Supplementary Information

Glyco-engineering of natural killer cells with CD22 ligands for enhanced anti-cancer immunotherapy

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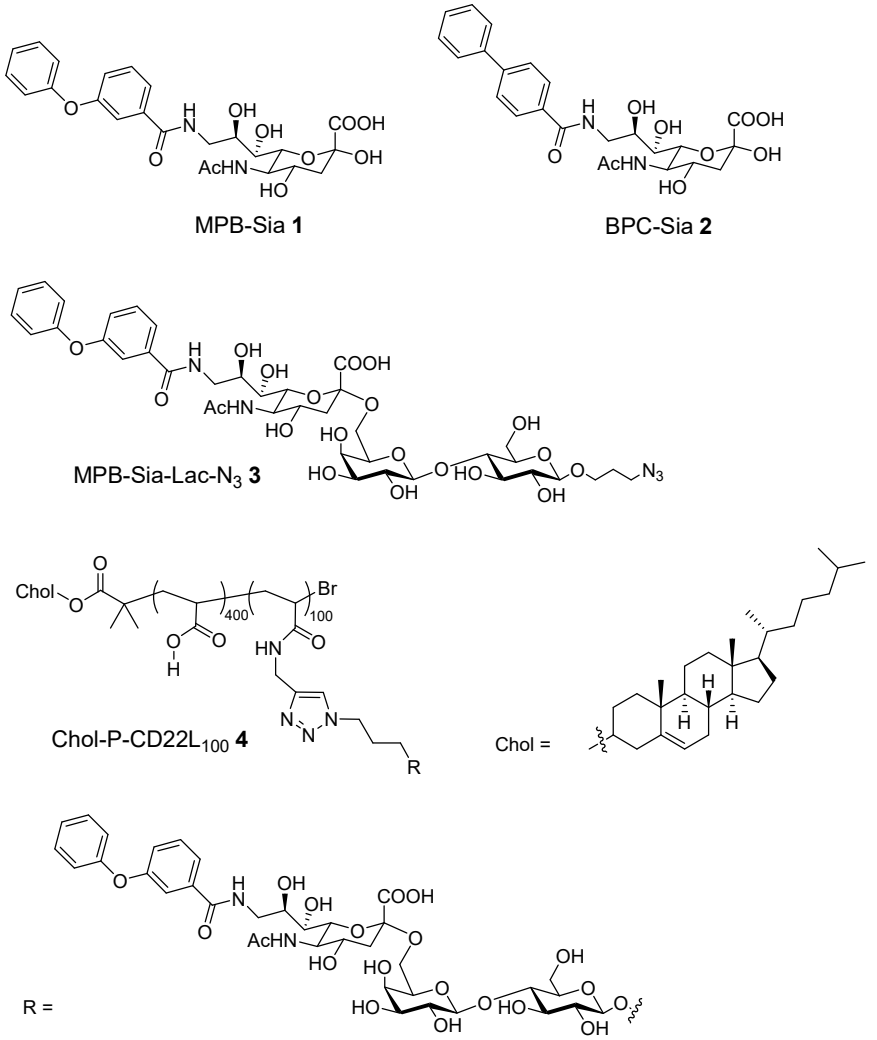
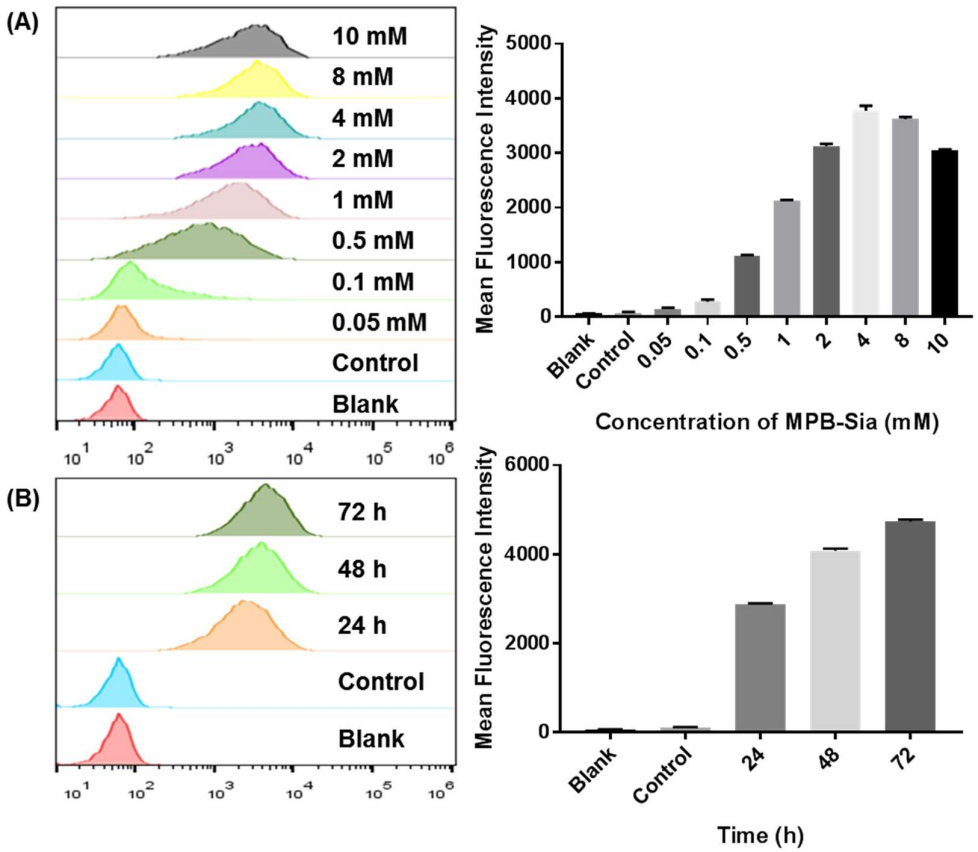
Table of Contents

Supplementary Figures

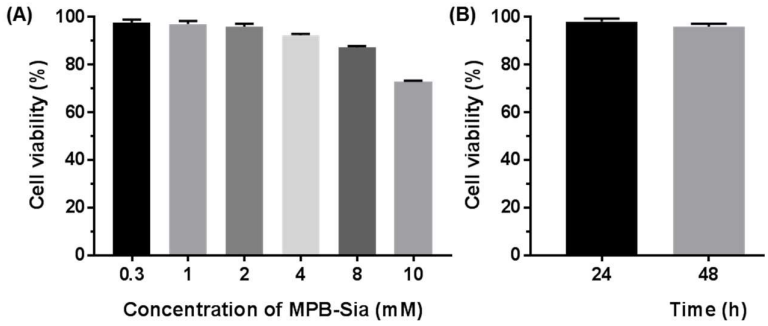
Supplementary Figures



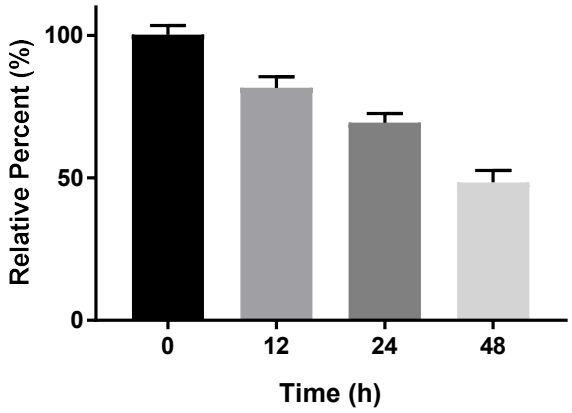
Supplementary Figure 1. Structures of sialic acid derivatives and glyco-polymer used in this study.



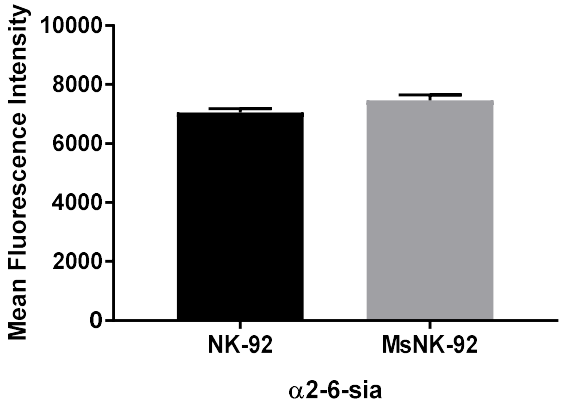
Supplementary Figure 2. Metabolic incorporation of MPB-sia **1** onto NK-92 cells. (A) Dose-dependence of metabolic incorporation of MPB-sia **1**. NK-92 cells (5×10^5 per sample) were incubated with different concentration of MPB-sia **1** (0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM or 10 mM) for 48 h. The cells were then incubated with human CD22-Fc protein followed by PE-anti-human CD22 antibody staining for FACS analysis. (B) Time-dependence of metabolic incorporation of MPB-sia **1**. NK-92 cells (5×10^5 per sample) were incubated with 2 mM MPB-sia **1** for different time periods (24 h, 48 h or 72 h). The cells were then incubated with human CD22-Fc protein followed by PE-anti-human CD22 antibody staining for FACS analysis. Mean with SD are presented for n = 3.



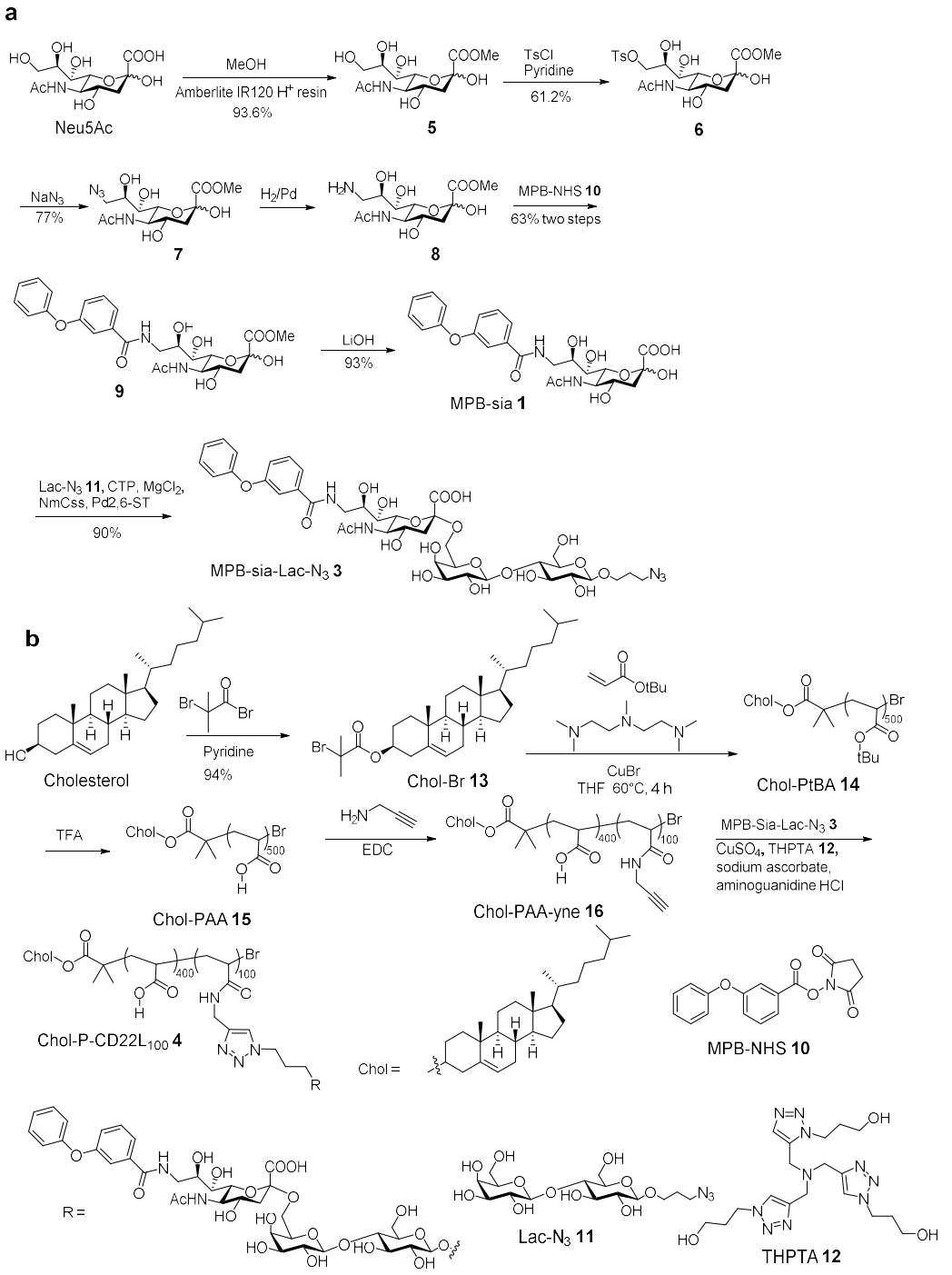
Supplementary Figure 3. The effect of MPB-sia **1** on NK-92 cell viability. (A) NK-92 cells (5×10^5 per sample) were incubated with different concentrations of MPB-sia **1** (0.3 mM, 1 mM, 2 mM, 4 mM, 8 mM or 10 mM) for 48 h. Cells were then stained with Calcein AM and the cell viability was analyzed by FACS. (B) NK-92 cells (5×10^5 per sample) were incubated with 2 mM of MPB-sia **1** for 24 h or 48 h. Cells were then stained with Calcein AM and the cell viability was analyzed by FACS. Mean with SD are presented for n = 3.



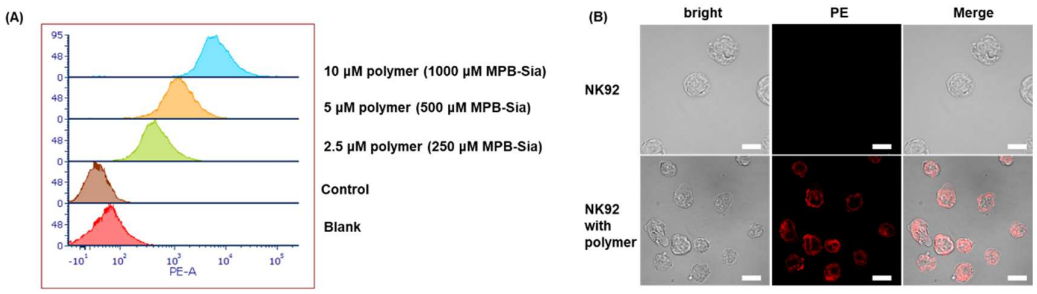
Supplementary Figure 4. Persistence of cell surface CD22 ligands after metabolic engineering. NK-92 cells were first incubated with 2 mM MPB-sia **1** for 48 h, followed by washing and incubating with fresh culture medium without MPB-sia **1** for various time periods (0 h, 12 h, 24 h or 48 h). The cells were further incubated with human CD22-Fc protein followed by PE-anti-human CD22 antibody staining for FACS quantification of surface CD22 ligand level. Mean with SD are presented for n = 3.



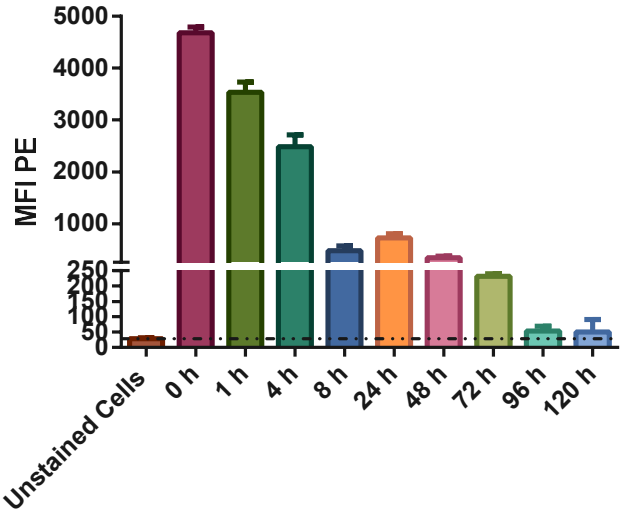
Supplementary Figure 5. The level of α2-6-sia linkage on NK-92 cell surface before and after metabolic engineering. Unmodified NK-92 cells and metabolic engineered NK-92 (MsNK-92) cells (5×10^5 per sample) stained by FITC-labeled α2-6-sia binding plant lectin Sambucus *nigra* lectin (SNA) followed by FACS analysis. Mean with SD are presented for n = 3.



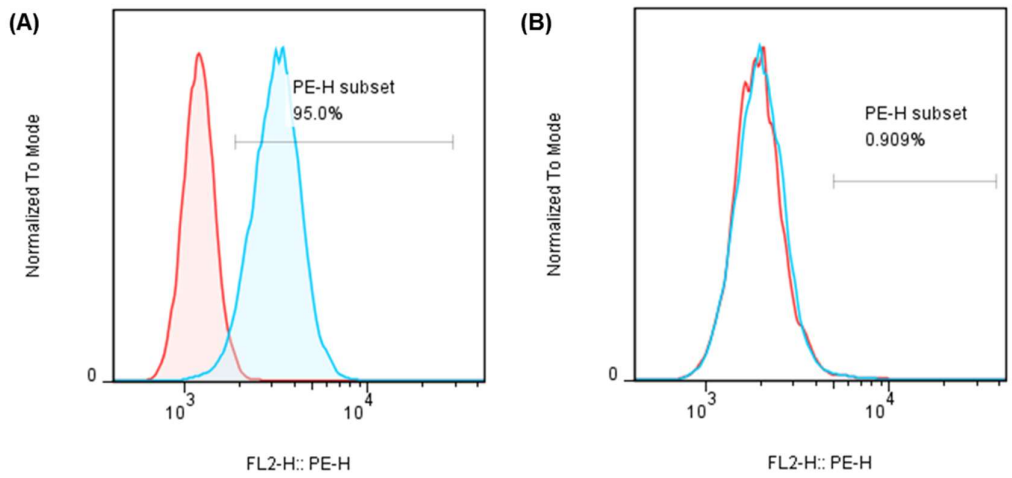
Supplementary Figure 6. a) Synthesis of MPB-sia **1** and MPB-sia-α2-6-Lac-N_3 **3**; b) synthesis of Chol-P-$CD22L_{100}$ **4.**



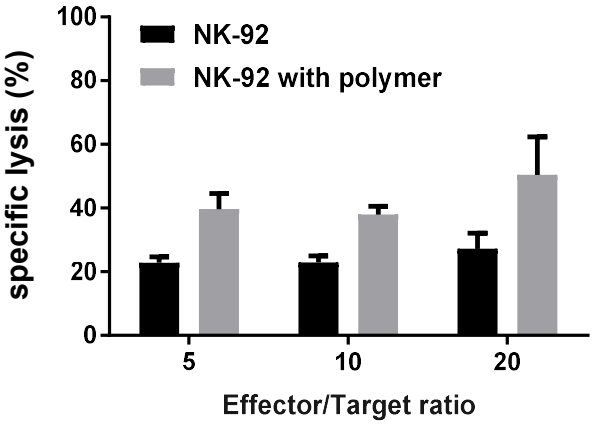
Supplementary Figure 7. Incubation of Chol-P-CD22L$_{100}$ **4** with NK-92 cells significantly enhanced the levels of CD22 ligands on cell surface. (A) FACS analysis of Chol-P-CD22L$_{100}$ **4** incubated NK-92 cells. NK-92 cells (5×10^5 per sample) were incubated with various concentrations of Chol-P-CD22L$_{100}$ **4** (2.5 μM, 5 μM or 10 μM) for 1 h in PBS buffer at room temperature. After washing, the cells were then incubated with human CD22-Fc protein followed by PE-anti-human CD22 antibody staining for FACS analysis. (B) Confocal image of Chol-P-CD22L$_{100}$ **4** treated NK-92 cells after incubated with 10 μM Chol-P-CD22L$_{100}$ **4** for 1 h in PBS buffer at room temperature.



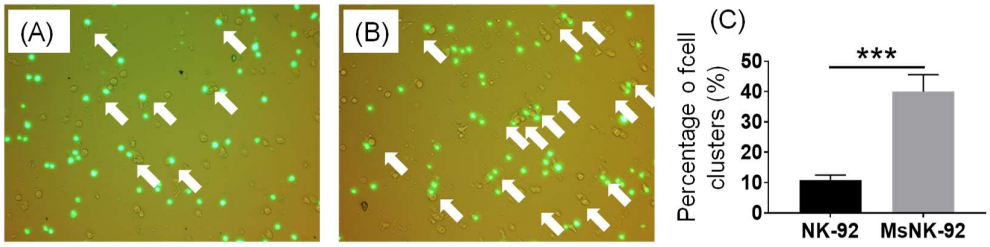
Supplementary Figure 8. Time-dependent persistence of CD22 ligands on cell surface after incubation with Chol-P-CD22L$_{100}$ **4**. NK92 cells were incubated with 10μM Chol-P-CD22L$_{100}$ **4** for 1h in PBS under room temperature. Cells were washed and cultured in fresh medium. An aliquot of cells was collected from the cell culture at different time points (0, 4, 8, 12, 24, 48, 72, 96 and 120 h) and incubated with human CD22-Fc protein followed by PE-anti-human CD22 antibody staining. FACS was used for detecting CD22 binding level. Polymer can last on cell surface for ~72h.



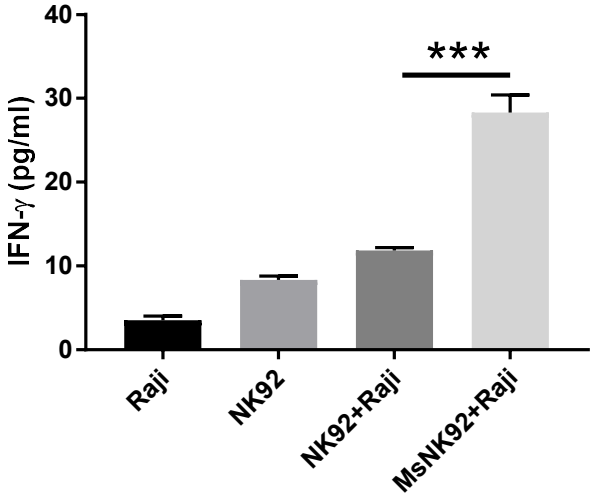
Supplementary Figure 9. Surface expression levels of CD22 on (A) Raji-luc vs (B) Hela cells as detected by flow cytometry. Raji-luc cells have much higher expression of CD22 compared to Hela cells.



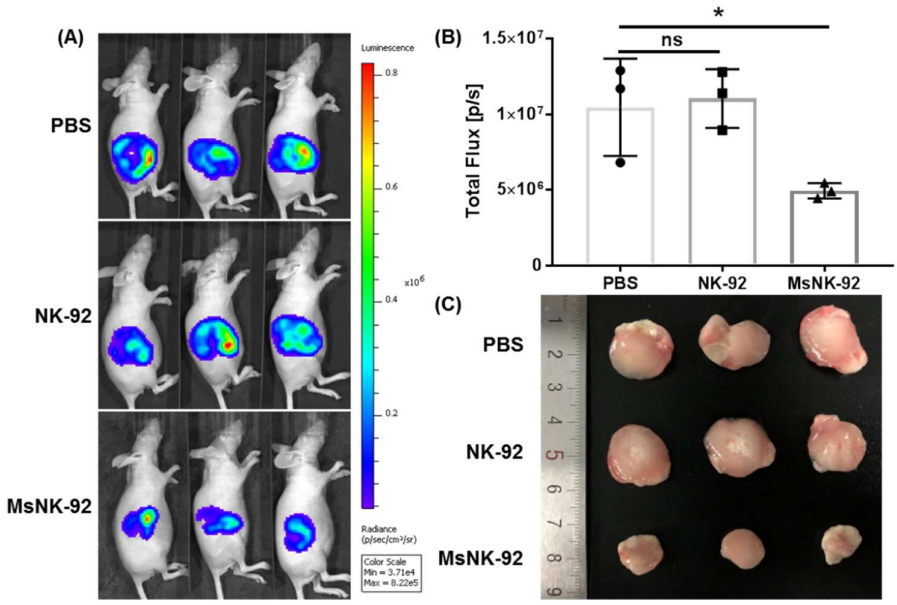
Supplementary Figure 10. Cell lysis of CHO-CD22 after incubation with Chol-P-CD22L$_{100}$ **4**. CFSE^{hi} labeled CHO-CD22 and CFSE^{lo} labeled CHO-WT cells were mixed at 1:1 ratio and co-cultured with NK92 or NK92+Chol-P-CD22L$_{100}$ **4** for 6h followed by FACS analysis. The specific lysis of CD22 was calculated based on the CFSE^{hi}/CFSE^{lo} cell ratio change before and after co-culture with NK cells. Mean with SD are presented for n = 3.



