APPLICATION OF FOURIER TRANSFORM ION CYCLOTRON RESONANCE MASS SPECTROMETRY TO OLIGOSACCHARIDES

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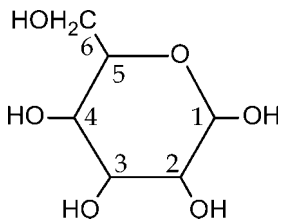
The application of Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) to the structural elucidation of oligosaccharides is described. This review covers the analyses of oligosaccharides in the context of the unique features of FTICR MS and the improvements in instrumentation that make it possible to study this class of compounds. It consists of work performed initially to understand the fundamental aspects of oligosaccharide ionization and unimolecular fragmentation. More recent investigation includes the application of the technique to samples of direct biological origin. Chemical and enzymatic degradation methods in conjunction with mass spectrometry (MS) and the use front-end methods with FTICR MS are also discussed. The current applications including the characterization of bacterial lipooligosaccharides and phosporylated carbohydrates are described. © 2004 Wiley Periodicals, Inc., Mass Spec Rev 24:232–264, 2005

Keywords: *Fourier transform ion cyclotron resonance mass spectrometry; oligosaccharides; CID; mucin-type; glycosaminoglycans; N-linked; O-linked*

I. INTRODUCTION

The development of mass spectrometric methods for the rapid and sensitive analysis of oligosaccharides has been a major topic of research in our laboratory. Oligosaccharides are in most cases conjugated with other biomolecules, which are known as glycoconjugates. Protein glycosylation is the most common form of post-translational modification. It is estimated that more than 50% of all proteins are glycosylated (Apweiler, Hermjakob, & Sharron, 1999). Oligosaccharides play a central role in many fundamental cellular processes such as cell–cell recognition, adhesion, control of cell division, cellular differentiation, and malignant transformation (Montreuil, 1980; Humphries et al., 1986; Ogier-Denis et al., 1989; Varki, 1993). For example, oligosaccharides that are present in mucin glycoproteins from egg jellies play important roles in the process of sperm-egg recognition and fertilization (Schmell, Gulyas, & Hedrick, 1983). Changes of *O*-linked oligosaccharide structures in cancer cells are often associated with cancerous transformation (Brockhausen, 1999). In addition, oligosaccharides in glycoproteins also play roles in maintaining protein structure and stability (Rudd & Dwek, 1997). They are responsible for some of the antigenic properties of proteins, and involved in complement pathways (Lisowska, 2002; Ritchie et al., 2002). With recent synthetic advances, glycoconjugates are emerging as targets for many therapeutic applications such as cancer chemotherapy, diabetes therapy, antibiotics, antivirals, anti-inflammatories, and immunostimulants (McAuliffe & Hindsgaul, 1997; Yarema & Bertozzi, 1998; Koeller & Wong, 2000; Bertozzi & Kiessling, 2001; Dove, 2001; Maeder, 2002). However, oligosaccharide analyses such as sequencing and structure characterization still remain very labor-intensive and complicated. Effective tools for oligosaccharide analyses will facilitate the understanding of their cellular functions as well as diseases. To this end, a research program was developed in this laboratory that initially involved the construction and optimization of Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) instrumentation, the study of fundamental gas-phase decomposition processes, and the analysis of biological samples to develop mass spectrometry (MS) as the primary tool for oligosaccharide analysis. MS offers the potential of both high sensitivity and speed, but to develop efficient schemes for sequencing oligosaccharides, a thorough understanding of the gas-phase chemistry of these compounds is also necessary.

Structural features of oligosaccharides contribute to make the mass spectrometric analyses of these compounds considerably more difficult than that of other biopolymers such as peptides and nucleotides. Knowing the sequence of an oligomeric chain is not sufficient for full characterization. The linkage, degree of branching, and stereochemistry must also be determined. Determination of the linkage alone, for example, is already a formidable task. A glycosidic bond linking two cyclic hexoses can be accomplished in one of five ways (neglecting stereochemistry) through the hydroxyl groups shown below. In comparison, amino acids are linked in the primary chain of a protein in only one way, by forming an amide bond. Branching also readily occurs with oligosaccharides but is seldom found in peptides or proteins. In addition, oligosaccharides generally have poorer MS sensitivities than, for example, peptides.



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Several features of FTICR make it ideal for oligosaccharide analysis. The high resolution and mass accuracy readily yields the composition in terms of numbers of fucose, hexose, *N*-acetylhexose, sialic acid, as well as degree of sulfation, etc. The simultaneous implementation of both electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), the two most useful ionization methods for oligosaccharides, was performed first in FTICR. The availability of reliable and consistent tandem MS events such as collision-induced dissociation (CID) and more recently infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD) with any ionization method in FTICR made it a rapid and key tool for structural elucidation.

This review is not meant to be comprehensive in oligosaccharide or carbohydrate analyses. There are already extensive reviews in this area (Harvey, 1999). Instead, it highlights the role FTICR MS has played in this area. As the early FTICR MS analyses of oligosaccharides were performed primarily in our laboratory, we begin with some historical perspectives including some early instrumentation development in our laboratory. The more recent studies performed in this laboratory and elsewhere were performed generally on commercial instruments.

II. INSTRUMENTATION

A. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

FTICR (Wanczek, 1984; Buchanan, 1987; Wilkins et al., 1989; Dienes et al., 1996; Marshall, Hendrickson, & Jackson, 1998) is performed by trapping ions in an analyzer cell composed of electrically conductive walls placed in a homogenous region of a magnet (Comisarow & Marshall, 1974a,b; Grosshans & Marshall, 1990). Detection is performed by exciting all the ions nearly simultaneously (depending on the excitation method, e.g., chirp versus impulse) and coherently to the same cyclotron radius (Schweikhard & Marshall, 1993). The image current produced by the motion of the coherent ions is digitized and Fourier transformed. The longer the ions remain in coherence, the longer the signal persists. This provides not only stronger signals but also better resolution (Grosshans & Marshall, 1990). Several factors determine the performance of the instrument. The most important of these are pressure, homogeneity (electric and magnetic field), and magnetic field strength. Collisions decrease the length of the transient by dephasing the motion of the ions (Marshall, Hendrickson, & Jackson, 1998). For this reason, the lowest pressure provides the best performance. In our system, cryopumps are used to produce a base pressure of 10^{-11} torr with MALDI and 10^{-9} torr with electrospray. Electric field and, to some extent, local magnetic field homogeneity is determined by the cell design and material (Comisarow, 1981; Caravatti & Allemann, 1991; Beu & Laude, 1992). Several groups and instrument manufacturers have implemented the cylindrical and open-end design as this yields better performance (Beu & Laude, 1992). We have implemented a double-cell design for the electrospray that consists of two complete analyzer cells in the same chamber. We used the second analyzer cell to monitor the temperature for performing thermal dissociation experiments (as in blackbody induced radiation dissociation or BIRD) (Price, Schnier, & Williams, 1996).

The magnetic field strength is important as ion retention, resolution, and high-mass capability all increase with the field strength (Ledford, Rempel, & Gross, 1984; Marshall, Hendrickson, & Jackson, 1998). The major limitation is that the price of superconducting magnets increases exponentially with the strength of the field. The overall cost of the instrument further increases as many of the components that are normally ''non-magnetic'' such as stainless steel electrodes, ion guides, vacuum chambers, etc., become magnetic. Many have been replaced with truly non-magnetic material such as aluminum or titanium. Turbo pumps and cryopumps as well as monitors are also affected unless the magnet is shielded. The majority of the studies performed in our laboratory have been on 4.7 and 5.0 T instruments up until now. These fields have provided an acceptable compromise between performance and price.

Oligosaccharides, and other biomolecules, require low vacuum or even atmospheric ionization sources to produce ions. The low vacuum interferes with the performance of FTICR. For this reason, most analytical instruments produce ions outside the analyzer chamber (externally) and transport the ions into the analyzer cell. The latest major advancement in FTICR is the development of ion guides to transport ions from a low vacuum source to the ultra-high vacuum analyzer chamber. This laboratory and several others including McIver (Hunt et al., 1985; McIver, Hunter, & Bowers, 1985; Lebrilla, Amster, & McIver, 1989) and Wanzek (Kofel et al., 1986) have proposed various methods of ion injection (Marshall & Schweikhard, 1992). These methods now include but are not limited to quadrupole and octupole ion guides and lens arrays whose purpose is to guide ions through the inhomogeneous region of the field.

The capability of FTICR to provide high masses (Chen et al., 1995), high resolution/accurate mass (Li, McIver, & Hunter, 1994; McIver, Li, & Hunter, 1994; McLafferty, 1994; Kelleher et al., 1995), and multiple stages of CID are well known. Less well known are differences between FTICR and other MS methods. Knowing these differences is important if the methods developed in FTICR are to be applied to other types of mass spectrometers. The time between ion formation and detection is 3 to 6 orders of magnitude longer in FTICR than TOF and sector instruments. The large difference in detection times is an important factor in ionization sources such as MALDI that produces a large fraction of ions as metastable species. However, the metastable ions can also be used to obtain more structurally relevant fragments. Delaying the detection time is an important method in TOF (i.e., post-source decay) (Spengler et al., 1994; Lemoine, Chirat, & Domon, 1996; Naven et al., 1997) for producing a greater number of structurally informative fragment ions. Differences in detection times are less of a factor when comparing quadrupole ion traps and FTICR. In many ways, these methods are similar. The major difference being the former is faster and less expensive, and the latter significantly higher in resolving power and mass accuracy.

At the beginning of this project, the laboratory was equipped with an external fast atom bombardment (FAB) ionization source coupled to a single set of quadrupole rods. The rods function as an ion guide that leads to the analyzer cell housed in the homogeneous region of a superconducting magnet (McCullough,

Gard, & Lebrilla, 1991). The reported performance of ESI (Yamashita & Fenn, 1984; Fenn et al., 1989) and MALDI (Karas & Hillenkamp, 1988; Stahl et al., 1991) for producing ions of large biomolecules encouraged us to proceed on a major construction effort to build ESI and MALDI source for our instrument.

We built ESI and MALDI sources that were interchangeable with the existing FAB source. The utility of MALDI and later ESI for the analysis of oligosaccharides was readily apparent but switching sources, particularly between ESI and MALDI, became a major limitation. Exchanging ionization sources required venting, removing the source, installing the other, pump-down, and bake-out; the entire procedure took up to 2 days. The necessity of having both sources forced us to devise a new method for rapidly exchanging them. The original chamber had been modified for electrospray with the addition of two additional stages of pumping. We decided to build a completely separate chamber with its own ion optics and analyzer cell that would house a MALDI source. The new chamber would share a common data system and magnet with the original chamber, resulting in considerable savings. This set-up had other advantages. One chamber could be used even when the other required service (Gard et al., 1996).

On the basis of our experience with the original chamber, we concluded that the three stages of differential pumping were unnecessary for either MALDI or FAB. We used a similar design for the new chamber, that is, to incorporate the quadrupole ion guide that had performed so effectively. However, we limited the number of pumping stages to only two. The resulting instrument contained two independent vacuum chambers that were mounted on wheels (Fig. 1). Each chamber could be positioned into the superconducting magnet and connected to the data system when needed (Carroll et al., 1996; Gard et al., 1996). MALDI with the new chamber worked as well as with the original one. The decreased pumping was not a limitation. Many of the results discussed below are obtained with both this instrument and a commercial instrument.

B. Ionization Methods

1. Fast Atom Bombardment (FAB)

FAB or more appropriately liquid secondary ion MS-LSIMS made it relatively straightforward to produce anionic and cationic species of oligosaccharides in the gas-phase with FTICR (Carroll et al., 1991; McCullough, Gard, & Lebrilla, 1991). Compounds with masses as large as $m/z = 1500$ were readily obtained, albeit decreasing quasimolecular ion abundances accompanied increasing mass. The quasimolecular ion was usually the proton coordinated species, although significant abundances of sodiated species were at times observed. We found that FTICR provided the same detection limits as other MS instruments for FAB (micromoles). The most notable difference between FTICR and sector, the most commonly used instrument with FAB at the time, was the nearly complete absence of matrix ions in the FTICR spectra. The absence of chemical background greatly simplified the spectra allowing the observation of even weakly abundant fragment ions.

In both anion and cation modes, extensive fragmentation was the norm with FAB in FTICR. Fragmentation was more extensive in the cation mode, but in the anion mode we observed significant cross-ring cleavages that yielded linkage information (Carroll & Lebrilla, 1992; Carroll et al., 1993; Carroll, Willard, & Lebrilla, 1995). Significant effort was placed on increasing sensitivity and ion yield but the best detection limit that could be obtained remained in the microgram and sometimes submicrogram range. This was not a limitation specifically of FTICR but rather that of the ionization method.

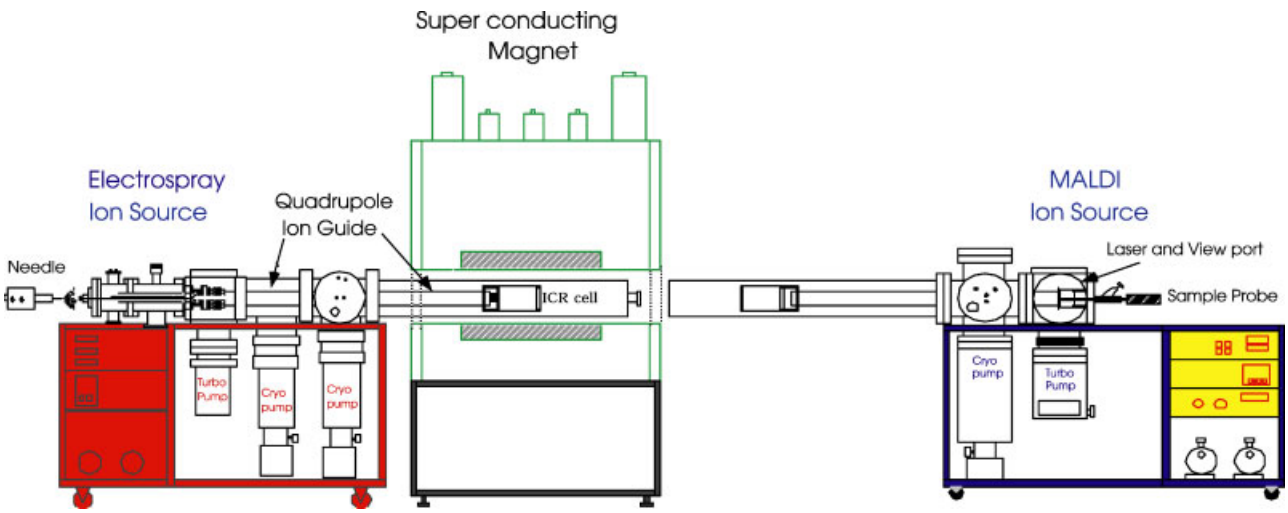


FIGURE 1. Schematic of FTICR instrument with dual champers for ESI and MALDI. This configuration provides rapid access to both ESI and MALDI. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ions produced by FAB are sufficiently vibrationally excited as to dissociate during the detection time scale of FTICR. We published the first reported study on metastable decay in the ICR (Ngoka & Lebrilla, 1993). Metastable decay of FAB-produced ions have been observed with other types of mass spectrometers (Grese, Cerny, & Gross, 1989; Thorne, Ballard, & Gaskell, 1990), but it was commonly thought that metastable decay could not be observed in the ICR because of the rapid rates of dissociation. However, the size of the molecules and the relatively low internal energy imparted by FAB, compared to electron impact, for example, produced ions whose metastable decay rates were in the order of milliseconds. Indeed, the absence of significant chemical noise in the FAB FTICR MS spectra can be attributed the metastable decay of matrix cluster ions. More recently, metastable decay of MALDI produced ions were also observed with FTICR for peptides, namely RPPGFSPF and PKPQQFFGLM (Ho & Fenselau, 2000). Metastable decay rates were dependent on a number of factors but especially laser fluences and MALDI matrices. They observed decay time constants on the order of 10 msec. Similar metastable decay may also contribute to the relatively lack of matrix ions observed in the MALDI spectra from FTICR MS.

The rates of metastable decay in FAB varied with the charge carrier (proton and alkali metal) and the charge type (Ngoka, Gal, & Lebrilla, 1994). The decay rates decrease as a function of cation ion size (i.e., $H^+ > Li^+ > Na^+ > K^+$) for linear and cyclic oligosaccharides (Table 1). There are at least two explanations for this behavior. The cation affinity of oligosaccharides increases with decreasing cation size. Thus, the effect could be because of the large internal energy associated with cationization. The second is that the small, densely charged cation may promote dissociation, in this case, glycosidic bond cleavage.

