Supporting Information

For

Structural Characterization and Analysis of Different Epimers of Neutral Glycosphingolipid LcGg4 by Ion Mobility Spectrometry-Mass Spectrometry

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Table S1. The LC retention time (Rt) of GalNAc-LcGg4 (d18:1/18:0), LcGg4 (d18:1/18:0), GlcNAc-LcGg4 (d18:1/18:0), GalNAc-LcGg4 (d16:1/18:0), and GalNAc-LcGg4-NBD. All the *m/z* values correspond to $[M + H]^+$ parent ions.

GSL ID	Rt (min)	Experimental (m/z)	Calculated (m/z)	Deviation (in ppm)
GalNAc-LcGg4 (d18:1/18:0)	27.7	1296.8249	1296.8150	7.63
LcGg4 (d18:1/18:0)	27.7	1296.8249	1296.8150	7.63
GlcNAc-LcGg4 (d18:1/18:0)	27.7	1296.8249	1296.8150	7.63
GalNAc-LcGg4 (d16:1/18:0)	23.2	1268.7892	1268.7838	4.26
GalNAc-LcGg4-NBD	10.6	1376.7267	1376.7182	5.59

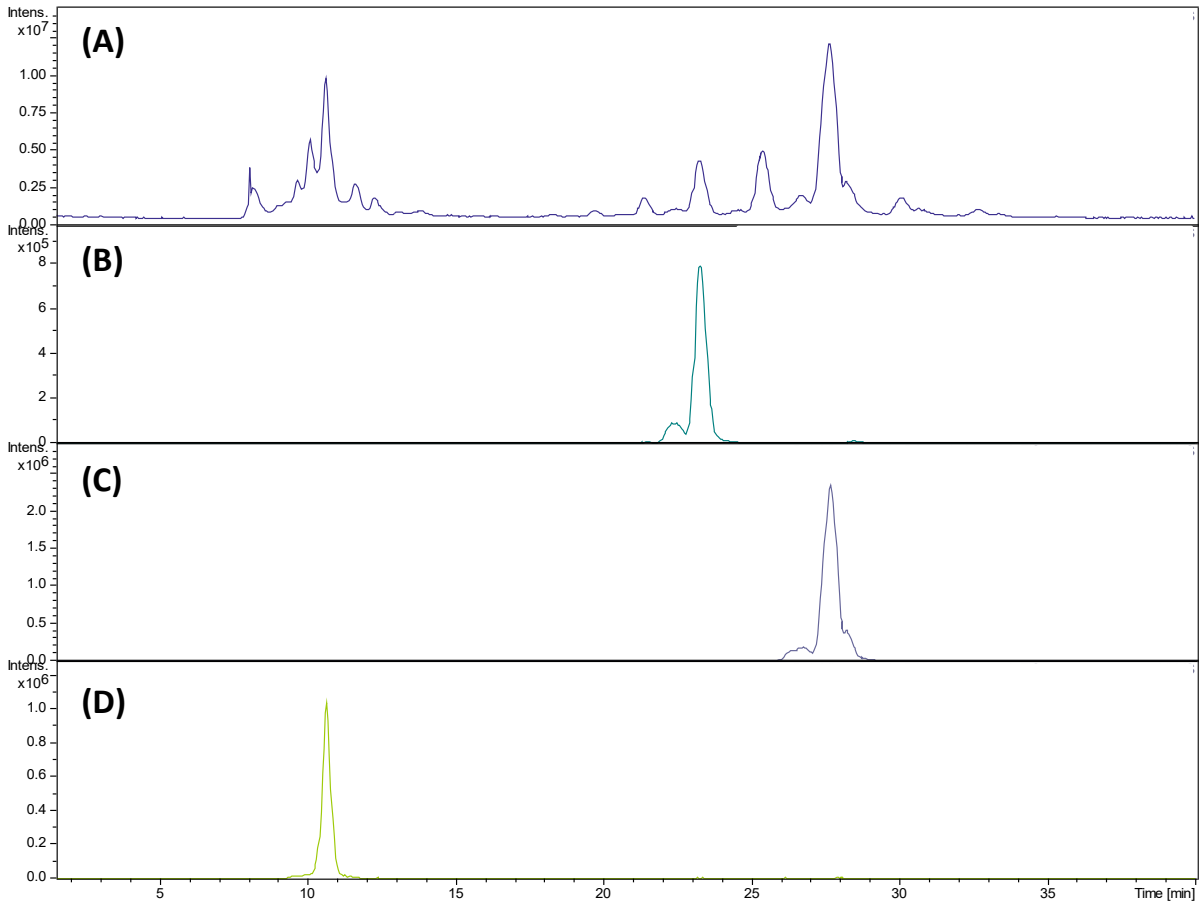

Figure S1. Total ion chromatogram (TIC) of the mixture of all 5 GSLs (**A**). Extracted ICs (EICs) of GalNAc-LcGg4 (d16:1/18:0) (**B**), LcGg4 (d18:1/18:0), GalNAc-LcGg4 (d18:1/18:0) and GlcNAc-LcGg4 (d18:1/18:0) (**C**), and GalNAc-LcGg4-NBD (**D**) from the LC-MS/MS data of their mixture. Different lipid forms can be easily distinguished by LC-MS, whereas all the epimers elude at the same retention time.

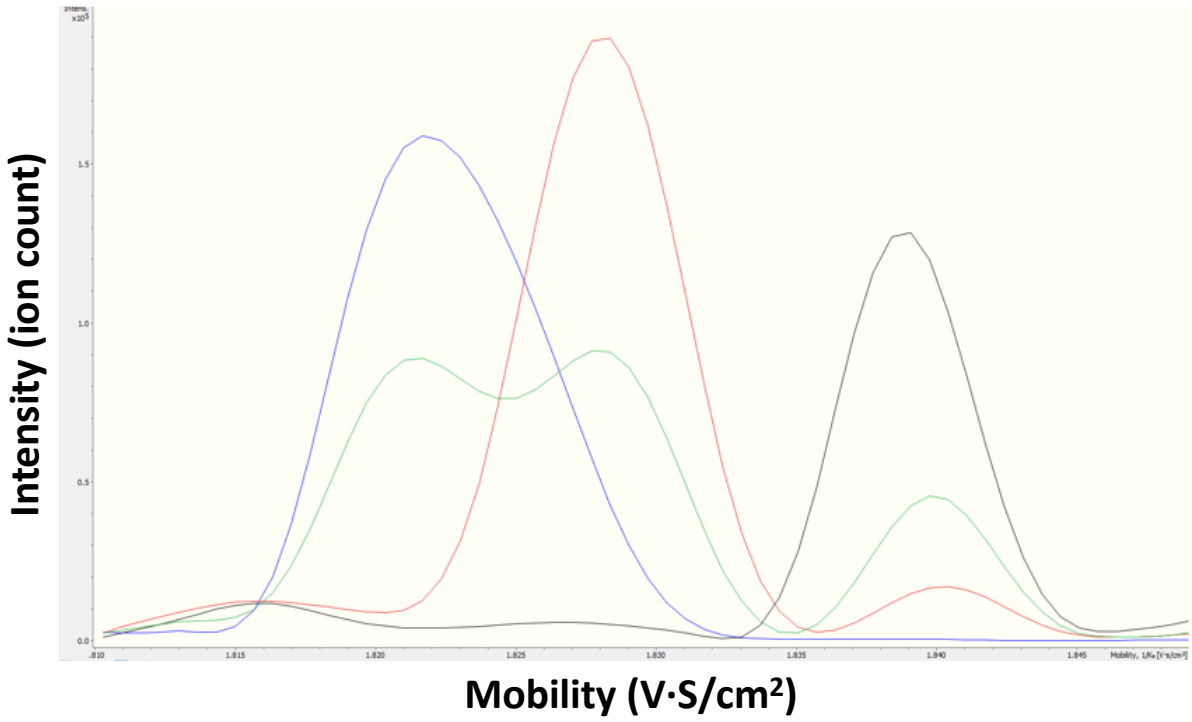

Figure S2. Stacked view of the EIMs for the $[M + H]^+$ ions of isomeric GalNAc-LcGg4 (blue), LcGg4 (red) and GlcNAc-LcGg4 (black) that were individually analyzed by the Bruker timsTOF flex system. The IMS result of their mixture (green) is in good accordance with the individual results.

