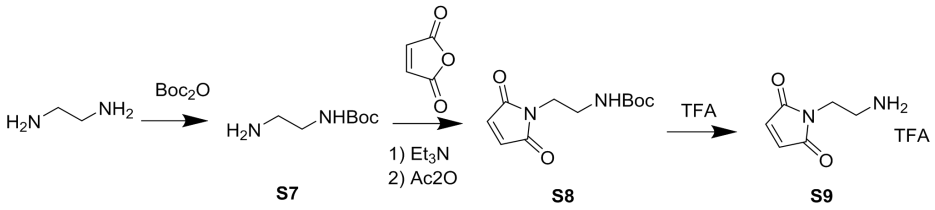
To a stirred solution of **S5** (650 mg, 2.26 mmol, 1.0 eq) in 10mL DCM was added propargylamine (186.17 mg, 3.38 mmol, 1.5 eq) followed by TEA (629 μL, 2.0 eq). The reaction was stirred at room temperature for 2 hours and monitored by TLC (EA/PE=1:1). The solution was then washed with water and brine respectively, and dried over anhydrous Na_2SO_4. The organic part was gathered and concentrated under reduced pressure to afford **S6b** as a yellow oil (462.8 mg, 90%) HRMS Calcd for $[M+Na]^+$ 251.1002, found $[M+Na]^+$ 251.1004.

2-Aminooxy-N-(propargyl)acetamide (10d)

To a stirred solution of **S6b** (462.8 mg) in 16.0 mL DCM was added TFA 4.0 mL (20% TFA/DCM solution) and stay at room temperature for 1 hour at which time TLC indicated complete assumption of the starting material. The reaction was concentrated *in vacuo* and concentrated to give **10d** as a slight brown solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.59 (t, J = 5.4 Hz, 1H), 4.36

(s, 2H), 3.93 (dd, J = 5.5, 2.5 Hz, 2H), 3.18 (t, J = 2.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d6) δ 167.90, 81.12, 73.78, 72.56, 28.11. HRMS Calcd for $[M+H]^+$ 129.0658, found $[M+H]^+$ 129.0659.

Synthesis of N-(2-Aminoethyl)maleimide[5]



Synthesis of N-Boc-Ethylenediamine (S7)

Ethylenediamine (7 mL, 100 mmol) was dissolved in 100 mL chloroform and to the solution was added di-tert-butyl bicarbonate (2.185 g, 10 mmol) dropwise in an ice bath over a period of 2 hour. The reaction mixture was stirred in the ice bath for another 2 hour before heated to and stirred at room temperature for 16 hour. The precipitate was filtered and the organic phase was washed with brine and water respectively. Dried over anhydrous Na_2SO_4 and concentrated to give a colorless oil (1.4 g, 87.5 %).

Synthesis of N-(2-[(t-Boc)amino]ethyl Maleimide (S8)

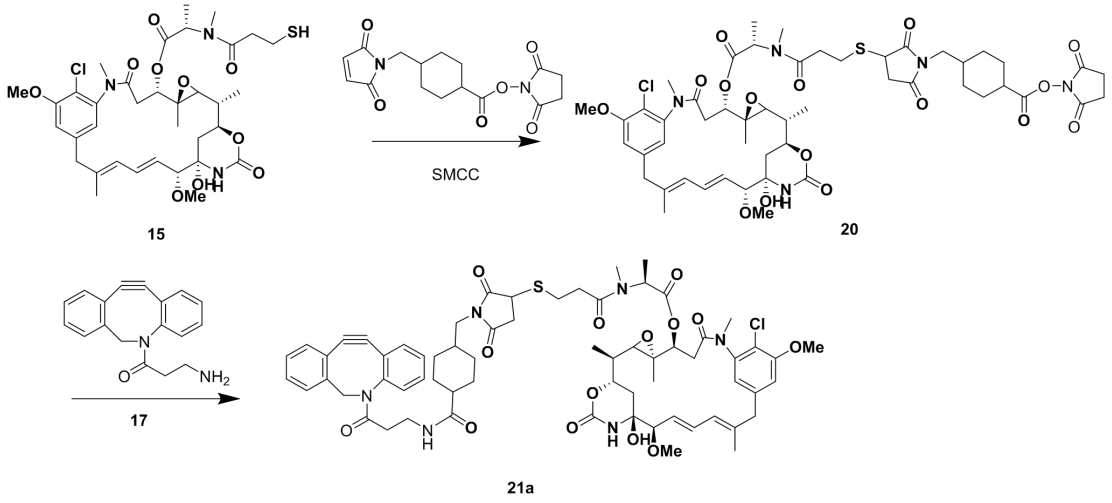
Step 1: N-Boc-Ethylenediamine (320 mg, 2 mmol, 1.2 eq) and triethylamine (278 μL, 1.2 eq) was dissolved in 30 mL ethanol and to the solution was added maleic anhydride (163 mg, 1.66 mmol, 1.0 eq) in 10 mL ethanol dropwise at an ice bath. The solution was stirred at 0 °C for another 4 hour before the complete consumption of starting material.

Step 2: The solvent was evaporated and the medium product was redissolved in 8 mL of acetic anhydride followed by sodium acetate (193 mg, 1.1 eq). Then the reaction was heated to 65 °C and stirred for 1 hour before cooled to room temperature. The solution was diluted with water and extracted with ethyl acetate. The organic phase was collected and washed with saturated $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified with flash chromatography (EA/PE, 1:2) to give a white solid (312.5 mg, 78.3 %). ^{1}H NMR (400 MHz, Chloroform-d) δ 6.73 (s, 2H), 4.74 (s, 1H), 3.68 (t, J = 8 Hz, 2H), 3.35 (q, J = 4 Hz, 2H), 1.43 (s,

9H).

Synthesis of N-(2-Aminoethyl)maleimide Trifluoroacetate Salt (S9)

N-(2-[(t-Boc)amino]ethyl maleimide (300 mg) was dissolved in 6 mL DCM with additional 2 mL TFA. The solution was stirred at room temperature for 2 hour and finally evaporated to give a yellow oil. The crude product was redissolved in 1 mL methanol and precipitated into excess ether. The product was filtered to give a white solid compound (272.7 mg, 92 %). ^{1}H NMR (400 MHz, DMSO-d6) δ 7.79 (s, 3H), 7.09 (s, 2H), 3.66 (t, J = 6.0 Hz, 2H), 2.99 (t, J = 5.8 Hz, 2H).



Synthesis of DM1-SMCC (20)

30.0 mg 0.04 mmol commercial DM1-SH **15** (1.0 eq) and 15.0 mg 0.045mmol N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, 1.1 eq) were stirred in a mixture of acetonitrile and pH 7.5 phosphate buffer (v:v =2:1) under argon atmosphere. The product was purified with preparative column after 2 hour at which time the RP-HPLC has indicated the complete consumption of DM1-SH. And the product was lyophilized to get a white powder (40.7 mg, 95%). HRMS Calcd for $[M+H]^+$ 1072.3992, found $[M+H]^+$ 1072.3974; $[M+Na]^+$ 1094.3811, $[M+Na]^+$ 1094.3797. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.30 (d, J = 15.2 Hz, 1H), 6.87 (dd, J = 5.2, 1.6 Hz, 1H), 6.67 (dd, J = 5.3, 1.6 Hz, 1H), 6.62 (d, J = 9.3 Hz, 1H), 6.45 (dd, J = 15.3, 11.1 Hz, 1H), 5.64 (dd, J = 15.1, 9.0 Hz, 1H), 5.35 (m, 1H), 4.76 (dt, J = 12 Hz, 1H), 4.36 (t, J = 11.4 Hz, 1H), 4.10 (s, 3H), 3.75 (ddd, J = 20.2, 9.1, 3.5 Hz, 1H), 3.67 (dd, J = 12.7, 5.2 Hz, 1H), 3.53 (d, J = 9.0 Hz, 1H), 3.39 (d, J = 3.3 Hz, 3H), 3.37 (s, 1H), 3.33-2.94 (m, 11H), 2.90 (s, 3H), 2.86 (s, 4H), 2.77-2.52 (m, 3H), 2.41 (ddd, J = 18.8, 9.3, 3.5 Hz, 1H), 2.33-2.22 (dd, J = 14.4, 2.8Hz, 1H), 2.16 (d, J = 13.3 Hz, 2H), 1.86-1.42 (m, 10H), 1.35 (d, J = 6.8 Hz, 3H), 1.30 (d, J = 6.4 Hz, 4H), 1.07 (m, 2H), 0.81 (s, 3H). ^{13}C NMR (126 MHz, CDCl3) δ 176.95, 176.85, 174.70, 171.01,

170.81, 170.53, 169.28, 169.24, 169.18, 156.02, 154.26, 154.20, 141.91, 141.08, 139.48, 133.33, 127.46, 125.17, 121.96, 118.70, 115.89, 113.23, 88.11, 80.80, 78.01, 77.28, 77.02, 76.77, 74.69, 66.99, 59.89, 56.71, 56.61, 46.61, 44.43, 44.36, 40.32, 39.58, 39.49, 38.81, 35.71, 35.66, 35.59, 35.41, 35.32, 32.44, 29.30, 29.22, 28.00, 27.11, 25.59, 15.53, 14.45, 13.44, 13.40, 12.11.

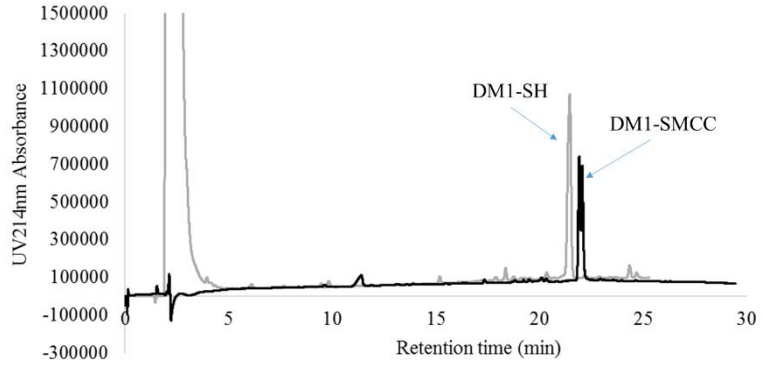


Figure S22. HPLC profiles (HPLC method A) of DM1-SMCC synthesis. The gray line was DM1-SH before reaction with SMCC. The black one was the sample removed from the reaction mixture.

Synthesis of DBCO-MCC-DM1 (21a)

A mixture of DM1-SMCC (**20**, 5.4 mg, 5.0 μmol, 1.0 eq), DBCO-NH_2 **17** (1.5 mg, 5.5 μmol, 1.1 eq) and Et_3N (0.8 μL, 5.5 μmol, 1.1 eq) was stirred at room temperature and monitored by RP-HPLC. The solution was subjected to preparative column after the end of reaction and the corresponding product was combined and lyophilized to get a white powder (5.8 mg, 94%). HRMS Calcd for $[M+H]^+$ 1233.4985, found $[M+H]^+$ 1233.5122; $[M+Na]^+$ 1255.4805, found $[M+Na]^+$ 1255.4941. ^1H NMR (500 MHz, DMSO-d6) δ 7.71 – 7.11 (m, 8H), 6.89 (s, 1H), 6.57 (m, 3H), 5.57 (t, 1H), 5.32 (m, 1H), 5.05 (d, J = 14.3 Hz, 1H, CH2 of DBCO), 4.54 (d, J = 12.0 Hz, 1H), 4.08 (t, J = 11.4 Hz, 1H), 4.01-3.84 (m, 4H), 3.63 (d, J = 13.9 Hz, 1H, CH2 of DBCO), 3.49 (d, J = 9.4 Hz, 1H), 3.25 (s, 3H), 3.22-2.97 (m, 4H), 2.72 (s, 3H), 2.45-2.29 (m, 2H), 2.21 (d, J = 18.2 Hz, 1H), 2.16-1.96 (m, 1H), 1.92-1.72 (m, 2H), 1.70-1.33 (m, 6H), 1.34-0.97 (m, 8H), 0.76 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 178.28, 176.28, 175.75, 171.67, 171.55, 171.33, 169.29, 159.56, 159.26, 156.42, 152.59, 152.39, 149.50, 142.39, 139.57, 133.76, 133.55, 130.66, 130.05, 129.62, 129.33, 129.19, 128.83, 127.97, 126.27, 123.68, 122.93, 122.52, 118.28, 117.63, 115.45, 115.32, 115.10, 109.27, 89.37, 81.15, 78.86, 74.32, 67.93, 61.19, 57.70, 57.28, 55.95, 52.83, 46.64, 45.07, 44.64, 41.28, 41.19, 41.11, 41.02, 40.95, 40.86, 40.78, 40.69, 40.52, 40.36, 40.19, 38.87, 37.51, 36.40, 36.24, 35.95, 35.38, 33.09, 30.90, 30.83, 30.44, 29.65, 29.63, 29.32, 29.31, 27.45, 16.25, 15.59, 14.25, 12.54.

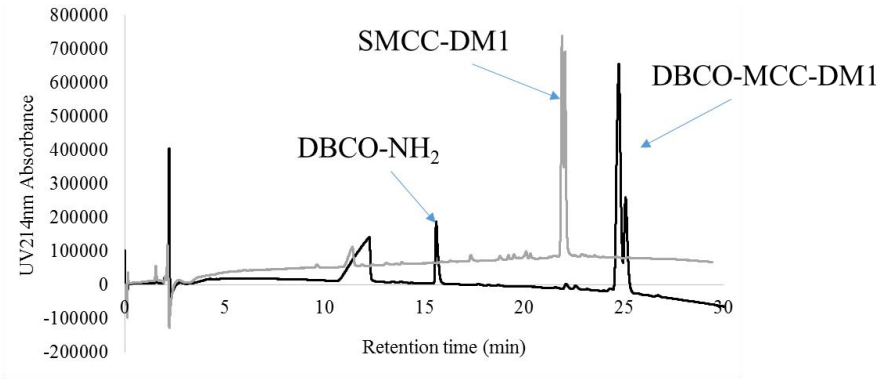
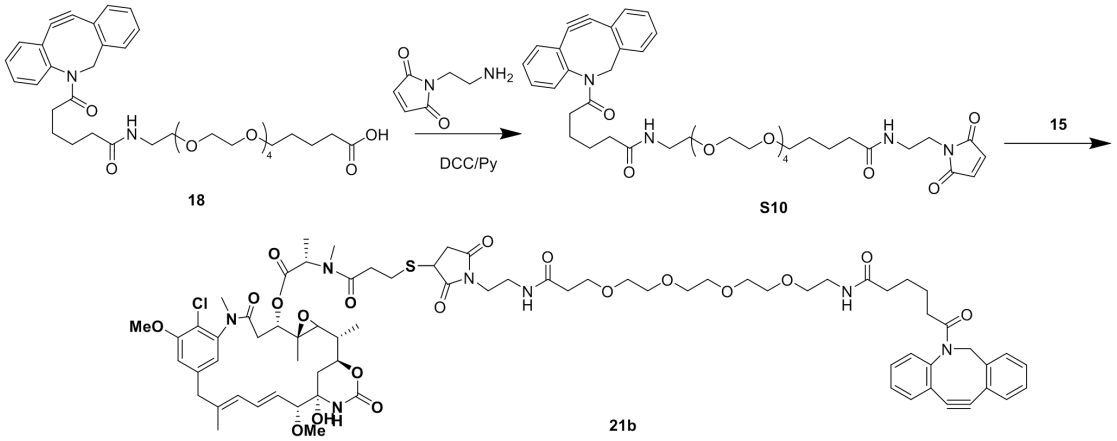


Figure S23. HPLC profiles (HPLC method A) of DBCO-MCC-DM1 synthesis. The gray line was the UV absorbance of SMCC-DM1. The black one was the sample removed from the reaction mixture. The corresponding peaks have been marked in the figure.

Synthesis of DBCO-PEG$_4$-DM1 (21b)



Synthesis of DBCO- PEG$_4$-Maleimide (S10)

To a solution of 2.0 mL DMF were added the **18** (5.8 mg, 10 μmol, 1.0 eq) and N-(2-Aminoethyl)maleimide (7.2 mg, 30 μmol, 3.0 eq) followed by the addition of Dicyclohexylcarbodiimide (DCC, 10.3 mg, 50 μmol, 5.0 eq) and Pyridin (24 μL, 30.0 eq). The reaction was stirred at room temperature for 40 hour and product was purified with preparative column to give DBCO-PEG$_4$-Maleimide (**S10**, 4.0 mg, 57%). HRMS Calcd for $[M+H]^+$ 703.3343, found 703.3352; $[M+Na]^+$ 725.3162, found 725.3174.

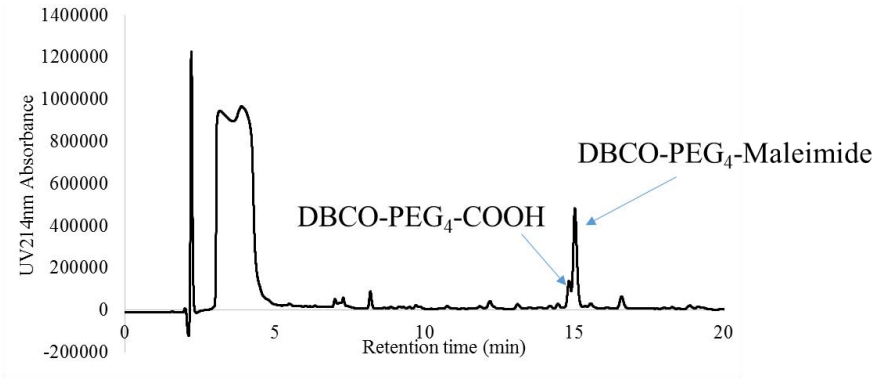


Figure S24. HPLC profile (HPLC method A) of **S10** synthesis

Synthesis of DBCO- PEG$_4$-DM1 (21b)

The collected **S18** (4.0 mg, 5.7 µmol, 1.0eq) was dissolved in acetonitrile and pH 7.5 phosphate buffer (v : v/2 : 1). To the stirred solution were added DM1-SH (4.6 mg, 6.27 µmol) and stirred at room temperature for 2 hour. The reaction mixture was subjected to preparative column and give DBCO- PEG$_4$-DM1 (**21b**) as a light white powder (5.3 mg, 65%). ^{1}H NMR (500 MHz, DMSO-d6, Deuterium Oxide (little)) δ 7.87 (q, J = 6 Hz, 1H), 7.66 (t, J = 5.5 Hz, 1H), 7.60 (d, J = 7.2 Hz, 1H), 7.55 (dd, J = 6.1, 2.9 Hz, 1H), 7.51-7.42 (m, 1H), 7.39-7.24 (m, 2H), 7.20-7.11 (dd, J = 12.5, 1.5Hz, 1H), 6.87 (s, 1H), 6.60-6.46 (m, 2H), 5.91 (s, 1H), 5.59-5.48 (m, 1H), 5.29 (dd, J = 6.8, 2.8 Hz, 1H), 5.03 (d, J = 14 Hz, 1H, H of DBCO), 4.51 (dd, J = 11.8, 1.5 Hz, 1H), 4.11-4.01 (t, J = 11Hz, 1H), 3.96-3.74 (m, 4H), 3.58 (d, J = 13.9 Hz, 1H, H of DBCO), 3.55-3.39 (m, 20H, 16H of PEG), 3.23 (s, 3H), 2.31 (dd, J = 18.3, 4.3 Hz, 1H), 2.25-2.08 (m, 5H), 2.08-1.97 (m, 2H), 1.82 (t, J = 7.1 Hz, 2H), 1.73 (m, 2H), 1.57 (s, 3H), 1.52-1.37 (m, 4H), 1.31-1.05 (m, 12H), 0.76 (d, J = 2.7 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 178.06, 178.00, 176.02, 172.97, 172.80, 171.69, 171.60, 171.49, 169.31, 169.28, 156.44, 156.39, 152.96, 152.40, 149.60, 142.46, 142.43, 142.40, 139.62, 133.76, 133.55, 130.60, 130.04, 129.63, 129.25, 129.15, 128.82, 127.95, 126.30, 123.64, 122.90, 122.55, 118.29, 115.11, 115.04, 89.35, 81.16, 78.85, 74.32, 70.91, 70.85, 70.80, 70.69, 70.64, 70.25, 67.94, 67.74, 61.19, 57.73, 57.70, 57.29, 52.82, 46.95, 46.55, 39.49, 37.46, 37.14, 36.01, 35.02, 33.14, 25.79, 25.63, 16.21, 15.63, 14.21, 12.49, 9.77.

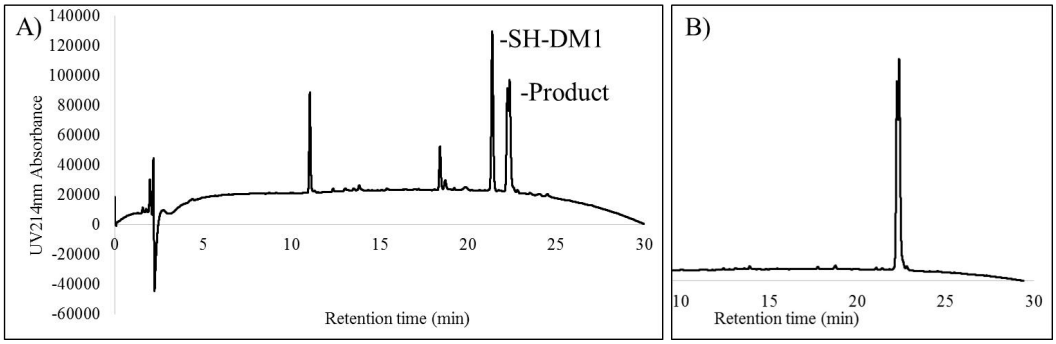
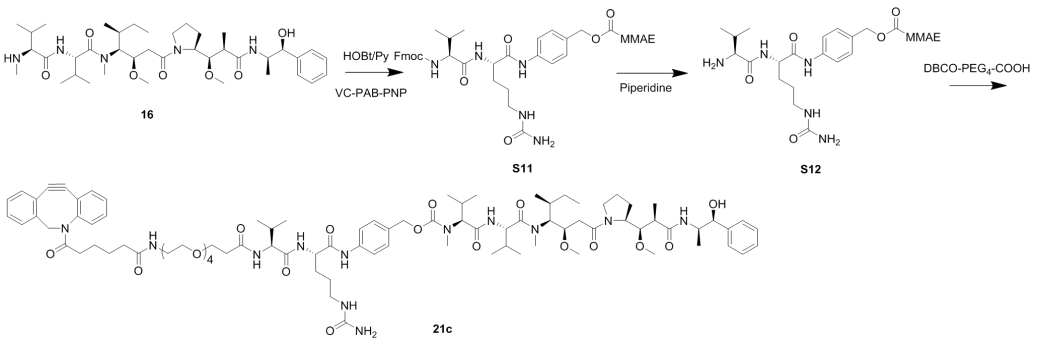


Figure S25. HPLC profiles (HPLC method A) of **21b** synthesis. A) Monitoring the reaction at 2 hour. B) The pure product after the preparative column purification.

Synthesis of DBCO-PEG$_4$-VC-PAB-MMAE (21c)



Synthesis of Fmoc-VC-PAB-MMAE (S11)

Fmoc-VC-PAB-PNP (20.0 mg, 26.1 μmol, 1.0 eq) and MMAE (28.0 mg, 39.0 μmol, 1.5 eq) were dissolved in 4.0 mL DMF. Then Hydroxybenzotriazole (HOBt, 0.8 mg, 5.2 μmol, 0.2 eq) and 60.0 μL Pyridine were added to the solution. The reaction was stirred at room temperature for 30 hour and monitored by RP-HPLC. Finally, the product was purified with preparative column to give a white powder (29.0 mg, 82.9%). HRMS Calcd for $[M+H]^+$ 1345.7812, found 1345.7743; $[M+Na]^+$ 1367.7631, found 1367.7556.

