Complete Mass Spectral Characterization of a Synthetic Ultralow-Molecular-Weight Heparin Using Collision-Induced Dissociation

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2012

Impact Factor: 5.64 · DOI: 10.1021/ac3015824 · Source: PubMed

5 AUTHORS, INCLUDING:

Muchena J Kailemia
University of California, Davis
5 PUBLICATIONS **80** CITATIONS
SEE PROFILE

Lingyun Li
Wadsworth Center, NYS Department of Health
64 PUBLICATIONS **1,060** CITATIONS
SEE PROFILE

Mellisa Ly
Rensselaer Polytechnic Institute
16 PUBLICATIONS **397** CITATIONS
SEE PROFILE

Ives Jonathan Amster
University of Georgia
129 PUBLICATIONS **3,964** CITATIONS
SEE PROFILE

analytical chemistry

Letter

pubs.acs.org/ac

Complete Mass Spectral Characterization of a Synthetic Ultralow-Molecular-Weight Heparin Using Collision-Induced Dissociation

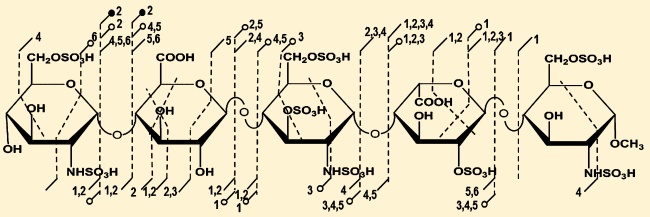
Muchena J. Kailemia,[†] Lingyun Li,[‡] Mellisa Ly,[‡,¶] Robert J. Linhardt,[‡,§,⊥,∥] and I. Jonathan Amster*[,†]

[†]Department of Chemistry, University of Georgia, Athens, Georgia, United States

[‡]Department of Chemistry and Chemical Biology, [§]Department of Chemical and Biological Engineering, [⊥]Department of Biology, [∥]Department of Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York, United States

Supporting Information

ABSTRACT: Glycosaminoglycans (GAGs) are a class of biologically important molecules, and their structural analysis is the target of considerable research effort. Advances in tandem mass spectrometry (MS/MS) have recently enabled the structural characterization of several classes of GAGs; however, the highly sulfated GAGs, such as heparins, have remained a relatively intractable class due their tendency to lose SO_3 during MS/MS, producing few sequence-informative fragment ions. The present work demonstrates for the first time the complete structural characterization of the highly sulfated heparin-based drug Arixtra. This was achieved by Na^+/H^+ exchange to create a more ionized species that was stable against SO_3 loss, and that produced complete sets of both glycosidic and cross-ring fragment ions. MS/MS enables the complete structural determination of Arixtra, including the stereochemistry of its uronic acid residues, and suggests an approach for solving the structure of more complex, highly sulfated heparin-based drugs.



Heparin (Hp) and structurally related heparan sulfate (HS) are biopolymers consisting of highly sulfated uronic acid and glucosamine repeating units and are found intracellularly, in the extracellular matrix, and on the cell surface of a wide variety of species.[1] To develop a deeper understanding of their biological function in angiogenesis,[2] tumor metastasis,[3] viral invasion,[4] cell growth and proliferation,[5] and anticoagulation,[6] elucidation of their molecular level structures is of great interest but remains a challenge.[7] Unlike biopolymers such as proteins or nucleic acids, the biosynthetic pathways for glycosaminoglycans (GAGs) are not based on a template mechanism; thus, they are heterogeneous in composition and highly polydisperse in molecular weight.[8] Although Hp is produced in large amounts as a drug, HS is often extracted from tissues in small quantities requiring sensitive analytical methods, such as mass spectrometry (MS).[8a] Negative mode electrospray ionization (ESI) is commonly used due to the highly acidic nature of Hp and HS,[9] and it provides multiply charged precursor ions, which facilitates MS/MS of higher mass molecules.

The presence of a high number of labile sulfate groups renders many MS/MS techniques insufficient for the structural characterization of Hp and HS.[10] Previously, methods such as collision induced dissociation (CID) and infrared multiphoton dissociation (IRMPD) were found to lead to sulfate decomposition (loss of SO_3) and to provide few sequence-informative cleavages.[8b,9,11] Electron detachment dissociation (EDD) and other electron-based methods have been used to characterize HS oligosaccharides with a low level of sulfation as well as chondroitin sulfate GAGs,[12] but these methods become less efficient as the number of sulfo groups per disaccharide increases.

Research on MS/MS methods for Hp analysis has been directed at the retention of labile sulfate groups during ion activation but with limited success. Increasing the charge state to a level where all the sulfate groups are ionized reduces sulfate loss and increases structurally informative cleavages,[11,13] but as the number of sulfate groups per disaccharide increases, charge−charge repulsion limits the ability to produce molecular ions with higher charge states with sufficient intensities for MS/MS.[8b,14] Even when the right charge state is selected, most of the cleavages obtained are glycosidic bond fragmentation, which cannot be used for locating the position of the sulfate groups within a monosaccharide unit. Multistage CID (MS^n) has provided structural information on these molecules but requires substantial amounts of sample and still is inefficient in Hp oligosaccharides having more than one sulfo group per disaccharide unit.[11]

Exchanging H^+ with metal cations such as Na^+ or Ca^{2+} has been shown to stabilize sulfate groups and to increase the formation of sequence-informative fragment ions,[11,15] but these previous studies showed few glycosidic and cross-ring cleavages and failed to provide comprehensive structural identification of analytes.[11] Here, we show that use of NaOH as a component of electrospray (ESI) enables the production of a precursor in

Received: June 8, 2012
Accepted: June 18, 2012

   dx.doi.org/10.1021/ac3015824 | *Anal. Chem.* XXXX, XXX, XXX−XXX

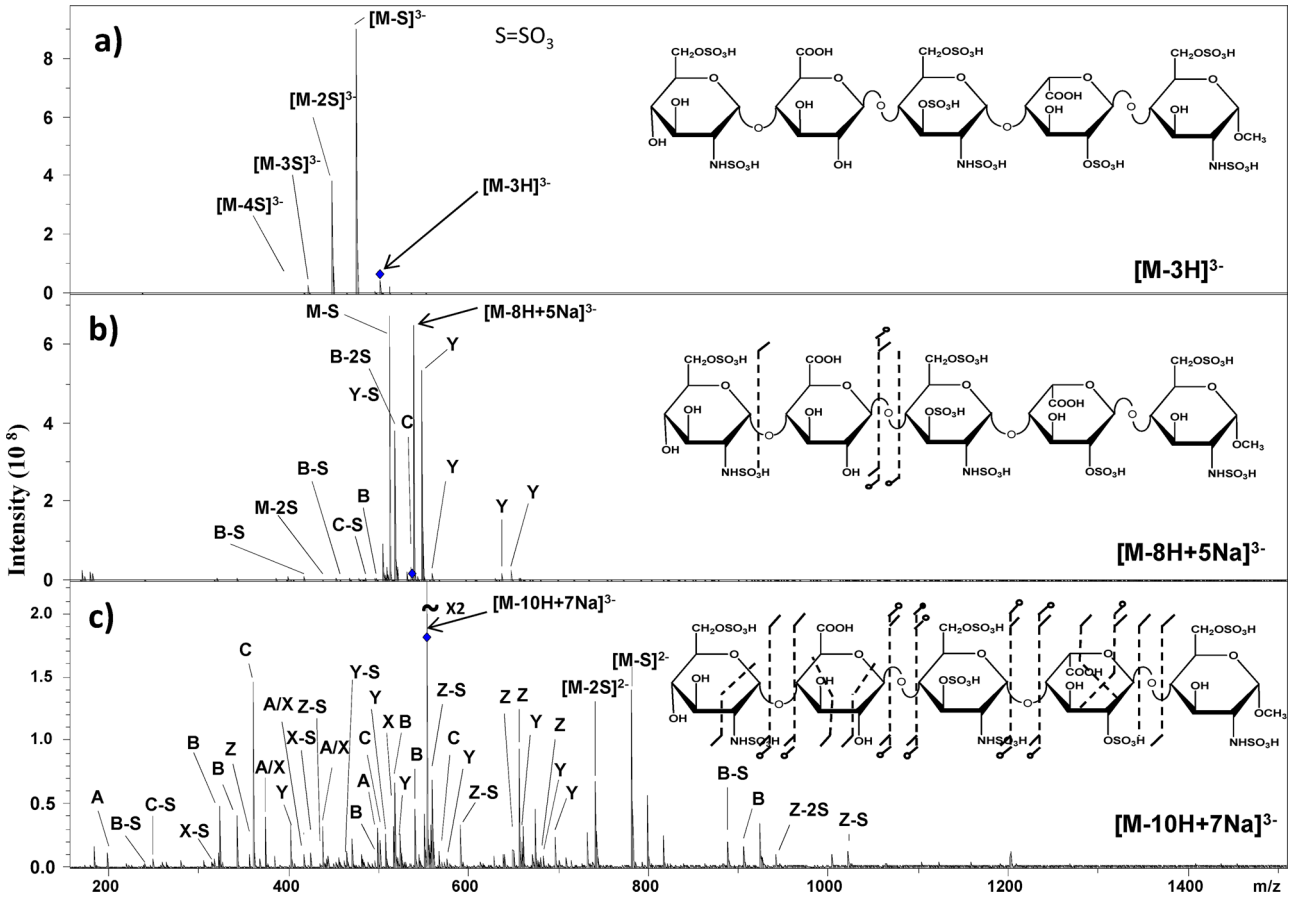


Figure 1. CID spectra for triply deprotonated molecular ions of Arixtra. (a) CID of $[M - 3H]^{3-}$ showing that a low charge state leads to the loss of SO_3 and little useful structural information. (b) Deprotonation of eight acidic groups, equal to the number of sulfo groups, through Na^+/H^+ exchange and charging, reduces SO_3 loss but provides few useful fragments. (c) When all 10 acidic groups are deprotonated, a large number of structurally informative glycosidic and cross-ring cleavages are observed.

which all ionizable protons are removed or replaced by Na^+. This precursor is found to be uniquely suitable for MS/MS analysis and leads to the production of abundant glycosidic and cross-ring fragments, enabling full characterization of a highly sulfated synthetic Hp oligosaccharide using a single CID spectrum.

Arixtra is a synthetic, ultralow-molecular-weight heparin based on the pentasaccharide sequence that comprises the antithrombin III binding site present in both Hp and HS that is responsible for anticoagulant activity.[16] Arrangement of the monosaccharide units, the position of the sulfo group substitution, and the stereochemistry of the uronic acid within this pentasaccharide are critical for its anticoagulant activity.[17] Arixtra $[C_{31}H_{53}O_{49}S_8N_3]$ has 8 sulfo groups and three carboxyl groups, for a total of 10 acidic sites. Higher charge states lead to less sulfate loss[11,13a] but charge−charge repulsion limits the ability to achieve a charge state in which all the sulfo groups are deprotonated.

The most abundant charge state observed in the mass spectrum of Arixtra was $[M - 3H]^{3-}$ (Supporting Information). In this case, only three sulfo groups were deprotonated, leaving the remaining seven acidic groups protonated. Mild CID activation of this molecular ion produces very intense SO_3 loss peaks, as shown in Figure 1a. As previously reported,[11] increasing the deprotonation of sulfo and carboxyl groups through metal cation/H^+ exchange reduces SO_3 loss and affords structurally informative cleavages. In Figure 1b, Na^+/H^+ exchange leads to deprotonation of eight acidic groups, $[M - 8H + 5Na]^{3-}$, which equals the number of sulfo groups in Arixtra. Although the most intense peaks in the resulting spectrum are the SO_3 loss peaks, a few glycosidic fragments are observed that provide limited structural information. When all the acidic groups were deprotonated, a CID spectrum for $[M - 10H + 7Na]^{3-}$ was obtained, which had a uniform distribution of both glycosidic, cross-ring cleavages with few low-intensity peaks resulting from neutral SO_3 loss (Figure 1c).

Due to the high density of assignable peaks within this spectrum, a simple annotation is used that does not include the entire ion nomenclature. In this annotation, A is used to denote any cleavage corresponding to the actual A fragment while A-S is used to denote loss of SO_3 from the corresponding A fragment. A complete peak list is included in the Supporting Information (S1). Higher charge states of the fully deprotonated sample were examined and found to give complementary data. Annotated structures for $[M - 10H + 6Na]^{4-}$ and $[M - 10H + 5Na]^{5-}$ show the observed fragmentations, which include an entire set of glycosidic bond cleavages and abundant cross-ring cleavages (Figure 2). For the molecular ion $[M - 10H + 6Na]^{4-}$, Z_1 places two sulfo groups on the reducing end residue, and the mass difference between $^{0,2}X_1$ and Y_1 places a sulfo group at the 2-position of the iduronic acid residue.

The mass difference between B_3 and C_2 shows there are three sulfo groups in the central glucosamine residue occupying all available sites of modification, i.e., 2-*N*-sulfo, 3-*O*-sulfo and

dx.doi.org/10.1021/ac3015824 | Anal. Chem. XXXX, XXX, XXX−XXX

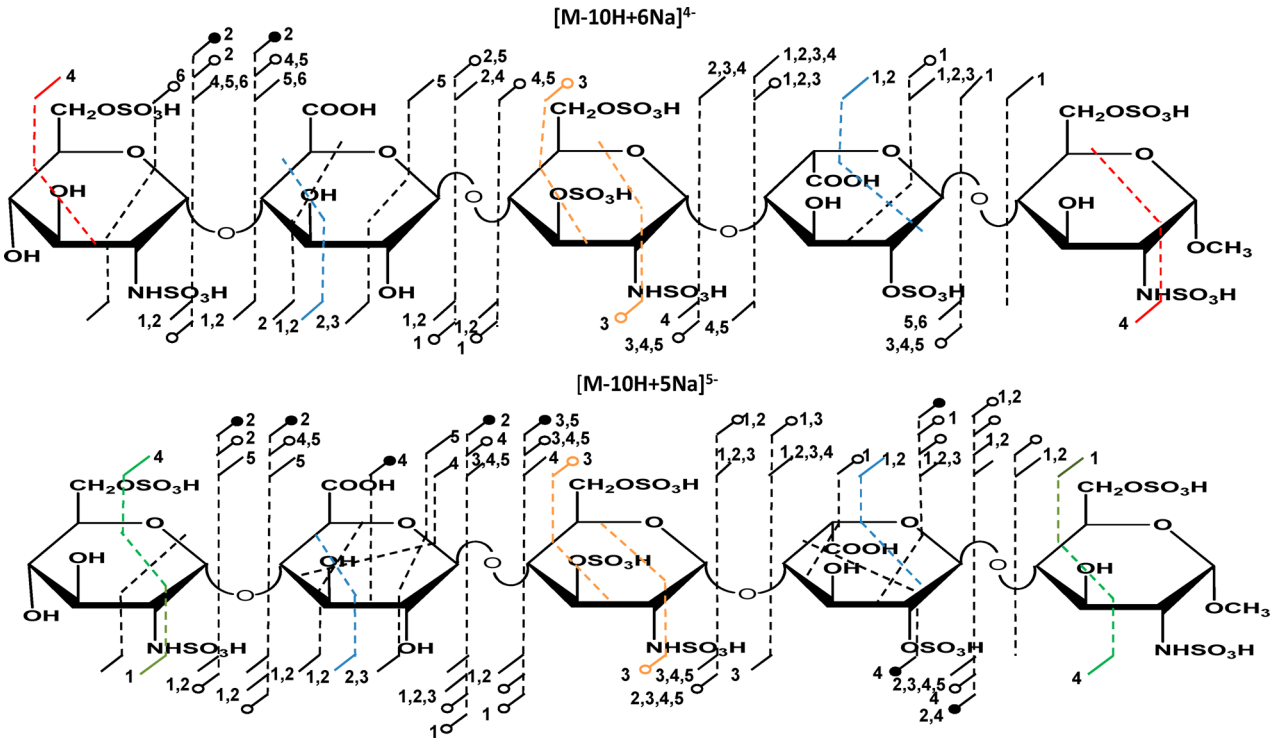


Figure 2. Annotated structures showing observed cleavages for Arixtra for two different charge states and levels of sodiation. Both $[M - 10H + 6Na]^{4-}$ and $[M - 10H + 5Na]^{5-}$ show similar fragmentation patterns affording more comprehensive structural information. The numbers adjacent to a cleavage indicate the number of sodium ions present in the fragment ion, open circles denote SO_3 loss accompanying the indicated fragmentation, and solid circles denote loss of two or more SO_3 groups. Colored lines indicate isobaric cleavages. For example $^{2,4}X_4$ and $^{1,5}A_4$, shown in red, have the same elemental composition, and are indistinguishable by mass measurement.

6-*O*-sulfo. The mass difference between Y_4 and the $^{2,4}X_4$ cleavage in the nonreducing end residue establishes sulfation on the 6-*O* and 2-*N* positions. The $^{2,4}X_4$ fragment is isobaric with a $^{1,5}A_5$ fragment, but 1,5 cleavage is unusual in the CID mass spectra of GAGs, whereas 2,4 cleavage is fairly common. Fragmentation of another fully deprotonated molecular ion of one higher charge state $[M - 10H + 5Na]^{5-}$ (Figure 2) produces cleavages similar to those observed for $[M - 10H + 6Na]^{4-}$ with additional fragments including $^{2,4}A_5$ that allow for the placement of the two sulfo groups on the 2-*N* and 6-*O* positions in the reducing end residue. The $^{2,4}A_5$ fragment is isobaric with a $^{1,5}X_4$ cleavage, but again, the former is common and abundant in CID spectra of GAGs and can be confidently assigned as such. A full mass list can be found in the Supporting Information.

The effectiveness of this method using an FTMS Orbitrap provided similar results, indicating that this approach can be applied using a wide variety of FT mass spectrometers. Moreover, the Orbitrap can afford additional low-mass fragments, such as $^{0,3}X_0$, $^{3,5}X_0$, $^{0,3}A_1$, $^{0,4}A_1$, and $^{1,3}A_1$ ions, using a higher collision dissociation (HCD) fragmentation approach (Supporting Information). An increase in SO_3 loss fragments is observed in the spectra as the charge state increases and the number of the sodium cations in the molecular ion decreases. This suggests that stabilizing the sulfo groups is more readily achieved by increasing the degree of sodiation of the molecular ion rather than by increasing the molecular ion charge.

Uronic acid C_5-stereochemistry (glucuronic acid/iduronic acid) is another important aspect of the Arixtra structure that determines how it interacts antithrombin III and other proteins to influence their functions.[16,18] Efforts to distinguish iduronic acid and glucuronic acid in Hp, HS, and chondroitin sulfate analytes is an ongoing challenge[12b,19] for analytical chemists. In the attempt to determine whether the abundant cross-ring cleavages generated by CID can aid in elucidating uronic acid stereochemistry, we examined the cross-ring cleavages of all of the uronic acid residues in a variety of GAG samples (chondroitin 4-sulfate, dermatan sulfate) dp4−dp10, Hp oligosaccharides dp4−dp8, and chemoenzymatically synthesized Hp (dp7) and HS dp10−dp12) oligosaccharides (dp is degree of polymerization). These results will be the subject of a future publication, which establishes the diagnostic nature of $^{2,4}A_n$ ions in characterizing uronic acid stereochemistry.

In all experiments, $^{2,4}A_n$ (where *n* is the uronic acid residue position in the analyte) cleavages appeared in all glucuronic acid residues but are absent (or present at very low intensity) in iduronic acid residues as long as all the acidic groups in the ion are deprotonated. In Arixtra, a $^{2,4}A_2$ fragment appears in the glucuronic acid residue but not in the iduronic acid residue. Although the $^{2,4}A_2$ fragment is isobaric with $^{1,5}X_1$ fragments, H/D exchange experiments confirmed the assignment of the $^{2,4}A_2$ fragment (Supporting Information).

Unlike the previously reported Q-TOF mass spectral data on Arixtra,[13] our study fully assigns all the fragments required for the complete establishment of primary structure. Additional studies are underway in the investigators' laboratories on longer oligosaccharides and even full-length Hp and HS chains to establish the effectiveness of this method on larger, highly sulfated GAGs.

It is interesting to compare this approach with prior efforts on GAGs and other polyanionic biomolecules, such as nucleic acids. Molecules with multiple acidic sites have a high

dx.doi.org/10.1021/ac3015824 | *Anal. Chem.* XXXX, XXX, XXX−XXX

propensity to pair with metal ions, which adversely impacts mass spectrometric analysis by introducing heterogeneity from incomplete replacement of the ionizable protons by metal ions. For this reason, researchers usually take great care to rigorously desalt anionic samples prior to MS analysis.[20] During the ESI experiments, reagents such as formic acid or ammonium hydroxide have been used to reduce metal/proton exchange and the consequential splitting of peaks that reduces the efficiency of both MS and MS/MS analysis.[13a,20c] In contrast to the standard approach for anionic biomolecules, the current work purposely introduces metal ions to exhaustively replace ionizable protons, which greatly improves the MS/MS analysis.

Prior investigations of the MS/MS behavior of highly sulfated GAGs have had limited success because they did not investigate the appropriate precursor species. When more than a single protonated acidic group remains in a precursor ion, MS/MS fragmentation is generally accompanied by the loss of SO_3. