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# Characterizing the electrospray-ionization mass spectral fragmentation pattern of enzymatically derived hyaluronic acid oligomers

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**Abstract**

Oligosaccharides derived from hyaluronic acid by action of bovine testicular hyaluronidase (BTH) (hyaluronate 4-glycanohydrolase, E.C. 3.2.1.35) were characterized by mass spectrometry (MS) with electrospray-ionization mass spectrometry (ESIMS) and compared with results obtained by matrix-assisted laser desorption/ionization. Both oligomers with an odd number and even number of sugar units with molecular masses up to 8 kDa were observed in the ESI spectra. However, the generation of odd-numbered oligomers is not consistent with the regiospecificity of the enzyme and with the MALDI results, which indicated even-numbered oligomers exclusively. In addition, a third method of characterization, high-performance anion-exchange chromatography (HPAEC), showed only even-numbered oligomers. Relative intensities of the odd-numbered oligomers demonstrated in ESIMS a cone-voltage dependence suggesting the odd-numbered oligomers resulted from collisional activation. In order to achieve results by ESI that mirror results from other techniques, the cone voltage must be kept low and precisely controlled. This study displays the usefulness and possible vulnerabilities of ESIMS when utilized for carbohydrate analysis without corroborating data from other methods of analysis.


*Keywords:* Electrospray-ionization; Oligosaccharides; ESIMS

## 1. Introduction

Hyaluronic acid, (HA, also termed hyaluronan, [→3)-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→4)-β-D-glucopyranuronosyl-(1→]$_n$) is a linear polymer (termed as glycosaminoglycan, GAG) composed of a disaccharide repeat unit of D-glucuronic acid (GlcA) and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc) (see Fig. 1). HA has numerous functions in the body, including its presence as the gelling substance of the vitreous humor of the eye, a constituent of the synovial fluid of the joints, as well as being a major component of the placenta.[1] Potentially, there are increasing number of uses for hyaluronic acid in medicine that are directly associated with the discovery of its many important biological functions. Studies have shown HA is involved in cancer metastasis, wound healing, degenerative disorders of the joints, and inflammation.[2–8]

Numerous studies have been reported on the enzymatic degradation of HA.[9–21] Many of these studies were aimed at developing methodology for the quantitation of oligosaccharides derived from the enzymatic depolymerization of HA. Two main types of enzymes are involved in these degradations: (1) hyaluronate lyase (E.C. 4.2.2.1) degradations that lead to oligosaccharides with a $\Delta^4$-unsaturated uronic acid nonreducing terminus (i.e., a nonreducing end of 4-deoxy-L-*threo*-hex-4-enuronic acid) to give oligosaccharides of the structure L-4dthrHex4enA-(1→3)-[D-GlcNAc-(1→4)-D-GlcA]$_n$-(1→3)-D-GlcNAc; and (2) hyaluronidase degradations (e.g., bovine testicular hyaluronidase (BTH), E.C. 3.2.1.35) that give structures of the type D-GlcA-(1→

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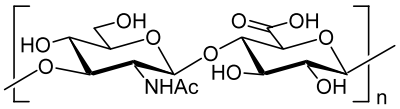


Fig. 1. Structure of hyaluronic acid composed of a disaccharide repeat unit of D-glucuronic acid (GlcA) and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc).

3)-[D-GlcNAc-(1→4)-D-GlcA]$_n$-D-GlcNAc with a fully saturated uronic acid at the nonreducing end. A common characteristic is that both enzymes produce fragments that contain an even number of saccharide units with a reducing terminus of GlcNAc.

In fact, nearly all the previous studies demonstrate absence of odd-numbered oligomers, the exceptions being the reports by Price and co-workers,[9] Shimada and co-workers,[10] and Yeung and co-workers,[11] who report conditions under which odd-numbered oligomers were observed in the product mixtures. Price and co-workers[9] and Shimada and co-workers[10] reported degradation conditions under which quantifiable amounts odd-numbered oligomers were formed and separated. Yeung and co-workers[11] reported the formation of odd-numbered oligomer fragments in the course of matrix-assisted laser-desorption ionizaton mass spectrometry (MALDIMS) experiments.

