

Supplemental material

Structural Characterization by MSⁿ of Human Milk Glycans Recognized by Human Rotaviruses

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and Vernon N. Reinhold¹**

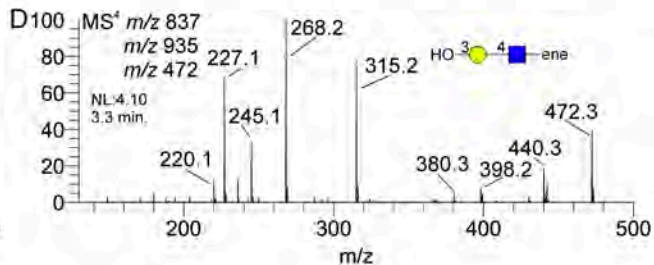
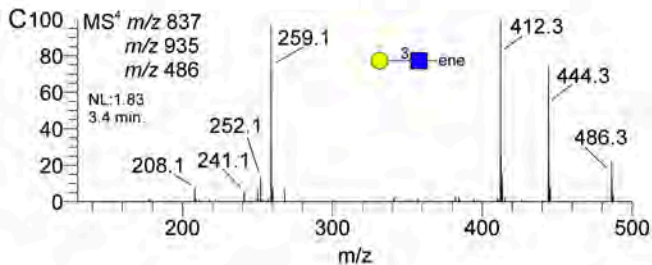
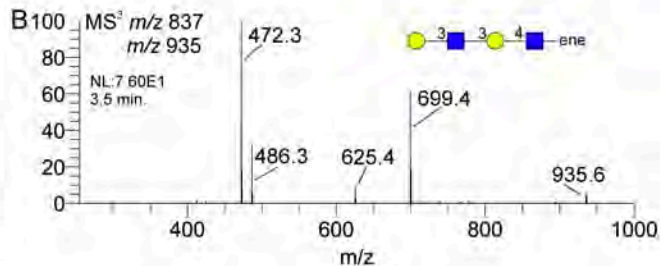
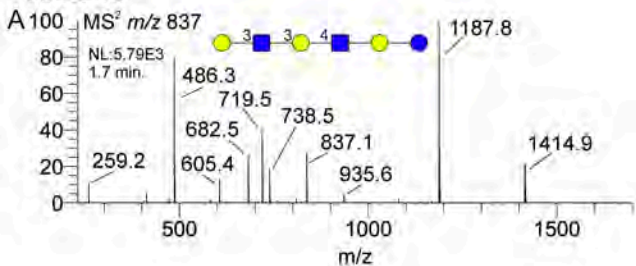
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The following supplemental figures show a selection of MSⁿ spectra acquired for each HMG sample. The proposed structures are shown in graphical form with empirically determined linkages explicitly indicated. For each MSⁿ spectrum, the precursor ion fragment structure is also shown graphically, with the former bond shown as a “tail” in the fragment structure, with the type of scar (-ene or -OH) explicitly shown.

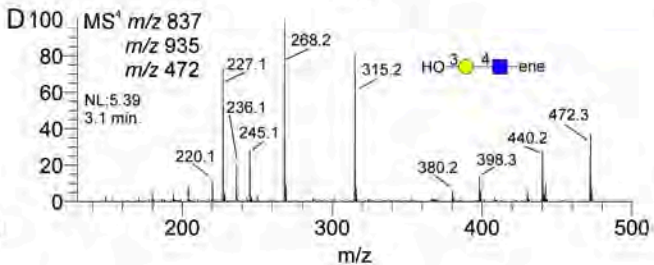
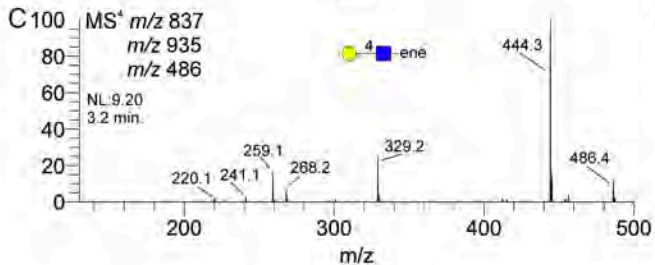
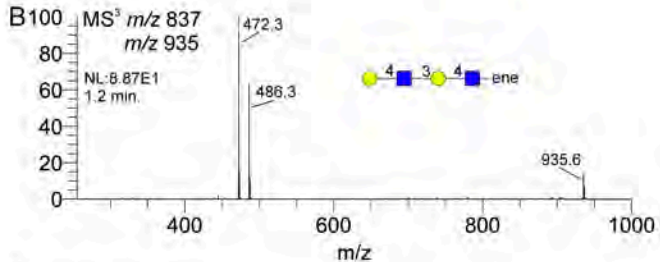
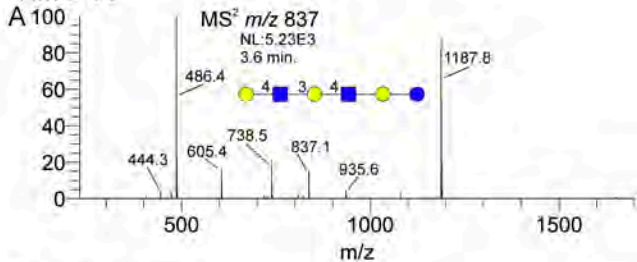
The fragmentation pathway is shown as the series of precursor ions chosen to obtain the given spectrum. Normalization level and signal averaging time are shown for each spectrum. Peak lists are included in a separate file. A short textual description of the sample data is given for each HMG.

Where appropriate, a table of spectrum matching scores is also shown. The standard structures and scoring algorithm have been described previously (12). The sialylated Lewis X trisaccharide standard fragment, m/z 646, was used for internal Lewis X comparisons. The 3-LN and 4-LN standards were both four-linked internal lactosamines, but differed in the linkage positions of substituents on the Gal, with 3-LN having a substituent at the 3-Gal and 4-LN having a substituent at the 4-Gal positions. The nature of the substituents seems not to affect the mass spectral fragmentation patterns.

HMG 13

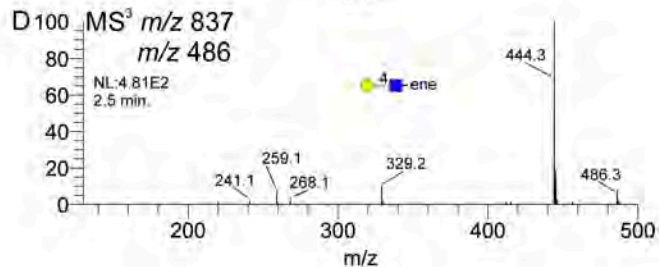
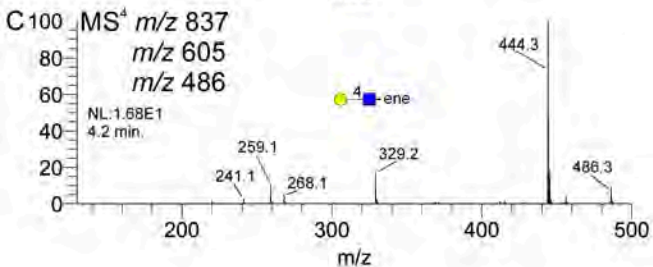
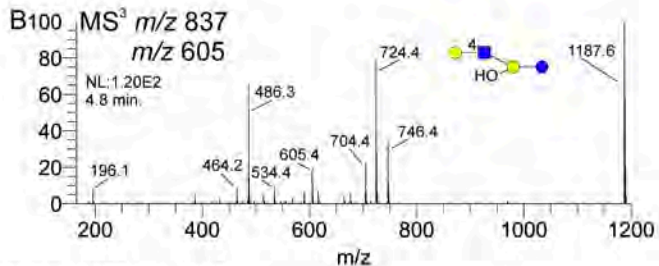
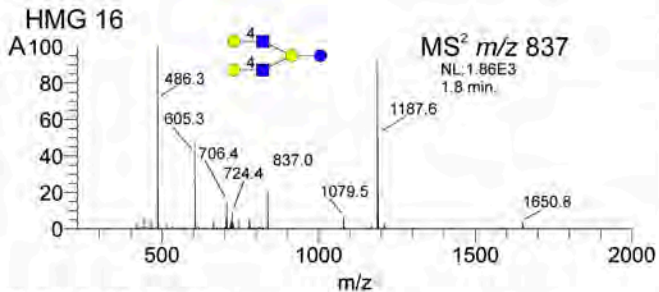


HMG 14

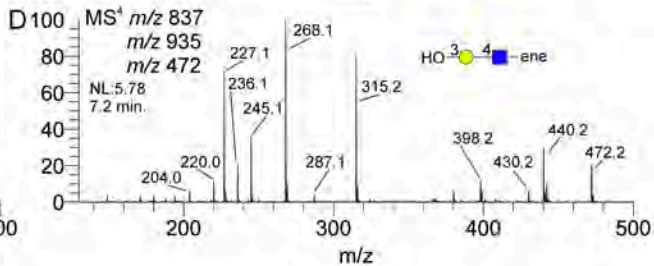
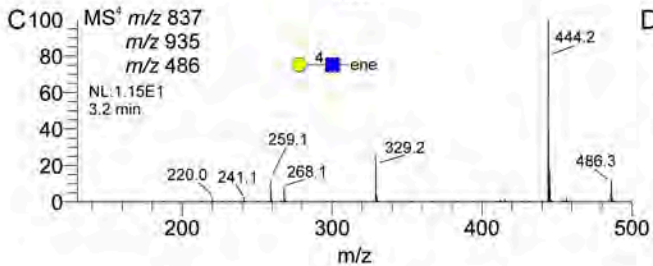
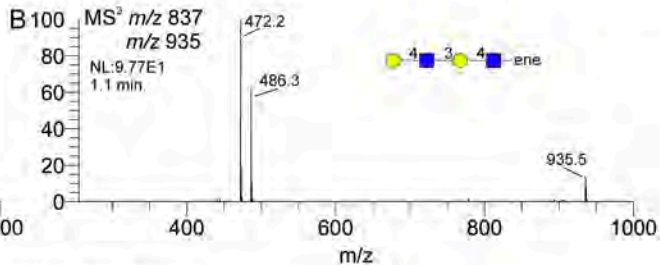
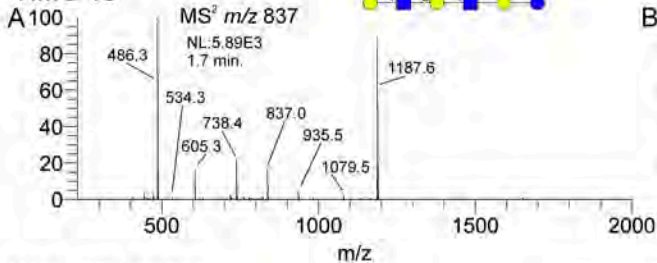


HMG 16

HMG 16 has the same molecular weight as HMG 13 and 14, but has a doubly-branched topology. Fragmentation reveals the core AEAB-lactose fragment mass of m/z 724, consistent with the presence of two substituents. The presence of the m/z 534 fragment, which is the core AEAB-glucose, positions both substituents on the core galactose, as expected. The terminal LacNAc branches exhibit fragmentation consistent with four-linkages (C) and (D).



HMG 18



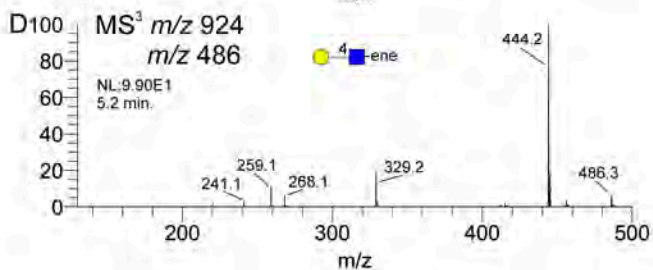
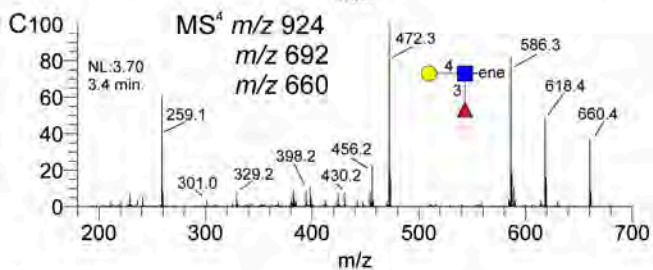
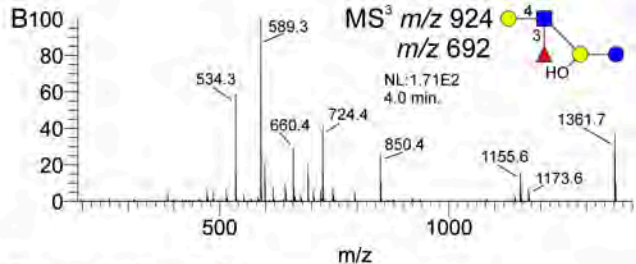
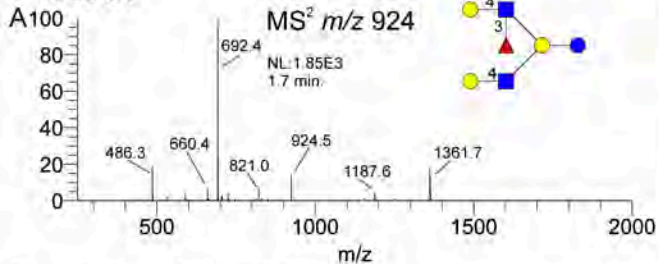
HMG 20

HMG 20 has the composition H₄N₂F₁. Disassembly reveals a doubly-branched core (m/z 724) with a fucosylated LacNAc fragment, m/z 660. Disassembly of the fucosylated LacNAc fragment (C) reveals a Lewis X structure based on spectral matching with standard materials. Figure D shows the terminal LacNAc branch to be exclusively four-linked.

HMG 20 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
8-12-13_HMG20_924_MS3_660_01.raw ms3 924.60-661.00					0.267	0.498	0.939				0.735
8-12-13_HMG20_924_MS3_660_02.raw ms3 924.60-661.00					0.267	0.496	0.938				0.747
8-12-13_HMG20_924_MS4_692_660_01.raw ms4 924.60-693.00-661.00					0.360	0.795	0.986				0.542

HMG 20

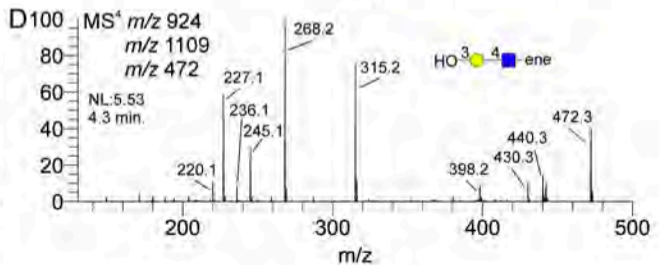
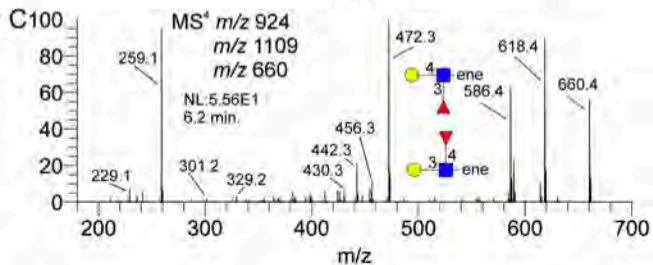
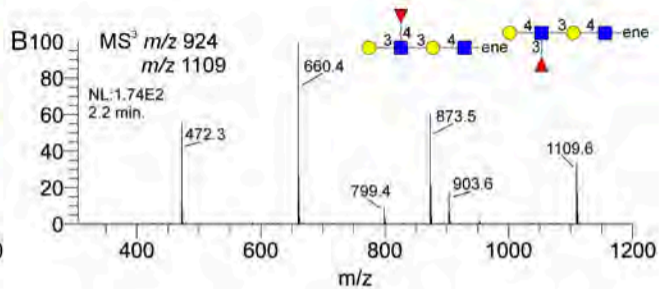
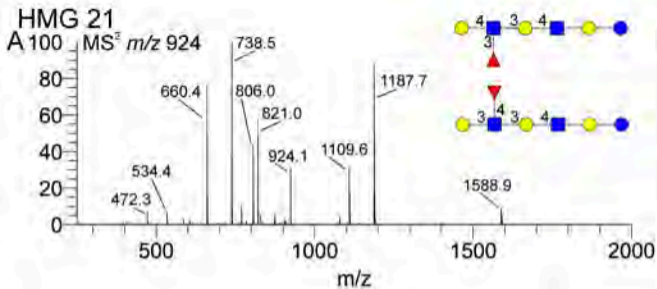


HMG 21

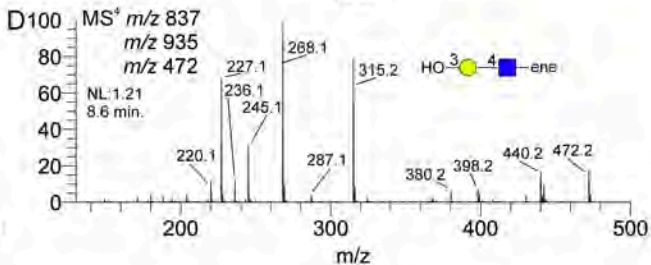
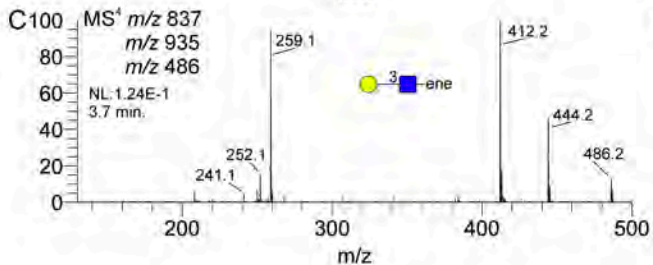
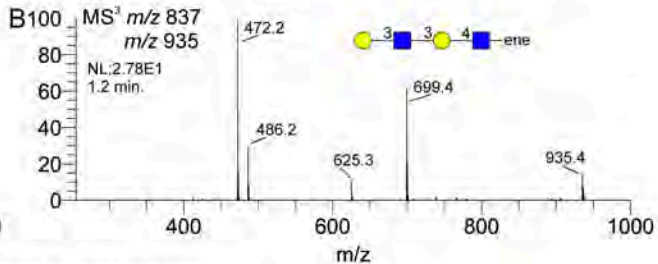
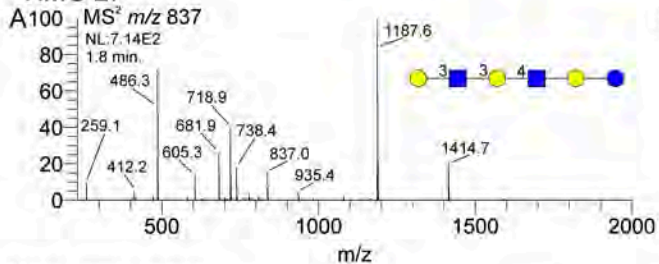
The molecular weight of HMG 21 indicates an H4N2F1 composition, like HMG 20. The core AEAB-lactose fragment, m/z 738, is indicative of a linear (or singly-branched) structure. A monofucosylated di-LacNAc fragment, m/z 1109, is consistent with this topology. Figure B indicates a terminal fucosylated LacNAc, m/z 660, and an internal LacNAc, m/z 472. Isolation and fragmentation of the terminal LacNAc (C) suggests a mixture of Lewis A and Lewis X. The internal LacNAc, as usual, is four-linked with the single substituent likely attached at the three position.