Metastable decay initially showed great promise for use as an analytical tool. The molecular ion and structurally informative fragments can be obtained merely by varying the detection time. It also eliminated a significant amount of chemical noise from the matrix as these peaks were often metastable and diminished in intensity at longer detection times. However, metastable decay proved to be source of several problems. It broadened the peaks severely thereby limiting mass resolution and accuracy. It was also not highly predictable and depended on many factors including the dissociation threshold and the internal energy of the molecules that apparently differed between molecules. Therefore, oligosaccharides with labile groups, such as sialic acids, fucose, and lipids, could not be produced intact in the gas phase. Furthermore, metastable decay limited the mass range as the rate appeared to scale with molecular size. The mass range limitation was the consequence of the internal energy that increased with molecular size. It became readily apparent, that rather than being a useful analytical tool, metastable decay and thus LSIMS would make it impossible to study large oligosaccharides with complicated structures.

TABLE 1. First order decay rate constants of linear and cyclic oligosaccharides coordinated to various cations

Oligosaccharide	cation	Rate Constants (s^{-1})
α-cyclodextrin	H	428 ± 44
	Li	385 ± 24
	Na	346 ± 36
	K	240 ± 25
maltohexaose	H	410 ± 81
	Li	346 ± 42
	Na	231 ± 18
	K	252 ± 28
α-cyclodextrin anion		193 ± 22

2. Electrospray Ionization (ESI)

This method employs a needle at a potential 2–3 kV above (or below depending on the desired polarity) the potential of the aperture to the first vacuum stage (Yamashita & Fenn, 1984; Fenn et al., 1989). Ions are produced as liquid flows out of the needle producing solvent clusters containing analyte. The specific mechanism for electrospray is still under contention. However, the desolvation of the droplets is the essential process (Kebarle & Tang, 1993). The clusters are desolvated with a heated capillary in our home made instrument. Other methods are used such as a counter flow of gas to achieve the same results (Yamashita & Fenn, 1984; Fenn et al., 1989). Electrospray can be used to analyze neutral and anionic oligosaccharide. ESI of neutrals is not known to produce detection limits as high as MALDI (Duffin et al., 1992; Fura & Leary, 1993; Huddleston, Bean, & Carr, 1993; Kohler & Leary, 1995; Viseux, Hoffmann, & Domon, 1997). Derivatization with a basic group (Takao et al., 1996; Yoshino et al., 1995) or more effectively, a positively charged group, such as Girards T (Naven & Harvey, 1996), increases the sensitivity of neutral oligosaccharides. Our experience with the ESI FTICR has been that neutral oligosaccharides do not produce strong signals in the cation mode as Na^+ ions were necessary to form the quasimolecular ions. Acidic oligosaccharides produce abundant signals with ESI (Reinhold, Reinhold, & Costello, 1995). The deprotonated ion is easier to form than the alkali metal coordinated species. Depending on the conditions, ESI can be tuned to give essentially no fragmentation. This is important when mixtures are analyzed and when it is not apparent which signals belong to fragments and which belong to quasimolecular ions.

An ESI source was built in-house, and designed with the ion simulation program SIMION. The design was based on one developed by Chait that employed a heated capillary for desolvation (Chowdhury, Katta, & Chait, 1990). As we were constructing our ESI source, several groups had incorporated ESI with the FTICR (Henry, Quinn, & McLafferty, 1991; Cheng et al., 1995). However, none have studied oligosaccharides. Other groups were exploring the utility of ESI for oligosaccharide analysis with quadrupole analyzer equipped instruments (Reinhold, Reinhold, & Costello, 1995; Yoshino et al., 1995; Naven & Harvey, 1996; Viseux, Hoffmann, & Domon, 1997). For neutral oligosaccharides, ESI offered no major advantages over MALDI. For anionic or acidic oligosaccharides, however, it showed great utility (Reinhold, Reinhold, & Costello, 1995). A comparison of gangliosides performed with ESI and MALDI on an FTICR

mass spectrometer has been published (Penn et al., 1997). Gangliosides with multiple sialic acids are difficult to analyze with MALDI but worked well with ESI-FTICR. When performed in the anion mode, ESI produced charge states that corresponded directly to the number of sialic acid. Other glycoconjugates with neutral oligosaccharides perform well with electrospray. The peptide moiety in glycopeptides may be capable of receiving the charge yielding protonated species with ESI (Alving et al., 1998). ESI employing both ion trap and FTICR was used to obtain oligosaccharide sequence and linkage information. Partial acid hydrolysis of oligosaccharides was performed by using an acid-exchange resin as the acid catalyst, which minimized the decomposition of monosaccharides and deacetylation of *N*-acetylhexosamines. In addition, Zn(dien)-Cl_2 was used for derivatization to determine stereochemistry and anomericity (Cancilla et al., 2000).

3. Matrix-Assisted Laser Desorption/Ionization (MALDI)

This method of ionization involves the co-crystallization of the analyte with a matrix that acts as a chromophore for the laser radiation (Karas & Hillenkamp, 1988). The mechanism for ion formation is initiated by the electronic excitation of the media (Bencsura & Vertes, 1995; Ehring & Sundqvist, 1995). However, collision dynamics rapidly assume a substantial role as energy propagates in the condensed-phase medium. Ions may be preformed in the solid state (Liao & Allison, 1995; Lehmann, Knochenmuss, & Zenobi, 1997) or produced by ion–molecule reactions in the gas phase (Wang et al., 1993; Liao & Allison, 1995). Furthermore, MALDI produces ions that are typically less energetic than FAB but more energetic than ESI (Demirev et al., 1987; Thorne, Ballard, & Gaskell, 1990; Spengler, Kirsch, & Kaufmann, 1991; Ngoka & Lebrilla, 1993; Ngoka, Gal, & Lebrilla, 1994; Karas et al., 1995).

Several groups including Hillenkamp showed the remarkable improvement of MALDI (coupled to a TOF instrument) over FAB for the analysis of oligosaccharides (Mock, Davey, & Cottrell, 1991; Stahl et al., 1991; Harvey, 1993). This encouraged us to immediately develop a MALDI source for our instrument. McIver was the first to implement MALDI to an external source FTICR instrument (Li, McIver, & Hunter, 1994; McIver, Li, & Hunter, 1994). They showed extraordinarily high mass and high resolution (10^6 FWHH) with peptides. Wilkins implemented MALDI in an internal source and showed the high-mass capability of the FTICR instrument (Castoro, Koester, & Wilkins, 1992; Koster, Castoro, & Wilkins, 1992; Castoro & Wilkins, 1993). Our FAB source was readily amenable to MALDI. We simply rotated the Cs^+ gun 90 degrees leaving an open area where the laser beam could enter and strike the probe. To obtain MALDI spectra, we placed a small nitrogen laser operating at 337 nm on the vacuum cart and pointed it towards the FAB source. This was sufficient to obtain the first MALDI spectra of oligosaccharides in FTICR (Carroll et al., 1996). The quadrupole ion guide worked well to collimate the ion beam. A nitrogen gas pulse trapped the translationally excited ions in the analyzer cell. The advantages of MALDI-FTICR in the analysis of oligosaccharide were immediately apparent. The quasimolecular ions of neutral oligosaccharides were always the base peak (Carroll et al., 1996). Fragmentation was minimal and the detection limit was increased by over 5 orders of magnitude compared to FAB. The ions were produced significantly cooler than with FAB and metastable decay was less of a problem. The MALDI spectrum obtained by FTICR was free of the chemical noise, because of the matrix, that is commonly observed in time-of-flight instruments (Cancilla et al., 1996; Carroll et al., 1996).

The choice of matrix is important as some produce more metastable decay than others. There are dozens of matrices and co-matrices that have been investigated for their effectiveness with oligosaccharides (Mohr, Boernsen, & Widmer, 1995; Kolli & Orlando, 1996; Krause, Stoeckli, & Schlunegger, 1996; Nonami, Fukui, & Erra-Balsells, 1997). An interesting study by Novotny used compounds (oxazone) that are structurally similar to oligosaccharides in the hope of inducing better co-crystallization (Chen, Baker, & Novotny, 1997). The best matrices in our hands are 2,5-dihydroxybenzoic acid (Strupat, Karas, & Hillenkamp, 1991) (DHB) and super-DHB (Karas et al., 1993) (a mixture composed of DHB and 2-hydroxy-5-methoxybenzoic acid) for neutral and 2,5-dihydroxyacetophenone (DHAP) (Krause, Stoeckli, & Schlunegger, 1996) for acidic oligosaccharides. We find that matrices that have been found to work well with TOF instruments do not necessarily work well with FTICR. The large time scale difference again enhances the effects of matrices that produce ions too energetically. Generally, MALDI works best for neutral oligosaccharides producing detection limits in the low picomolar and sub-picomolar levels. To increase detection limits, several groups have employed derivatization (Dell, 1987; Naven & Harvey, 1996; Powell & Harvey, 1996; Pitt & Gorman, 1997). Even a single derivative on the reducing terminus can significantly increase the sensitivity of MALDI. We introduced 9-aminofluorene to label oligosaccharides by reductive amination to the reducing terminus for MALDI-FTICR characterization with increased sensitivity (Franz, Molinski, & Lebrilla, 2001). Y- and B-fragments were observed in the presence of sodium dopant, and the protonated of the labeled compounds showed only Y-fragments, which allowed for complete sequence analysis. Interestingly, protonated benzylamine-labeled and 9-aminofluorene-labeled lacto-*N*-fucopentaose I (LNFP I) and lacto-*N*-difucohexaose I (LNDFH I) showed a long-range glycosyl transfer reaction in CID-FTICR mass spectra (Franz & Lebrilla, 2002). L-Fucose was preferentially involved in the transfer reaction and molecular modeling studies supported the proton-catalyzed mechanism for explaining the transfer reaction in gas phase.

We characterized the instrument further with respect to MALDI specifically for oligosaccharides by probing the limits of detection and the resolution. The limits of detection for a diverse group of oligosaccharides were found to be in the pico- to femtomole per liter range; similar to the detection limits reported on TOF instruments (Mock, Davey, & Cottrell, 1991; Stahl et al., 1991; Mohr, Boernsen, & Widmer, 1995). Derivatization increased the sensitivity to the low femtomolar range as it does with TOF instruments (Lemoine, Chirat, & Domon, 1996; Naven & Harvey, 1996; Powell & Harvey, 1996; Takao et al., 1996). The resolution on a routine analysis is in the order of 10^5 (FWHH) and, with additional effort, it is easily increased to 10^6. In addition, the mass accuracy for MALDI and ESI was unmatched by any other types of mass analyzer. With external calibration,

McIver showed that mass accuracies of less than 20 ppm could be reliably obtained with on a relatively low field magnet (Li, McIver, & Hunter, 1994). We published mass accuracies of less than 5 ppm obtained routinely with internal calibration on a similarly low field magnet (Wu et al., 1995).

To analyze neutral and acidic oligosaccharides simultaneously, we developed a new anion dopant for neutral oligosaccharides to detect in negative ion mode (Wong et al., 1999). Dilute H_2SO_4 solution was used as a dopant. A sulfate adduct $[M+HSO_4]^-$ was formed under mild conditions, and a sulfate derivative $[M+HSO_4\text{-}H_2O]^-$ was produced under more concentrated H_2SO_4 solutions ($\sim 10^{-2}$ M). This method allowed simultaneous detection of neutral and acidic oligosaccharides mixtures by the combination of complex formation (with neutral oligosaccharides) and the deprotonation of acidic oligosaccharides. Later, alkylsulfonates were examined as anion dopants to quantify desialylation reactions (Wong, Wang, & Lebrilla, 2000). They produced quasimolecular ions composed of the oligosaccharides and the deprotonated alkylsulfonates. It was found that sulfated oligosaccharides suppressed sialylated oligosaccharides in negative ion mode MALDI-FTICR (An & Lebrilla, 2001). These results were important as both oligosaccharides are found together in many biological preparations. To minimize suppression effects, sialylated oligosaccharides were converted into their methyl ester forms and sulfanilic acid was used as a dopant. This allowed both sialylated and sulfated oligosaccharides to be successfully analyzed in negative ion mode, while minimizing the effect of suppression. To characterize the fragmentation behavior of acidic oligosaccharide metal complexes, sialylated oligosaccharides were doped with two different alkali metals (Cs^+, Na^+, and Li^+) simultaneously. This formed gas-phase mixed metal complexes in MALDI-FTICR (Penn, Cancilla, & Lebrilla, 2000). It was determined that a sialic acid residue is capable of solvating two large alkali metals, such as, cesium, simultaneously. However, two small alkali metals could not be accommodated because of having higher charge densities.

A high pressure MALDI-FTICR was introduced for thermal stabilization of labile biomolecules to significantly decrease fragmentation (O'Connor & Costello, 2001). An ion source was designed where gas pressure (1–10 mbar) was pulsed in the source region during desorption. This work was extended to the analyses of highly labile gangliosides containing up to five sialic acid residues; the molecular ion species were detected with minimal loss of sialic acid residues (O'Connor, Mirgorodskaya, & Costello, 2002). In Figure 2a, a trisialylated ganglioside (G_{T1b}) lost all three sialic acid residues during typical MALDI ionization process. However, fragmentation was reduced when collisional cooling of MALDI ions performed (Fig. 2b). The sodiated molecular ion peak appeared as the base peak in the spectrum without loss of sialic acid residues.

The combination of electrophoresis and MS was successfully introduced in this laboratory in which open-access channel electrophoresis (ROACHE) was used in conjunction with MALDI-FTICR MS (Liu et al., 2001). Separation was conducted electrophoretically in the open channel, and the chip was directly placed into the MALDI source after solvent evaporation. The running buffer for electrophoresis contained matrix (2,5-dihydroxy benzoic acid). Therefore the sample was ready for analysis after solvent evaporation.

C. Gas-Phase Dissociation Methods

1. Collision-Induced Dissociation (CID)

CID continues to play a prominent role in the analyses of oligosaccharides. The advantage of FTICR (and ion traps in general) is that CID is temporally resolved rather than spatially. It is further easily implemented with either MALDI- or

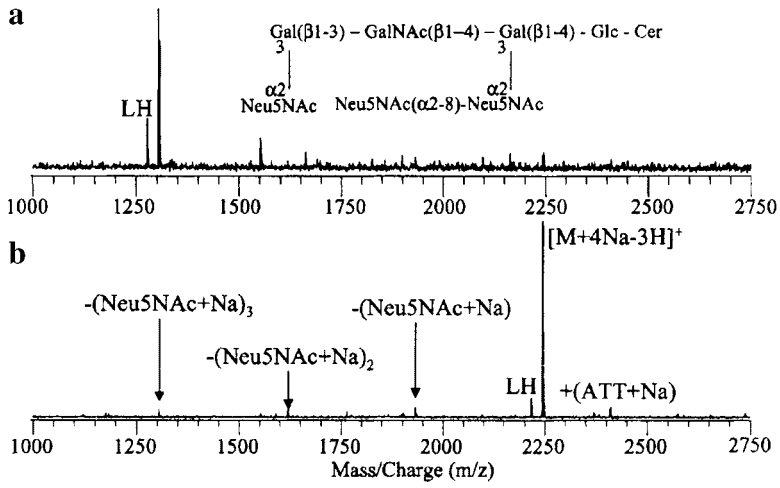


FIGURE 2. Ganglioside G_{T1b} desorbed from 6-azo-2-thiothymine (ATT) matrix (**a**) without collision gas, and (**b**) with collision gas. (Reprinted from O'Connor and Costello, *Rapid Communication in Mass Spectrometry* 2001;15:1862–1868. Copyright © 2001 John Wiley & Sons, Ltd.)

ESI-produced ions. CID in FTICR is performed by isolating the desired ion. This procedure involves the resonance excitation of all other ions to the point where they leave the cell or collide with the cell walls. In the past, ejection of unwanted ions was performed with selective resonance ejection of individual ions. More sophisticated methods have been developed using arbitrary waveform generators that can be programmed for the retention of desired masses (Guan & Marshall, 1996).

