Supplementary Information

For

Synthesis and Evaluation of Protein Conjugates of GM3 Derivatives Carrying Modified Sialic Acids as Highly Immunogenic Cancer Vaccine Candidates

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Experimental

General Methods. NMR spectra were recorded on a 400 or 500 MHz instrument with chemical shifts reported in ppm (δ) in reference to Me_4Si if not specified otherwise and coupling constants (*J*) in hertz (Hz). High resolution electron spray ionization mass spectra (HR ESI MS) were obtained with a Waters Micromass-LCTPremier-XE mass spectrometer, and matrix-assisted laser desorption ionization time of fly (MALDI-TOF) MS were obtained with a Bruker Ultraflex instrument. Thin layer chromatography (TLC) was performed on silica gel GF254 plates with detection by phosphomolybdic acid in EtOH or 1% H_2SO_4 in EtOH. Molecular sieves were dried under high vacuum at 170-180 ^oC for 6-10 h just before use. Commercial anhydrous solvents and other reagents were used without further purification. GM3NPhAc conjugates **1a** and **2a** and the HSA conjugate of GM3 were previously prepared by our laboratory.[1]

2-Azidoethyl (Methyl 4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-trifluoroacetamido-D-*glycero*-*α*-D-*galacto*-non-2-ulopyranosylonate)-(2→3)-(2,6-di-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-D-glucopyranoside (5). A mixture of glycosyl donor **3** (1.0 g, 1.69 mol), acceptor **4** (500 mg, 0.805 mmol), and activated molecular sieves (4Å, 2.0 g) in anhydrous acetonitrile (5.0 mL) was stirred at rt for 24 h under argon. After the mixture was cooled to -35 ^{o}C, NIS (764 mg, 3.38 mmol) was added, and 30 min later, TfOH (74.5 µl, 0.17 mmol) was added. The mixture was kept at -35 ^{o}C for 1 h and then diluted with dichloromethane (DCM). The solid material was filtered off and washed with DCM. The combined filtrates were washed with aqueous NaS_2O_3 (20%) and water. The organic phase was dried over Na_2SO_4 and concentrated in vacuum. Silica gel column chromatography of the residue afforded the desired trisaccharide **5** (410 mg, 63%). ^{1}H NMR($CDCl_3$, 400 MHz): δ 6.46 (d, 1H, J = 8.0 Hz, NH), 5.51-5.49 (m, 1H, H-8''), 5.36 (dd, 1H, J = 8.8, 2.4 Hz, H-7''), 5.17 (t, 1H, J = 8.8 Hz , H-2), 5.00-4.98 (m, 1H, H-2'), 4.94 (d, 1H, J = 8.8 Hz, H-1), 4.94-4.90 (m, 1H, H-4''), 4.54 (d, 1H, J = 8.4 Hz, H-1'), 4.46-4.44 (m, 1H, H-6), 4.42-4.40 (m, 1H, H-9''), 4.30-4.27 (m, 1H, H-3'), 4.23 (d, 1H, J = 6.8 Hz, H-1'), 4.19-4.15 (m, 1H, H-6), 4.08-4.04 (m, 1H, H-9''), 4.03-4.00 (m, 1H, H-5''), 3.99-3.95 (m, 1H, -OC$\underline{H_2}$CH_2N_3), 3.87-3.82 (m, 1H, H-3), 3.79 (s, 3H, -OCH_3), 3.83-3.79 (m, 1H, H-5'), 3.70-3.68 (m, 1H, -OC$\underline{H_2}$CH_2N_3), 3.67-3.63 (m, 1H, H-5), 3.62-3.59 (m, 1H, H-4), 3.50-3.44 (m, 1H, -OCH_2C$\underline{H_2}N_3$), 3.38 (bs, 1H, H-4'), 3.29-3.25 (m, 1H, -OCH_2C$\underline{H_2}N_3$), 2.69 (dd, 1H, J = 9.2, 4.8 Hz, H-3''e), 2.44 (bs, 1H, OH), 2.21, 2.13, 2.09, 2.09, 2.08, 2.05, 2.03, 2.03, 2.01 (9s, 9x3H, OAc), 1.78-1.84 (m, 1H, H-3''a). ^{19}F NMR ($CDCl_3$): δ -76.61 (s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.2, 170.9, 170.9, 170.8, 170.7, 170.2, 170.0, 169.7, 168.4, 101.1, 100.7, 97.1, 76.5, 74.1, 73.2, 73.0, 71.9, 71.9, 71.7, 69.8, 68.8, 67.3, 67.1, 62.9, 62.4, 53.5, 50.7, 50.0, 37.8, 21.6, 21.1, 21.0, 20.9, 20.9, 20.8, 20.7. MALDI-TOF MS Calcd for $C_{44}H_{59}F_3N_4NaO_{28}$ $[M+Na]^+$: 1171.3; Found: 1171.3.

***N*-{2-*O*-[(Methyl 4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-trifluoroacetamido-D-*glycero*-*α*-D-*galacto*-non-2-ulopyranosylonate)-(2→3)-(2,6-di-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl]-ethyl} 4-Pentenamide (6).** After a solution of **5** (140 mg, 0.122 mmol) in CH_3OH (4.0 mL) was stirred with Pd/C (100 mg) under a H_2 atmosphere at rt for 2 h, 4-pentenoic anhydride (50 µL, 0.244 mmol) and triethyl amine (50 µL) were added. The mixture was stirred at rt overnight, and then the solid material was filtered off. The filtrate was condensed under reduced pressure. The residue was purified by column chromatography to afford **6** (91 mg, 62%, 2 step overall) as a white solid. ^{1}H NM R($CDCl_3$, 400 MHz): δ 6.96 (d, 1H, J = 9.6 Hz, NH), 6.00 (t, 1H, J = 5.2 Hz,

NH), 5.84-5.74 (m, 1H, CH=CH_2), 5.52-5.48 (m, 1H, H-8''), 5.36 (dd, 1H, *J* = 8.8, 2.4 Hz, H-7''), 5.15 (t, 1H, *J* = 9.2Hz, H-2), 5.07-5.02 (m, 1H, H-2'), 4.98 (d, 1H, *J* = 8.0 Hz H-1), 4.97-4.85 (m, 1H, H-4''), 4.55 (d, 1H, *J* = 8.0 Hz, H-1'), 4.47-4.44 (m, 1H, H-6), 4.42-4.38 (m, 1H, H-9''), 4.27 (dd, 1H, *J* = 9.6, 2.4 Hz, H-3'), 4.22 (d, 1H, *J* = 5.6 Hz, H-1'), 4.18-4.14 (m, 1H, H-6), 4.10-4.07 (m, 1H, H-9''), 4.04-4.02 (m, 1H, H-5''), 4.01-3.94 (m, 1H, -OC$\underline{H_2}CH_2N_3$), 3.83-3.78 (m, 1H, H-3), 3.77 (s, 3H, -OCH_3), 3.77-3.75 (m, 1H, H-5'), 3.70-3.67 (m, 1H, -OC$\underline{H_2}CH_2N_3$), 3.66-3.62 (m, 1H, H-5), 3.60-3.57 (m, 1H, H-4), 3.46-3.41 (m, 1H, -$OCH_2C\underline{H_2}N_3$), 3.39-3.37 (m, 1H, -$OCH_2C\underline{H_2}N_3$), 3.37 (d, 1H, *J* = 3.6 Hz, H-4'), 2.71-2.66 (m, 1H, H-3''e), 2.38-2.33 (m, 2H, -$\underline{CH_2}CH_2CH=CH_2$), 2.26-2.24 (m, 2H, -$\underline{CH_2}CH=CH_2$), 2.21, 2.12, 2.08, 2.07, 2.07, 2.04, 2.02, 2.02, 1.99 (9s, 9x3H, OAc), 1.78 (t, 1H, *J* = 12.8 Hz, H-3''a). ^{19}F NMR ($CDCl_3$): δ -76.58 (s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.4, 170.7, 170.6, 170.5, 170.4, 169.8, 169.8, 169.6, 169.4, 168.1, 136.8, 76.0, 73.7, 72.9, 72.8, 71.6, 69.5, 69.2, 68.3, 68.0, 67.0, 66.8, 62.6, 62.1, 53.2, 49.7, 39.1, 37.5, 35.6, 29.5, 21.3, 20.8, 20.7, 20.6, 20.5, 20.5, HR ESI MS (*m*/*z*) Calcd. for $C_{49}H_{67}F_3N_2NaO_{29}$ $[M+Na]^+$: 1227.3679; Found: 1227.3717.

General procedure for the synthesis of compounds 7b-e. Compound **6** (30 mg, 0.025 mmol) was dissolved in 0.5 N aq. NaOH (2.0 mL), and the solution was stirred at rt for 10 h. After neutralization and condensation under reduced pressure, the crude product was directly used for the acylation. To a solution of the resultant amine (20 mg, 0.03 mmol) in 2.5 mL of MeOH and 0.5 mL of NaOH (0.5 N) was added 0.1 mL of an acyl anhydride dropwise in an ice-water bath. After the reaction is finished (in 6 h) as indicated by TLC, the mixture was condensed under reduced pressure. The residue was purified on a Biogel P-2 column with H_2O as the eluent. Fractions containing the expected product were combined and freeze-dried to afford compound **7b-e** after lyophilization as a white solid.

***N*-{2-*O*-{[3,5-Dideoxy-5-(*p*-methylphenylacetamido)-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Pentenamide (7b).** ^{1}H NMR (D_2O, 400 MHz): δ 7.23-7.21 (m, 4H, aromatic H), 5.86-5.76 (m, 1H, $\underline{CH}=CH_2$), 5.08-5.00 (m, 2H, CH=$\underline{CH_2}$), 4.50-4.46 (m, 2H), 4.08-4.06 (m, 2H), 3.97-3.90 (m, 3H), 3.83-3.37 (m, 20H), 3.32-3.27 (m, 2H), 2.73 (dd, 1H, *J* = 12.0, 4.0 Hz, H-3''e), 2.33-2.31 (m, 4H, $CO\underline{CH_2}\underline{CH_2}CH=CH_2$), 2.30 (s, 3H, $Ph\underline{CH_3}$), 1.76 (t, 1H, *J* = 12.0 Hz, H-3''a). ^{13}C NMR (D_2O, 100 MHz): δ 176.7, 176.1, 174.1, 137.8, 137.3, 132.2, 129.8, 129.3, 115.9, 102.9, 102.5, 100.0, 78.5, 74.5, 73.1, 73.1, 72.1, 69.6, 68.9, 68.4, 68.4, 67.7, 62.9, 61.3, 60.3, 52.0, 42.5, 40.0, 39.5, 35.3, 29.6, 20.4. HR ESI MS (*m*/*z*) Calcd. for $C_{37}H_{55}N_2O_{20}$ $[M - H]^+$: 847.3348; Found: 847.3332.

