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Accelerated Articles

Alkaline Degradation of Oligosaccharides Coupled with Matrix-Assisted Laser Desorption/Ionization Fourier Transform Mass Spectrometry: A Method for Sequencing Oligosaccharides

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A new technique for determining sequence and linkage information of underivatized oligosaccharides is developed using alkaline degradation and matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS). Alkaline degradation (also known as the "peeling" reaction) is a chemical degradation technique that only cleaves the glycosidic bond at the reducing end by β-elimination to yield a new reducing end. The reaction products are sampled directly with minimal cleanup and monitored by MALDI-FTMS to elucidate the oligosaccharide sequence. Linkage information is provided by cross-ring cleavage fragmentation of the new reducing ends, created by either MALDI source fragmentation or sustained off-resonance irradiation collision-induced dissociation. This method is illustrated by the successful sequence and linkage determination of neutral, branched, fucosylated, and sialylated oligosaccharides. Experiments on differently linked disaccharides are also performed to determine the specificity of the cross-ring cleavage reactions. The power of this technique is enhanced by the Fourier transform mass analyzer, which provides high-resolution, exact mass, and facile tandem mass spectrometry experiments of MALDI-produced ions.

Oligosaccharides have many diverse functions in biological systems. Some examples are aiding in the conformation and stability of proteins, modulating the functions of proteins, acting as target structures for microorganisms, toxins, and antibodies, serving as ligands for specific binding events that mediate protein targeting, and acting as ligands for cell−matrix or cell−cell interactions.[1] The majority of oligosaccharide functions in biological systems are mediated by the carbohydrate's primary structure.[2] The ability to sequence oligosaccharides is of utmost importance for determining the structure/function relationship in biological systems. Sequencing methods need to resolve slight variations in structure that can cause major alterations in biological function. Unfortunately, the determination of oligosaccharide sequences presents problems that are more difficult than those commonly encountered in protein sequencing. Because of the variety of monomers, the different linkages between them, and the anomeric carbon configuration, there is no single method like Edman degradation for peptides[3] that has been devised for oligosaccharide sequencing.

In recent years, mass spectrometry has increasingly become a key tool for the characterization of oligosaccharides.[4−7] This is the result of the invention of novel ionization techniques such as electrospray ionization[8] (ESI) and matrix-assisted laser desorption/ionization[9] (MALDI) that allows the production of ions from large, thermally labile oligosaccharides. These ionization methods also

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allow detection of femtomolar amounts of material.[10−13]

Since these ionization techniques produce minimal fragmentation, the oligosaccharide ions must be activated to produce fragments and obtain sequence information. This is typically achieved using collision-induced dissociation[14−18] (CID) or in the case of MALDI-time-of-flight, postsource decay[19,20] (PSD). However, there are limitations in current activation techniques that complicate sequence and linkage assignments in carbohydrates. The first is nonselective glycosidic bond cleavage, such as loss of fucose or *N*-acetylneuraminic acid (sialic acid). This makes it difficult to obtain a complete sequence when either of the two monosaccharides is present. The second is the lack of cross-ring cleavages in many component monosaccharides. Cross-ring cleavages occur most abundantly at the reducing end of the oligosaccharide. Linkage determination is limited to only those residues having undergone cross-ring cleavage.

To fully sequence oligosaccharides with mass spectrometry, a combination of mass spectrometry and chemical degradation is necessary. Currently, there are a variety of carbohydrate degradation methods. Enzymatic degradation is promising for the characterization of oligosaccharides.[21] It offers very specific glycosidic bond cleavage that provide the full sequence, linkage, and configuration of the anomeric carbon. Unfortunately, enzymatic techniques are hampered by expensive reagents, the highly specific nature of the reactions, and the tedious and extensive analyte purification needed for MALDI.[22,23] There are many chemical degradation techniques, such as acid hydrolysis,[24] methanolysis of permethylated oligosaccharides,[25,26] periodate oxidation, reduction, and methylation (ORM),[5,18,25,26] and chromium trioxide,[27] that have been used in conjunction with mass spectrometry. Sequence, linkages, and even configuration at the anomeric carbon are obtained. Recently, Nakamura and coworkers developed a new chemical sequential degradation analysis of reducing oligosaccharides using 8-aminonaphthalenesulfonic acid coupled to HPLC.[28] With this technique, the authors were able to sequence small linear oligosaccharides. Due to the great variation in oligosaccharide structures not one general analytical method has emerged. Instead it has become necessary to have an arsenal of tools for analysis.

In this work, we create a new method for sequencing oligosaccharides by coupling alkaline degradation (AD) with matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS). Alkaline degradation of oligosaccharides is a very old reaction and is commonly known as the "peeling" reaction. Alkaline degradation has been used as a degradation method for mono-, oligo-, and polysaccharide analyses for over 80 years.[29] It involves the treatment of carbohydrates with a strong base, usually NaOH, at somewhat elevated temperatures. The reaction successively dissociates the monosaccharide units starting with the reducing end.[29−31] Alkaline degradation is also used to liberate oligosaccharides from proteins, but the reaction is often intercepted with sodium borohydride reduction to form an alditol at the reducing end.[32] Ironically, most biochemists working with oligosaccharides try to avoid this reaction.

The sequential removal of monosaccharides from reducing end oligosaccharides in alkaline solution proceeds through a β-elimination reaction. The products of the reaction are aldonic acids and the nascent reducing end oligosaccharide.[24] The aldonic acid products released during the degradation reaction were the species that were previously analyzed and have themselves been widely studied.[29,33] The interest in the aldonic acids rather than the remaining carbohydrate was due to the limitation of the analytical methods of the time. Instead, we analyze the prevailing oligosaccharide by mass spectrometry. Despite the considerable amount of work on alkaline degradation reactions, it has not been used in direct conjunction with MALDI-MS analysis to the best of our knowledge.

MALDI-FTMS represents a promising new technique for the analysis of oligosaccharides.[17,34−36] This MS technique offers several capabilities that make it highly suitable for evaluating alkaline degradation products. The accurate mass capability[37] facilitates the identification of fragment ions from the possibly large amount of chemical noise due to impurities and byproducts. FTMS possesses features such as ease of ion isolation and multiple MS stages that are temporally separated,[17,38,39] providing ample

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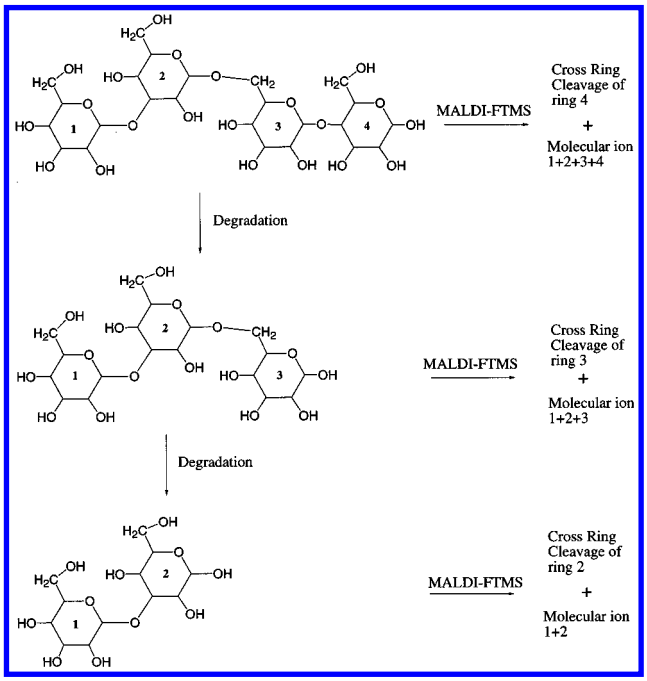
Scheme 1



opportunities to produce cross-ring cleavages. Furthermore, the high sensitivity of the method is valuable for nanoscale applications as sample availability is often a limitation.

For the analysis, it is important that the reaction reveals a nascent reducing ring so that MALDI and CID can be employed to produce cross-ring cleavages. The reaction is allowed to progress until every monosaccharide residue within the oligosaccharide becomes a reducing ring that can be fragmented by MALDI or CID, providing complete linkage information. For example, in the hypothetical tetrasaccharide shown in Scheme 1, the glycosidic bond of the reducing sugar 4 is cleaved first to yield a new reducing sugar 3. MALDI, and if necessary, CID, is then used to promote cross-ring cleavages at the nascent reducing ring to yield the linkage between residue 3 and residue 2. By allowing the reaction to continue, the oligosaccharide is fully sequenced with all linkage information obtained. Moreover, the degradation method is nonselective, allowing linkage and sequence determination of even sialylated and fucosylated oligosaccharides.

EXPERIMENTAL SECTION

Reagents. All disaccharides were acquired from Sigma Chemical Co. (St. Louis, MO). The larger oligosaccharides were obtained from Oxford Glycosystems (Oxford, U.K.). 2, 5-Dihydroxybenzoic acid (DHB) and granular NaOH were purchased from Aldrich Chemical Co. (Milwaukee, WI). All materials were used without purification.

Alkaline Degradation. The procedure used for the alkaline degradation of oligosaccharides is as follows. To 10 μL of a 0.1−1 mg/mL solution of oligosaccharide dissolved in water, 10 μL of 0.01 M NaOH was added in a Teflon-capped, 0.1-mL glass reaction vial. Conditions were optimized to find the best NaOH concentration. Lower concentrations peeled too slowly while higher concentrations were detrimental to MALDI. The reaction vial was placed in a constant temperature aluminum heating block at approximately 60 °C for a period lasting between 3 and 24 h.

Matrix-Assisted Laser Desorption/Ionization. Mass spectrometry was performed with an external source Fourier transform mass spectrometer (HiResMALDI, IonSpec Corp., Irvine, CA) equipped with a 4.7-T magnet and a 337-nm nitrogen laser. The instrumental conditions for the analysis of oligosaccharides have been described in detail in an earlier publication.[40] DHB was used for all analyses at a concentration of 50 mg/mL in ethanol. MALDI samples were prepared directly from the reaction mixture. A 2-μL aliquot of solution was obtained from the reaction vial and placed into a 0.5-mL polypropylene microtube. The sample was acidified to a pH of approximately 5 by adding 2 μL of 0.01 M HCl. Acidification was performed for two reasons: first, to arrest the peeling reaction and, second, to enhance the MS intensities. DHB produced poor signals when crystallized with the analyte under basic solutions. At pH 5, the solution is not too acidic to induce acid hydrolysis. The resulting 4-μL acidified solution was deposited onto the probe tip and the solvent was evaporated with a warm stream of air. The sample was then redissolved on the probe tip with 2 μL of 50:50 H_2O/methanol. Finally, 1 μL of DHB was placed on the probe tip and the solution was evaporated by rapid drying with a stream of warm air.

Collision-Induced Dissociation. Sustained off-resonance irradiation collision-induced dissociation (SORI CID) was performed on nascent fragments that were liberated by alkaline degradation to obtain cross-ring cleavage information. MALDI-produced ions were first trapped and then translationally cooled by an argon pulse in the analyzer cell. The ion of interest was isolated using a series of radio frequency sweeps and bursts created by an arbitrary wave form generator (IonSpec Corp.). The translational energies of the ions were increased by applying a radio frequency pulse between 1.5 and 3.0 V base to peak at 1−2 kHz below the cyclotron frequency for a duration of 1 s. Two argon pulses, in 500-ms intervals, maintained the pressure in the analyzer cell at approximately 5×10^{-6} Torr during CID. A 3-s detect delay allowed the argon to be pumped out of the ICR cell to a base pressure of approximately 1×10^{-11} Torr. The entire CID experiment lasts 5 s. A total of 5−10 experiments were combined to produce the resulting spectra.

RESULTS AND DISCUSSION

Comparison of AD-MALDI, MALDI, and CID Fragmentation of Disaccharides. To demonstrate that cross-ring cleavages of specific linkages are distinct, a group of variably linked disaccharides were studied by subjecting them to the conditions of AD and recording their MALDI-FTMS spectra. We further show that cross-ring fragmentation is produced by MALDI or CID rather than during alkaline degradation. Figure 1 shows the mass spectra of β-D-GlcNAc(1→4)D-GlcNAc with (a) normal MALDI-FTMS conditions, (b) CID of $[M + Na]^+$ isolated from (a), and (c) MALDI-FTMS of the reaction mixture consisting of the disaccharide in 0.01 M NaOH, after a 24-h reaction period. There remain sufficient intensities of the quasi-molecular ion $[M + Na]^+$ and of monosaccharide products resulting from direct glycosidic bond cleavages (B_1 and C_1, Domon and Costello nomenclature for carbohydrate fragmentation is used[41]) to provide the sequence even after the long reaction period. Alkaline degradation does

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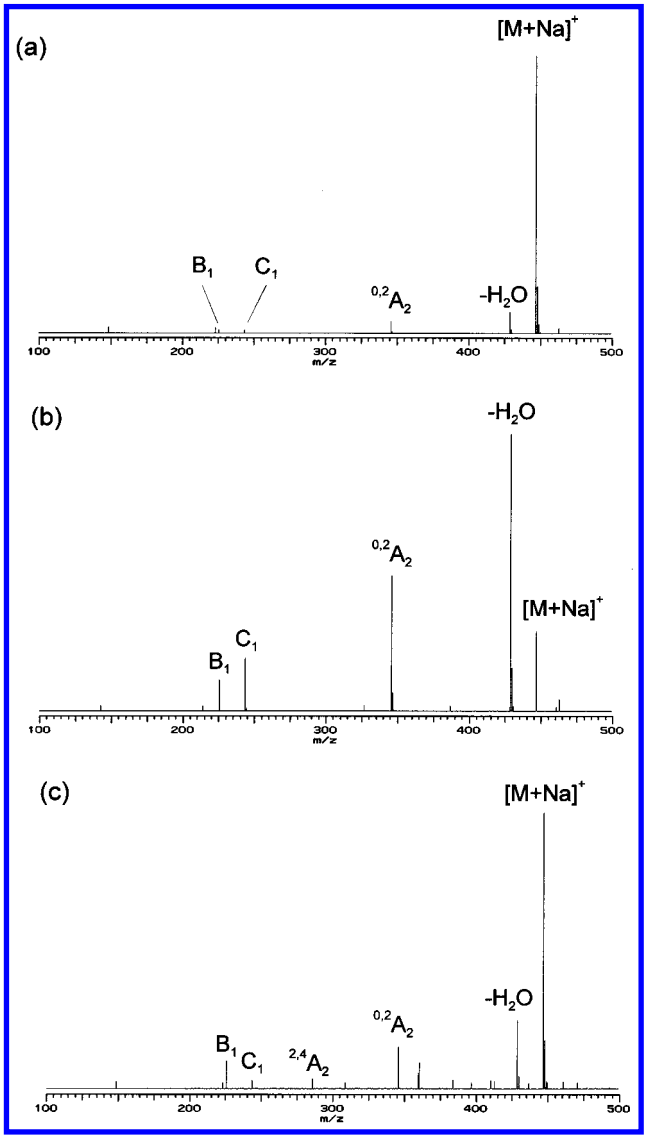


Figure 1. (a) MALDI-FTMS spectrum of untreated β-D-GlcNAc-(1→4)D-GlcNAc. (b) SORI CID of the quasi-molecular ion isolated from (a). (c) MALDI-FTMS spectrum of the disaccharide after 24 h of alkaline degradation. Reaction conditions do not appear to alter the MALDI spectrum. The smaller peaks are side products from the degradation reaction.

not proceed to completion due to the stable intermediates that form during the elimination reaction. This leads to the accumulation of nascent reducing end species in solution that can be analyzed simultaneously. Therefore, the analysis is not limited by having to determine precise reaction times. The quasi-molecular ion is the dominant peak, with the loss of water as the major fragment. The cross-ring cleavage products, $^{0,2}A_2$ (m/z 346.111) and $^{2,4}A_2$ (m/z 286.090), correspond to the dissociation of the reducing ring and are characteristic of the (1→4)-linkage. The minor peaks observed in Figure 1c are due to side reactions in the alkaline degradation. This chemical noise does not generally interfere with the analysis. Accurate masses can be determined (typically to 10 ppm) allowing rapid identification of fragment ions. (vida supra)

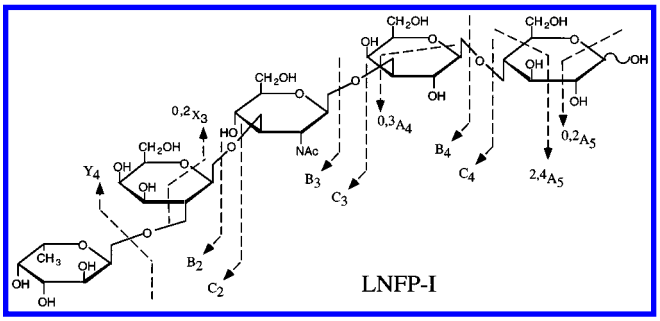
Table 1 summarizes the fragments observed with MALDI of untreated disaccharides (M), CID of the quasi-molecular ions (C), and MALDI of the corresponding alkaline degradation reaction

Table 1. Disaccharide Fragments Observed through MALDI-FTMS, CID, and AD-MALDI-FTMS[a]

disaccharide	−18 $-H_2O$	−60 $^{0,2}A_2$	−90 $^{0,3}A_2$	−120 $^{0,2}X_1$(a), $^{2,4}A_2$(b)	−162 C_1
β-D-Glc(1→2)D-Glc	A, C			A, C, M[(a)]	
β-D-Glc(1→3)D-Glc	A, C, M		A, C, M		A, C
β-D-Glc(1→4)D-Glc	A, C	A, C, M			A, C
β-D-Glc(1→6)D-Glc	A, C, M	A, C, M	A, C, M	A, C, M[(b)]	
	−18	−101		−161	−203
β-D-GlcNAc(1→4)-D-GlcNAc	A, C, M	A, C, M		A[(b)]	A, C, M
β-D-Gal(1→3)D-GlcNAc	A, C, M				A, C, M

[a] A, alkaline degradation conditions; C, collision-induced dissociation of untreated samples; M, MALDI of untreated samples.

Chart 1



mixtures (A). Distinct differences are evident in the fragments of various linkages. This behavior has been observed with alkali metal-coordinated and deprotonated oligosaccharides formed by FAB.[14,42−49] All disaccharides lose H_2O from the parent in the alkaline degradation mixtures. For the (1→2)-linked disaccharide sophorose, MALDI of the reaction mixture produces two fragments corresponding to the loss of water and loss of 120 u (cross-ring cleavage product $^{0,2}X_1$). The (1→3)-linked disaccharide laminarabiose yields three fragments corresponding to a loss of water, a loss of 90 u due to the cross-ring cleavage $^{0,3}A_2$, and a loss of 162 u due to glycosidic bond cleavage. The (1→4)-linked disaccharide cellobiose produces loss of water, loss of 60 u ($^{0,2}A_2$), and a glycosidic bond cleavage. The (1→6)-linked disaccharide gentobiose shows the greatest number of fragments corresponding to the loss of water, and three cross-ring cleavage products corresponding to $^{0,2}A_2$, $^{0,3}A_2$, and $^{2,4}A_2$. Note that glycosidic bond cleavage is not observed with both the (1→2), and (1→6)-linked disaccharides under all the conditions. Also note that the disaccharide β-D-Gal(1→3)D-GlcNAc did not produce cross-ring cleavages under these conditions. As will be shown later, a hexose

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Chart 2

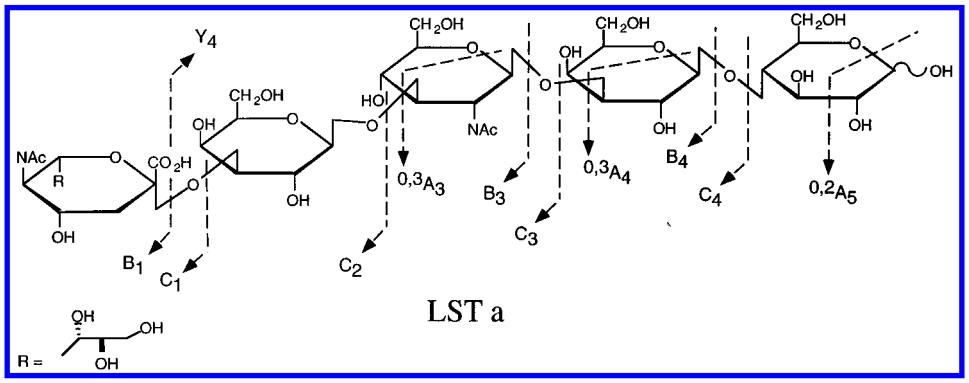
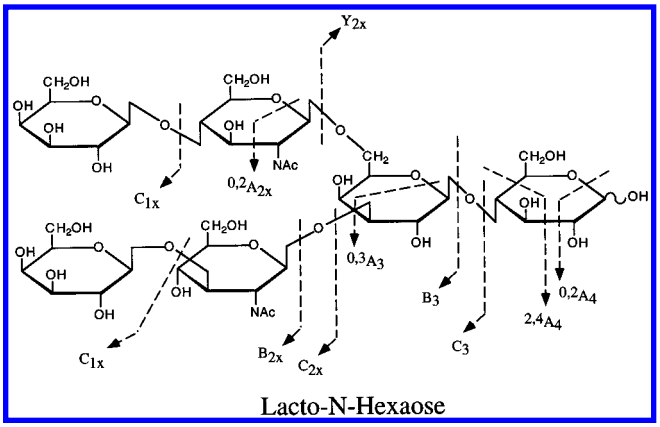


Chart 3



(1→3)-linked to a *N*-acetylhexosamine does not readily produce cross-ring cleavages even in larger oligosaccharides. Furthermore, both β-D-Glc(1→4)D-Glc and β-D-GlcNAc(1→4)D-GlcNAc produce similar fragments with the exception of the $^{2,4}A_2$ cross-ring cleavage, which is observed only with the latter compound.