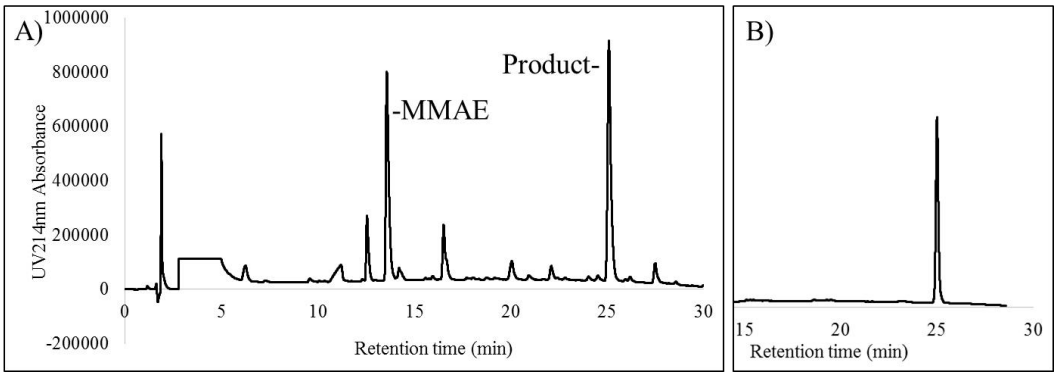


Figure S26. HPLC profiles (HPLC method A) of **S11** synthesis. A) Aliquots taken from reaction mixture, and B) The product after purified with preparative column.

Synthesis of NH$_2$-VC-PAB-MMAE (S12)

S11 was dissolved in acetonitrile containing 20.0 % piperidine and was stirred at room temperature for 1 hour after which time the RP-HPLC indicated the completion of de-Fmoc process. Then the solution was concentrated under pressure and redissolved in 50.0 % acetonitrile before subjected to the preparative column. Product was combined and lyophilized to give a white powder (23.3 mg, 96.2%). HRMS Calcd for $[M+H]^+$ 1123.7131, found 1123.7280; $[M+Na]^+$ 1145.6950, found 1145.7001.

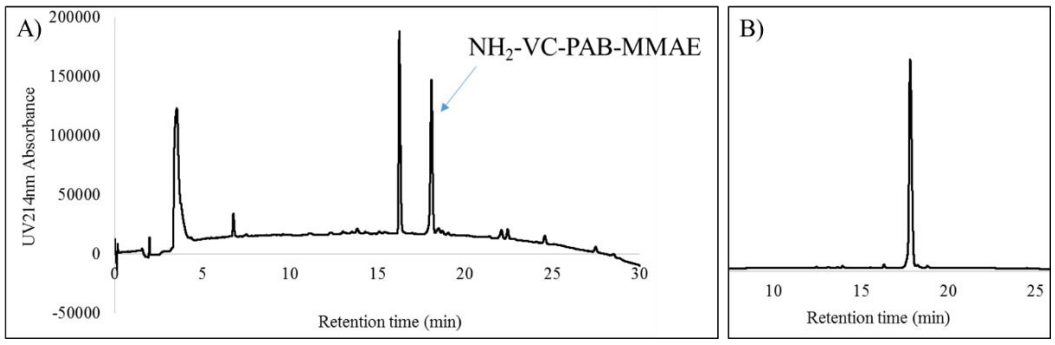


Figure S27. HPLC profiles (HPLC method A) of **S12** synthesis. A) Aliquots taken from reaction mixture, and B) The product after purified with preparative column.

Synthesis of DBCO-PEG$_4$-VC-PAB-MMAE (21c)

To a solution of 2.0 mL DMF were added NH_2-VC-PAB-MMAE and DBCO-PEG$_4$-COOH. The solution was stirred at room temperature over 40 hours and monitored by RP-HPLC. Finally the corresponding product was purified with preparative column to give**21c** as a white powder. HRMS Calcd for $[M+2H]^{2+}$ 843.4944, found $[M+2H]^{2+}$ 843.5011. ^{1}H NMR (500 MHz, DMSO-d6) δ 8.12 (d, J = 7.4 Hz, 1H), 7.92-7.84 (m, 2H), 7.72-7.66 (m, 1H), 7.66-7.54 (m, 3H), 7.53-7.23 (m, 9H), 7.22-7.15 (m, 1H), 5.04 (d, J = 14.0 Hz, 2H), 4.50 (d, J = 5.8 Hz, 1H), 4.41 (dd, J = 24.3, 6.0 Hz, 3H), 4.32-4.20 (m, 3H), 4.06-3.92 (m, 3H), 3.79 (d, J = 9.3 Hz, 0H), 3.64-3.56 (m, 3H), 3.48 (d, J = 7.8 Hz, 6H), 3.33 (t, J = 6.0 Hz, 2H), 3.27-3.16 (m, 4H), 3.11 (dd, J = 10.4, 4.4 Hz, 2H), 2.98 (s, 1H), 2.87 (d, J = 18.0 Hz, 2H), 2.42 (ddd, J = 36.2, 14.1, 6.6 Hz, 4H), 2.33-2.22 (m, 1H), 2.21-2.06 (m, 3H), 1.97 (dd, J = 13.7, 6.8 Hz, 2H), 1.85 (t, J = 7.1 Hz, 2H), 1.75 (dd, J = 16.6, 7.7 Hz, 2H), 1.64-1.23 (m, 2H), 1.18 (dd, J = 13.6, 6.9 Hz, 2H), 1.02 (td, J = 16.2, 14.2, 6.7 Hz, 4H), 0.95-0.72 (m, 13H).

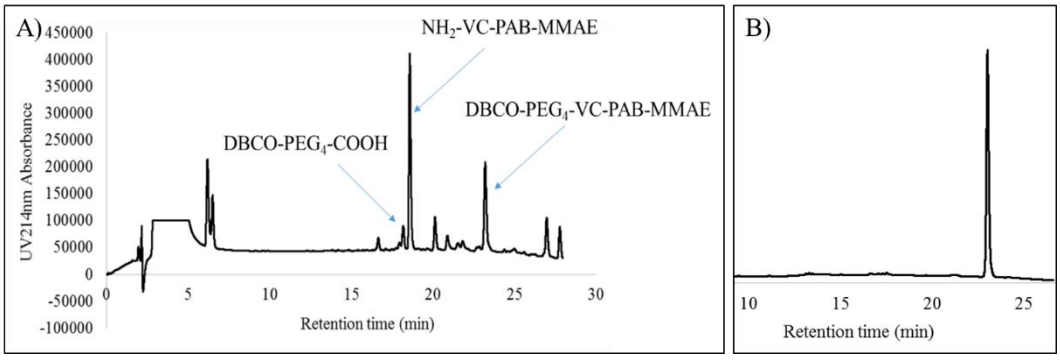
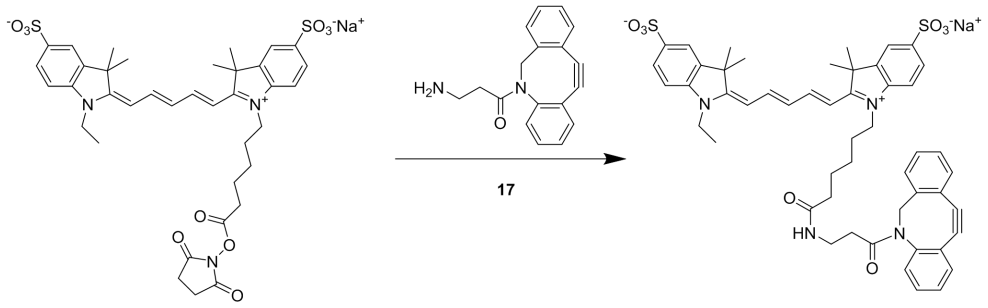


Figure S28. HPLC profiles (HPLC method A) of **21c** synthesis. A) Aliquots taken from reaction mixture, and B) The product after purified with preparative column.

Synthesis of DBCO-Cy5 (21d)



1.0 mg 1.3μmol Cy5-NHS (1.0 eq), 0.4 mg 1.42μmol DBCO-NH_2 (1.1 eq) and 0.5 μL 2.7eq Et_3N were dissolved in a mixture of acetonitrile and water (1:1). The mixture was stirred at room temperature and protected from light for 4 hour. The solution was subjected to a semi-preparative column when the RP-HPLC has indicated the complete transformation of Cy5-NHS to DBCO-Cy5. The corresponding product was combined and lyophilized to get a dark black solid (1.1 mg, 92%). HRMS Calcd for $[M-Na+2H]^+$ 915.3467, found $[M-Na+2H]^+$ 915.3488.

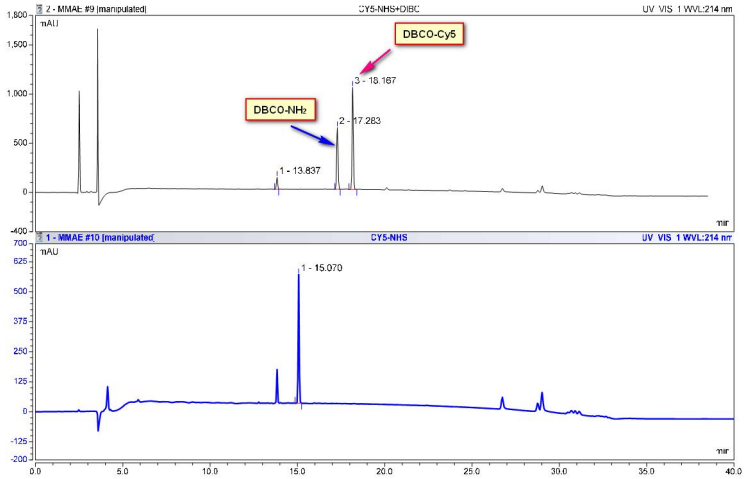
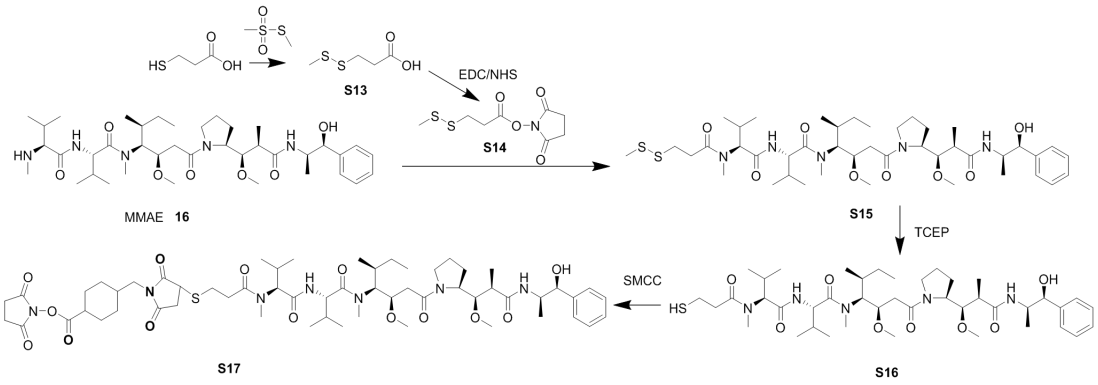


Figure S29. HPLC profiles (HPLC method C) of DBCO-Cy5 synthesis. The blue one was the commercial Sulfo-Cy5-NHS ester. The gray one was the sample removed from the reaction mixture. The corresponding peaks have been labeled with abbreviations.

Synthesis of SMCC-MMAE



Synthesis of 3-(Methyldithio)propanoic Acid (S13)

3-Mercaptopropanoic acid (1.00 g, 9.4 mmol) was dissolved in water (30 mL) and was cooled in an ice bath, to the solution was added methyl methanethiolsulfonate (1.31 g, 10.4 mmol) in absolute ethanol (15 mL). The mixture was then diluted with saturated brine (80mL) after stirring overnight at room temperature and extracted with ether. The combined organic parts were then washed with saturated brine and finally dried over anhydrous Na_2SO_4, concentrated, and then distilled to afford a colorless liquid.

Synthesis of 2, 5-dioxopyrrolidin-1-yl 3-(methylsulfinothioyl)propanoate (S14)

3-mercaptopropanoic acid (200mg 1.32mmol) was dissolved in dichloromethane (5 mL) and stirred magnetically as N-hydroxysuccinimide (227mg , 1.98 mmol) was added followed by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 380 mg, 1.98 mmol). The mixture was stirred under an argon atmosphere for 2 h. The reaction mixture was poured into a 125 mL separatory funnel, 40 mL of ethyl acetate was added, and the solution was washed with 50 mM potassium phosphate buffer at pH 6.0 (2×20 mL) and saturated sodium chloride (20 mL). The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed by rotary evaporation under vacuum to give the desired product, which was used without further purification.

Synthesis of 3-(Methyldithio)propanoic MMAE (S15)

MMAE (30 mg, 0.042 mmol) was dissolved in acetonitrile (5 mL) and stirred magnetically as 2,5-dioxopyrrolidin-1-yl 3-(methylsulfinothioyl)propanoate (52.3 mg, 0.21 mmol) was added. Then, 50 mM sodium phosphate buffer at pH=7.5 (2.5 mL) was added in the reaction. The reaction mixture was stirred for 48h at room temperature. The reaction was monitored by HPLC. Eventually the product was separate by semi-preparation column. HRMS. Calcd for $[M+H]^+$ 852.4979., found 852.4940; Calcd for $[M+Na]^+$ 874.4798, found 874.4762.

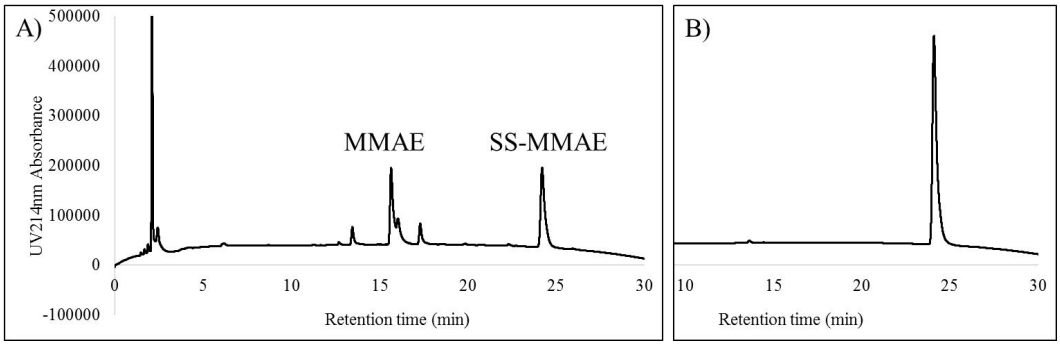


Figure S30. HPLC profiles (HPLC method A) of **S15** synthesis. A) Aliquots taken from the reaction mixture, and B) The product after purified with preparative column.

Synthesis of 3-mercaptopropanoic MMAE (S16)

S15 (10mg, 0.0124 mmol) was dissolved in acetonitrile (2 mL) and stirred magnetically was added Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 7.1 mg, 0.0248 mmol) in neutral aqueous solution. The reaction was stirred for 2h at room temperature. The reaction was monitored by HPLC. Eventually the product was separate by semi-preparation column. HRMS. Calcd for $[M+H]^+$ 806.5102., found 806.5143; Calcd for $[M+Na]^+$ 828.4921, found 828.4964.

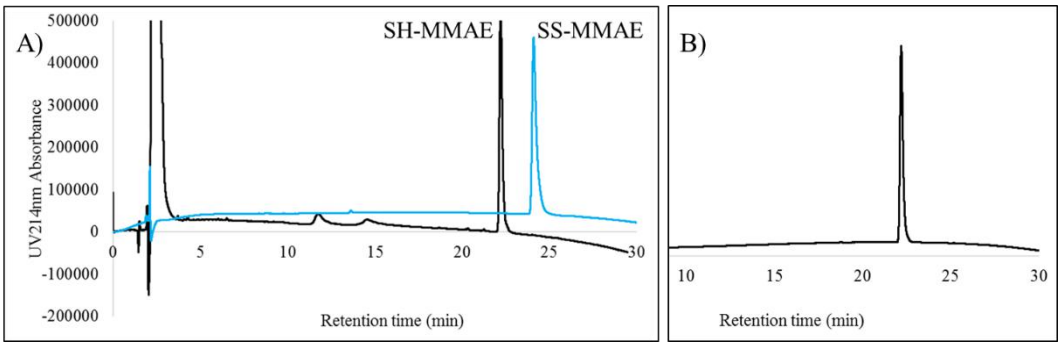


Figure S31. HPLC profiles (HPLC method A) of **S16** synthesis. A) Aliquots taken from the reaction mixture, and B) The product after purified with preparative column.

Synthesis of MMAE-SMCC (S17)

To a stirred solution of **S16** (5 mg, 0.0062 mmol) in a mixture of acetonitrile (2 mL) and 50 mM sodium phosphate buffer at pH=7.5 (1 mL) was treated with N-Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, 2.27 mg, 0.0068 mmol) under argon at room temperature. After 1 h, samples was removed from the reaction buffer and was monitored by HPLC. Finally the product was separate by semi-preparation column. HRMS. Calcd for $[M+H]^+$ 1140.6266., found 1140.6211; Calcd for $[M+Na]^+$ 1162.6086, found 1162.6037.

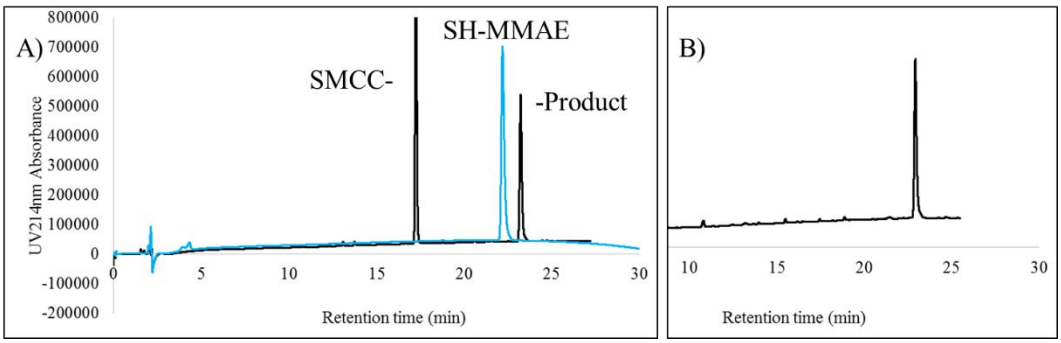
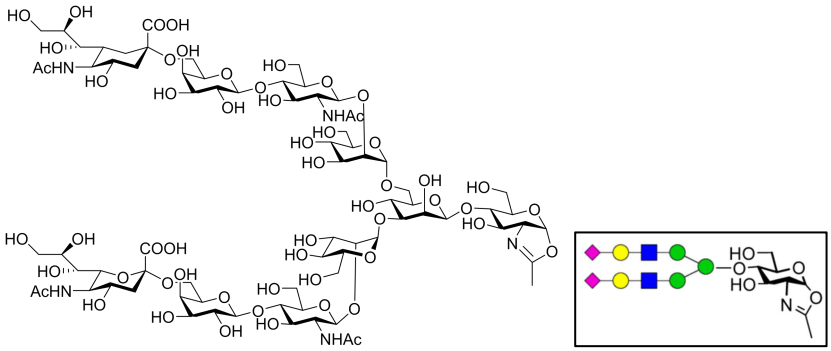


Figure S32. HPLC profiles (HPLC method A) of **S17** synthesis. A) Aliquots taken from the reaction mixture, and B) The product after purified with preparative column.

4. NMR and HPAEC data of oxazoline 2-5

One-pot semi-synthesis of SCT-ox (2)



^{1}H NMR (400 MHz, Deuterium Oxide) δ 5.91 (d, J = 7.3 Hz, 1H, H1 of oxa), 4.95 (s, 1H, H1c), 4.78 (s, 1H, H1c'), 4.57 (s, 1H, H1b), 4.49-4.38 (m, 2H, H1d, H1d'), 4.26 (d, J = 8.0 Hz, 2H, H1e, H1e'), 4.21 (s, 1H, H3a), 3.99 (d, J = 13.0 Hz, 4H, H2a, H2b, H2c, H2c'), 2.48 (dd, J = 12.1, 4.0 Hz, 2H, H3f$_{eq}$, H3f'$_{eq}$), 1.98-1.77 (m, 15H), 1.54 (t, J = 12.1 Hz, 2H, H3f$_{ax}$, H3f'$_{ax}$).

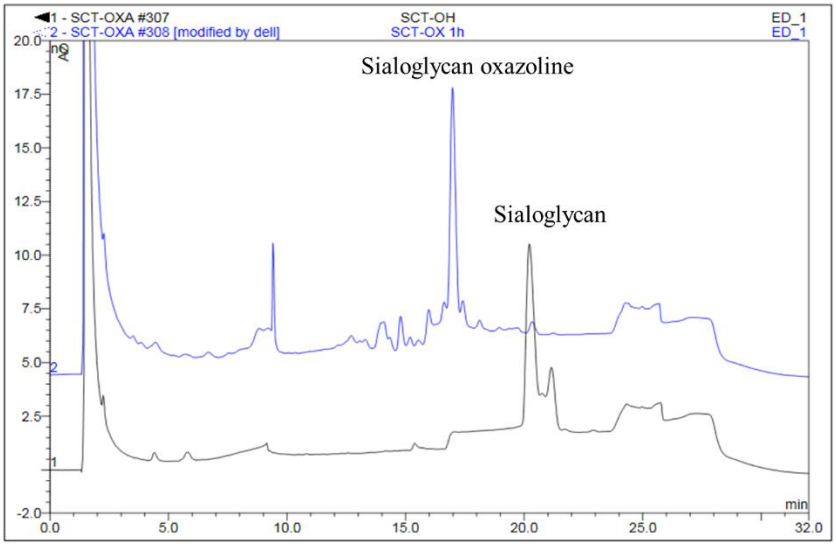
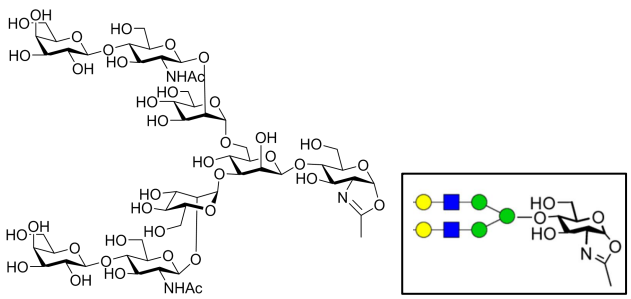


Figure S33. HPAEC chromatography of SCT-ox synthesis process. The black line (down) is the sialylated oligosaccharides released from SGP. The blue line (up) is the reaction buffer after reaction with DMC/Et_3N

One-pot semi-synthesis of CT-ox (3)



^{1}H NMR (400 MHz, Deuterium Oxide) δ 5.97 (d, J = 7.1 Hz, 1H, H1 of oxa), 4.99 (s, 1H, H1c), 4.83 (s, 1H, H1c'), 4.62 (s, 1H, H1b), 4.47 (m, 2H, H1d, H1d'), 4.35 (d, J = 8.3 Hz, 2H, H1e, H1e'), 4.27 (s, 1H, H3a), 4.05 (m, 4H, H2a, H2b, H2c, H2c'), 1.93 (d, J = 4.9 Hz, 9H, Ac).

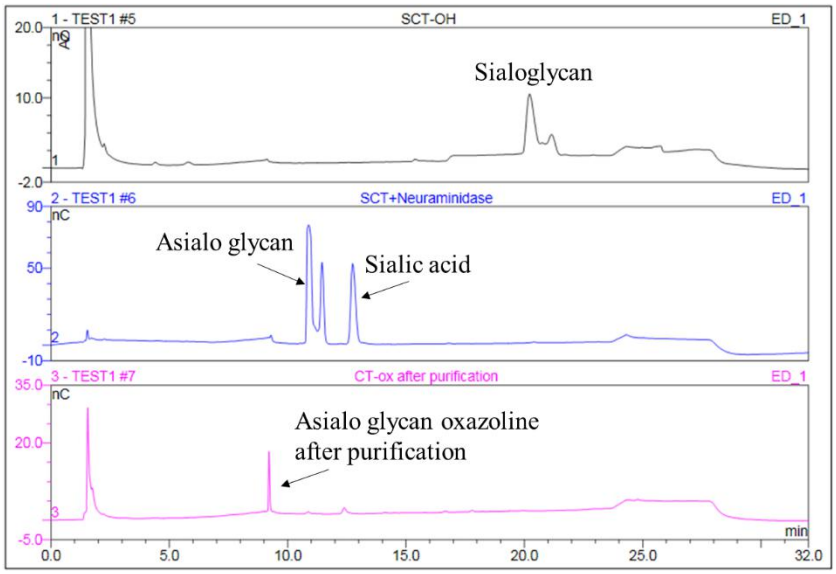
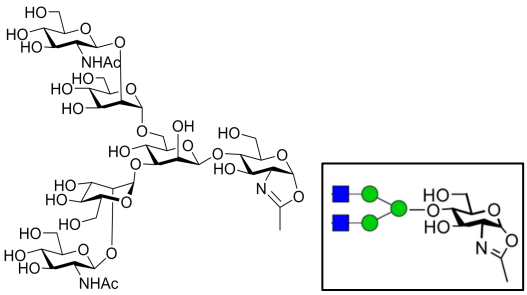


Figure S34. HPAEC chromatography of one-pot CT-ox synthesis. The first black line is the sialylated oligosaccharides released from SGP. The second blue line is sample removed from the reaction buffer after incubation with neuraminidase. The third pink line is the final product purified with PGC after incubation with DMC/Et_3N.