Previous investigations in our laboratory focused on the degradation of HA by *Streptomyces* hyaluronate lyase (E.C. 4.2.2.1) which afforded an α,β-unsaturated carboxylic acid moiety at the nonreducing terminus.[9] The oligosaccharides were readily quantitated by gel-permeation chromatography (GPC) with UV detection as well as by electrospray-ionization mass spectrometry (ESIMS). Degradation gave not only the preponderant even-numbered oligosaccharides, but also minor amounts of odd-numbered oligosaccharides. Most troubling was the fact that when subjected to ESIMS, the ratios of odd-numbered oligomers to their even-numbered counterparts changed as a function of cone voltage. In contrast to UV data from the GPC, ESIMS indicated a preponderance of odd-numbered oligosaccharides in some of the fractions. Odd-numbered oligomers were also detected in the ESI spectra when HA oligomers derived from BTH were analyzed. However, as the products of BTH degradation of HA do not contain unsaturated end-groups, UV confirmation of the presence of odd-numbered oligomers following GPC is not possible, but determination of the ratios of odd- to even-numbered oligosaccharides in a given fraction from the GPC was possible via high-performance anion-exchange chromatography (HPAEC) using pulsed amperometric detection (PAD). Clearly, at this juncture, a more detailed investigation was needed to assess the identity and purity of BTH-derived oligomers. MALDI was thus employed in an effort to confirm the absence or presence of odd-numbered oligomers.

MALDI is gaining acceptance as a tool for probing the structural characteristics of sugars.[22] The rise in popularity and applications is due to a small sample requirement and simplicity of instrumentation. Reasonable tolerance toward salts and buffers and high sensitivity have made MALDI amenable to the analysis of complex carbohydrate compounds.

The growing recognition of the role of carbohydrates in cellular processes, including cell–cell recognition, as well as in the rapidly growing field of glycomics, has created a need for rapid, reliable, and sensitive methods for carbohydrate analysis. Therefore, it was of interest to assess the reliability of the abundance of odd-numbered oligomers as displayed in the ESIMS.

## 2. Experimental

### 2.1. Materials

Bovine testicular hyaluronidase (BTH; E.C. 3.2.1.35) and sodium hyaluronate were purchased (Sigma Chemical Co., St. Louis, MO). HPLC-grade MeOH (Fisher Scientific Co., Atlanta, GA) and 2,5-dihydroxybenzoic acid (2,5-DHB) (Aldrich Chemical Co., Milwaukee, WI) were used as received. Water was deionized and filtered using a Nanopure 550 system.

### 2.2. Enzyme degradations and preparative column chromatography

For the BTH digestions, sodium hyaluronate (50 mg) was suspended in aq buffer (10 mL, 200 mM in NaCl and 50 mM in NaOAc, adjusted to pH 6.0), and the mixture was stirred overnight at 0 °C, resulting in a clear solution to which BTH (1000 U) was added, with stirring at 37 °C. Reaction time was varied to generate oligosaccharides of differing size ranges. Reaction times of ~36, ~3, and ~2 h were utilized to produce oligomer ranges 4–14-, 12–30-, and 20–42-mers, respectively. The reaction was quenched by the addition of EtOH, the mixture was concentrated on a rotary evaporator (40 °C) to remove the EtOH, and the aqueous solution was lyophilized. The resulting white powder (~200 mg) was subjected to GPC (BioGel P-30), using 0.25 M pyridinium acetate as eluent. Collected fractions (~2 mL each) were lyophilized then analyzed as described in the following sections.

### 2.3. Mass spectrometry

**2.3.1. Negative-ion ESIMS.** Negative-ion ESIMSs were acquired using a Quattro-II triple-quadrupole instrument from Micromass (Manchester, UK) having a mass-to-charge range of 4 kDa, and equipped with a 'Z-source' and a coaxial probe. Data acquisition and

deconvolution was accomplished with the Micromass MassLynx software package, v. 3.4, including MaxEnt data-analysis software (Waters Corp., Milford, MA). Sample solutions in 1:1 MeOH–water ( ~10 μg/mL) were infused at a flow rate of 5 μL/min. Nitrogen was used as both the nebulizing (20 L/h) and drying (300 L/h) gas. The source block and desolvation temperatures were maintained at 110 and 150 °C, respectively. Spectra were acquired in the multichannel-acquisition mode from scans encompassing $m/z$ 50–1700 at 10 s/scan.

**2.3.2. Matrix-assisted laser-desorption ionizaton mass spectrometry (MALDIMS).** MALDIMS was carried out using a Voyager DE, RP time-of-flight instrument (Applied Biosystems, Foster City, CA, USA) equipped with a high-current detector. Oligosaccharides ( ~2 mg/mL) were dissolved in water and mixed with the matrix solution (10 mg 2,5-DHB/mL water) in a 1:4 (v/v) ratio. Samples (0.5 μL) were spotted twice per sample well, and allowed to dry at room temperature. Mass analysis was done in the linear negative-ion mode with delayed extraction. The acceleration voltage was set at 20 kV, and grid voltage, delay time, and guide wire voltage varied depending on the mode and mass range in use. Fifty mass spectra were acquired and summed for each sample spot. Mass calibrations were performed with angiotensin and bovine serum albumin with 2,5-DHB as the matrix.

### 2.4. High-performance anion-exchange chromatography (HPAEC)

Conditions for HPAEC were as described previously by Price and co-workers.[9] Briefly, a Dionex DX-500 system with a Carbopac PA-1 guard precolumn and column, and a type II PAD detector interfaced to a Hewlett–Packard 3390A integrator was operated to obtain HPAEC data on the fractions previously separated as described in Section 2.2 above. Sodium acetate buffer concentrations of 0.28, 0.40, and 0.55 M at pH of 7.5 were utilized for eluting the HA oligomers.

## 3. Results and discussion

### 3.1. General observations for ESIMS and MALDIMS of oligosaccharide fractions

Fig. 2 presents a typical negative-ion ESI spectrum, at negative cone voltage (−CV) of 30 V, from a GPC fraction containing the 14- and 16-mers (Sample 1) derived from BTH digestion of HA. Analysis of the data shows the existence of multiple charge states, which are expected with ESIMS of acidic oligosaccharides. Application of the MaxEnt software[23] to the data of Fig. 2 deconvolutes the charge state envelopes and calculates the underlying molecular weight distribution (zero charge state), shown in Fig. 3. The major peak in Fig. 3 represents the 16-mer (theoretical average molecular weight 3052.5) with the two peaks at 3074.7 and 3096.6 being sodiated derivatives ([M−H+Na] and [M−2H+2Na]). The salient peaks at lower mass represent the 14- and 15-mers (having theoretical masses of 2672.4 and 2850.6), respectively.

Fig. 4 shows the MALDI mass spectrum of Sample 1 in the negative-ion mode. Comparison with the ESIMS result (Fig. 3) discloses several differences, the most striking of which is the absence of a signal for the 15-mer. Other differences include the relative absence of sodiated derivatives of the 16-mer, and a wider range of observed even-numbered oligomers, i.e., the 12- and 18-mers at 2292.6 and 3429.9 Da, respectively. The appearance of the complete even-numbered oligomer series to the exclusion of odd-numbered species in the MADLI mass spectrum is indicative of the absence of odd-numbered species in the GPC fraction prior to ESIMS.

Further evidence that the odd-numbered oligomers are not present in the analyte is found in the HPAEC. Prior to the analysis, a calibration curve was constructed using the procedure described by Price and co-workers[9] for BTH-derived oligomers. Contrary to previous observations[9] for HA oligomers, no odd-numbered oligomers were detected by HPAEC. This was likely due to a change in quenching procedure. It has been reported that boiling the enzyme-reaction mixture can give rise to degradation of HA.[24] The odd-numbered oligomers observed in previous studies prompted a change in the digestion procedure to quench with ethanol instead of boiling. The results obtained with HPAEC thus agreed with the MALDI results (presented above) and confirmed the absence of odd-numbered oligomers in the GPC fractions.