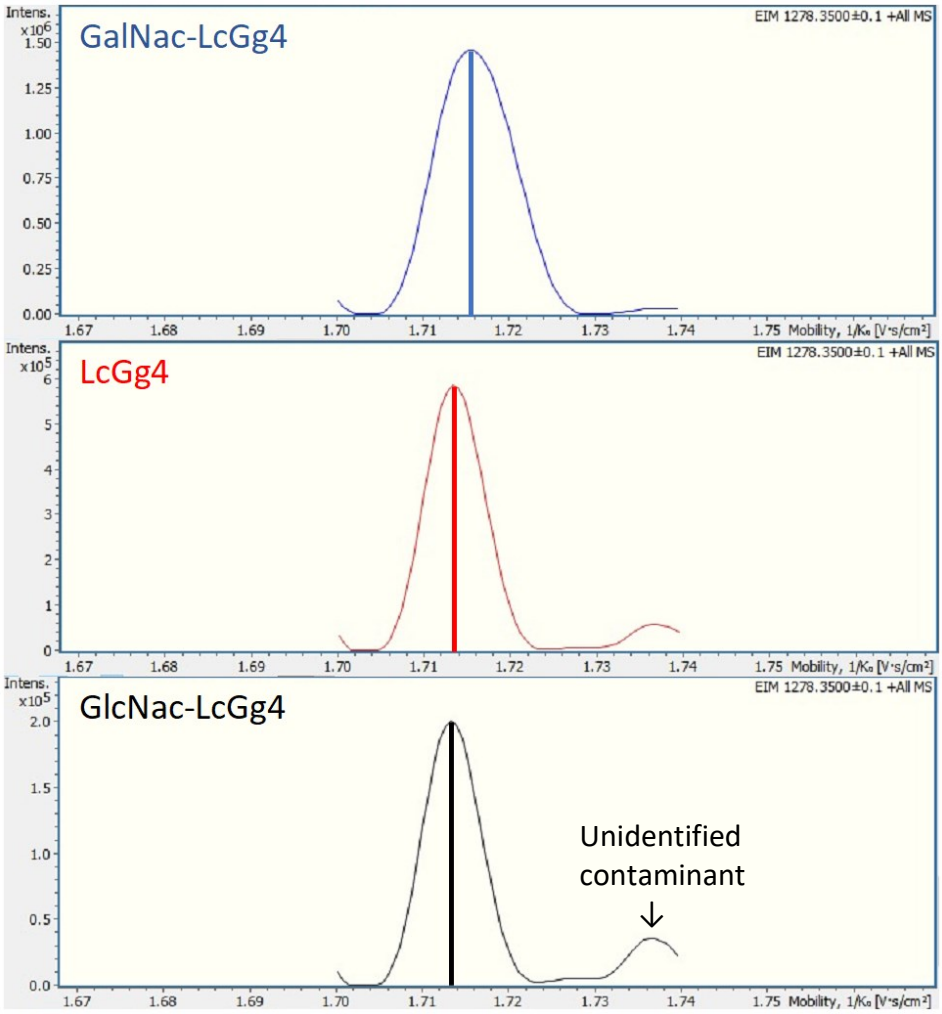

Figure S3. Extracted ion mobilograms (EIM) for the $[M - H_2O + H]^+$ ion of each isomer obtained from the Bruker timsTOF flex system. Differences in mobility were not observed for LcGg4 and GlcNAc-LcGg4 (both at ~1.714 V·S/cm^2), while GalNAc-LcGg4 had a slightly different mobility value (~1.716 V·S/cm^2). The minor peak at ~1.735 V·S/cm^2 belongs to an unidentified contaminant.