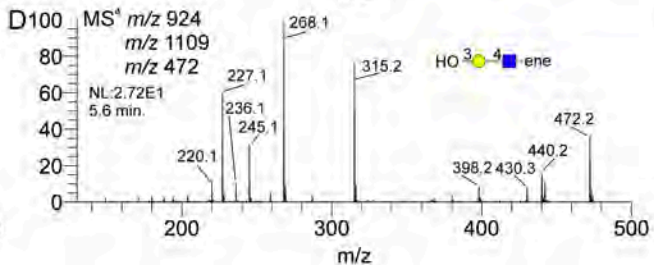
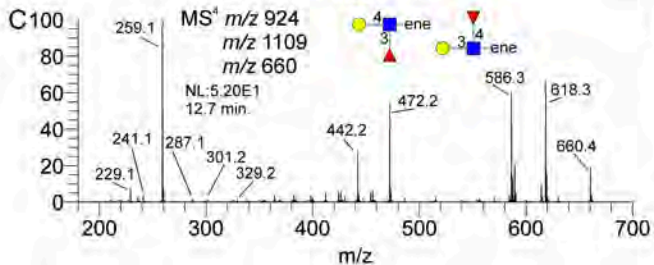
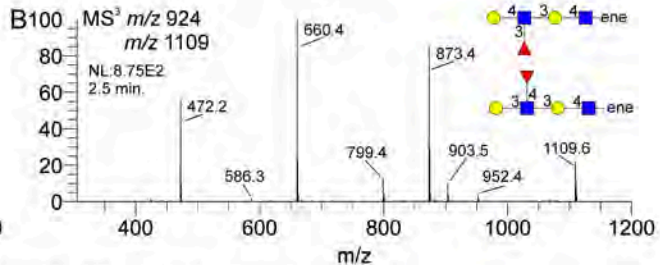
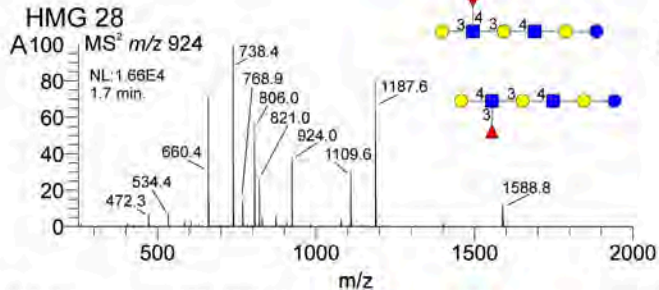
HMG 21 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
7-2-13_HMG21_924_MS3_660_01.raw ms3 924.60-661.00					0.357	0.896	0.943				0.567
7-2-13_HMG21_924_MS3_660_02.raw ms3 924.60-661.00					0.357	0.897	0.943				0.566
7-2-13_HMG21_924_MS3_660_03.raw ms3 924.60-661.00					0.355	0.891	0.945				0.581
7-2-13_HMG21_924_MS4_1109_472_01.raw ms4 924.60-1110.00-473.00	0.935	0.633									
7-2-13_HMG21_924_MS4_1109_660_01.raw ms4 924.60-1110.00-661.00					0.309	0.797	0.950				0.685
7-2-13_HMG21_924_MS4_1109_660_02.raw ms4 924.60-1110.00-661.00					0.311	0.798	0.954				0.677



HMG 27





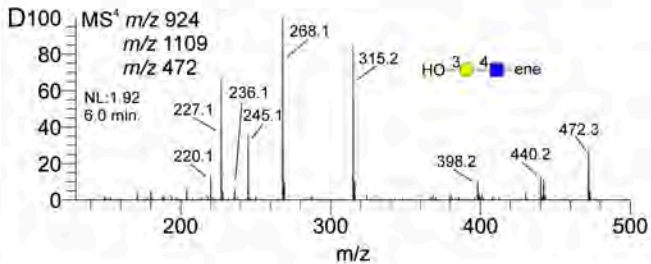
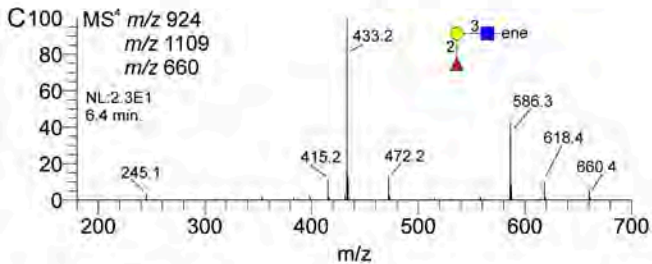
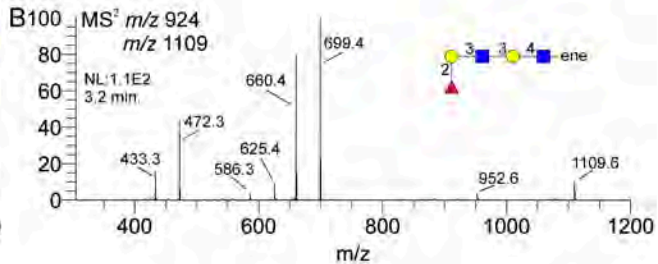
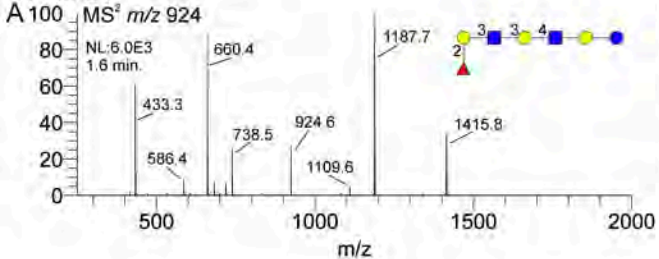
HMG 29

HMG 29 has the composition H₄N₂F₁, just as HMG 21 and 28, but MSⁿ fragmentation reveals that the terminal fucosylated Lactosamine is an H1 structure. Like HMG 21 and 28, this structure is linear (singly-branched) with a 4-linked internal LacNAc.

HMG 29 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
8-12-13_HMG29_924_MS3_472_01.raw ms3 924.60-473.00	0.596	0.552									
8-12-13_HMG29_924_MS4_1109_472_01.raw ms4 924.60-1110.00-473.00	0.934	0.591									
8-12-13_HMG29_924_MS4_1109_472_02.raw ms4 924.60-1110.00-473.00	0.930	0.618									
8-12-13_HMG29_924_MS4_1109_660_01.raw ms4 924.60-1110.00-661.00					0.992	0.307	0.284				0.208
8-12-13_HMG29_924_MS4_1109_660_02.raw ms4 924.60-1110.00-661.00					0.996	0.323	0.306				0.236

HMG 29

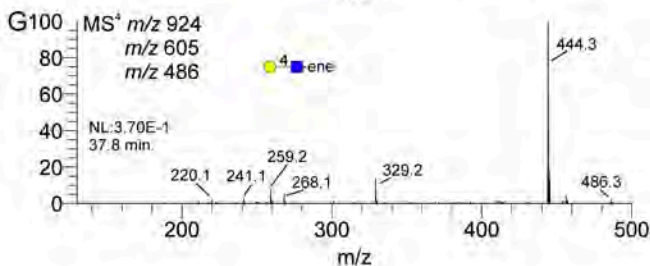
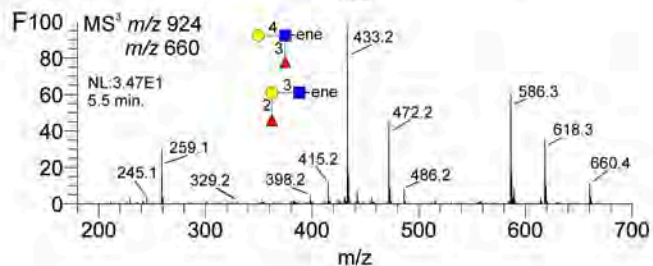
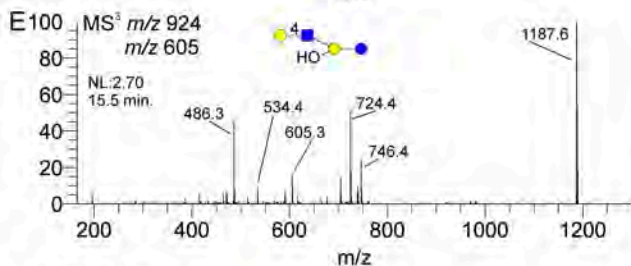
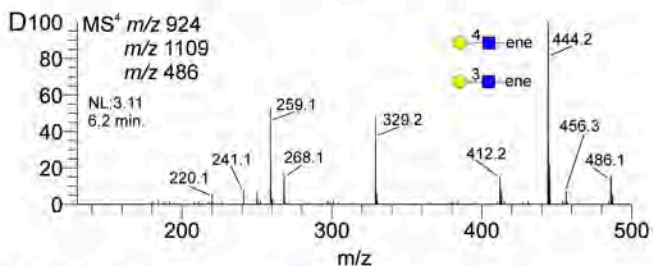
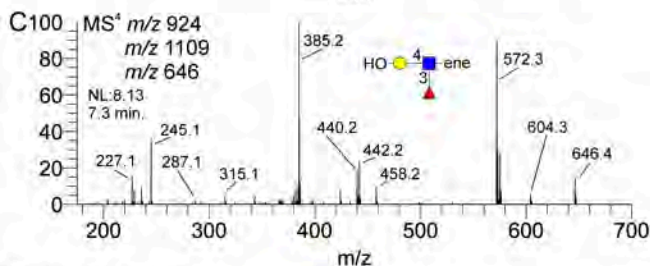
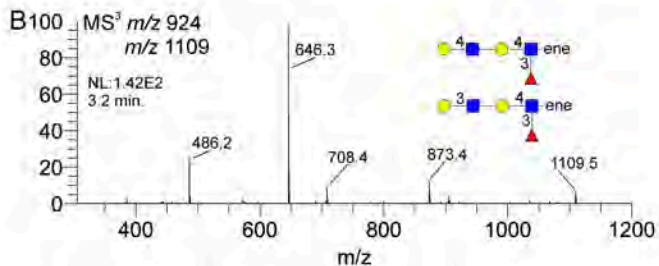
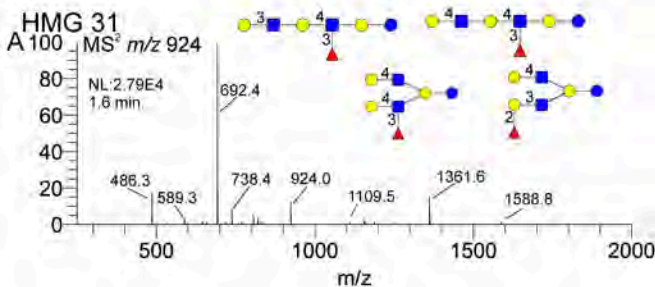


HMG 31

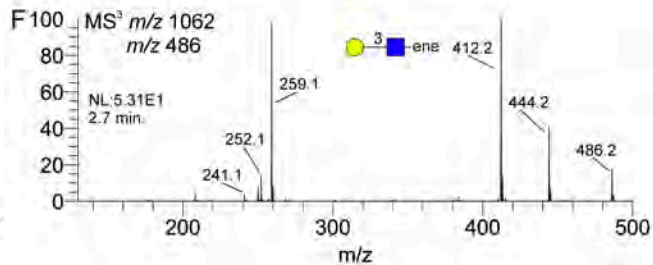
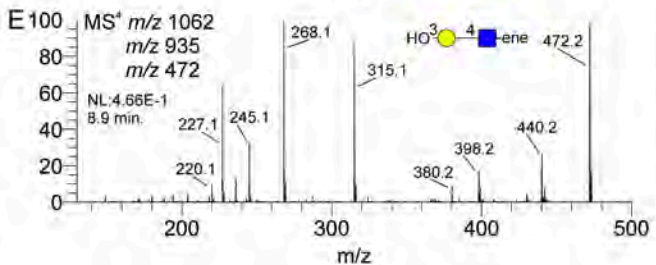
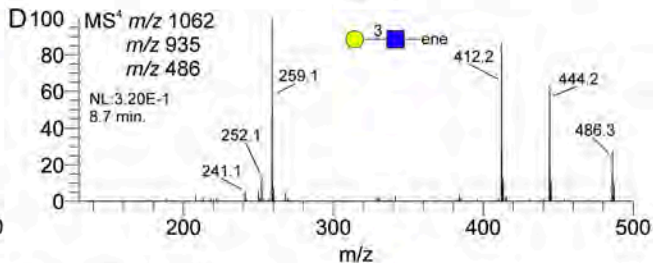
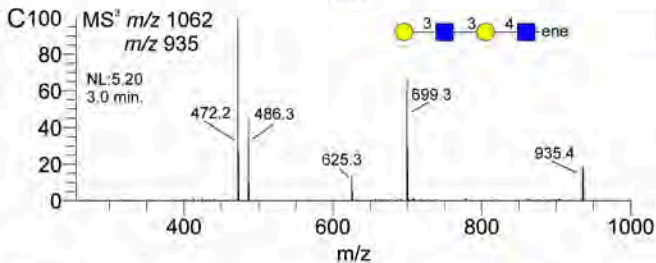
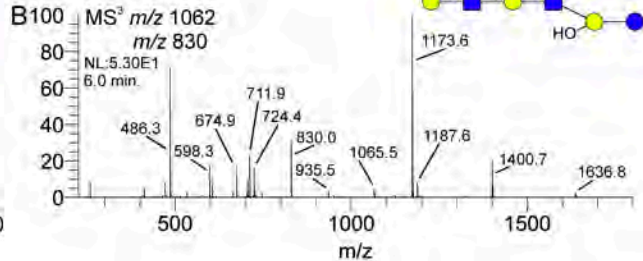
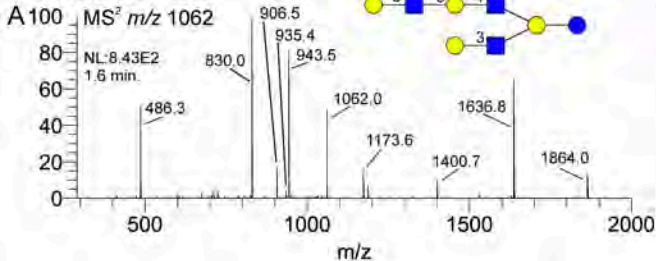
HMG 31 has the composition H₄N₂F₁ and MSⁿ analysis reveals a mixture consisting of at least four structures. Two linear (singly-branched) structures are indicated by the m/z 738 core fragment and m/z 1109 monofucosylated di-LacNAc fragments. Disassembly reveals that each isomer has an internal Lewis X but differ in the linkage of the terminal lactosamine, as (D) indicates a mixture of three- and four-linked LacNAc. The branched structures are revealed through isolating the m/z 605 fragment, formed by loss of a terminal fucosylated LacNAc fragment. This spectrum (E) shows a doubly-branched core fragment, m/z 724, and a terminal LacNAc. The terminal fucosylated LacNAc (F) is consistent with a mixture of H1 and Lewis X. The terminal LacNAc of the branched isomers seems to be exclusively four-linked. Importantly, the disassembly pathways chosen for the branched and linear isomers isolate the terminal LacNAcs for those respective isomers separately. For that reason, (G) and (D) represent the LacNAc fragments of different parent structures and are different spectra. Isolating the m/z 486 ion at the MS³ level would not provide this distinction.

HMG 31 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
8-16-13_HMG31_924_MS3_660_01.raw ms3 924.40-661.00					0.931	0.592	0.635				0.452
8-16-13_HMG31_924_MS3_660_02.raw ms3 924.40-661.00					0.933	0.592	0.630				0.451
8-16-13_HMG31_924_MS4_1109_646_01.raw ms4 924.40-1110.00-647.00				0.448				0.953		0.332	
8-16-13_HMG31_924_MS4_1109_646_02.raw ms4 924.40-1110.00-647.00				0.444				0.983		0.397	
8-16-13_HMG31_924_MS4_692_646_01.raw ms4 924.40-692.60-647.00				0.483				0.991		0.391	



HMG 33



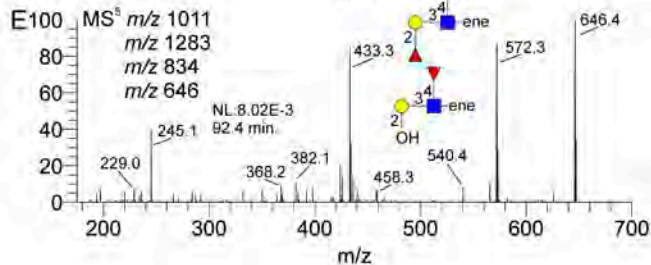
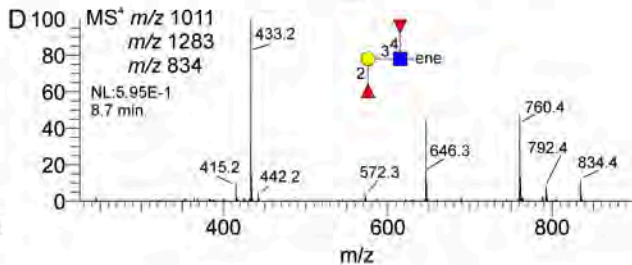
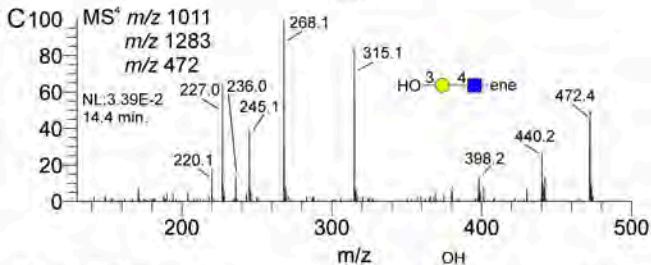
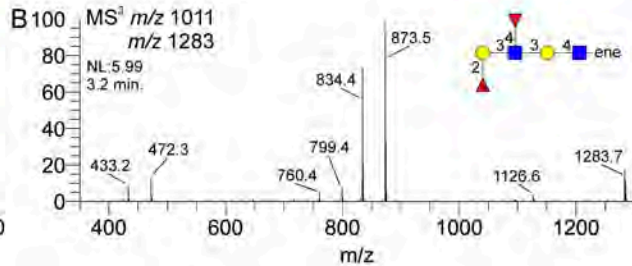
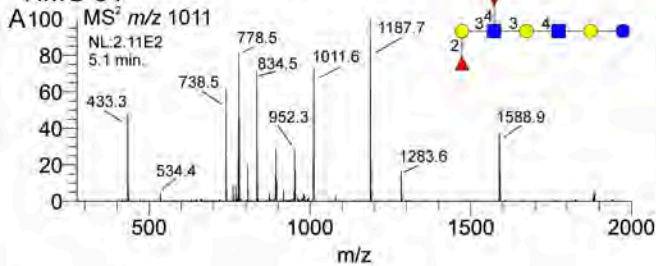
HMG 34

HMG 34 has the composition H₄N₂F₂. Disassembly reveals a linear (singly-branched) structure, as indicated by the m/z 738 core fragment. A terminal difucosylated lactosamine and difucosylated diLacNAc are also interesting features. Fragmentation of the difucosylated diLacNAc (B) shows the terminal difucosylated LacNAc, m/z 834, and the internal LacNAc, m/z 472. Fragmentation of the internal LacNAc shows the typical four-linkage with a likely three-linked substituent. Spectra D and E show the MS⁴, m/z 834, and MS⁵, m/z 646, spectra, which are consistent with the Lewis B structure.

HMG 34 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
12-18-13_HMG34_1011_MS3_834_01.raw ms3 1011.60-835.00			0.971						0.778		
12-18-13_HMG34_1011_MS4_1283_472_01.raw ms4 1011.60-1284.00-473.00	0.953	0.638									
12-18-13_HMG34_1011_MS4_1283_834_01.raw ms4 1011.60-1284.00-835.00			0.985						0.895		
12-18-13_HMG34_1011_MS4_834_646_01.raw ms4 1011.60-835.00-647.00				0.994				0.491		0.222	
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12-18-13_HMG34_1011_MS5_1283_834_646_02.raw ms5 1011.60-1284.00-835.00-647.00				0.972				0.515		0.228	
12-18-13_HMG34_1011_MS5_1283_834_646_03.raw ms5 1011.60-1284.00-835.00-647.00				0.929				0.591		0.206	

HMG 34



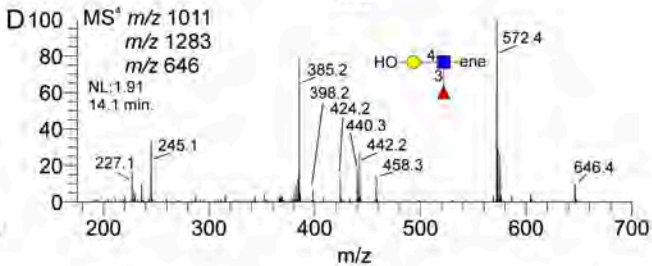
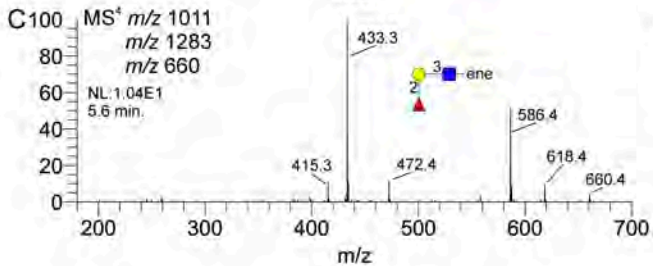
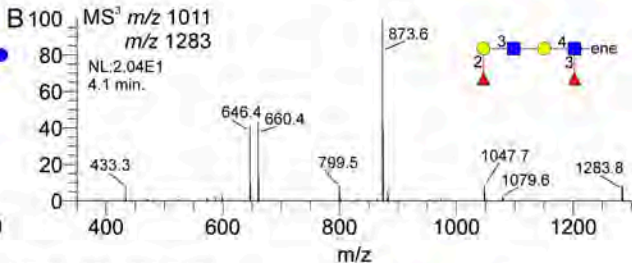
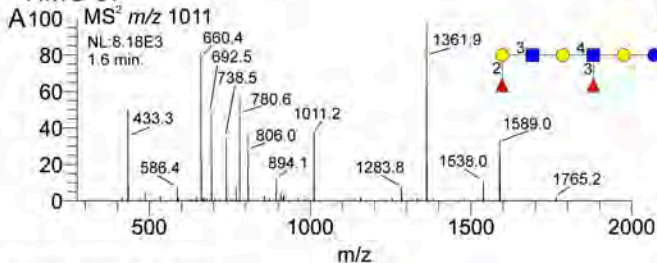
HMG 37

HMG 37 has the composition H₄N₂F₂, just as HMG 34. However, the MSⁿ analysis reveals a different fucosylation pattern. HMG 37 has the linear (singly-branched) core, as indicated by the m/z 738 fragment. It also has a difucosylated diLacNAc fragment, m/z 1283, but does not have a difucosylated LacNAc fragment. Dissociation of the difucosylated diLacNAc fragment reveals terminal, m/z 660, and internal, m/z 646, fucosylated LacNAc fragments. Further disassembly shows a terminal H1 structure and an internal Lewis X.

HMG 37 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472	472	834	646	660	660	660	646	834	646	660
	3-LN	6-LN	Leb	Leb	H1	Lea	Lex	Slex	Ley	Ley	H2
6-26-13_HMG37_1011_MS3_660_01.raw ms3 1011.60-661.00					0.995	0.339	0.281				0.183
6-26-13_HMG37_1011_MS4_1109_646_01.raw ms4 1011.60-1110.00-647.00				0.531				0.984		0.366	
6-26-13_HMG37_1011_MS4_1283_646_01.raw ms4 1011.60-1284.00-647.00				0.506				0.988		0.363	
6-26-13_HMG37_1011_MS4_1283_660_01.raw ms4 1011.60-1284.00-661.00					0.980	0.403	0.342				0.215
6-26-13_HMG37_1011_MS4_1361_646_01.raw ms4 1011.60-1362.00-647.00				0.587				0.942		0.256	
6-26-13_HMG37_997_MS4_1283_646_01.raw ms4 997.80-1284.00-647.00				0.521				0.970		0.339	
6-26-13_HMG37_997_MS4_1283_660_01.raw ms4 997.80-1284.00-661.00					0.984	0.375	0.321				0.232

HMG 37

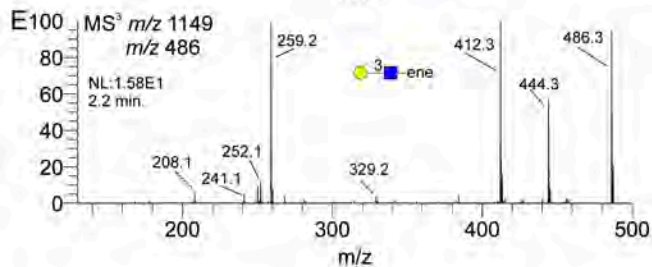
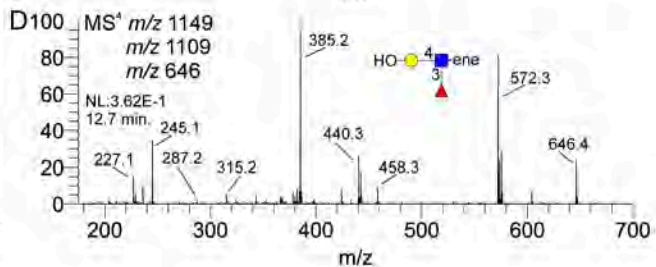
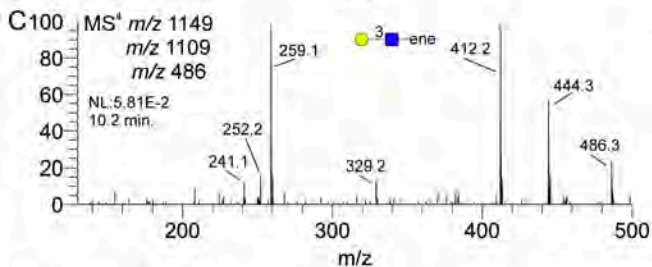
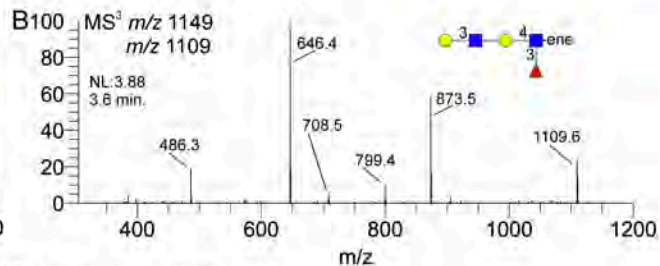
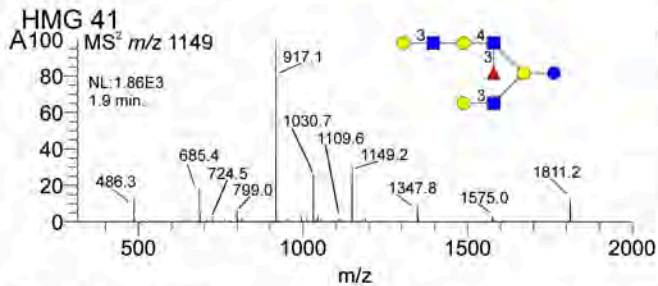


HMG 41

HMG 41 has the composition H₅N₃F₁. The doubly-branched core lactose is indicated by the m/z 724 fragment. A fucosylated diLacNAc fragment is observed at the MS² level and was selected for deeper interrogation. The MS³ for this fragment shows a terminal LacNAc, m/z 486, and an internal fucosylated LacNAc, m/z 646. Fragmentation of these ions shows an internal Lewis X structure and a predominantly three-linked terminal LacNAc. The presence of the ^{3,5}A fragment, m/z 329, in both MS³ and MS⁴ spectra of m/z 486 precursors suggests a small but detectable amount of four-linked LacNAc is also present.

HMG 41 MSⁿ spectral matching scores

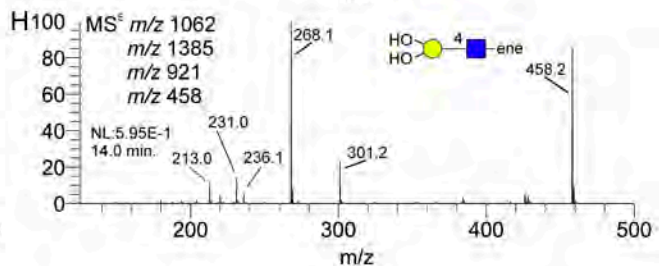
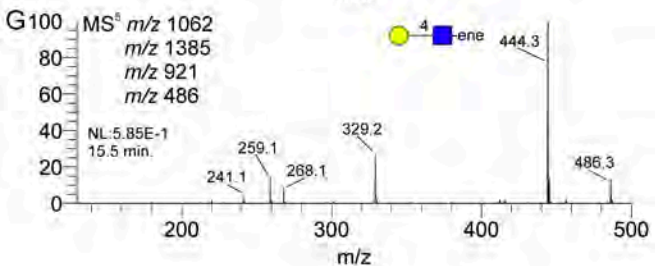
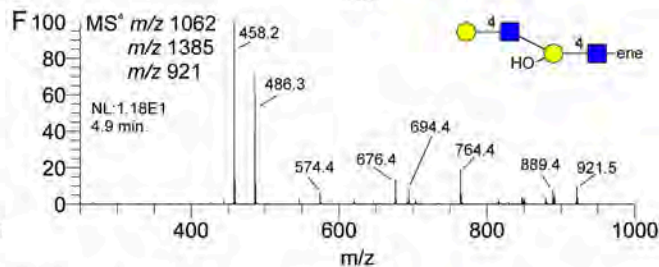
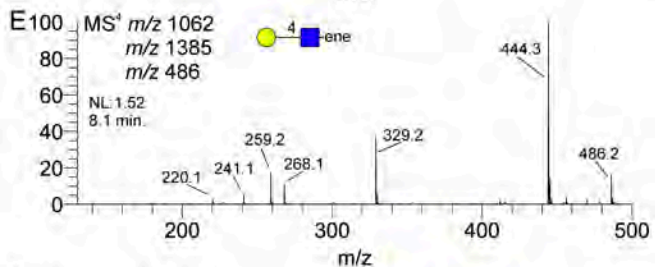
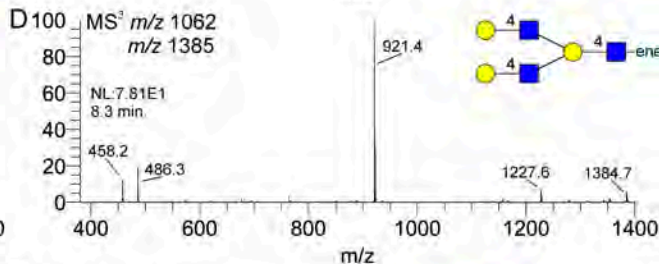
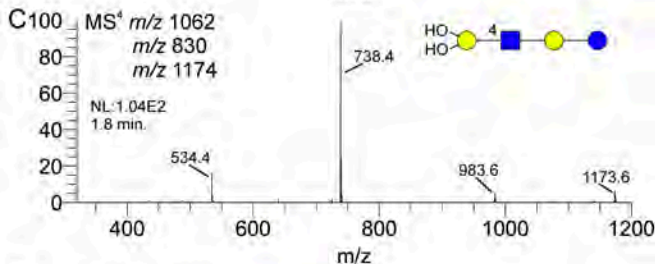
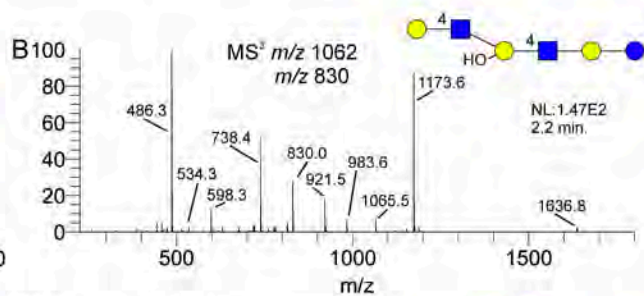
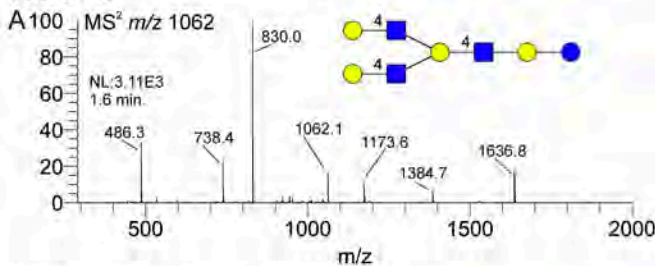
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472	472	834	646	660	660	660	646	834	646	660
	3-LN	6-LN	Leb	Leb	H1	Lea	Lex	Slex	Ley	Ley	H2
6-27-13_HMG41_1149_MS4_1109_646_01.raw ms4 1149.00-1110.00-647.00				0.442				0.964		0.431	
6-27-13_HMG41_1149_MS4_1109_646_02.raw ms4 1149.00-1110.00-647.00				0.425				0.965		0.411	
6-27-13_HMG41_1149_MS4_917_646_01.raw ms4 1149.00-918.00-647.00				0.300				0.883		0.445	



HMG 45

HMG 45 has the composition H₅N₃. Unexpectedly, the MSⁿ fragmentation of this HMG indicated a singly-branched core, based on the presence of the m/z 738 AEAB-lactose fragment. Further MSⁿ disassembly indicated that the structure was branched at a lactosamine unit. Multiple fragmentation pathways were employed to confirm this. The first disassembly pathway is shown in spectra B and C. These represent successive losses of two terminal LacNAc units, followed by a branched LacNAc leading to the singly-branched AEAB-lactose core fragment. Permethylolation is essential for this analysis and interpretation, as the mass differential of the methyl groups enables the distinction of terminal (m/z 486), internal with one substituent (m/z 472), and branched with two substituents (m/z 458). Note that the mass differences are multiples of 14 mu. The branched tri-LacNAc, m/z 1385, was isolated and disassembled to produce spectra D, E, F, G, and H. These data show two four-linked terminal LacNAc units, m/z 486, and the four-linked doubly-branched LacNAc unit, m/z 458. This motif is also found on several larger HMGs, where it is expected to occur.

HMG 45

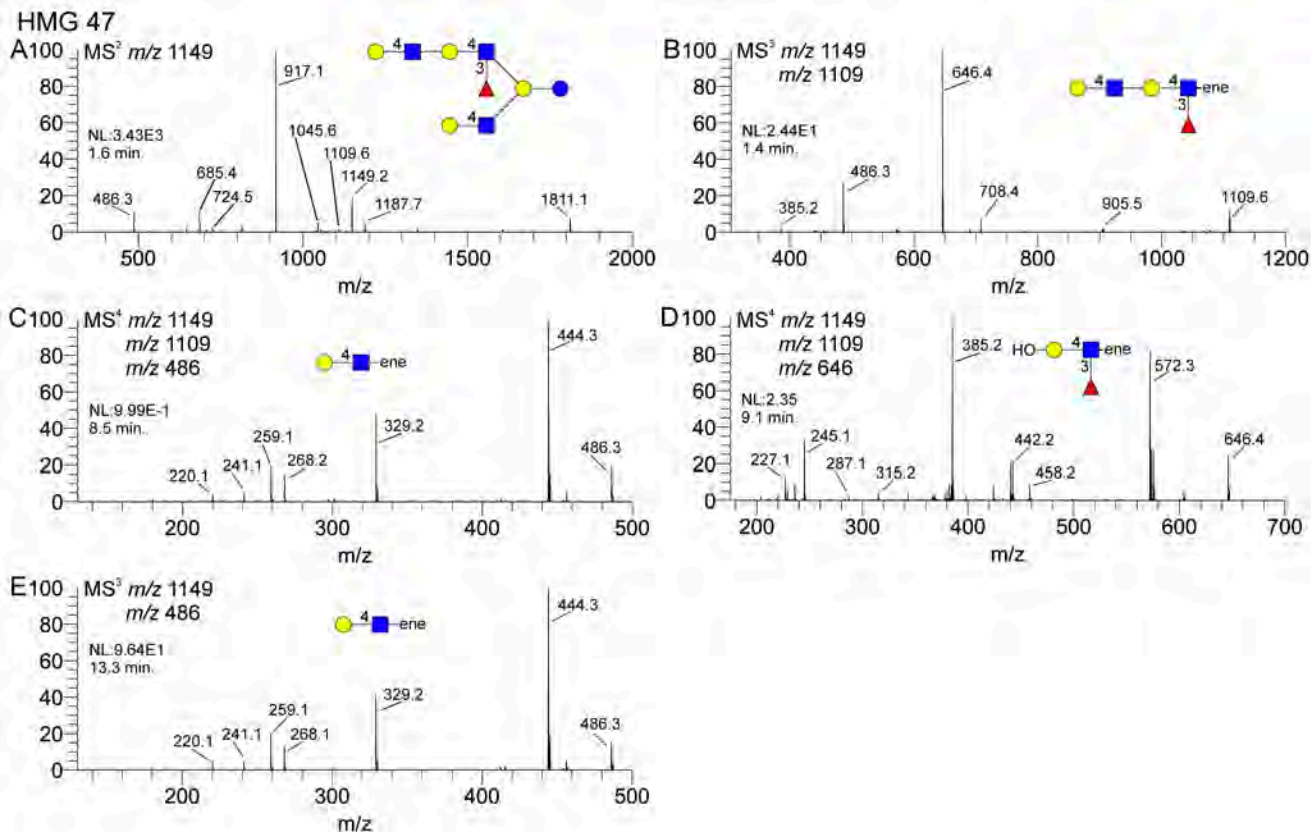


HMG 47

HMG 47 has the composition H₅N₃F₁. Spectrum A shows the doubly-branched core AEAB-lactose, m/z 724, and a monofucosylated diLacNAc, m/z 1109. Spectra B, C, and D show that the monofucosylated diLacNAc consists of a four-linked terminal LacNAc and an internal Lewis X motif. Spectra E, showing an MS³ of the terminal LacNAc, m/z 486, shows that both terminal LacNAcs are likely four-linked.

HMG 47 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
8-22-13_HMG47_1148_MS4_1109_646_01.raw ms4 1149.00-1110.00-647.00				0.413				0.968		0.402	
8-22-13_HMG47_1148_MS4_1109_646_02.raw ms4 1149.00-1110.00-647.00				0.426				0.975		0.399	
8-22-13_HMG47_1148_MS5_917_685_646_01.raw ms5 1149.00-917.60-686.00-647.00				0.479				0.992		0.400	
8-22-13_HMG47_1148_MS5_917_685_646_02.raw ms5 1149.00-917.60-686.00-647.00				0.478				0.992		0.396	



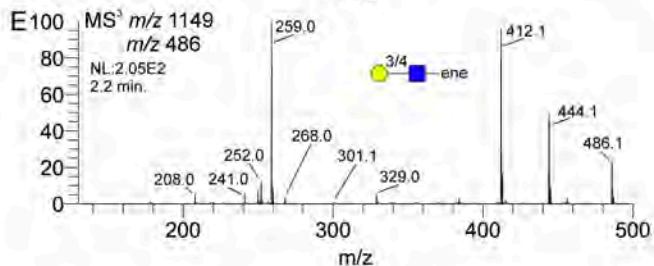
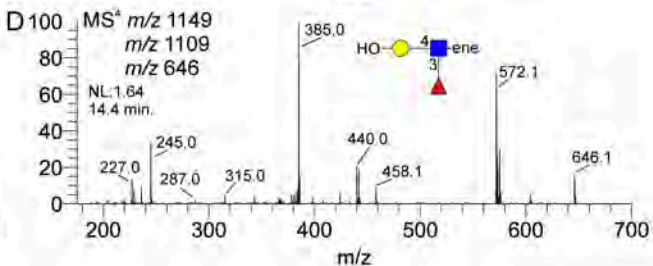
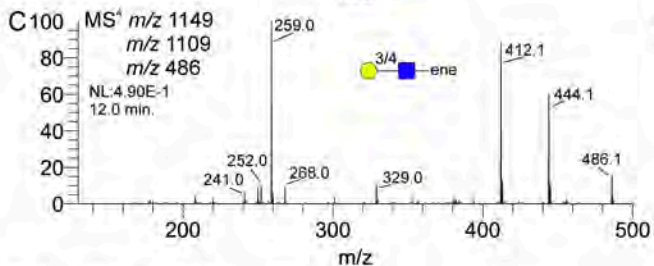
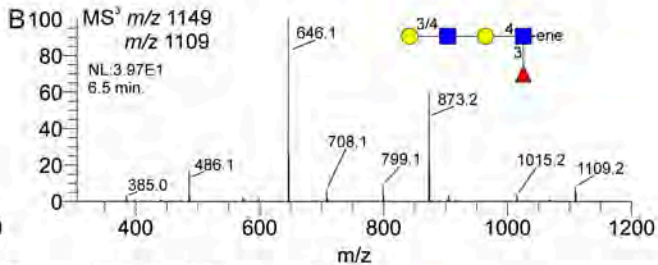
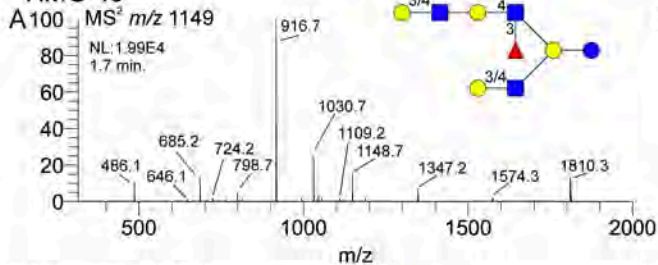
HMG 49

HMG 49 has the composition H5N3F1. MSⁿ fragmentation shows similar data to HMG 41, including a small but detectable amount of four-linked terminal LacNAcs.

HMG 49 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
4-26-13_HMO-49_1134_MS5_902_1109_660_01.raw ms5 1135.00-903.60-1110.00-661.00					0.090	0.364	0.217				0.004
4-26-13_HMO-49_1148_MS3_646_01.raw ms3 1149.40-647.00				0.349				0.908		0.379	
4-26-13_HMO-49_1148_MS4_1109_646_01.raw ms4 1149.40-1110.00-647.00				0.380				0.953		0.421	
4-26-13_HMO-49_1148_MS4_1109_646_02.raw ms4 1149.40-1110.00-647.00				0.400				0.962		0.410	

HMG 49



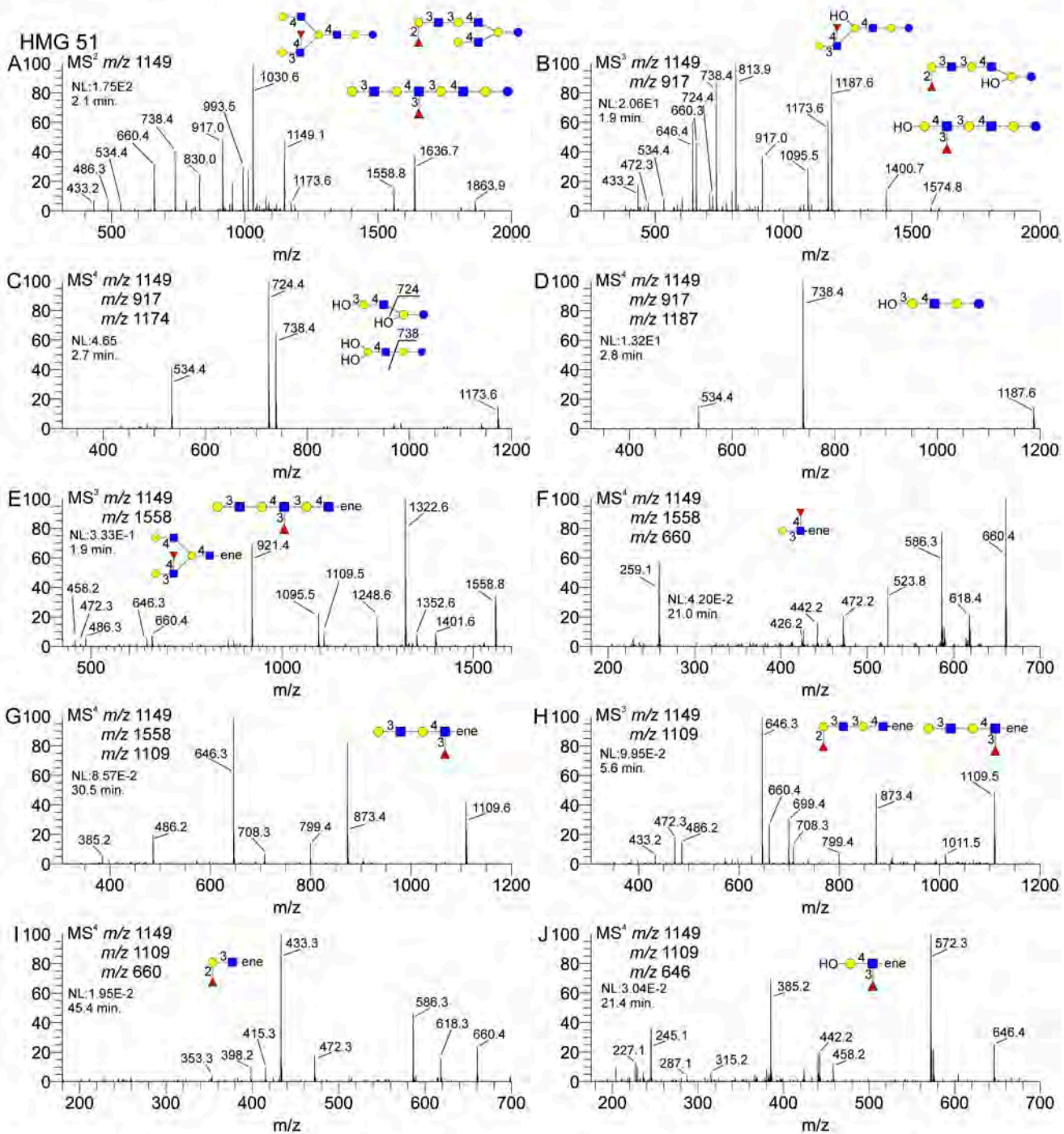
HMG 51 has the composition H₅N₃F₁. MSⁿ analysis reveals a mixture of at least three isomers. Spectra B, C, and D show the successive disassembly to the AEAB-lactose core for this sample. Spectra B and C show the successive losses of terminal LacNAc and terminal fucosylated LacNAc. Spectrum C shows AEAB-lactose fragment ions of both m/z 724 and 738, indicating singly-branched and doubly-branched cores. The AEAB-glucose fragment, m/z 534, indicates that all substituents are attached to the core galactose, as expected. The neutral losses in spectrum C indicate one isomer with an internal LacNAc (loss of 449), leading to the doubly-branched core (m/z 724), and another isomer with a branched LacNAc (loss of 436), leading to the singly-branched core (m/z 738); both of these isomers also have a terminal LacNAc and a terminal fucosylated LacNAc. Spectra B and D show the successive losses of a terminal LacNAc and an internal fucosylated LacNAc, followed by an internal LacNAc. Spectrum B shows fragment contributions from all three of these isomers. Spectrum C contains contributions from two of these isomers, those possessing terminal LacNAc and terminal fucosylated LacNAc. Spectrum D shows fragments only from the single isomer containing a terminal LacNAc and an internal fucosylated LacNAc.

Further fragmentation confirms and clarifies these structures. Spectrum B shows the fucosylated tri-LacNAc fragment, m/z 1558, which consists of two different structures, found on the isomers having the singly-branched core lactose. Spectrum E shows the MS³ spectrum of this ion, showing two different isomeric topologies. Note the different LacNAc masses, m/z 458, 472, and 458, and the different fucosylated LacNAc masses, m/z 646 and 660; these indicate the components of the different topology and branching isomers. Spectrum F shows the terminal fucosylated LacNAc, from the singly-branched core-lactose isomer, as being a Lewis A structure, based on spectrum matching with known standards. Spectrum G shows the MS⁴ spectrum of the fucosylated diLacNAc fragment from one of the singly-branched core-lactose isomers; this shows a topology with a terminal LacNAc and an internal fucosylated LacNAc. Sensitivity limitations prevented deeper interrogation, but the structure could be determined through alternative pathways. Spectrum H shows the MS³ spectrum of a fucosylated diLacNAc fragment, m/z 1109. At this stage, ions are contributed from two different isomers, one of the singly-branched core isomers and the doubly-branched core isomer. Note that two different topologies are represented in this spectrum, one with terminal LacNAc and internal fuc-LacNAc (m/z 486 and 646) and another with a terminal fuc-LacNAc and internal LacNAc (m/z 472 and 660). Probing further, spectrum I shows the disassembly of this terminal fuc-LacNAc as an obvious H1 structure, which is distinctly different from the terminal fuc-LacNAc shown in spectrum F. These fragmentation pathways have effectively separated the isomeric structures to provide distinct, pure spectra consistent with comparable standards. The internal fuc-LacNAc of the other isomer is shown in spectrum J as an internal Lewis X, as all internal fuc-LacNAcs found so far have been.

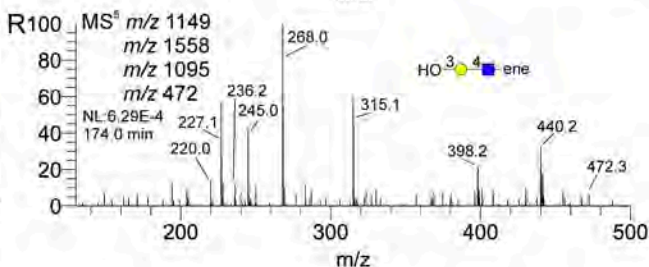
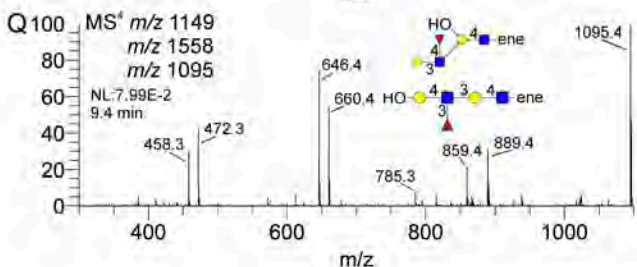
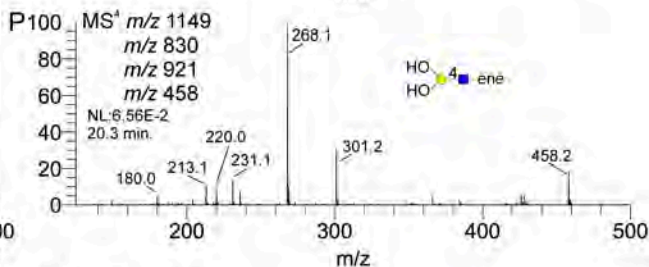
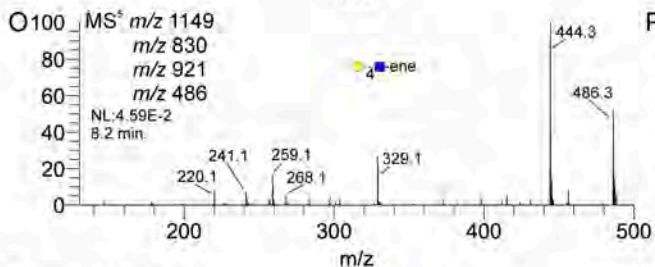
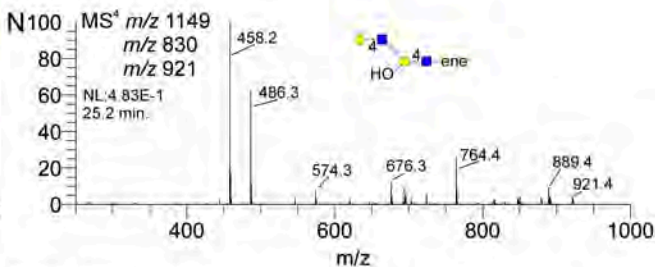
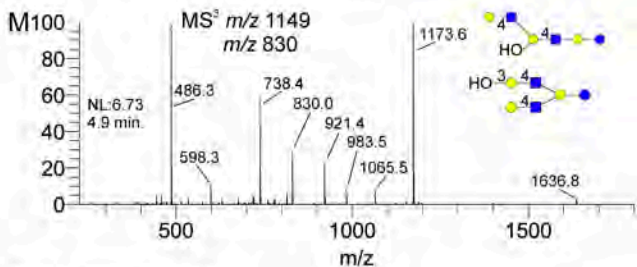
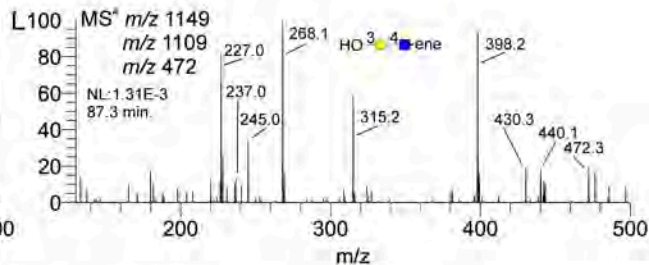
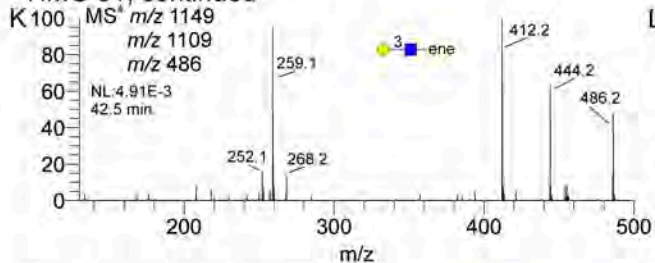
Spectrum K shows the CID spectrum of the terminal LacNAc component of the isomer having the internal Lewis X; this spectrum is consistent with a three linkage. Spectrum L shows CID spectrum of the internal LacNAc complement of the terminal H1 structure; this spectrum is consistent with a four-linkage and with the H1 being attached at the three position of the Gal residue.

Determining the terminal LacNAc linkage for the other isomers required a different fragmentation pathway. Spectrum M shows the MS³ fragmentation of the m/z 830 ion, formed by loss of a terminal fucosylated LacNAc epitope. This separates these isomers from the one containing an internal fuc-LacNAc. Selecting the m/z 921 ion isolates the branched LacNAc containing isomer from the doubly-branched core isomer and provides a pathway to isolate the terminal LacNAc for that structure, shown in spectrum O. Note that this fragmentation is consistent with a four-linkage and provides a clean separation and a distinctly different spectrum than the three-linked LacNAc shown in spectrum K. Isolation of the branched LacNAc, m/z 458, in spectrum P is consistent with a four-linkage.

Direct interrogation of the internal LacNAc of the long linear (singly-branched) structure with the internal fuc-LacNAc required yet another fragment pathway, shown in spectra Q and R, which were consistent with a four-linkage with the extension attached at the three position. This spectrum is of marginal quality due to sensitivity limitations, but is sufficient to provide a matching score with the 3-position substituted lactosamine standard.



HMG 51, continued



HMG 54

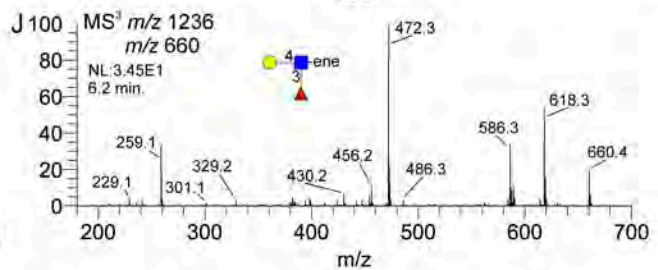
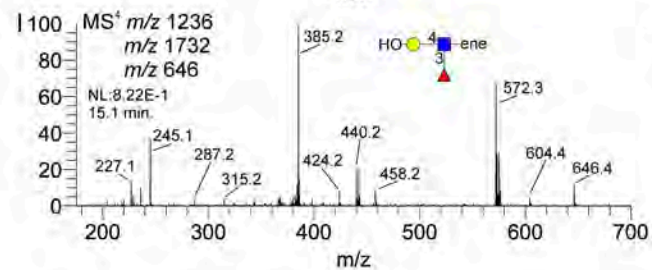
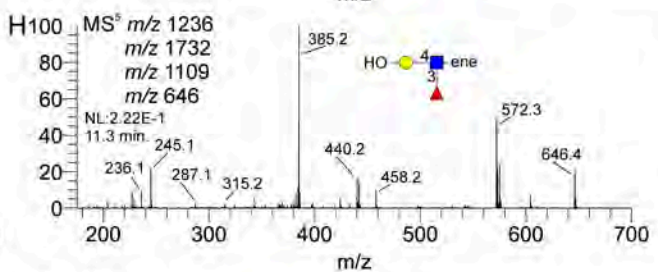
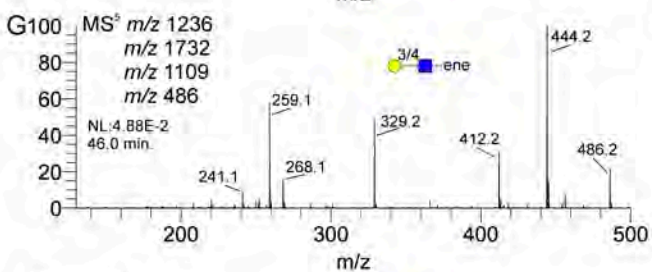
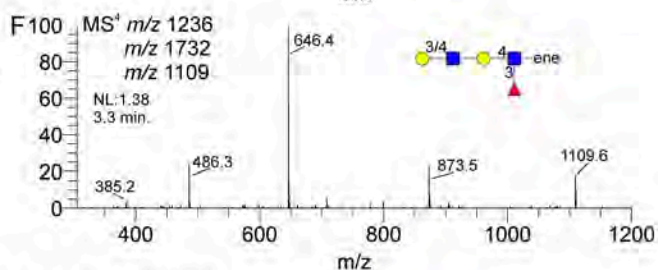
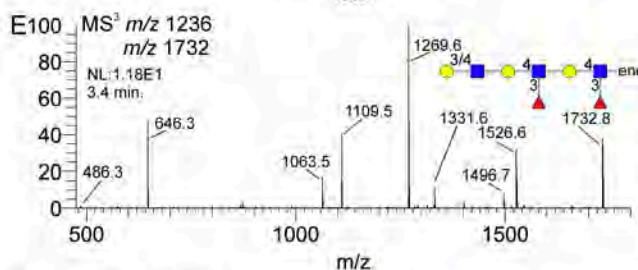
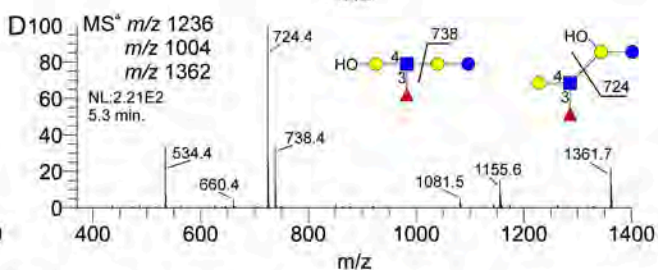
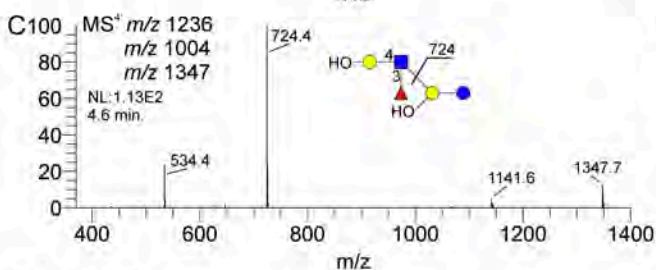
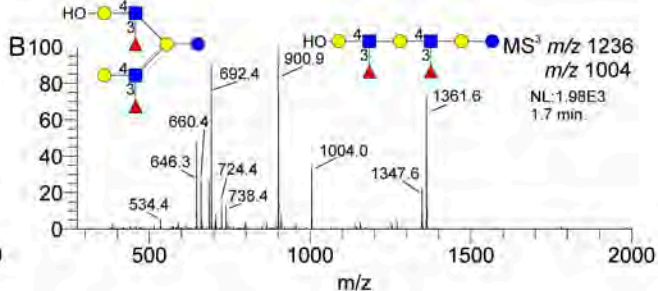
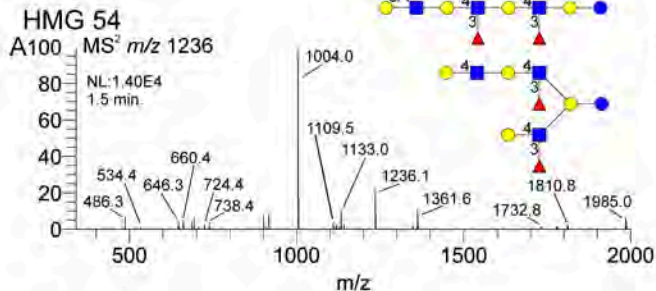
HMG 54 has the composition H₅N₃F₂. This sample contained several isomers. Spectra A, B, C, and D show disassembly pathways clarifying topology and branching. Both singly- and doubly-branched lactose cores are present. The doubly-branched lactose core isomer, shown in spectra A, B, and D, revealed the loss of a terminal LacNAc, internal fuc-LacNAc, and a terminal fuc-LacNAc leading to the m/z 724 doubly-branched AEAB-lactose core ion. The singly-branched core isomer is shown in spectra B, C, and D, through two fragmentation pathways showing losses of terminal LacNAc and two internal fuc-LacNAc fragments to lead to the singly-branched AEAB-lactose core fragment, m/z 738.

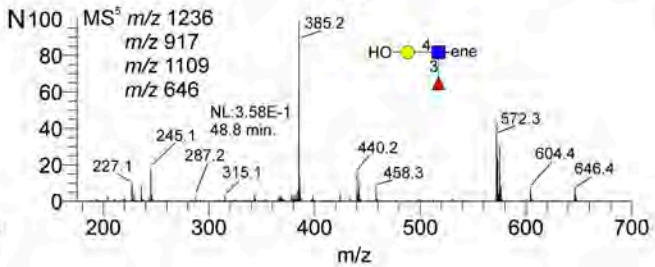
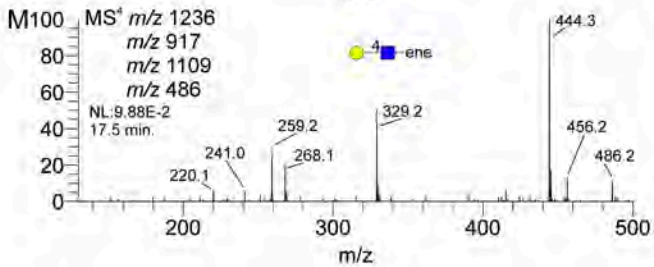
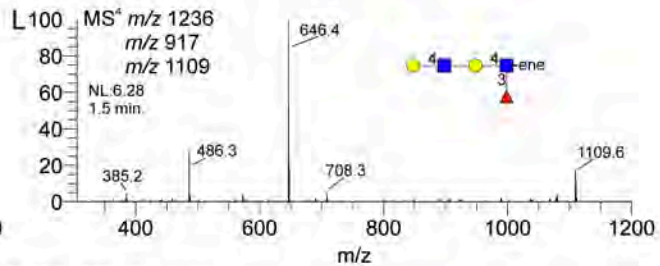
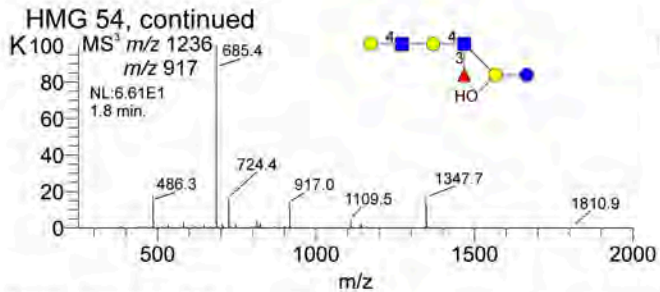
For the singly-branched core isomer, the complementary ion to the core is the m/z 1732 ion present in spectrum A. By complementary, the m/z 738 and m/z 1732 ions are formed by the breaking of a single bond in the precursor; taking the sum of the masses, and adjusting for charge state/adduct, yields the precursor m/z. Spectrum E shows the CID spectrum of the m/z 1732 ion, revealing the terminal LacNAc (m/z 486) and internal fuc-LacNAc (m/z 646), along with various combinations of these units. Interrogation of the fucosylated di-LacNAc (m/z 1109) fragment is shown in spectra F, G, and H; these data are consistent with an internal Lewis X and a mixture of three- and four-linked terminal LacNAc units. Spectrum I would be expected to represent both internal fuc-LacNAc units. The spectrum is consistent with an internal Lewis X, so both units are likely the same.

Spectrum J shows the terminal fuc-LacNAc spectrum; this fragmentation pattern is consistent with Lewis X. In order to isolate the fuc-diLacNAc fragment specifically from the doubly-branched core isomer, the m/z 917 fragment was isolated; this ion is formed by loss of a terminal fuc-LacNAc, a motif that the singly-branched core isomer does not have. Spectra K, L, M, and N show these data. Unlike the terminal LacNAc from the other isomer, this structure seems to contain only the four-linked LacNAc. The internal fuc-LacNAc again seems to be an internal Lewis X.