One-pot semi-synthesis of GlcNAc2Man3-ox (4)



^{1}H NMR (500 MHz, Deuterium Oxide) δ 6.01 (d, J = 7.3 Hz, 1H, H1 of oxa), 5.05 (s, 1H, H1c), 4.87 (s, 1H, H1c'), 4.67 (s, 1H, H1b), 4.49 (dd, J = 14.4, 8.4 Hz, 2H, H1d, H1d'), 4.32 (d, 1H, H3a), 4.16-4.03 (m, 4H, H2a, H2b, H2c, H2c'), 2.02-1.94 (m, 9H, Ac).

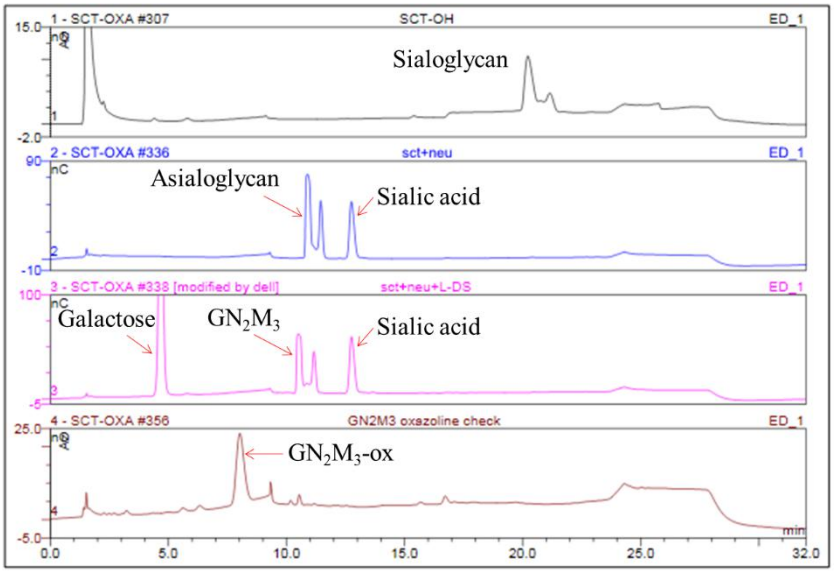
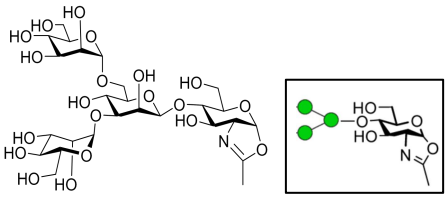


Figure S35. HPAEC chromatography of one-pot GlcNAc2Man3-ox synthesis. The first black line is the sialylated oligosaccharides released from SGP. The second black line is sample removed from the reaction buffer after incubation with neuraminidase. The third pink line is sample removed from the reaction buffer after incubation with additional Lactase DS. The last orange one is the pure GN_2M_3-oxazoline after purification.

One-pot semi-synthesis of Man3-ox (5)



^{1}H NMR (400 MHz, Deuterium Oxide) δ 5.95 (d, J = 7.3 Hz, 1H, H1 of oxa), 4.97 – 4.93 (s, 1H, H1c), 4.80 (s, 1H, H1c'), 4.59 (s, 1H, H1b), 4.24 (s, 1H, H3a), 4.05 (m, 1H, H2a), 4.01 (d, J = 2.7 Hz, 1H, H2b), 3.92 (dd, J = 3.3, 1.7 Hz, 1H, H1c), 3.87 (d, J = 3.4 Hz, 1H, H1c'), 1.92 (d, J = 1.7 Hz, 3H, Ac of oxa).

5. Expression and purification of enzymes

Material:

The Endo-S and Endo-M genes and primers were synthesised by GENEWIZ (Suzhou, China). PNGase-F plasmid is a gift from Dr Zhao, Qiang's lab in Shanghai Institute of Materia Medica. The vectors were purchased from Invitrogen (USA). BL21(DE3) Chemically Competent Cell was purchased from TransGen (China). The Ni-NTA agarose used in purification was purchased from QIAGEN. Other materials used in expression were purchased from Sangon Biotech (China).

Construction of expression vectors:

The nucleotide sequence encoding for Endo-S gene (ndoS) was obtained from GenBank (No. AF296340). The DNA sequence bearing NdeI and NotI cleaving sites and was synthesized after optimized for expression in *E. coli* by GENEWIZ (Suzhou, China). The synthetic ndoS gene was further subcloned from cloning vector pUC57 into expression vector pET-30a(+) (Invitrogen, USA) using NdeI and NotI (NEB, USA) restriction endonucleases and T4 DNA ligase (Thermo Fisher, USA). The constructed plasmid, was then transformed into BL21(DE3) (TransGen, China).

Original protein sequence of *ndoS* gene[6]

```
1   ATGGAGGAGA AGACTGTTCA GGTTCAGAAA GGATTACCTT CTATCGATAG CTTGCATTAT
61  CTGTCAGAGA ATAGCAAAAA AGAATTTAAA GAAGAACTCT CAAAAGCGGG GCAAGAATCT
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181 GCCAAAATGA AAATTCCTGA GAAAATACCG ATGAAACCGT TACATGGTCC TCTCTACGGT
241 GGTTACTTTA GAACTTGGCA TGACAAAACA TCAGATCCAA CAGAAAAAGA CAAAGTTAAC
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541 GCTATTGTTG ATGAATATGT TTATAAATAC AACCTTGATG GCTTAGATGT GGATGTTGAA
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2761AAAATAAAAG AGATGGCTTT AGAAACTTCT TTGAACAGTA AGATTTTTGA TGTAACTGCT
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The synthetic sequence of Endo-S after optimization for *E. coli* expression:

NdeI highlighted in Green
NotI highlighted in Yellow
His-tag highlighted in Turquoise
Start and stop codon shown in Red
NdeI and NotI excluded during optimization

1 CATATGGAAG AGAAGACAGT TCAGGTTCAG AAGGGCCTGC CGAGCATCGA CAGTCTGCAT
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121 AGCCAGAAAG TTAAGGAGAT TCTGGCAAAA GCCCAGCAGG CCGATAAACA GGCACAGGAA
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241 GGTGGCTATT TTCGCACCTG GCATGACAAA ACCAGCGACC CGACCGAGAA AGACAAGGTG
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661 ATCCAGGTTT TTGAGGAAAT CGGCAAGCTG ATCGGTCCGA AAGGCGTTGA CAAGAGCCGC
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2341GAAGATGGTT TAATCAAGCA CTGGCGCTTT TTCAATGACA GCGCCCGTAA CCCTGAAACA
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2821GCCATCAACG CCAATGCAGG CGTTCTGAAA GACTGTATTG AAAAGCGCCA GCTGCTGAAG
2881AAACATCATC ATCATCATCA TCATCACCAC CACTAAGCGG CCGC

The Amino acid sequence expressed from pET-30a(+):ndoS. (D233 is the D198 in below sequence because the signal peptide was removed)

```
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81      G  Y  F  R  T  W  H  D  K  T  S  D  P  T  E  K  D  K  V  N
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601     P  T  G  H  Y  P  D  T  Y  G  K  T  S  L  R  L  P  V  A  N
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821		N	L	L	E	N	P	N	K	F	D	D	E	K	Y	W	I	T	V	D	T
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901		T	A	K	E	L	S	Q	Q	K	D	K	F	S	Q	K	M	L	D	E	L
921		K	I	K	E	M	A	L	E	T	S	L	N	S	K	I	F	D	V	T	A
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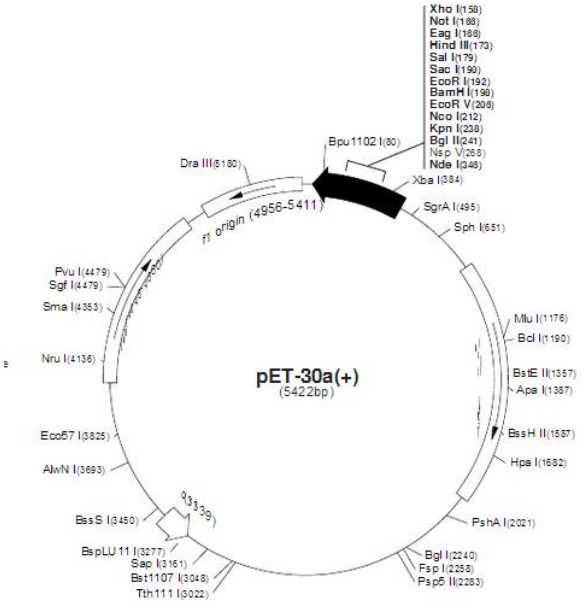


pET-30a(+) sequence landmarks

T7 promoter	419-435
T7 transcription start	418
His•Tag coding sequence	327-344
S•Tag coding sequence	249-293
Multiple cloning sites	
(*Nco* I-*Xho* I)	158-217
His•Tag coding sequence	140-157
T7 terminator	26-72
*lacI*coding sequence	826-1905
pBR322 origin	3339
Kan coding sequence	4048-4860
f1 origin	4956-5411

The maps for pET-30b(+) and pET-30c(+) are the same as pET-30a(+) (shown) with the following exceptions: pET-30b(+) is a 5421bp plasmid; subtract 1bp from each site beyond *Bam* H I at 198. pET-30c(+) is a 5423bp plasmid; add 1bp to each site beyond *Bam* H I at 198.

Site-directed mutagenesis of Endo-S D233A and D233Q were generated by SOE PCR, using vectors containing Endo-S wild type as the template. The forward primers were 5'-GGCCTGGACGTTGCCGTGGAACACGATAGCATTCCGAAAGTG-3' (for D233A) and 5'-GGCCTGGACGTTCAGGTGGAACACGATAGCATTCCGAAAGTG-3' (for D233Q). The reverse primers were 5'-TTCCACGGCAACGTCCAGGCCATCCAGGTTGTACTTGTACAC-3' (for D233A) and 5'-TTCCACCTGAACGTCCAGGCCATCCAGGTTGTACTTGTACAC-3' (for D233Q). Then we use Dpn I (NEB, USA) to eliminate original template. All the constructs were confirmed by DNA sequencing. The constructed plasmid, was then transformed into BL21(DE3) (TransGen, China).

By the similar procedure, the Endo-M gene sequence was obtained from GenBank. The sequence containing NdeI and XhoI cleaving sites was synthesized by GENEWIZ (Suzhou, China). The synthetic gene was subcloned into expression vector pET-23b(+) (Invitrogen, USA) with NdeI, XhoI, and T4 DNA ligase. We then transformed it into BL21(DE3) (TransGen, China).

The PNGase F plasmid obtained from Dr Zhao's laboratory (Shanghai Institute of Materia Medica) was transformed into BL21(DE3) (TransGen, China).

Expression and purification procedures[7]

E. Coli BL21 (DE3) bacteria expressing the wild type pET-30a (+)-ndoS expression vector were first grown in 50 mL LB/Kan+ medium including 0.1 mg/mL Kanamycin at final concentration. Control the temperature at 37 ºC, after a 4 hours pre-culture step (220 rpm) OD600 = 0.6-1.0 can be reached. Then add all the bacterial into 1L LB/Kan+ medium and culture for more than 12hours. When OD600=0.4-0.6 was attained, the expression bacterial was incubated by the addition of IPTG (isopropyl β-D-1 thiogalactopyranoside) with 0.1mM as the final concentration. The cultures were incubated at 16 ºC overnight to get soluble protein. Cells were harvested by centrifugation at 4 ºC and 10000×g for 20 min. Then the bacterial were suspended in 10 mM phosphate buffer (10mM, pH =7.4) to a total volume of 50 mL. Cells were lysed by ultrasonication and the extract was centrifuged at 4 ºC and 18000×g for 20min to get supernatant.

Here we use 5mL column volume of Ni-NTA agarose (QIAGEN) to purify 1L cells lysate, and before purification procedure, the Ni-NTA affinity column should be pre-processed with several steps for equilibration. The lysate solution was loaded onto it for more than one time. After washing with 10 column volumes of phosphate buffer (10mM, pH =7.4), we can remove the non-specific binding of protein. Then wash with 10 column volumes of phosphate buffer (10mM, pH =7.4) containing 20 mM imidazole gently. This operation can remove most impurity protein and a gradient elution procedure can also be used as optimized protocol. Target protein can be eluted by 250 mM imidazole. Results of expression and purification from SDS-PAGE and western blot analysis suggested that the protein were effectively purified.

Expression and purification of Endo-M

E. Coli BL21(DE3)bacteria were first grown in 5 mL LB/Amp+ medium including 0.1 mg/mL ampicillin at final concentration. Control the temperature at 37 ºC, after a 12 hours pre-culture step (220 rpm) OD600=0.6-1.0 can be reached. Then add all the bacterial into 1L LB/ Amp + medium and culture for more than 12hours. When OD600 = 0.4-0.6 was attained, the expression bacterial was incubated without IPTG. The cultures were incubated at 30 ºC for 3 days. Cells were harvested by centrifugation at 4 ºC and 10000×g for 20 min. Then the bacterial were suspended in 10 mM phosphate buffer (10mM, pH =7.4) to a total volume of 50 mL. Cells were lysed by ultrasonication and the extract was centrifuged at 4 ºC and 18000×g for 20min to get supernatant.

Here we use 5mL column volume of Ni-NTA agarose (QIAGEN) to purify 1L cells lysate, and before purification procedure, the Ni-NTA affinity column should be pre-processed with several steps for equilibration. The lysate solution was loaded onto it for more than one time. After washing with 10 column volumes of phosphate buffer (10mM, pH =7.4) we can remove the non-specific binding of protein. Then wash with 10 column volumes of phosphate buffer (10mM, pH =7.4) containing 50 mM imidazole gently. Target protein can be eluted by 100 mM imidazole. Results of expression and purification from SDS-PAGE and western blot analysis suggested that the protein were effectively purified.

Expression and purification of PNGase F

E. Coli BL21(DE3)bacteria were first grown in 5 mL LB/Amp+ medium including 0.1mg/mL ampicillin at final concentration. Control the temperature at 37 ºC, after a 12 hours pre-culture

step (220 rpm) OD600 = 0.6-1.0 can be reached. Then we get 1L bacterial through enlarged culture and when OD600 = 0.4-0.6 was attained, the expression bacterial was incubated with IPTG with 0.5mM as final concentration. The cultures were incubated at 16 ^{o}C overnight. Cells were harvested by centrifugation at 4 ^{o}C and 10000×g for 20 min. Then the bacterial were suspended in 10 mM phosphate buffer (10mM, pH =7.4) to a total volume of 50 mL. Cells were lysed by ultrasonication and the extract was centrifuged at 4 ^{o}C and 18000×g for 20min to get supernatant.

Here we use 5mL column volume of Ni-NTA agarose (QIAGEN) to purify 1L cells lysate, and before purification procedure, the Ni-NTA affinity column should be pre-processed with several steps for equilibration. The lysate solution was loaded onto it for more than one time. After washing with 10 column volumes of phosphate buffer (10mM, pH =7.4) we can remove the non-specific binding of protein. Then wash with 10 column volumes of phosphate buffer (10mM, pH =7.4) containing 20 mM imidazole gently. We can also wash with 50, 60 and 70mM imidazole as gradient elution. Target protein can be eluted by 250 mM imidazole. Results of expression and purification from SDS-PAGE and western blot analysis suggested that the protein were effectively purified.

6. Other procedures

Non-enzymatic glycation of oxazoline 2 onto 6:

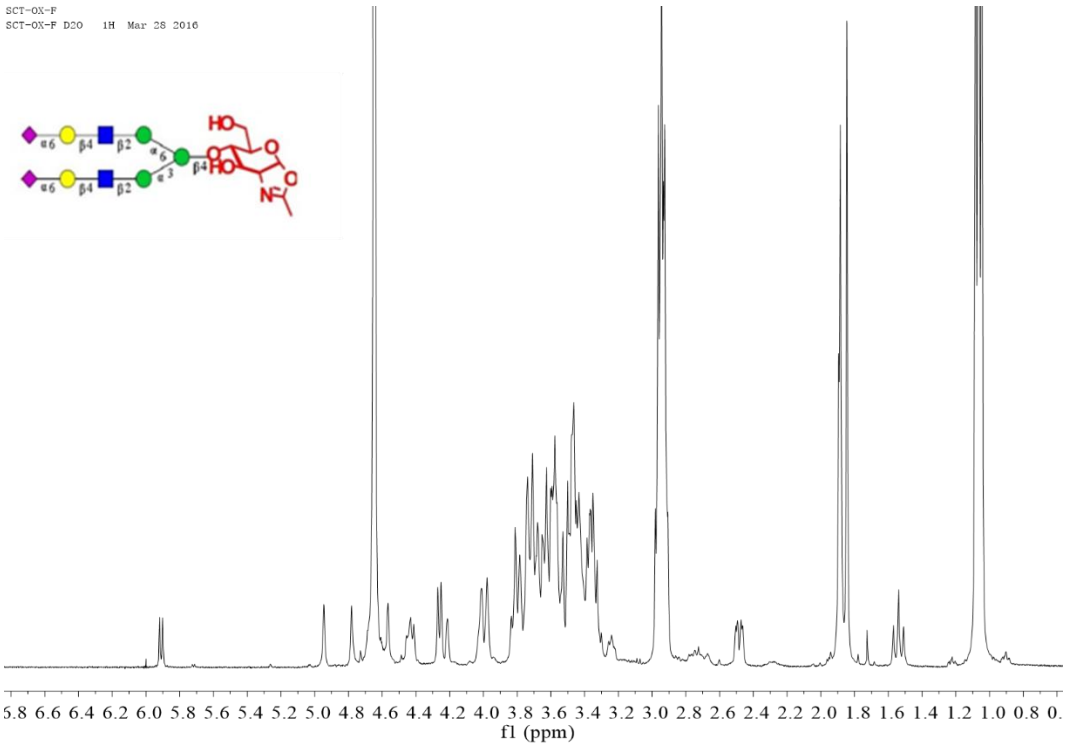
Before the studies of one-pot transglycosylation of (Fucα1,6)GlcNAc-Herceptin **6**, the non-enzymatic reaction such as "glycation" process was conducted. First, pure SGP (50mM) was incubated with Endo-M to give a solution of sialo-glycan mixed with GlcNAc-peptide. The released glycan was then transformed to oxazoline **2** by additional DMC (15 eq) and Et3N (40.0 eq). With the *in situ* oxazoline **2**, we studied the non-enzymatic glycation using 1.0 mM, 1.5 mM, 2.5 mM and 5.0 mM of **2** and **6** (final concentration 5.0 mg/mL) in a Tris buffer (pH 7.4). Aliquots (1 μL each) were taken from reaction cells at 1h, 2h, 3h, 4h and 12h respectively and diluted in SDS sample buffer for SDS-PAGE analysis. The results is shown in Fig. S6.

Synthesis of T-DM1. To a solution of wide-type Herceptin (5 mg) in PB (50 mM, pH 7.5, 5 mL) was added DM1-SMCC **20** (0.54 mg, 15.0 eqiv,). The reaction mixture was incubated at 37 ^{o}C. After 1-2 hour, LC-MS monitoring indicated the random ligation of 3.2 DM1 onto Herceptin which had been incubated with EndoS before subjecting to the LC-MS column. The solution was immediately subjected to Protein A resin (1mL) and eluted as former procedure. Tubes containing the products were combined and buffer exchanged to Tris or phosphate buffer (50 mM, pH 7.5).

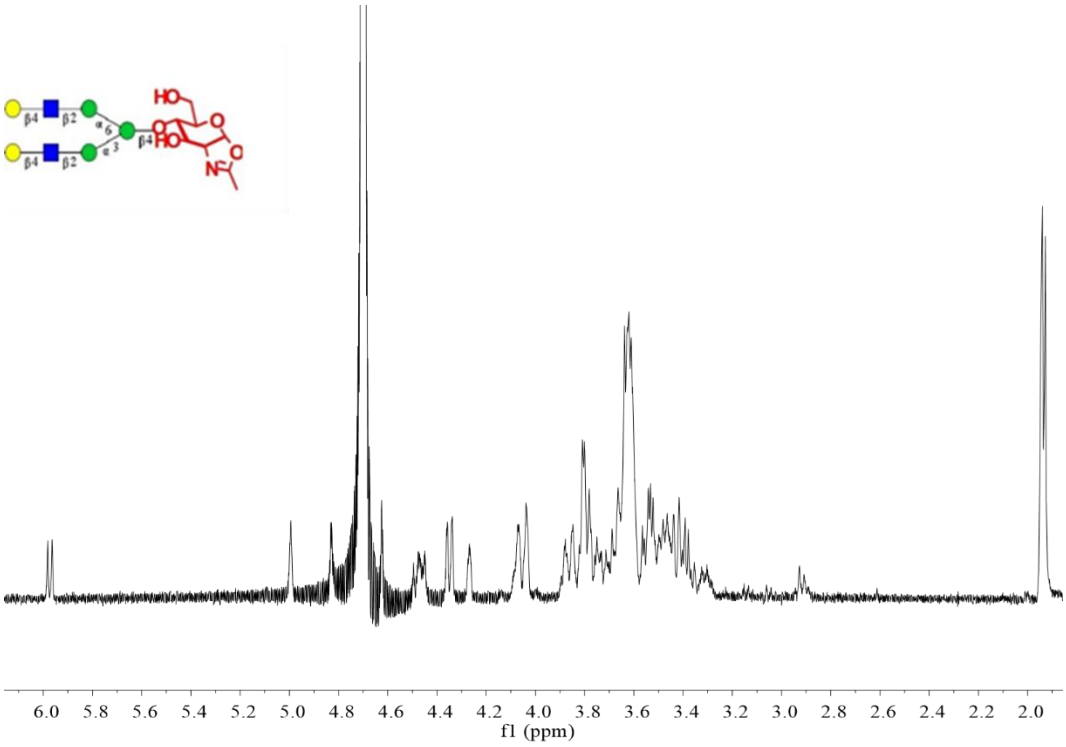
Synthesis of T-MMAE. Successfully synthesis of T-DM1 made us the similar procedure of T-MMAE synthesis, which was composed of the same linker SMCC to T-DM1 together with 3-mercaptopropionic acid. DAR is 3.5.

Randomly Cy5 Labeling of commercial Herceptin. To a solution of commercial Herceptin (1.0 mg/mL, 0.5 mL, 1.0 eqiv) in 50 mM pH 7.5 phosphate buffer was added Sulfo-Cy5-NHS **19** (38.75 μg, 15.0 eq, Little-PA Sciences Co., Ltd) and was incubated 30 ^{o}C for 2 hours to give the Cy5 randomly linked Herceptin **26**. The labeled Herceptin was purified via affinity chromatography with a protein A column.