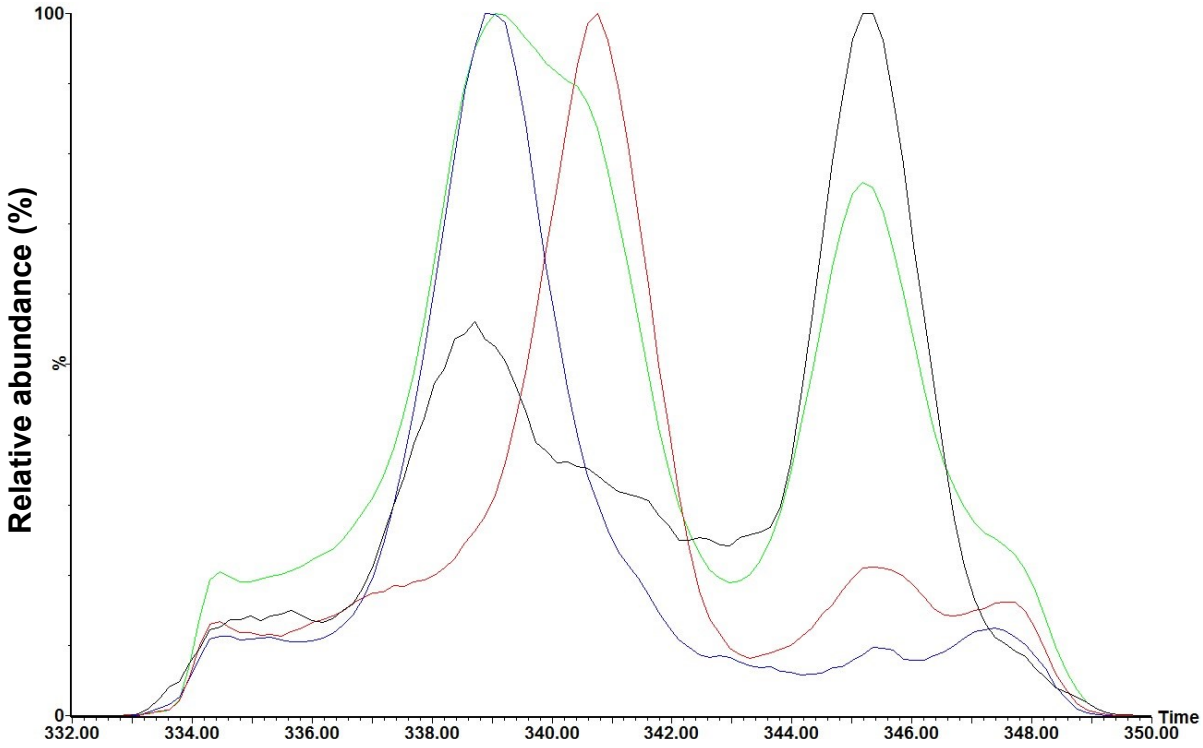

Figure S4. A stacked view of the IMS-MS arrival time distributions of GalNAc-LcGg4 (blue), LcGg4 (red), and GlcNAc-LcGg4 (black) analyzed individually, and as a mixture (green), obtained from the Waters Cyclic IMS system.