***N*-{2-*O*-{[3,5-Dideoxy-5-(*p*-methoxyphenylacetamido)-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Pentenamide (7c).** ^{1}H NMR (D_2O, 400 MHz): δ 7.25 (d, 2H, J = 8.8 Hz, aromatic H), 6.97 (d, 2H, J = 8.0 Hz, aromatic H), 5.86-5.79 (m, 1H, $\underline{CH}$=CH_2), 5.09-5.00 (m, 2H, CH=$\underline{CH_2}$), 4.68-4.48 (m, 2H), 4.09-4.05 (m, 1H), 3.98-3.90 (m, 2H), 3.81 (s, 3H, PhO$\underline{CH_3}$), 3.79-3.27 (m, 24H), 2.73 (dd, 1H, J = 12.0, 4.0 Hz, H-3''e), 2.35-2.31 (m, 4H, CO$\underline{CH_2}\underline{CH_2}$CH=$CH_2$), 1.76 (t, 1H, J = 12.0 Hz, H-3'a). ^{13}C NMR (D_2O, 100 MHz): 176.7, 176.2, 174.2, 158.3, 137.3, 130.6, 127.9, 115.9, 114.7, 103.0, 102.6, 100.1, 78.6, 74.6, 73.2, 73.1, 72.1, 69.7, 68.9, 68.5, 68.4, 67.7, 63.0, 61.3, 60.3, 55.7, 52.0, 42.0, 40.1, 39.5, 35.3, 29.6. HR ESI MS (*m*/*z*) Calcd. for $C_{37}H_{55}N_2O_{21}$ $[M - H]^+$: 863.3297; Found: 863.3317.

***N*-{2-*O*-{[5-(*p*-Acetophenylacetamido)-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Pentenamide (7d).** ^{1}H NMR (D_2O, 400 MHz): δ 7.97 (d, 2H, J = 8.0 Hz, aromatic H), 7.45 (d, 2H, J = 8.0 Hz, aromatic H), 5.88-5.80 (m, 1H, $\underline{CH}$=CH_2), 5.09-5.01 (m, 2H, CH=$\underline{CH_2}$), 4.50-4.46 (m, 2H), 4.10-4.06 (m, 1H), 3.98-3.93 (m, 3H), 3.86-3.28 (m, 24H), 2.74 (dd, 1H, J = 12.0, 4.0 Hz, H-3''e), 2.65 (s, 3H, CO$\underline{CH_3}$), 2.34-2.33 (m, 4H, CO$\underline{CH_2}\underline{CH_2}$CH=$CH_2$), 1.78 (t, 1H, J = 12.0 Hz, H-3'a). ^{13}C NMR (D_2O, 100 MHz): δ 203.9, 176.8, 175.0, 174.2, 141.6, 137.4, 135.7, 129.8, 129.4, 115.9, 103.0, 102.6, 78.6, 74.6, 73.1, 72.1, 69.7, 68.9, 68.5, 68.4, 67.8, 62.9, 61.3, 60.4, 52.1, 42.9, 40.1, 39.5, 35.3, 29.7, 26.6. HR ESI MS (*m*/*z*) Calcd. for $C_{38}H_{55}N_2O_{21}$ $[M - H]^+$: 875.3297; Found: 875.3315.

***N*-{2-*O*-{[5-(*p*-Chlorophenylacetamido)-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Pentenamide (7e).** ^{1}H NMR (D_2O, 400 MHz): δ 7.29 (d, 2H, J = 8.8 Hz, aromatic H), 7.01 (d, 2H, J = 9.2 Hz, aromatic H), 5.92-5.83 (m, 1H, $\underline{CH}$=CH_2), 5.12-5.04 (m, 2H, CH=$\underline{CH_2}$), 4.53-4.48 (m, 2H), 4.12-4.09 (m, 1H), 4.01-3.94 (m, 3H), 3.87-3.31 (m, 21H), 2.77 (dd, 1H, J = 12.0, 4.8 Hz, H-3''e), 2.37-2.35 (m, 4H, CO$\underline{CH_2}\underline{CH_2}$CH=$CH_2$), 1.80 (t, 1H, J = 12.0 Hz, H-3'a). ^{13}C NMR (D_2O, 100 MHz): δ 176.7, 175.5, 174.2, 137.3, 133.9, 132.8, 130.9, 129.1, 115.9, 102.9, 102.5, 100.1, 78.5, 74.6, 73.1, 72.1, 69.6, 68.9, 68.5, 68.4, 67.7, 62.9, 61.3, 60.3, 52.0, 42.2, 40.1, 39.5, 35.3, 29.6. HR ESI MS (*m*/*z*) Calcd. for $C_{36}H_{52}ClN_2O_{20}$ $[M - H]^+$: 867.2802; Found: 867.2805.

General procedure for the synthesis of compounds 8b-e. To a stirred solutions of **7b-e** (18 mg) in MeOH (5 mL) at -78 ^{o}C, ozone was bubbled until a blue color appeared and remained at -78 °C for 0.5 h. After introducing nitrogen to remove the remaining ozone, Me_2S (0.5 mL) was added at -78 ^{o}C.

The resultant solutions were allowed to warm to rt over a period of 1 h and stand for another 1 h before they were condensed in vacuum. The crude products were purified by a Biogel P-2 column using distilled water as the eluent to give aldehydes **8b-e** after lyophilization as white solids, which were used in the following conjugation reactions without further purification.

***N*-{2-*O*-{[3,5-Dideoxy-5-(*p*-methylphenylacetamido)-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Oxo-butanamide (8b).** ^{1}H NMR (D_2O, 400 MHz): δ 8.46 (bs, 1H, -CHO), 7.24-7.22 (m, 4H, aromatic H), 4.50-4.42 (m, 2H), 4.11-4.06 (m, 1H), 3.95-3.86 (m, 2H), 3.76-3.50 (m, 16H), 3.38-3.33 (m, 2H), 2.75 (dd, 1H, J = 12.0, 4.8 Hz, H-3''e), 2.46-2.40 (m, 4H, CO$\underline{CH_2CH_2}$CHO), 2.33 (s, 3H, Ph$\underline{CH_3}$), 1.79 (t, J = 12.0 Hz, 1H, H-3'a). HR ESI MS (m/z) Calcd. for $C_{36}H_{53}N_2O_{21}$ $[M - H]^+$: 849.3141; Found: 849.3170.

***N*-{2-*O*-{[3,5-Dideoxy-5-(*p*-methoxyphenylacetamido)-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Oxo-butanamide (8c).** ^{1}H NMR (D_2O, 500 MHz): δ 8.39 (s, 1H, -CHO), 7.18 (d, J = 6.8 Hz, 2H, aromatic H), 6.90 (d, 2H, J = 6.8 Hz, aromatic H), 4.44-4.37 (m, 3H), 4.02-3.84 (m, 7H), 3.73 (s, 3H, O$\underline{CH_3}$), 3.71-3.38 (m, 33H), 2.65 (dd, 1H, J = 10.0, 3.6 Hz, H-3''e), 2.52-2.30 (m, 4H, CO$\underline{CH_2CH_2}$CHO), 1.68 (t, J = 9.2 Hz, 1H, H-3'a). HR ESI MS (m/z) Calcd. for $C_{36}H_{53}N_2O_{22}$ $[M - H]^+$: 865.3090; Found: 865.3101.

***N*-{2-*O*-{[5-(*p*-Acetophenylacetamido)-3,5-Dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Oxo-butanamide (8d).** ^{1}H NMR (D_2O, 500 MHz): δ 8.35 (s, 1H, -CHO), 7.89 (d, 2H, J = 6.4 Hz, aromatic H), 7.37 (d, 2H, J = 6.8 Hz, aromatic H), 4.42-4.38 (m, 2H), 4.01-3.99 (m, 1H), 3.89-3.85 (m, 3H), 3.77-3.21 (m, 23H), 2.66 (dd, 1H, J = 12.0, 4.5 Hz, H-3''e), 2.57 (s, 3H, CO$\underline{CH_3}$), 2.38-2.35 (m, 4H, CO$\underline{CH_2CH_2}$CHO), 1.69 (t, 1H, J = 12.0 Hz, H-3'a). MALDI-TOF MS (m/z) Calcd. for $C_{37}H_{54}N_2NaO_{22}$ $[M + Na]^+$: 901.3; Found: 901.3.

***N*-{2-*O*-{[5-(*p*-Chlorophenylacetamido)-3,5-Dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid]-(2→3)-*β*-D-galactopyranosyl-(1→4)-D-glucopyranosyl}-ethyl} 4-Oxo-butanamide (8e).** ^{1}H NMR (D_2O, 400 MHz): δ 8.45 (bs, 1H, -CHO), 7.41 (d, 2H, J = 8.0 Hz, aromatic H), 7.30 (d, 2H, J = 8.8 Hz, aromatic H), 4.70-4.47 (m, 2H), 4.01-3.99 (m, 1H), 3.89-3.85 (m, 3H), 3.77-3.21 (m, 23H), 3.96-3.52 (m, 21H), 3.35-3.33 (m, 2H), 2.76 (dd, 1H, J = 12.4, 4.8 Hz, H-

3''e), 2.47-2.40 (m, 4H, CO$\underline{CH_2CH_2}$CHO), 1.79 (t, 1H, J = 12.0 Hz, H-3'a). HR ESI MS (m/z) Calcd. for $C_{36}H_{50}ClN_2O_{21}$ $[M - H]^+$: 869.2595; Found: 869.2624.

Procedure for the coupling between 8b-e and KLH or HSA. The solutions of **8b-e** (7 mg), KLH or HSA (7 mg), and $NaBH_3CN$ (7 mg) in 0.1 M aq. $NaHCO_3$ (0.1 mL, pH 7.5-8.0) were allowed to stand at rt in the dark for 4 days with occasional shaking. The reaction mixtures were then purified by a Biogel A 0.5 column using 0.1 M phosphate buffered saline (PBS) buffer (I = 0.1, pH =7.8) as the eluent. The fractions containing the glycoconjugates, characterized by bicinchoninic acid (BCA) assay for proteins, were combined and dialyzed against distilled water for 2 days. They were lyophilized to give a white powder of the expected glycoconjugates **1b-e** and **2b-e** (~ 6-7 mg).

Analysis of the carbohydrate loading levels of the glycoconjugates 1b-e and 2b-e.[2] The solution of an exactly weighed glycoconjugate (0.35-0.6 mg) in distilled water (1.0 mL) was mixed with the resorcinol reagent (2.0 mL) and the mixture was heated in a boiling water bath for 30 min. After it was cooled to rt, was added an extraction solution (1-butanol acetate and 1-butanol, 85:15 v/v, 3.0 mL). The mixture was shaken vigorously before it was allowed to stand still for ca. 10 min to allow the organic layer to separate well from the inorganic layer. The organic layer was transferred to a 1.0-cm cuvette, and its absorbance at 580 nm was determined by an UV-Vis spectrometer, using a blank extraction solution as the control. The sialic acid content of the glycoconjugates was determined against a calibration curve created with the solution of an individual standard NeuNPhAc derivative analyzed under the same condition. The carbohydrate loading of each glycoconjugate was calculated according to the following equation.

$$\text{Derivatized GM3PhAcs loading (\%)} = \frac{\text{derivatized GM3NPhAc content (mg) in the sample}}{\text{weight of the glycoconjugate sample (mg)}} \times 100\%$$

Immunization of Mouse. A total of 0.1 mL of the emulsion of **1a-e** (containing 3 μg of carbohydrate antigen) and Titermax Gold adjuvant (Sigma Chemical, St. Louis, MO) were intramuscularly injected to each group of five female C57BL/6 mice at the age of 6-8 weeks (Jackson Laboratories, Bar Harbor, ME) on day 0, 14, 21 and 28, respectively. The mice were bled prior to the initial immunization on day -1 and after immunization on day 27 and day 37. Blood samples collected at each time point were clotted to obtain antisera and stored at -80 ^oC before assays.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA plates were treated respectively with 100 μL solution of conjugates **2a-e** and GM3-HSA (2 μg/mL) in the coating buffer (0.1 M bicarbonate, pH

9.6) overnight at 4 °C, followed by washing 3 times with PBS containing 0.05% Tween-20 (PBST). Individual or pooled antisera from mice inoculated with **1a-e** were diluted 1:300 to 1:72900 in serial half-log dilutions in PBS and incubated for 2 h at 37 ^{o}C in the coated ELISA plates (100 µL/well). The plates were washed and then incubated with 1:1000 dilution of alkaline phosphatase linked goat anti-mouse kappa, IgM or IgG2a antibody or with 1:2000 dilution of alkaline phosphatase linked goat anti-mouse IgG1 or IgG3 antibody for 1 h at rt. Finally, the plates were washed and developed with 100 µL of PNPP solution (1.67 mg/mL in PNPP buffer) for 30 min at rt for colorimetric readout using a BioRad 550 plate reader at 405 nm wavelength. For titer analysis, optical density (OD) values were plotted against dilution numbers, and a best-fit line was obtained. The equation of this line was used to calculate the dilution number at which an OD value of 0.5 was achieved, and the antibody titer was calculated at the inverse of this dilution number.

References

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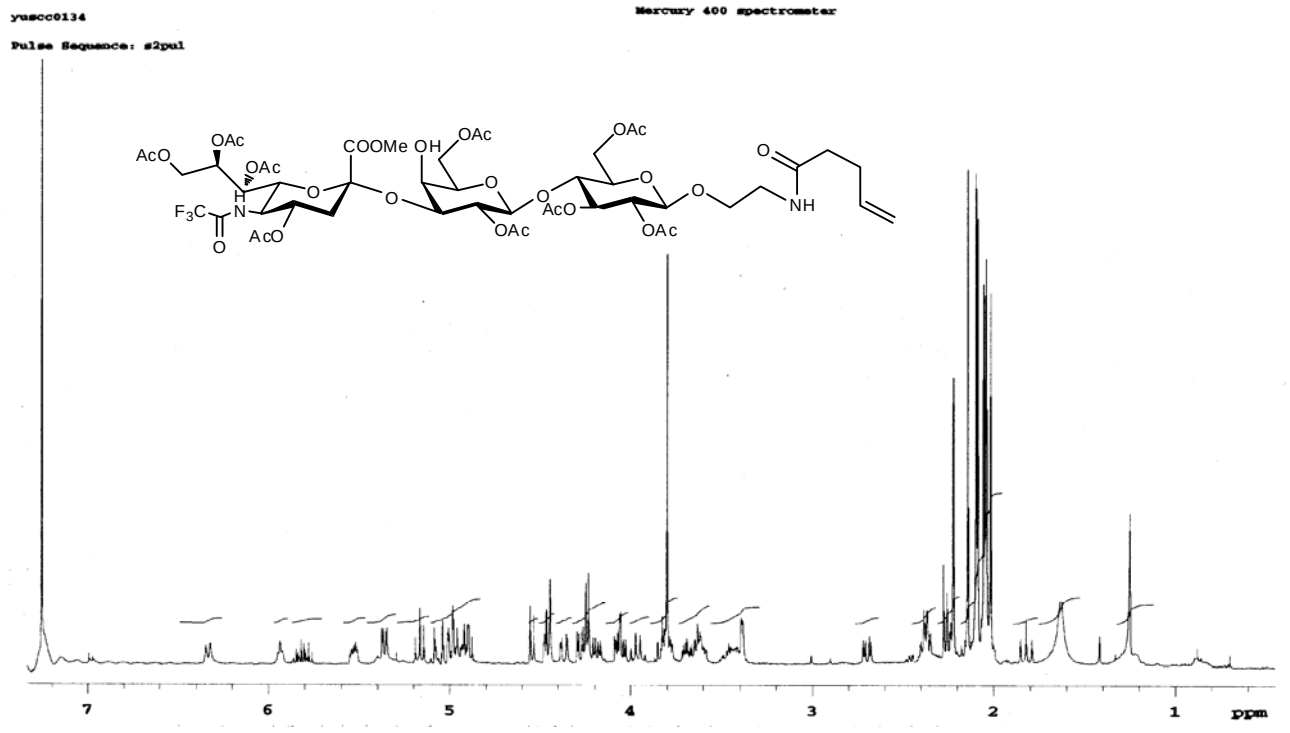
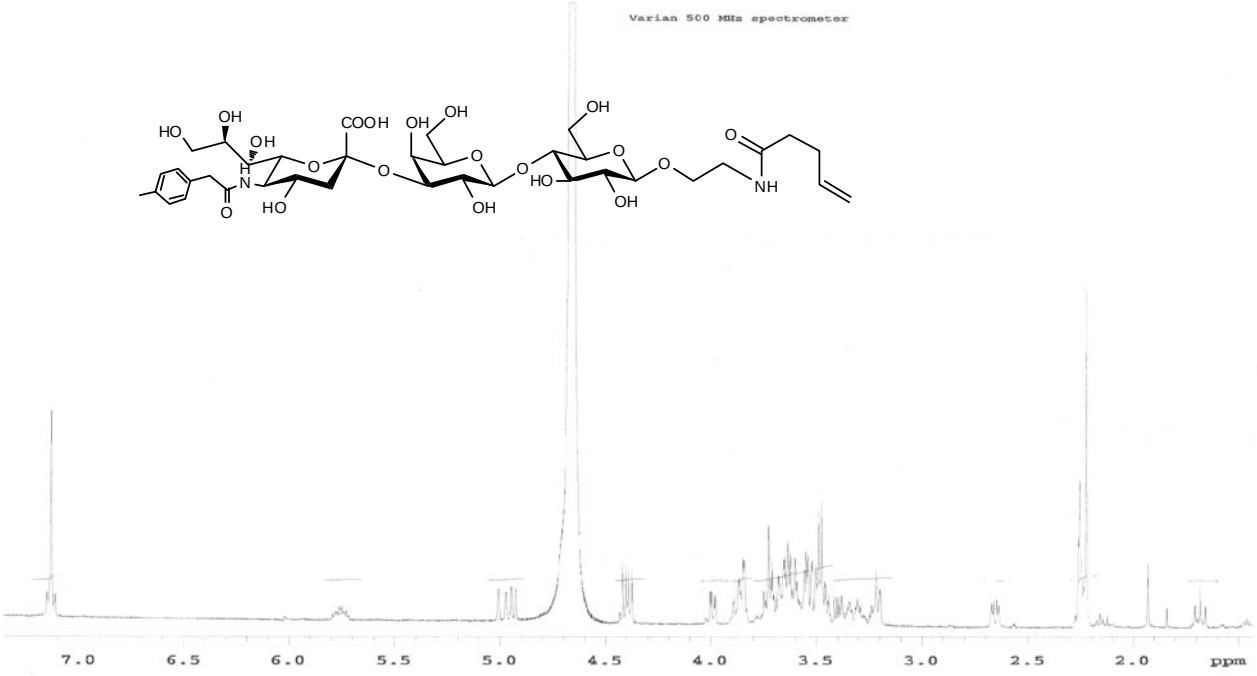
2. L. Svennerholm, *Biochim Biophys Acta*, 1957, **24**, 604-611.

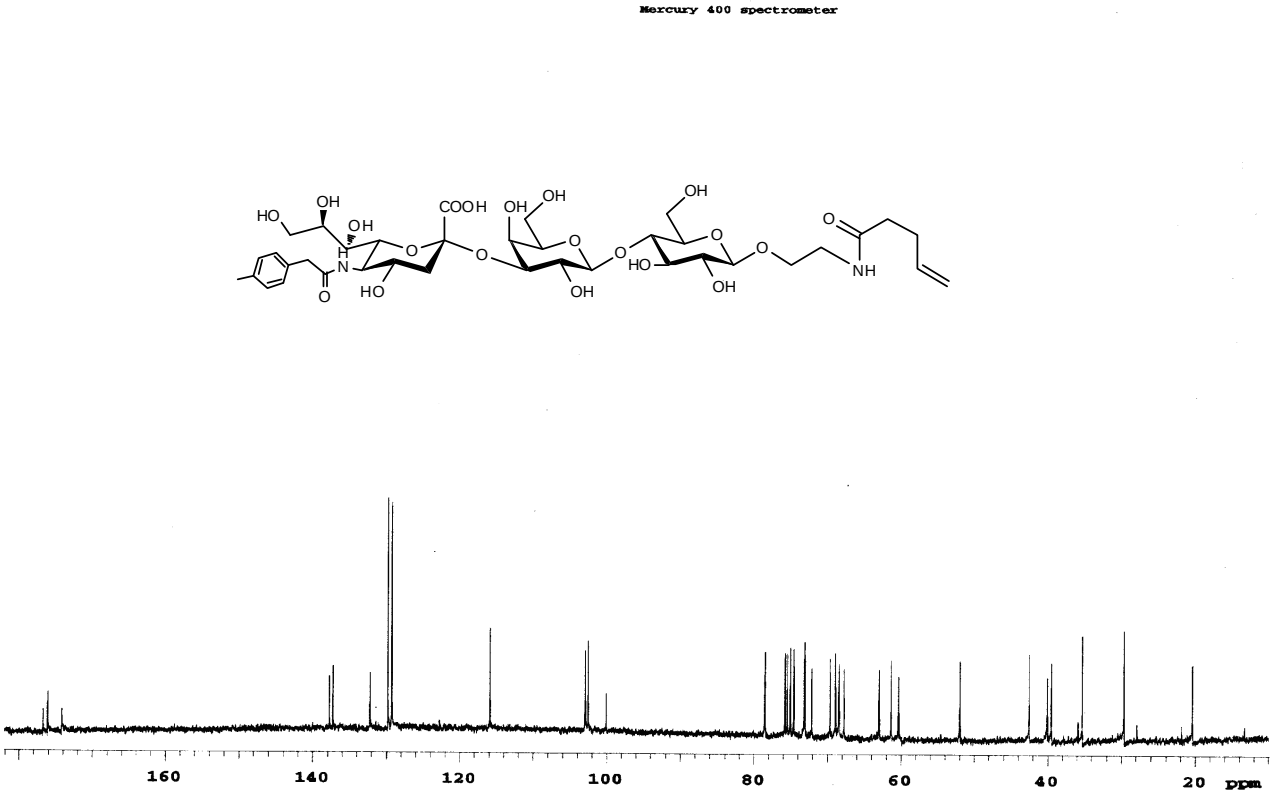
NMR and MS spectra



^{1}H NMR spectrum of **6** ($CDCl_3$, 400 MHz)



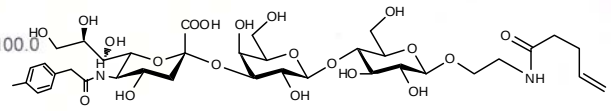
^{1}H NMR spectrum of **7b** (D_2O, 500 MHz)



^{13}C NMR spectrum of **7b** (D_2O, 100 MHz)

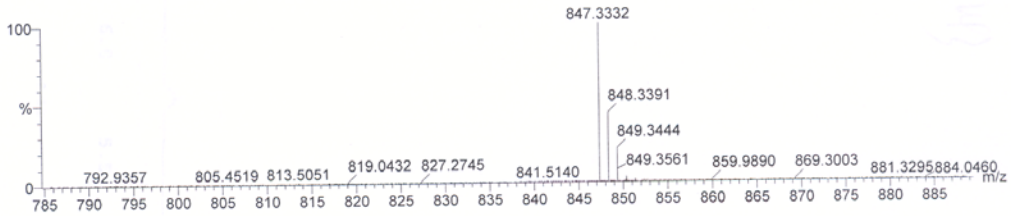
Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = 0.0, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3



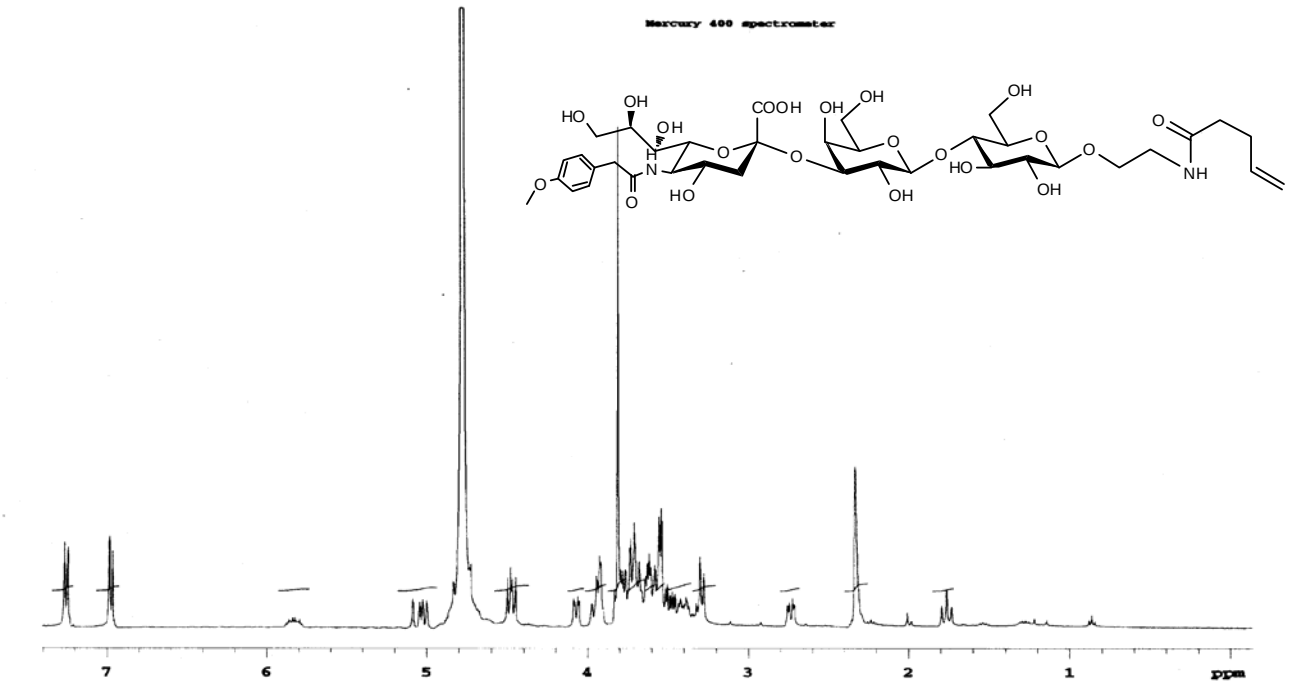
Monoisotopic Mass, Even Electron Ions
1815 formula(e) evaluated with 12 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-100 H: 0-200 N: 0-4 O: 0-20 Na: 0-1
SHI CHONG YU YUSCC 0143
Lew 2008-07b.pro
2009_0921_0596 17 (0.334) Cm (14:21-(3:9+38:47))

LCT Premier 21-Sep-2009 11:11:57
1: TOF MS ES-
5.50e+002

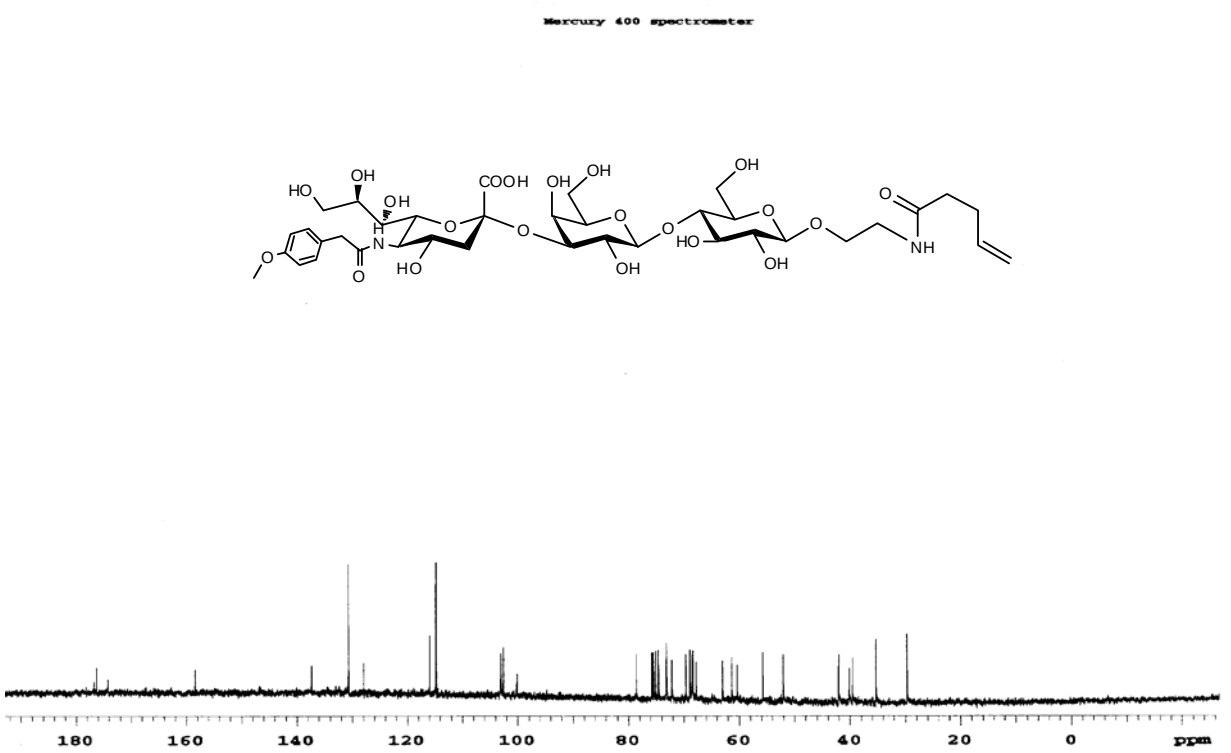


Minimum:		5.0	5.0	0.0			
Maximum:				100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
847.3332	847.3324	0.8	0.9	8.5	100.3	2.5	C35 H56 N2 O20 Na
	847.3348	-1.6	-1.9	11.5	99.2	1.5	C37 H55 N2 O20 ←
	847.3364	-3.2	-3.8	12.5	99.4	1.6	C40 H56 O18 Na
	847.3306	2.6	3.1	21.5	100.9	3.2	C47 H52 O13 Na
	847.3319	1.3	1.5	26.5	100.6	2.8	C48 H48 N4 O9 Na
	847.3330	0.2	0.2	24.5	99.7	1.9	C49 H51 O13
	847.3343	-1.1	-1.3	29.5	99.9	2.1	C50 H47 N4 O9
	847.3359	-2.7	-3.2	30.5	100.3	2.6	C53 H48 N2 O7 Na
	847.3300	3.2	3.8	39.5	103.1	5.4	C60 H44 N2 O2 Na
	847.3325	0.7	0.8	42.5	101.8	4.1	C62 H43 N2 O2
	847.3341	-0.9	-1.1	43.5	101.5	3.7	C65 H44 Na
	847.3365	-3.3	-3.9	46.5	102.2	4.4	C67 H43

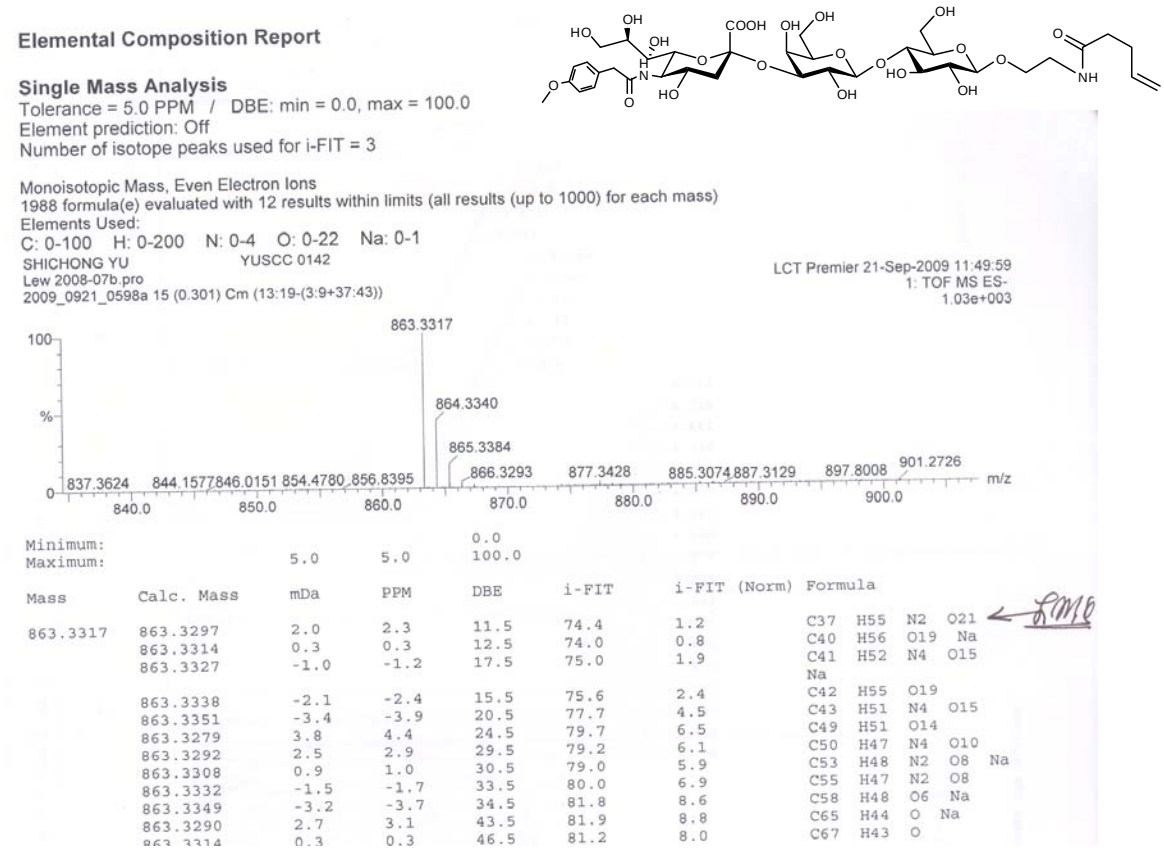
HR ESI MS spectrum of **7b**



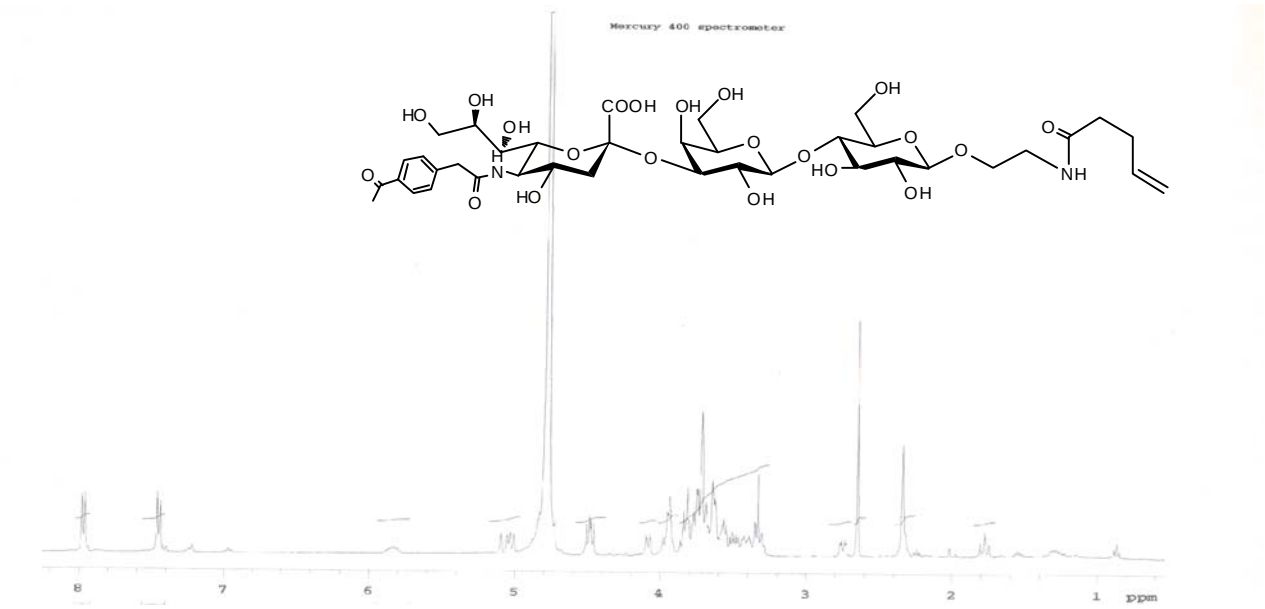
^{1}H NMR spectrum of **7c** (D_2O, 400 MHz)



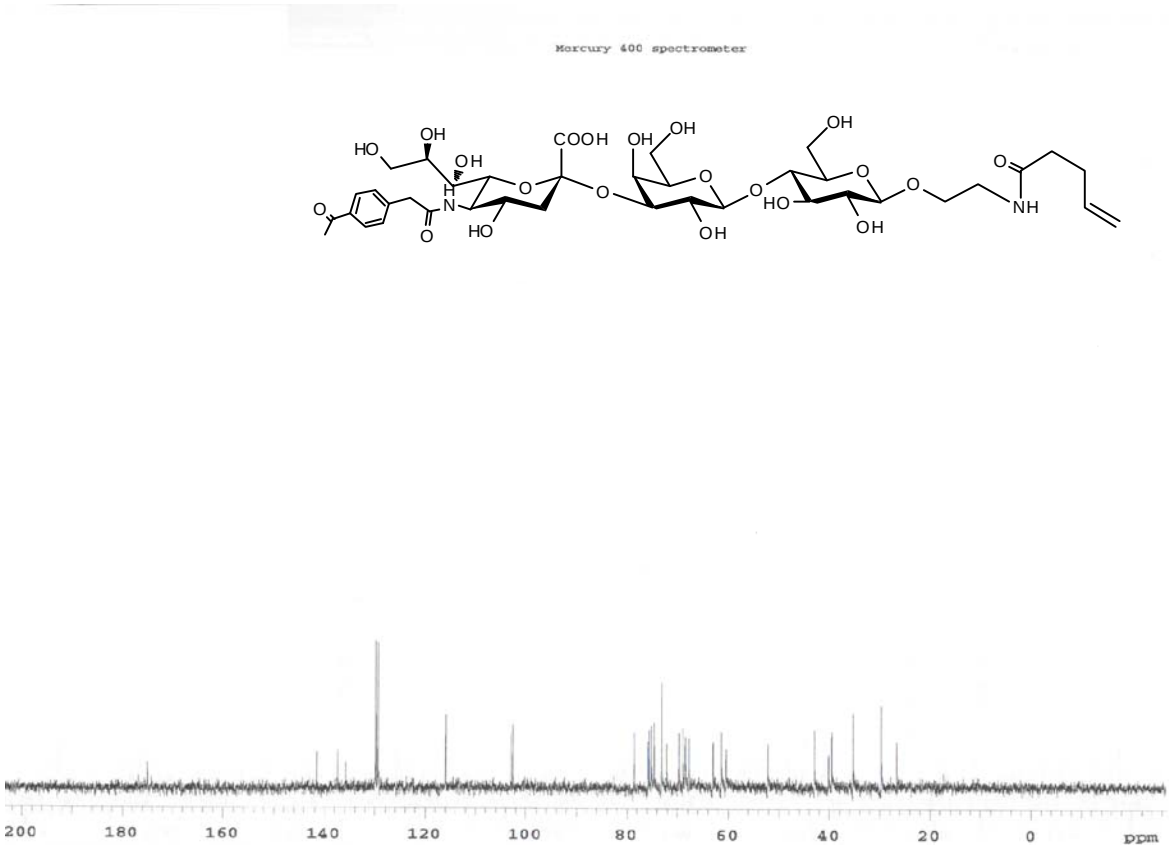
^{13}C NMR spectrum of **7c** (D_2O, 100 MHz)



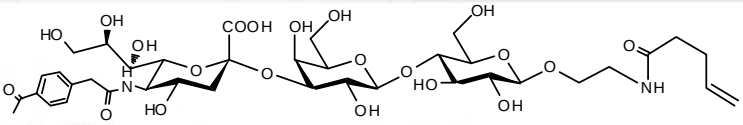
HR ESI MS spectrum of **7c**



^{1}H NMR spectrum of **7d** (D_2O, 400 MHz)



^{13}C NMR spectrum of **7d** (D_2O, 100 MHz)

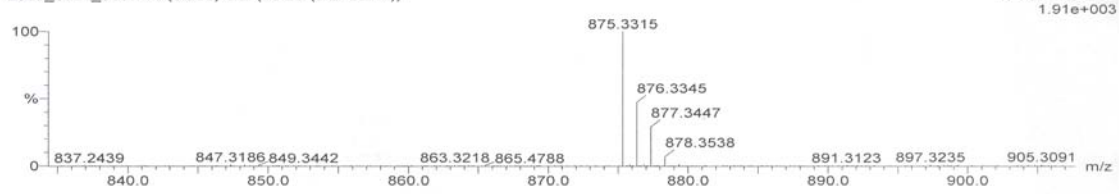


Elemental Composition Report

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = 0.0, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

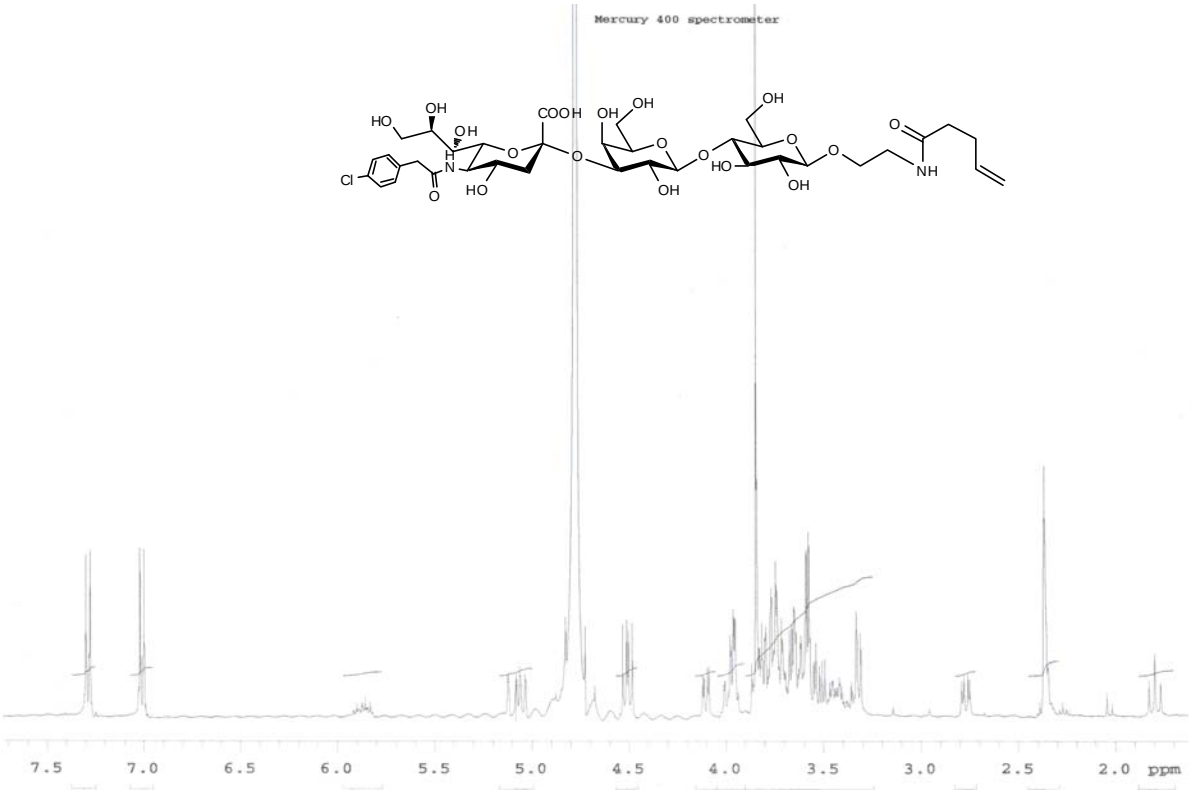
Monoisotopic Mass, Odd and Even Electron Ions
2021 formula(e) evaluated with 22 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-100 H: 0-200 N: 0-4 O: 0-22 Na: 0-1
SHICHONG YU YUSCC 0147
Lew 2008-07b.pro
2009_0921_0597 14 (0.283) Cm (13:20-(3:9+39:47))

LCT Premier 21-Sep-2009 11:22:44
1: TOF MS ES-
1.91e+003

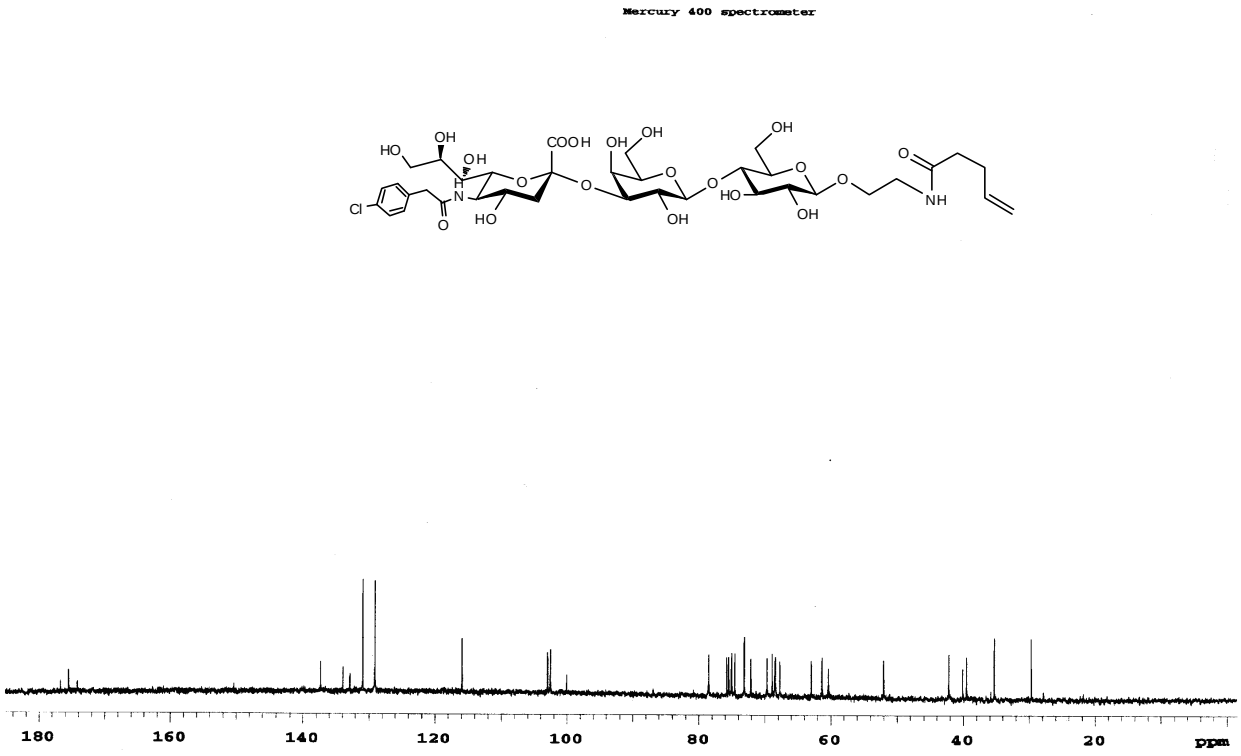


Minimum:		5.0	5.0	0.0						
Maximum:				100.0						
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula			
875.3315	875.3273	4.2	4.8	9.5	135.8	4.5	C36 Na	H56	N2	O21
	875.3297	1.8	2.1	12.5	134.9	3.6	C38	H55	N2	O21
	875.3300	1.5	1.7	14.0	134.8	3.5	C39 Na	H54	N3	O18
	875.3324	-0.9	-1.0	17.0	134.2	2.9	C41	H53	N3	O18
	875.3314	0.1	0.1	13.5	134.3	2.9	C41	H56	O19	Na
	875.3327	-1.2	-1.4	18.5	134.2	2.9	C42 Na	H52	N4	O15
	875.3338	-2.3	-2.6	16.5	133.9	2.6	C43	H55	O19	

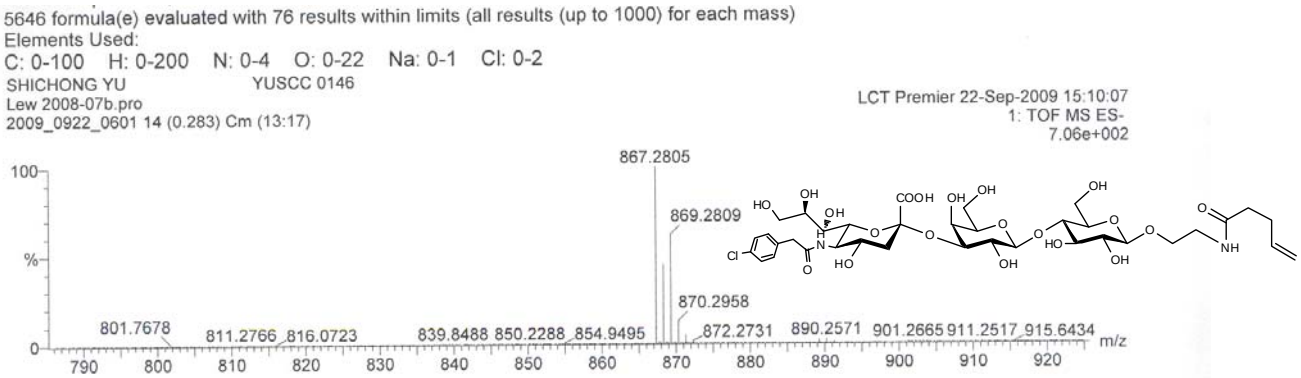
HR ESI MS spectrum of **7d**



^{1}H NMR spectrum of **7e** (D_2O, 400 MHz)

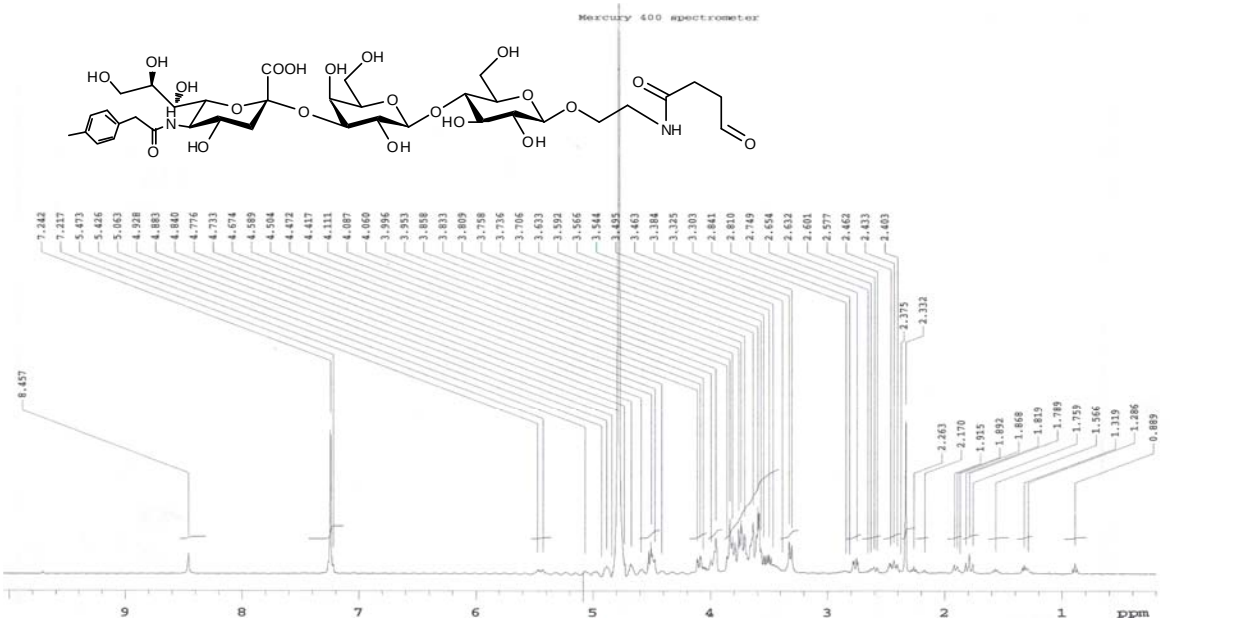


^{13}C NMR spectrum of **7e** (D_2O, 100 MHz)

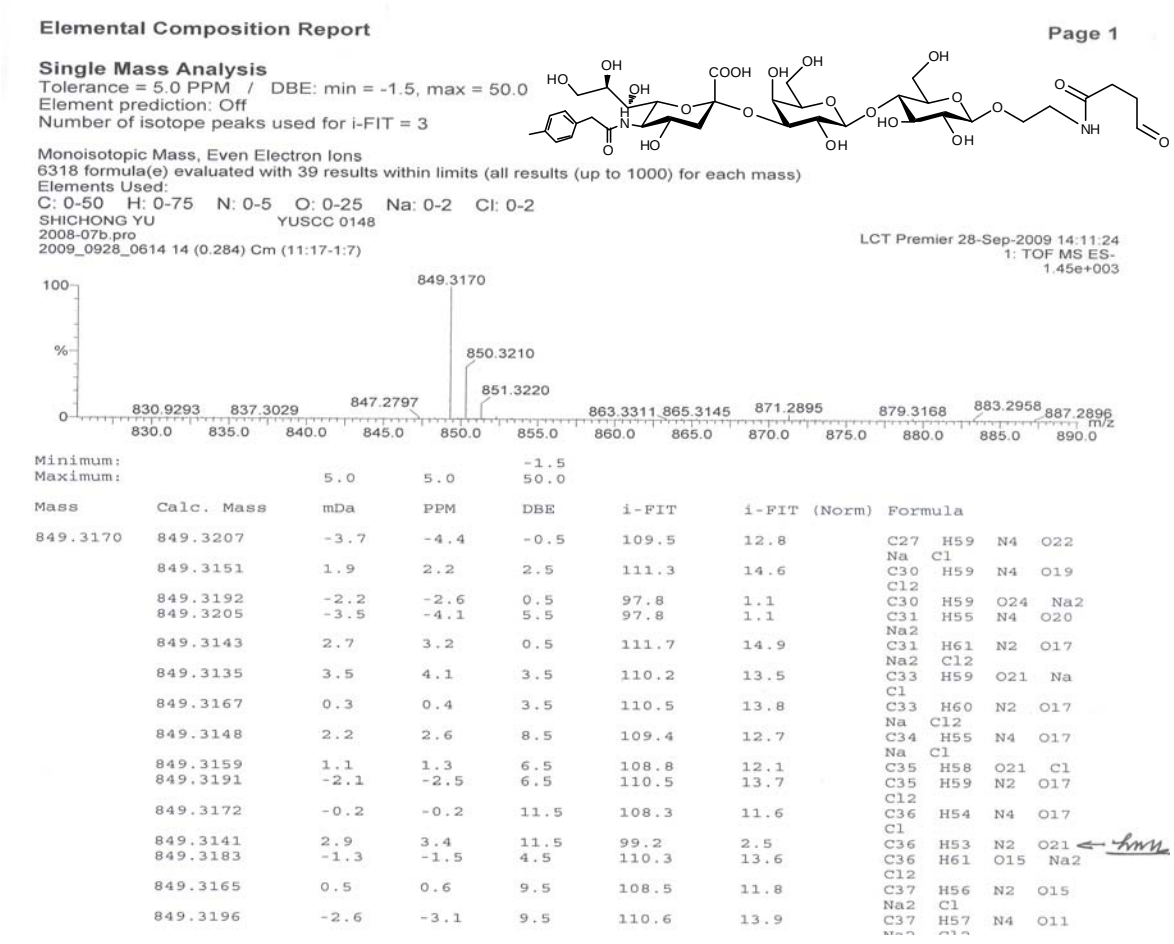


Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
Minimum:				0.0			
Maximum:		5.0	10.0	100.0			
867.2805	867.2805	0.0	0.0	33.5	91.5	8.9	C54 H43 O11
	867.2802	0.3	0.3	11.5	85.7	3.0	C36 H52 N2 O20 Cl
	867.2800	0.5	0.6	17.5	91.4	8.7	C40 H48 N2 O18 Na
	867.2800	0.5	0.6	51.5	91.5	8.9	C67 H35 N2
	867.2810	-0.5	-0.6	8.5	85.6	2.9	C34 H54 N4 O16 Na Cl2
	867.2813	-0.8	-0.9	30.5	86.7	4.1	C52 H45 N2 O7 Na Cl
	867.2797	0.8	0.9	29.5	86.3	3.6	C49 H44 N4 O9 Cl
	867.2797	0.8	0.9	37.5	87.0	4.4	C60 H45 O2 Cl2
	867.2796	0.9	1.0	3.5	86.1	3.4	C33 H58 O20 Na Cl2
	867.2815	-1.0	-1.2	24.5	85.7	3.0	C48 H49 N2 O9 Cl2

HR ESI MS spectrum of **7e**

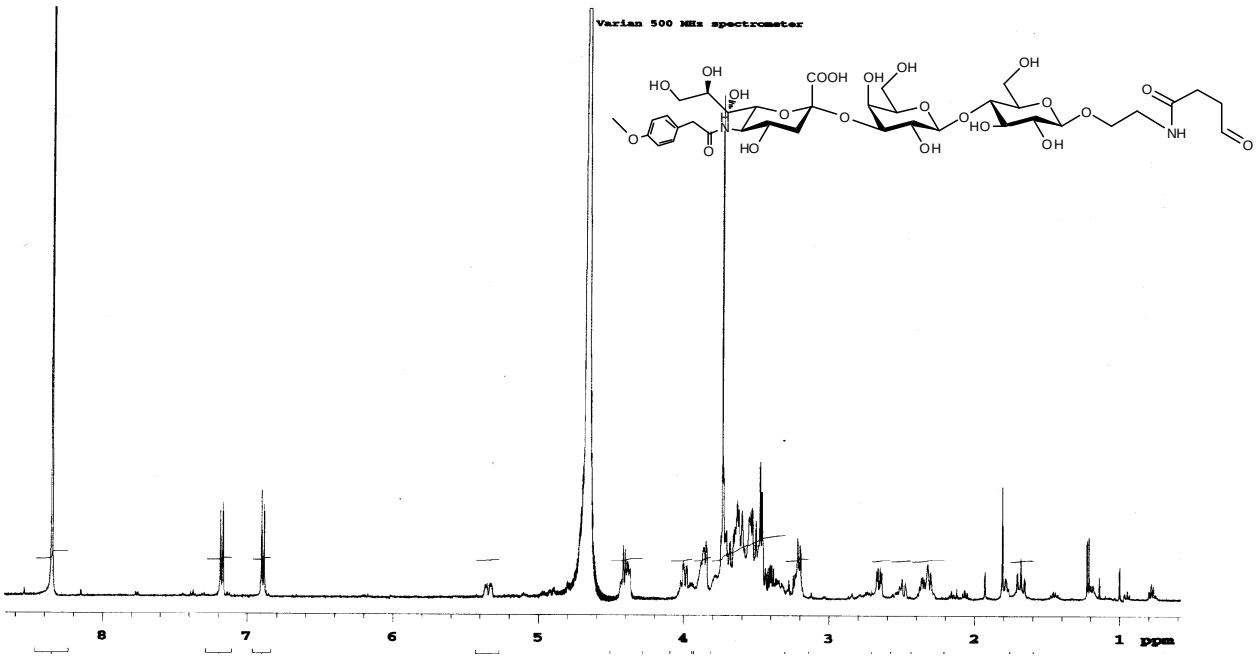


^{1}H NMR spectrum of **8b** (D_2O, 400 MHz)



Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
849.3170	849.3207	-3.7	-4.4	-0.5	109.5	12.8	C27 H59 N4 O22 Na Cl
	849.3151	1.9	2.2	2.5	111.3	14.6	C30 H59 N4 O19 Cl2
	849.3192	-2.2	-2.6	0.5	97.8	1.1	C30 H59 O24 Na2
	849.3205	-3.5	-4.1	5.5	97.8	1.1	C31 H55 N4 O20 Na2
	849.3143	2.7	3.2	0.5	111.7	14.9	C31 H61 N2 O17 Na2 Cl2
	849.3135	3.5	4.1	3.5	110.2	13.5	C33 H59 O21 Na Cl
	849.3167	0.3	0.4	3.5	110.5	13.8	C33 H60 N2 O17 Na Cl2
	849.3148	2.2	2.6	8.5	109.4	12.7	C34 H55 N4 O17 Na Cl
	849.3159	1.1	1.3	6.5	108.8	12.1	C35 H58 O21 Cl
	849.3191	-2.1	-2.5	6.5	110.5	13.7	C35 H59 N2 O17 Cl2
	849.3172	-0.2	-0.2	11.5	108.3	11.6	C36 H54 N4 O17 Cl
	849.3141	2.9	3.4	11.5	99.2	2.5	C36 H53 N2 O21 ←
	849.3183	-1.3	-1.5	4.5	110.3	13.6	C36 H61 O15 Na2 Cl2
	849.3165	0.5	0.6	9.5	108.5	11.8	C37 H56 N2 O15 Na2 Cl
	849.3196	-2.6	-3.1	9.5	110.6	13.9	C37 H57 N4 O11

HR ESI MS spectrum of **8b**



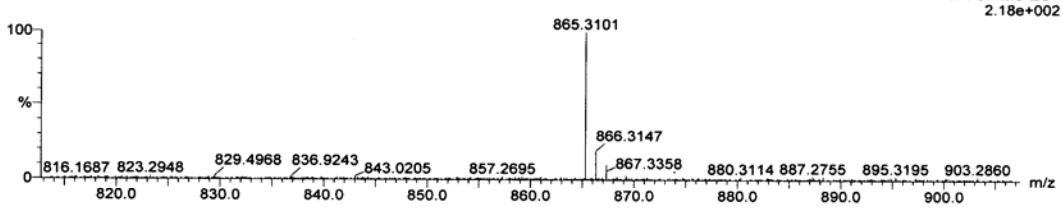
^{1}H NMR spectrum of **8c** (D_2O, 500 MHz)

Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

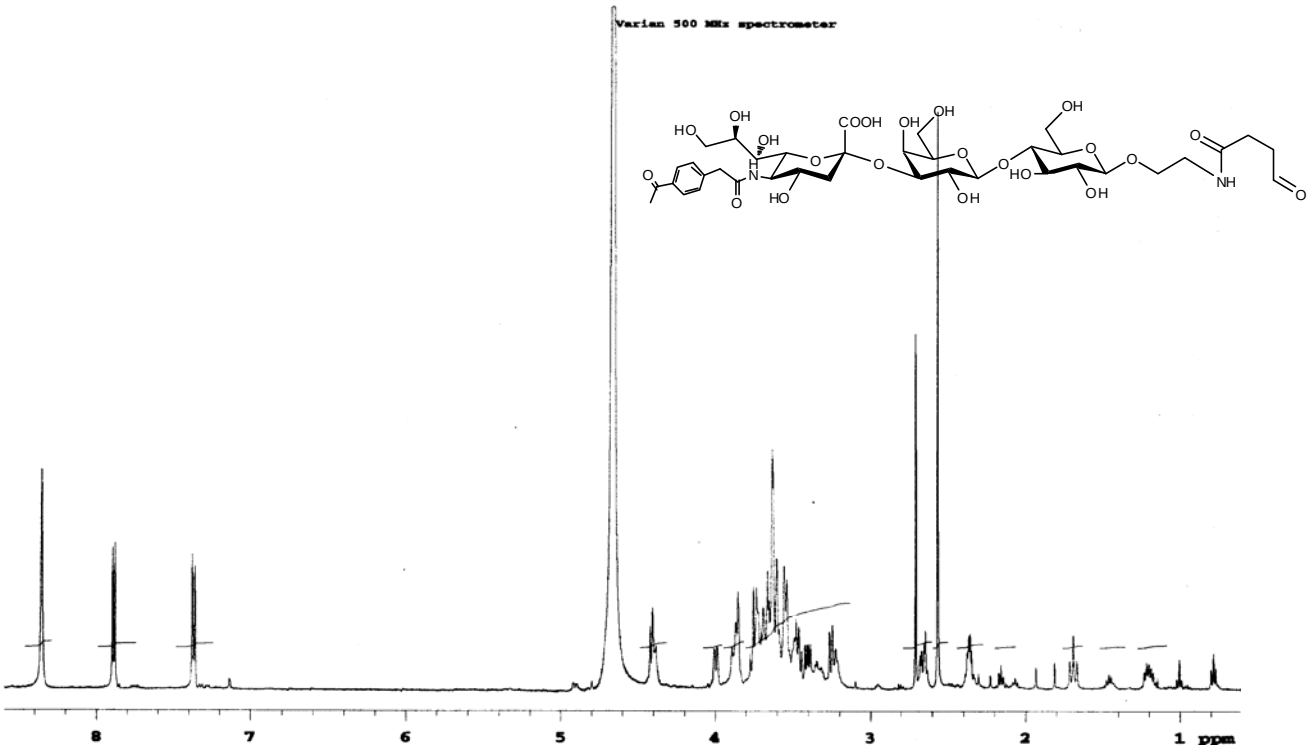
Monoisotopic Mass, Even Electron Ions
1861 formula(e) evaluated with 14 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-50 H: 0-75 N: 0-5 O: 0-25 Na: 0-2
SHICHONG YU YUSCC 0150
2008-07b.pro
2009_0929_0618a 14 (0.283) Cm (14:17-1:8)

LCT Premier 29-Sep-2009 13:59:56
1: TOF MS ES-
2.18e+002

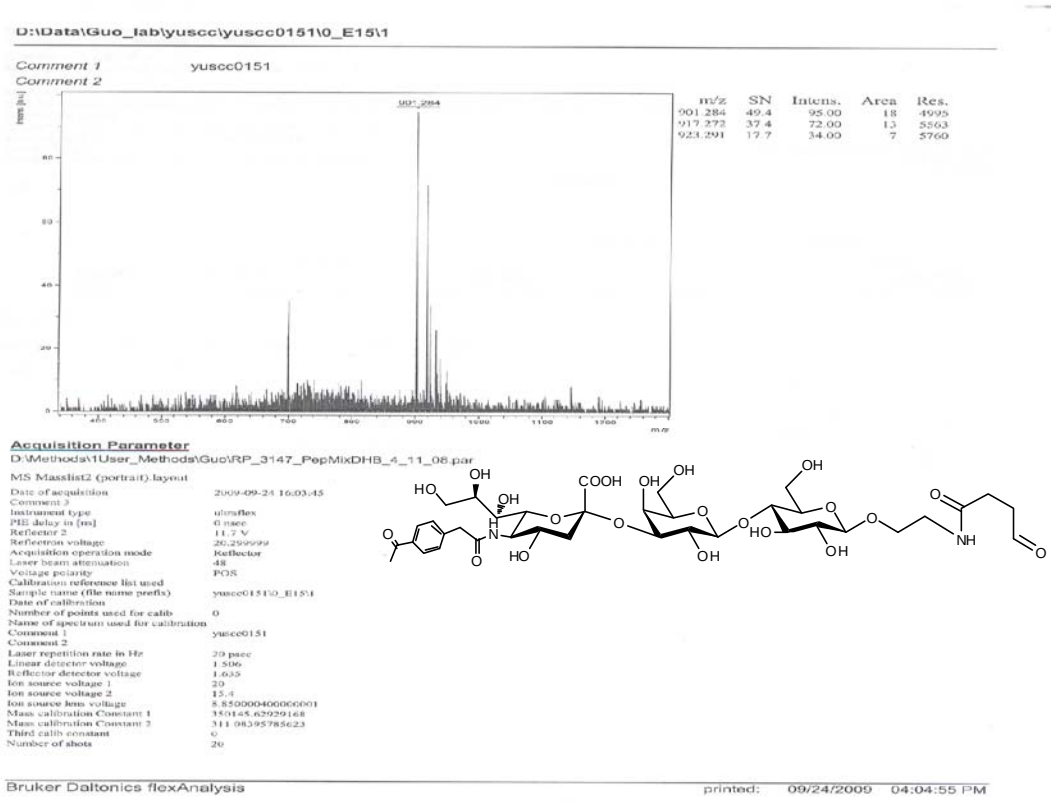


Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
865.3101	865.3106	-0.5	-0.6	12.5	73.9	2.1	C39 H54 O20 Na
	865.3095	0.6	0.7	14.5	74.1	2.3	C38 H51 N4 O16 Na2
	865.3090	1.1	1.3	11.5	74.0	2.2	C36 H53 N2 O22
	865.3085	1.6	1.8	29.5	75.4	3.6	C49 H45 N4 O11
	865.3082	1.9	2.2	9.5	74.2	2.4	C37 H55 O20 Na2
	865.3120	-1.9	-2.2	17.5	74.3	2.5	C40 H50 N4 O16 Na
	865.3077	2.4	2.8	27.5	75.7	3.9	C50 H47 N2 O9 Na2
	865.3130	-2.9	-3.4	15.5	74.5	2.7	C41 H53 O20
	865.3071	3.0	3.5	24.5	75.8	4.0	C48 H49 O15
	865.3136	-3.5	-4.0	18.5	75.0	3.2	C43 H51 N2 O14 Na2
	865.3066	3.5	4.0	8.5	74.7	2.9	C34 H54 N2 O22 Na
	865.3061	4.0	4.6	26.5	76.4	4.6	C47 H46 N4 O11 Na
	865.3141	-4.0	-4.6	0.5	73.2	1.5	C30 H59 O25 Na2
	865.3144	-4.3	-5.0	20.5	75.3	3.5	C42 H49 N4 O16

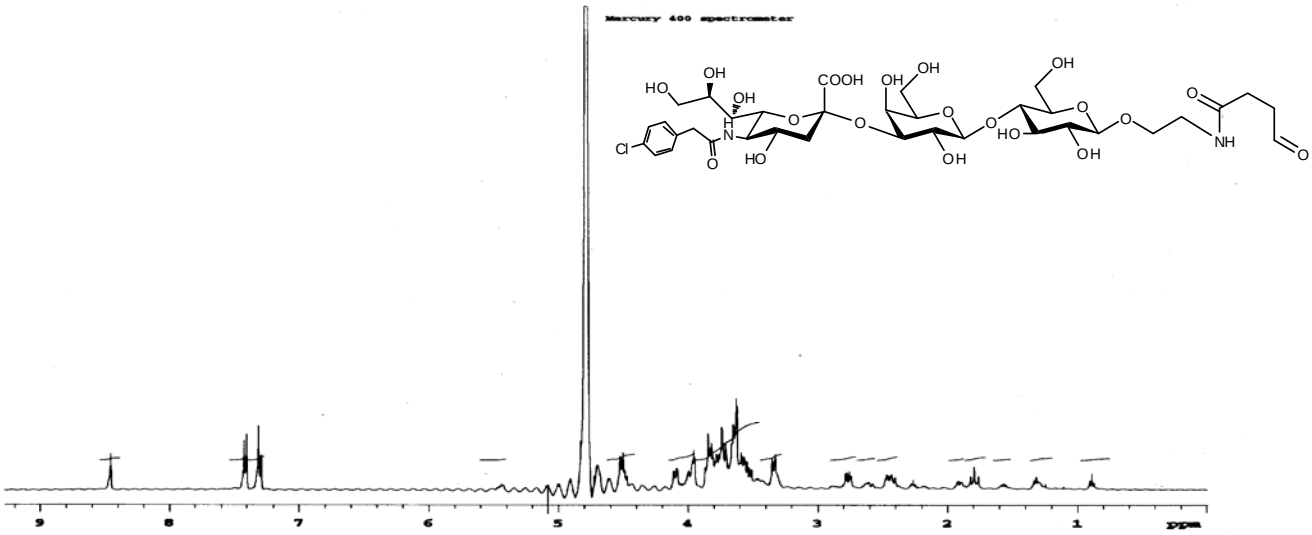
HR ESI MS spectrum of **8c**



^{1}H NMR spectrum of **8d** (D_2O, 500 MHz)



MALDI spectrum of **8d**



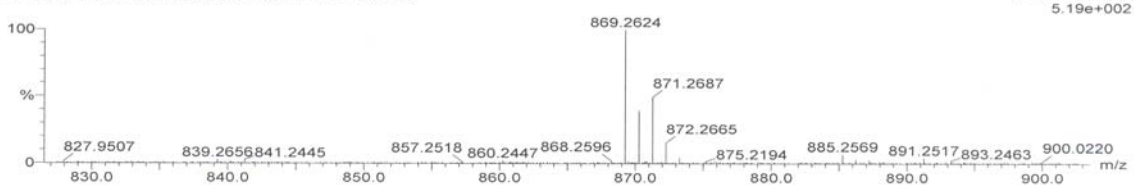
^{1}H NMR spectrum of **8e** (D_2O, 400 MHz)

Elemental Composition Report Page 1

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
6027 formula(e) evaluated with 38 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-50 H: 0-75 N: 0-5 O: 0-25 Na: 0-2 Cl: 0-2
SHICHONG YU YUSCC 0149
2008-07b.pro
2009_0928_0613a 17 (0.335) Cm ((10:11+17:22)-1:7)

LCT Premier 28-Sep-2009 13:30:31
1: TOF MS ES-
5.19e+002



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
869.2624	869.2661	-3.7	-4.3	-0.5	93.7	6.9	C26 H56 N4 O22 Na Cl2
	869.2645	-2.1	-2.4	0.5	88.7	2.0	C29 H56 O24 Na2 Cl
	869.2627	-0.3	-0.3	5.5	92.4	5.7	C30 H51 N2 O24 Na2
	869.2659	-3.5	-4.0	5.5	88.5	1.7	C30 H52 N4 O20 Na2 Cl
	869.2589	3.5	4.0	3.5	94.6	7.8	C32 H56 O21 Na Cl2
	869.2651	-2.7	-3.1	8.5	93.3	6.6	C32 H50 N2 O24 Na
	869.2602	2.2	2.5	8.5	94.0	7.2	C33 H52 N4 O17 Na Cl2
	869.2613	1.1	1.3	6.5	93.7	6.9	C34 H55 O21 Cl2
	869.2667	-4.3	-4.9	9.5	94.6	7.9	C35 H51 O22 Na2
	869.2595	2.9	3.3	11.5	89.3	2.5	C35 H50 N2 O21 Cl
	869.2626	-0.2	-0.2	11.5	93.2	6.4	C35 H51 N4 O17 Cl2
	869.2587	3.7	4.3	9.5	89.8	3.0	C36 H52 O19 Na2 Cl
	869.2618	0.6	0.7	9.5	93.3	6.6	C36 H53 N2 O15 Na2 Cl2
	869.2600	2.4	2.8	14.5	89.8	3.0	C37 H48 N4 O15 Na2 Cl
	869.2642	-1.8	-2.1	12.5	92.9	6.1	C38 H52 N2 O15 Na Cl2
	869.2611	1.3	1.5	12.5	89.1	2.3	C38 H51 O19 Na Cl
	869.2624	0.0	0.0	17.5	89.3	2.5	C39 H47 N4 O15

HR ESI MS spectrum of **8e**