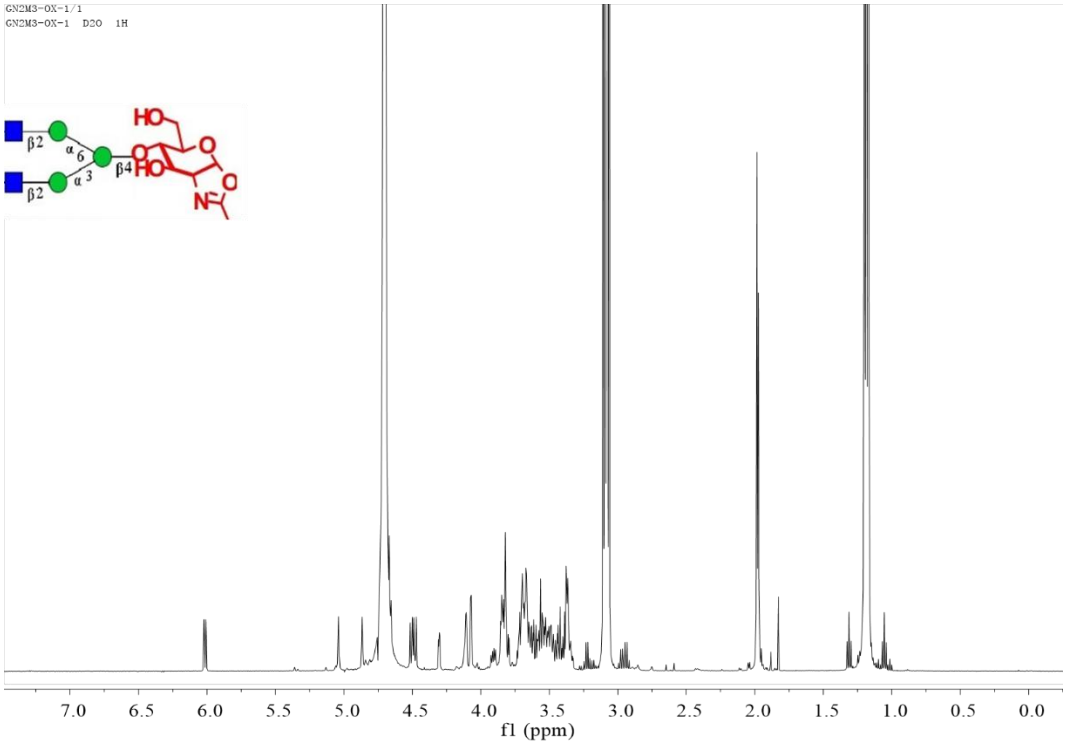
7. NMR spectra



^{1}H NMR of **2**



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



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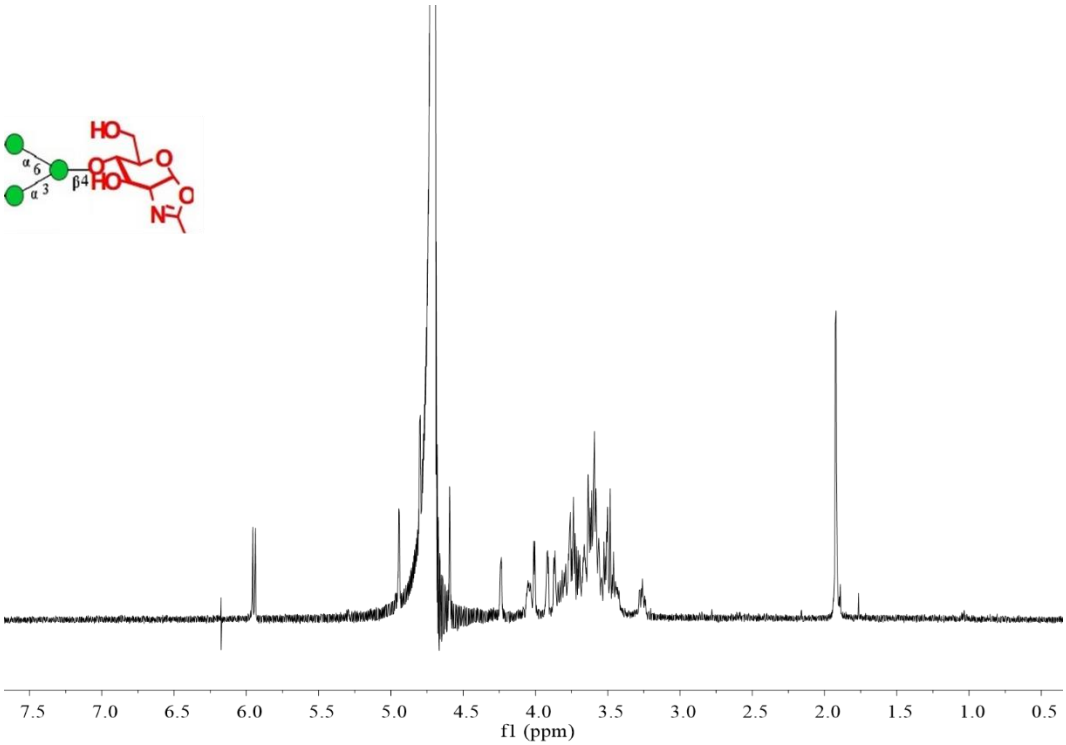
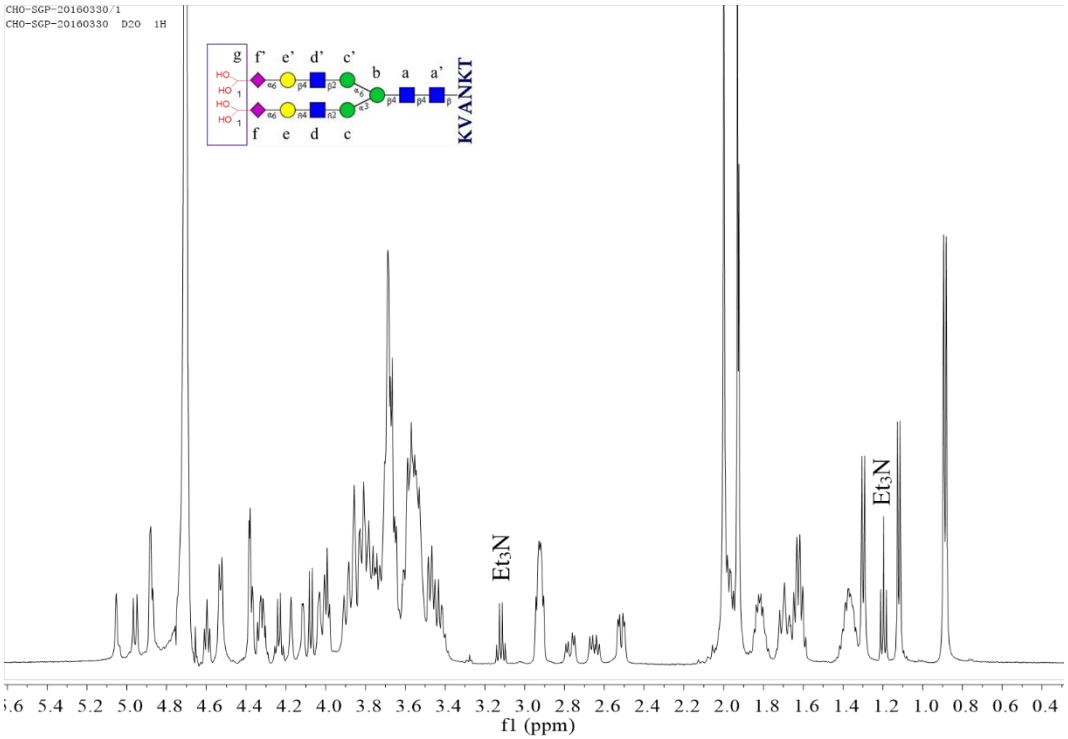
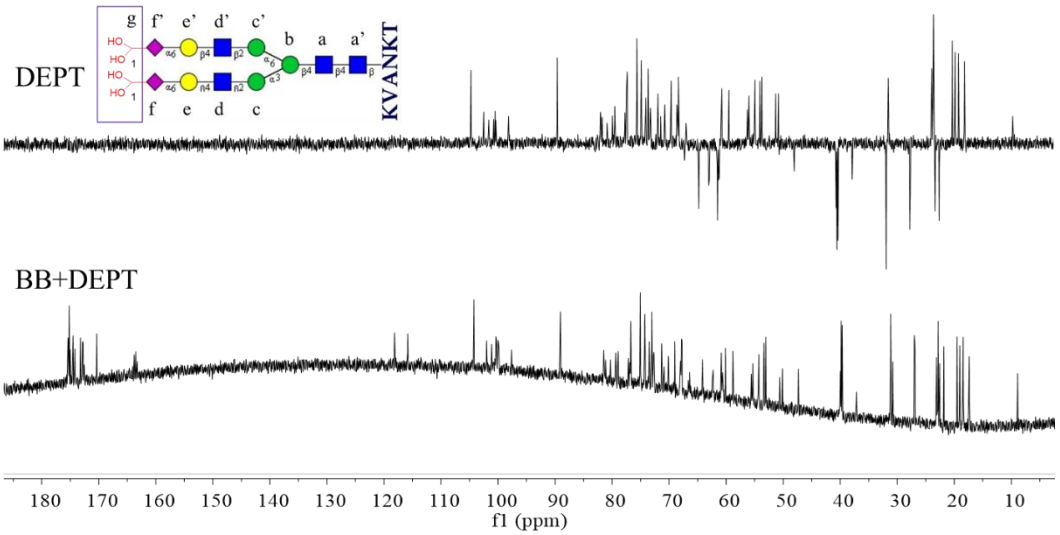


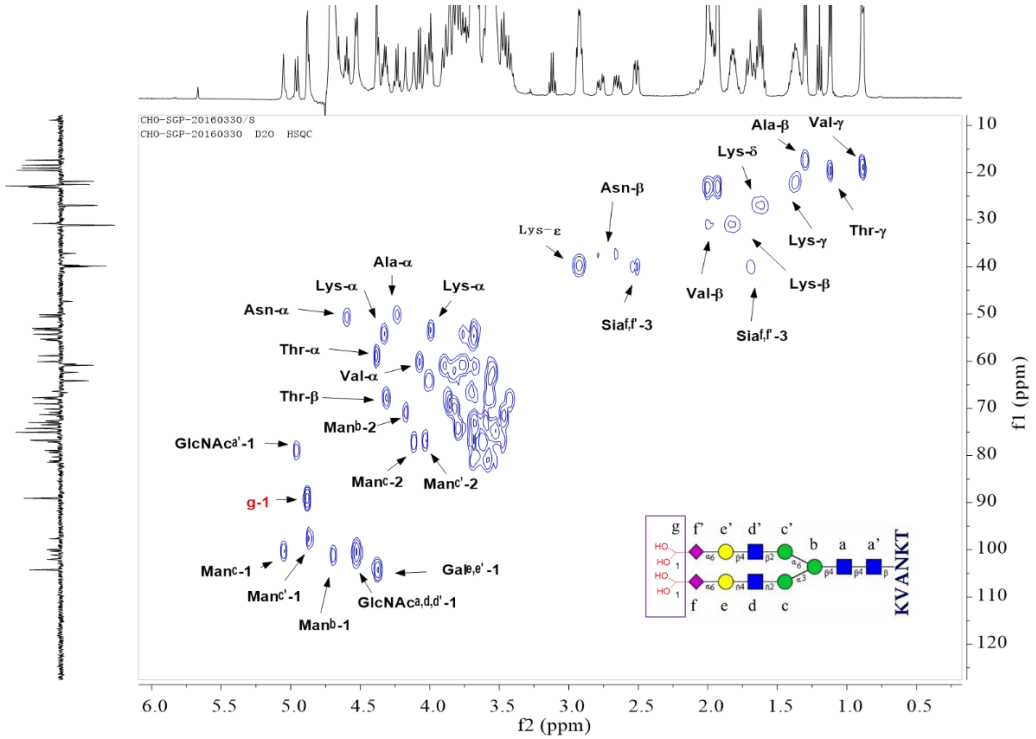
^{1}H NMR of **5**



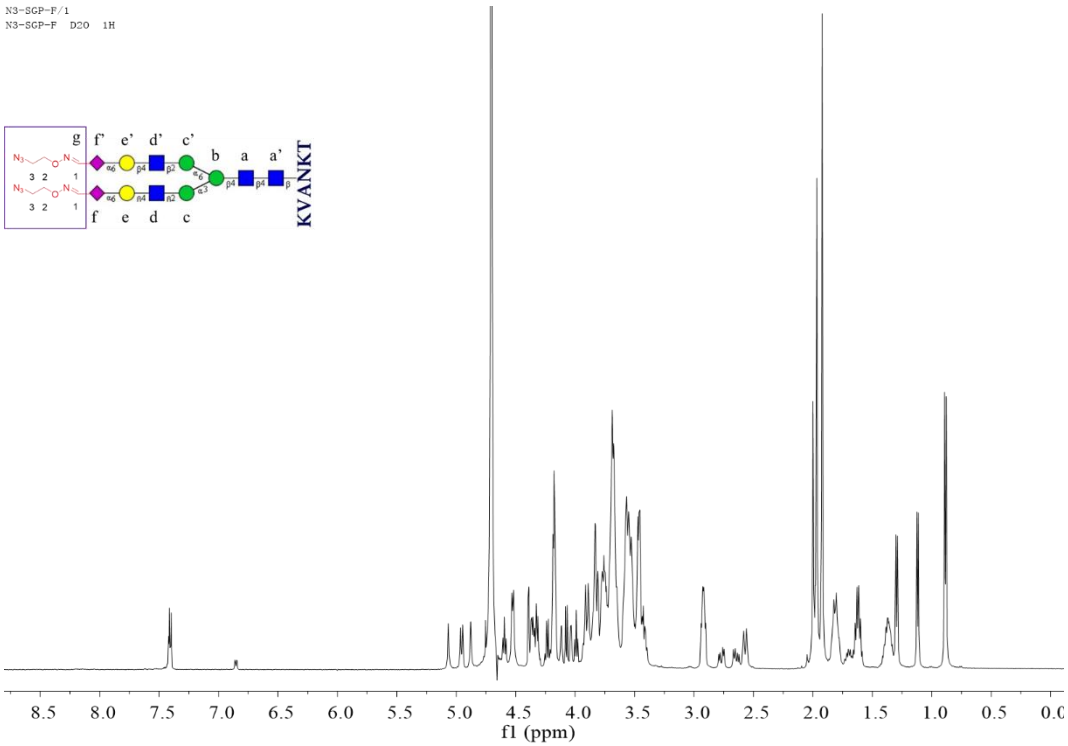
^{1}H NMR of CHO-SGP (**8**)



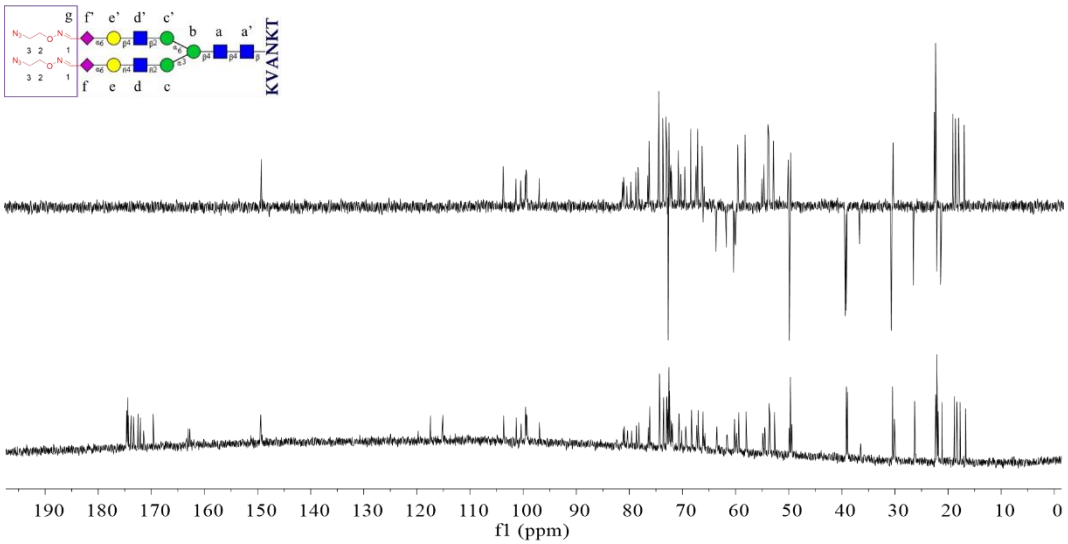
^{13}C NMR of CHO-SGP (**8**)



HSQC of CHO-SGP (**8**)



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



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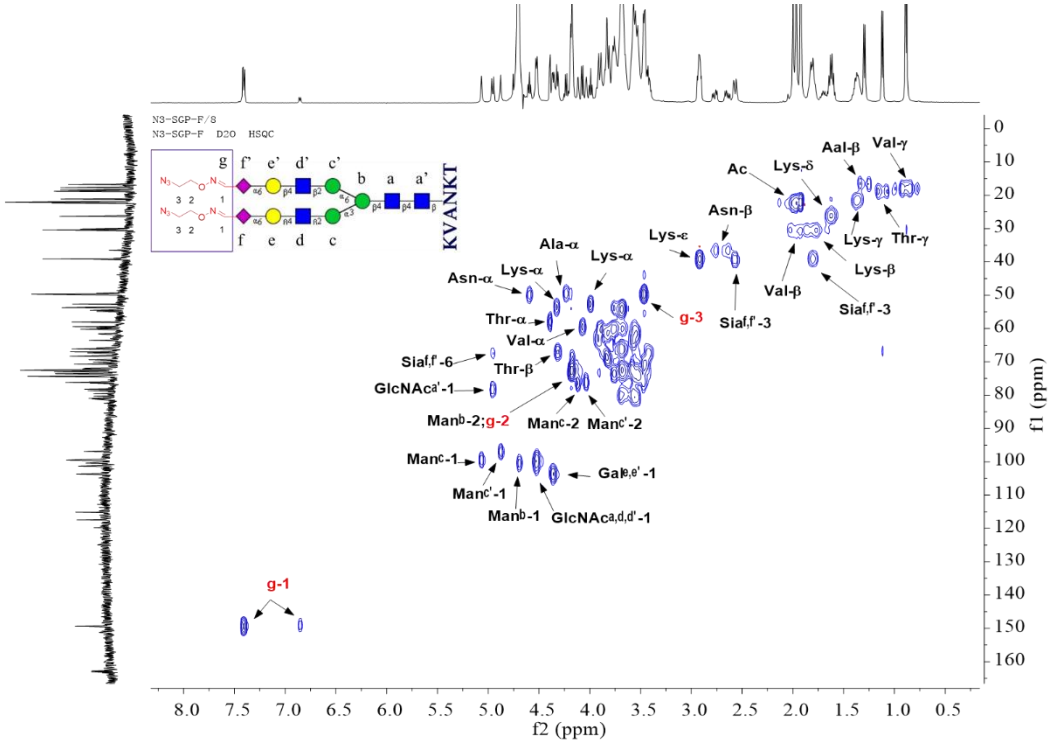

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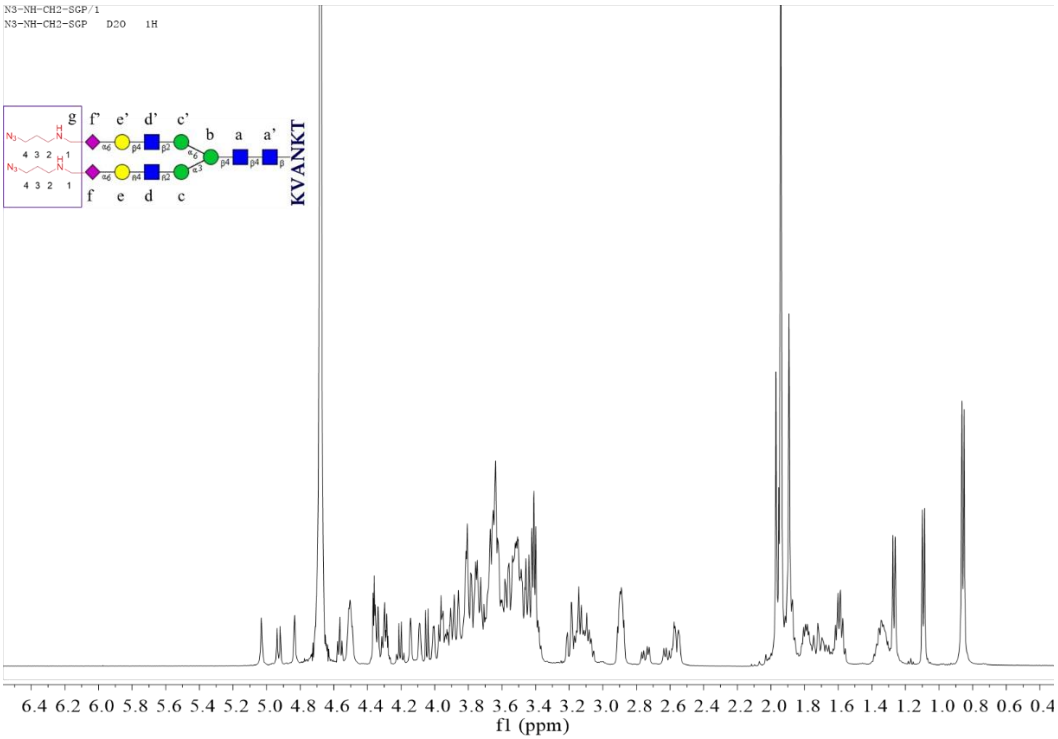
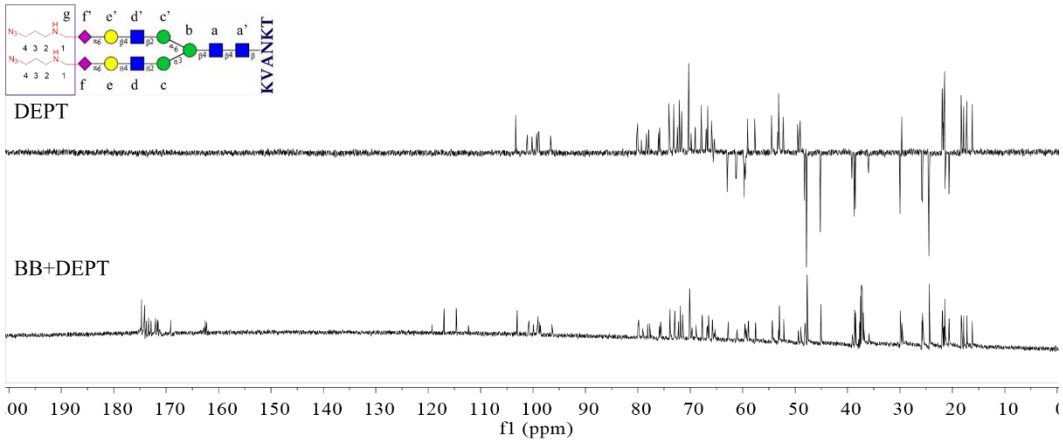


^{1}H NMR of **11b**



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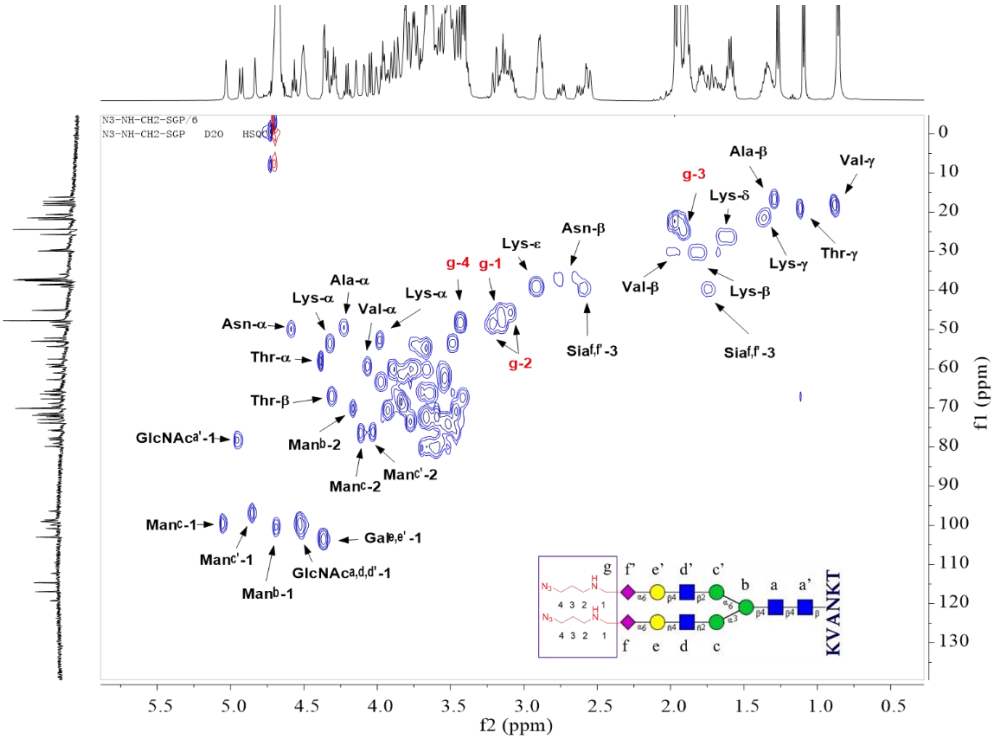

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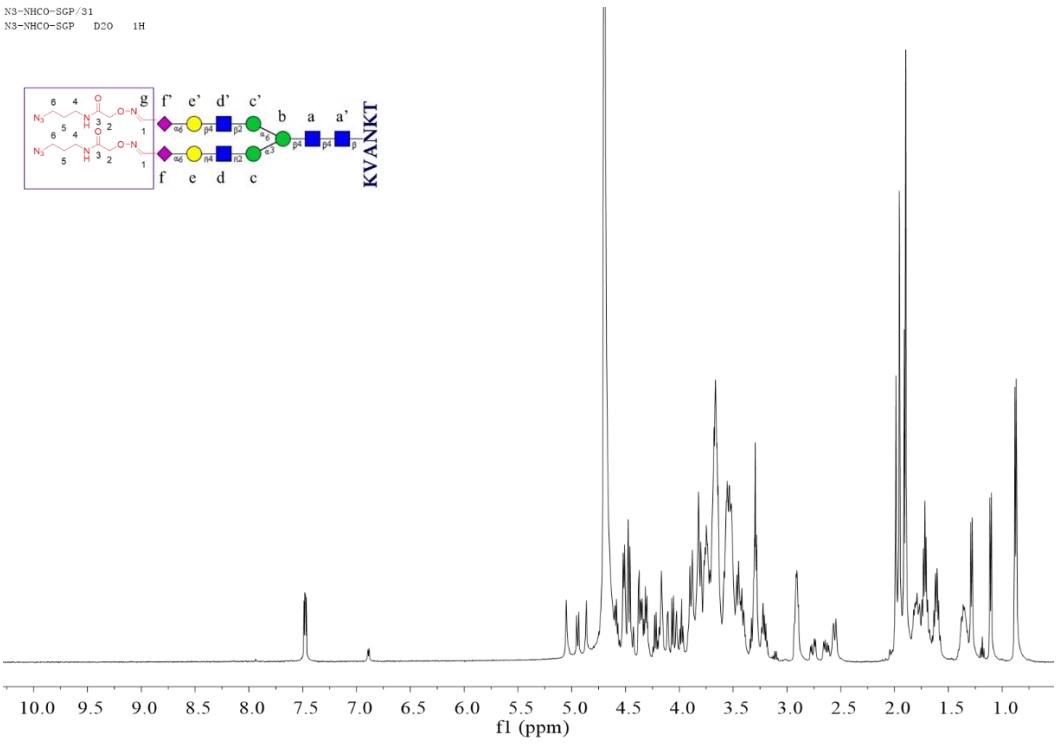

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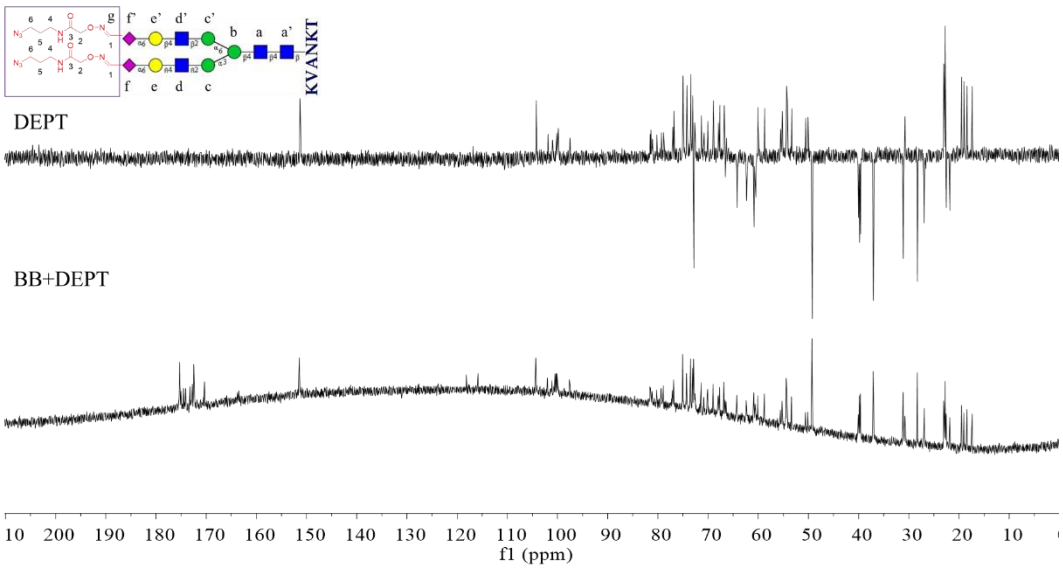

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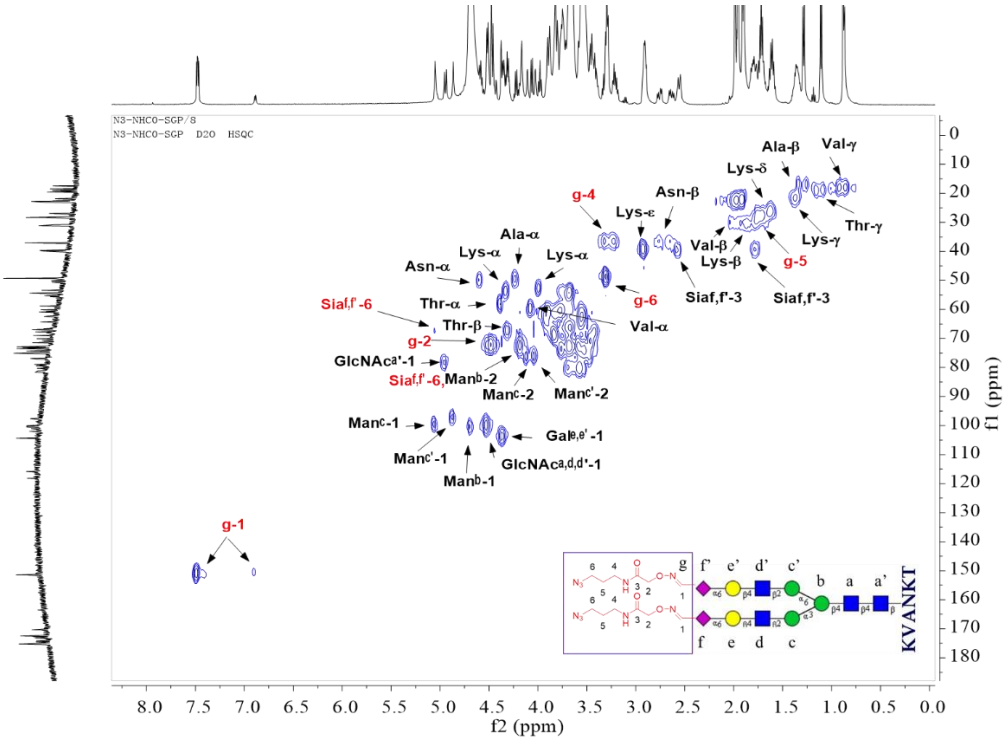

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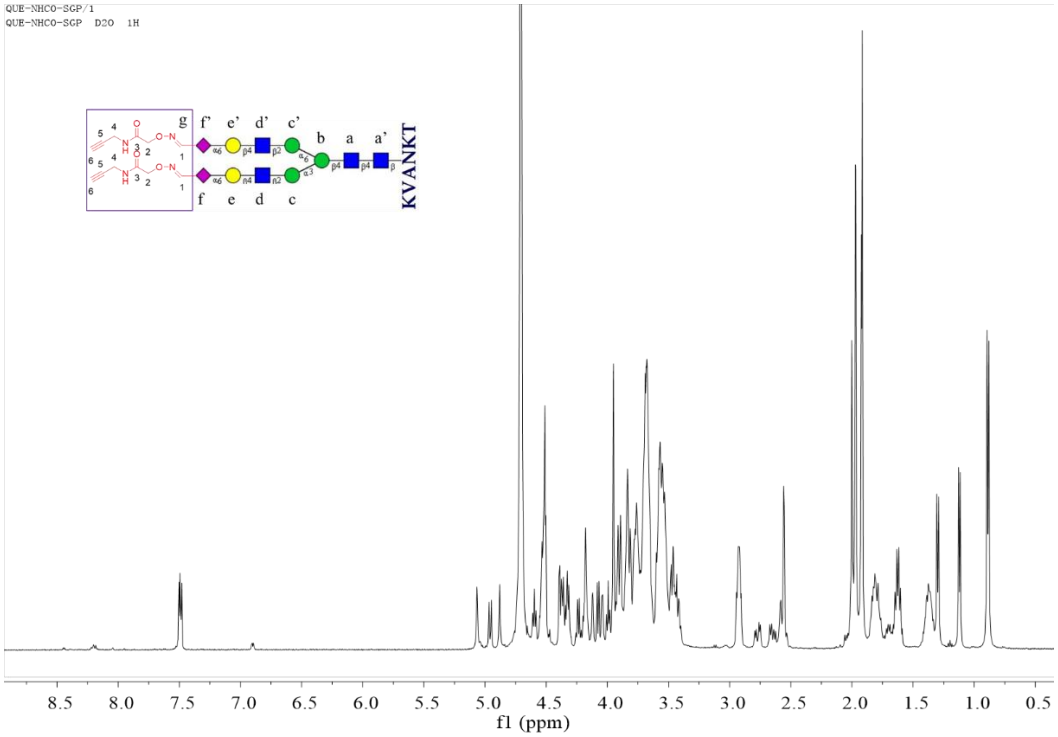

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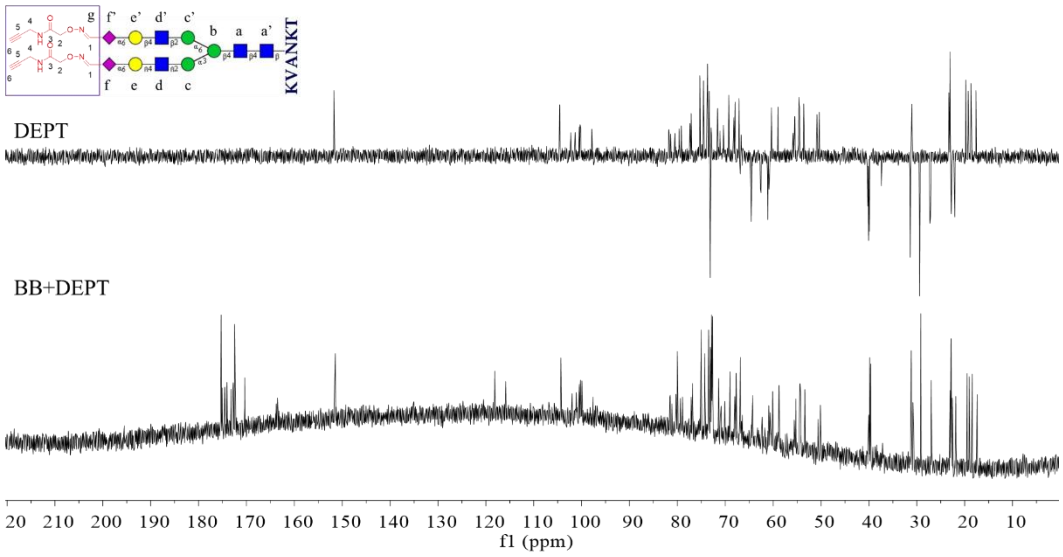

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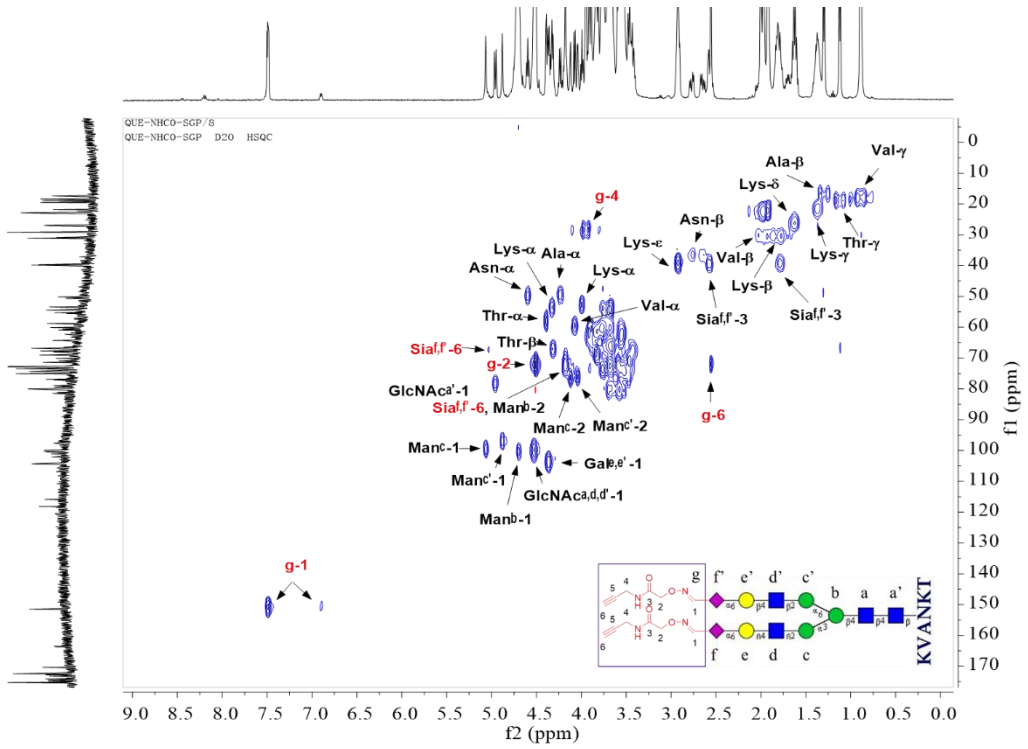

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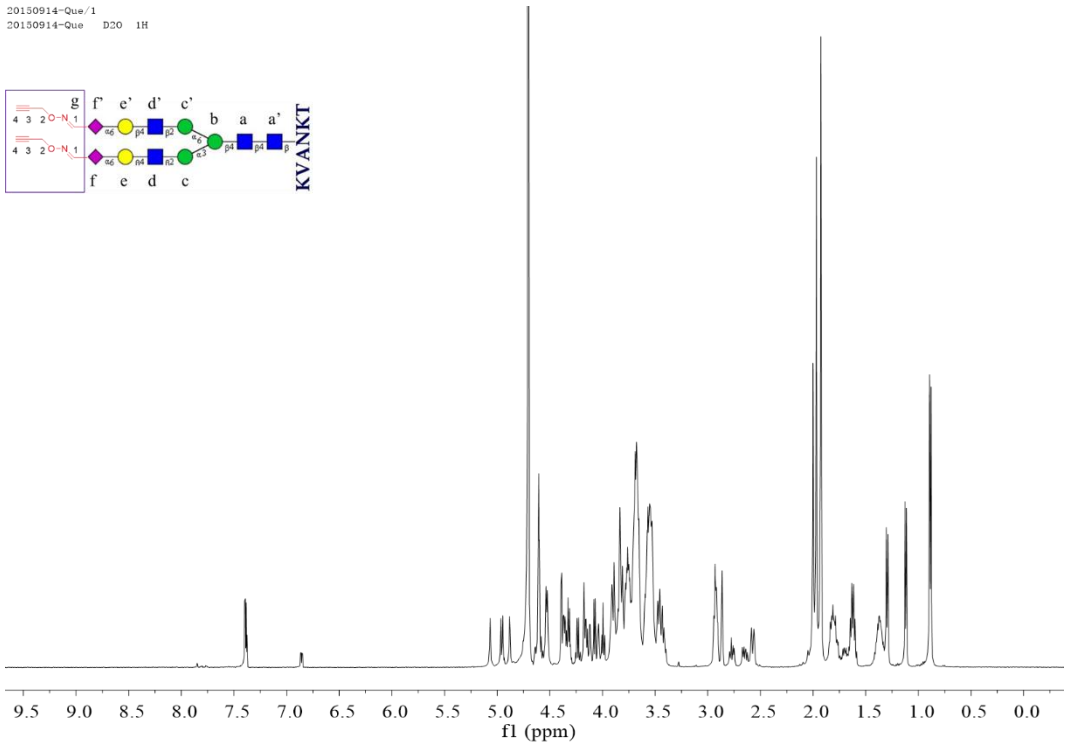

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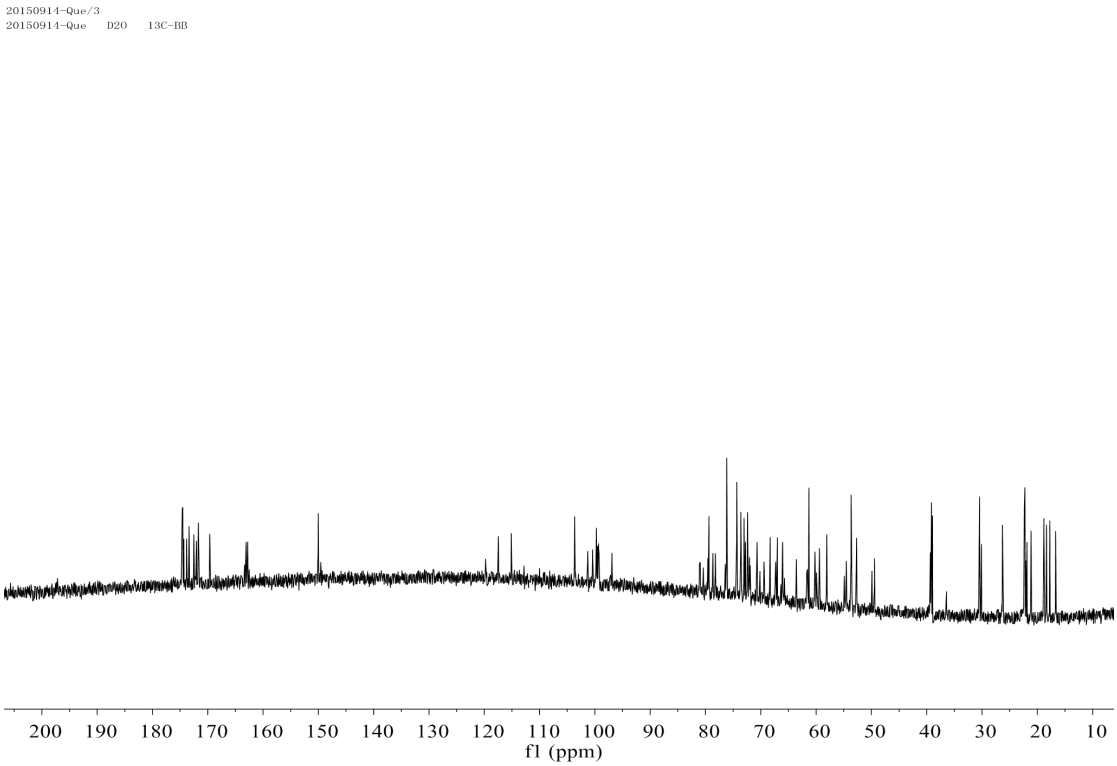
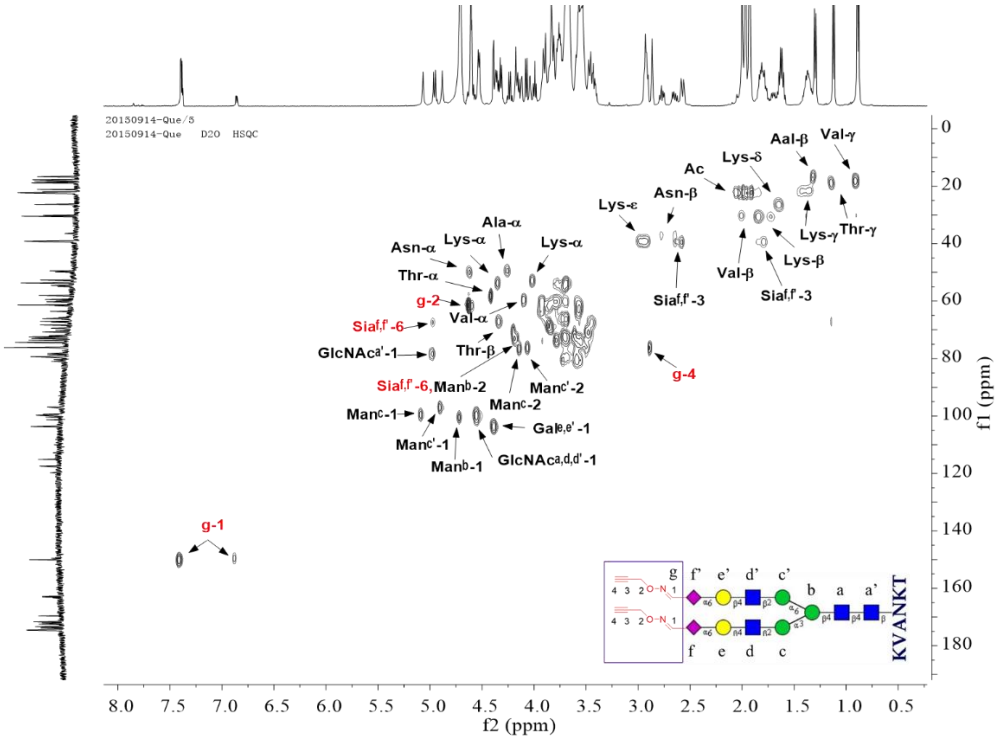
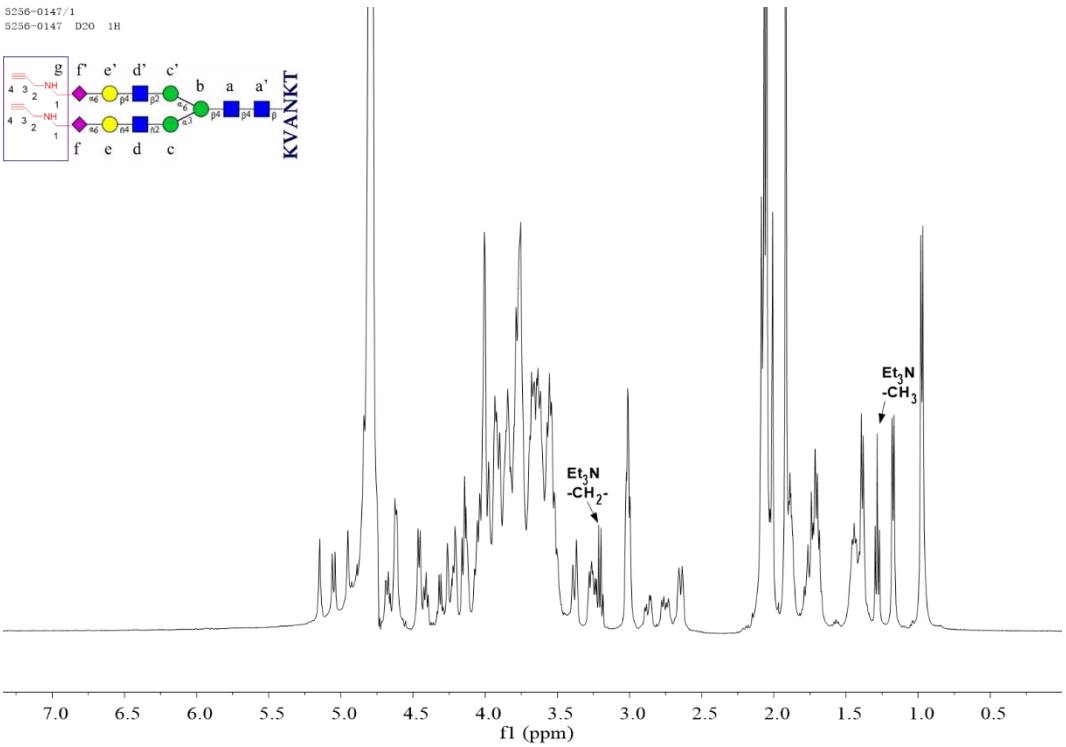


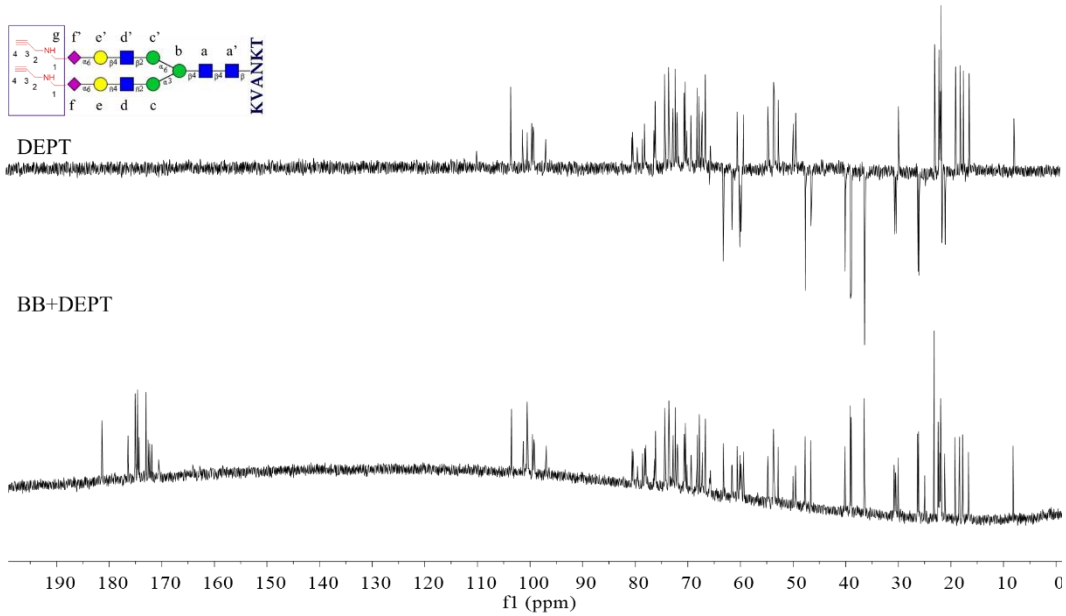
^{13}C NMR of **11e**



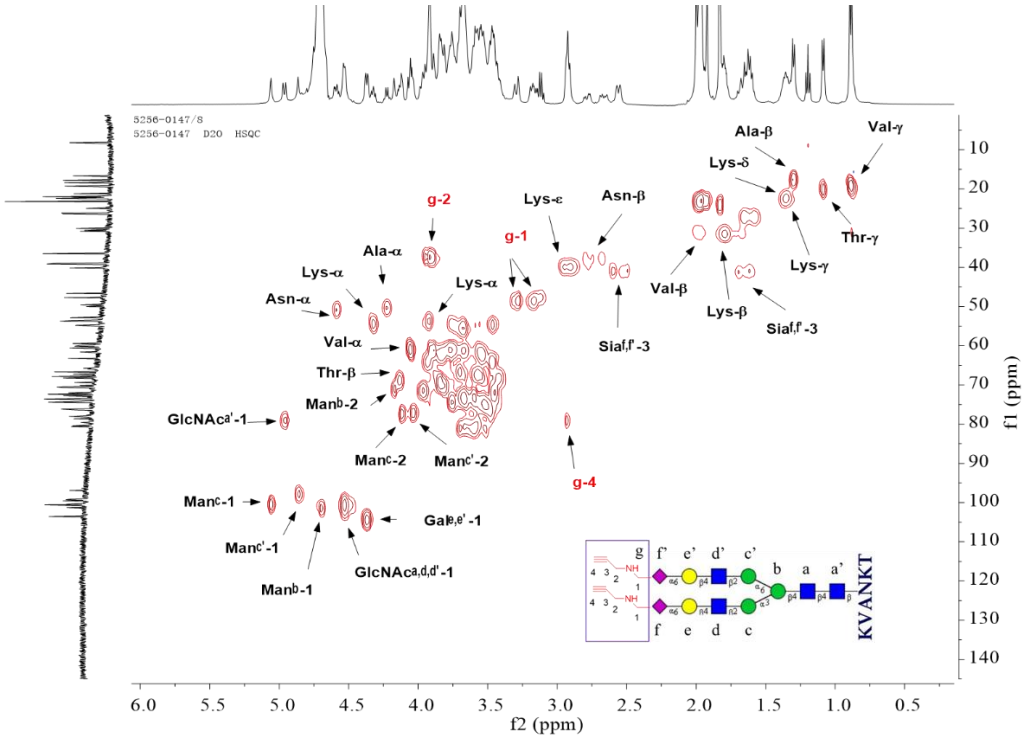
^{1}H-^{13}C HSQC NMR of **11e**



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

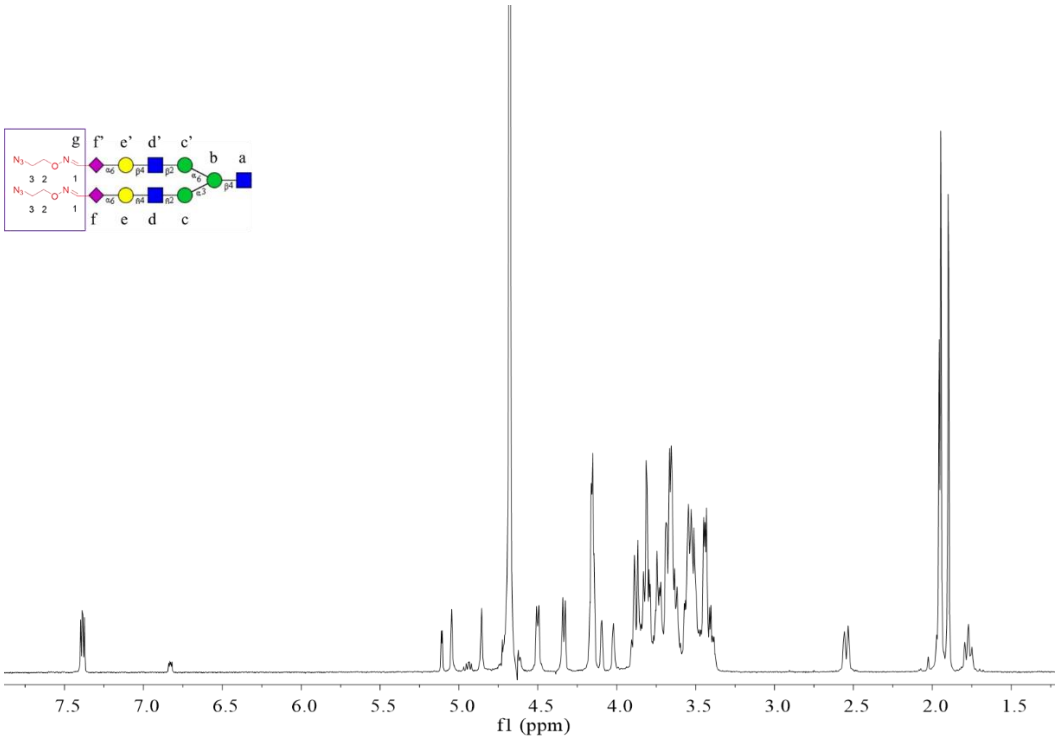


^{13}C NMR of **11f**

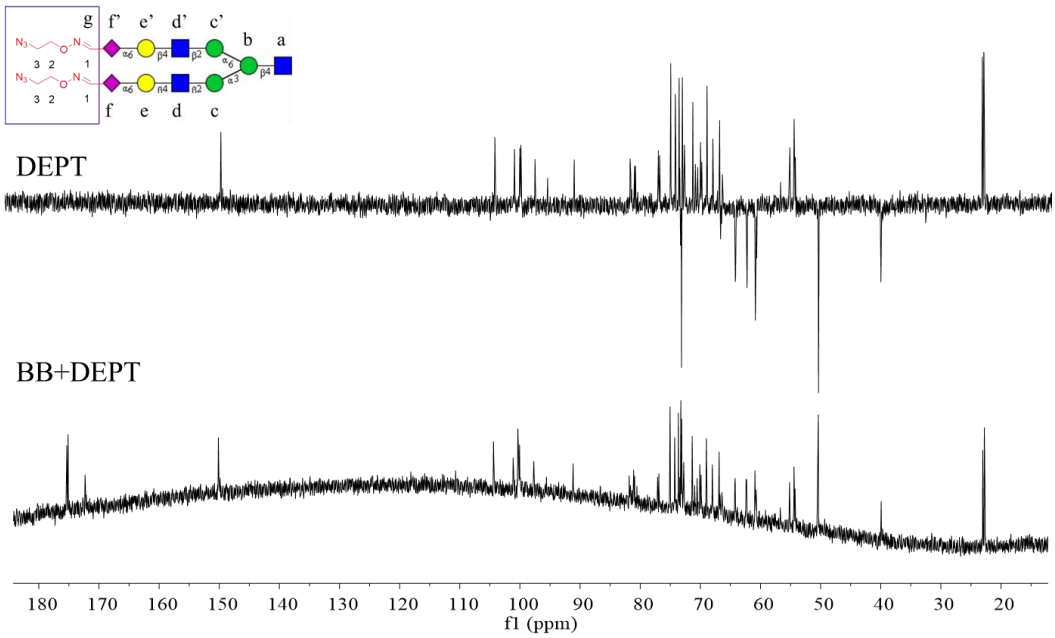


^{1}H-^{13}C HSQC NMR of **11f**

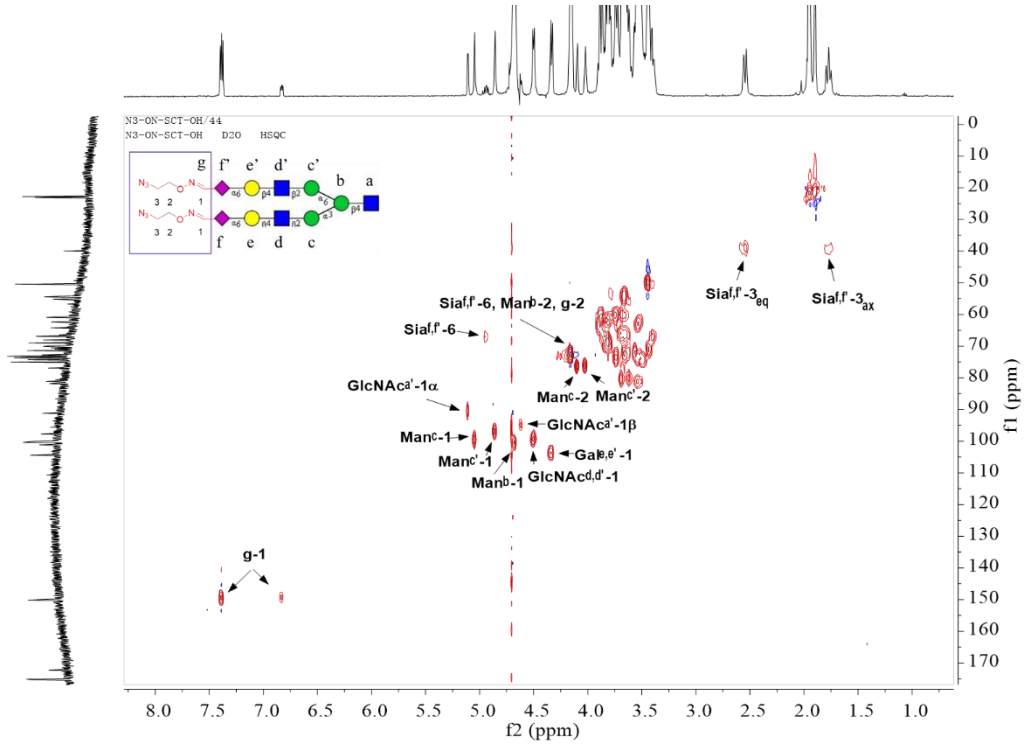
NMRs of N3-SCT (12) and N3-SCT-ox (13)