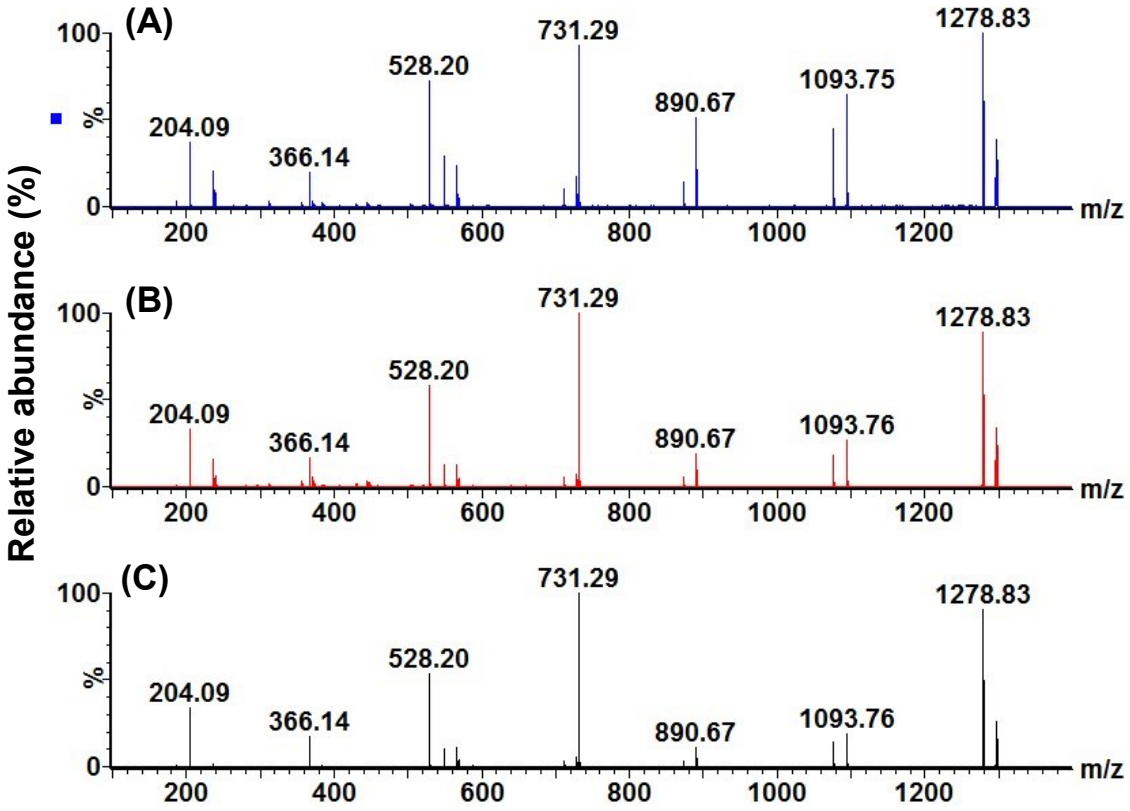

Figure S5. MS/MS spectra of GalNAc-LcGg4 (**A**), LcGg4 (**B**), and GlcNAc-LcGg4 (**C**) obtained from the Waters Cyclic IMS-MS/MS system at a 30 eV collision energy using $[M + H]^+$ ion as the parent ion.

Studies on collisional cross section in nitrogen

We also used the timsTOF and Cyclic IMS systems to discover the collisional cross section in nitrogen (CCS_{N2}) for each GSL isomer. CCS_{N2} is typically a reproducible physical property for each specific ion, determined by the surface area of an ion to interact with carrier gas. The results listed in Table S2 clearly suggest an observable difference among the three isomers. Also, in both systems, the CCS values of three isomers are different by the similar amounts (CCS of GlcNAc-LcGg4 is 2.2 larger than that of LcGg4 and 3.4 larger than that of GalNAc-LcGg4). However, it should be noted that different tune mixtures were utilized for Cyclic IMS and timsTOF following each vendors' recommendations of the best conditions (details about the tune mixtures are given in the experimental section), thus these two sets of CCS values may not be comparable.

Table S2. Observed CCS_{N2} values for LcGg4, GalNAc-LcGg4, and GlcNAc-LcGg4.

GSL	Observed CCS by Cyclic IMS	Observed CCS by timsTOF flex
GalNAc-LcGg4	378.2	368.8
LcGg4	379.4	370.0
GlcNAc-LcGg4	381.6	372.3

Table S3. Matched product ions and fragmentation assignments for GlcNAc-LcGg4 (18:1/18:0). The relative peak intensity (I%) shows the percentage of each ion against the combination of all identified carbohydrate and glycolipid ions.

Fragmentation Assignment	Experimental m/z	Theoretical m/z	ppm	I%
Carbohydrate fragments				
B1	204.0920	204.0872	23.5	19.53
B2Y2a/2b	366.1480	366.1395	23.2	7.24
B3Y2a/2b	528.2029	528.1923	20.1	15.32
B2	569.2300	569.2188	19.7	1.58
B3	731.2856	731.2717	19.0	26.63
Glycolipid fragments				
Z0	548.5493	548.5407	15.7	4.34
Y0	566.5629	566.5512	20.7	3.72
Z1	710.6075	710.5935	19.7	1.75
Y1	728.6185	728.6035	20.6	2.04
Y2aZ2	872.6605	872.6463	16.3	1.58
Y2ab	890.6719	890.6563	17.5	3.98
Z2	1075.7433	1075.7251	16.9	5.49
Y2a/Y2b	1093.7538	1093.7357	16.5	6.81

Table S4. Matched product ions and fragmentation assignments for LcGg4 (18:1/18:0). The relative peak intensity (I%) shows the percentage of each ion against the combination of all identified carbohydrate and glycolipid ions.