HMG 54 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
12-18-13_HMG54_1235_MS3_660_01.raw ms3 1236.00-661.00					0.285	0.568	0.964				0.725
12-18-13_HMG54_1235_MS4_1109_646_01.raw ms4 1236.00-1110.00-647.00				0.348				0.907		0.366	
12-18- 13_HMG54_1235_MS5_1732_1109_646_01.raw ms5 1236.00-1733.40-1110.00-647.00				0.326				0.911		0.447	





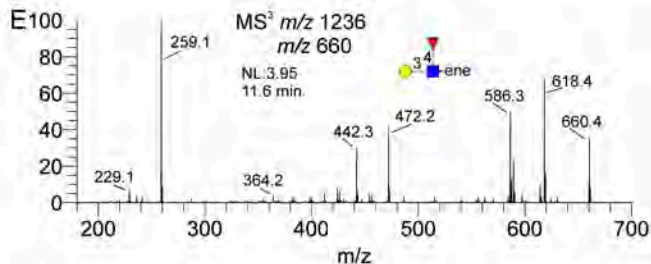
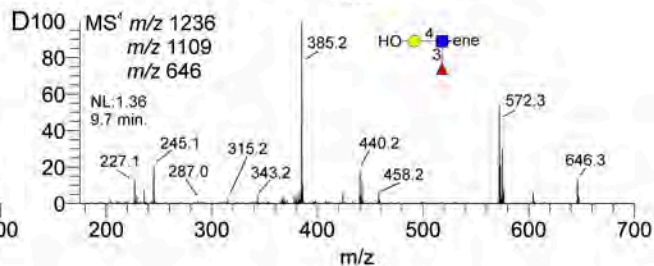
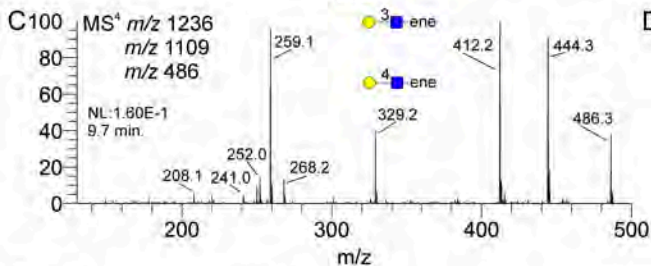
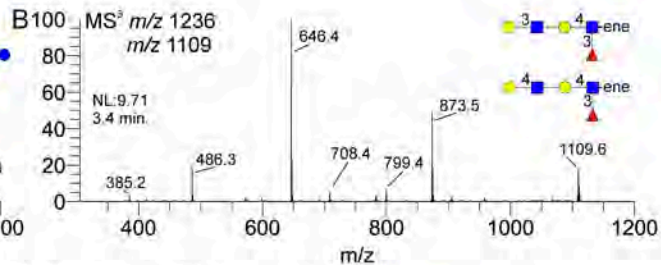
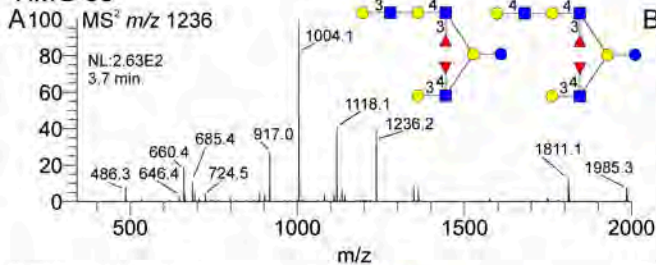
HMG 55

HMG 55 has the composition H₅N₃F₂. There are two isomers here, differing in the terminal LacNAc linkage. Both isomers have doubly-branched lactose cores with a terminal Lewis A on one arm, as indicated in spectrum E. The other arm is a monofucosylated diLacNAc with a terminal LacNAc and an internal Lewis X. Both three-linked and four-linked terminal LacNAc are present, as indicated by the mixture shown in spectrum C.

HMG 55 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
7-22-13_HMG55_1236_MS3_646_01.raw ms3 1236.10-647.00				0.355				0.938		0.425	
7-22-13_HMG55_1236_MS3_660_01.raw ms3 1236.10-661.00					0.258	0.869	0.797				0.549
7-22-13_HMG55_1236_MS4_1109_646_01.raw ms4 1236.10-1110.00-647.00				0.334				0.913		0.431	
7-22-13_HMG55_1236_MS5_917_686_646_01.raw ms5 1236.10-917.40-686.00-647.00				0.450				0.970		0.434	

HMG 55

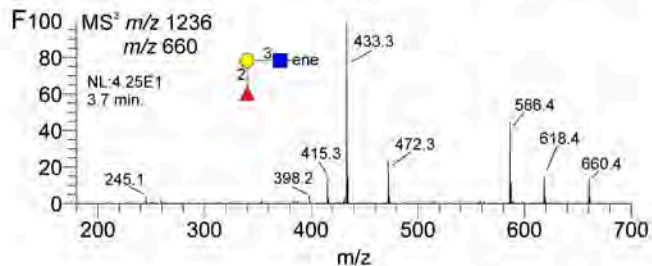
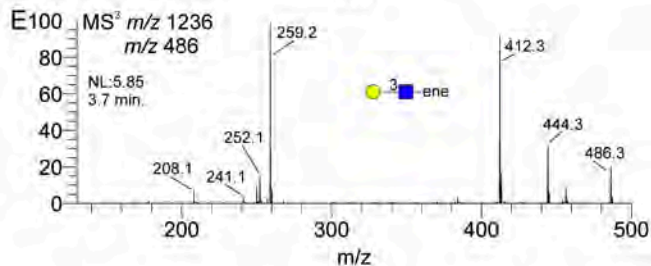
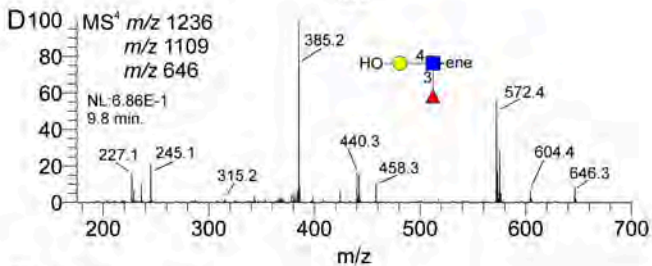
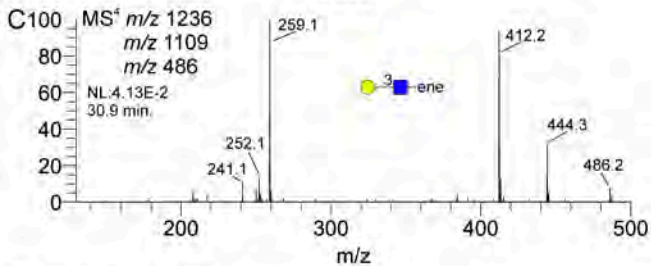
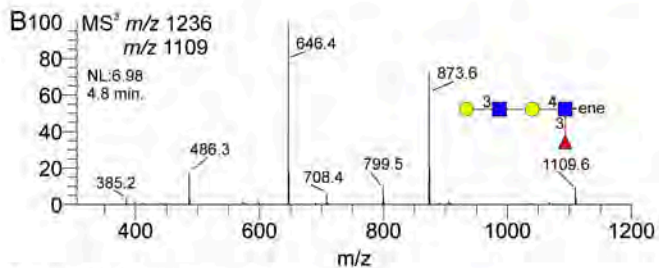
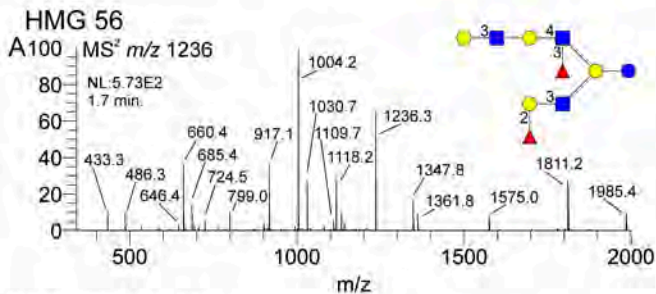


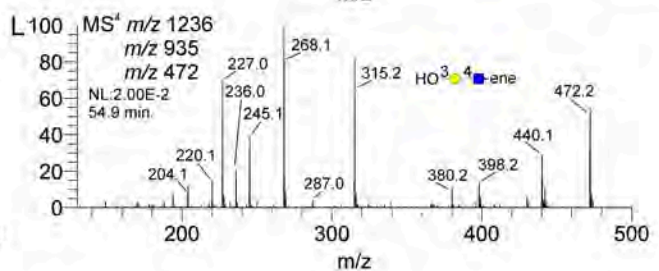
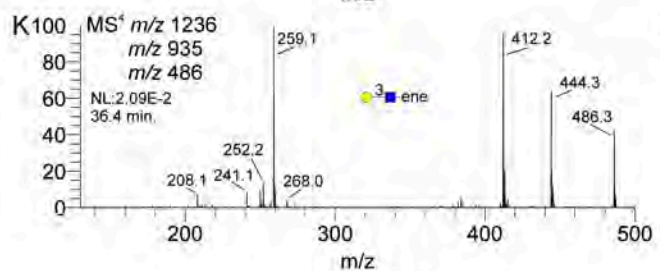
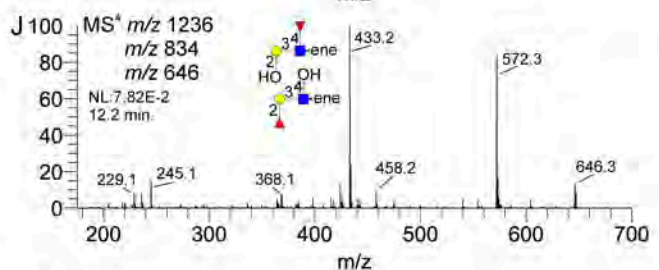
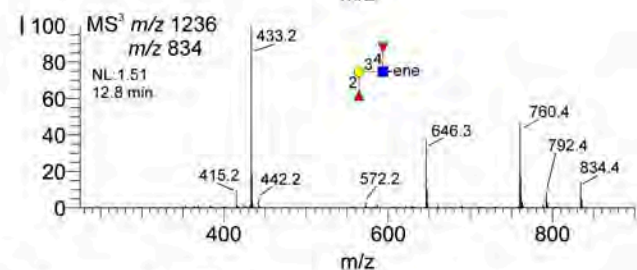
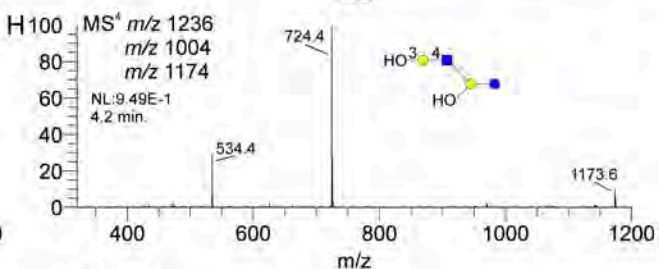
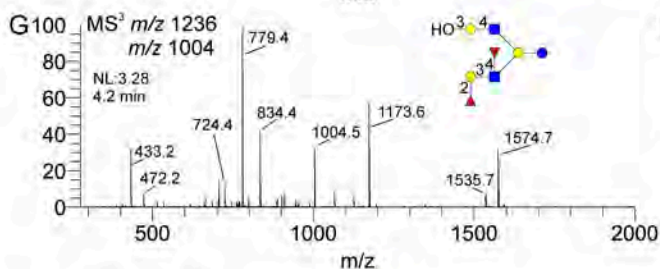
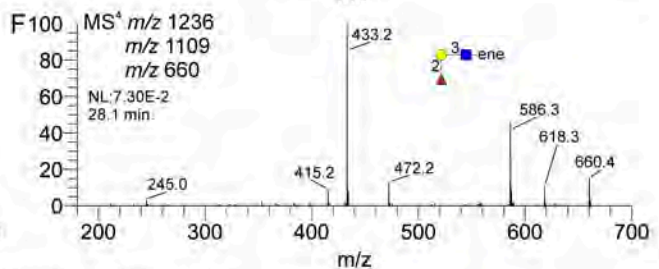
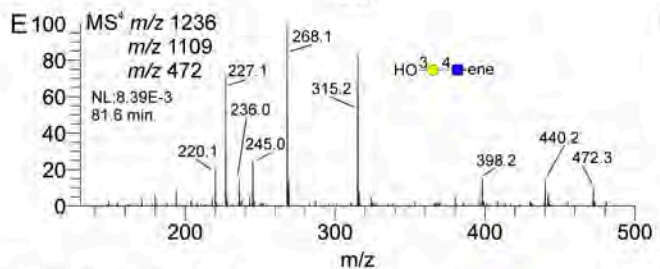
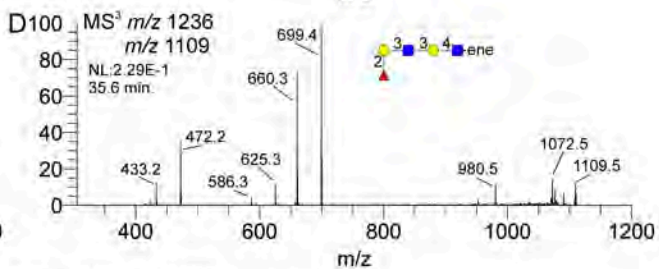
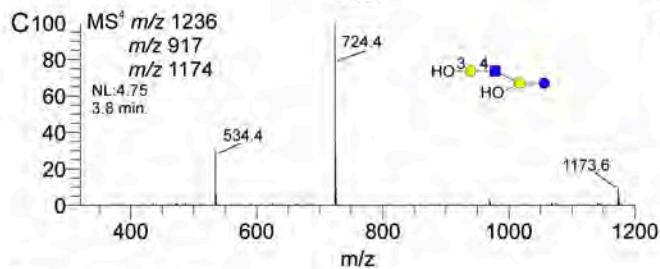
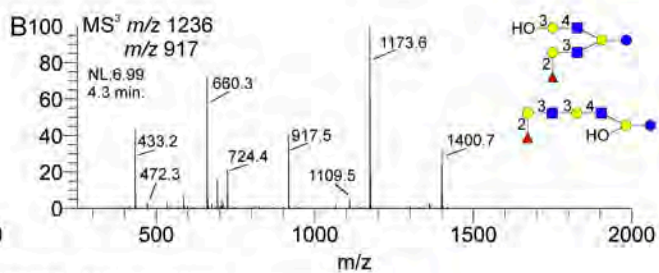
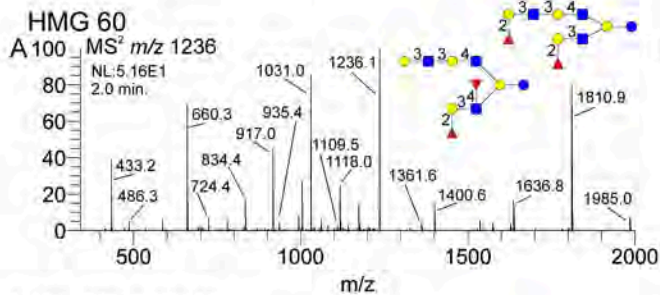
HMG 56

HMG 56 has the composition H5N3F2. This sample seems to contain a single structure, distinctly different from HMG 55. There is a doubly-branched lactose core, with a fuc-LacNAc on one arm and a fuc-diLacNAc on the other arm. The terminal fuc-LacNAc arm was determined to be an H1 structure, as indicated by the comparison of spectrum F to standard spectra. The fuc-diLacNAc arm was made up of terminal LacNAc and internal fuc-LacNAc units. The terminal LacNAc unit seemed to be exclusively three-linked, as shown in spectra C and E. The internal fuc-LacNAc was consistent with an internal Lewis X, as shown in spectrum D.

HMG 56 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
6-27-13_HMG56_1235_MS3_660_01.raw ms3 1236.00-661.00					0.995	0.355	0.381				0.293
6-27-13_HMG56_1235_MS4_1109_646_01.raw ms4 1236.00-1110.00-647.00				0.341				0.919		0.439	
6-27-13_HMG56_1235_MS4_1283_834_01.raw ms4 1236.00-1284.00-835.00			0.791						0.677		
6-27-13_HMG56_1235_MS5_1004_1348_646_01.raw ms5 1236.00-1004.80-1348.60-647.00				0.566				0.963		0.314	





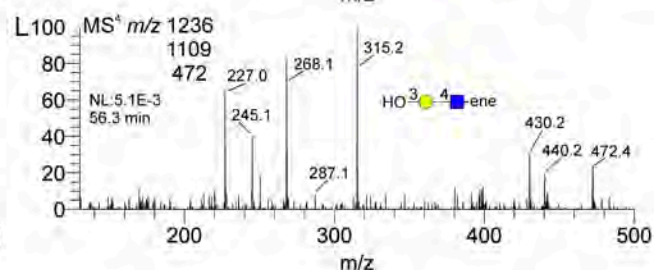
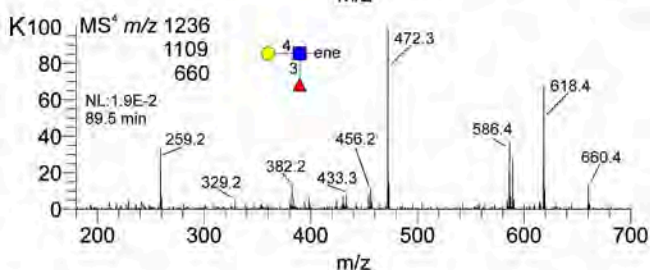
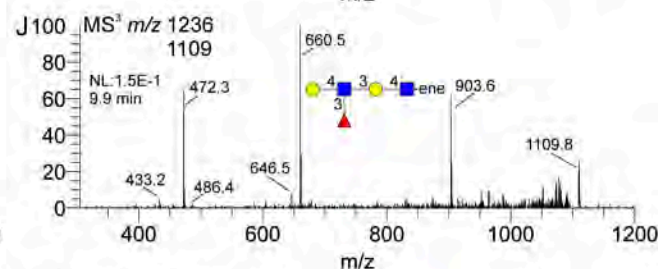
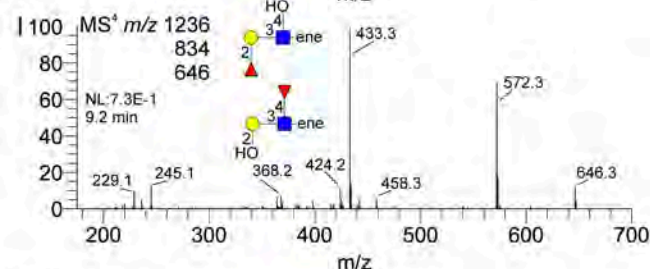
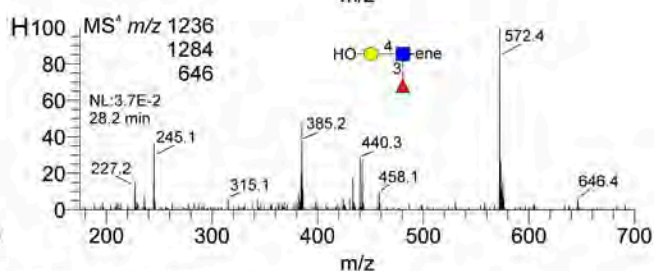
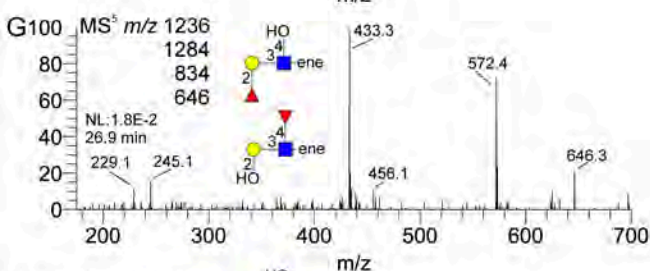
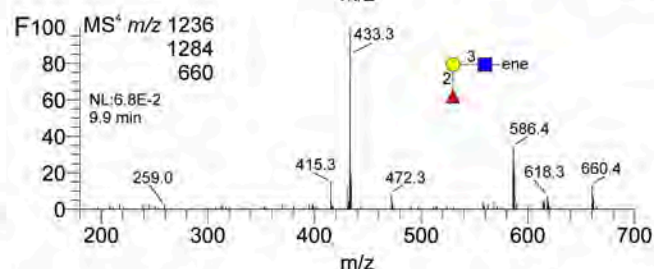
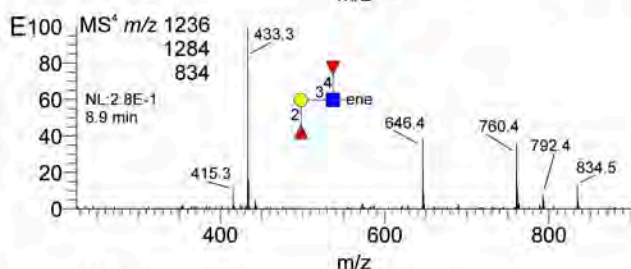
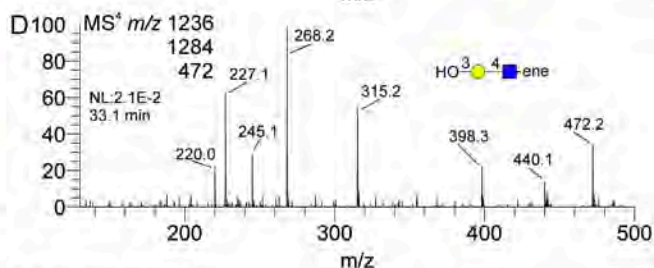
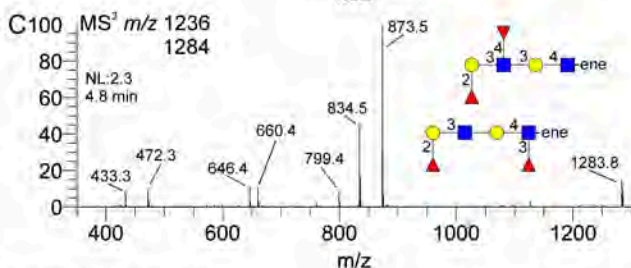
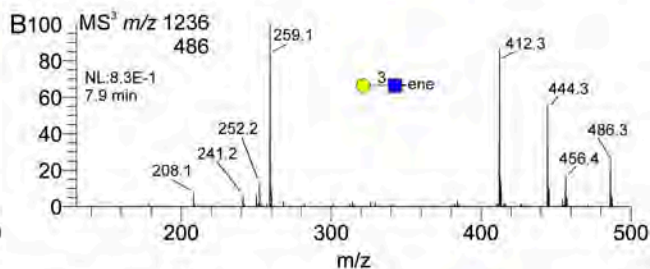
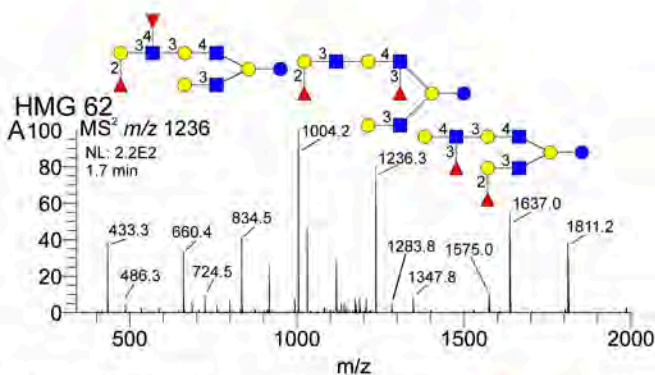
HMG 62

HMG 62 also has the composition H₅N₃F₂. There seem to be three isomers here, differing in fucose positions. The MS² spectrum (A) shows fragments consistent with terminal LacNAc (m/z 486), terminal fucosylated LacNAc (m/z 660), terminal difucosylated LacNAc (m/z 834), and a difucosylated diLacNAc (m/z 1283), among others. The terminal LacNAc MS³ spectrum (B) seems to be exclusively three-linked. Disassembly of the difucosylated diLacNAc, shown in spectrum C, shows two different isomers here. One has a terminal difucosylated LacNAc (m/z 834) with a complementary internal LacNAc (m/z 472); the other has a terminal fuc-LacNAc (m/z 660) complemented with an internal fuc-LacNAc (m/z 646). Further interrogation (spectra D-H) show an isomer with a terminal Lewis B attached to a four-linked internal LacNAc and another isomer with a terminal H1 attached to an internal Lewis X. Since the only core fragment mass detected was the doubly-branched AEAB-lactose, m/z 724, these structures would both likely contain the three-linked terminal LacNAc on the other arm.

While these two isomers seem to be most abundant, there was a small but detectable monofucosylated-diLacNAc ion, m/z 1109, which was abundant enough to isolate and fragment. The MS⁴ spectra are shown in K and L, and indicate an arm with a terminal Lewis X and a four-linked internal LacNAc. The terminal fucosylated LacNAc, which would be the other arm, would be a component of an MS³ spectrum of the m/z 660 ion. This spectrum, not shown, was identical to that of H1.

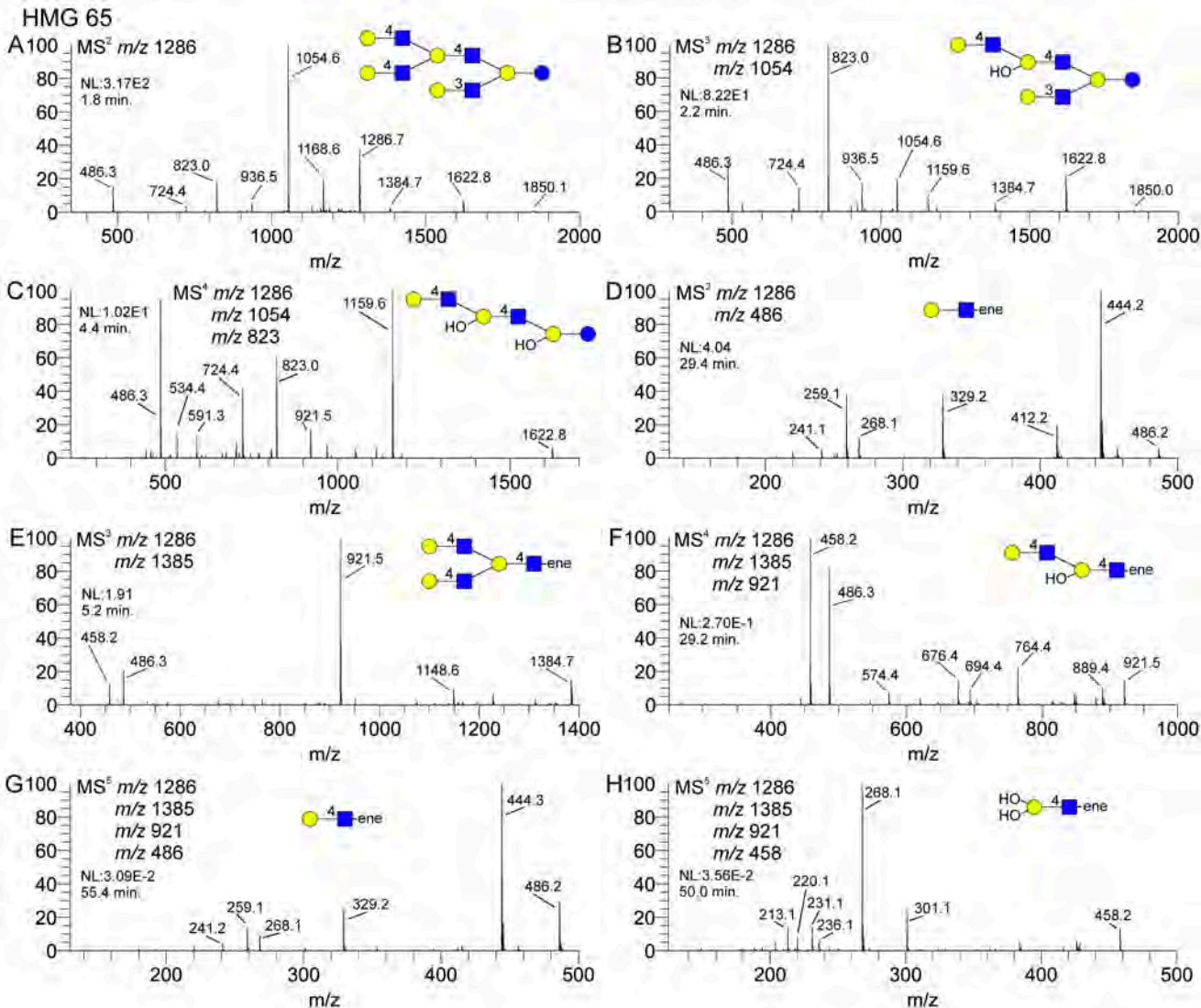
HMG 62 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
6-28-13_HMG62_1235_MS3_660_01.raw ms3 1236.50-661.00					0.990	0.318	0.264				0.182
6-28-13_HMG62_1235_MS3_834_01.raw ms3 1236.50-835.00			0.941						0.802		
6-28-13_HMG62_1235_MS4_1109_472_01.raw ms4 1236.50-1110.40-473.00	0.882	0.713									
6-28-13_HMG62_1235_MS4_1109_660_01.raw ms4 1236.50-1110.40-661.00					0.332	0.589	0.939				0.744
6-28-13_HMG62_1235_MS4_1283_646_01.raw ms4 1236.50-1284.40-647.00				0.582				0.950		0.346	
6-28-13_HMG62_1235_MS4_1283_646_02.raw ms4 1236.50-1284.40-647.00				0.638				0.948		0.317	
6-28-13_HMG62_1235_MS4_1283_660_01.raw ms4 1236.50-1284.40-661.00					0.967	0.291	0.251				0.201
6-28-13_HMG62_1235_MS4_1284_834_01.raw ms4 1236.50-1284.40-835.00			0.954						0.896		
6-28-13_HMG62_1235_MS4_834_646_01.raw ms4 1236.50-835.00-647.00				0.993				0.480		0.223	
6-28-13_HMG62_1235_MS5_1284_472_01.raw ms4 1236.50-1284.40-473.00	0.908	0.568									
6-28-13_HMG62_1235_MS5_1284_834_646_01.raw ms5 1236.50-1284.40-835.00-647.00				0.958				0.499		0.230	



HMG 65

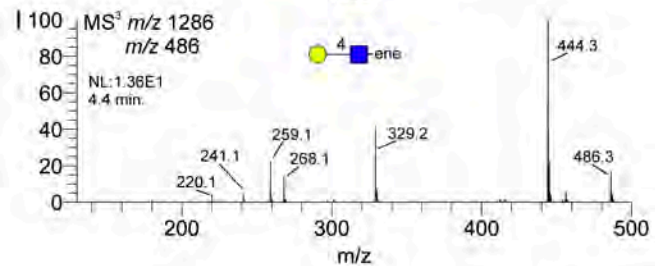
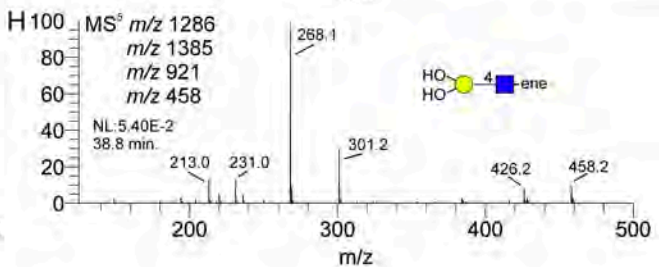
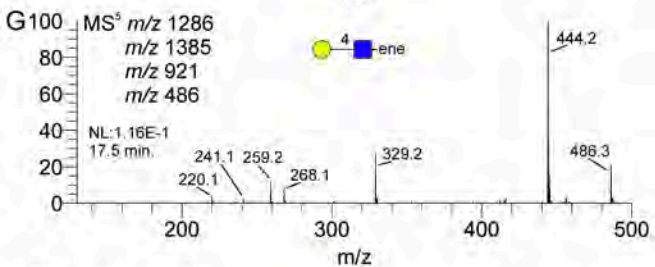
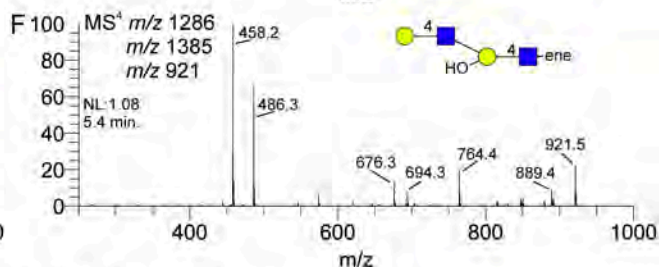
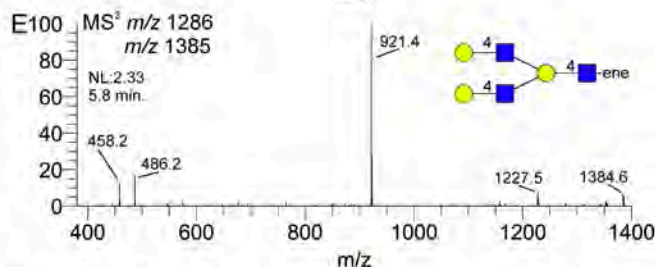
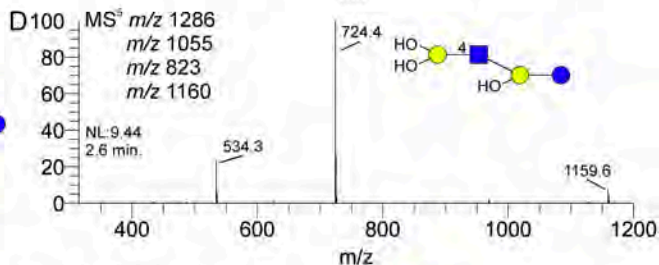
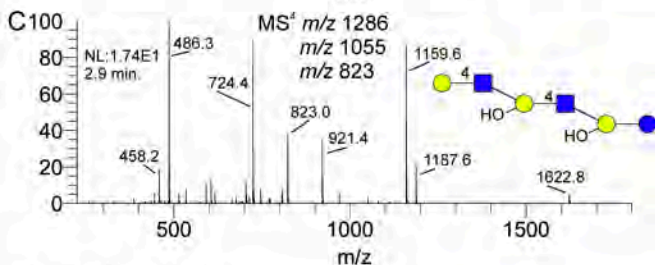
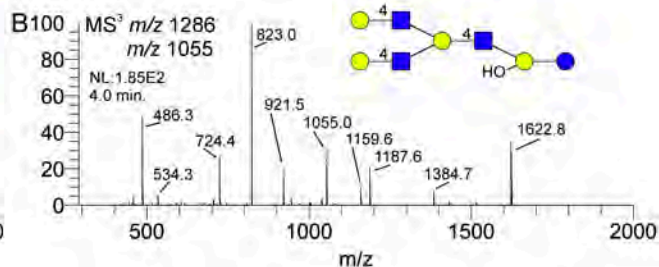
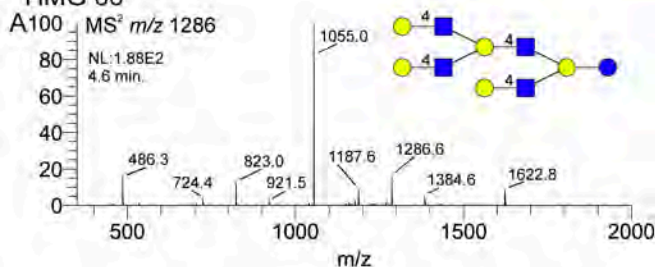
HMG 65 has the composition H₆N₄. This structure seems to have two arms, a single LacNAc and a triLacNAc, m/z 1384 in spectrum A. Spectrum D, MS³ m/z 486, indicates that this structure seems to contain a mixture of three- and four-linked terminal LacNAc units. Isolating the triLacNAc fragment, spectra E-H, shows the branching motif, with terminal LacNAc (m/z 486) and doubly-branched LacNAc (m/z 458) units. Spectrum G indicates that the terminal LacNAc units of the branched arm seem to be exclusively four-linked. This suggests that the three-linked LacNAc detected in spectrum D is probably located on the single LacNAc arm.