Supplementary Figure 11. Glyco-engineered NK-92 binding with CD22^{+} cells. Fluorescence microscopy image of cell clusters between Raji cells stained with Calcein-AM and (A) NK-92 or (B) MsNK-92. (C) Percentage of cell clusters, P=0.0010. Mean with SD are presented for n = 3. Statistical significance was assessed using Student`s t-test. In all figures, ns, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.



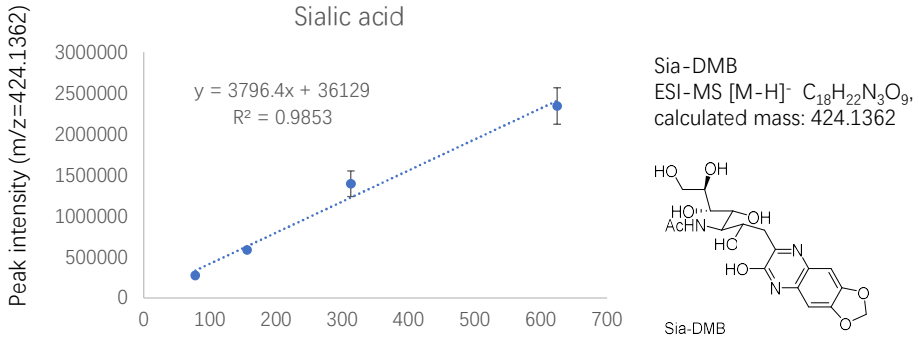
Supplementary Figure 12. IFN-γ production by glycoengineered NK-92 cells when stimulated with $CD22^+$ Raji cells. P=0.0002. Mean with SD are presented for n = 3. Statistical significance was assessed using Student`s t-test. In all figures, ns, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

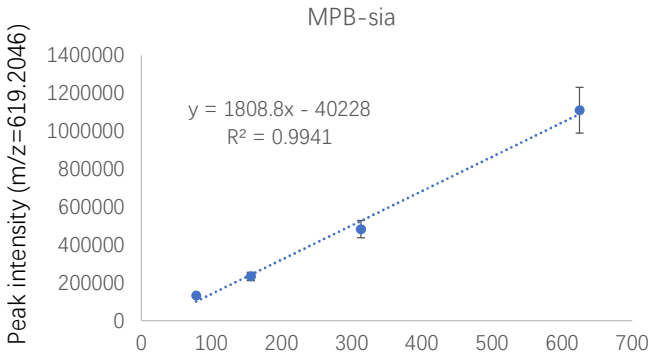


Supplementary Figure 13. *In vivo* anti-tumor activity of NK-92 cells in mice. 10^7 Raji-luc cells were injected subcutaneously into the flanks of Balb/c nude mice. Fifteen days later, the mice were treated with intratumoral injection of PBS, 10^7 NK-92 cells, or MsNK-92 cells in 50 µL PBS once a week. Bioluminescence images were acquired with IVIS® Lumina II imaging system. (A) BLI measurements of the Raji-luc in mice. (B) Quantitative BLI of Raji-luc activity. (C) Tumor takeout from mice. P=0.0425 (B). Mean with SD are presented for n = 3. Statistical significance was assessed using Student`s t-test. In all figures, ns, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001. These experiments were repeated one more time with another group of 3 mice. The results from the repeat experiments were similar to those presented here.

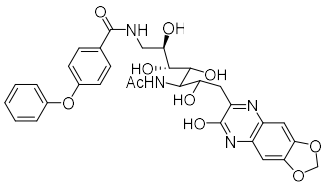
Table S1. Quantification of sialic acid and derivatives from engineered NK-92 cells. Following incubation with sialic acid, MPB-sia, and BPC-sia respectively, the corresponding NK-92 cells (cell + Sia, cell + MPB-sia, cell + BPC-sia) were thoroughly washed and then treated with an α2-3,6,8 neuraminidase. The supernatant was incubated with 1,2-diamino-4,5-methylenedioxybenzene (DMB). The amounts of sialic acid, MPB-sia, BPC-sia were quantified by comparing the MS intensities of the corresponding DMB adducts with the standard samples. N/A indicates no significant peaks in MS. For cell + BPC-sia group, while BPC-sia-DMB adduct was detected by MS, its amount was too small to be accurately quantified. The calibration curves and the structures of the DMB adducts are shown below the table.

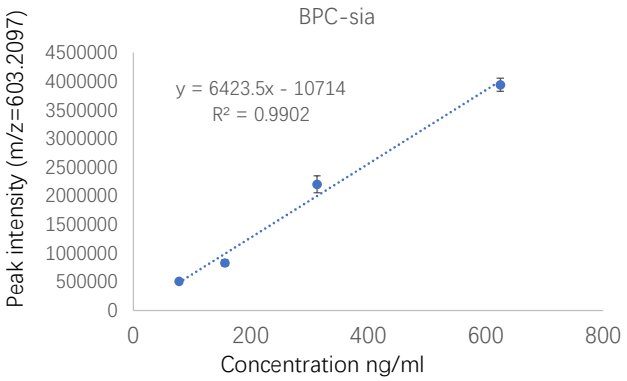
	Sialic acid amount (ng) /10^7 cells	Number of sialic acid molecules (x10^6) per cell	MPB-sia amount (ng) /10^7 cells	Number of MPB-sia molecules (x10^6) per cell	BPC-sia amount (ng) /10^7 cells	Number of BPC-sia molecules (x10^6) per cell
cell only	4.2	0.8	N/A	N/A	N/A	N/A
cell+ Sia	16	3.1	N/A	N/A	N/A	N/A
cell+ MPB-sia	5.2	1.0	44	5.2	N/A	N/A
cell+ BPC-sia	5.9	1.1	N/A	N/A	peak detected	peak detected



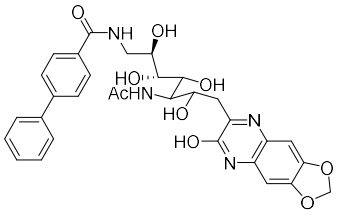


MPB-sia-DMB
ESI-MS $[M-H]^-$ $C_{31}H_{31}N_4O_{10}$,
calculated mass: 619.2046





BPC-sia-DMB
ESI-MS $[M-H]^-$ $C_{31}H_{31}N_4O_9$,
calculated mass: 603.2097



Experimental Methods

Reagents

Sialic acid, Amberlite IR 120 H+ resin, m-phenoxybenzoic acid (MPB), cytidine-5'-triphosphate (CTP), propargyl amine, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 4% paraformaldehyde were purchased from Millipore-Sigma. Toluenesulfonyl chloride, sodium azide, $Pd(OH)_2/C$ (10-15% loading), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl), *N*-hydroxysuccinimide (NHS), lithium hydroxide, α-bromoisobutyryl bromide, t-butylacrylate (tBA), *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA), CuBr, sodium ascorbate, amino guanidine were purchased from Acros Organics. Cholesterol, 4-dimethylaminopyridine (DMAP) were purchased from Alfa Aesar. Anhydrous methanol, anhydrous ethanol, diethyl ether, dichloromethane (DCM), anhydrous pyridine, acetic acid, dimethylformamide (DMF), diisopropylethylamine (DIPEA), magnesium chloride, triethylamine (TEA), sodium chloride, sodium sulfate, tetrahydrofuran (THF), trifluoroacetic acid (TFA) were purchased from Fisher Scientific. *E. coli* Recombinant CMP-Sialic acid synthetase from *Neisseria meningitides* (NmCss), *Photobacterium damselae* α2−6-sialyltransferase (Pd2,6ST) were purchased from Chemily Glycoscience. Human CD22-Fc Chimera protein was purchased from R&D Systems. PE-mouse anti-human CD22 monoclonal antibody (Clone HIB22) was purchased from BD Pharmingen™. FITC-labeled α2,3-sialic acid binding plant lectin Maackia Amurensis Lectin I (FITC-MAL I) and FITC-labeled α2,6-sialic acid binding plant lectin Sambucus Nigra Lectin (FITC-SNA) were purchased from Vector Laboratories. Firefly luciferase reporter gene assay kit, Immunostaining permeabilization buffer with Triton X-100, DAPI were purchased from Beyotime Biotechnology. BPC-Sia **2** was purchased from Xiamen Nuokangde Biological Technology Co. Ltd. Perforin-specific antibody δG9 was purchased from Santa Cruz Biotechnology. Alexa Fluor 594-coupled anti-mouse antibody was purchased from Life Technologies.

No unexpected or unusually high safety hazards were encountered.

Mice

BALB/c nude mice and NOD SCID mcie (female, 6-8 weeks) were purchased from model animal research center of Nanjing university (Nanjing, China) and all animal experiments were conducted under the animal use and care regulations approved by Institutional Animal Care and Use Committee at Xiamen University.

Synthesis of compound 5

Sialic acid (20 g, 64.7 mmol) and Amberlite IR 120 H^+ resin (15 g) were suspended in anhydrous methanol (300 ml). The reaction mixture was stirred under room temperature for 72 h. After filtering off the resin, the solvent was concentrated by

rotavapor to around 30 ml. Then diethyl ether (300 ml) was added to precipitate out the product. The resulting solid product **5** (19.63 g, yield = 93.6 %) was collected by filtration and dried under nitrogen gas overnight. The product was used without further purification. ^{1}H NMR (500 MHz, Methanol-d4) δ 4.08 – 3.96 (m, 2H), 3.86 – 3.76 (m, 2H), 3.77 (s, 3H), 3.70 (ddd, J = 9.3, 5.7, 2.9 Hz, 1H), 3.61 (dd, J = 11.4, 5.7 Hz, 1H), 3.47 (dd, J = 9.3, 1.5 Hz, 1H), 2.21 (dd, J = 12.9, 4.9 Hz, 1H), 2.01 (s, 3H), 1.89 (dd, J = 12.9, 11.4 Hz, 1H). ^{13}C NMR (126 MHz, Methanol-d4) δ 175.29, 171.94, 96.82, 72.20, 71.78, 70.31, 68.00, 64.97, 54.45, 53.34, 40.84, 22.83. ESI-MS: $[M+Na]^+$ $C_{12}H_{21}NaNO_9$ calculated 346.1109; observed: 346.1120. The NMR data matched previously reported data[1].

Synthesis of compound 7

The synthesis of compound **7** followed literature report with modifications[2]. Compound **5** (3.23 g, 10 mmol) was dissolved in anhydrous pyridine (20 ml) and cooled on ice for 10 min. Tosyl chloride (2.5 g, 13 mmol) was dissolved in anhydrous pyridine (10 ml) and added to the solution of compound **5** dropwise. The reaction mixture was allowed to reach room temperature after addition of tosyl chloride was completed followed by stirring overnight. After reaction, the solvent was removed by rotavapor and the residue was collected and purified by silica gel column with DCM: MeOH = 10: 1 elution to obtain product **6** (2.92 g, yield = 61.2 %). ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.75 (d, *J* = 8.3 Hz, 2H), 7.33 (d, *J* = 8.3 Hz, 2H), 7.17 – 6.98 (m, 1H), 4.38 – 4.18 (m, 1H), 4.18 – 4.04 (m, 3H), 4.03 – 3.91 (m, 1H), 3.89 – 3.79 (m, 1H), 3.76 (s, 3H), 3.48 (s, 1H), 2.42 (s, 3H), 2.31 – 2.16 (m, 1H), 2.04 (s, 3H), 2.00 – 1.92 (m, 1H).

Compound **6** (2.92 g. 6.1 mmol) was dissolved in methanol (50 ml). NaN_3 (2 g, 30.8 mmol) was added to the reaction. Then the reaction mixture was heated to reflux and stirred until compound **6** was completely consumed (monitored by TLC, DCM: MeOH = 10: 1). After the reaction mixture was cooled down to room temperature, the unreacted NaN_3 was carefully filtered off. The filtrate was collected and concentrated by rotavapor followed by silica gel column purification (DCM: MeOH = 10: 1) to yield pure compound **7** (1.64 g, yield = 77%).^{1}H NMR (500 MHz, Methanol-d_4) δ 3.92 (ddd, *J* = 11.4, 10.1, 4.9 Hz, 1H), 3.87 – 3.79 (m, 2H), 3.73 (s, 3H), 3.73 – 3.65 (m, 1H), 3.51 (dd, *J* = 12.8, 2.8 Hz, 1H), 3.38 – 3.29 (m, 2H), 2.21 (dd, *J* = 13.0, 4.9 Hz, 1H), 1.98 (s, 3H), 1.90 (dd, *J* = 13.0, 11.4 Hz, 1H). ^{13}C NMR (126 MHz, Methanol-d_4) δ 178.11, 174.36, 99.29, 74.42, 73.23, 72.95, 70.34, 58.24, 57.14, 56.63, 43.01, 26.11. ESI-MS: $[M+Na^+]$ $C_{12}H_{10}NaN_4O_8$ calculated: 371.1179, observed: 371.1178.

Synthesis of MPB-Sia 1

Compound **7** (1.5 g, 4.3 mmol) was dissolved in methanol (20 ml), $Pd(OH)_2$/C (10-15% loading, 200 mg) and acetic acid (1 ml) were added to the flask. The reaction mixture was then stirred under H_2 atmosphere until the compound **7** was completely

consumed (monitored by TLC, DCM: MeOH = 10: 1). The reaction mixture was then filtered to remove the $Pd(OH)_2/C$. The resulting filtrate was collected and concentrated to yield compound **8** (1.26 g, 91.3 %). The product was used without further purification. ^{1}H NMR (500 MHz, Methanol-d_4) δ 4.06 (ddd, J = 11.4, 10.1, 5.0 Hz, 1H), 3.97 – 3.89 (m, 2H), 3.84 – 3.73 (m, 4H), 3.55 – 3.47 (m, 1H), 3.25 (dd, J = 12.8, 3.9 Hz, 1H), 3.03 (dd, J = 12.8, 7.5 Hz, 1H), 2.27 (dd, J = 13.0, 5.0 Hz, 1H), 2.03 (s, 3H), 1.88 (dd, J = 13.0, 11.3 Hz, 1H).

MPB-NHS **10** was synthesized by the following procedure: MPB (2.14 g, 10 mmol) was dissolved in DMF (20 ml), followed by adding EDC•HCl (2 g, 10.5 mmol) and NHS (1.2 g, 10.5 mmol). The reaction mixture was stirred under room temperature overnight. After the reaction, the solvent was removed by rotavapor and the residue was purified by silica gel column (hexane: ethyl acetate =3: 1 to 2: 1) to yield MPB-NHS **10** (2.56 g, 82.3 %).^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.88 (ddd, J = 8.0, 1.5, 0.9 Hz, 1H), 7.73 (dd, J = 2.5, 1.5 Hz, 1H), 7.48 (t, J = 8.0 Hz, 1H), 7.41 – 7.36 (m, 2H), 7.32 (ddd, J = 8.0, 2.5, 0.9 Hz, 1H), 7.19 – 7.13 (m, 1H), 7.06 – 7.00 (m, 2H), 2.89 (s, 4H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 169.18, 161.41, 157.92, 156.13, 130.34, 130.11, 130.09, 126.66, 125.09, 124.22, 120.01, 119.42, 25.68.

Compound **8** (1.26 g, 3.9 mmol) was dissolved in DMF (20 ml) followed by the addition of DIPEA (1.1 ml, 0.81 g, 6.3 mmol) and MPB-NHS **10** (1.8 g, 5.8 mmol). The reaction mixture was stirred overnight under room temperature. After removal of solvent by rotavapor, the residue was purified by silica gel column (DCM: MeOH= 10: 1 to 2: 1) to yield compound **9** (1.40 g, yield = 69.5 %). ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.56 (ddd, J = 7.7, 1.7, 0.9 Hz, 1H), 7.50 – 7.41 (m, 2H), 7.41 – 7.34 (m, 2H), 7.18 – 7.11 (m, 2H), 7.04 – 6.98 (m, 2H), 4.07 – 3.96 (m, 2H), 3.89 – 3.77 (m, 3H), 3.76 (s, 3H), 3.57 – 3.36 (m, 2H), 2.20 (dd, J = 12.9, 4.9 Hz, 1H), 1.97 (s, 3H), 1.91 – 1.81 (dd, J = 12.9, 11.5 Hz, 1H).

An aqueous solution of LiOH (324 mg, 13.5 mmol in 20 ml DI water) was added to the flask containing compound **9** (1.40 g, 2.7 mmol). The reaction was stirred under room temperature for 2 h. The reaction mixture was neutralized with Amberlite IR 120 H^+ resin to pH 5. After removal of resin by filtration, the solution was collected and lyophilized to yield MPB-Sia **1** without further purification (1.26 g, yield = 93 %). ^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.54 – 7.46 (m, 2H), 7.46 – 7.39 (t, J = 8.5 Hz, 2H), 7.42 – 7.30 (m, 1H), 7.27 – 7.18 (m, 2H), 7.11 – 7.05 (d, J = 8.5 Hz, 2H), 4.07 – 3.95 (m, 2H), 3.95 – 3.78 (m, 2H) 3.72 (dd, J = 14.2, 3.1 Hz, 1H), 3.53 – 3.38 (m, 2H), 2.25 (dd, J = 12.9, 4.9 Hz, 1H), 1.97 (s, 3H), 1.81 (dd, J = 12.9, 11.6 Hz, 1H). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 174.62, 173.91, 170.48, 156.96, 156.19, 135.42, 130.34, 130.08, 124.11, 122.07, 119.00, 117.11, 95.40, 70.10, 69.42, 68.73, 66.69, 53.73, 52.01, 43.19, 38.84, 21.93. ESI-MS: $[M-H]^-$ $C_{24}H_{27}N_2O_{10}$ Calculated: 503.1671 Found: 503.1671. The NMR data matched previously reported data[3].

Synthesis of MPB-Sia-Lac-N_3 3

Lac-N_3 **11**[4] (1.06 mg, 2.5 μmol), MPB-Sia **1** (5.0 mg, 10 μmol), CTP (7.25 mg, 15 μmol), $MgCl_2$ (3.5 mg, 37.5 μmol), Tris buffer (pH = 8.8, 150μl of 1.5 M stock solution), *E. coli* Recombinant CMP-Sialic acid synthetase from Neisseria meningitides (NmCss, 0.2 U) and Photobacterium damselae α2−6-sialyltransferase (Pd2,6ST, 0.08 U) were mixed in a vial and Milli Q water was added to a total volume of 750 μl. The reaction mixture was kept under 37 ℃ for 1 h. Then ice-cold ethanol (750 μl) was added to the reaction mixture, followed by centrifuge at 5000 rpm for 5 min to remove enzyme precipitates. The supernatant was collected and passed through Bio-gel P2 column to obtain the product MPB-Sia-Lac-N_3 **3** in 90% yield. ^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.47 – 7.33 (m, 2H), 7.32 – 7.22 (m, 3H), 7.15 – 7.05 (m, 2H), 7.00 – 6.88 (m, 2H), 4.25 (dd, *J* = 12.9, 7.9 Hz, 2H), 3.88 (ddd, *J* = 8.9, 7.3, 3.3 Hz, 1H), 3.84 – 3.75 (m, 4H), 3.72 – 3.67 (m, 1H), 3.66 – 3.55 (m, 5H), 3.52 – 3.33 (m, 9H), 3.29 (t, *J* = 6.7 Hz, 2H), 3.16 – 3.11 (m, 1H), 2.54 (dd, *J* = 12.4, 4.7 Hz, 1H), 1.82 (s, 3H), 1.79 – 1.70 (m, 2H), 1.57 (t, *J* = 12.2 Hz, 1H). ^{13}C NMR (126 MHz, Deuterium Oxide) δ 174.74, 173.36, 170.26, 156.99, 156.24, 135.56, 130.36, 130.12, 124.13, 122.17, 122.04, 119.05, 117.26, 103.09, 101.88, 100.25, 79.54, 74.53, 73.60, 72.59, 72.31, 72.26, 70.66, 70.08, 69.96, 68.43, 68.21, 67.18, 63.58, 60.19, 51.67, 47.76, 42.79, 39.99, 29.48, 28.14, 21.92. ESI-MS: $[M-H]^-$ $C_{39}H_{52}N_5O_{20}$ calculated: 910.3211, observed: 910.3220.

Synthesis of Chol-Br 13

Cholesterol (1 g, 2.6 mmol), DMAP (0.93 g, 7.6 mmol) and trimethylamine (0.73 ml, 0.53 g, 5.3 mmol) were dissolved in DCM (20 ml) and cooled on ice for 15 min. α-Bromoisobutyryl bromide (1.6 ml, 2.98 g, 12.9 mmol) in DCM (5 ml) was then added dropwise. Subsequently, the reaction was stirred on ice for 1 h under room temperature for another 12 h. The reaction mixture was diluted with DCM (50 ml) and washed 3 times with saturated NaCl aqueous solution. The organic phase was collected and dried with Na_2SO_4, then concentrated by rotavapor to ~ 3 ml. Ethanol (50 ml) was used to precipitate out the desired product Chol-Br **13**. The product was collected by filtration and dried under N_2 overnight (1.02 g, yield = 74 %).^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.45 – 5.32 (m, 1H), 4.73 – 4.60 (m, 1H), 2.44 – 2.32 (m, 2H), 2.05 – 1.80 (m, 11H), 1.73 – 1.28 (m, 12H), 1.22 – 0.94 (m, 12H), 0.93 – 0.84 (m, 9H), 0.69 (s, 3H). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 171.04, 139.34, 122.91, 75.59, 56.66, 56.32, 56.11, 49.97, 42.30, 39.70, 39.51, 37.55, 36.86, 36.59, 36.17, 35.79, 31.91, 31.83, 30.70, 28.23, 28.01, 27.27, 24.28, 23.82, 22.83, 22.57, 21.03, 19.35, 18.71, 11.85.

Synthesis of Chol-PAA 15

Chol-Br **13** (5 mg, 9.4 μmol) and ′butyl acrylate (0.75 g, 5.86 mmol) were dissolved in THF (5 ml). PMDETA (2 μl, 1.6 μg, 9.4 μmol) was added, followed by three freeze-pump-thaw cycles to remove oxygen. CuBr (1.4 mg, 9.4 μmol) was then added under N_2 protection. The reaction was kept under 60 ℃ for 3 h. After polymerization, 50 μl

of the reaction mixture was taken for NMR analysis (Monomer conversion= 95% based on NMR analysis). The remaining solvent was concentrated to ~2 ml by rotavapor after the reaction and H_2O: MeOH = 1: 1 (20 ml) was added. The precipitate was collected and re-dissolved in THF (2 ml). The precipitation process was repeated 3 times to yield crude Chol-PtBA **14** (0.58 g).

Chol-PtBA **14** (0.58 g) was dissolved in TFA: DCM = 1: 1 (2 ml) and stirred under room temperature for 1 h. The solvent was then removed by rotavapor and the residue was re-dissolved in water and dialyzed against pure water (MWCO = 3500 Da) for 24 h followed by lyophilization to yield Chol-PAA **15** (0.29 g, yield = 68% (two steps)). ^{1}H NMR (500 MHz, Deuterium Oxide) δ 4.98 – 4.96 (m, 1H), 2.42 – 2.11 (m, 500H), 1.88 – 1.37 (m, 1000H), 0.95 – 0.94 (m, 3H), 0.73 – 0.69 (m, 3H).

Synthesis of Chol-PAA-yne 16

Chol-PAA **15** (0.1 g, ~2.8 μmol) was dissolved in water (5 ml) followed by the addition of EDC•HCl (100 mg, 0.52 mmol) and propargyl amine (18 mg, 0.33 mmol). The reaction was stirred under room temperature for 24 h. The reaction mixture was then collected and dialyzed against 0.1x PBS buffer for 48 h then in pure water for another 24 h (MWCO = 3500). The solution in dialysis tubing was collected and lyophilized to yield Chol-PAA-yne **16** (87 mg, yield= 74%). ^{1}H NMR (500 MHz, Deuterium Oxide) δ 4.03 – 3.82 (m, 200H), 2.71 – 2.49 (m, 100H), 2.35 – 1.15 (m, 1500H), 0.67 (s, 3H).

Synthesis of Chol-P-CD22L$_{100}$ 4

MPB-Sia-Lac-N_3 **3** (6 mg, 6.5 μmol), $CuSO_4$ (0.5 mg, 3.25 μmol), THPTA **12** (5 mg, 13 μmol), sodium ascorbate (11 mg, 52 μmol), amino guanidine (6 mg, 52 μmol), Chol-PAA-yne **16** (2 mg, containing 6.5 μmol alkyne) were dissolved in water (1 ml) in a sealed vial and stirred under room temperature for 24 h. The reaction mixture was passed through G25 column to yield purified **4**. ^{1}H NMR (500 MHz, Deuterium Oxide) δ 7.31 (s, 900H), 4.41 – 4.09 (m, 100H), 4.09 – 3.20 (m, 2300H), 3.11 – 2.87 (m, 100H), 2.80 – 0.63 (m, 2000H).

General procedure of metabolic glyco-engineering of NK-92 cells

NK-92 cells (5×10^5 cells/mL) were incubated with culture medium supplemented with MPB-sia **1**, BPC-sia **2** or free sialic acid at different final concentrations for various time periods. In this study, "MsNK-92" refers the NK-92 cells incubated with 2 mM MPB-sia 1 for 48 h if not specified. After incubation, the cells were washed three times with PBS buffer. These cells were collected and used in this study. The binding ability of these engineered NK-92 cells toward human CD22 protein was studied by FACS and confocal imaging. 5×10^5 of cells were suspended in 0.1 ml PBS buffer and incubated with 0.5 μg human CD22-Fc on ice for 30 min. The cells were washed twice with PBS buffer after incubation followed by PE mouse anti-human CD22 mAb (clone

HIB22) staining on ice in dark for another 30 min. The cells were washed twice with PBS buffer and analyzed by FACS directly. For confocal imaging study, the cells were fixed by 4% paraformaldehyde after mAb staining and washing, then stained by DAPI before imaging.

Mass spectrum analysis of surface sialic acid and derivatives on NK92 cells after metabolic glycoengineering

NK92 cells (1 x 10^7 cells) were incubated with medium only (10 mL), or medium (10 mL) containing 2 mM sialic acid, 2 mM MPB-sia, 2 mM BPC-sia respectively for 24 h followed by thorough washing with PBS buffer for 3 times to remove all free ligands. 1x10^7 of NK92 cells from each group were collected and resuspended in 200 µl PBS buffer.

1 µl enzyme stock solution containing 50 units of α-2-3,6,8 neuraminidase (New England Biolabs) was added to each group and the resulting cell-enzyme mixtures were kept under 37℃ on a shaker for 1 h. After the incubation, the enzyme was deactivated by heating to 65 ℃ for 15 min. The samples were centrifuged at 5,000 rpm for 5 min and the supernatants were collected and lyophilized. On the same day of mass spectrum analysis, the lyophilized samples were dissolved in 50 µl DI water followed by addition of 50 µl of DMB reagent stock (3.5 mg DMB hydrochloride, 172 µl acetic acid, 112 µl 2-mercaptoethanol and 4.9 mg sodium hydrosulfite dissolved in 1.5 ml DI water, prepared right before use). For standard curve measurement, 50 µl of sialic acid, MPB-sia **1** and BPC-sia **2** (1 mg/ml in DI water) were mixed with 50 µl of the DMB reagent stock. All reaction mixtures were kept in dark in 60 ℃ water bath for 2 h. The samples were then cooled on ice and diluted with acetonitrile : water (1:1) for mass spectrum analysis. For standard curves and sialic acid quantification results, see **Table S1**.

Insertion of Chol-P-CD22L$_{100}$ 4 onto NK-92 cells

NK-92 cells (1×10^7 cells/ml) were incubated with Chol-P-CD22L$_{100}$ **4** in PBS buffer at various final concentrations (2.5 µM, 5 µM or 10 µM) for 1 h under room temperature. After incubation, the cells were washed three times with PBS buffer. These cells were collected and used for further study.

Dose and time dependent metabolic incorporation of MPB-Sia 1 on NK-92 cells

To study the dose dependence of MPB-Sia **1** metabolic incorporation, NK-92 cells (5×10^5 per sample) were incubated with NK-92 medium (1 ml) supplemented with different concentrations of MPB-sia **1** for 48 h. After incubation, the cells were collected and washed three times with PBS buffer. The cells were then incubated with human CD22-Fc followed by PE-mouse anti-human CD22 mAb staining and FACS analysis as described above. The data represent the mean of triplicate samples.

To study the metabolic incorporation of MPB-Sia **1** during different time periods, NK-92 cells (5×10^5 per sample) were incubated with NK-92 medium (1 ml) supplemented with 2 mM MPB-sia **1** for 24 h, 48 h or 72 h. After incubation, the cells

were collected and washed three times with PBS buffer. The cells were then incubated with human CD22-Fc followed by PE-mouse anti-human CD22 mAb staining and FACS analysis as described above. The data represent the mean of triplicate samples.

The level of α2-6-sia linkage on NK-92 cell surface before and after metabolic engineering

NK-92 cells (5×10^5 per sample) were suspended in 100 μl Hanks buffer containing 4% BSA and 0.1% NaN_3. 1 μg FITC-labeled α2,6-SA binding plant lectin Sambucus Nigra Lectin (SNA) was added to each sample. After incubation on ice in dark for 30 min, the cells were collected and washed 3 times with Hanks buffer. Unmodified NK-92 cells were stained by the same procedure. An unstained NK-92 sample was included as the negative control for analysis. The samples were analyzed by FACS. The data represent the mean of triplicate samples.

The influence of MPB-sia 1 on NK-92 cell viability

NK-92 cells (5×10^5 per sample) were incubated with different concentrations of MPB-Sia **1** for 48 h, or with 2 mM MPB-Sia **1** for different time periods (24 h, 48 h or 72h). After incubation, the cells were collected and washed twice with PBS buffer. The cells were resuspended in 1 ml of PBS buffer containing 1μM Calcein AM and were incubated under 37℃ for 15 min. The samples were analyzed by FACS and the cell viability was calculated based on fluorescent cell counts per 100 μl sample.

Time dependent decrease of CD22 binding after metabolic glyco-engineering

Metabolic engineered or polymer engineered NK-92 cells (5×10^5 cells per sample) were collected and washed 3 times with PBS buffer to remove free MPB-Sia **1** or Chol-P-$CD22L_{100}$ **4**. The CD22 binding at 0 h was obtained by incubating 5 x 10^5 cells with human CD22-Fc followed by PE-mouse anti-human CD22 mAb staining and FACS analysis immediately after the removal of MPB-Sia **1** or Chol-P-$CD22L_{100}$ **4**. Other cells were re-suspended in fresh culture medium without MPB-Sia **1** or Chol-P-$CD22L_{100}$ **4** and incubated at 37 ºC for another 12 h, 24 h or 48 h. At each time point, 5×10^5 cells were collected, stained and analyzed by FACS. An unmodified NK-92 sample was incubated with CD22 protein and stained with PE-mouse anti-human CD22 mAb as the negative control. The mean fluorescence intensities of PE were normalized to 0 h (100%).

Measurement of surface CD22 expression level on different cell lines

Raji or Hela cells (1×10^6 cells per sample) were collected and washed with PBS for three times. The cells were re-suspended in 100 μl PBS buffer and stained with PE mouse anti-human CD22 on ice in dark for 30 min, followed by FACS analysis. An unstained cell sample was included as the negative control.

Imaging analysis of NK-92 cell and Raji cell binding

Raji cells (1×10^6/ml) were incubated with 1μM Calcein AM for 15 min at 37℃ and then washed three times with 1640 medium containing FBS, followed by incubation with NK-92 cells or MsNK-92 cells at a 1: 1 ratio. Two hours later, the cells mixture was imaged by fluorescent microscope. The percentages of cell complexes were calculated as the proportion of the Raji cells attached to the NK-92 cells over the total number of Raji cells. The data represent the mean of triplicate samples.

***In vitro* NK cytotoxicity assay against Raji cells**

Raji cells stably transfected with firefly luciferase (Raji-luc, 1×10^4 per well) were co-incubated with unmodified NK-92 or MsNK-92 at different effector to target cell ratios (E/T = 0.5, 1, 5) at 37 ºC in 200 μL of mixture medium (RPMI-1640 : NK-92 medium = 1: 1). After 5 h incubation, the cells were centrifuged, and the supernatant was discarded. The cells were lysed, and bioluminescence was detected by firefly luciferase reporter gene assay kit according to manufacturer's instruction. The luminescence intensity of Raji-luc cells was recorded. The cell lysis of each sample was normalized to the luminescence intensity of Raji-luc only (100 %) and buffer only (0 %) samples. The data represent the mean of triplicate samples. To study the influence of CD22 binding on specific killing against Raji, a competition experiment was performed. NK-92 or MsNK-92 (1×104 per well) were first incubated with various amounts of human CD22-Fc (0 μg/mL, 1 μg/mL, 5 μg/mL) on ice for 30 min. Then Raji-luc cells (1×104 per well) were added to NK-92 or MsNK-92 to reach E/T = 1: 1. After 5 h incubation under 37 ºC, the cells were centrifuged, and the supernatant was discarded. The cells were lysed, and bioluminescence was detected by firefly luciferase reporter gene assay kit according to manufacturer's instruction. The luminescence intensity of Raji-luc cells was recorded. The cell lysis of each sample was normalized to the luminescence intensity of Raji-luc only (100 %) and buffer only (0 %) samples. The data represent the mean of triplicate samples.

CD22 specific *in vitro* killing assay on CHO-WT and CHO-hCD22 cells

CHO WT cells and CHO hCD22 cells were incubated with 0.5 μM and 5 μM CFSE respectively in 1 mL of PBS buffer containing 0.1% BSA under 37 ºC for 10 min. After incubation, 10 mL of complete culture medium were added to each tube respectively and the cells were incubated under room temperature for another 10 min. The cells were collected respectively and washed once with complete culture medium. The cell densities of both CFSE-lo-CHO WT cells and CFSE-hi-CHO hCD22 cells were adjusted to 1×10^5/mL. The two type of cells were mixed at a 1:1 ratio, and co-incubated with NK-92 or MsNK-92 cells at different effector to target ratios (E/T = 5: 1, 10: 1, 25: 1, and 50: 1) under 37 ºC for 5 h. The cell mixtures were analyzed by FACS after co-incubation. The percentages of lysis of CHO-hCD22 cells were calculated based on the CFSE-hi/CFSE-lo cell ratio changes before and after incubating with NK-

92 or MsNK-92.

In vitro NK cytotoxicity assay against patient-derived leukemic samples

Patient-derived leukemic samples were from to be discarded samples following routine diagnostic procedures. The white blood cells were enriched by density gradient centrifugation and then treated with red blood cell lysis buffer. The purified cells were treated with 5 μL PE mouse anti-human CD22 in 0.1 mL volume on ice for 30 min to identify the CD22 expression levels. For the cell-mediated cytotoxicity assay, the purified cells were labeled with 1 μM Calcein AM and incubated with NK-92 cells or MsNK-92 cells for 5h at E/T=5:1 under 37 ºC. The samples were analyzed by FACS and the cell viability was calculated based on fluorescent cell counts per 100 μL sample.

Perforin imaging

Cell-cell complex formation and redistribution of cytotoxic granules containing perforin were analyzed by confocal microscopy. NK (5×10^5) cells and Raji cells (5×10^5) were co-incubated at a 1: 1 ratio for 1 h at 37 ºC, then fixed for 10 min with 4% polyformaldehyde solution. Immunostaining permeabilization buffer with Triton X-100 was added for cell permeabilization. Cells were washed and blocked for 30 min with 10% FBS in PBS to avoid unspecific binding. The cells were incubated for 75 min at room temperature with perforin-specific antibody δG9, followed by Alexa Fluor 594-coupled anti-mouse antibody. Cell nuclei were counterstained with DAPI. Cells were wash twice with PBS, then dropped onto the slide to make microscopic slides and analyzed with Olympus FV1000MPE-B.