Fragmentation Assignment	Experimental m/z	Theoretical m/z	ppm	I%
Carbohydrate fragments				
B1	204.0913	204.0872	20.1	17.74
B2Y2a/2b	366.1476	366.1395	22.1	5.80
B3Y2a/2b	528.2023	528.1923	18.9	15.48
B2	569.2296	569.2188	19.0	1.43
B3	731.2852	731.2717	18.5	26.72
Glycolipid fragments				
Z0	548.5502	548.5407	17.3	3.68
Y0	566.5617	566.5512	18.5	3.38
Z1	710.6058	710.5935	17.3	1.58
Y1	728.6182	728.6035	20.2	2.33
Y2aZ2	872.6614	872.6463	17.3	1.90
Y2ab	890.6722	890.6563	17.9	5.52
Z2	1075.7443	1075.7251	17.8	5.62
Y2a/Y2b	1093.7549	1093.7357	17.6	8.81

Table S5. Matched product ions and fragmentation assignments for GalNAc-LcGg4 (18:1/18:0). The relative peak intensity (I%) shows the percentage of each ion against the combination of all identified carbohydrate and glycolipid ions.

Fragmentation Assignment	Experimental m/z	Theoretical m/z	ppm	I%
Carbohydrate fragments				
B1	204.0905	204.0872	16.2	15.94
B2Y2a/2b	366.1451	366.1395	15.3	4.70
B3Y2a/2b	528.2000	528.1923	14.6	13.99
B2	569.2259	569.2188	12.5	1.01
B3	731.2822	731.2717	14.4	18.69
Glycolipid fragments				
Z0	548.5482	548.5407	13.7	4.57
Y0	566.5587	566.5512	13.2	4.08
Z1	710.6023	710.5935	12.4	1.88
Y1	728.6141	728.6035	14.5	3.28
Y2aZ2	872.6582	872.6463	13.6	2.59
Y2ab	890.6689	890.6563	14.1	8.09
Z2	1075.7380	1075.7251	12.0	7.53
Y2a/Y2b	1093.7494	1093.7357	12.5	13.63

Table S6. Matched product ions and fragmentation assignments for GalNAc-LcGg4 (d16:1/18:0). The relative peak intensity (I%) shows the percentage of each ion against the combination of all identified carbohydrate and glycolipid ions.

Fragmentation Assignment	Experimental m/z	Theoretical m/z	ppm	I%
Carbohydrate fragments				
B1	204.0892	204.0872	9.80	18.89
B2Y2a/2b	366.1415	366.1395	5.46	6.04
B3Y2a/2b	528.1941	528.1923	3.41	14.83
B2	569.2210	569.2188	3.86	1.13
B3	731.2689	731.2717	-3.83	16.57
Glycolipid fragments				
Z0	520.5107	520.5094	2.50	4.64
Y0	538.5182	538.5199	-3.16	4.16
Z1	682.5627	682.5622	0.73	1.87
Y1	700.5706	700.5722	-2.28	2.71
Y2aZ2	844.6159	844.6150	1.07	2.23
Y2ab	862.6263	862.6250	1.51	7.97
Z2	1047.6907	1047.6938	-2.96	6.86
Y2a/Y2b	1065.7009	1065.7044	-3.28	12.08

Table S7. Matched product ions and fragmentation assignments for GalNAc-LcGg4-NBD. The relative peak intensity (I%) shows the percentage of each ion against the combination of all identified carbohydrate and glycolipid ions.

Fragmentation Assignment	Experimental m/z	Theoretical m/z	ppm	I%
Carbohydrate fragments				
B1	204.0892	204.0872	9.80	5.83
B2Y2a/2b	366.1415	366.1395	5.46	1.29
B3Y2a/2b	528.1941	528.1923	3.41	5.44
B2	569.2210	569.2188	3.86	0.61
B3	731.2744	731.2717	3.69	13.96
Glycolipid fragments				
Z0	628.4421	628.4432	-1.75	10.87
Y0	646.4542	646.4527	2.32	11.40
Z1	790.4930	790.4961	-3.92	3.18
Y1	808.5074	808.5066	0.99	3.00
Y2aZ2	952.5522	952.5489	3.46	8.25
Y2ab	970.5573	970.5595	-2.27	16.61
Z2	1155.6256	1155.6283	-2.34	9.05
Y2a/Y2b	1173.6396	1173.6388	0.68	10.51