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Delivery of an Active Lysosomal Enzyme Using GNeosomes

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Materials

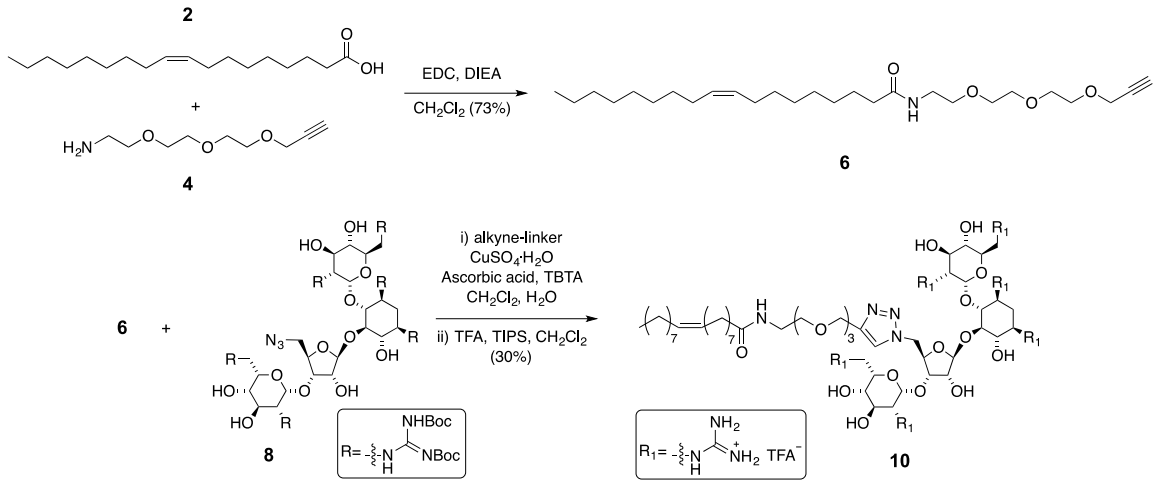
Materials obtained from commercial suppliers were used without further purification. Chemicals and reagents were purchased from Sigma Aldrich. Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phospho-ethanolamine), and cholesterol were purchased from Avanti Polar Lipids. PBS (Dulbecco's phosphate buffered saline), F-12 Nutrient Mixture (Ham), DMEM, and F12/DMEM were purchased from Thermo Scientific. Trypsin/EDTA was purchased from VWR. 4-methylumbelliferyl α-L-iduronide was purchased from CarboSynth. α-L-Iduronidase (Aldurazyme) was manufactured by BioMarin Pharmaceuticals and purified via FPLC on a heparin column prior to use.

Instrumentation

NMR spectra were recorded on either a Varian 400 MHz or 500 MHz spectrometers. Mass spectra were recorded at UCSD Chemistry and Biochemistry Mass Spectrometry Facility utilizing an Agilent 6230 HR-ESI-TOF mass spectrometer. Reverse-phase HPLC purification (CLIPEUS, C18, 5μm, 10x250 mm, Higgins analytical and SepaxGP, C4, 5μm, 10x250 mm 5μm, 4.6x150 mm column) and analysis (Eclipse, XDB-C18, 5μm, 4.6x150 mm and SepaxGP, C4, 5μm, 4.6x150 mm) were carried out on an Agilent 1200 series instrument. Fluorescence spectroscopy measurements were performed using a Horiba fluorimeter. Particle size, polydispersity, and surface charge of the lipid vesicles were measured by dynamic light scattering on a Zetasizer Nano ZS (model ZEN3600 from Malvern Instruments). Flow cytometry studies were performed on a BD FACSCalibur.

Synthesis of Lipid-GNeo Derivatives

Amino-alkyne linker (**4**), N_3-BocGNeo (**8**), and stearyl-GNeo (**9**) were synthesized according to previously published procedures.[1-3]



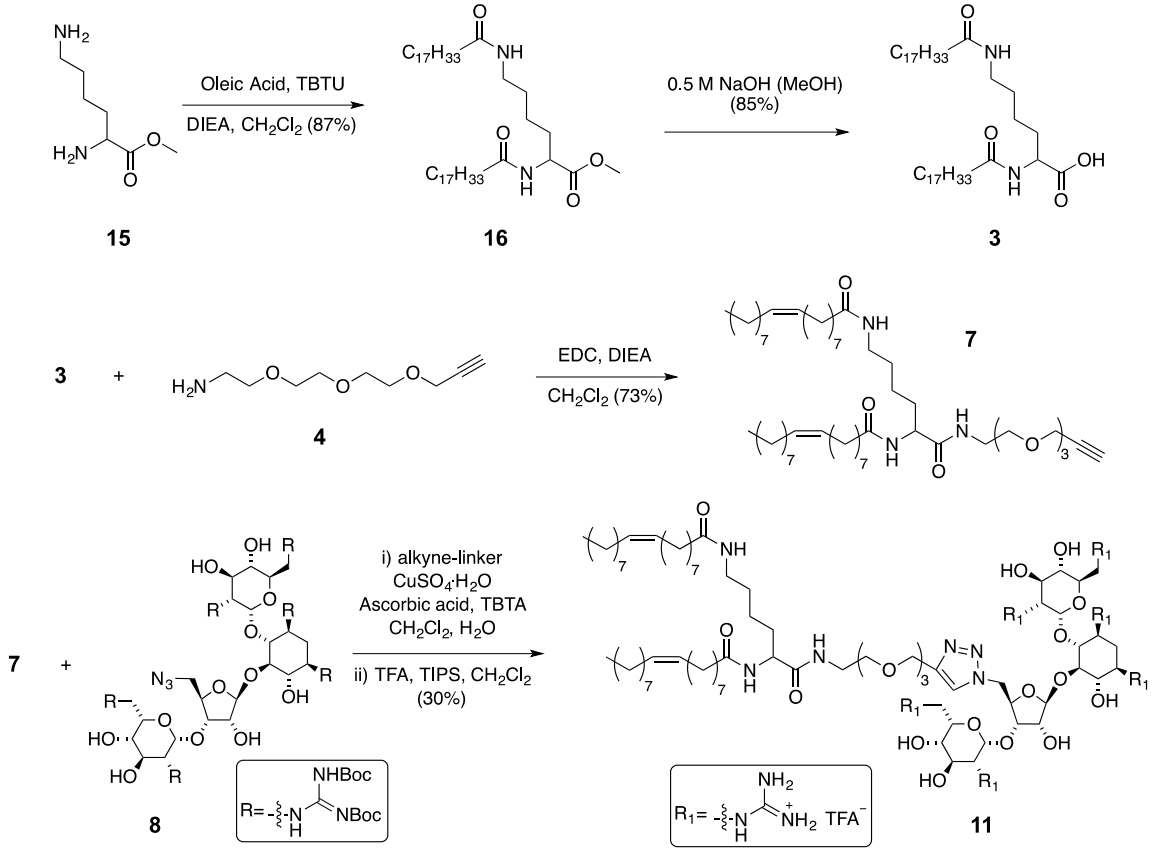
Scheme S1. Synthesis of oleyl-GNeo.