IFN-γ release assay

IFN-γ release by NK-92 cells was determined by using BD Cytometric Bead Array Flex Set for Human IFN-γ according to the manufacturer`s protocol. Briefly, 2×10^4 Raji cells were incubated with equal numbers of NK-92 cells in the wells of 96-well V-bottom plates for 5 h at 37 ºC. IFN-γ concentrations in supernatants were measured using BD Cytometric Bead Array Flex Set for Human IFN-γ.

Tumor challenge

For intratumoral administration of NK cells

BALB/c nude mice (female, 6-8 weeks) were purchased from model animal research center of Nanjing University (Nanjing, China). Prior to tumor implantation, mice received cyclophosphamide (2 mg in 0.1 mL PBS) intraperitoneally for three days. 1×10^7 Raji-luc cells in 0.2 mL were injected subcutaneously in the right flank of the mice on day 0. Mice were treated on day 15, 22 and 29 with intratumoral injections of 1×10^7 NK-92 cells or MsNK-92 cells (n =3 per group) in 50 μL PBS. The tumor size was measured before intratumoral injections with Vernier caliper and the tumor

volumes were calculated by the formula length×width×width×0.5. Animals were sacrificed at day 36 and xenografts were surgically excised and weighed. For *in vivo* imaging, the mice were injected with 100 μL D-luciferin (30 mg/mL) intraperitoneally and the bioluminescence signal in mice was measured using IVIS® Lumina II system. For *ex vivo* imaging, the excised tumor was immersed in 10 mL D-luciferin (3 mg/mL) for about 5 min, then the bioluminescence signal was measured using IVIS® Lumina II system. These experiments were repeated with n=5 mice per group. The results from the repeat experiments were similar to those in **Fig. 5**.

For intravenous administration of NK cells

NOD SCID mice (female, 6-10 weeks) were purchased from model animal research center of Nanjing University (Nanjing, China). 1×10^6 Raji-luc cells were injected intravenously on day 0. At days 2, 5, 8, 12, 15, animals were administrated with 1×10^7 NK-92 cells or MsNK-92 cells via the tail vein (each group n = 10). The control group received PBS only. The body weight and survival of the mice were continuously monitored. Mice that developed hind-limb paralysis in the course of the experiments were euthanized. Kaplain-Meier curves were constructed.

Supplementary References

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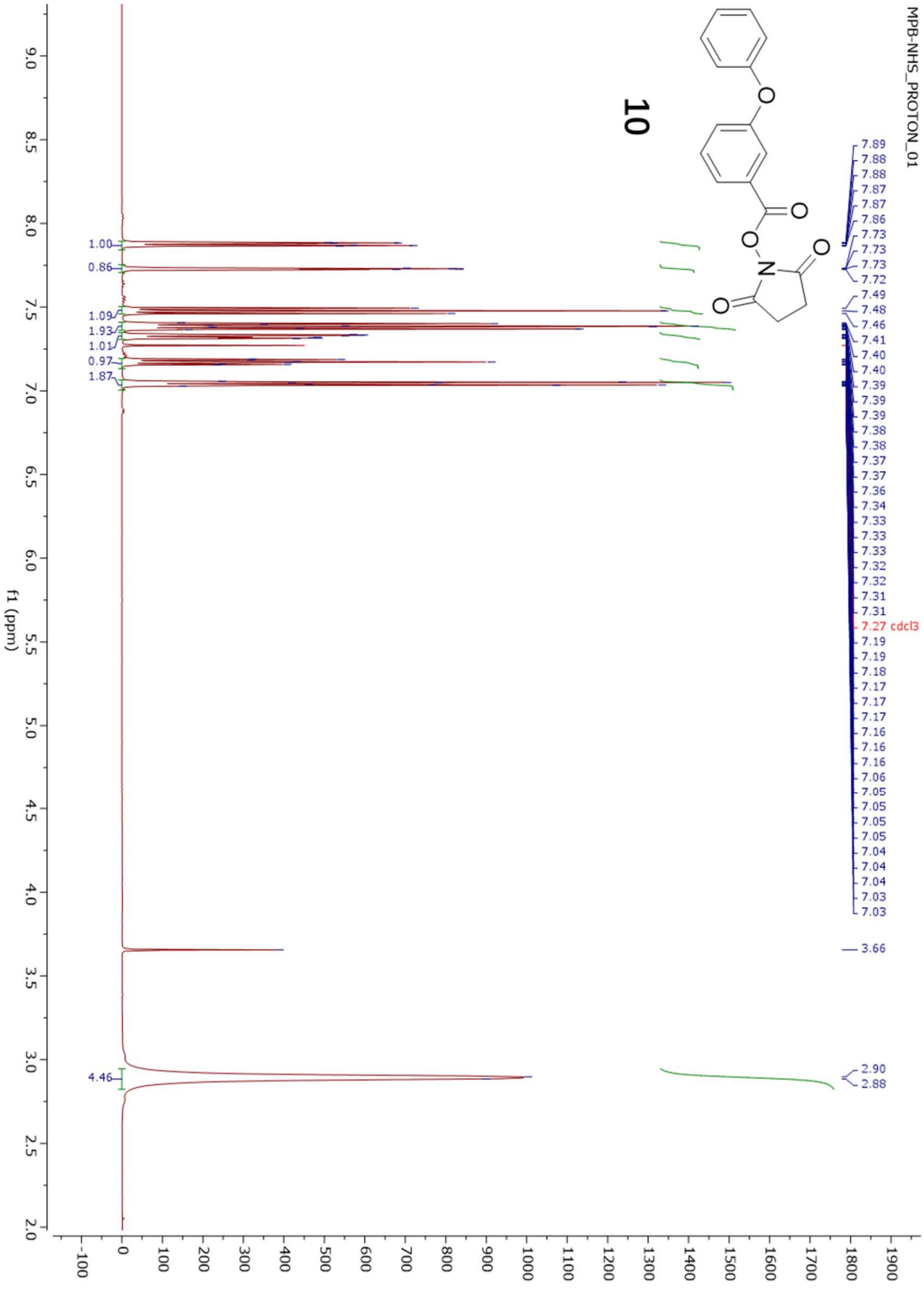
^{1}H NMR for compound **10**, $CDCl_3$, 500MHz



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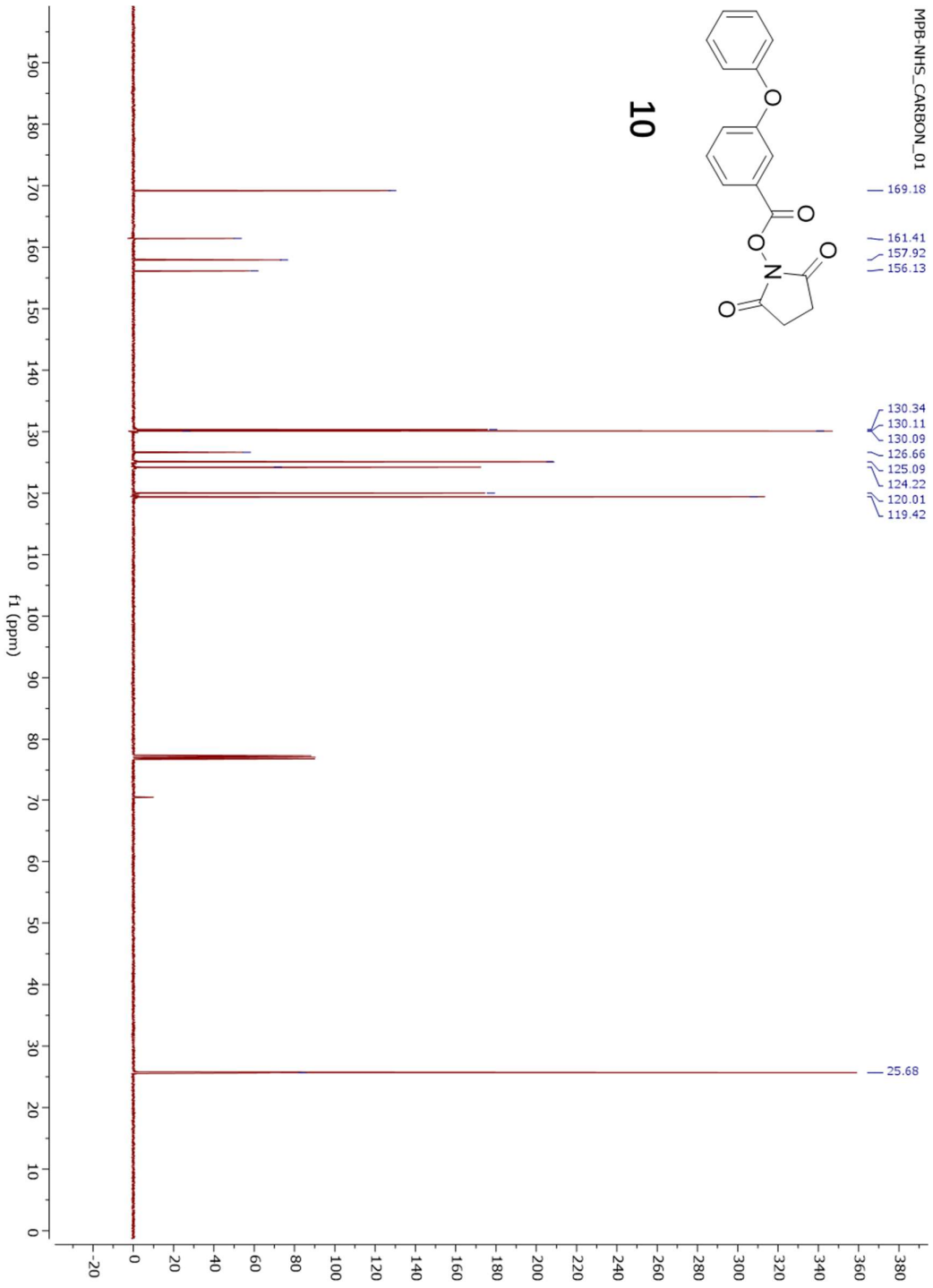

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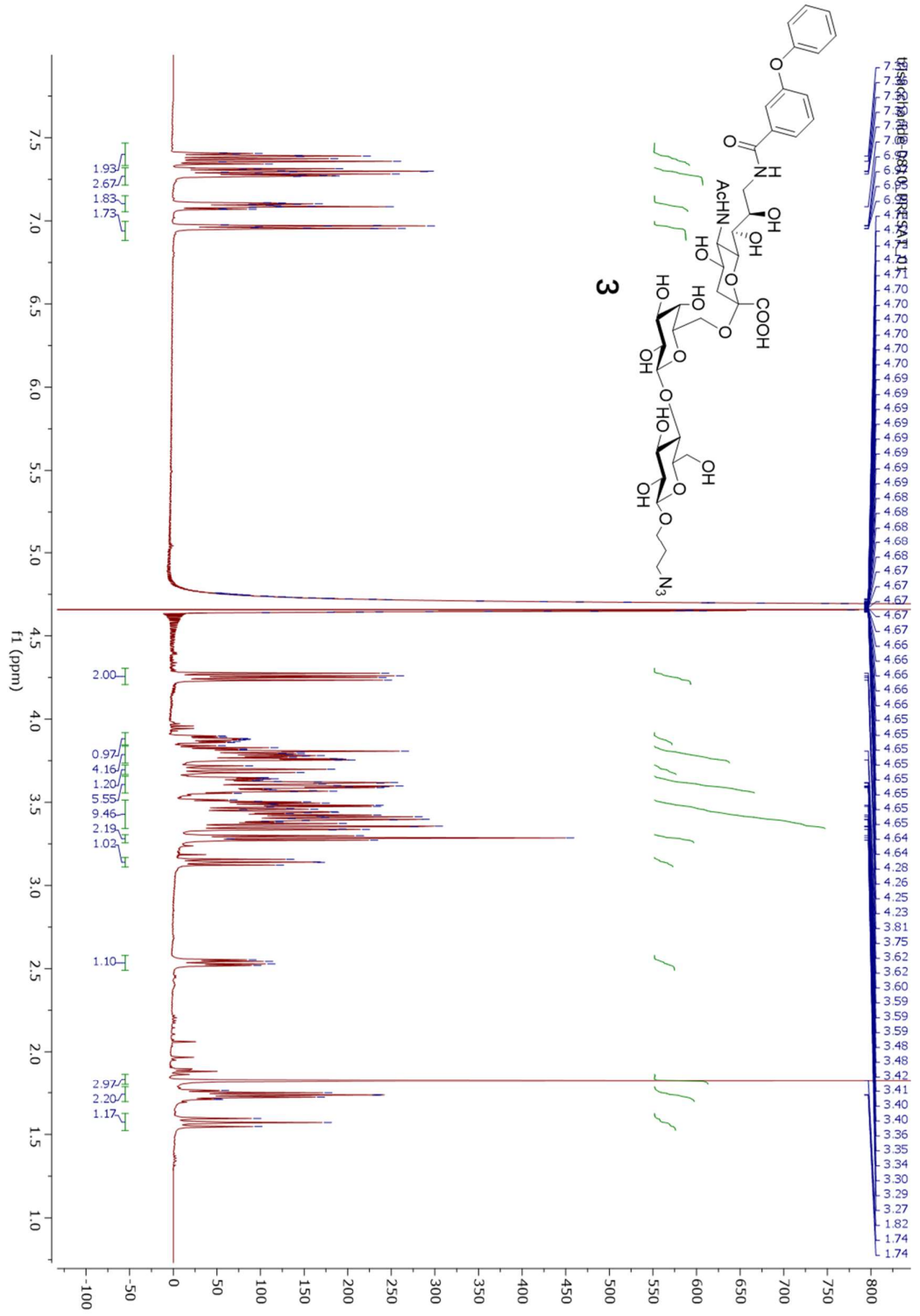

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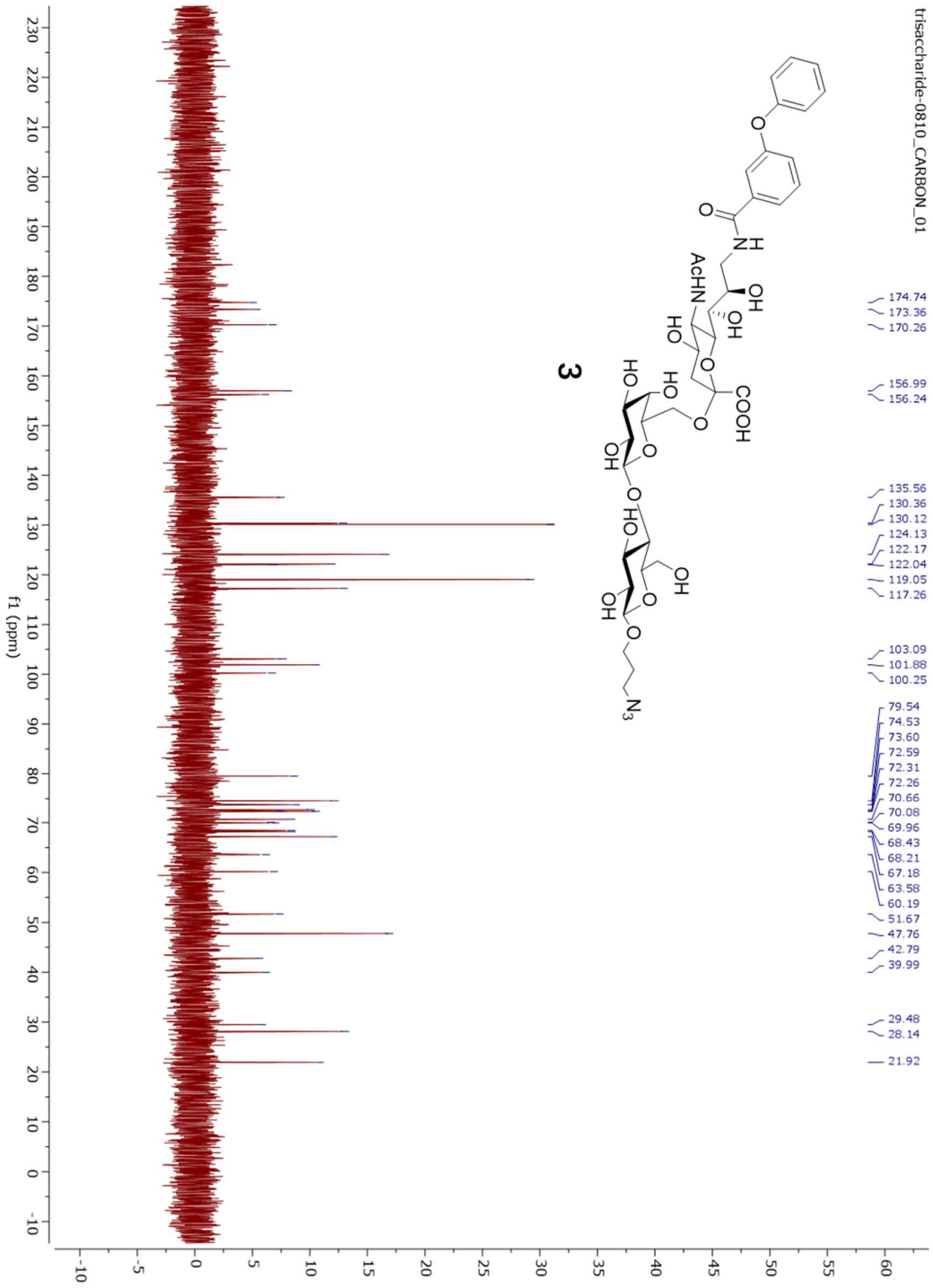
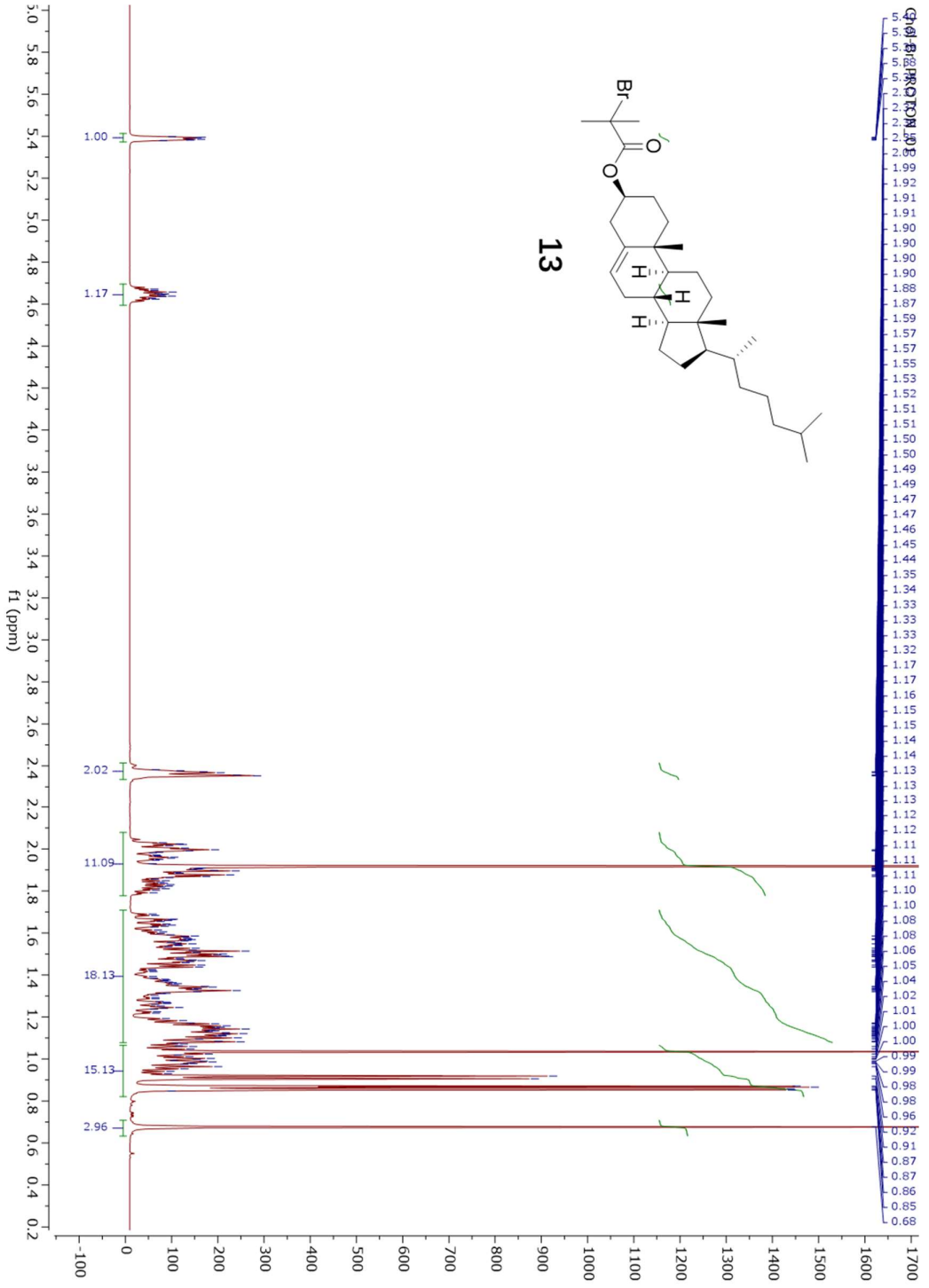


^{1}H NMR for compound **13**, $CDCl_3$, 500MHz



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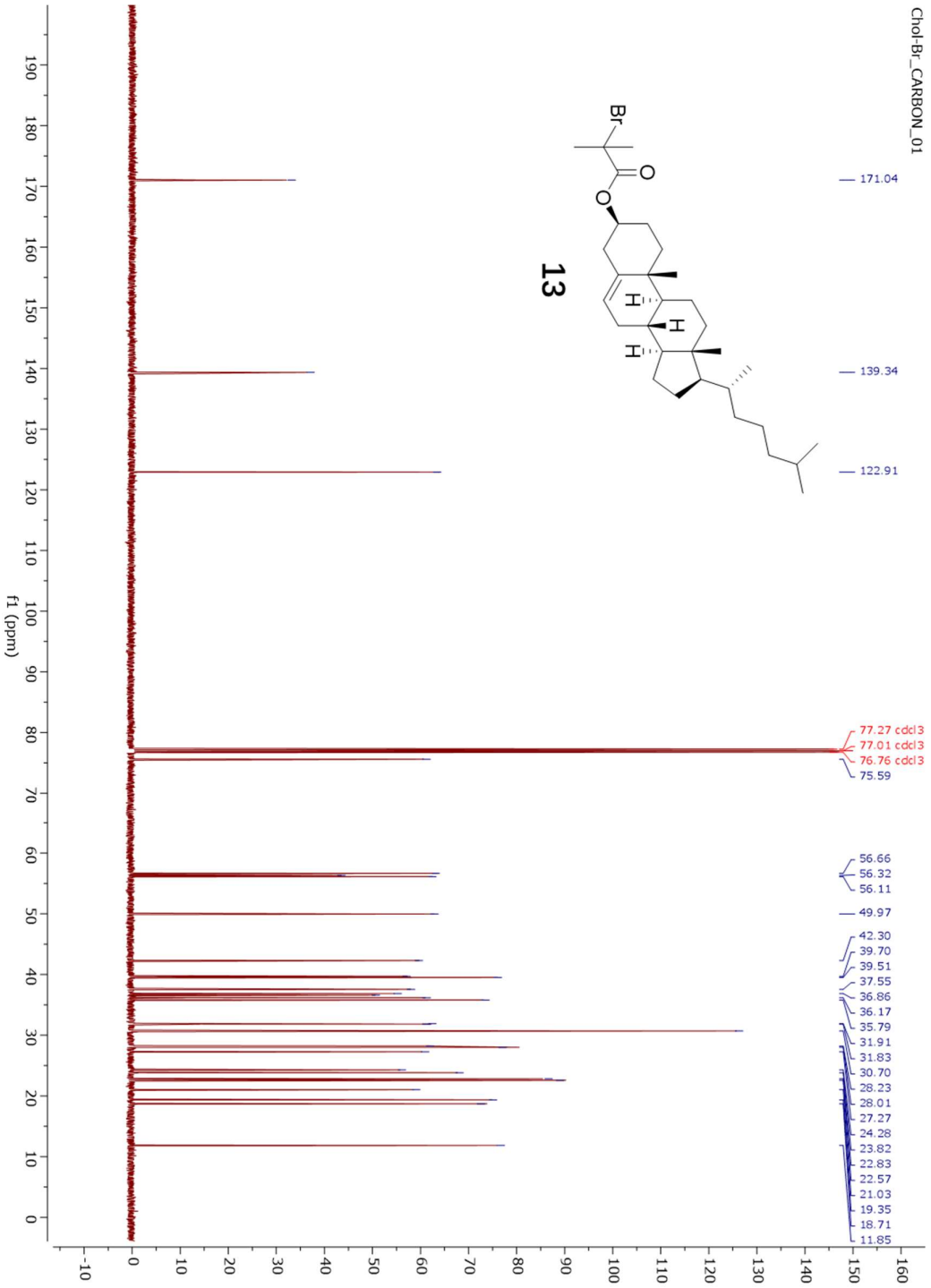

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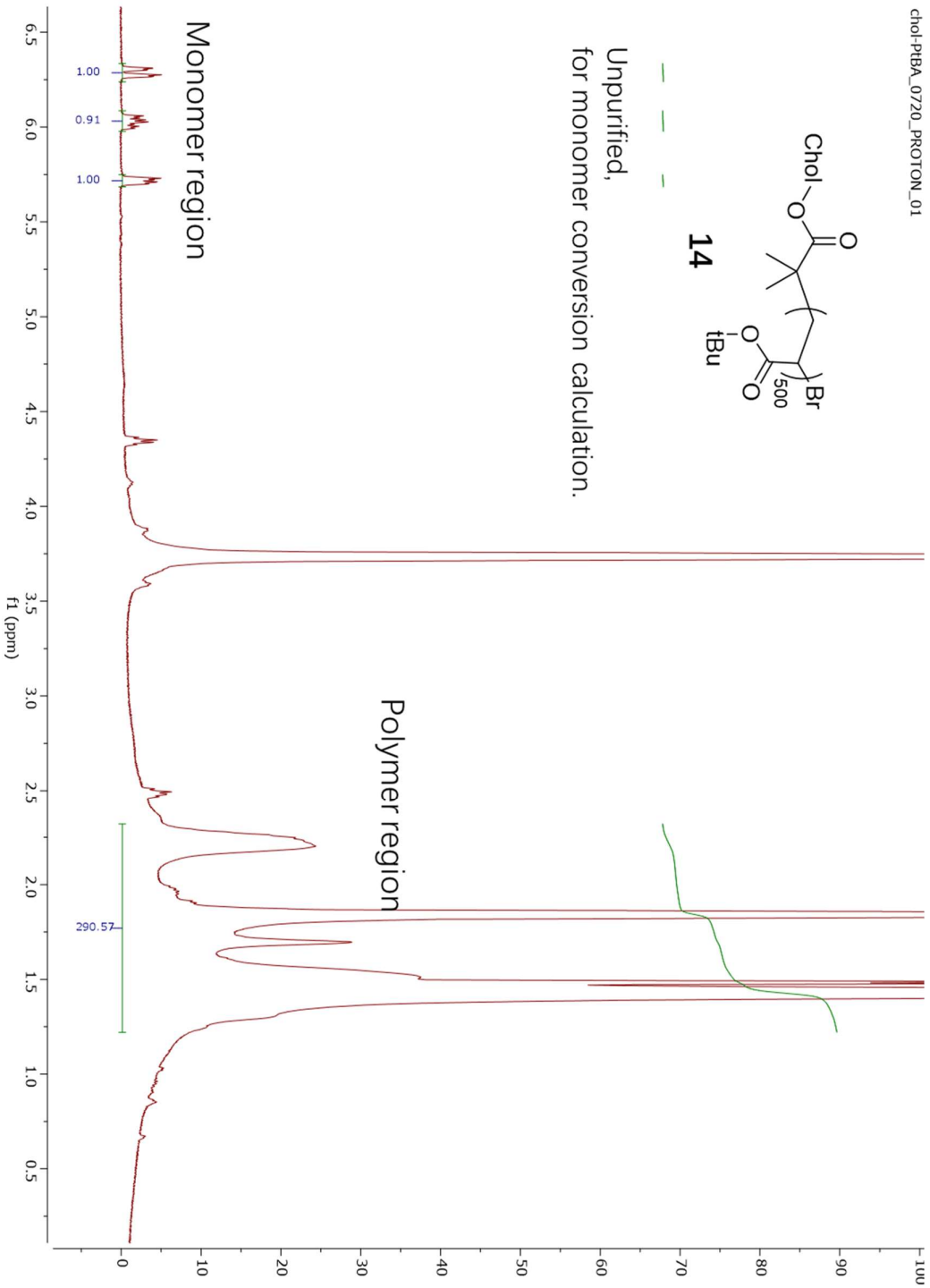
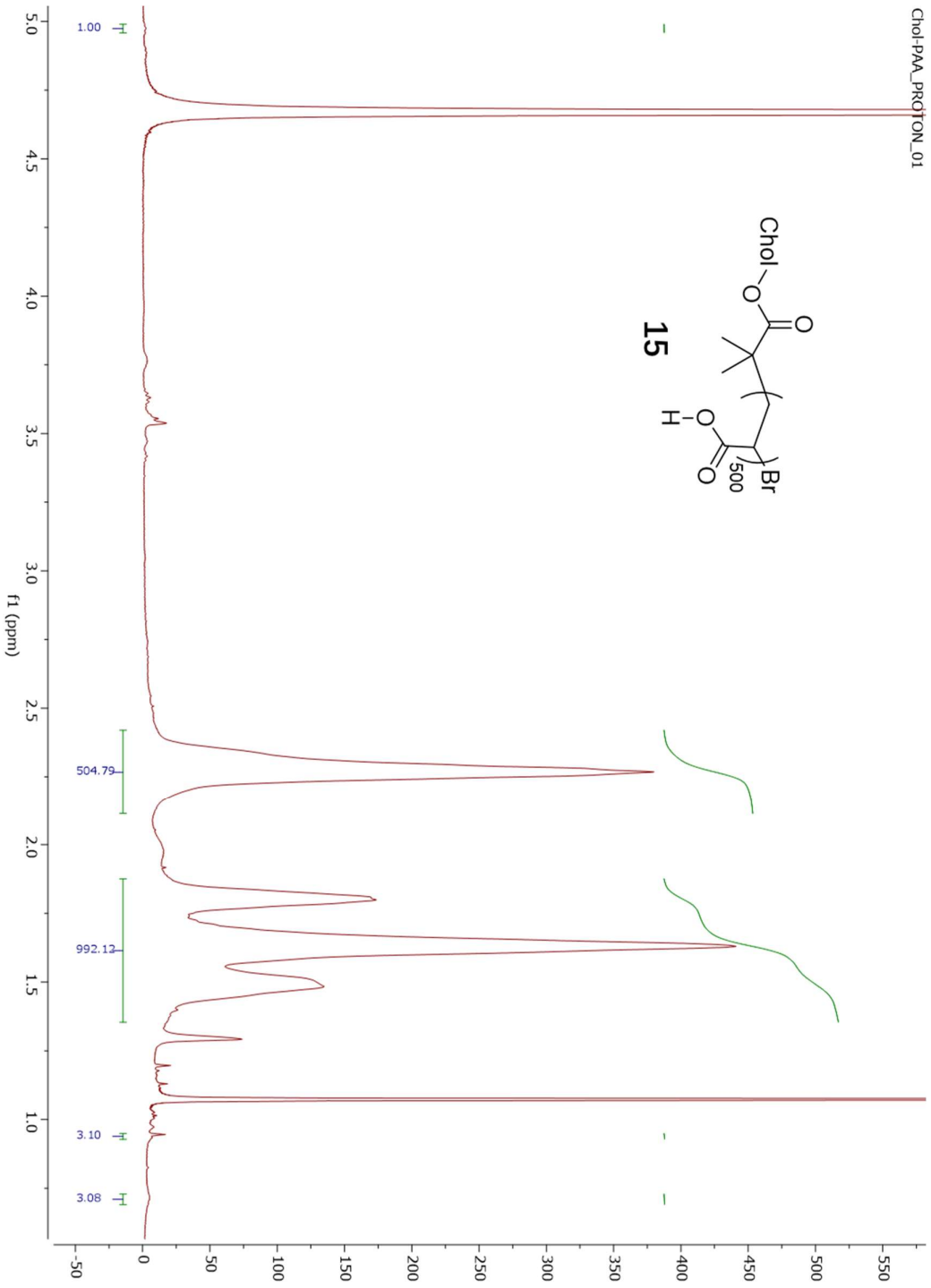
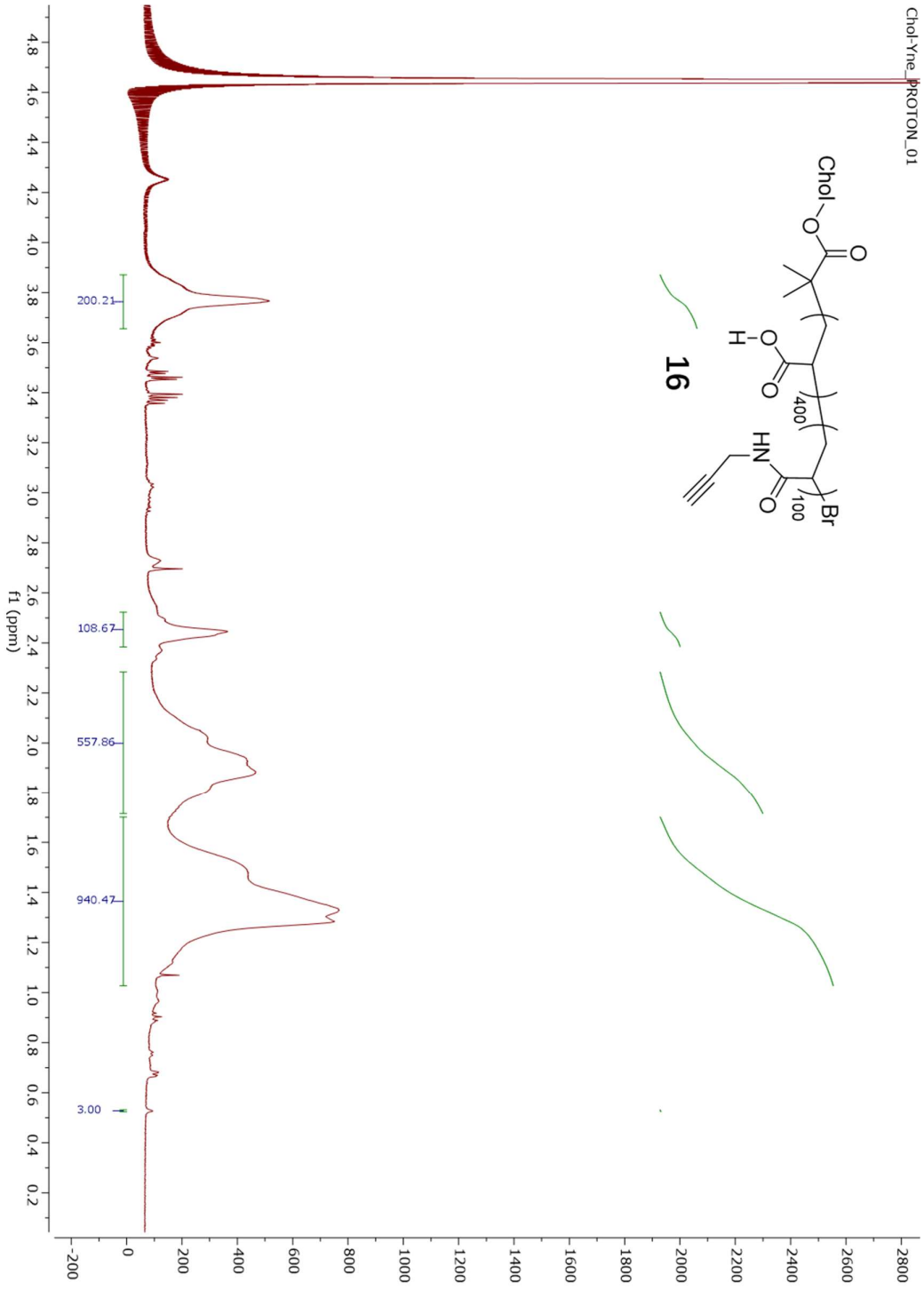


^{1}H NMR for compound **15**, D_2O, 500MHz



^{1}H NMR for compound **16**, D_2O, 500MHz



^{1}H NMR for compound **4**, D_2O, 500MHz