If odd-numbered oligomers are observed in the ESIMS, but are not present in the GPC fractions, it follows that those odd-numbered oligomers must be artifacts of the ESI process. There are two possible ways in which an odd-numbered oligomer may be derived from the next higher even-numbered precursor during ESI: (1) as a chemical reaction within the charged electrospray droplet prior to complete evaporation of the droplet; and/or (2) as a collision-induced dissociation of the molecular anion after the droplet has completely evaporated and the ion has entered the intermediate pressure region of the electrospray ion source (i.e., that region between the atmospheric pressure of the spray chamber and the high vacuum of the mass analyzer). Each of these two possibilities will be discussed individually below.

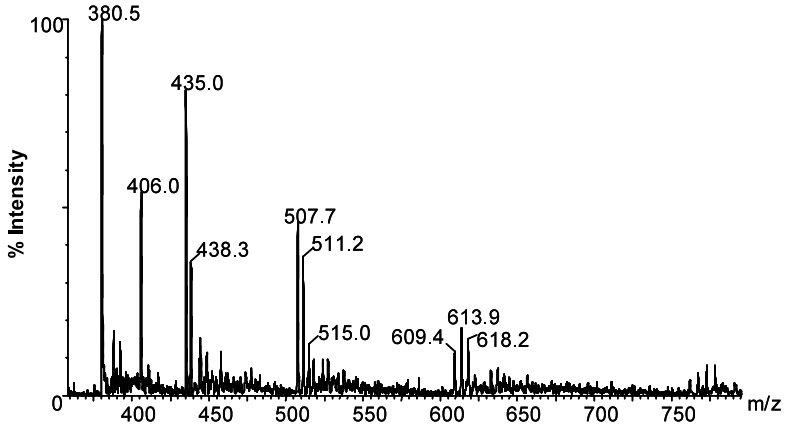


Fig. 2. Negative-ion electrospray mass spectrum acquired at $-CV = 30$ V observed for GFC fraction containing the 16-mer, enzymatically derived with BTH, sample 1.

### 3.2. pH-Dependent chemical degradation of HA oligomers in the electrospray droplet

HA consists of essentially two types of glycosidic linkages: (1) a β-D-GlcNAc-(1→4)-D-GlcA linkage; and (2) a β-D-GlcA-(1→3)-D-GlcNAc linkage, each of which has distinctive cleavage characteristics. At pH 9 it has been shown[10] that odd-numbered fragments can be produced among oligosaccharide products from both *Streptomyces* hyaluronidase (EC 4.2.2.1) and from the BTH (EC 3.2.1.35), which is supported by results from this laboratory.[9] The mechanism is that of a facile β-elimination reaction that is characteristic of 3-*O*-substituted 2-acetamido-2-deoxyhexosamines.[25] Such eliminations have been extensively observed in anion-exchange chromatography of GAG molecules[26] and can be suppressed by lowering the pH.

It is known that positive-ion ESI conditions produce a $10^3$–$10^4$ increase in the acidity from that of the neutral bulk solution (pH ~7, moving to pH ~2.6–3.3 in the positive-ion mode) to the electrospray droplets.[27] This suggests changes in the pH, specifically an increase in the pH, would be expected under these conditions in the negative-ion mode.[28] An increase from pH ~7 (water–MeOH mixture) to around pH 9–10 could promote a β-elimination analogous to the known bulk solution process[25] described above.

This proposed β-elimination reaction would occur within the ES droplet, prior to its complete evaporation, and prior to insertion into the intermediate vacuum region of the ion source where the cone voltage accelerates the ions into the mass analyzer. The appearance and abundance of odd-numbered oligomers generated by this process would be independent of the CV

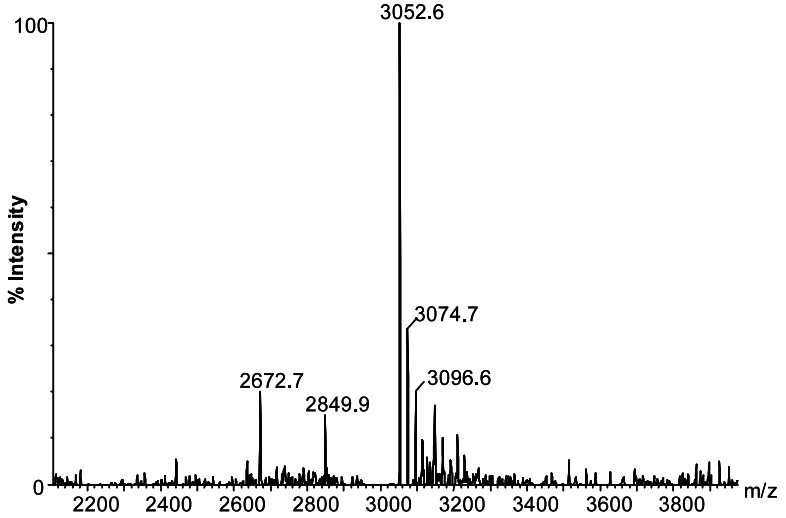


Fig. 3. Max-Ent generated molecular weight distribution for the raw data of sample 1, shown in Fig. 2.

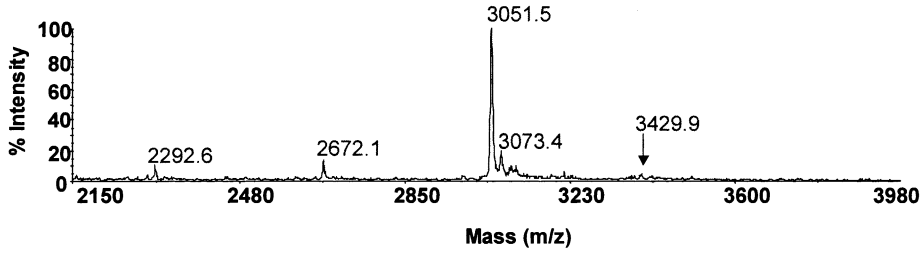


Fig. 4. MALDI mass spectrum for sample 1 run in the negative-ion linear mode.

applied. As indicated below, the generation of odd-numbered oligomers emphatically *is* dependent upon CV, thus arguing against this pH-dependent chemical process as a significant genesis of the odd-numbered oligomers.

### 3.3. Cone voltage-dependent fragmentation of even-numbered oligomer anions

Changing the ESI cone voltage (CV) typically induces a shift in the measured charge state distribution of ions capable of carrying multiple charges.[29] In general, higher CVs produce charge-state envelopes centered at lower charge-states (i.e., at higher *m/z* values). This spreads out the mass spectrum and often reduces ambiguity in assigning structures and molecular weights. In addition, higher CVs impart more internal energy into ions through collision with neutral species (nitrogen and solvent molecules) present in the intermediate pressure region of the ion source. This internal energy can give rise to collisionally activated 'in-source fragmentation'. The amount of internal energy imparted to the ion can be controlled by altering the CV.

In order to assess the effect of CV on the generation of odd-numbered oligomers in the HA oligomers, GPC fractions containing the following oligomers were used: a mixture of 14- and 16-mers (sample 1), a mixture of 8- and 10-mers (sample 2), and a mixture of 22- and 24-mers (sample 3). Each sample was subjected to ESIMS using a range of CVs from 5 to 45 V.

Data for one of the oligosaccharides, sample 1, are presented in Fig. 5. This plot represents the intensity as a function of the CV for each of the oligomers observed in the spectra. It can be seen that the spectrum of sample 1 displays a small abundance of the 15-mer even at the lowest CV. This suggests either a contribution of the pH-dependent solution reaction proposed above, or a residual collisionally activated fragmentation, even at 5 V. The 15-mer contribution increases steadily with increasing CV. By contrast, Fig. 6, which represents similar data for sample 2 (consisting mainly of the 10-mer), shows the onset of fragmentation (appearance of the 9-mer) only above 15 V. Thus, the prevalence of the fragmentation reaction to the next lower oligomer is dependent not only on the CV, but also on the size of the original oligomer.

A plausible explanation of this observation follows. For increasingly large oligomers, the number of carboxylic acid groups increases (one extra carboxylate for each two monosaccharide units; see Fig. 1). The charge-state of the molecular anions generated in the ESI

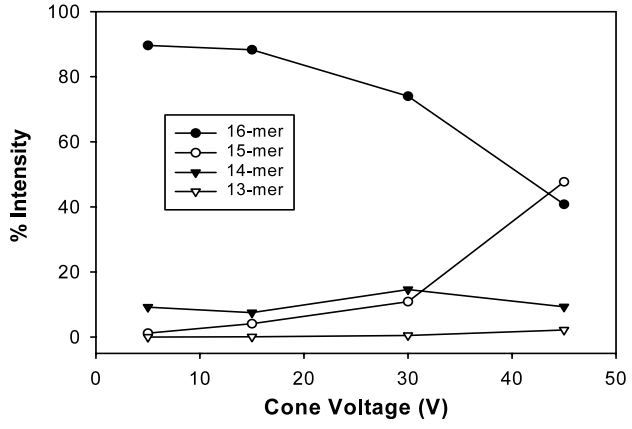


Fig. 5. Plot of the normalized negative-ion ESI ion intensity vs. CV for even- and odd-numbered oligomers generated from sample 1.

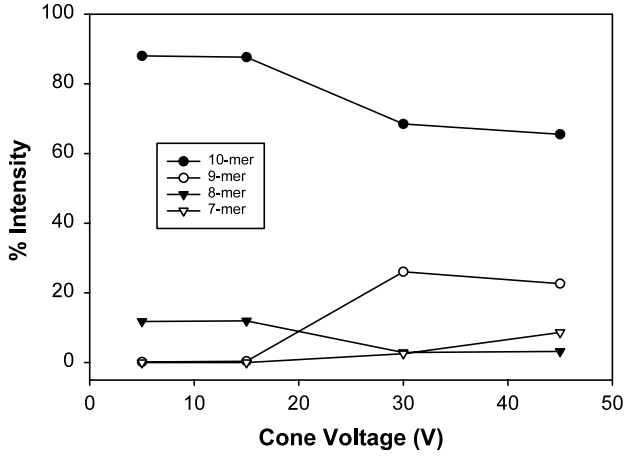


Fig. 6. Plot of the normalized negative-ion ESI ion intensity vs. CV for even- and odd-numbered oligomers generated from sample 2.

process is therefore potentially greater for the larger oligomers. (Note that for the 16-mer, the most abundant peak observed in the ESIMS is the one having all eight carboxylic acid moieties stripped of their protons; i.e., *m/z* 380.5 in Fig. 2.) The amount of internal energy that can be imparted to an ion by collision is proportional to the magnitude of the electrical field through which it is accelerated (i.e., proportional to the CV) and proportional to the electrical charge upon which that field operates (i.e., proportional to the charge-state of the ion). Thus, the same CV operating on a more highly charged ion will generate more internal energy within the ion than it would for a less highly charged ion. This greater internal energy translates into a greater propensity for fragmentation to the next lower oligomer, resulting in a greater abundance of that odd-numbered oligomer observed in the ESIMS.

For samples 1 and 2, the spectra at the lowest CV exhibit negligible ($<2\%$) abundances of the odd-numbered oligomers. For sample 3, however, (data not shown) the CV cannot be lowered sufficiently to eliminate the odd-numbered oligomer as a significant salient peak in the spectrum. (Setting the CV below 5 V is not practicable because insufficient ions are steered into the mass analyzer to produce a useable mass spectrum.)

## 4. Conclusions

ESIMS affords a good technique for the characterization of moderately sized acidic oligosaccharides if appropriate care is taken in the choice of a proper cone voltage. The ionic nature of acidic oligosaccharides, coupled with the ability of electrospray to sample pre-formed ions, makes characterization of these materials by ESIMS a powerful probe for the researcher. However, if purity is to be assessed, it is of critical importance that the propensity of these fragile molecules to undergo in-source fragmentation during the electrospray process be kept in mind. The likelihood for such fragmentation increases with the number of charges the molecule can carry i.e., with the number of carboxylic acid moieties present. For higher oligomers it may not be possible to avoid in-source fragmentation altogether, even using the lowest practical cone voltage. In such cases, MALDIMS may be the better method (as it was for these examples) for characterization.

The methodology described herein confirms the absence of odd-numbered oligomers from samples derived by BHT digestion with ethanol quenching. However, odd-numbered oligomers can be generated if digestion procedures similar to those reported previously are followed.[9,10]

## Acknowledgements

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## References

1. Nelson, D. L.; Cox, M. M. *Lehninger: Principles of Biochemistry*; 3rd ed.; Worth: New York, 2000; pp 309–310.
2. Noble, P. W.; McKee, C. M.; Horton, M. R. *J. Med. Chem.* **1998**, *41*, 5336–5352.
3. Ziebell, M. R.; Zhao, Z.-G.; Luo, B.; Luo, Y.; Turley, E. A.; Prestwich, G. D. *Chem. Biol.* **2001**, *8*, 1081–1094.
4. Lynn, B. D.; Li, X.; Cattini, P. A.; Turley, E. A.; Nagy, J. I. *J. Comp. Neurol.* **2001**, *439*, 315–330.
5. Jiang, H.; Knudson, C. B.; Knudson, W. *Arthritis Rheum.* **2001**, *44*, 2599–2610.
6. Nishida, Y.; D'Souza, A. L.; Thonar, E. J.; Knudson, W. *Arthritis Rheum.* **2000**, *43*, 1315–1326.
7. Knudson, W.; Casey, B.; Nishida, Y.; Eger, W.; Kuettner, K. E.; Knudson, C. B. *Arthritis Rheum.* **2000**, *43*, 1165–1174.
8. Sy, M. S.; Liu, D.; Schiavone, R.; Ma, J.; Mori, H.; Guo, Y. *Curr. Top. Microbiol. Immunol.* **1996**, *213*, 129–153.
9. Price, K. N.; Tuinman, A.; Baker, D. C.; Chisena, C.; Cysyk, R. L. *Carbohydr. Res.* **1997**, *303*, 303–311.
10. Shimada, E.; Matsumura, G. *J. Biochem. (Tokyo)* **1984**, *96*, 721–725.
11. Yeung, B.; Marecak, D. *J. Chromatogr., A* **1999**, *852*, 573–581.
12. Shimada, E.; Matsumura, G. *J. Biochem. (Tokyo)* **1980**, *88*, 1015–1023.
13. Schiller, J.; Arnhold, J.; Benard, S.; Reichl, S.; Arnold, K. *Carbohydr. Res.* **1999**, *318*, 116–122.
14. Inoue, Y.; Nagasawa, K. *Carbohydr. Res.* **1985**, *141*, 99–110.
15. Shimada, E.; Matsumura, G. *J. Chromatogr.* **1985**, *328*, 73–80.
16. Nebinger, P. *J. Chromatogr.* **1985**, *320*, 351–359.
17. Nebinger, P.; Koel, M.; Franz, A.; Werries, E. *J. Chromatogr.* **1983**, *265*, 19–25.
18. Cowman, M. K.; Balazs, E. A.; Bergmann, C. W.; Meyer, K. *Biochemistry* **1981**, *20*, 1379–1385.
19. Ulrich, H. P.; Klein, U.; Von Figura, K. *Hoppe-Seyler's Z. Physiol. Chem.* **1979**, *360*, 1457–1463.
20. Takagaki, K.; Munakata, H.; Nakamura, W.; Matsuya, H.; Mijima, M.; Endo, M. *Glycobiology* **1998**, *8*, 719–724.
21. Takagaki, K.; Kojima, K.; Majima, M.; Nakamura, T.; Kato, I.; Endo, M. *Glycoconjugate J.* **1992**, *9*, 174–179.
22. Harvey, D. J. *Mass Spectrom. Rev.* **1999**, *18*, 349–450.

23. Ferrige, A. G.; Seddon, M. J.; Green, B. N.; Jarvis, S. A.; Skilling, J. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 707–711.
24. Bothner, H.; Waaler, T.; Wik, O. *Int. J. Biol. Macromol.* **1988**, *10*, 287–291.
25. Lee, Y. C. *Anal. Biochem.* **1990**, *189*, 151–162.
26. Hicks, K. B. *J. Chromatogr. Libr.* **1995**, *58*, 361–387.
27. Gatlin, C. L.; Turecek, F. *Anal. Chem.* **1994**, *66*, 712–718.
28. Van Berkel, G. J.; Zhou, F.; Aronson, J. T. *Int. J. Mass Spectrom. Ion Processes* **1997**, *162*, 55–67.
29. Ashton, D. S.; Beddell, C. R.; Cooper, D. J.; Green, B. N.; Oliver, R. W. A. *Org. Mass Spectrom.* **1993**, *28*, 721–728.