Oleyl-alkyne linker (6). To a solution of oleic acid (424 mg, 1.5 mmol) in dichloromethane, was added *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (370 mg, 2 mmol) and the solution was stirred at room temperature for 30 min. Compound **4** (187 mg, 1.0 mmol) and DIEA (178 μL, 1 mmol) were dissolved in CH_2Cl_2 and added to the reaction. After stirring overnight at room temperature, the reaction was diluted with CH_2Cl_2 and washed with aqueous citric acid (5%) and brine. The organic phase was dried over sodium sulfate and evaporated. The residue was purified by flash chromatography to afford the desired compound (340 mg, 0.75 mmol, 73% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.12 (s, 1H), 5.3 (m, 2H), 4.21 (d, J = 2.4 Hz, 2H), 3.71–3.64 (m, 8H), 3.56 (t, J = 4.8 Hz, 2H), 3.45 (q, J = 5.1 Hz, 2H), 2.44 (t, J = 2.4 Hz, 1H), 2.18 (t, J = 7.5 Hz, 2H), 2.00 (m, 4H), 1.63 (m, 2H), 1.35–1.25

(m, 24H), 0.87 (t, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 173.32, 129.96, 129.74, 79.47, 74.64, 70.50, 70.34, 70.16, 69.95, 69.09, 58.41, 39.13, 36.70, 31.89, 29.75, 29.72, 29.51, 29.30, 29.16, 27.21, 27.18, 25.74, 22.66, 14.10. ESI-HR-MS calculated $[M+H]^+$ 452.3734, found 452.3735.

Oleyl-GNeo (10). To a solution of **8** (1 eq) and **6** (2 eq) in methanol/tetrahydrofuran/water (2:1:0.3, 3.3 mL/0.1μmol) was added $CuSO_4 \cdot 5H_2O$ (2 eq) and sodium ascorbate (2 eq) at room temperature. The mixture was vigorously stirred at room temperature for 18 hours and then diluted with CH_2Cl_2 and water. The organic phase was washed twice with EDTA (0.1 M), aqueous KCN (5%), and brine. The organic phase was dried over Na_2SO_4 and evaporated. The residue was dissolved in CH_2Cl_2 (2 mL) and triisopropylsilane (100 μL) and trifluoroacetic acid (2 mL) were added. The reaction was stirred 12 h at room temperature, concentrated under vacuum and coevaporated with toluene (3×). The residue was dissolved in water, washed extensively with CH_2Cl_2, lyophilized, and on reverse phase HPLC to obtain the desired compound as an amorphous fluffy white powder (4 mg, 30% yield). ^{1}H NMR (500 MHz, D_2O) δ: 7.90 (s, 1H), 5.63 (s, 1H), 5.28 (m, 2H), 4.97 (s, 2H), 4.34 (m, 1H), 4.26 (m, 2H, 4.03 (m, 2H), 3.68-3.38 (m, 26H), 3.26 (m, 2H), 2.10 (m, 3H), 1.89 (m, 4H), 1.45 (m, 3H), 1.14 (m, 22H), 0.74 (m, 3H). ^{13}C NMR (125 MHz, D_2O) δ: 176.66, 163.20, 162.92, 162.64, 162.36, 157.68, 157.26, 157.17, 157.03, 156.46, 143.70, 130.53, 130.20, 125.59, 119.81, 117.48, 115.16, 112.84, 111.37, 97.73, 95.81, 85.21, 78.82, 77.68, 77.21, 74.40, 72.78, 72.61, 71.87, 70.87, 69.60, 69.46, 69.26, 69.12, 68.92, 66.71, 63.14, 55.39, 53.29, 51.94, 50.43, 41.85, 41.69, 38.82, 35.79, 32.08, 32.06, 31.47, 29.12, 28.88, 28.78, 28.67,

28.54, 28.35, 26.68, 25.46, 22.24, 22.21. ESI-HR-MS calculated $[M+2H]^{2+}$ 672.4151, found 672.4149.



Scheme S2. Synthesis of di-oleyl-GNeo.

Di-oleyl-ester (**16**) and di-oleyl-acid (**3**) were prepared according to literature procedures.[4]

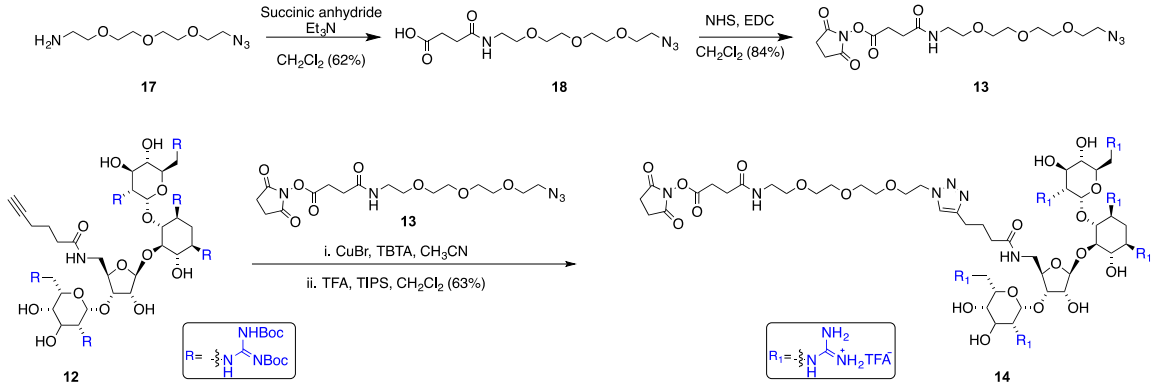
Di-oleyl-alkyne linker (7). To a solution of **3** (152 mg, 0.225 mmol) in dichloromethane, was added EDC (56 mg, 0.3 mmol) and the solution was stirred at room temperature for 30 min. Compound **4** (28 mg, 0.15 mmol) and DIEA (27 µL, 0.15 mmol) were dissolved in CH_2Cl_2 and added to the reaction. After stirring overnight at room temperature, the reaction was diluted with CH_2Cl_2 and washed with aqueous citric acid (5%) and brine. The organic phase was dried over sodium sulfate and evaporated. The residue was purified by flash chromatography to afford the desired compound (90

mg, 0.11 mmol, 73% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.97 (s, 1H), 6.57 (s, 1H), 6.23 (s, 1H), 5.34 (m, 4H), 4.42 (m 1H), 4.22 (d, *J* = 2.4 Hz, 2H), 3.75–3.68 (m, 5H), 3.64 (m, 4H), 3.56 (m, 2H), 3.46 (m, 2H), 3.25 (m, 2H), 2.49 (t, *J* = 2.4 Hz, 1H), 2.21 (m, 4H), 2.01 (m, 8H), 1.80 (m, 1H), 1.62 (m, 8H), 1.24–1.27 (m, 42H), 0.88 (t, *J* = 6.8 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 174.64, 174.40, 174.27, 130.01, 129.70, 129.67, 52.10, 38.79, 36.68, 36.40, 31.92, 31.18, 29.77, 29.55, 29.34, 29.31, 29.22, 29.20, 28.96, 27.23, 25.84, 25.71, 22.70, 21.97, 14.15. ESI-HR-MS calculated $[M+H]^+$ 844.7137, found 844.7132.

Di-oleyl-GNeo (11). To a solution of **8** (1 eq) and **7** (2 eq) in methanol/tetrahydrofuran/water (2:1:0.3, 3.3 mL/0.1μmol) was added $CuSO_4 \cdot 5H_2O$ (2 eq) and sodium ascorbate (2 eq) at room temperature. The mixture was vigorously stirred at room temperature for 18 hours and then diluted with CH_2Cl_2 and water. The organic phase was washed twice with EDTA (0.1 M), aqueous KCN (5%), and brine. The organic phase was dried over Na_2SO_4 and evaporated. The residue was dissolved in CH_2Cl_2 (2 mL) and triisopropylsilane (100 μL) and trifluoroacetic acid (2 mL) were added. The reaction was stirred 12 h at room temperature, concentrated under vacuum and coevaporated with toluene (3×). The residue was dissolved in water, washed extensively with CH_2Cl_2, lyophilized, and on reverse phase HPLC to obtain the desired compound as an amorphous fluffy white powder (35% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.01 (s, 1H), 5.90 (d, *J* = 3.6 Hz, 1H), 5.35 (t, *J* = 5 Hz, 2H), 5.13 (s, 1H), 5.10 (m, 1H), 5.07 (s, 1H), 4.70 (m, 7H), 4.40 (m, 1H), 4.26 (m, 4H), 4.12 (m, 1H), 4.01 (m, 1H), 3.75–3.52 (m, 38H), 3.42-3.35 (m, 8H), 3.16 (m, 3H), 2.27 (m, 2H), 2.18 (t, *J* = 7.5 Hz, 2H), 2.04 (m, 6H), 1.70-1.51 (m, 16H), 1.39-1.27 (m, 50H), 0.91 (t, *J* = 6.6 Hz, 3H).

^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.10, 174.90, 173.48, 161.70, 158.21, 157.89, 157.79, 157.67, 157.10, 144.03, 129.49, 129.33, 125.37, 111.91, 97.73, 95.57, 85.93, 79.97, 70.01, 77.65, 76.39, 74.97, 73.05, 72.76, 72.68, 70.61, 69.94, 69.89, 69.79, 69.71, 69.47, 69.07, 67.48, 63.46, 55.56, 53.91, 53.58, 53.54, 52.44, 51.58, 50.26, 42.17, 41.75, 38.89, 38.54, 35.81, 35.46, 33.31, 32.21, 31.65, 31.55, 31.19, 29.43, 29.20, 29.03, 29.00, 28.94, 28.89, 28.85, 28.68, 26.73, 25.71, 25.57, 24.69, 22.86, 22.32, 13.04. ESI-HR-MS calculated $[M+3H]^{3+}$ 579.3926, found 579.3925.

Synthesis of GNeo-NHS



Scheme S3. Synthesis of NHS-N_3-linker and GNeo-NHS.

Alkyne-BocGNeo (**12**) was prepared according to a previously published procedure.[5]

Acid-N3-Linker (18). Succinic anhydride (110 mg, 1.1 mmol) and triethylamine (153 µL, 1.1 mmol) were added to a solution of 11-azido-3,6,9-trioxaundecan-1-amine (200 mg, 0.92 mmol) in dichloromethane (1 mL). The reaction was allowed to stir for 12 hours at room temperature. The reaction was diluted in CH_2Cl_2, washed with 0.1N HCl and brine. The organic layer was dried over sodium sulfate, concentrated under reduced pressure and further purified by flash chromatography (9% CH_3OH in CH_2Cl_2) to afford an oil (180mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.57 (broad, 1H), 3.71–3.66 (m,

8H), 3.63(m, 2H), 3.55 (t, *J* = 5.2 Hz, 2H), 3.46 (m, 2H), 3.39 (t, *J* = 4.9 Hz, 2H) 2.69 (t, *J* = 6.2 Hz, 2H), 2.52 (t, *J* = 6.3 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 174.48, 172.65, 70.75, 70.57, 70.44, 70.18, 69.95, 69.52, 50.66, 39.53, 31.09, 30.44. ESI-HRMS: calculated $[M+Na]^+$ 341.1432, found: 341.1434.

NHS-N_3-Linker (13). N-hydroxysuccinimde (32 mg, 0.232 mmol) and **18** (75 mg, 0.23 mmol) were dissolved in dichloromethane (2 mL) and treated with *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride (32 mg, 0.28 mmol). The reaction was stirred at room temperature for 12 hours. The reaction was diluted with CH_2Cl_2 and washed with water, brine, and dried over sodium sulfate. The organic layer was concentrated under reduced pressure and further purified by flash chromatography (4% CH_3OH in CH_2Cl_2) providing the product as an oil (57mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.37 (s, 1H), 3.68–3.64 (m, 8H), 3.62 (m, 2H), 3.56 (t, *J* = 4.9 Hz, 2H), 3.46 (m, 2H), 3.39 (t, *J* = 5.1 Hz, 2H), 2.98 (t, *J* = 7.3 Hz, 2H), 2.82 (s, 4H), 2.60 (t, *J* = 7.3 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.13, 169.09, 168.35, 70.80, 70.73, 70.65, 70.35, 70.14, 69.83, 50.81, 39.58, 30.66, 26.90, 25.71. ESI-HRMS: calculated $[M+Na]^+$ 438.1595, found: 438.1597.

GNeo-NHS (14). Compounds **12** (200 mg, 0.0925 mmol) and **13** (57 mg, 0.139 mmol) were dissolved in acetonitrile (2 mL). 20 mol % CuBr (462 μL of 0.04 M solution in acetonitrile) and 20 mol % *tris*[(1-benzyl-1*H*-1,2,3-triazol-4yl)methyl]amine (462 μL of 0.04 M solution in acetonitrile) were added to the reaction mixture. The reaction was allowed to stir at room temperature under argon for 24 hours. The solvent was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and washed with aqueous KCN (5%) and EDTA (0.1 M). The organic layer was dried over sodium sulfate and

concentrated under reduced pressure followed by flash chromatography purification (3% CH_3OH in CH_2Cl_2) to afford a white solid. The compound was then dissolved in CH_2Cl_2 (2 mL) and treated with triisopropylsilane (20 μL) and trifluoroacetic acid (2 mL) and stirred for 2 hours at room temperature. The reaction was concentrated in vacuo and coevaporated with toluene (3×) to remove the trifluoroacetic acid. The residue was dissolved in cold water, filtered, and lyophilized to afford a white solid (119 mg, 63%). ^{1}H NMR (500 MHz, DMSO–d_6) δ: 8.14 (b, 1H), 8.04 (m, 2H), 7.80 (s, 1H), 7.61 (b, 1H), 7.57–7.04 (m, 26H), 6.90 (d, J = 8.9 Hz, 1H) 5.93 (b, 1H), 5.77 (b, 1H), 5.61 (d, J = 3.6 Hz, 1H), 5.41 (b, 2H), 4.96 (s, 1H), 4.82 (s, 1H), 4.46 (t, J = 5.3 Hz, 2H), 4.19 (b, 1H), 4.08 (t, J = 5.8 Hz, 1H), 3.90 (t, J = 6.8 Hz, 1H), 3.85 (b, 1H), 3.79 (t, J = 5.4 Hz, 2H), 3.72 (m, 1H), 3.64 (m, 1H), 3.60–3.55 (m, 2H), 3.54–3.42 (m, 12H), 3.41–3.30 (m, 7H), 3.30–3.10 (m, 4H), 2.85 (t, J = 6.9 Hz, 1H), 2.79 (s, 4H), 2.49–2.30 (m, 3H), 2.20 (t, J = 7.3 Hz, 2H), 1.96 (m, 1H), 1.82 (m, 3H), 1.60–1.48 (m, 2H). ^{13}C NMR (125 MHz, DMSO–d_6) δ: 173.10, 171.08, 170.78, 170.11, 169.78, 168.63, 159.13, 158.86, 158.60, 158.33, 157.63, 157.45, 157.15, 157.06, 156.71, 146.30, 122.20, 110.41, 97.79, 95.45, 85.37, 79.20, 78.77, 75.88, 74.28, 73.03, 72.13, 70.08, 69.89, 69.60, 69.54, 69.50, 69.45, 69.05, 69.00, 68.75, 66.51, 55.22, 53.16, 51.65, 51.26, 50.09, 49.21, 41.40, 34.87, 29.91, 29.72, 29.15, 29,11, 28.76, 27.95, 25.90, 25.22, 25.10, 24.60. ESI-HRMS: calculated $[M+2H]^{2+}$ 688.3429 found: 688.3408.

Preparation of liposomes

Lipid films were prepared from DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), and cholesterol (73:11:16) dissolved in chloroform. The solvent was evaporated and further

dried under high vacuum to form a lipid film. The resulting film was rehydrated with phosphate buffered saline (PBS, pH 7.4) containing the cargo to be encapsulated (either 100 μM Cy5 or 5mg/ml α-L-iduronidase). The lipid suspension was subjected to sonication, freeze and thaw cycles, and extrusion 17 times through 100 nm polycarbonate membranes. Extravesicular cargo was removed by gravitational gel filtration (Sephadex G-50 for small molecules or Sepharose 4B for enzyme), eluting with PBS. Lipid concentration was determined by adapting the Stewart method.[6]

Preparation of GNeo-liposomes: Pre-inserted

Liposomes were prepared as described above except the lipid film was rehydrated with PBS containing 0.9 mol % lipid-GNeo and the cargo to be encapsulated.

Preparation of GNeo-liposomes: Post-inserted

Unmodified liposomes (3 mg/mL), as described above, were stirred for 1 h at room temperature with the lipid-GNeo derivative (0.9 or 1.8 mol %). Unincorporated lipid-GNeo was removed via centrifuge gel filtration (Sephadex G-50).[7]

Preparation of GNeo-liposomes: Post-modification

Unmodified liposomes (3 mg/mL), as described above, were stirred for 1 h at room temperature with GNeo-NHS (10 or 20 mol %). Remaining GNeo-NHS was removed via centrifuge gel filtration (Sephadex G-50).[7]

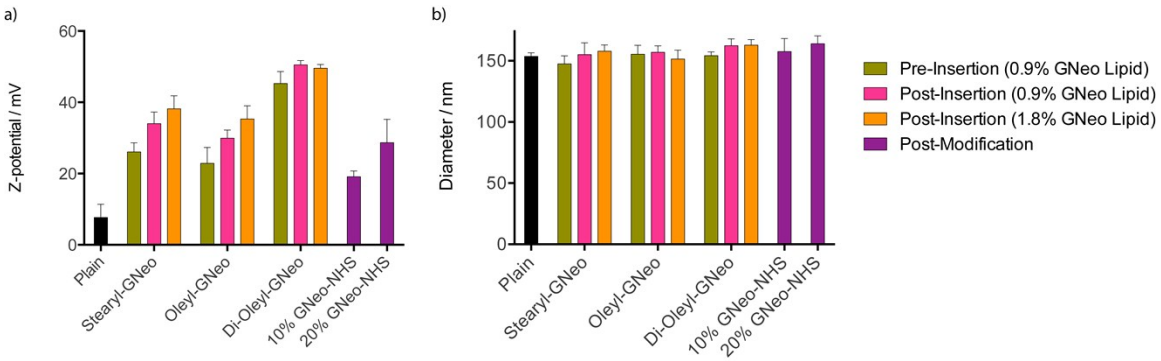


Fig. S1 Characterization of liposomes. (a) Zeta-potential of the evaluated liposomes. (b) Average

diameter of the evaluated liposomes. Four different batches of liposomes were each measured in triplicate.

Determination of lipid concentration in liposomal suspension

Lipid concentration was determined by adapting the Stewart method.[6] Briefly, diluted liposomes (50 μL) were vortexed with chloroform (1.5 mL) for 10 s. Ammonium ferrothiocyanate (1.5 mL, 0.1 M) was added and the biphasic systems was vortexed for 15 s then centrifuged for 1 min. The optical density of the organic phase was measured at 480 nm against chloroform as a blank. The amount of lipids present was estimated by comparison to a calibration curve generated using liposomal suspensions with a known lipid content.

Enzyme activity assay

α-L-Iduronidase activity was measured by assaying the conversion of 4-methylumbelliferyl α-L-iduronide (Carbosynth, Belkshire, UK) into the fluorochrome 4-methylumbelliferone (4-MU). The assay was performed in 96-well plates, using sodium citrate buffer (80 μL of 0.1 mol/L, pH 4.5) containing (NaCl 150 mmol/L), substrate (50 nmol) substrate, and enzyme (10 μL). After 1 h at 37 °C, fluorescent product was measured (Ex/Em 340 and 485 nm, respectively) and quantified using a standard curve of 4-MU. One unit (U) of activity is defined as the liberation of 1 μg 4-MU per hour at pH 4.5, 37 °C.

Cy5 encapsulation efficiency

To estimate the encapsulation efficiency of Cy5, the fluorescence intensity (640/672) of the liposome solution was measured in 1 mL 0.075 N HCl in isopropanol:water (9:1) before and after size exclusion purification (Figure S2).

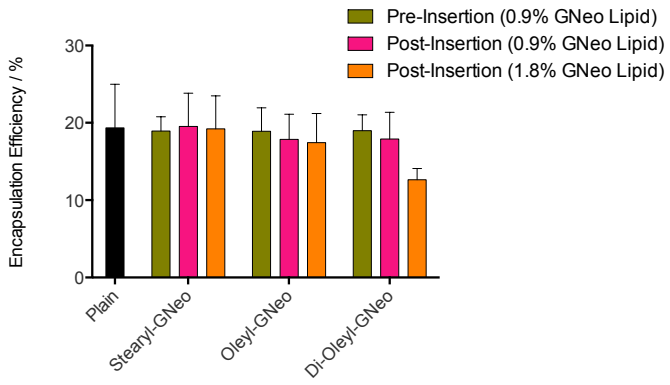


Fig. S2 Cy5-Encapsulation Efficiency.

Enzyme encapsulation efficiency

To estimate the amount of enzyme encapsulated, liposomes were lysed with 3% Tween 20 and analyzed for enzyme concentration by protein gel and for enzyme activity using the fluorescent 4-MU-α-L-iduronide substrate. Protein gel was run on a NuPage 4-12% Bis-Tris gel (Novex by Life Technologies) for 35 min at 200 V with MES buffer. Protein bands were visualized on an Odyssey Infrared imaging system (Li-Cor Biosciences) and quantitated by densitometry (Figure S2). Enzyme encapsulation efficiency was calculated as the ratio of IDUA before and after SEC.

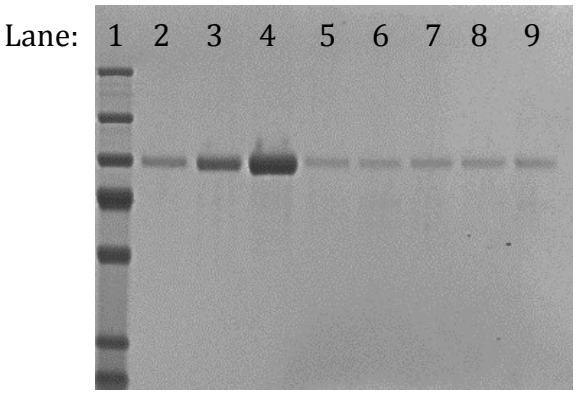


Fig. S3 IDUA-Encapsulation and Concentration. Opened liposomes and known concentrations of IDUA were loaded onto NuPage 4-12% Bis-Tris gel and run for 35 min at 200 V with MES buffer. Protein bands were imaged on an Odyssey Infrared imaging system. Lane 1: PageRuler Plus Prestained Protein Ladder (Life Technologies). Lane 2-4: 50, 100, and 500 ng IDUA. Lane 5: Plain liposomes. Lane 6: 0.9% stearyl-GNeo post-inserted. Lane 7: 1.8% stearyl-GNeo post-inserted. Lane 8: 0.9% di-oleyl-GNeo post-inserted. Lane 9: 0.9% di-oleyl-GNeo pre-inserted.

Cell culture

All cells were grown at 37 °C under an atmosphere of 5% CO_2 in air and 100% relative humidity. Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61). CHO-K1 cells were grown in F-12 medium supplemented with fetal bovine serum (10% v/v), penicillin/streptomycin. HFF and fibroblasts from MPS I patients were obtained from Coriell (GM00200 and GM00338) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin, sodium pyruvate, and glutamine.

Cellular uptake of Cy5-containing liposomes

Wild-type CHO-K1 cells were seeded onto 24-well tissue culture plates (100,000 cells/well, 0.4 mL) and grown for 24 h to about 80% confluence. Cells were washed with PBS and incubated with 300 μL of the liposomal suspension diluted in F-12 growth medium to 300 μg/mL at 37 °C for 1 h. Cells were washed twice with PBS, detached with trypsin/EDTA, diluted with PBS containing 0.1% BSA and analyzed by flow cytometry.

Cellular uptake of IDUA-containing liposomes

Normal HFF and MPS I fibroblasts were seeded onto 24-well plates (80,000 cells/well, 0.4 mL) and grown for 48 h. Cells were washed with PBS and incubated with liposome suspensions diluted in DMEM growth medium at the concentrations indicated. The cells were incubated at 37 °C for 1 h, washed twice with PBS, treated with trypsin/EDTA, and then combined with complete medium to inhibit the trypsin. Cells were sedimented by centrifugation, washed with PBS, and resuspended in 30 μL of RIPA lysis buffer. Enzyme activity in the cell extracts was measured as described above in

triplicate using 10 μL of cell lysate. Total protein concentration in the cell lysate was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific).

Turnover of [^{35}S]glycosaminoglycans

Normal and MPS fibroblasts were seeded in 12-well plates, and at confluence 50μCi of $H_2[^{35}S]O_4$ (PerkinElmer) was added in 1 mL DMEM/F12 medium supplemented with 10% fetal bovine serum. After 48 hours, the MPS I cells were washed with PBS and incubated with IDUA-containing liposomes or GNeo-IDUA at 37 °C for 1 h. All cells were then washed twice with PBS and chased for 24 h with 1 mL fresh DMEM/F12. Cells were harvested with trypsin, centrifuged (2400 rpm, 5 min), and washed once with PBS. The sedimented cells were then lysed with 0.1 M NaOH and purified over DEAE column. Total [^{35}S]glycosaminoglycan was counted by liquid scintillation spectroscopy using Scintillator Ultima Gold XR (PerkinElmer).

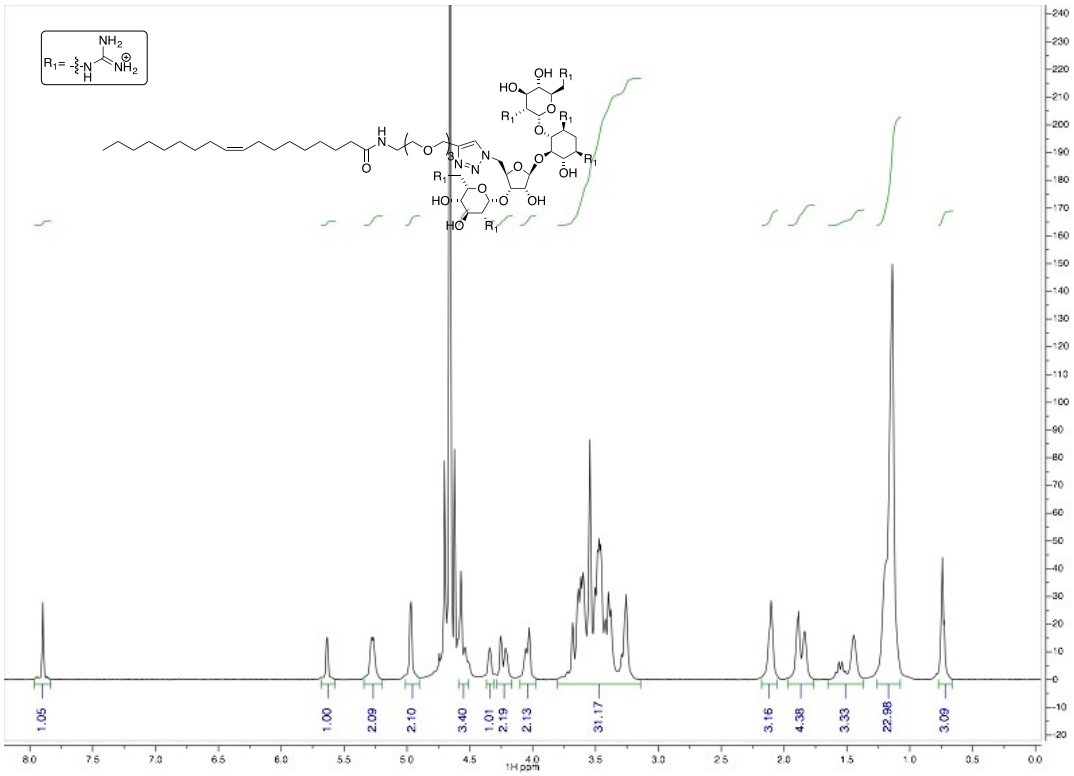


Fig. S4 ^{1}H NMR of of Oleyl-GNeo (**10**, D_2O, 500 MHz).

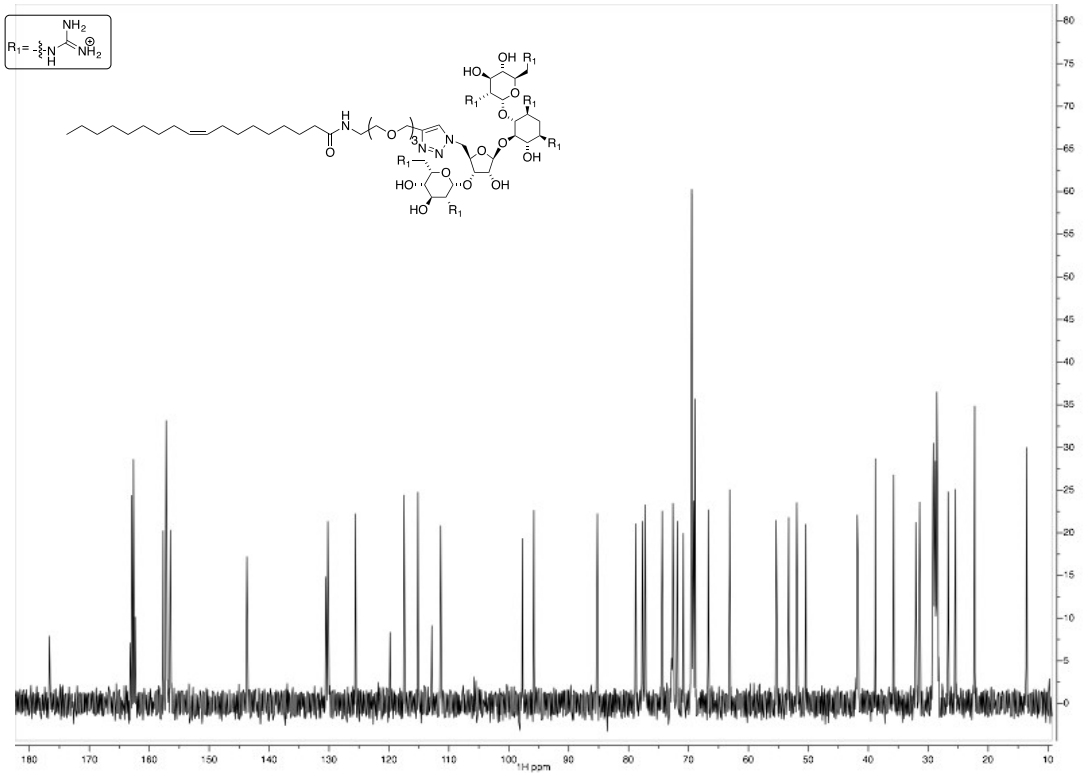


Fig. S5 ^{13}C NMR of of Oleyl-GNeo (**10**, D_2O, 500 MHz).

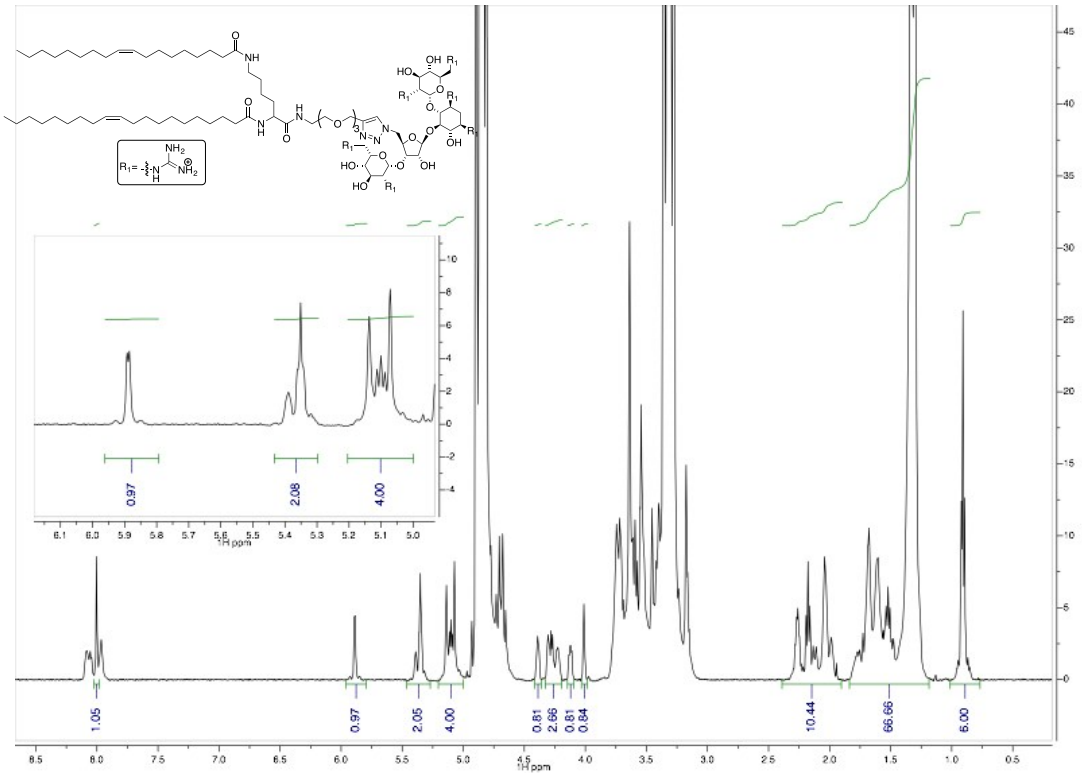


Fig. S6 ^{1}H NMR of of Di-Oleyl-GNeo (**11**, CD_3OD, 500 MHz).

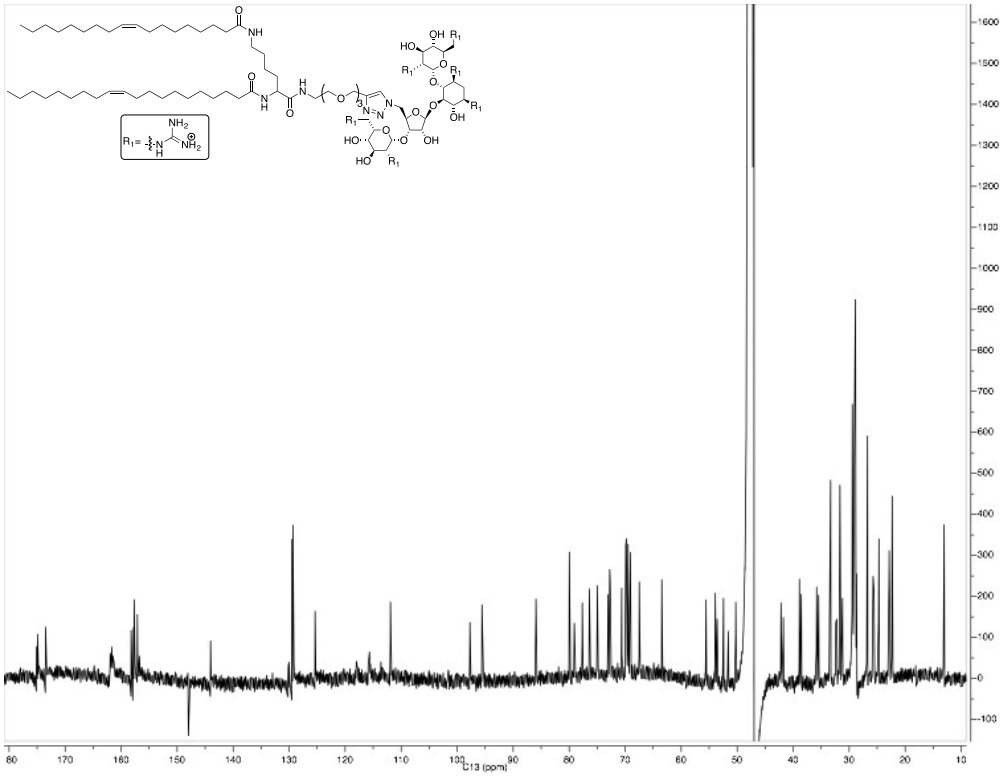


Fig. S7 ^{13}C NMR of of Di-Oleyl-GNeo (**11**, CD_3OD, 500 MHz).

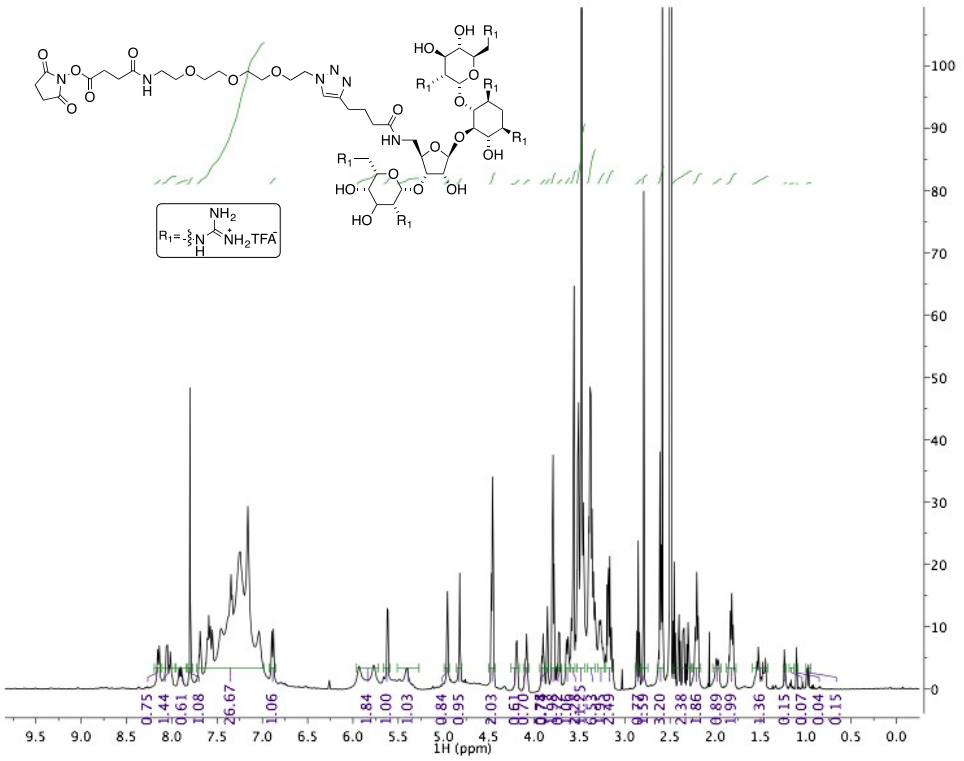


Fig. S8 ^{1}H NMR of of NHS-GNeo (**13**, DMSO-*d*6, 500 MHz).

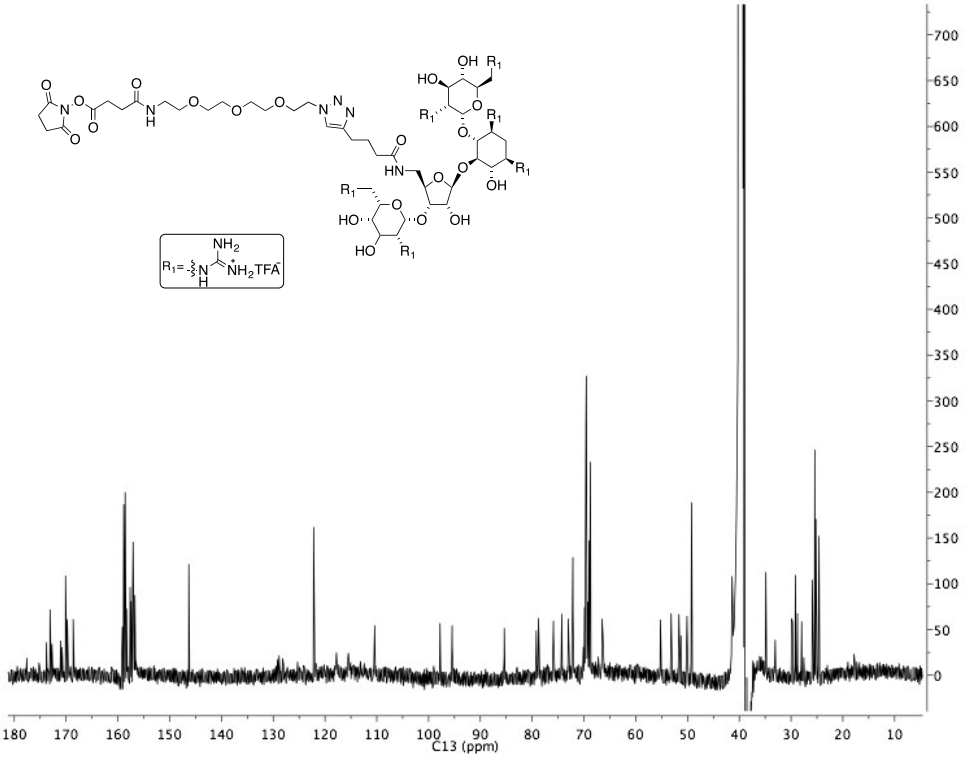


Fig. S9 ^{13}C NMR of of NHS-GNeo (**13**, DMSO-*d*6, 500 MHz).

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