Two methods of CID are currently used with FTICR. On-resonance CID uses an excitation frequency equal to the ion's cyclotron frequency to translationally excite the ion (Cody et al., 1982; Cody, Burnier, & Freiser, 1982; Cody & Freiser, 1982). In this method, the ions are translationally excited to a larger orbit. Collision gas is pulsed into the analyzer chamber producing collisions, in which translational energy is converted into vibrational energy. Off-resonance excitation (or sustained off-resonance excitation SORI (Gauthier, Trautman, & Jacobson, 1991)) employs a frequency slightly offset from the ion's cyclotron frequency. A constant RF level is applied to the excite electrodes throughout the CID event. A constant background pressure of collision gas is typically employed. The advantage of this method is that the ions are excited to a smaller radius and de-excited back to the cell center. Thousands of cycles are used and multiple collisions occur throughout the process. The ions are vibrationally excited continuously by relatively low energy collisions. The precursor and fragment ions hover near the center of the cell allowing additional stages of CID and stronger intensities during detection. SORI is the main mode of CID used in this investigation.

2. *Infrared-Multiphoton Dissociation (IRMPD)*

Although CID is invaluable for structural analyses, it has inherent limitations. The total energy imparted on the ion is limited by instrumentation design. The conversion of translational energy to internal energy is not uniform and varies considerably with the size of the ion, which in turn leads to variable fragmentation efficiencies. Ion loss because of scattering decreases the sensitivity of the method (Williams, Furlong, & McLafferty, 1990). Multiple MS events that are often necessary to provide extensive fragmentation are rendered ineffective by the extensive material losses during each CID event.

In Fourier transform MS, CID further limits the accumulation time as each event adds an additional period to allow the removal of the collision gas. IRMPD provides essentially the same fragment ions as CID (Gauthier, Trautman, & Jacobson, 1991), but the technique has distinct advantages. CID is limited to a maximum kinetic energy that depends on the magnetic field strength and the geometry of the analyzer cell. The energy in IRMPD is limited primarily by the power supplied by the laser. IRMPD experiments have higher duty cycles than CID as the latter employs a collision gas that requires a pump-down period.

IRMPD is well suited for FTICR MS and other ion-storage mass analyzers, but it has not been widely applied for bioanalyses. IRMPD analyses of several small compounds have long ago been reported (Morgenthaler & Eyler, 1979; Dunbar, 1984; Thorne & Beauchamp, 1984; Bensimon, Rapin, & Gaumann, 1986; Gauthier, Trautman, & Jacobson, 1991; Dunbar et al., 1995). Studies of IRMPD of proteins have also been reported (Little et al., 1994; Dufresne, Wood, & Hendrickson, 1998). *De novo* sequencing of proteins, often with 100% sequence coverage, was demonstrated with IRMPD (Little et al., 1994). Similar capabilities with DNA and nucleotides were also demonstrated (Little & McLafferty, 1995, 1996; Little et al., 1996). Nucleotides were also dissociated in an external ion reservoir of an FTICR mass spectrometer (Hofstadler, Sannes-Lowery, & Griffey, 1999). Phosphorylated peptides were similarly examined in the negative mode (Flora & Muddiman, 2001).

Costello and co-workers showed that IRMPD provides fragments of the glycan components of glycopeptides, leaving the peptide portion intact under certain conditions (Hakansson et al., 2001). An earlier study used IRMPD to validate the structures of saccharomicins, which are heptadecaglycoside antibiotics (Shi et al., 1999). These studies involved primarily protonated species of glycoconjugates. However, free oligosaccharides are typically coordinated to alkali metal ions for ionization.

To perform IRMPD experiments, minor modifications may be necessary on some commercial instruments. The modification performed on our instrument involved replacing an internal electron impact source on the trapping plate. The trapping plate with the electron filament block was removed and replaced with a copper plate containing a 13-mm hole in the center. Four copper wires (0.24 gauge) were fixed by screws on the plate, two horizontally and two vertically, over the hole and set 3.6-mm apart. The existing aluminum vacuum chamber was fitted with a 70-mm diameter BaF_2 window (Bicron Corp., Newbury, OH). A Parallax continuous wave CO_2 laser (Waltham, MA) with 20-W maximum power was mounted onto the magnet and aimed directly at the center of the analyzer cell.

The IRMPD of alkali metal coordinated oligosaccharides are illustrated in Figure 3. These compounds were isolated from biological sources, that is, mucin-type oligosaccharides from extracelluar matrix (Tseng et al., 1997; Tseng, Hedrick, & Lebrilla, 1999; Xie et al., 2001). These *O*-linked glycans are most conveniently removed as alditols making the reducing end residue unique. Alditols often fragment from the non-reducing end first, thereby leaving the alditol residue as the smallest observable mass (Tseng et al., 1997; Tseng, Hedrick, & Lebrilla, 1999). This allows the determination of the reducing end as being either a hexaose or an *N*-acetylhexaosamine. We have posited that the open alditol residue coordinates the alkali metal more strongly than the pyranose residues, making it often the smallest fragment in the CID spectra.

The IRMPD spectrum of an *O*-linked oligosaccharide alditol ($m/z = 1065.3$) isolated from the egg-jelly glycoproteins of South African toad, *Xenopus laevis*, is shown in Figure 3a. This spectrum contains nearly all the same ions as the CID spectrum (Fig. 3b). For comparison, the fragment ion spectra produced from elevated MALDI laser fluence, a type of "in-source dissociation" (ISD), is shown in Figure 3c. Note the absence of cross-ring cleavages in all the spectra; they are typically not observed with reduced oligosaccharides. Generally, we find ISD to provide the most structurally useful fragment ions. For example, fragments $Y_{1\beta}/Y_{2\alpha'}/Y_{2\alpha'}$ ($m/z = 408.2$) and $B_{3\alpha}/Y_{2\alpha'}/Y_{3\alpha'}$ ($m/z = 347.1$), which are two key fragments for determination

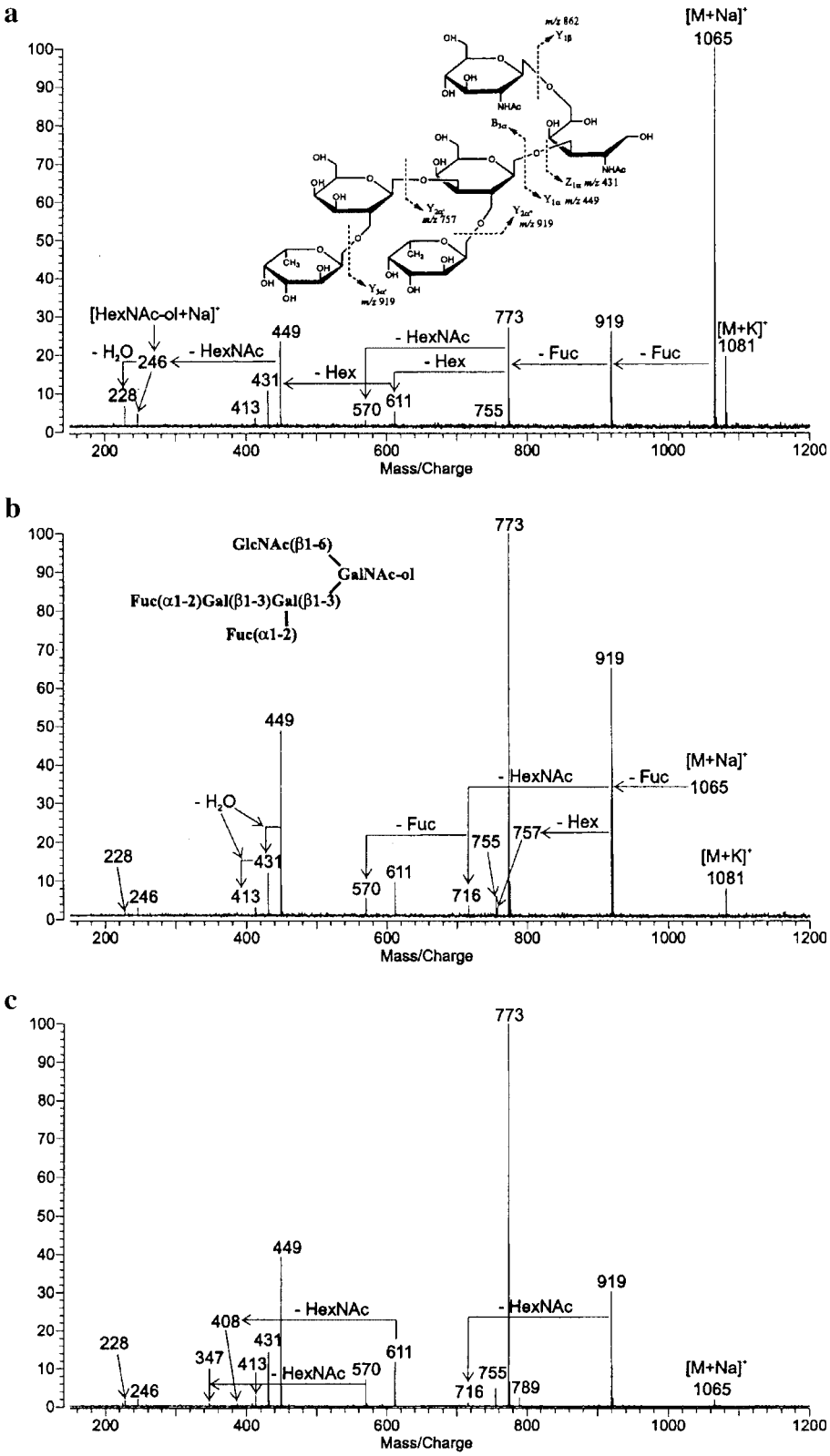


FIGURE 3. **a**: IRMPD spectrum, (**b**) CID spectrum, and (**c**) fragmentation ion spectra produced from ISD (in-source decay) of an *O*-linked oligosaccharide alditol ($m/z = 1065.3$) isolated from the egg-jelly glycoproteins of *Xenopus laevis*.

of the structure, were missing in both IRMPD and CID spectra (Fig. 3a,b). A major limitation of the ISD spectra is that structural elucidation is possible only with pure compounds.

A weakly abundant $Y_{2\alpha'}$ ($m/z = 757.2$) fragment, diagnostic of the Gal(β1-3)Gal linkage (Tseng, Hedrick, & Lebrilla, 1999), was not observed in the IRMPD but was observed as a weak signal in the CID spectrum. However, small fragments, like alditol residue ions ($m/z = 228.1$ and 246.1) were clearly and consistently observed in the IRMPD spectra but were often absent in the CID spectra. In our experience, we found that compounds with molecular weight greater than 1,300 Da generally required more than two MS stages of CID to yield small fragments that correspond to the alditol residue. However, the IRMPD readily yielded the monosaccharide alditol residue in a single MS/MS event.

The two major reaction pathways under IRMPD are the loss of the metal (dissociation) and the fragmentation of the oligosaccharides. For the small alkali metals (Li^+ and Na^+), the major reaction products were because of fragmentation. For the large alkali metal ions, K^+, Rb^+, and Cs^+, the prominent reaction products observed were the alkali metal ions, corresponding primarily to dissociation. Similar behavior was observed in an extensive CID investigation reported earlier (Cancilla et al., 1999). However, the differentiation between the two reaction channels were not as distinct in CID. Fragmentation reactions were observed prominently in CID even with K^+, Rb^+, and Cs^+, albeit the relative abundance of the bare metal ion increased with the size of the alkali metal.

The binding energy of the alkali metal to oligosaccharides decrease with the order $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$ (Cancilla et al., 1996). For this reason, alkali metal ion loss is greater for Cs^+ and less for Li^+. This order is also the same for the ability of the metal to catalyze charge-site fragmentation. Li^+ and Na^+ produce more fragments than the larger alkali metal ions. The energy diagram in Figure 4 describes the two types of behavior. For Li^+ and Na^+, the reaction threshold is higher for metal ion loss than for fragmentation (Fig. 4a). For the larger alkali metal ions, K^+, Rb^+, and Cs^+, the reverse is true. The metal ion dissociation has a lower threshold than fragmentation (Fig. 4b).

There are mainly similarities in the CID and IRMPD spectra of oligosaccharides. However, there are minor but critical differences between the two dissociation methods. The larger variations in energy transferred during collisions provide dissociation pathways that are not accessible to IRMPD. CID therefore may provide characteristic ions that allow better differentiation of stereoisomers and linkages. However, for the oligosaccharides, we found very few variations in the ions produced. IRMPD provides energy not only to the isolated ions but also to the fragment ions to produce a cascade of fragments down to the last residue. For the oligosaccharide alditols, it allows the rapid determination of the reducing end. This information is accessible by CID often only through multiple tandem MS events. In this regard, IRMPD and CID offer complementary information. IRMPD provides another method in the toolbox needed for oligosaccharide analyses.

In situations when high laser power is used, IRMPD can be used to isolate specific masses from the analyzer cell. Infrared laser isolation (IRLI) was used to isolate ions by exciting the

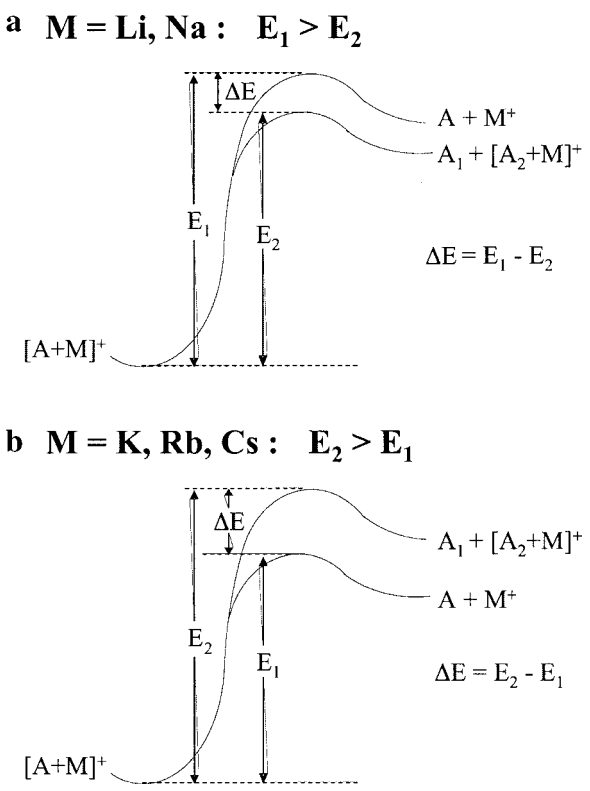


FIGURE 4. Energy diagrams for (**a**) small alkali metal ions (Li^+ and Na^+), and (**b**) large alkali metal ions (K^+, Rb^+, and Cs^+).

desired ions to a radius larger than the beam radius (Xie, Schubothe, & Lebrilla, 2003). The ions in the center of the analyzer cells decompose in the highly degradative beam to yield protons or alkali metal ions that are readily lost through ion evaporation from the analyzer cell. This concept was illustrated for mixtures of oligosaccharides where the isolated species was further dissociated by CID and IRMPD.

3. Electron Capture Dissociation (ECD)

The use of low energy electron to yield fragmentation in multiply positively charged analytes is the main concept behind ECD. The process can in principle be applied to any trapped analyzer but it has been primarily applied to FT ICRMS. The method has found wide applications in the so-called ''top-down'' approach where the whole intact protein is simultaneously sequenced (Fridriksson et al., 2000). A more intriguing use of this dissociative method is to search for post-translation modification such as glycosylation. Zubarev and co-workers used ECD to localize *O*-glycosylation sites on peptides (Mirgorodskaya, Roepstorff, & Zubarev, 1999). Large glycopeptides derived from immunoglubilin were also analyzed in the similar manner (Fridriksson et al., 2000) with the site determined and the heterogeneity of five high-mannose glycoforms characterized.

Trypsin-derived glycopeptides from a lectin were analyzed by Nilsson and co-workers (Hakansson et al., 2001). IRMPD and ECD were compared directly with ECD yielding predominantly fragmentation along the peptide chain and IRMPD fragmentation along the oligosaccharide chain. While ECD shows considerable promise for proteins and peptides, it may be of limited use to oligosaccharides. Neutral oligosaccharides are typically observed as sodium-coordinated species whereas multiply anionic oligosaccharides, though multiply charged, are often observed as anions and would not be readily amenable to ECD.

III. FUNDAMENTAL STUDIES OF OLIGOSACCHARIDE FRAGMENTATION

A. Dissociation of Oligosaccharides

The study of metastable decay of FAB produced ions was informative and helped us realize the limitations associated with this ionization method. An understanding based on fundamental knowledge of ion dissociation mechanisms and processes are key towards developing useful analytical tools. To this end, we have pursued greater understanding of the unimolecular dissociation of oligosaccharides with MALDI.