MALDI and SORI CID typically yield similar fragment ions with variations only in the relative abundances. The similarities between the fragments resulting from MALDI of the untreated samples and CID of the quasi-molecular ion indicate that CID is useful for enhancing fragment ion yields in systems where minimal cross-ring fragments are obtained with MALDI. The fragment yields of MALDI can also be increased by selecting the proper ionization conditions. For example, laser power, matrix, and extraction voltages all affect the relative amounts of fragment ions.[50,51] It must be emphasized that the similarities in the three types of spectra, A, C, and M, indicate cross-ring cleavages generally occur during MALDI but not during alkaline degradation. The results further show that the reaction mixture does not suppress the MALDI spectrum.

Alkaline Degradation of Selected Oligosaccharides. The following are results for six oligosaccharides of various constituents including neutral, acidic, and fucosylated oligosaccharides. CID is an important component of the analysis because in several occasions MALDI is not sufficient to provide cross-ring cleavages even at the reducing ring. Exact mass determination is also an important feature of the analysis. AD produces several side products that complicate the mass spectra. The advantage of exact mass determination is in allowing an unambiguous determination of the elemental composition of the fragment ions.

Note that in the ion cleavage diagrams (Charts 1−5), the Domon−Costello nomenclature is used. This system is used to label all fragments regardless of their source. We have not yet formulated a nomenclature to specify alkaline degradation cleavages. In addition, some fragments may be formed from the combination of AD and MALDI fragmentation. It is not possible, in many instances, to differentiate between gas-phase and solution-phase dissociations.

(1) LNFP-I. This compound is a pentasaccharide derived from human milk and contains one fucose at the nonreducing end. As mentioned above, fucose is often highly labile during MALDI making its position and linkage difficult to determine. MALDI-FTMS of the untreated compound shows primarily cleavage of the fucose glycosidic bond and a $^{0,2}A_5$ cross-ring cleavage of the reducing ring (Figure 2a). No other structural information was obtained under the MALDI-FTMS experimental conditions. Instrumental conditions were optimized to minimize fragmentation so as to allow differentiation of glycosidic bond cleavages from alkaline degradation rather than from source fragmentation.

After a 4-h reaction period, considerable amounts of glycosidic bond cleavage near the reducing end becomes apparent with no further increase in anhydrofucose loss (Figure 2b). This demonstrates that the peeling reaction is progressing through the oligosaccharide, only dissociating monosaccharide units from the reducing end. This proves further that nascent reducing ends are produced in solution and not during the MALDI process.

After a 24-h reaction period, there are sufficient fragment ions to completely sequence the oligosaccharide and provide most of the linkage information (Figure 2c). Table 2 summarizes the mass and the cleavages for the structurally informative fragments. Chart 1 shows the cleavage pattern observed from AD-MALDI-FTMS. From the mass spectrum, numerous glycosidic bond cleavages are readily observed corresponding to C_4 (*m*/*z* 714.245), C_3 (*m*/*z* 552.190), and C_2 (*m*/*z* 349.109). In addition, the complementary fragments such as B_4 (*m*/*z* 696.233), B_3 (*m*/*z* 534.180), and B_2 (*m*/*z* 331.099) are obtained. There are also combinations of cleavage reactions. For example *m*/*z* 388.120 corresponds to a $B_3 + Y_4$ cleavage (designated as B_3/Y_4), *m*/*z* 406.131 to C_3/Y_4, and *m*/*z* 568.186 to C_4/Y_4. In addition, the cross-ring cleavage $^{0,3}A_4$ provides the (1→3)-linkage at this saccharide position. The advantages of AD for fucose-containing oligosac-

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Chart 4

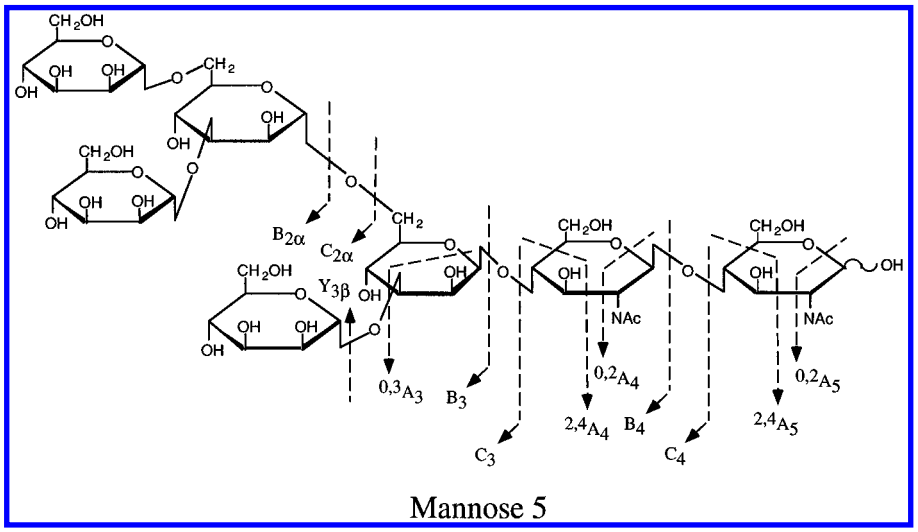
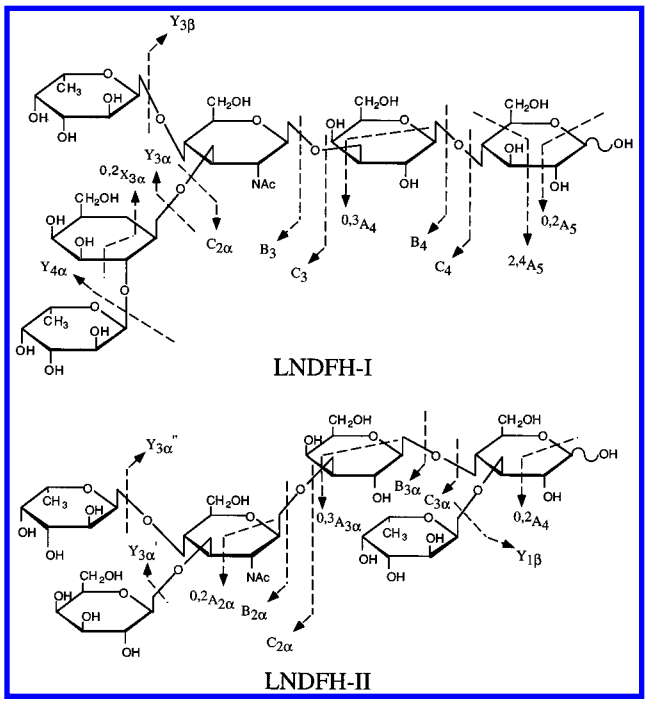


Chart 5



charides is further illustrated in this example. The uninformative loss of anhydrofucose from the quasi-molecular ion is still observed (Y_4, m/z 730.238) during MALDI. However, the molecule remains sufficiently intact so that the position of the fucose is unambiguously identified at the end of the chain. The C_2 fragment (m/z 349.109) is the disaccharide that makes up the nonreducing end and identifies the position of the fucose.

CID is effective for obtaining cross-ring cleavages of the nascent reducing end when MALDI fragmentation is insufficient. No cross-ring cleavages were observed to designate the linkage between the α-L-Fuc(1→2)D-Gal and between the β-D-Gal(1→3)-D-GlcNAc in the mass spectra even after alkaline degradation. The fragment due to C_2 cleavage (m/z 349.109) was isolated and subjected to CID (Figure 3). From the spectrum, a $^{0,2}X_3/C_2$ (m/z 229.069) cleavage is observed yielding the (1→2)-linkage between the fucose and the galactose. Both the C_3 and C_3/Y_4 ions did not produce cross-ring cleavages when subjected to CID after isolation; mainly water loss and glycosidic bond cleavages were observed. This is somewhat expected as the disaccharide β-D-Gal(1→3)D-GlcNAc also shows no cross-ring cleavages (Table 1). The lack of cross-ring cleavages in this system does not necessarily prohibit linkage assignment. Their absences allow one to speculate the (1→3)-linkage in this position.

To illustrate the utility of CID to produce cross-ring cleavages at the nascent reducing ring, a CID map was constructed and shown in Figure 4. This 2-D plot shows the MALDI of the alkaline degradation reaction mixture after 14 h on the *X*-axis and the CID of selected glycosidic bond cleavages (C_n fragments) on the *Y*-axis. Each C_n-type ion was isolated and SORI CID was performed yielding a new mass spectrum which is represented along the *Y*-axis. The CID spectra provide supporting evidence for the MALDI assignments and confirm the identity of the proposed linkages. This type of 2-D plot demonstrates the power of performing CID on the alkaline degradation products to determine sequence and linkage information quickly and easily from a reaction mixture.

Based solely on the mass spectra of the alkaline degradation reaction mixture and the CID, one could predict the linkage and sequence for LNFP-I as

dHex(1→2)Hex(1→3)HexNAc(1→3)Hex(1→4)Hex

The underlined linkage represents ambiguity in the assignment. The symbols Hex, HexNAc, and dHex stand for hexose, *N*-acetylhexosamine, and deoxyhexose, respectively.

(2) LST a. LST a is an acidic oligosaccharide found in human milk and contains one sialic acid residue on the nonreducing end. The MS analysis of sialylated oligosaccharides is problematic even under the most favorable conditions. The sialic acid glycosidic bond is extremely labile during ionization so that the loss of the sialic acid is often the major fragment observed.

A MALDI-FTMS spectrum of the reaction mixture after 14 h is shown in Figure 5. The structure and the observed fragments from MALDI-FTMS and CID after alkaline degradation are shown in Chart 2. The important fragments are provided in Table 2.

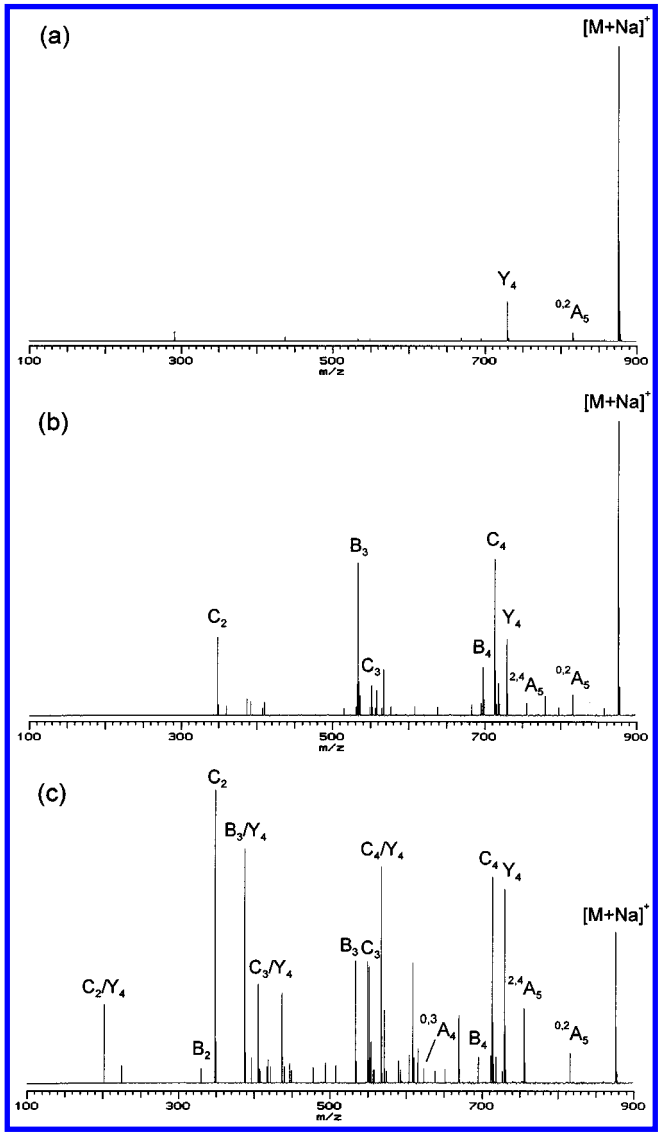


Figure 2. (a) Alkaline degradation MALDI-FTMS spectrum of LNFP-I at reaction time 0. (b) Spectrum after 4 h of alkaline degradation. There are significant C- and B-type cleavages due to the chemical degradation. No increased loss of fucose is observed. This spectrum determines the position of the fucose on the nonreducing end. (c) Spectrum after 24 h of alkaline degradation. The oligosaccharide is fully sequenced, and nearly all linkage information is obtained.

The prominent peak at m/z 730.247 is the Y_4 cleavage which corresponds to the loss of the sialic acid. This fragment is a consequence of the ionization and not the chemical degradation. The molecular ion is no longer present but is observed in the spectrum at earlier times (m/z 1021.353, spectrum not shown). The fragments corresponding to glycosidic bond cleavage C_4 (present earlier in the analysis at m/z 859.292), C_3 (m/z 697.235), C_2 (m/z 494.151), and B_1 (m/z 314.085) yield the sequence and the position of the sialic acid. Linkage is obtained on the reducing ring by a $^{0,2}A_5/Y_4$ (m/z 670.226) cleavage which designates the (1→4)-linkage. A $^{0,3}A_3/Y_4$ cleavage at m/z 316.101 produced by MALDI provides the (1→3)-linkage between the β-D-Gal and the β-D-GlcNAc after a 24-h reaction period (spectrum not shown). With MALDI alone, cross-ring cleavage was not obtained to designate the β-D-GlcNAc(1→3)D-Gal linkage. Linkage was obtained from CID of the isolated C_4/Y_4 fragment to yield the $^{0,3}A_4/Y_4$ fragment at m/z 478.152 (spectrum not shown). The linkage between the galactose and the sialic acid could not be determined. The C_2 fragment containing the sialic acid readily dissociated to produce glycosidic bond cleavage rather than cross-ring cleavage. This could be a common problem with sialylated oligosaccharides and may limit the effectiveness of alkaline degradation in determining the linkages of sialic acids. Attempts were made to convert the carboxylic acid to the methyl ester, using the method by Harvey,[52] to stabilize the residue during the MALDI process. However, in alkali solution, the resulting compound demethylated to produce the underivatized sialic acid.