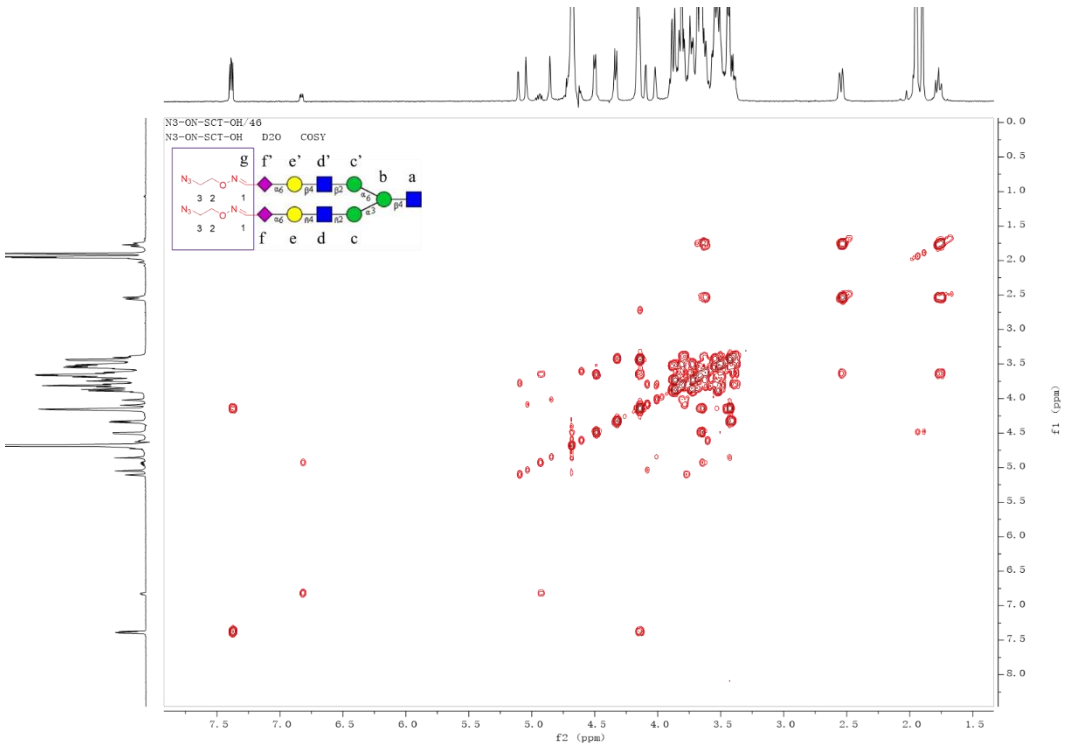
^{1}H NMR of **12**



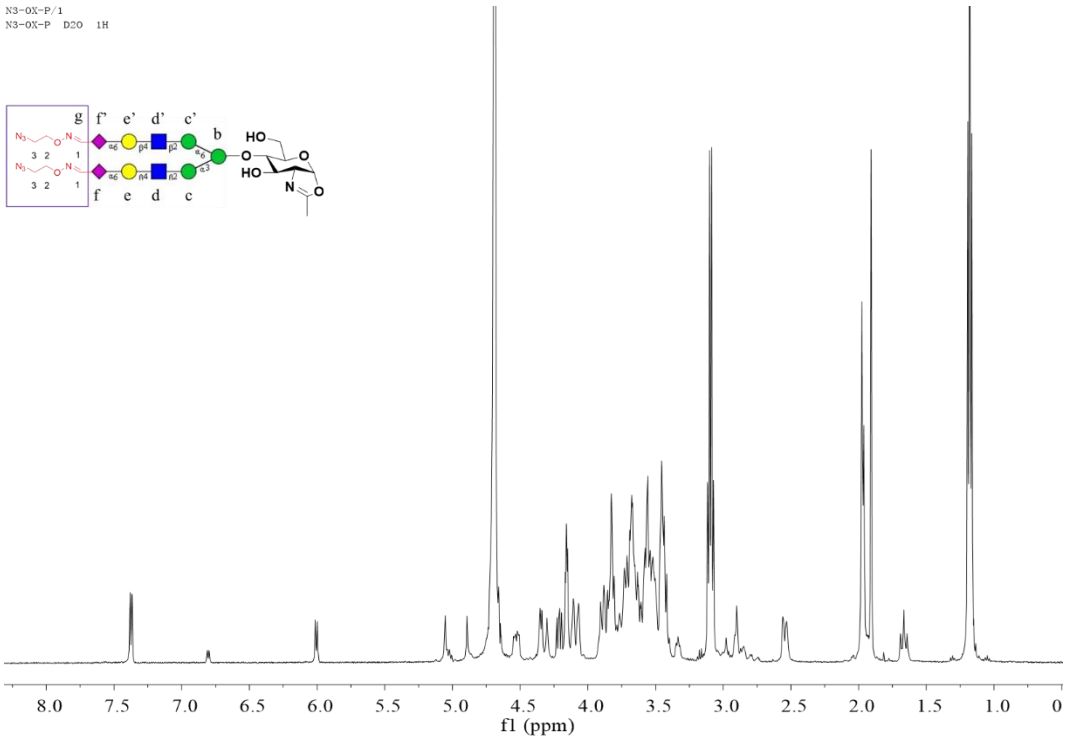
^{13}C NMR of **12**



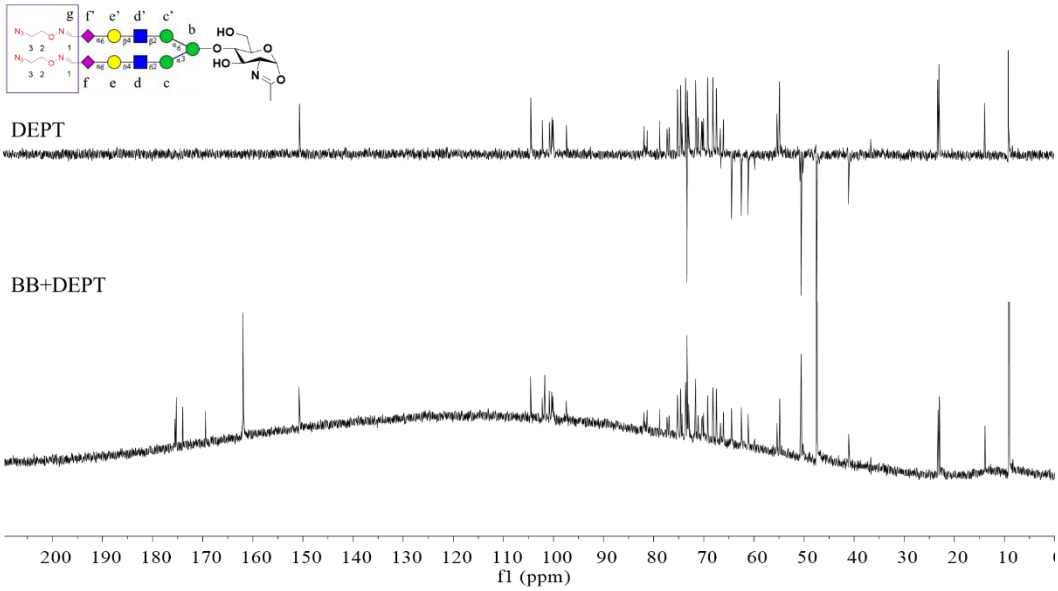
^{1}H-^{13}C HSQC NMR of **12**



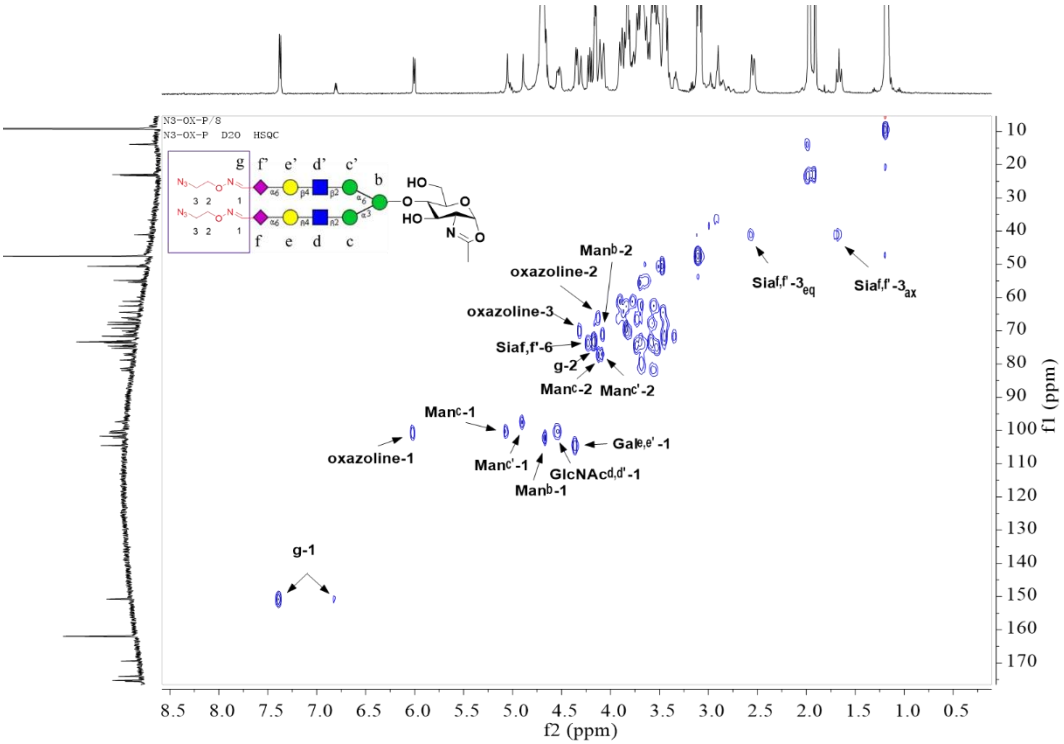
^{1}H-H COSY NMR of **12**



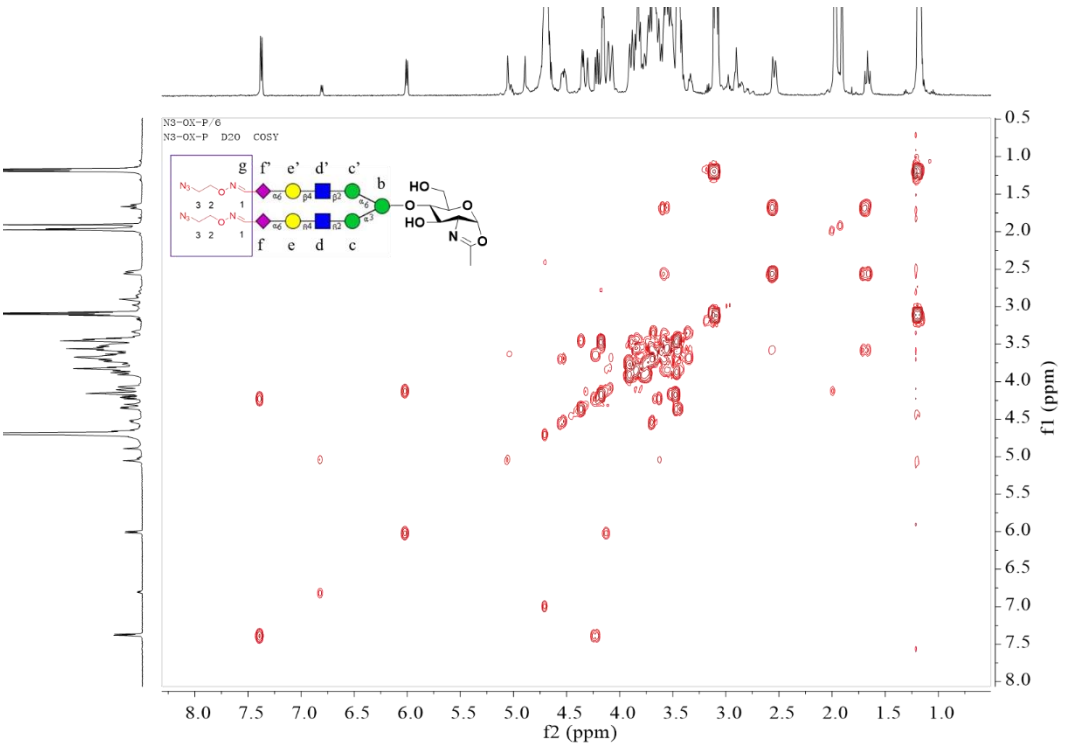
^{1}H NMR of **13**



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

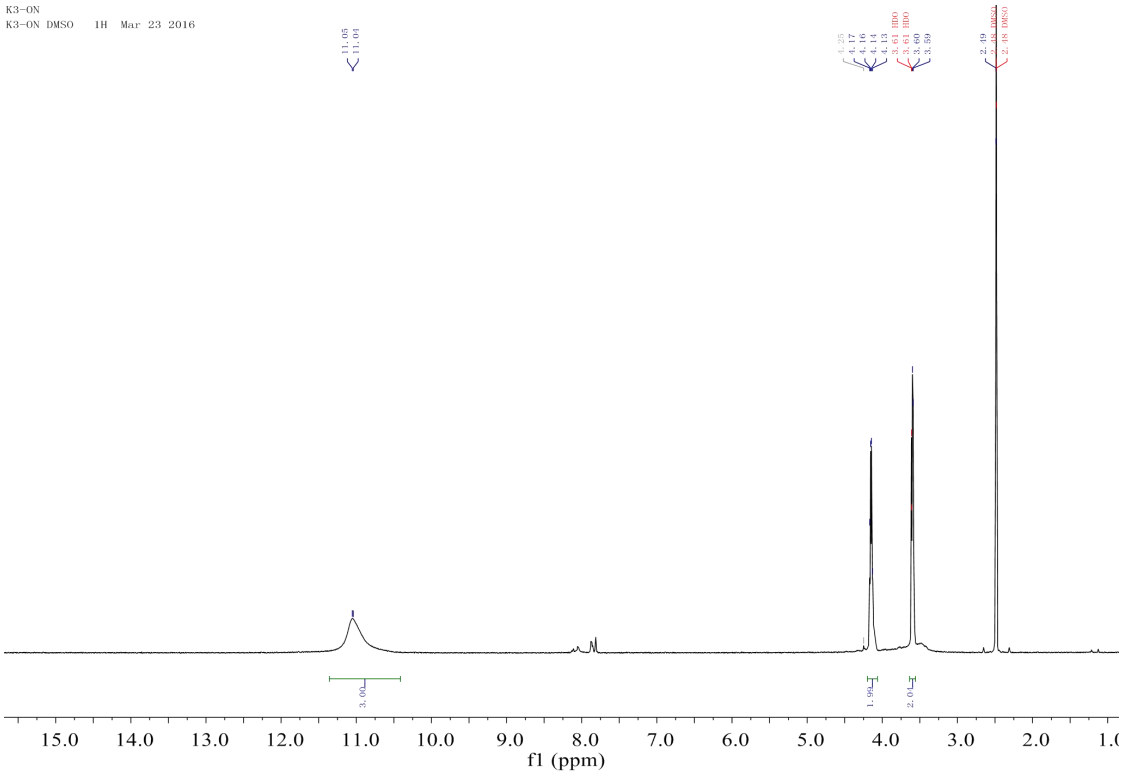


^{1}H-^{13}C HSQC NMR of **13**

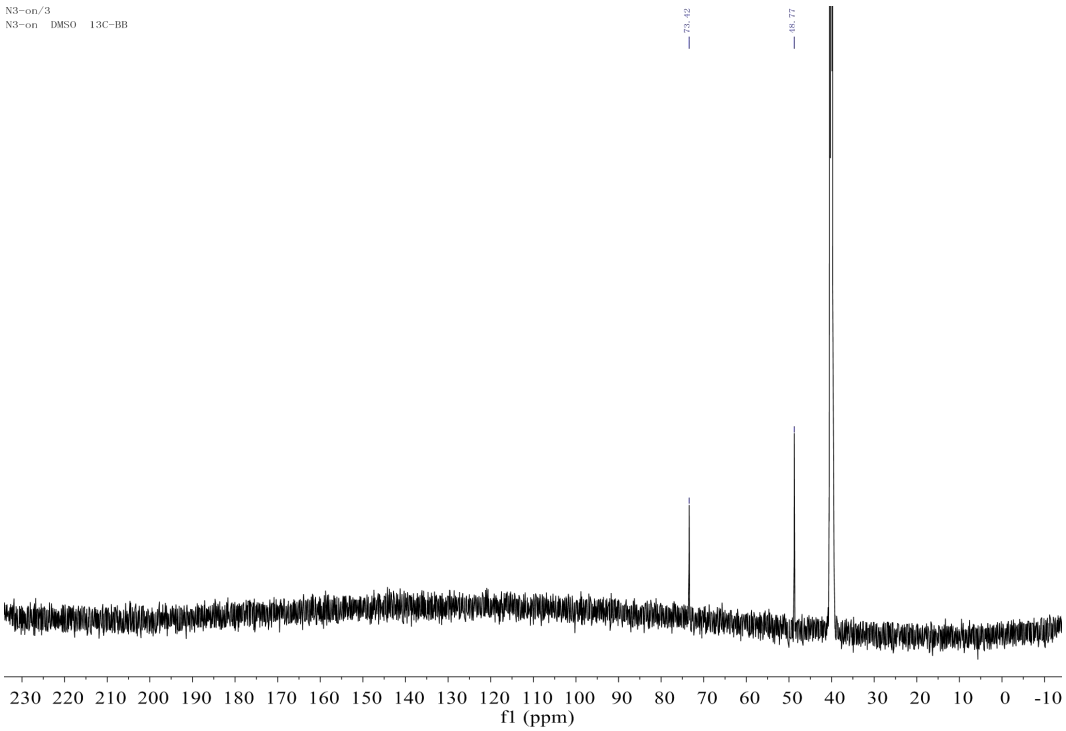


^{1}H-H COSY NMR of **13**

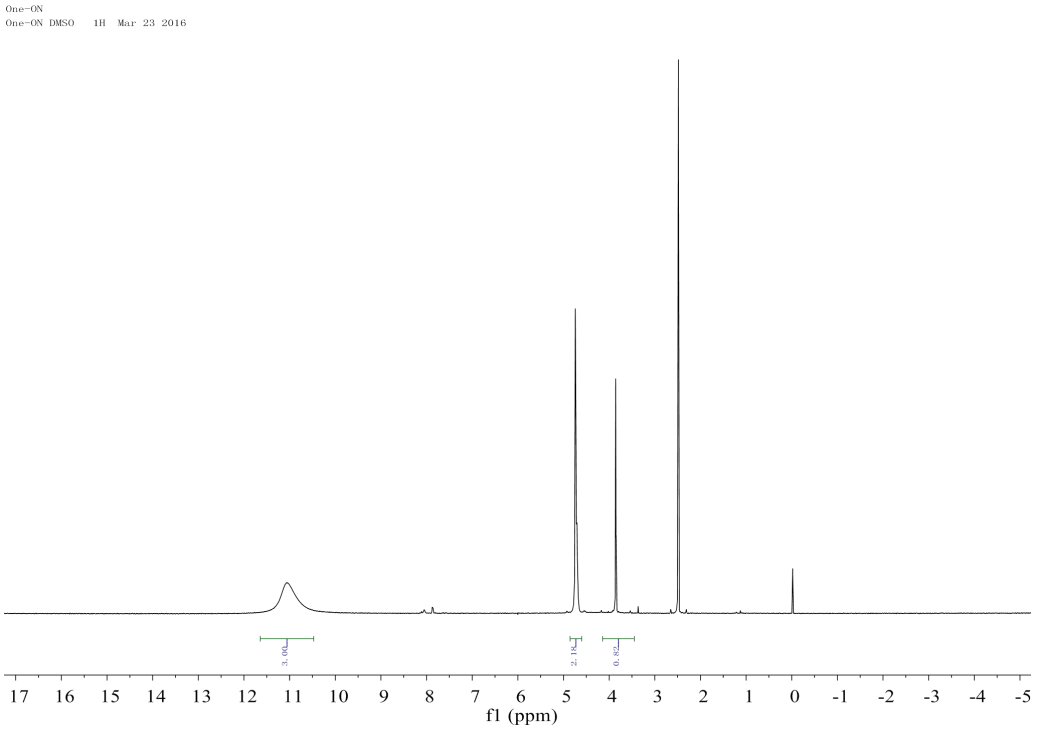
NMRs of the small molecules



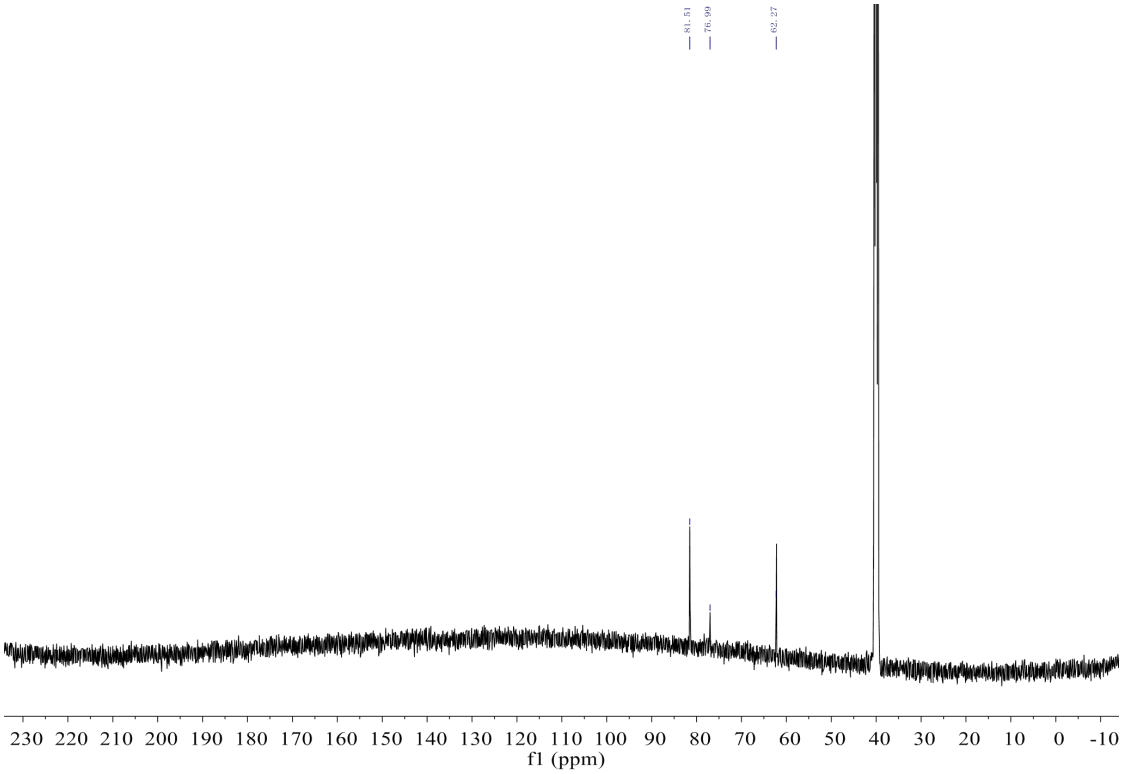
^{1}H NMR of **10a**



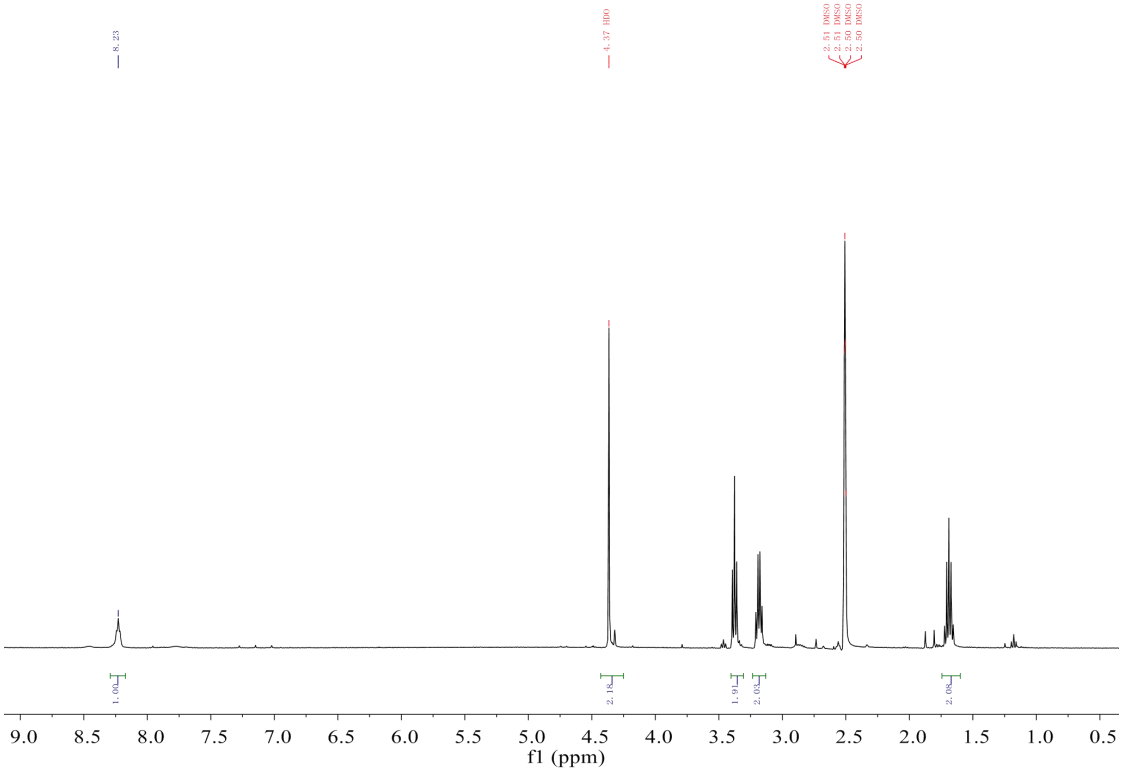
^{13}C NMR of **10a**



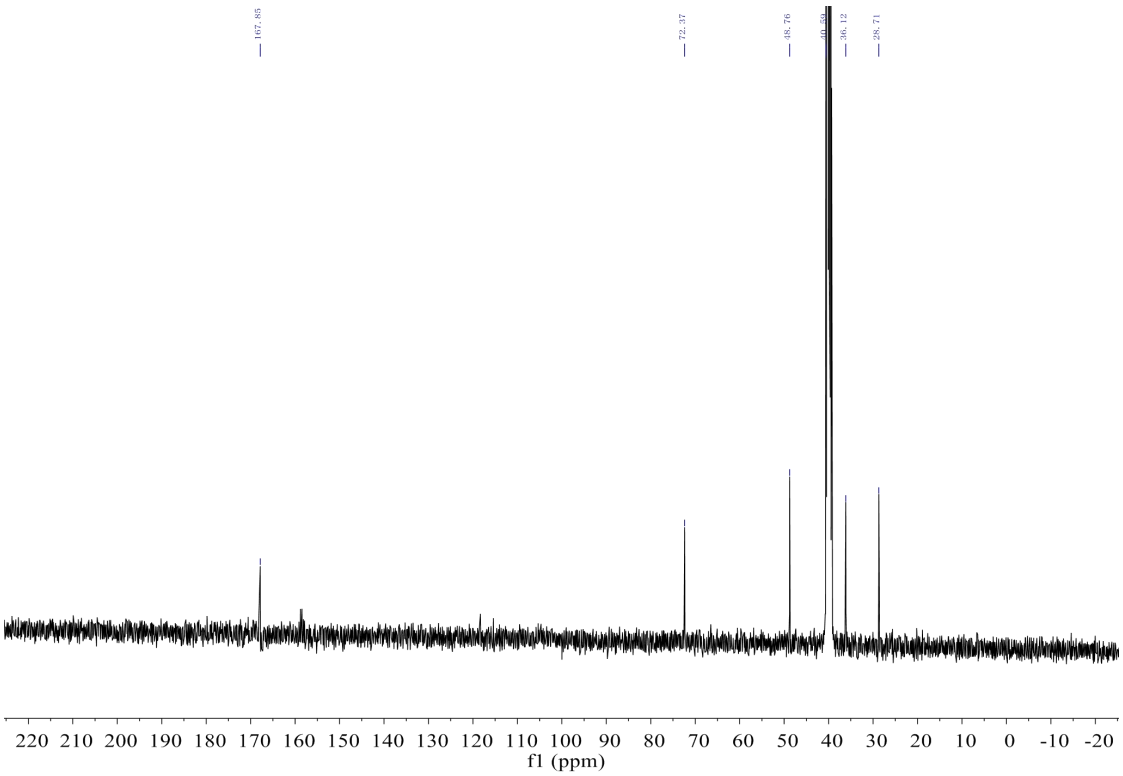
^{1}H NMR of **10b**



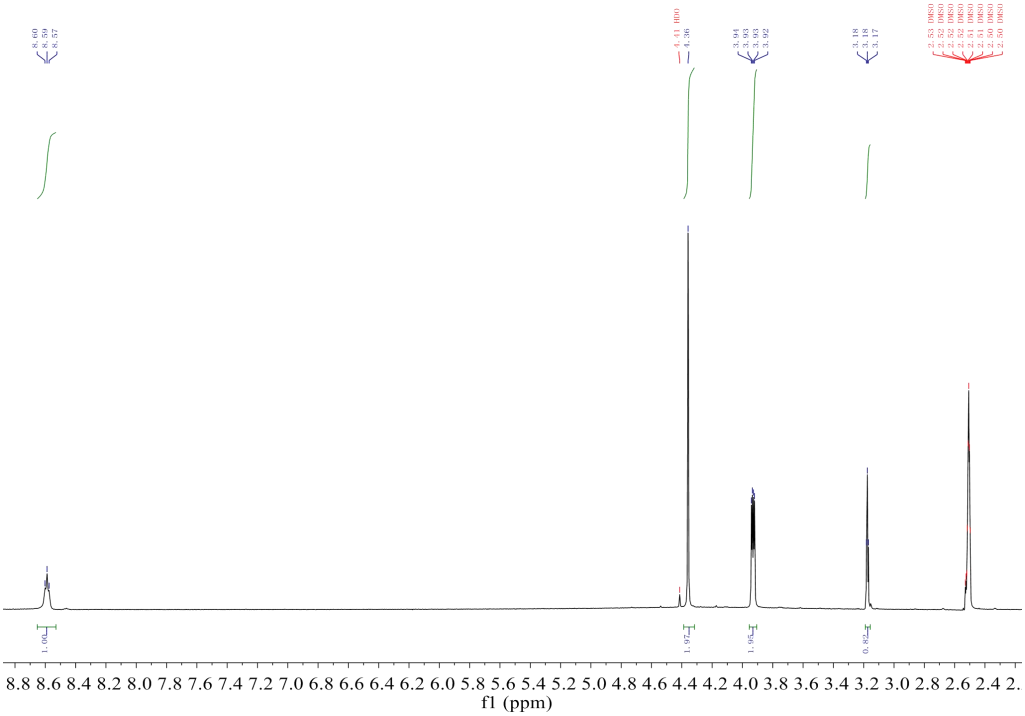