A systematic study employing MALDI on neutral oligosaccharides with varying structures and the alkali metals yielded several interesting trends regarding oligosaccharide structure and the alkali metal ion (Cancilla et al., 1996). First, a correlation was found between the degree of branching and the fragment ion yield (Fig. 5). The amount of fragment ions decreased with increased branching. With molecular dynamics simulations, we demonstrated that the preferred site of binding by the alkali metals (Na^+, K^+, Rb^+, and Cs^+) was on the branched points of the oligosaccharides. Shown in Figure 6 is the result of structure minimization calculations using molecular dynamics of Cs^+ coordinated to a highly branched oligosaccharide. It appears that the metals are less likely to promote cleavages of glycosidic bonds when they are positioned at the branched sites. This may be related to how the metal is coordinated. If the metal is highly coordinated then it is less apt to produce fragmentation.

Second, the amount of fragments decreased with increasing alkali metal ion sizes (Cancilla et al., 1996). This phenomenon was illustrated by the MALDI-FTICR study of LiCl and CsCl doped difucosyllato-*N*-hexaose (spectra not shown). The Cs^+-doped oligosaccharide samples produced significantly fewer fragment ions (<5%) with MALDI-FTICR than Li^+ doped samples (90%). As we will show below, the difference in fragmentation aptitude is useful for elucidating mixtures and differentiating molecular ions from fragments. The phenomenon is a general one and was observed with every oligosaccharide that was analyzed. We proposed two possible reasons for the observed behavior. One is related to the strength of the binding between the metal and the oligosaccharide. Studies with crown ethers show that Li^+ is more strongly bound than Cs^+ (Zhang et al., 1991; Katritzky et al., 1992). Gas-phase cationization, which is known to occur during MALDI, may produce more energetically excited quasimolecular ions with Li^+ because it forms the stronger bond. Alternatively, Li^+ coordination may produce a lower dissociation barrier because it has a higher charge density than Cs^+.

Third, there exists a minimum number of monosaccharide units necessary to observe a quasimolecular ion during MALDI-FTICR (Cancilla et al., 1996). This conclusion is based on the study of a homologous series of oligosaccharides ranging in size from maltose to maltoheptaose. For Li^+ and Na^+, two hexoses are necessary to observe the complex. For K^+, maltotriose is the smallest oligosaccharide that will produce a

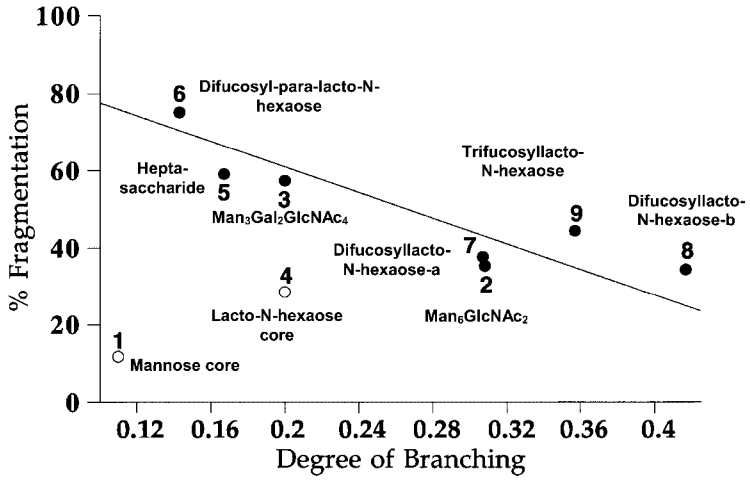


FIGURE 5. Plot of fragmentation versus branching. Each number corresponds to a specific oligosaccharides. The branching value is assigned according to the procedure described (Cancilla et al., 1996). The empty circles belong to oligosaccharides that compose the core branched group. These oligosaccharides are particularly stable.

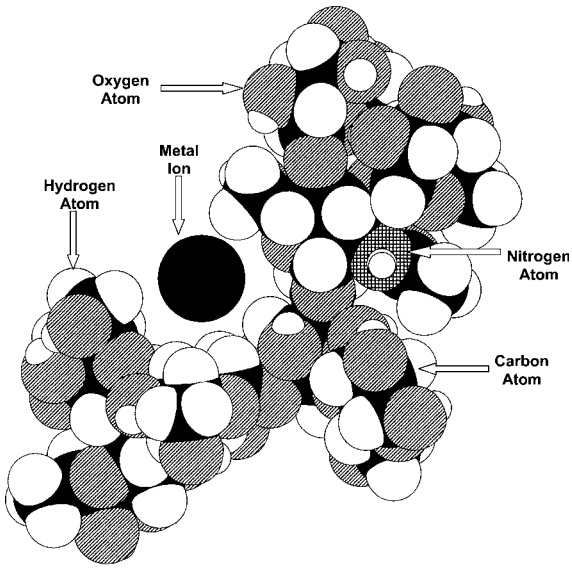


FIGURE 6. Molecular modeling result of Cs^+ coordinated to a branched OS. Coordination is preferred near the branched site where the interaction between the metal and the oligosaccharide is greatest.

quasimolecular ion. Quasimolecular ions with Rb^+ are observed with oligosaccharides as large as maltotetraose, whereas Cs^+ is observed only with oligosaccharides that are at least as large as maltopentaose.

CID is readily implemented in MALDI-FTICR (Penn, Cancilla, & Lebrilla, 1996). Glycosidic bond and cross-ring cleavages are obtained on alkali metal coordinated species. The advantages of CID coupled to FTICR were readily apparent. High resolution, high-mass accuracy of the fragment ions, and multiple MS stages (up to four have been performed so far in our laboratory) are readily obtained.

B. Energetics of Dissociation

CID was recently used to determine factors that affect the fragmentation of oligosaccharides. A method was developed to determine relative dissociation thresholds using off-resonance CID and multiple collision conditions. It was shown that although absolute values could not be obtained, this method provides relative values that reproduced the qualitative trends in a series of crown ethers. Relative appearance energies were determined for the three major gas-phase fragmentation reactions of oligosaccharides complexed to alkali metal ions using MALDI-FTICR. Three fragmentation reactions were examined including: metal ion dissociation, glycosidic bond cleavage, and cross-ring cleavage.

The relative strengths of alkali metal binding to *N*-*N*′diacetylchitobiose (chitobiose) and *N*,*N*′,*N*′-triacetylchitotriose (chitotriose) were determined. Chitobiose provided a suitable model for alkali metal dissociation. It is a disaccharide that binds all the alkali metal ions because the amide groups of the two *N*-acetylhexoses act as strong ligands to alkali metal ions. In contrast, disaccharides that are composed only of hexoses do not readily bind K^+, Rb^+, and Cs^+, as discussed in the above section. The dissociation of alkali metal ions decreased in the order $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$; the order follows increasing alkali metal ion size (Table 2) (Cancilla et al., 1999). A similar, although less obvious trend, was observed for chitotriose. For the trisaccharide and all other larger oligosaccharides, dissociation of alkali metal ions becomes a dominant process and the preferred mode of reaction for K^+, Rb^+, and Cs^+.

Relative dissociation thresholds for common glycosidic bond fragmentation reactions were also determined as a function of the alkali metal ion. As a model for glycosidic bond cleavage, loss of fucose was examined for a set of isomeric trisaccharides and isomeric hexasaccharide. From Table 3, it is shown that increasing the alkali metal ion size also increases the dissociation threshold. The results further suggest that glycosidic bond cleavage is a charge-induced process rather than a charge-remote one. Larger alkali metal ions make it more difficult to produce glycosidic bond cleavage than smaller metals.

In contrast to glycosidic bond cleavage, cross-ring cleavages are charge-remote. Threshold studies involving the alkali metal ions and isomeric tetrasaccharides, maltotetraose and isomaltotetraose, show that cross-ring cleavages are independent of the alkali metal ion for Li^+, Na^+, and K^+ (Table 4). The reaction monitored is the loss of 60 mass units from the quasimolecular ion corresponding to the $^{1,3}A$ cleavage.

IV. CHEMICAL AND ENZYMATIC DEGRADATION METHODS OF OLIGOSACCHARIDES

A. Chemical Degradation Methods

We reintroduced the method of alkaline degradation and coupled it with MALDI-FTICR for the sequence and linkage determination of oligosaccharides (Cancilla, Penn, & Lebrilla, 1998). Alkaline degradation, or the ''peeling'' reaction, has long been used as a degradative method for oligosaccharide analysis (Whistler & BeMiller, 1958; BeMiller, 1965; Kennedy &

TABLE 2. Relative appearance energies of alkali metal ions bound chitobiose

Alkali Metal Ion	AE	Internal Energy (300K)	AE_{cor} (300 K)
Cs^+	0.5	0.8	1.3
Rb^+	1.0	0.8	1.8
K^+	1.3	0.8	2.1
Na^+	1.7 - 2.5[a]	0.8	2.5 - 3.3
Li^+	>2.5[a]	0.8	>3.3

All values in eV. Estimated error is ±0.2 eV.
[a]Estimated, dissociation of alkali metal ion was not observed.

TABLE 3. Relative appearance energies for dissociation of anhydro-fucose from two pairs of fucosylated, isomeric oligosaccharides

Alkali Metal	2-FL[a] AE	2-FL[a] AE_{cor} (300 K)	3-FL[a] AE	3-FL[a] AE_{cor} (300 K)	LNDFH-I[b] AE	LNDFH-I[b] AE_{cor} (300 K)	LNDFH-II[b] AE	LNDFH-II[b] AE_{cor} (300 K)
Li^+	1.3	2.2	1.1	2.0	2.4	4.2	2.3	4.1
Na^+	1.4	2.3	1.3	2.2	2.5	4.3	2.5	4.3
K^+	NO[c]	-	NO	-	2.8	4.6	2.8	4.6
Rb^+	NO	-	NO	-	2.9	4.7	2.8	4.6
Cs^+	NO	-	NO	-	3.1	4.9	2.9	4.7

All values in eV. Estimated error is ±0.2 eV.
[a]Calculated internal energy = 0.9 eV (300 K).
[b]Calculated internal energy = 1.8 eV (300 K).
[c]NO = Quasimolecular ion not observed.

White, 1979; Knirel et al., 1995; Yang & Montgomery, 1996a,b). The reaction has been known for over 80 years; it is one of the earliest methods used to determine linkages of oligosaccharides.

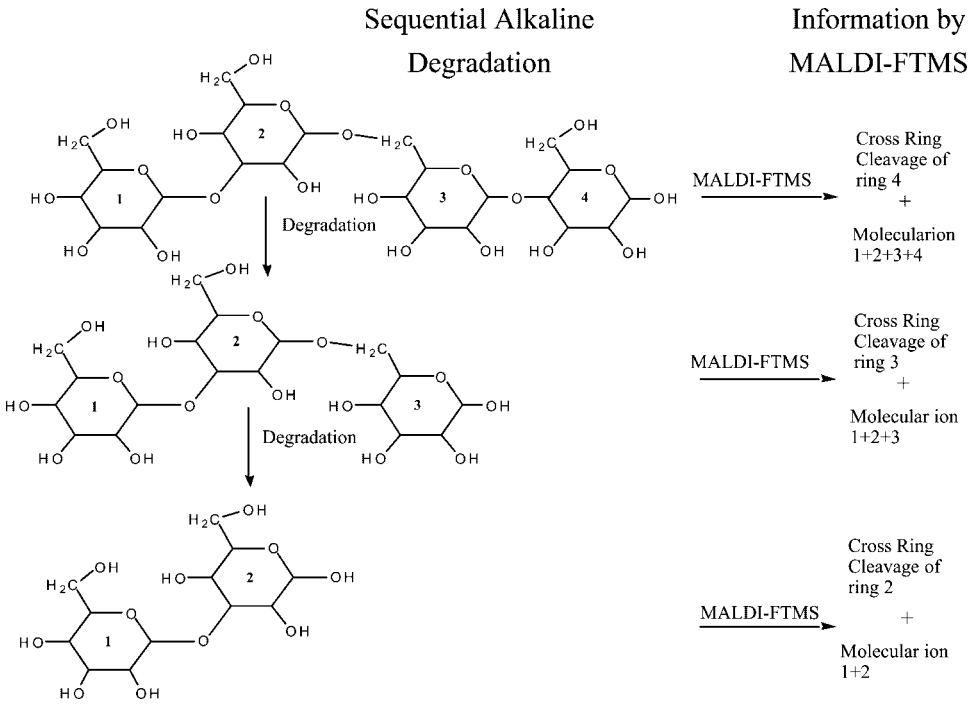
Alkaline degradation works by sequentially dissociating (or depolymerizing) the oligosaccharide beginning with the reducing ring. In this way, it has similarities to the commonly known Edman degradation for peptides which provides sequence information beginning with the N-terminus. Sequence information is obtained because alkaline degradation is an inefficient process. It has long been known that alkali-stabilized intermediates are produced during the course of the reaction. These intermediates are typically rearrangement products. These reactions and other side reactions, such as epimerization, are fortunately often transparent to the MS analysis. The method has several advantages over existing chemical degradative techniques. It employs inexpensive and readily available reagents; only NaOH is used. It does not contain reagents that unnecessarily complicate the MS analysis. It also involves minimal sample work-up; the addition of dilute HCl suffices.

Despite the large number of studies on alkaline degradation (Whistler & BeMiller, 1958; BeMiller, 1965; Aspinall, 1977; White & Kennedy, 1988), the majority of these have been on the aldonic acids (the small monosaccharide fragment). The larger fragment was often ignored because of limitations in the methods of analysis. Linkage information was inferred from the rate of the reactions. The order of decreasing reaction rate is known to follow 1,3 > 1,4 > 1,6 > 1,2. The 1,3 linkage is the most reactive. Cleavage of this bond is known to occur in cold, dilute, alkali solutions. The 1,4-linked oligosaccharide also does not cleave to completion because of a side reaction that forms an alkali stable oligosaccharide complex. The 1,6-linked disaccharide is less

TABLE 4. Relative appearance energies for the cross-ring cleavage fragment loss of $C_2H_4O_2$ (−60 u) from the respective quasimolecular ion

Alkali Metal Ion	Maltotetraose[a] AE	Maltotetraose[a] AE_{cor} (300 K)	Isomaltotetraose[a] AE	Isomaltotetraose[a] AE_{cor} (300 K)
Li^+	1.6	2.8	1.3	2.5
Na^+	1.6	2.8	1.3	2.5
K^+	1.7	2.9	1.2	2.4
Rb^+	b	-	b	-
Cs^+	b	-	b	-

All values in eV. Estimated error is ±0.2 eV.
[a]Calculated internal energy = 1.2 eV (300 K).
[b]Metal ion loss is predominant dissociation pathway.



SCHEME 1. Sequential alkaline degradation.

reactive than 1,4-linked but also cleaves at elevated temperatures. The 1,2 linkage does not react even under the most vigorous conditions. With a branched oligosaccharide that has, for example, both 1,3 and 1,6 linkage at the branch point, the reaction will proceed via the 1,3-linked antenna first. The most important feature of this reaction to MS is that the degraded oligosaccharides contain nascent reducing rings. Cross-ring cleavages, whether with MALDI or CID, occur most readily on the reducing ring. The method is summarized in Scheme 1. Sequential degradation is obtained to produce nascent reducing rings that are fragmented by MALDI or CID. Sequence is obtained because the reaction is inefficient and representative fragments corresponding to nearly all degradative cleavages are observed.

For MALDI, the net result of alkaline degradation is that glycosidic bonds are cleaved beginning with the reducing ring. Each cycle reveals a new reducing ring so that MALDI and MALDI-CID fragmentation can be employed to produce cross-ring cleavages at the nascent reducing end. Furthermore, because the quasimolecular ions are sodium coordinated, cross-ring cleavages are produced to yield the specific linkages (Coates & Wilkins, 1985; Garozzo et al., 1990; Spengler, Dolce, & Cotter, 1990; Carroll & Lebrilla, 1992; Carroll, Willard, & Lebrilla, 1995). Both linkage and sequence information are obtained with less than 100 pmol.

The result obtained with LNFP-1 illustrates in some detail the utility of AD coupled to MALDI-FTICR. The MALDI-FTICR spectrum of the untreated compound shows only the quasimolecular ion and the prominent loss of fucose (spectrum not shown). No other structural information is obtained. CID of the quasimolecular ion produces only more fucose loss until the ion is totally defucosylated.

After treatment of the oligosaccharide for 24 hr with sodium hydroxide at 60°C, there are sufficient fragment ions in the MALDI-FTICR spectrum to sequence the oligosaccharide and determine most of the linkages (Fig. 7). The cleavage pattern for LNFP-1 obtained with AD-MALDI is shown (Scheme 2). 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 ($m/z = 696.233$) and B_3 ($m/z = 534.180$), are also obtained. There are combinations of cleavages such as $m/z = 388.120$ corresponding to a $B_3 + Y_4$ cleavage (designated as B_3/Y_4), $m/z = 406.131$ because of C_3/Y_4, and $m/z = 568.186$ because of C_4/Y_4. In addition, cross-ring cleavages such as $^{0,3}A_4$ show the 1,3-linkage at the fourth saccharide position. The advantages of AD for fucose containing oligosaccharides are further illustrated in this example. The uninformative loss of fucose is still obtained, $m/z = 730.238$, during MALDI. However, the molecule remains sufficiently intact so that the placement 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 non-reducing end and identifies the position of fucose.

CID is useful for obtaining cross-ring cleavages at the nascent reducing end when they are absent in the MALDI

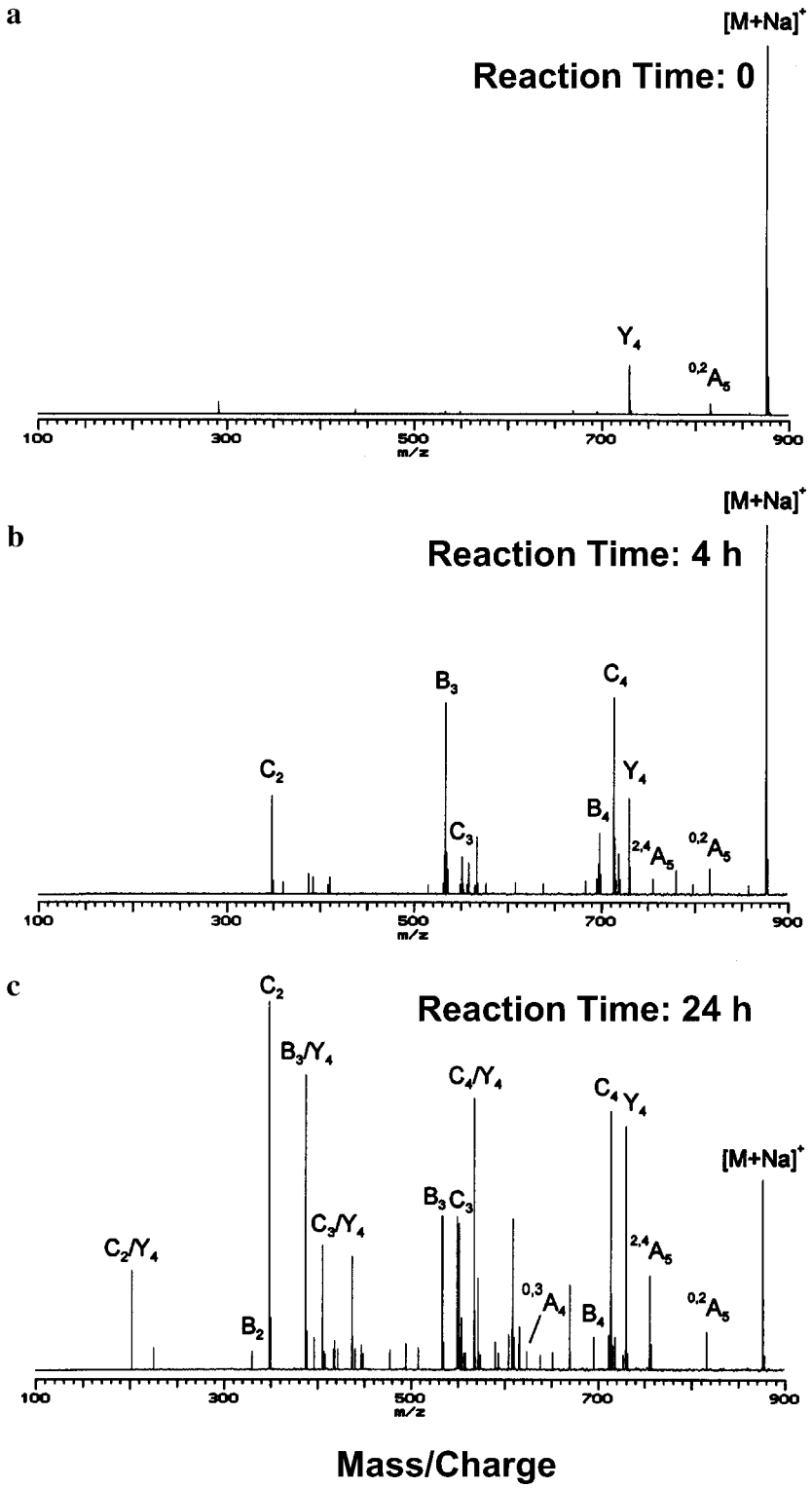
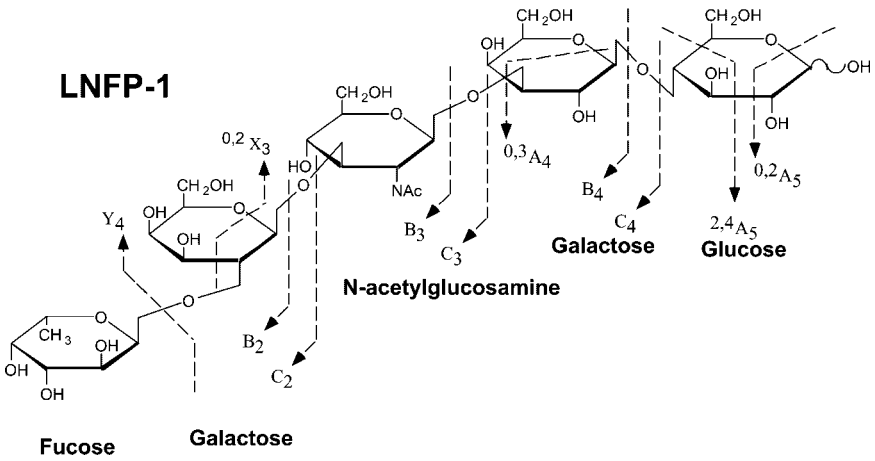


FIGURE 7. MALDI-MS spectrum of LNFP-1 after 24 hr under alkaline degradation conditions.



SCHEME 2. Cleavage pattern for LNFP-1 after treatment of alkaline degradation and analyzed by MALDI-FTICR.

spectrum. No cross-ring cleavages are observed for saccharides 2 and 3 (1 is the non-reducing ring) in the MALDI spectrum. The fragment because of C_2 cleavage ($m/z = 349.109$) was subjected to CID (spectrum not shown). From the spectrum we observe an $^{0,2}X_3$ ($m/z = 229.069$) cleavage yielding the 1,2 linkage at saccharide 4. The saccharide 3 does not yield cross-ring cleavages even with CID. This linkage combination (i.e., Glc1 → 3GlcNAc) appears to be unique in this regard. Similar linkage combinations in other oligosaccharides also do not produce cross-ring cleavages.

To illustrate that CID can be used to produce cross-ring cleavages in nearly every nascent reducing ring, a CID map was constructed and is shown in Figure 8. This 2-D plot shows the MALDI of the alkaline degradation reaction mixture on the X-axis and the CID of selected glycosidic bond cleavage products (C_n fragments) on the Y-axis. The CID spectra, along

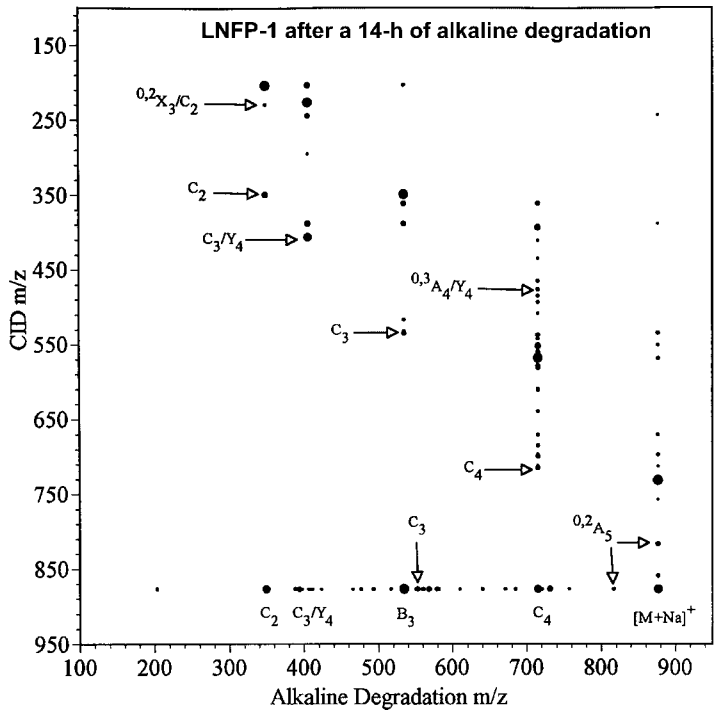


FIGURE 8. CID map of LNFP-1 from alkaline degradation.

the Y-axis, provide further supporting evidences for the MALDI assignments and confirm the identity of the proposed linkages.

Based on the MS analysis of the alkaline degradation reaction mixture, one can predict the linkage and sequence for this compound to be:

$$\text{Fuc1} \rightarrow \text{2Hex1} \rightarrow \text{(2 or 3)HexNAc1} \rightarrow \text{3Hex1} \rightarrow \text{4Hex}.$$

The only ambiguity is in the third saccharide residue where the linkage may be either 1-2 or 1-3. Both Hex1-2HexNAc and Hex1-3HexNAc combinations do not appear to produce cross-ring cleavages.

The results of the five other oligosaccharides are summarized schematically below (Scheme 3). All compounds were peeled by AD and analyzed by MALDI-FTICR. Despite the large diversity in structures, there were sufficient fragmentations to fully sequence each compound and obtain nearly all the linkage information. Fucosylated oligosaccharides are readily sequenced as shown with LNFP-I, LNDFH-I, and LNDFH-II. Even sialylated oligosaccharides do not appear to pose a problem as shown with LSTa. Branched points are easily being determined as shown by the fragmentation pattern in the AD-MALDI-FTICR spectra of Man-5, LNH, LNDFH-I, and LNDFH-II (spectra not shown).

B. Enzymatic Degradation Methods

Enzymatic degradation has been one of the most important tools for the characterization of oligosaccharide structures amongst biologists. Because of the heterogeneity and structural diversity of oligosaccharides and polysaccharides, it is necessary to use enzymes to degrade them. Exo- and endoglycosidases are commonly used to sequence oligosaccharide structures in combination with other analytical methods (Prime et al., 1996). Johnson et al. characterized *O*-linked oligosaccharide structures purified from serum alpha-fetoprotein using sequential exoglycosidase digestion in combination with fluorescence labeling, HPLC, and MALDI-TOF (Johnson et al., 1999). Examples of commonly used exo- and endoglycosidases are α-D-sialidase, β-D-galactosidase, α-D-galactosidase, α-D-mannosidase, α-L-fucosidase, and endoglycosidase (H, F1, F2, and F3) (Prime et al., 1996). In the author's laboratory, sequential exoglycosidase digestion was used to refine the structure and remove structural ambiguities from mucin *O*-linked neutral oligosaccharides that were released by reductive β-elimination from the jelly coat of the *Xenopus laevis* eggs (Xie et al., 2001). We developed the optimal conditions of exoglycosidase (buffer, temperature, and pH) that are compatible with MALDI-FTICR. This idea was extended to characterize mucin *O*-linked oligosaccharides released from the egg-jelly coats of the *Xenopus borealis* and *X. tropicals* eggs in combination with the catalog-library approach (this will be discussed in Section VI(B)) in our laboratory.

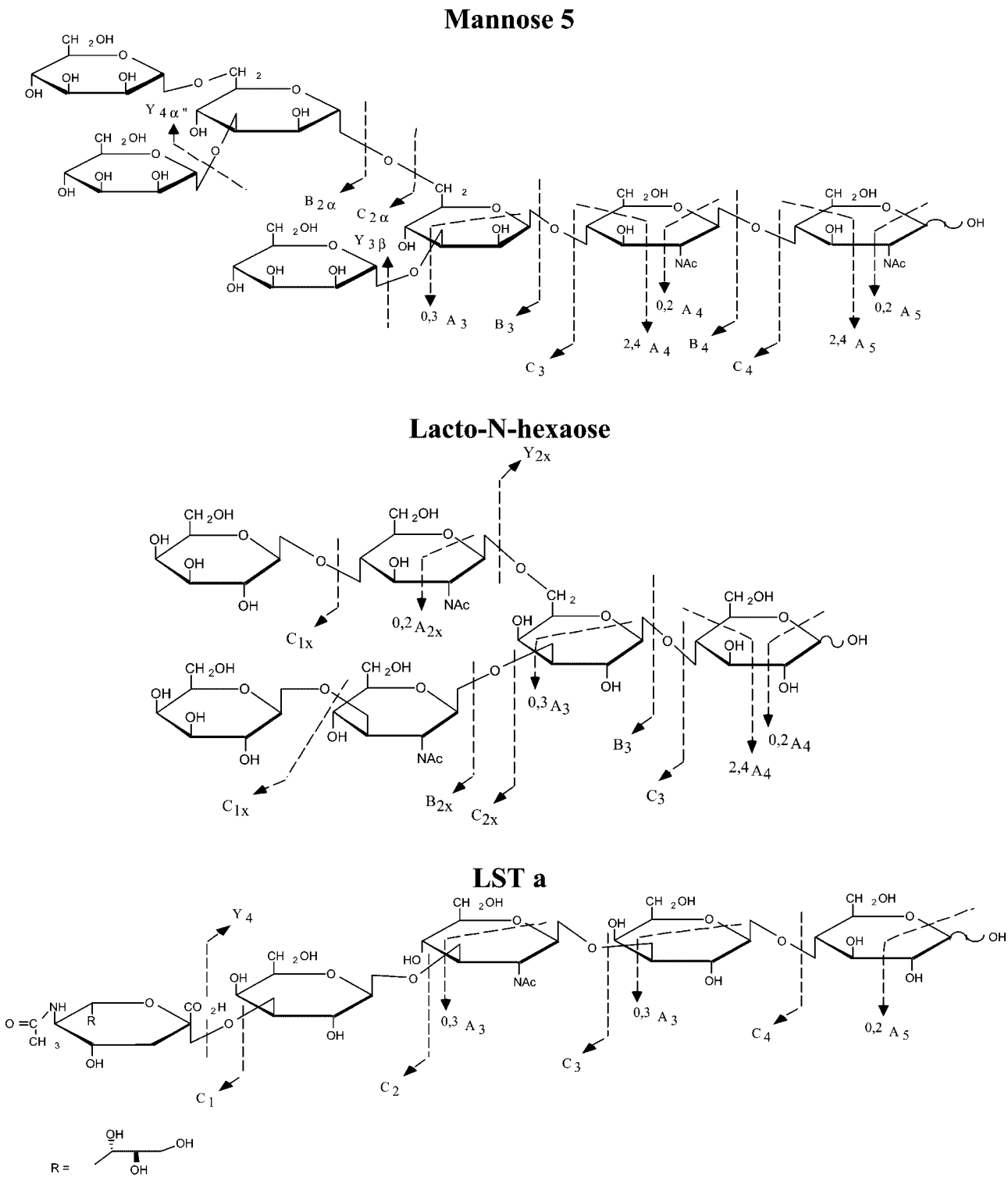
MS in the characterization of glycosaminoglycan structures has emerged. Glycosaminoglycans are generally sulfated except for hyaluronic acid, and the sulfation position/sequence may play important roles in their biological activities. To obtain a better understanding of the roles glycosaminoglycan play, it is important to be able to characterize the position of sulfation and the sequence. Prior to MS analysis, glycosaminoglycans were degraded by enzymes to smaller units for analyses with, for example, HPLC. For degrading glycosaminoglycans, either lyases or hydrolases can be used. Lyases are generally obtained from bacterial sources and act through an eliminase mechanism. Following the lyase digestion, a final product containing a 4,5 unsaturated uronic acid at non-reducing terminus was obtained (Linhardt, Galliher, & Cooney, 1986). For the preparation of chondroitin sulfate-derived oligosaccharides by lyases, chondroitinase ABC, AC I, AC II, C, and B are commercially available. Commercially prepared chondroitinase ABC is comprised of endolytic and exolytic enzymes (Hamai et al., 1997), and chondroitinase such as AC I and II that act endolytically (Hiyama, 1976; Hiyama & Okada, 1976). Heparin/heparan sulfate glycosaminoglycans are degraded by heparin lyases from *Flavobacterium*: heparin lyase I, II, and III (Lohse & Linhardt, 1992). Hydrolases from mammalian sources have a hydrolytic mechanism which generates saturated oligosaccharides at a non-reducing terminus.

ESI-ion trap MS was employed to determine structural characterization of chondroitin sulfate and heparin/heparan sulfate by employing lyase digestion (Desaire, Sirich, & Leary, 2001; Saad & Leary, 2003). Two-step enzymatic digestion protocol was conducted to characterize the sequence type of two chondroitin sulfates polymers (Desaire, Sirich, & Leary, 2001). In the first step, chondroitnase C was used as a selective enzyme, and then in the second step chondroitinase ABC was used as a non-selective enzyme. By heparin lyase treatment on heparin/heparan sulfate, compositional analysis and quantification were achieved by ESI-ion trap tandem MS (Saad & Leary, 2003).

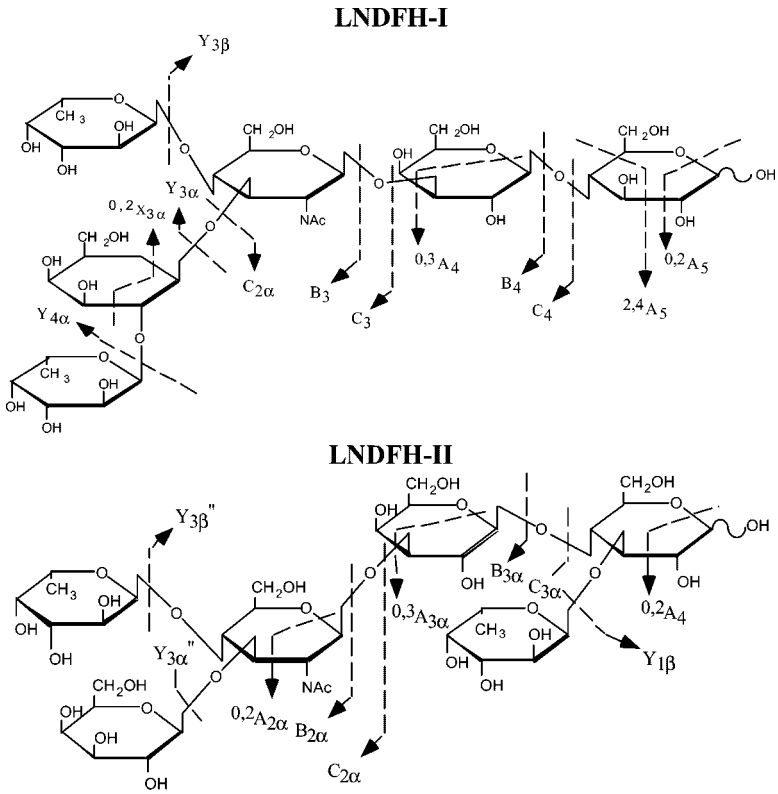
Zaia et al. reported negative ion-ESI using a triple quadrupole instrument to differentiate between positional sulfation isomers (4-*O*- or 6-*O*-sulfation in *N*-acetylgalactosamine) of chondroitin sulfate disaccharides. After digestion of proteoglycans, bovine articular cartilage decorin and biglycan, with chondroitinase ABC exhaustively, sulfation isomers in disaccharides were quantified (Zaia & Costello, 2001). The same group has employed the method using ESI-FTICR tandem MS to determine the 4-*O*-/6-*O*-sulfation sequence in chondroitin sulfate oligosaccharides. Both chondroitinase AC I and testicular hyaluronase were used to study the influence of charge state on the product ion spectra for the determination of the sulfate position on chondroitin sulfate oligosaccharides (McClellan et al., 2002). Their results suggest that even-numbered B*n*, C*n*, Y*n*, and Z*n* ions are predominant when the charge (z) is less than the number of sulfates. On the other hand, the odd-numbered fragmentation ions are predominant when z is equal to the number of sulfates.

V. BIO-AFFINITY MALDI FOR OLIGOSACCHARIDE ANALYSES

Biotin-avidin technology is currently employed to develop a rapid method for the isolation and MS analysis of oligosaccharides.



SCHEME 3. Five oligosaccharides (mannose 5, lacto-N-hexaose, LSTa, LNDFH-I, and LNDFH-II) after treatment with alkaline degradation and analyzed by MALDI-FTICR.



SCHEME 3. (*Continued*)

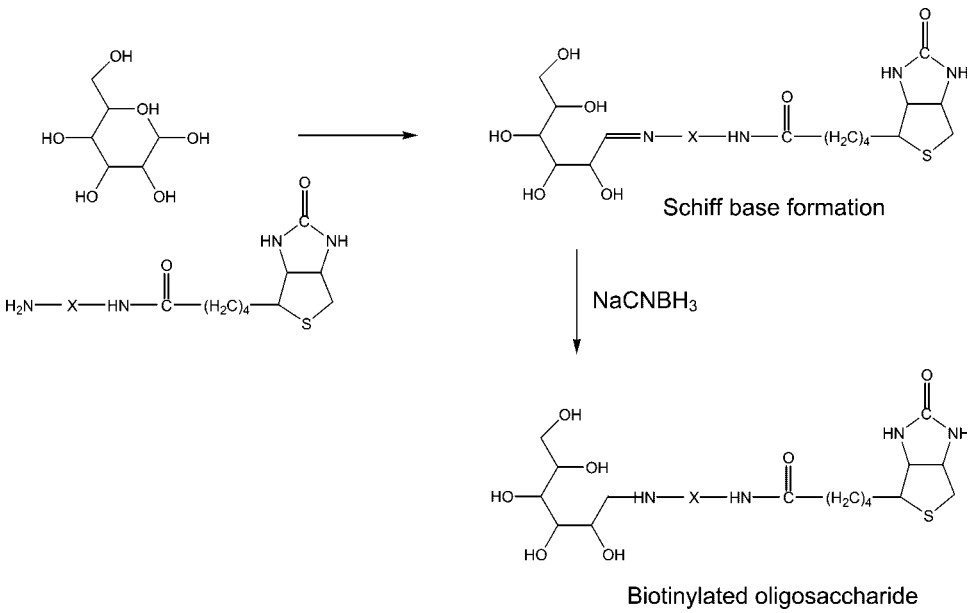
The concept of biologically active probes for selective MALDI has been present for several years. Hutchens has suggested the use of probe surfaces that are designed to extract specific molecules from unfractionated biological fluids and extracts (Hutchens & Yip, 1993). In one application, agarose beads with attached single-strand DNA were used to capture lactoferrin from preterm infant urine and placed directly on the MALDI probe for analysis. Orlando developed probe affinity MS to immobilize monoclonal antibody IgG1 directly on the probe so that biotinylated insulin can be captured and analyzed (Brockman & Orlando, 1995). To determine the specific components of the protein human basic fibroblast growth factor (bFGF) that interact with mouse mAB 11.1, Chait used agarose beads with the antigen to concentrate partially digested components of the protein (Zhao et al., 1996). Li and co-workers used agarose beads containing avidin to extract biotinylated peptides and proteins (bradykinin and insulin) (Schriemer & Li, 1996). The researchers further found that the condition for MALDI preparation caused the degradation of the complex so that the biotin-labeled compound can be deposited on the probe whereas the agarose beads are physically removed. They noted that agarose beads on the probe surface produce deleterious effects on the MALDI spectrum (Nelson et al., 1995).

We produced a number of bioaffinity probes specifically to analyze oligosaccharides to be examined with MALDI-FTICR MS. In the first series of bioaffinity probes, avidin was immobilized directly on the probe surface and the oligosaccharides were biotinylated. In the second series, lectins (oligosaccharide binding proteins) were directly immobilized to a microprous surface to concentrate oligosaccharides on the probe surface.

A. Avidin Affinity Probe

A simple method was developed to immobilize avidin on the MALDI probe, thereby eliminating the use of the agarose beads (Wang, Tseng, & Lebrilla, 1999). An avidin solution was simply applied to the microporous surface and dried. Drying the solution fixes the protein on the surface. Research by Li and co-workers showed that avidin dried on a surface remains active towards biotin (Schriemer & Li, 1996). Once the avidin is immobilized, MALDI preparation involves taking the solution containing the biotin labeled compounds and depositing it on the probe. The biotin-avidin interaction was allowed to take place and the probe was washed to remove non-biotin labeled material. Matrix was then applied and the solvent evaporated.

Derivatization of oligosaccharides by biotin was relatively straightforward. Several types of biotin derivatives are commercially available (Savage et al., 1992). They are functionalized with carboxylic acids or amines and can have long and short spacers ranging in length from 10 to 24 Å. The longer spacer arms were important for this study as they have been shown to allow greater access to avidin immobilized on surfaces (Schriemer & Li, 1996). We adapted a procedure developed by Takao and co-workers for attaching 4-aminobenzoic acid



SCHEME 4. Derivatization of oligosaccharides with biotin by reductive amination. X refers to a variable length spacer.

2-(diethylamino)ethyl ester to neutral oligosaccharides (Yoshino et al., 1995; Takao et al., 1996). This involved forming the Schiff base and reducing the intermediate with sodium cyanoborohydride to produce the corresponding amine (Scheme 4). The "X" in Scheme 4 refers to a variably length spacer.

Several polymer films were examined including the microporous polyethylene, used by Worrall et al. (Worrall, Cotter, & Woods, 1998), as MALDI probe to purify peptides and proteins directly on the probe surface. The best polymer surface for immobilizing avidin, as measured by the intensity of a standard biotinylated oligosaccharide, is a common transparency film (Cannon®) (Table 5). The film was treated simply by wiping the surface with an ethanol dampened tissue. To illustrate the method, two pieces (each consisting of a 2×3-mm probe surface) were prepared. One piece was left untreated whereas the other was treated with a 1 mg/mL solution of NeutrAvidin (a cheaper deglycosylated form of avidin) and allowed to dry. An aqueous solution containing 50:50 labeled (biotinylated) and unlabeled LNDFH-1 was applied to the treated and the untreated surfaces. The sample on the untreated surface (no avidin) was dried and matrix solution was added. Figure 9a shows the MALDI-FTICR spectrum showing the native ($m/z = 1022.37$) and biotinylated LNDFH-1 ($m/z = 1264.49$) deposited on the polymer film. The sample on the avidin treated surface was further washed with 1 mL water to remove the uncomplexed component, dried, and treated with the matrix solution. Figure 9b shows the MALDI-FTICR spectrum of the avidin treated surface. The unlabeled oligosaccharide (arrow) is not observed and is presumably washed away, whereas the biotin labeled oligosaccharide is retained as the major peak in the spectrum.

TABLE 5. Relative intensity (normalized to the transparency film spectrum) of LNDFH-I with bio-affinity MALDI probes produces from several polymer films

Membrane Material	Relative Intensity of Quasimolecular Ion
Transparency Film	1.0
Microporous Polyethylene	0.94
Low-density Polyethylene	0.59
High-density Polyethylene	No Signal
Polyester	0.25
Polyvinylidene Fluoride (PVDF)	No Signal

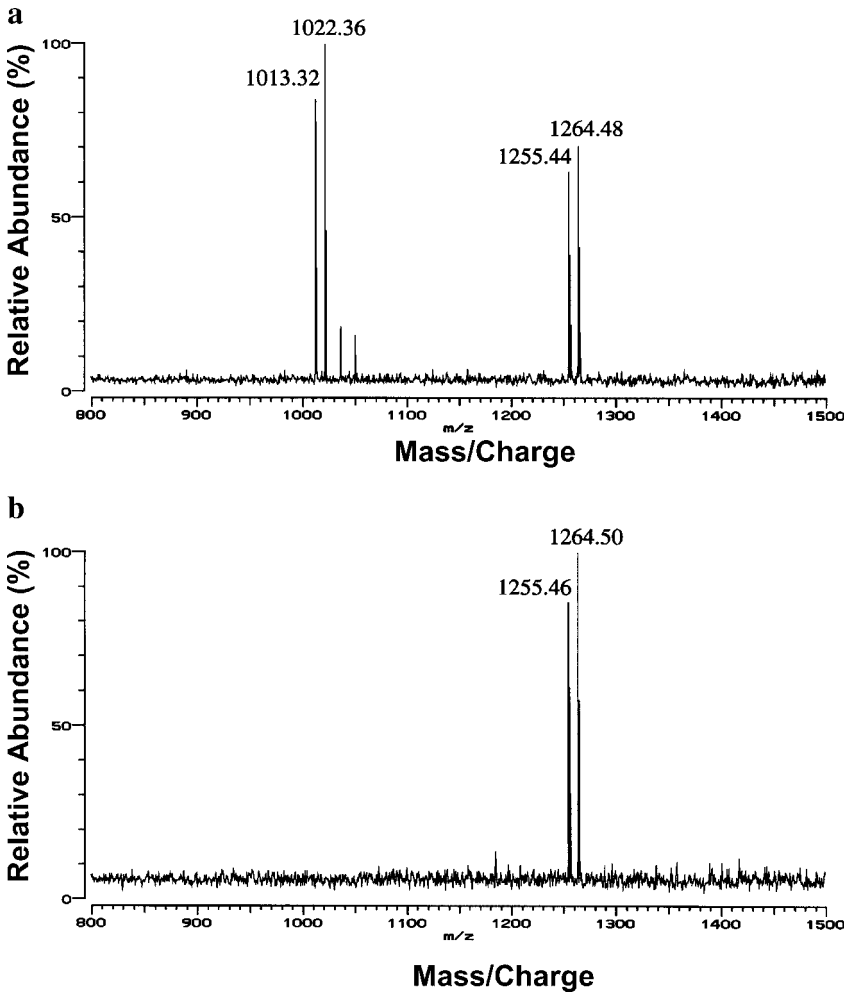


FIGURE 9. **a**: MALDI-MS of biotinylated and native LNDFH-1 applied to a polymer film. Both biotinylated ($m/z = 1264.49$) and native ($m/z = 1022.37$) are observed. The other peaks are because of losses of fucose from the corresponding quasimolecular ion. **b**: The same sample applied to a polymer film treated with avidin. Only the biotinylated compound ($m/z = 1264.50$) is observed.

Biotinylated oligosaccharides of different structures (linear and branched) and polarity (neutral and anionic) have all been examined and found to be retained by the avidin probe without noticeable structural discrimination.

B. Lectin Affinity MALDI

The Fenselau group introduced lectin affinity MALDI technique for bacteria biocapture by MALDI-TOF (Bundy & Fenselau, 1999, 2001; Afonso & Fenselau, 2003). They immobilized conconavalin A to a gold foil via a self-assembled monolayer to generate gold-thiolate linkage (Bundy & Fenselau, 1999). The prepared lectin probe was used for the isolation of bacteria from physiological samples in MALDI-TOF. Afterwards, their work was extended to analyze a variety of microorganisms from physiological buffers, urine, milk, and processed chicken samples by preparing both lectin affinity and carbohydrate affinity MALDI probes (Bundy & Fenselau, 2001). Lectin (conconavalin A and wheat germ) affinity probes were prepared via primary amine using a commercially available affinity membrane. Carbohydrate affinity probes were prepared by immobilizing streptavidin to the membrane surface, followed by reacting with the biotinylated carbohydrates. The authors reported that carbohydrate affinity probes were more sensitive than lectin affinity probes. Recently, the same group used inexpensive thin glass microscope slides that were compatible with MALDI (Afonso & Fenselau, 2003). Biotinylated lectins were immobilized onto the streptavidin-coated slides for purifying agglutinated *Bacillus* spores with reduced fragmentations.

In the author's laboratory, lectins were immobilized on the probe surface using the same method employed for avidin. To characterize oligosaccharides that bind to cortical granule lectin (CGL), CGL was immobilized on the surface of the MALDI

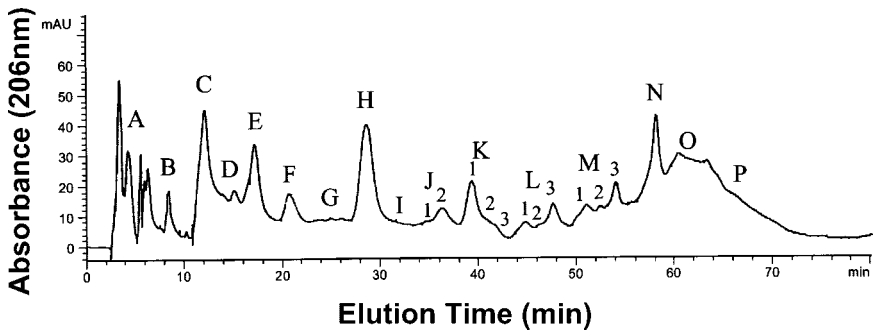


FIGURE 10. HPLC of the jelly coat of *Xenopus laevis*. The sample contains the neutral oligosaccharides.

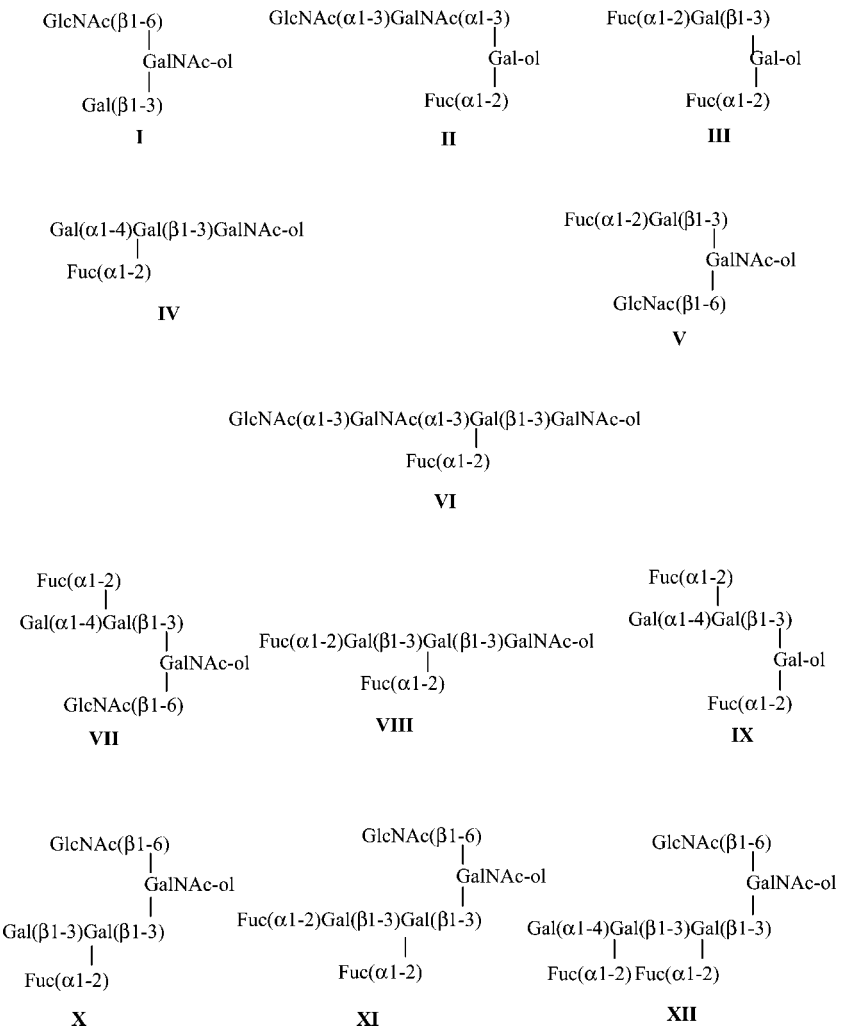


CHART 1. The structure of 12 oligosaccharides elucidated by NMR (Strecker et al., 1995). The structures are confirmed by mass spectrometry (See Table 6).

probe (Tseng et al., 2001). CGL is a lectin that is released from eggs of the South African toad *Xenopus laevis* upon fertilization to prevent polyspermy. The structures of oligosaccharides were elucidated by CID. It was determined that oligosaccharides with the sulfate esters on the non-reducing termini bonded more favorably with CGL, suggesting further that sulfate esters were responsible for preventing polyspermy.

VI. ANALYSIS OF COMPOUNDS FROM BIOLOGICAL SOURCES

We are currently developing analytical methods based on FTICR MS in the elucidation of structures of oligosaccharides in the jelly coat of *Xenopus laevis*. The egg of *X. laevis* has long been a model to study the early events of fertilization (Yurewicz, Oliphant, & Hedrick, 1975). It is surrounded by an extracellular matrix (ECM) through which the fertilizing sperm must pass before fusing with the egg plasma membrane. Mature fertilized eggs of most animals are surrounded by a similar ECM (Yang & Hedrick, 1997). The ECM of *X. laevis* is composed of two morphologically and functionally distinct regions, namely, the egg-jelly coat and the underlying vitelline envelope. The study of *X. laevis* provides insight into human reproduction. It has been shown that the molecular mechanism of fertilization is phylogenetically preserved (Schmell, Gulyas, & Hedrick, 1983). More specifically, significant identity is found between the important glycoproteins in the vitelline envelope of amphibians and the similar glycoproteins in the zona pellucida of mammals. Two classes of oligosaccharides are present in the egg-jelly coat. Anionic oligosaccharides contain sulfates and sialic acids. Neutral oligosaccharides contain neither residues.

A. Characterization of Neutral Oligosaccharides in the Jelly Coat of *Xenopus laevis*

Oligosaccharides were released as alditols using alkaline sodium borohydrate. HPLC on an amine column is used to separate the oligosaccharide components. The mixtures are highly complex containing nearly a hundred different structures. The HPLC chromatogram of only the neutral component showed in Figure 10. The twelve most abundant components of the *O*-linked neutral oligosaccharides in the jelly coat have been identified earlier by Strecker et al. using 2-D NMR (Strecker et al., 1995). The twelve structures, obtained by NMR, are shown in Chart 1. MS verification was not performed in the original publication. Furthermore, because of limitations in sensitivity, several of the minor components that are observable in the HPLC trace could not be identified by NMR. We estimate that the unknown oligosaccharides range in amounts a few picomoles to 500 picomoles. MALDI-FTICR was performed on all discernible eluant peaks. By matching the HPLC traces in the two experiments, we confirmed the identities of the 12 structures with their mass assignments (Table 6) (Tseng et al., 1997). In addition, investigation of the minor components found at least seven additional oligosaccharides. From the mass measurements, the monosaccharide compositions were determined, that is, the number of hexose, *N*-acetylhexosamine, and fucose. Tandem MS (MS^n, $n = 2–4$) experiments were performed to verify further the monosaccharide compositions (Table 7).

TABLE 6. Theoretical and observed mass of oligosaccharide structures assigned by mass spectrometry

Structure	HPLC Label	m/z (Theor.) $[M+Na]^+$	m/z (Obs.) $[M+Na]^+$
I	C1	611.23	611.23
II	C1	757.29	757.29
III	C1	659.24	659.24
IV	C1	716.26	716.26
V	C2	757.29	757.29
VI	D2	960.37	960.37
VII	E	919.34	919.34
VIII	E	862.32	862.32
IX	E	821.29	821.30
X	F	919.34	919.35
XI	H	1065.40	1065.40
XII	Not shown	1227.46	1227.46

The analysis of oligosaccharide mixtures by MS is complicated by the labile nature of the glycosidic bond particularly in the positive mode, which produces numerous cleavage reactions during MALDI. This makes the identification of fragments from quasimolecular ions problematic. We use a method developed in this laboratory for elucidating mixtures of oligosaccharides with MS (Tseng et al., 1997). The method involves doping the sample with CsCl and observing peaks that shift to higher masses. Based on our studies of the fragmentation behavior of oligosaccharides complexed to different alkali metal ions (Cancilla et al., 1996), we know that sodiated (or natriated) compounds dissociate more readily during MALDI than oligosaccharides coordinated to Cs^+. Furthermore, the sodiated species cleave to produce Na^+ coordinated fragments, whereas cessiated species cleave to produce mainly Cs^+. The method is illustrated with an HPLC fraction containing two oligosaccharides. The MALDI of the eluent shows a series of peaks with the quasimolecular ions being $m/z = 1373$ $[1350 + Na^+]$ and 1389 $[1366 + Na^+]$. Also present fragment ions correspond to the loss of fucose ($m/z = 1211$ and 1227) (Fig. 11a). The same sample is doped with CsCl and the analysis is repeated again. The mass of two components have shifted, by the mass difference between Cs^+ and Na^+, to $m/z = 1499$ $[1366 + Cs^+]$ and 1483 $[1350 + Cs^+]$ (Fig. 11b). Unknowns **4**, **5**, and **6** (Table 7) found in G1 (see Fig. 10) were also elucidated in this manner.

B. Catalog-Library Approach to the Structural Elucidation of Unknown Components

The limitations stated earlier with CID and MALDI fragmentations, that is, non-structure specific fragmentation, appear to

TABLE 7. Unknown neutral oligosaccharides determined by mass spectrometry

Unknown	HPLC Label	m/z (Theor.) $[M+Na]^+$	m/z (Exp.) $[M+Na]^+$	Composition Hex:Fuc:HexNAc
1	D1	903.34	903.35	1:2:2
2	D3	903.34	903.35	1:2:2
3	F	1065.40	1065.40	2:2:2
4	G1	919.34	919.34	2:1:2
5	G1	1024.37	1024.36	3:2:1
6	G1	1389.50	1389.48	4:2:2
7	G2	1065.40	1065.40	2:2:2

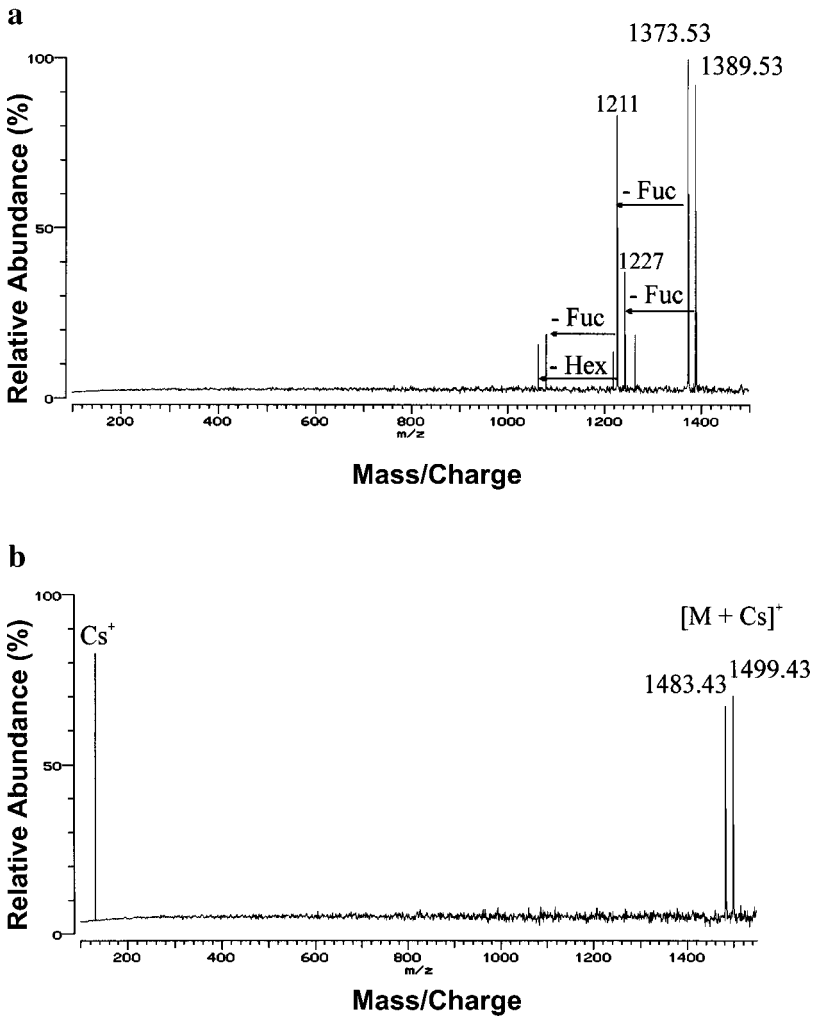


FIGURE 11. **a**: MALDI-MS spectrum of eluant G1 from HPLC. **b**: The same sample doped with Cs^+.

apply mainly to the aldehydes. Unfortunately, many CID studies were performed on aldehydes, which often give non-specific fragmentation. For example, fucoses are more labile during CID producing fragment ions with no information as to the origin of the fucose residue. There is therefore a misconception that CID of oligosaccharides often yields non-structure specific fragmentation. We have performed a systematic study on the CID of all the neutral oligosaccharide alditols released from *X. laevis* (the twelve with known and seven with unknown structures) (Tseng et al., 1997). Our studies show that the fragmentation patterns of the alditols containing fucoses are distinct for each structure. More importantly, specific structural motifs produce distinct fragmentation patterns that are conserved between different compounds. For example, the trisaccharide corresponding to both an *N*-acetylglucosamine $\beta(1 \rightarrow 6)$ and a galactose $\beta(1 \rightarrow 3)$-linked to an *N*-acetylgalactosaminitol (**I**) produces the MALDI-FTICR spectrum shown (Fig. 12a). A compound containing the same structural core as **I** (Structure **VII** in Chart 1) exhibits similar fragmentation behavior at the low masses (Fig. 12b). Note that the same peaks with similar abundances are found in both spectra below $m/z = 700$. This structural motif is found in three other compounds and each shows the same fragmentation pattern in that mass range. We have identified five substructural motifs (Chart 2) that have distinct fragmentation patterns. The five substructural motifs compose the catalog.

The construction of the catalog provides a method to elucidate structures of unknown oligosaccharides based solely on CID spectra. The presence of specific subtructural motifs is determined from the CID spectra. The unknown structure is

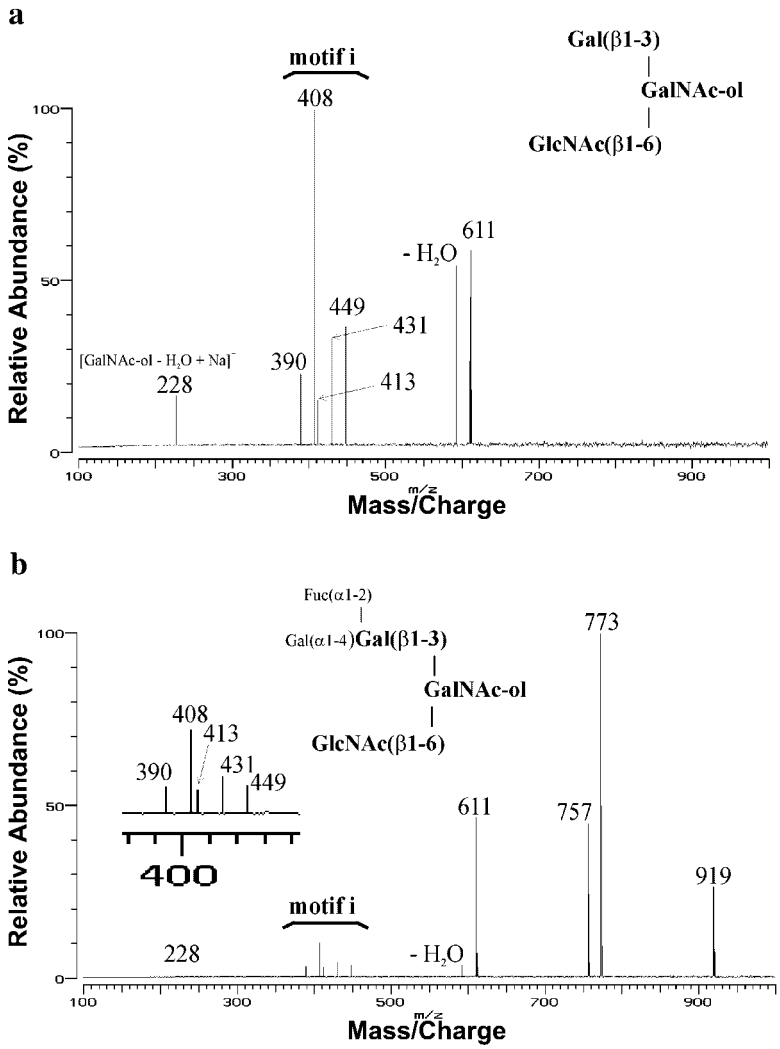


FIGURE 12. **a**: MALDI-MS spectrum of Compound **I** (Chart 1). **b**: MALDI-MS/MS of Compound **VII**. This compound contains a similar structural motif found in **I**. The MS/MS spectrum produces a pattern that is also found in (a).