Table 2. AD-MALDI-FTMS Fragments Observed

LNFP-I		LST a		lacto-*N*-hexaose		mannose 5	
m/z	cleavage	m/z	cleavage	m/z	cleavage	m/z	cleavage
858.282	$-H_2O$	859.292	C_4	1077.376	$-H_2O$	1239.142	$-H_2O$
816.278	$^{0,2}A_5$	730.247	Y_4	1035.354	$^{0,2}A_4$[a]	1156.385	$^{0,2}A_5$
756.259	$^{2,4}A_5$	697.235	C_3	975.327	$^{2,4}A_4$	1096.359	$^{2,4}A_5$
730.241	Y_4	670.226	$0,2A_5/Y_4$	933.322	C_3	1054.349	C_4
714.245	C_4	568.190	Y_4/C_4	915.310	B_3[a]	1036.339	B_4
696.233	B_4	550.178	Y_4/B_4	568.188	C_3/Y_{2x}	953.306	$^{0,2}A_4$
624.215	$^{0,3}A_4/C_4$	494.151	C_2	478.158	$^{0,3}A_3/Y_{2x}$[a]	893.283	$^{2,4}A_4$
552.190	C_3	478.152	$^{0,3}A_4/Y_4$[a]	406.133	C_{2x}	851.273	C_3
534.180	B_3	406.134	Y_4/C_3	388.126	B_{2x}	833.264	B_3
349.109	C_2	388.123	Y_4/B_3	305.085	$^{0,2}A_{2x}$[a]	689.219	$C_3/Y_{3\beta}$
331.099	B_2	332.096	C_1	203.054	C_{1x}	671.206	$B_3/Y_{3\beta}$
229.069	$^{0,2}X_3/C_2$[a]	316.101	$^{0,3}A_3/Y_4$			599.182	$^{0,3}A_3/Y_{3\beta}$
203.052	C_2/Y_4	314.085	B_1			527.164	$C_{2\alpha}$
						509.151	$B_{2\alpha}$

[a] Fragments obtained from CID.

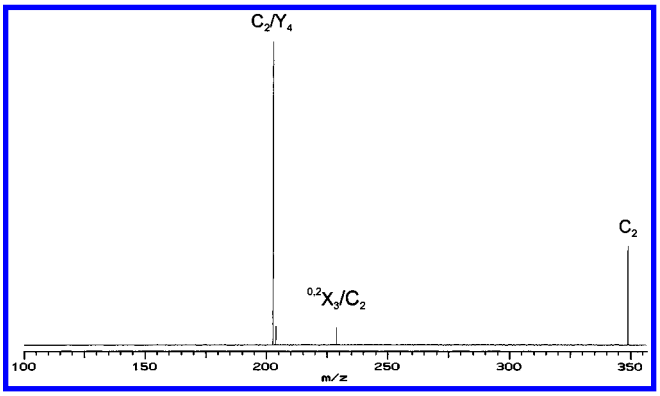


Figure 3. SORI CID of isolated C_2 ion from Figure 2c. The $^{0,2}X_3/C_2$ fragment identifies the (1→2)-linkage between the terminal fucose and the galactose in LNFP-I.

The MALDI-FTMS and CID data of the degradation products yield all but one linkage. The predicted structure of LST a based solely on the MS data is

NeuNAc(2→?)Hex(1→3)HexNAc(1→3)Hex(1→4)Hex

(3) Lacto-*N*-Hexaose. Since alkaline degradation begins at the reducing end, it is also a useful tool for determining branch points of the oligosaccharide.[24] At a branch point, the peeling reaction tends to favor one antenna. Each linkage type has a

(52) Powell, A. K.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1027−1032.

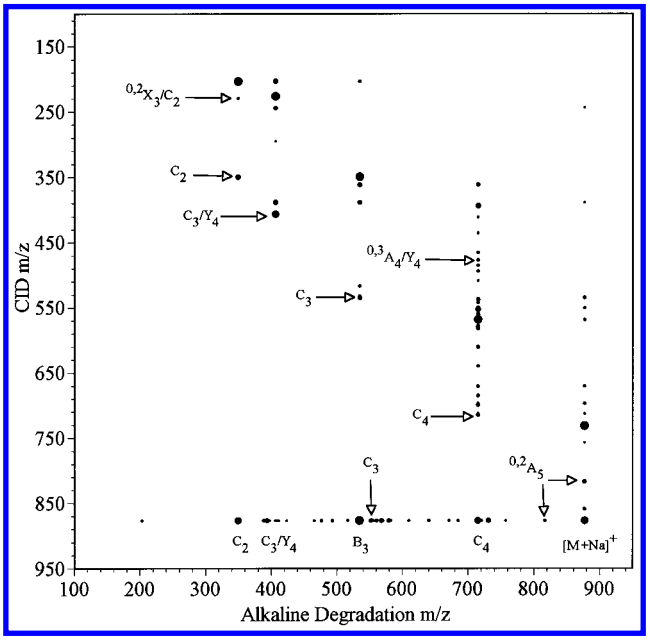


Figure 4. Alkaline degradation MALDI-FTMS 2-D plot. The spectrum on the *X*-axis represents the AD-MALDI-FTMS of LNFP-I after a 14-h of reaction period. On the *Y*-axis are the SORI CID spectra of each new reducing end (C-type ions) isolated from the 14-h reaction mixture.

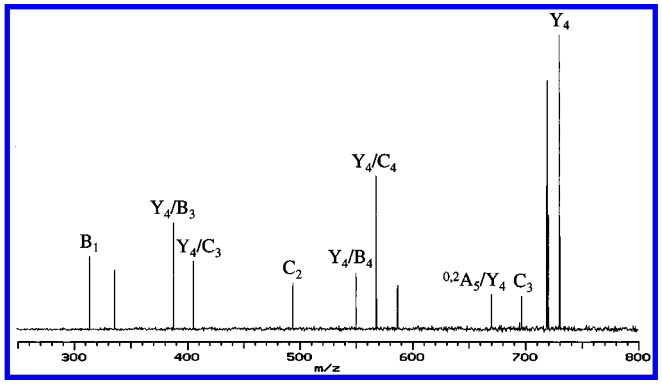


Figure 5. MALDI-FTMS spectrum of LST a after a 14-h reaction period. The molecular ion is not present due to loss of neuraminic acid during ionization. Strong abundances of C- and B-type cleavages readily sequence the oligosaccharide and determine position of the neuraminic acid at the nonreducing end.

different β-elimination rate due to the formation of stable intermediates.[29] At a branch point, the linkage with the greater elimination rate is cleaved first. The degradation rates are as follows: (1→3)-linkage > (1→4)-linkage > (1→6)-linkage > (1→2)-linkage. The (1→2)-linkage is relatively stable under alkaline conditions.[29]