^{13}C NMR of **10b**



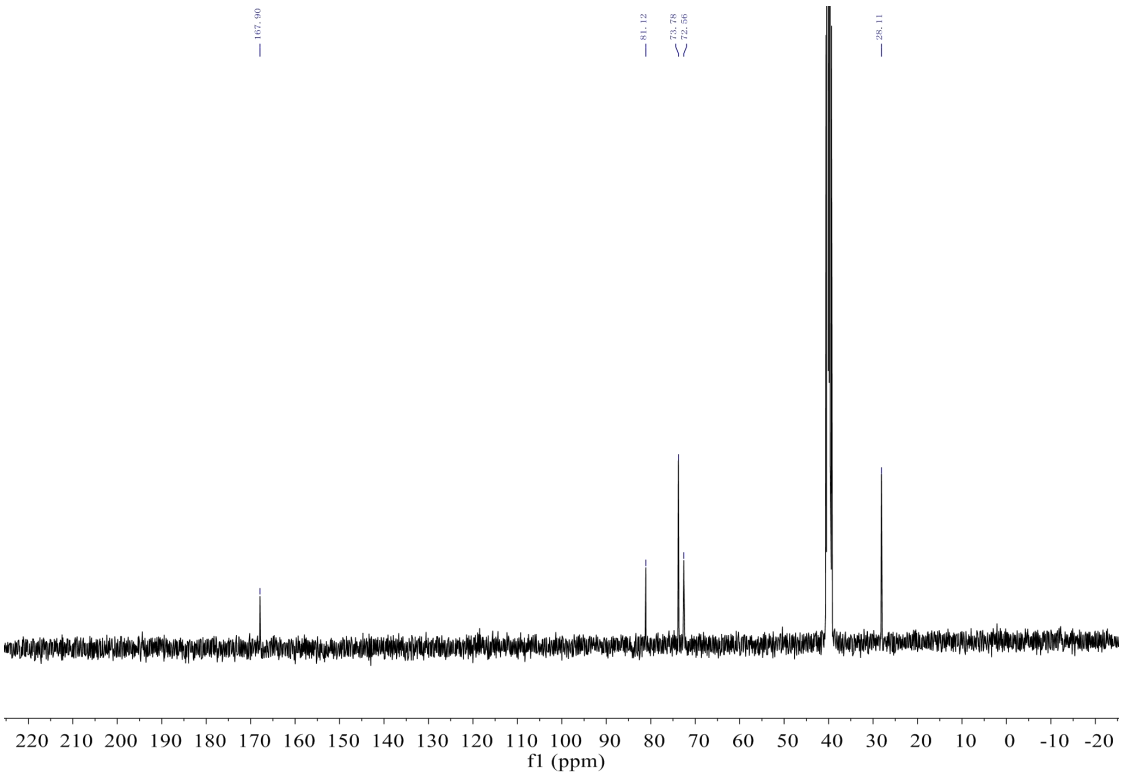
^{1}H NMR of **10c**



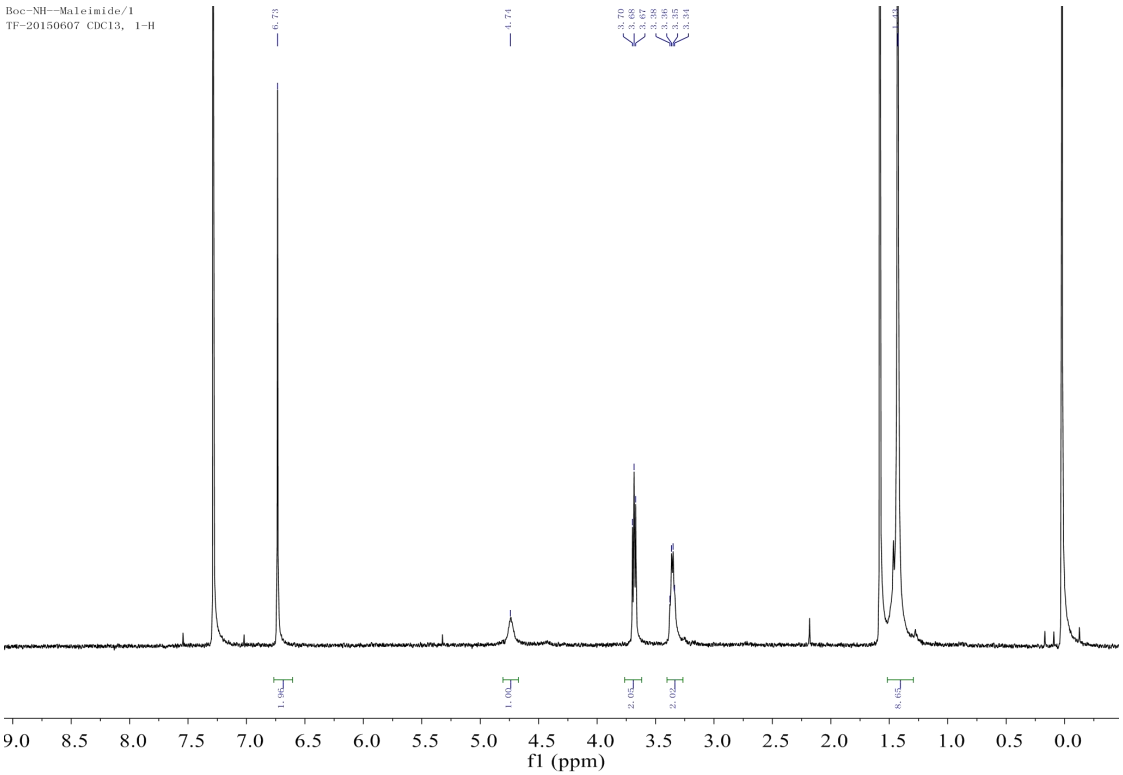
^{13}C NMR **10c**



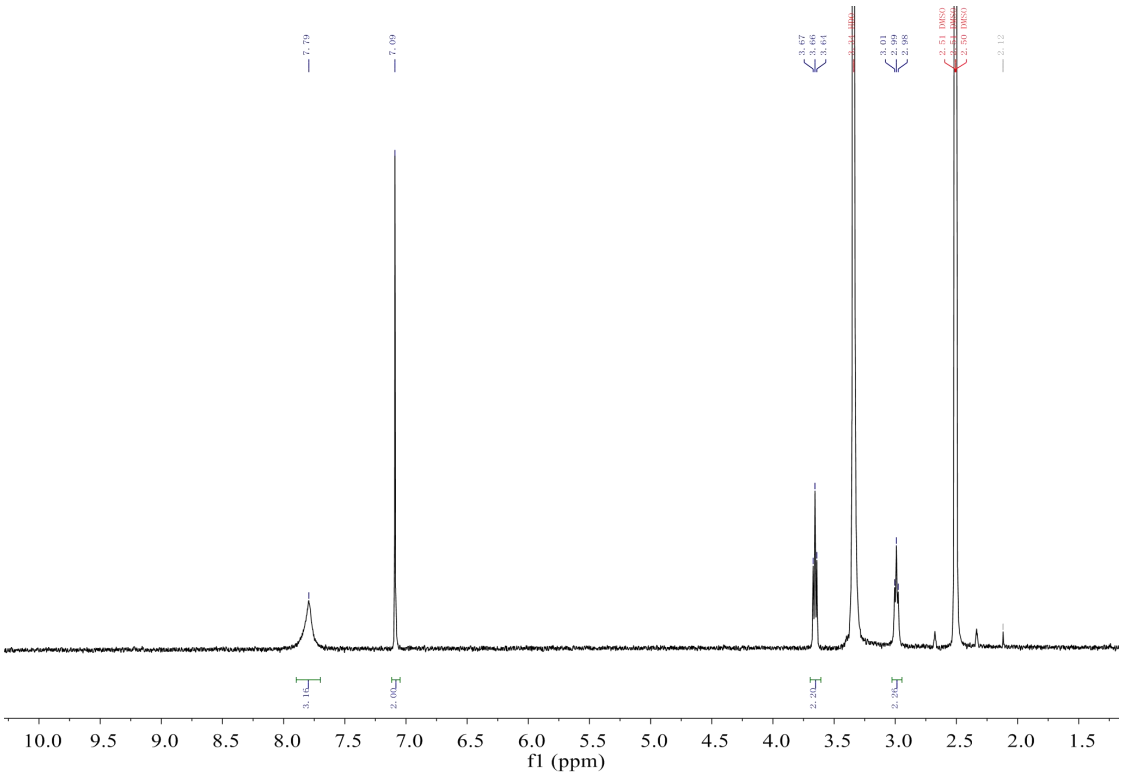
^{1}H NMR of **10d**



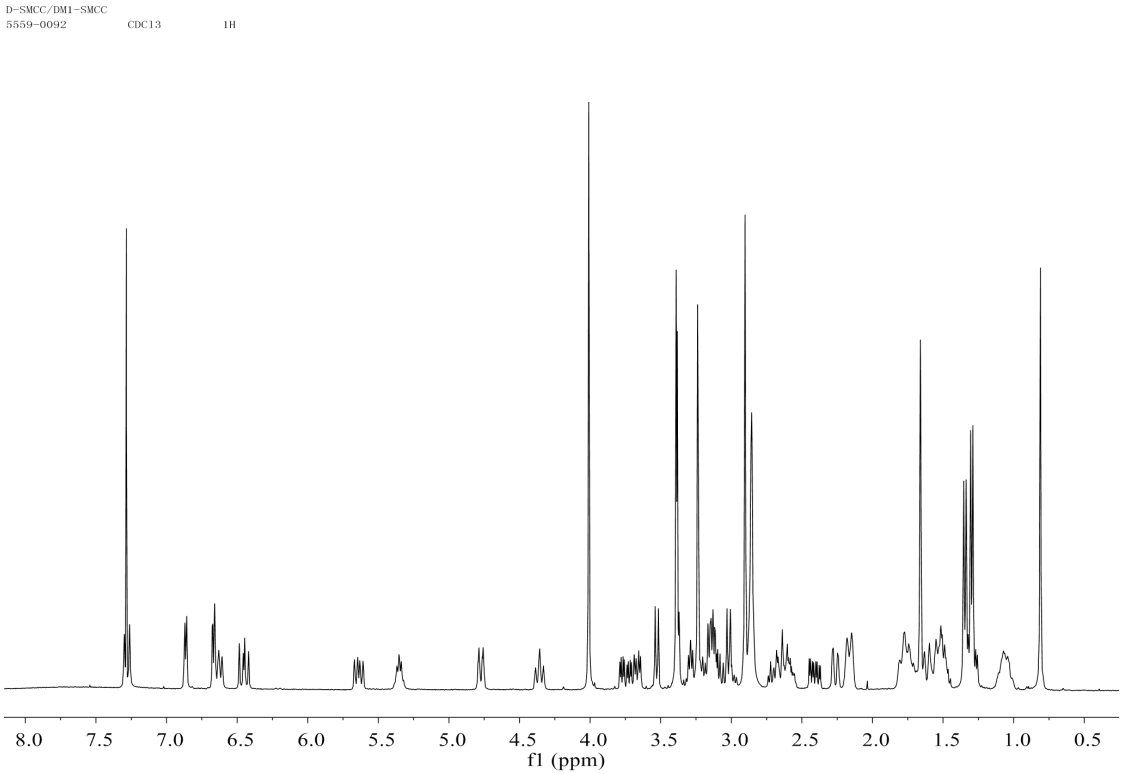
^{13}C NMR of **10d**



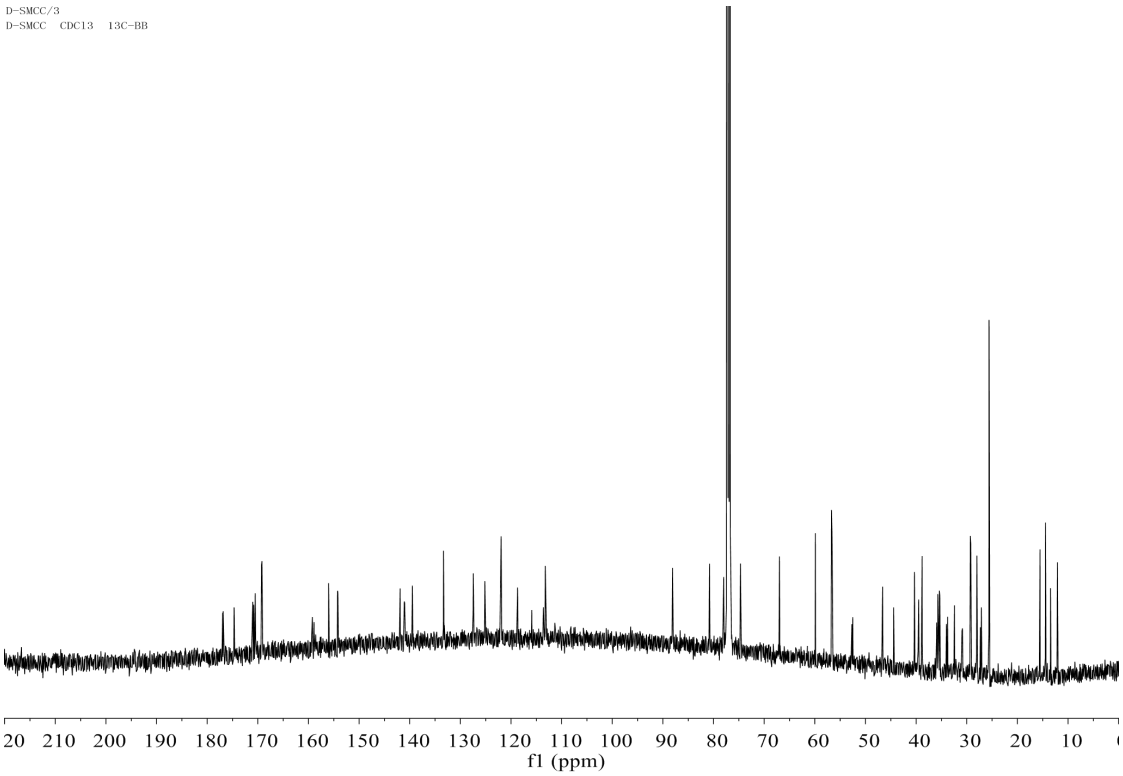
^{1}H NMR of **S8**



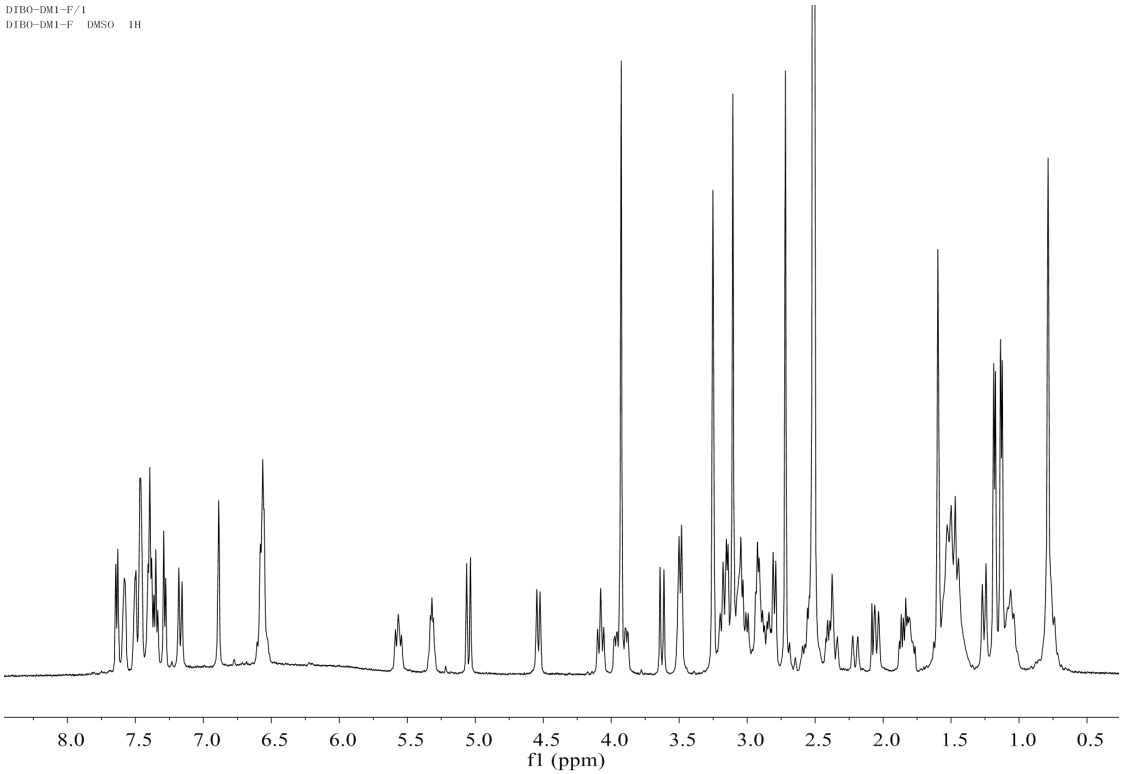
^{1}H NMR of **S9**



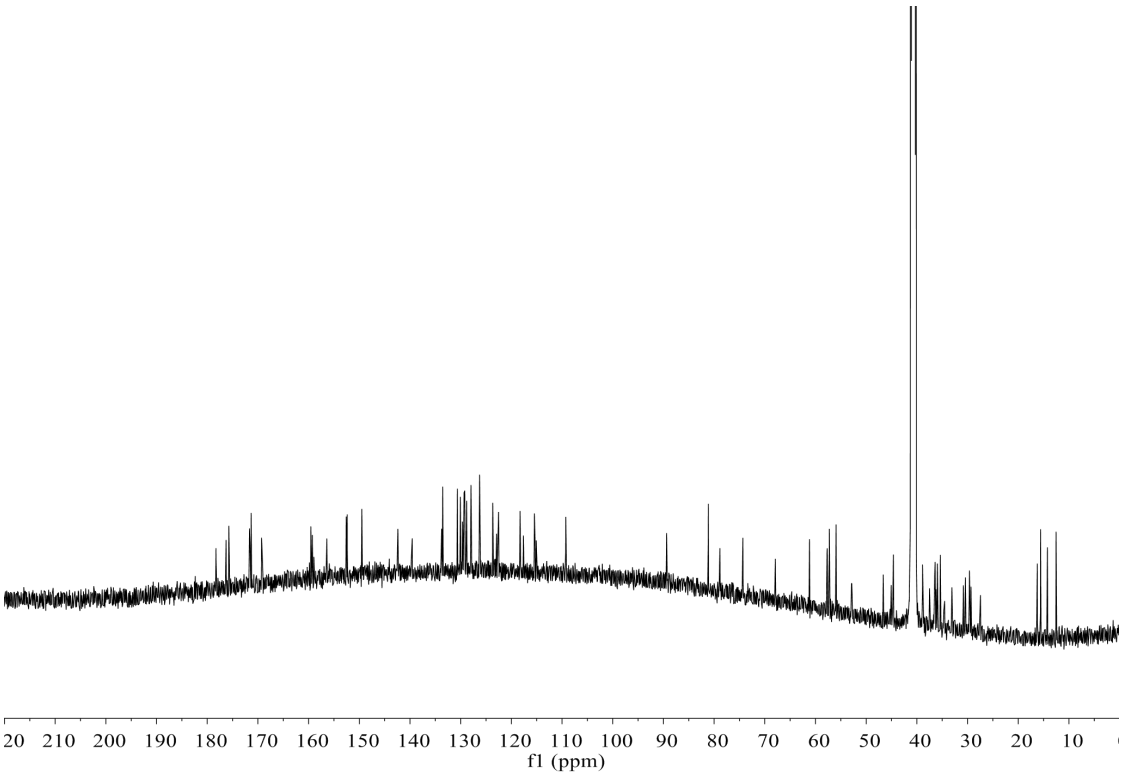
^{1}H NMR of **15**



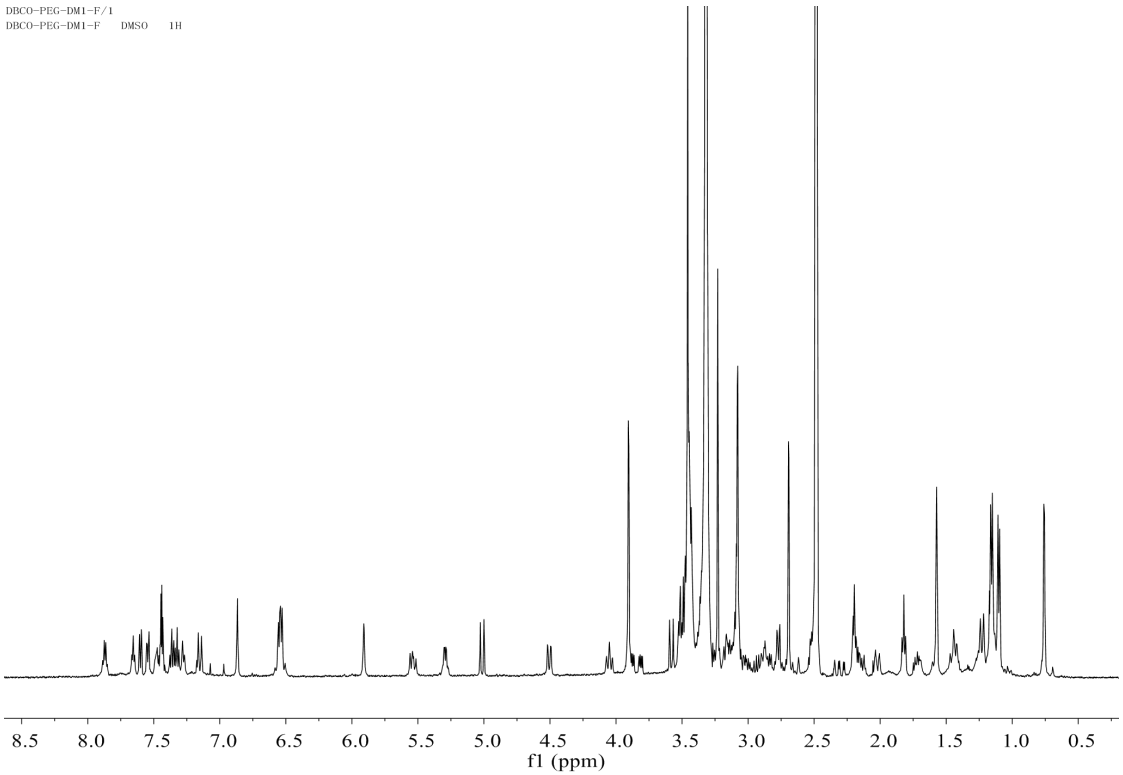
^{13}C NMR **15**



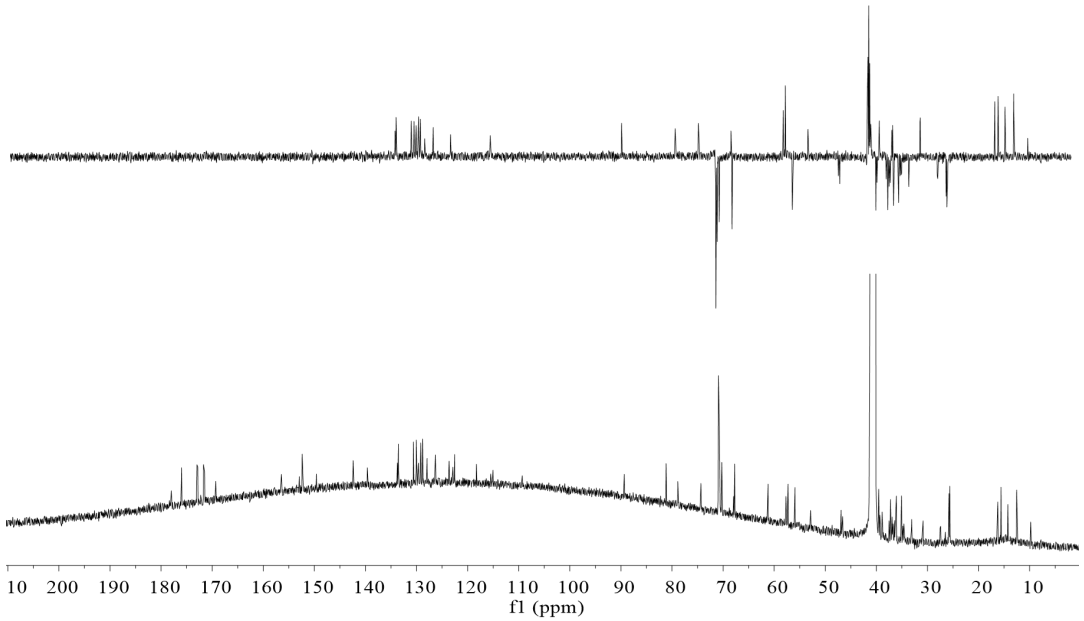
^{1}H NMR of **21a**



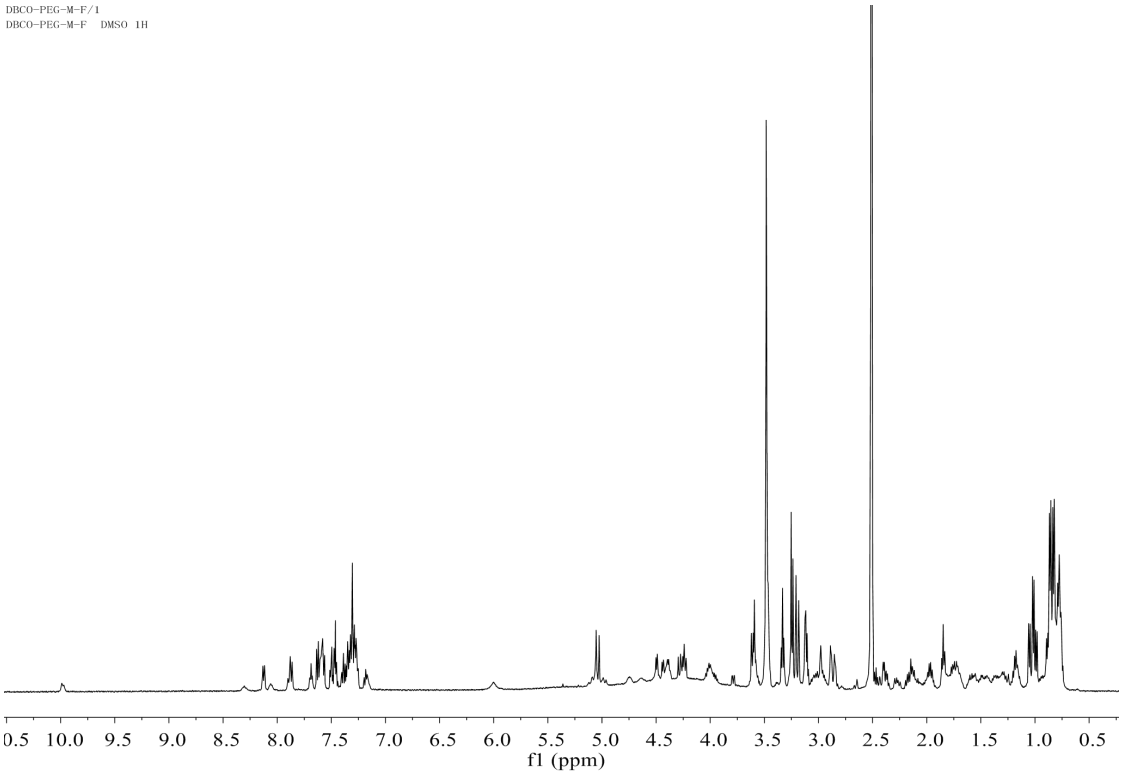
^{13}C NMR **21a**



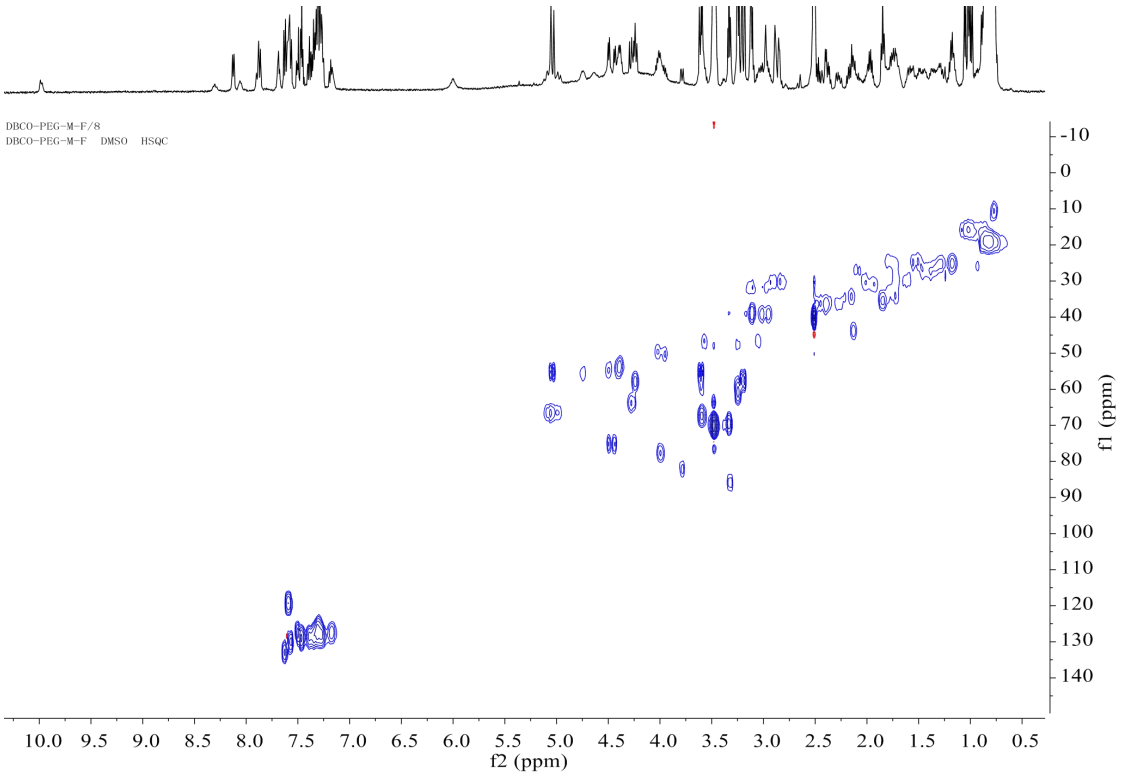
^{1}H NMR **21b**



^{13}C NMR **21b**



^{1}H NMR **21c**



HSQC **21c**

8. Reference

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