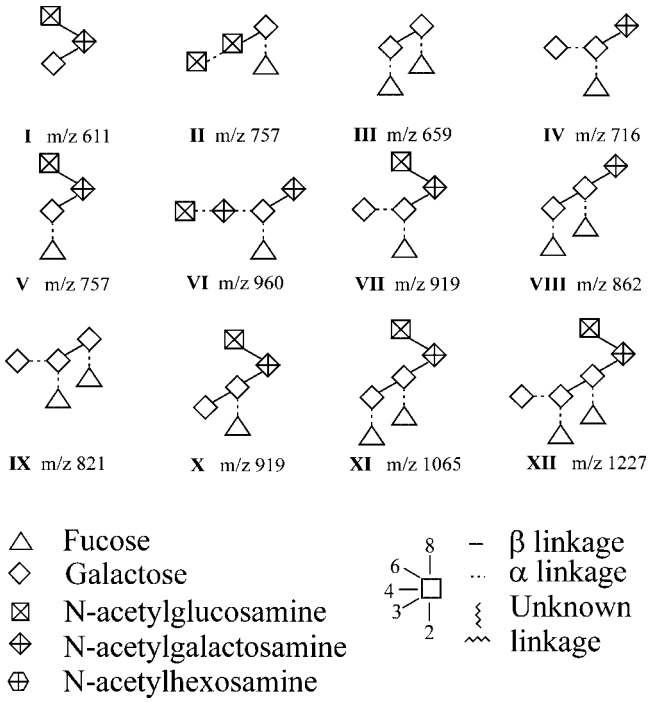


CHART 2. Five substructural motifs identified by CID. Each substructural motifs are identified with the CID fragmentation pattern. The structures of unknown oligosaccharides are reconstructed based on the presence of specific patterns in the CID spectrum.

pieced together by combining the motifs. This is illustrated with an oligosaccharide that is a minor component of the mixture and whose structure is unknown. Unknown **3** found in F (Fig. 10) of the HPLC trace produces the CID spectrum (MS^2) shown in Figure 13a. The MS^4 experiment was also performed ($m/z = 1065 \rightarrow 773/757 \rightarrow 611$) and is shown in Figure 13b. Note the presence of motifs **i** and **ii** (See Chart 2) in the two spectra. We reconstruct a structure containing both motifs **i** and **ii** and we reconcile the difference in mass by the addition of fucose. The result is the structure summarized in Figure 13. The additional fucose is placed on the Gal simply because it is not usually found linked to another fucose or to a GlcNAc. Note that this compound is similar in structure to **VII**. The two differ by only a fucose residue.

This method has been used to elucidate at least nearly all the remaining structures in the mixture of neutral oligosaccharides (Chart 3). The possibility of obtaining complete structural elucidation with only a few picomoles of material (in this case approximate 10 picomoles), makes this method a potentially powerful tool for analysis. This method will have wide applications in the study of other oligosaccharide libraries where several of the structures are known by NMR. It can also be used to determine the structures of less abundant component or changes in composition when an external stimulus is applied to the organism.

C. Characterization of Bacterial Lipooligosaccharides

Recent events have placed renewed emphasis on the rapid determination and characterization of microorganisms. MALDI mass spectrometry has been used for the detection and characterization of intact microorganisms since the first demonstration in 1975 (Fenselau & Demirev, 2001). Intact protein biomarkers from *Bacillus cereus* T spores (Demirev, Ramirez, & Fenselau, 2001), and lipopeptide biomarkers from *Bacillus globigii* (Williams, Hathout, & Fenselau, 2002) were characterized by MALDI-FTICR. Simple and rapid microorganism identification method was reported by employing on-slide proteolytic digestion followed by MALDI-FTICR tandem mass spectrometry and database searching (Yao, Afonso, & Fenselau, 2002).

Bacterial lipooligosaccharides play important roles in virulence and pathogenesis to the host. Therefore, elucidation of the precise structure of lipooligosaccharides is necessary for understanding the pathogenic process. Many mass spectrometric applications have been developed to characterize structures of bacterial lipooligosaccharides. In earlier studies, plasma desorption mass spectrometry provided the information regarding oligosaccharide sequence, molecular weight, and the location of acyl residues (Jardine et al., 1986, 1989). Also ESI mass spectrometry was introduced to investigate the structural heterogeneity of lipooligosaccharides (Gibson et al., 1993; Mühlecker et al., 1999). In conjunction with capillary electrophoresis, the structure of lipooligosaccharides from *Moraxella catarrhalis* was characterized by ESI mass spectrometry and tandem mass spectrometry (Kelly et al., 1996). Delayed extraction MALDI with a TOF has been used to analyze bacterial lipooligosaccharide structures (Bradford et al., 1997). Delayed extraction improved both mass resolution and stability of the molecular ions.

ESI-FTICR was used to characterize the structures of lipooligosaccharides (Gaucher et al., 2000; Leavell, Leary, & Yamasaki, 2002). Sequence and linkage information of unknown lipooligosaccharide glycoforms were obtained from *Haemophilus influenza* 2019 using tandem MS (MS^n) analysis on FTICR and quadrupole ion trap instruments in combination with alkali metal ion coordination without permethylation or peracetylation (Gaucher et al., 2000). To get structural information about oligosaccharide moiety, especially linkage and connectivity, MS^n was performed on the sodiated complexes in the positive mode. Their results suggested that the lipooligosaccharides extracts from *Haemophilus influenza* have extensive microhetrogeniety with structural isomers having different connectivities. As another application of FTICR, the structure of lipooligosaccharides from *Neisseria gonorrhoeae* 302 lipooligosaccharides has been reported (Leavell, Leary, & Yamasaki, 2002). With the mass accuracy of FTICR, the lipooligosaccharide composition was confirmed through exact mass measurements. In addition, tandem mass spectrometry in the negative ion mode was conducted to determine the topology of the glycoform, and confirmed the topology using a computer program, saccharide topology analysis tool (STAT).

D. Phosporylated Carbohydrates

The phosphate position of hexoses phosphate monosaccharides was determined by using ion/molecule reactions in

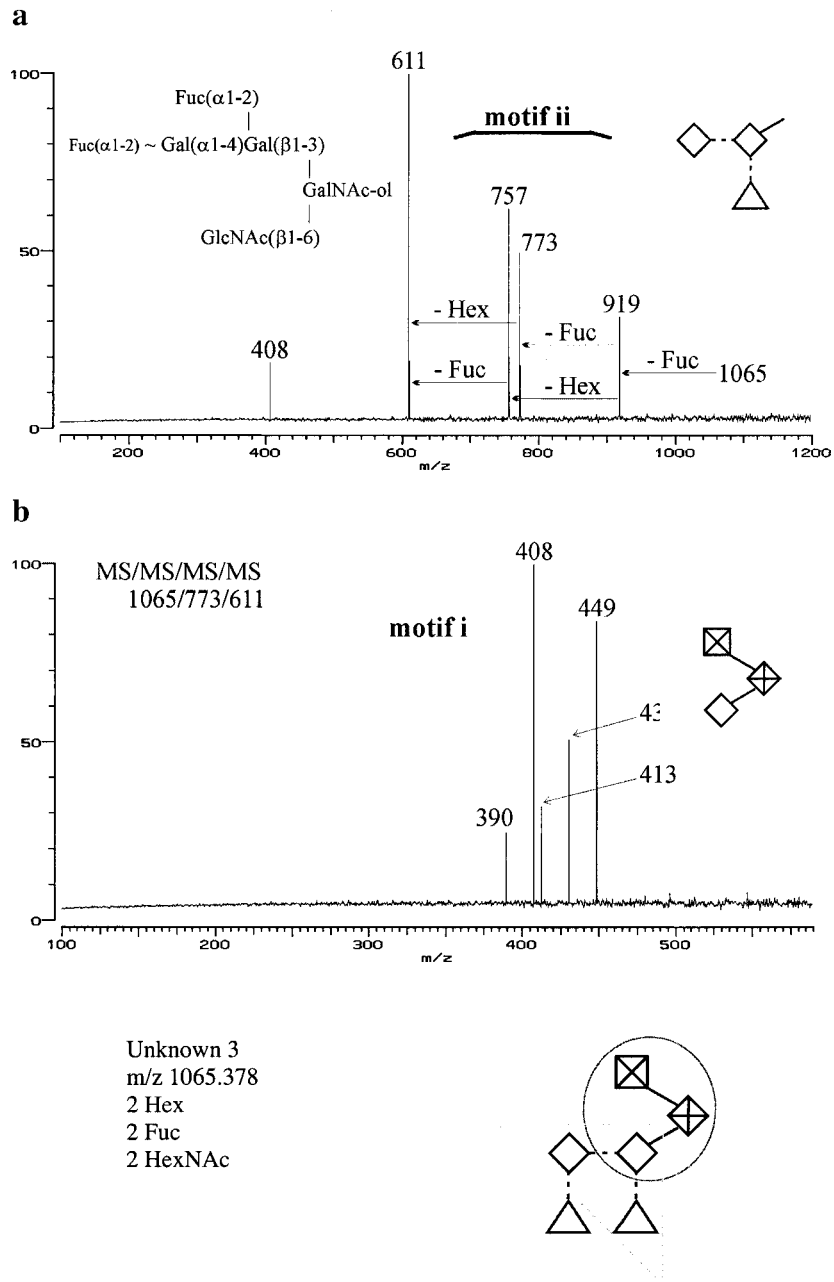


FIGURE 13. **a**: MALDI-MS/MS of unknown **3**. **b**: MALDI-MS/MS/MS/MS of unknown **3**. Motifs **i** and **ii** are found in unknown **3**, yielding the structure presented in **Chart 3**.

combination with FTICR tandem mass spectrometry (Leavell, Kruppa, & Leary, 2002, 2003; Leavell & Leary, 2003). Chlorotrimethylsilane (TMSCl) was used as an ion/molecule reaction reagent to react with phosporylated hexoses (Leavell, Kruppa, & Leary, 2002). Diagnostic product ions were generated for two isomers (glucose-1-phosphate (Glc1P) and glucose-6-phosphate (Glc6P)) to differentiate the position of phosphate (Fig. 14). The most important diagnostic peaks between Glc1P and Glc6P were $m/z = 313.1$ (loss of H_2O, Fig. 14a) and $m/z = 271.0$ (loss of $C_2H_4O_2$, Fig. 14b), respectively. The idea was extended from monosaccharide to oligosaccharides and synthetic lipophosphoglycan analog by employing TMSCl as an ion/molecule reaction reagent (Leavell & Leary, 2003).

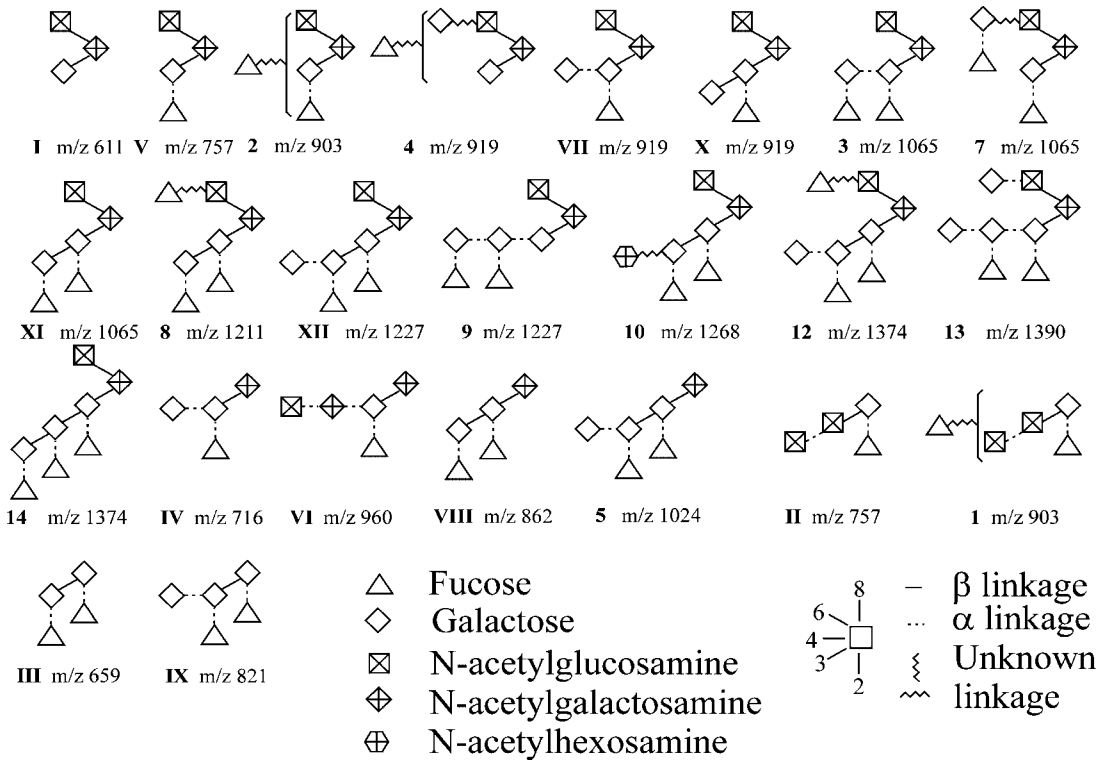


CHART 3. Structures of nearly all neutral components in the egg jelly of *Xenopus laevis*. The structures major components were determined by NMR (See Chart 1) whereas the minor components were determined solely by MS.

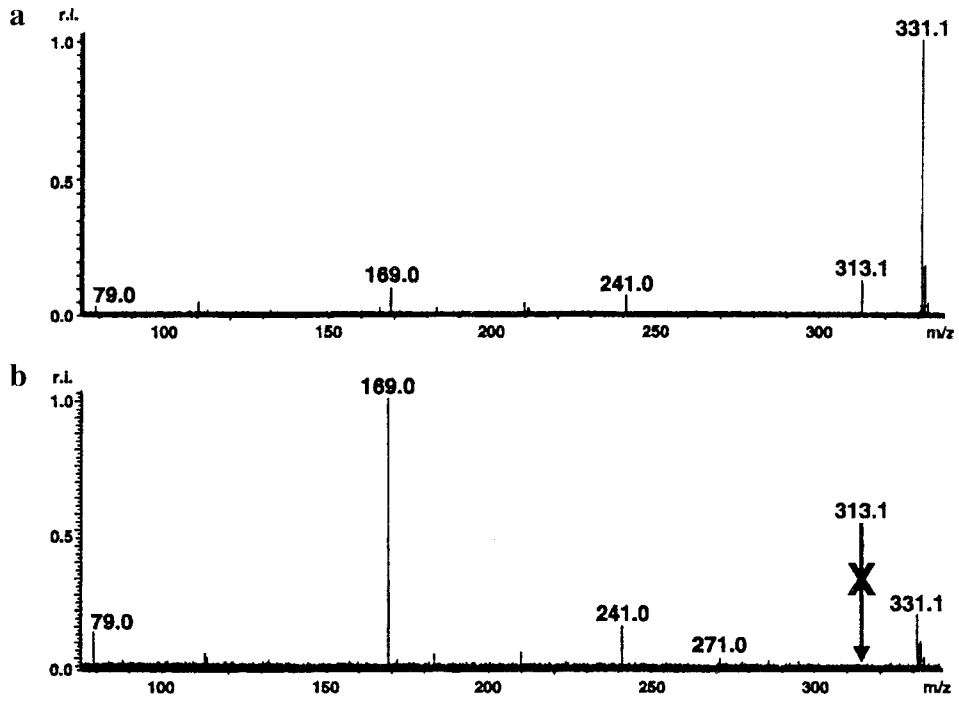


FIGURE 14. Product ion spectrum of (**a**) $[Glc1P/TMSCL\text{-}HCl]^-$ and (**b**) $[Glc6P/TMSCL\text{-}HCl]^-$, illustrating the diagnostic ions for the glucose-phosphate isomers. (Reprinted from Leavell and Leary, *Journal of American Society for Mass Spectrometry* 2003;14:323–331. Copyright © 2003 American Society for Mass Spectrometry. Published by Elsevier Science, Inc.)

VII. CONCLUSION

FTICR is highly suited for the structural elucidation of oligosaccharides. Important features including high resolution, high-mass accuracy, and facile implementation of CID help to overcome many of the difficulties in analyzing this class of compounds. However, complete structural elucidation in the picomolar and even subpicomolar level may not be possible with mass spectrometry alone. Several tools are also important including NMR, chemical, and enzymatic methods. When used in combination with mass spectrometry, these methods will be able to elucidate structures in complex mixtures in amounts that are biologically important. It is further clear that mass spectrometry, and specifically FTICR, will play a central role in the general structural analyses of these compounds.

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