Lacto-*N*-hexaose is a hexasaccharide that is also derived from human milk and contains one branch point at the residue adjacent to the reducing end. The first product observed after 3 h was the C_3 fragment (m/z 933.322), spectrum not shown. A representative spectrum after a 14-h degradation period is shown in Figure 6. The quasi-molecular ion (m/z 1095.376) is still readily observed. The MALDI-FTMS analysis of the reaction mixture produced glycosidic bond cleavages that readily identified the position of the branch point (Chart 3, Table 2). The C_3 and B_3 (m/z 915.310) cleavages (Figure 6) are unequivocal evidence for the reducing end to be a hexose sugar. Another major fragment

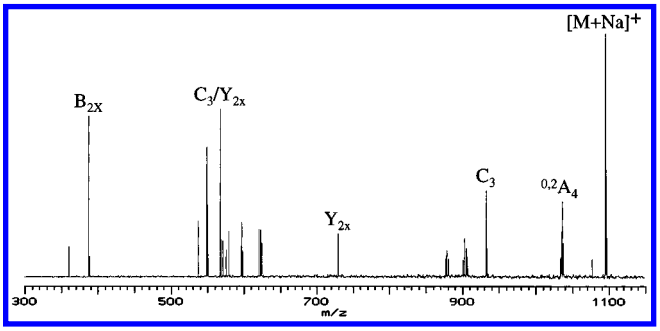
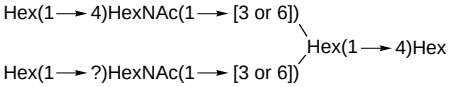


Figure 6. MALDI-FTMS spectrum of Lacto-*N*-hexaose after a 14-h reaction period. The branch position is elucidated by the C_3/Y_{2x} fragment. See text for further discussion.

was due to the C_3/Y_{2x} cleavage. The *x* designates that it could be from either the α or β antenna. However, the cleavage is likely due to the elimination of the (1→3)-linked antenna at the branch point because this linkage is the most reactive in alkaline degradation. This cleavage combination (C_3/Y_{2x}) identifies the position of the branch point.

The $^{0,2}A_4$ cleavage yields the (1→4)-linkage at the reducing end. CID of the quasi-molecular ion also produces the $^{2,4}A_4$ cleavage at m/z 975.327 (spectrum not shown). The C_3/Y_{2x} fragment was isolated and subjected to SORI CID to produce the $^{0,3}A_3/Y_{2x}$ cleavage at m/z 478.158 (spectrum not shown). This cleavage identifies the (1→3)- and (1→6)-linkage at the branching galactose. After a 24-h reaction period the C_{2x} (m/z 406.133) and C_{1x} (m/z 203.054) fragments grew in intensity to allow isolation and CID. SORI CID of the C_{2x} fragment produced one cross-ring cleavage at m/z 305.085 corresponding to $^{0,2}A_{2x}$. This fragment identifies the (1→4)-linkage between the galactose and the *N*-acetylglucosamine of one antenna. As previously stated, the disaccharide β-D-Gal(1→3)D-GlcNAc does not readily produce cross-ring cleavages. Note that the C_{2x} cleavage produces isomeric ions and CID of the product ions produce cleavages for the (1→4)-linked only and not for the (1→3)-linked. In a true unknown, the absence of cross-ring cleavages for one antenna would produce some amount of ambiguity.

Based on the MALDI-FTMS and CID data of the degradation products, the predicted structure is



(4) Mannose 5. Mannose 5 is a triantennary high-mannose heptasaccharide from porcine thyroglobulin (Chart 4). MALDI-FTMS of the reaction mixture after 3.5 h produced the spectrum in Figure 7a. The $^{0,2}A_5$ and $^{2,4}A_5$ cleavages (m/z 1156.385 and 1096.359, respectively) identify the (1→4)-linkage of the reducing end. The onset of the C_4 (m/z 1054.349) and B_4 (m/z 1036.339) glycosidic bond cleavages is also present.

Figure 7b shows the MALDI-FTMS spectrum of the reaction mixture after 14 h. The C_3 fragment indicates that the reducing end and the adjacent saccharide are two *N*-acetylhexosamines. These assignments are supported by the B_4 and B_3 fragments. The (1→4)-linkage of the second GlcNAc is revealed by the $^{0,2}A_4$ (m/z 953.306) and the $^{2,4}A_4$ (m/z 893.283) fragments. The $C_3/$

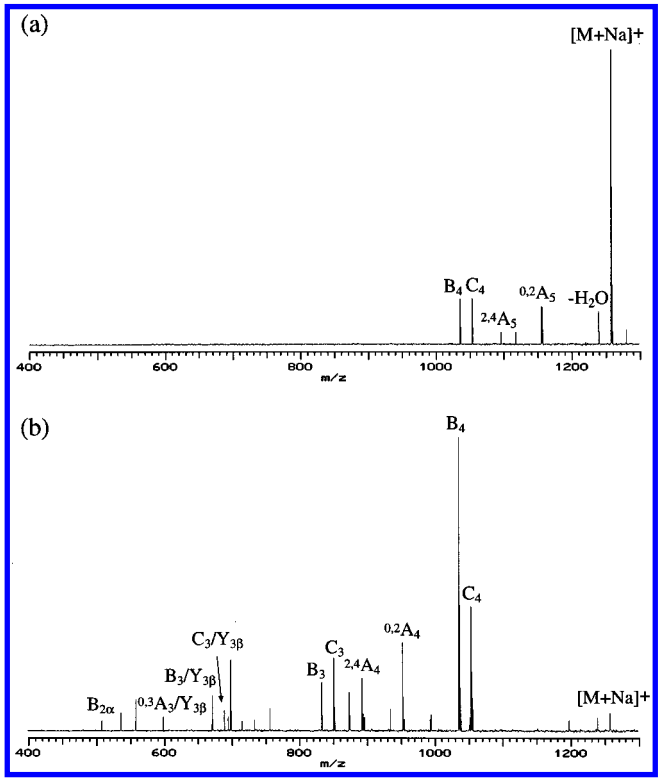


Figure 7. Alkaline degradation MALDI-FTMS spectra of mannose 5 after (a) 3.5 and (b) 14 h. See text for discussion.

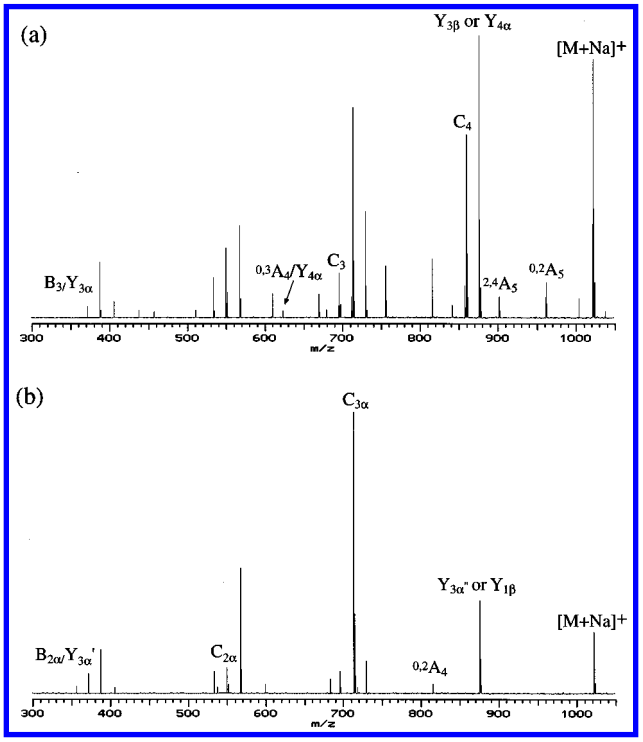
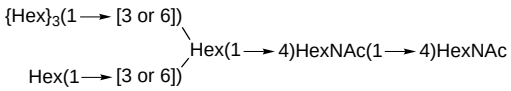


Figure 8. AD-MALDI-FTMS spectra of (a) LNDFH-I and (b) LNDFH-II after a 14 h reaction period. Only peaks important to the discussion are labeled for clarity.

$Y_{3\beta}$ and $B_{2\alpha}$ fragments indicate a branch point at the third saccharide that is a hexose. The linkages at the first branch point are revealed by the cleavage $^{0,3}A_3/Y_{3\beta}$ to yield the (1→3)- and (1→6)-linkages. The linkages in the second branch point could not be identified after a 24-h reaction period. The yield of $C_{2\alpha}$–cleavage was too poor to obtain further cross-ring cleavages. The $C_{2\alpha}$ fragment intensity rose slowly due to the slower elimination rate of the (1→6)-linkage. The weak abundances of the $C_{2\alpha}$ fragment prohibit linkage determination at the second branch point. The list of structurally informative fragments is given in Table 2. From the cleavage products, one can construct most of the sequence and the important linkages as



(5) LNDFH-I and (6) LNDFH-II. The two isomers LNDFH-I and LNDFH-II (structures in Chart 5) would normally be indistinguishable by MALDI-FTMS alone. Their spectra are nearly identical and show intense losses of anhydrofucose from the sodiated quasi-molecular ion and minor cross-ring cleavages of the reducing end (spectra not shown). SORI CID of the quasi-molecular ions produces more anhydrofucose loss as expected for fucosylated compounds. There is no other structural information obtained until the molecule is fully defucosylated in the gas phase.

Alkaline degradation products analyzed by MALDI-FTMS and CID yielded the fragments designated in Chart 5. Although, LNDFH-I and LNDFH-II are structural isomers, they degrade to produce distinct products at different rates. The spectra after a 14-h reaction period are shown in Figure 8 (for clarity not all fragments have been labeled). The cleavages are unique and clearly differentiate the two isomers. For example, all of the glycosidic bond cleavages C_4, C_3, and $B_3/Y_{3\alpha}$ of LNDFH-I pinpoint the position of both fucoses. Whereas, cleavages $C_{3\alpha}$, $C_{2\alpha}$, and $B_{2\alpha}/Y_{3\alpha}'$ all indicate the separation of the two fucoses between the reducing and the nonreducing ends in LNDFH-II.

Advantages of Exact Mass Determinations. Alkaline degradation produces other side reactions and side products. These products in turn produce complicated mixtures that may interfere with the analysis if the fragment ion peaks are not well resolved. The exact mass determinations and high resolving power of FTMS make it the ideal mass detector for alkaline degradation products.

Figure 9 shows the expanded view of the C_3 fragment ion region of LNDFH-I after 6 h of degradation. The asterisk peaks mark unknown chemical byproducts from the reaction. The C_3 fragment at m/z 698.256 and the $^{13}C-C_3$ peak at m/z 699.259 are clearly resolved from the byproduct peaks. The spectrum was acquired in broad-band (low-resolution) mode which provides a resolution of approximately 10 000 and easily resolves the group of ions. The exact mass of the C_3 fragment is 698.248 amu. This differs from the predicted mass by 11 ppm. This accuracy is readily obtained even with external calibration. The signal unambiguously identifies the C_3 fragment from the peaks due to side products. This example demonstrates the utility of FTMS to precisely distinguish sequence fragments from complicating side reactions. A TOF instrument with suitable resolution and possibly postsource decay can also easily be adapted for the analysis.

CONCLUSIONS

A new technique for determining the sequence and linkage of oligosaccharides has been demonstrated. The method character-

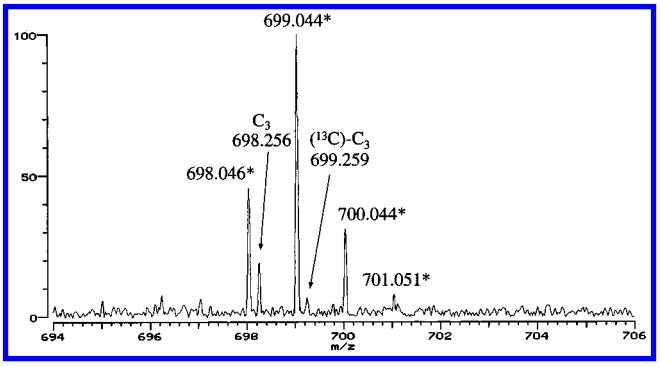


Figure 9. C_3 fragment ion region of LNDFH-I after a 6-h reaction period. Asterisks (*) designate an unknown chemical byproduct from chemical degradation. The C_3 fragment and its $^{13}C-C_3$ isotope are clearly resolved from the mixture. The exact mass determination confirms the identity of the C_3 fragment within 11 ppm with external calibration only.

izes the remaining portion of the carbohydrate by MALDI-FTMS after alkaline degradation. Both sequence and linkage information are readily obtained. Linkage information is acquired from cross-ring cleavages generated either by MALDI source fragmentation or from SORI CID of the isolated nascent reducing end. Although the limits of detection for this method have not been rigorously determined, picomolar quantities of oligosaccharides have been sequenced successfully.

Since alkaline degradation is sequential and nonspecific, it unambiguously determines the position of labile saccharides such as fucose and sialic acid. This could not be determined using MALDI-MS or CID alone. In addition, alkaline degradation also offers a unique analysis into oligosaccharide structure by the sequential degradation from the reducing end compared to exoglycosidase enzymatic techniques which cleave from the nonreducing end. The method is simple, requires almost no sample preparation, and uses inexpensive reagents.

There are areas in this method that need improvement. These include (1) the lack of cross-ring cleavages in some instances and (2) the long reaction times. For example with LNFP-I, cross-ring cleavages were not observed in the GlcNAc residue. The absence of some cross-ring cleavages may be addressed with a better understanding of oligosaccharide fragmentation during MALDI and CID. When activated, HexNAc units produce more B-type than C-type ions,[5,26] which do not readily produce linkage characteristic cross-ring cleavages. A B-type ion is not a reducing end and therefore is not able to open to form the acyclic hydroxyaldehyde proposed by Cotter et al.[53] which is the first step in the preferred cross-ring cleavage mechanism. The long reaction time is necessitated by the relatively low NaOH concentration. Higher concentrations produce faster reactions,[54,55] but the MS signals are severely attenuated with higher Na^+ levels. To accommodate the higher concentrations, we plan to perform microdialysis before MALDI to decrease the amount of Na^+ in solution.

These results display the utility of alkaline degradation as a rapid technique for the structural elucidation of oligosaccharides. It provides information on sequence and linkages that can be used in conjunction with NMR or enzymatic analyses to help obtain the complete structure. Finally, its advantage is in providing sequencing information for situations where only nano- to picomolar amounts of material are available.

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