HMG 66

HMG 66 is another decaose, H₆N₄, isomer. The branching and topology seem to be similar to HMG 65. This structure seems to have no detectable three-linked terminal LacNAc units, as spectra G and I seem to be exclusively four-linked.

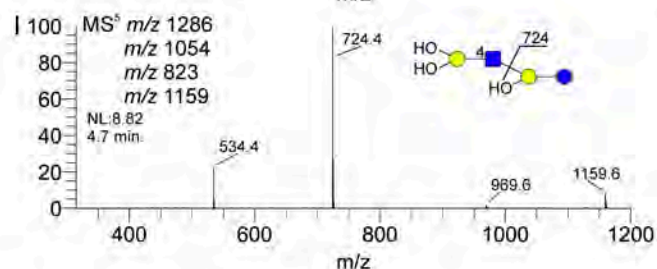
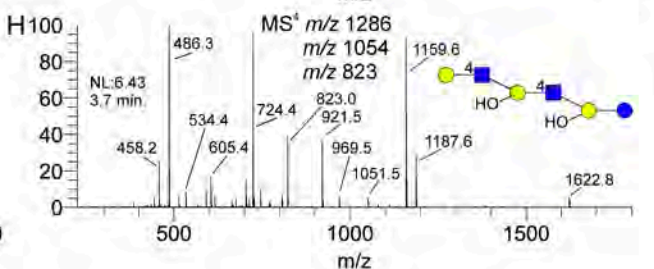
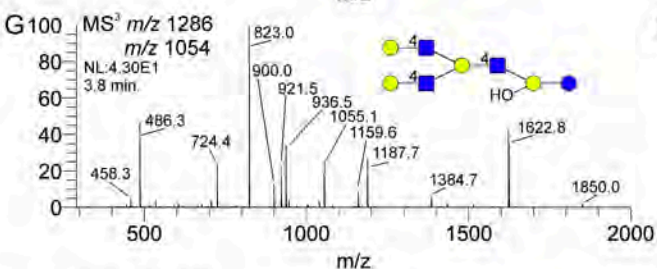
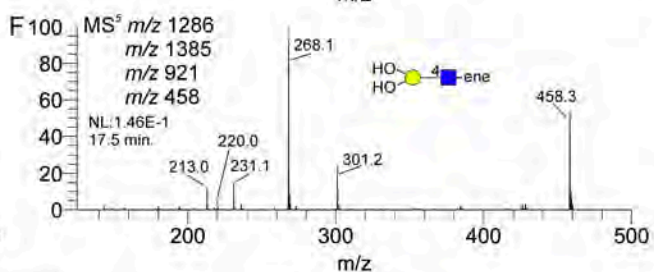
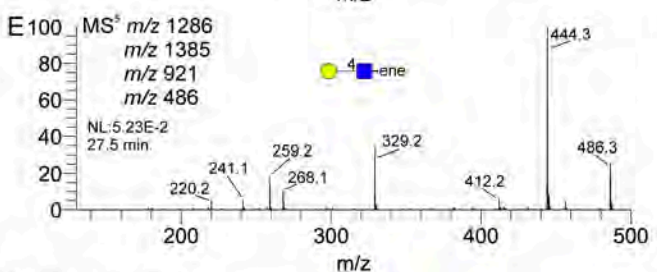
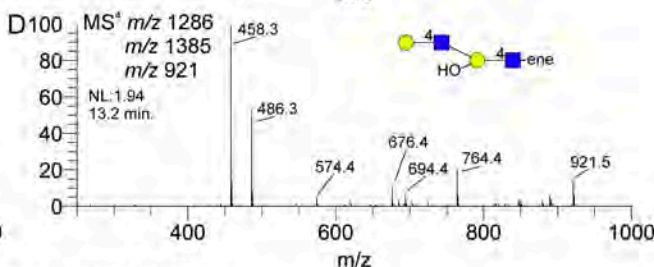
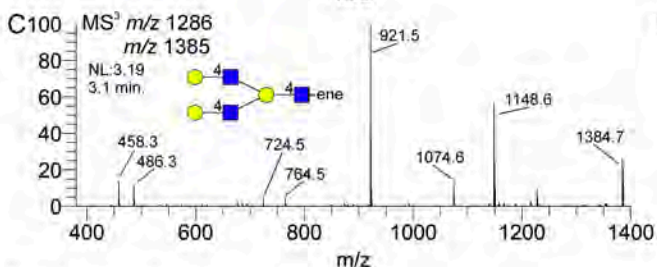
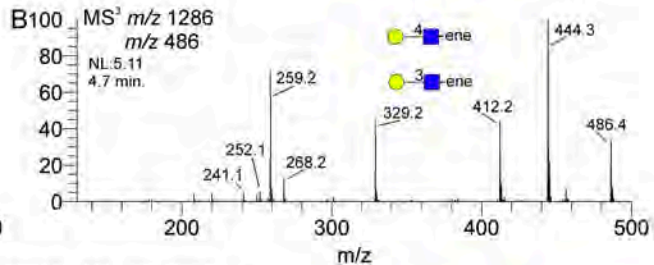
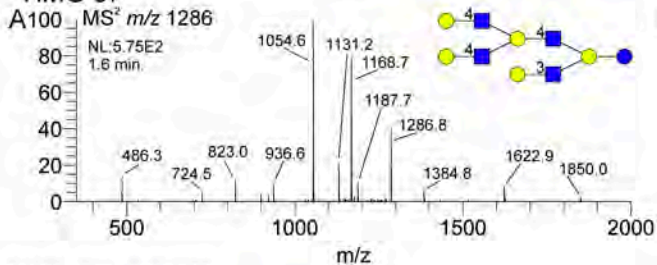
HMG 66



HMG 67

HMG 67 has the composition H₆N₄. The MSⁿ data suggest a similar branching pattern as HMGs 65 and 66, with some differences in terminal LacNAc linkages and distribution. The branched arm of HMG 67 seems to contain predominantly four-linked terminal LacNAc, though there is a detectable amount of three-linked LacNAc on this arm, as shown in spectrum E. The proportion of three-linked LacNAc here seems to be much lower than in the MS³ spectrum (B) suggesting that most of the three-linked LacNAc is on the single-LacNAc arm.

HMG 67



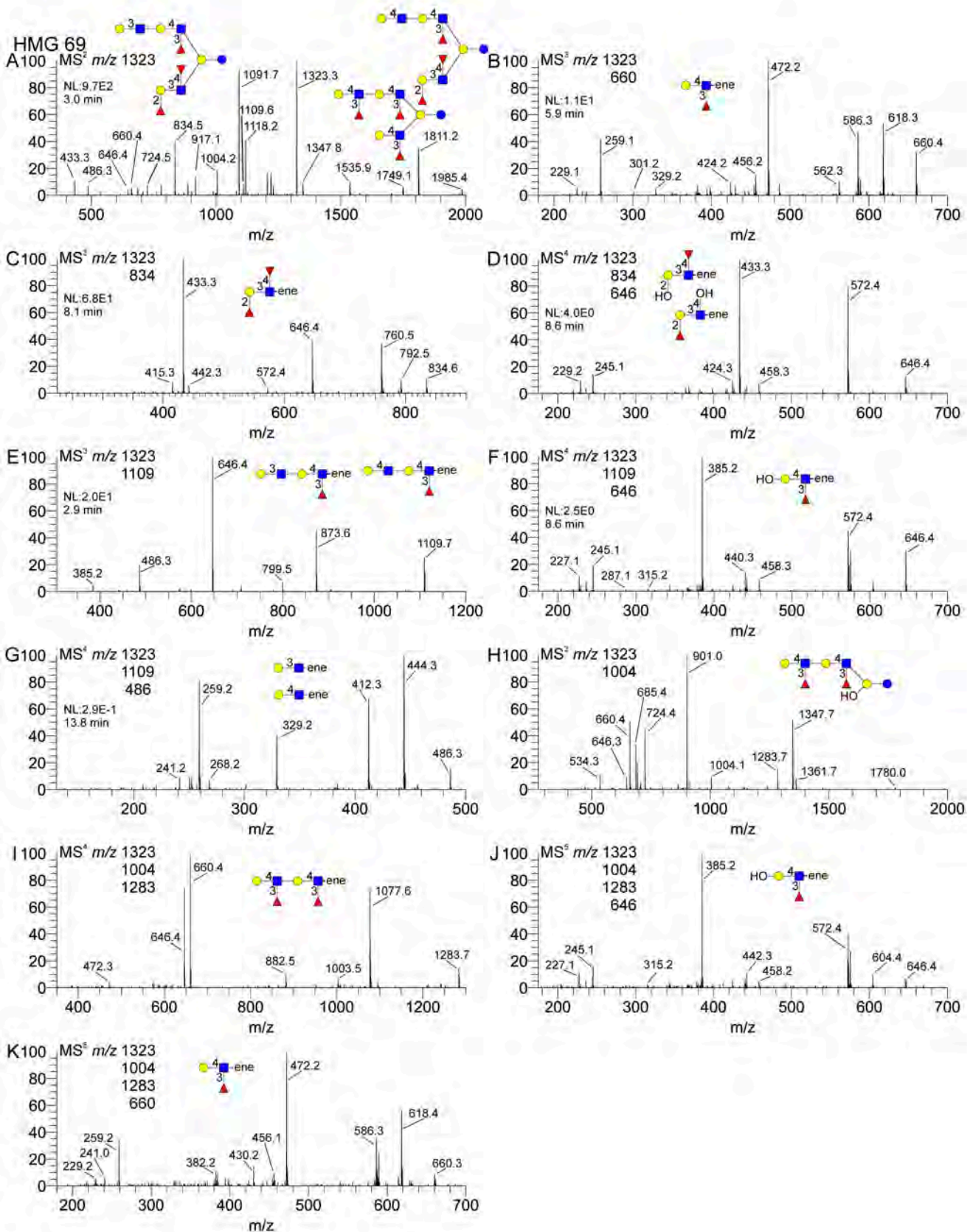
HMG 69

HMG 69 has the composition H₅N₃F₃. This sample seems to have three isomers, differing in fucose localization and terminal LacNAc linkage. All structures in this sample have doubly-branched lactose cores, as evidenced by the m/z 724 AEAB-lactose ion. The terminal monofucosylated LacNAc, m/z 660 is consistent with Lewis X, as shown in spectrum B. The terminal difucosylated LacNAc, m/z 834, is consistent with Lewis B, as shown in spectra C and D. The singly fucosylated diLacNAc arm, m/z 1109, is composed of a terminal LacNAc and an internal fuc-LacNAc, as shown in spectra E, F, and G. This arm does not seem to have a terminal fuc-LacNAc unit, since there is no detectable m/z 660 ion in spectrum E. The internal fuc-LacNAc is consistent with an internal Lewis X. The terminal LacNAc seems to be a mixture of three- and four-linked LacNAc units. Based on the composition of this sample, the singly-fucosylated diLacNAc arm would be coupled with a difuc-LacNAc unit on the other arm, both coupled to the usual doubly-branched lactose core.

Spectrum H shows the CID spectrum of the m/z 1004 ion, formed by loss of a terminal fuc-LacNAc unit. At this stage, we can see a doubly-fucosylated diLacNAc fragment, m/z 1283. Disassembly reveals a structure with a single fucose on each LacNAc, consisting of a terminal Lewis X and an internal Lewis X, as shown in spectra I, J, and K.

HMG 69 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472 3-LN	472 6-LN	834 Leb	646 Leb	660 H1	660 Lea	660 Lex	646 Slex	834 Ley	646 Ley	660 H2
7-22-13_HMG69_1323_MS3_834_01.raw ms3 1323.00-835.00			0.983						0.897		
7-22-13_HMG69_1323_MS4_1109_646_01.raw ms4 1323.00-1110.00-647.00				0.300				0.882		0.443	
7-22-13_HMG69_1323_MS4_834_646_01.raw ms4 1323.00-835.00-647.00				0.985				0.526		0.233	
7-23-13_HMG69_1323_MS3_660_01.raw ms3 1323.00-661.00					0.328	0.651	0.980				0.666
7-23-13_HMG69_1323_MS4_1004_646_01.raw ms4 1323.00-1004.50-647.00				0.320				0.900		0.429	
7-23-13_HMG69_1323_MS4_1004_660_01.raw ms4 1323.00-1004.50-661.00					0.286	0.563	0.962				0.669
7-23-13_HMG69_1323_MS5_1004_1283_646_01.raw ms5 1323.00-1004.50-1284.00-647.00				0.304				0.882		0.431	
7-23-13_HMG69_1323_MS5_1004_1283_646_02.raw ms5 1323.00-1004.50-1284.00-647.00				0.269				0.856		0.454	
7-23-13_HMG69_1323_MS5_1004_1283_660_01.raw ms5 1323.00-1004.50-1284.00-661.00					0.279	0.596	0.953				0.723
7-23-13_HMG69_1323_MS5_1004_1283_660_02.raw ms5 1323.00-1004.50-1284.00-661.00					0.281	0.592	0.946				0.694



HMG 76

HMG 76 has the composition H₆N₄F₂. This structure is doubly-branched with each branch being a singly-fucosylated diLacNAc. One can follow a disassembly pathway where units are successively lost as two terminal LacNAc units and two internal fucosylated LacNAc units leading to the doubly-branched lactose core. Spectra B and C show part of one possible fragmentation pathway; spectrum H shows the final spectrum of an alternative pathway. These differ in the chosen charge state of the precursor, but the units lost are the same. Isolation and fragmentation of the singly-fucosylated diLacNAc arms, shown in spectra E-G, indicate a terminal LacNAc and an internal fuc-LacNAc. The terminal LacNAc shows a mixture of three- and four-linked LacNAc; the internal fuc-LacNAc is an internal Lewis X.

HMG 76 MSⁿ spectral matching scores

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
	472	472	834	646	660	660	660	646	834	646	660
	3-LN	6-LN	Leb	Leb	H1	Lea	Lex	Slex	Ley	Ley	H2
10-4-13_HMG76_981_MS3_660_01.raw ms3 981.80-661.00					0.548	0.402	0.385				0.255
10-4-13_HMG76_981_MS3_660_02.raw ms3 981.80-661.00					0.592	0.424	0.448				0.319
10-4-13_HMG76_981_MS5_827_997_646_01.raw ms5 981.80-827.40-997.60-647.00				0.321				0.901		0.445	
10-4-13_HMG76_981_MS6_827_997_894_646_01.raw ms6 981.80-827.40-997.60-895.00-647.00				0.347				0.919		0.439	
10-4- 13_HMG76_981_MS7_827_997_894_1348_646_01.raw ms7 981.80-827.40-997.60-895.00-1348.20-647.00				0.006				0.010		0.004	
7-23-13_HMG76_981_MS4_1109_646_01.raw ms4 981.80-1109.60-647.00				0.362				0.918		0.430	
7-23-13_HMG76_981_MS5_1229_997_646_01.raw ms5 981.80-1229.00-997.50-647.00				0.256				0.861		0.439	
7-23-13_HMG76_981_MS5_1229_997_646_02.raw ms5 981.80-1229.00-997.50-647.00				0.337				0.916		0.440	

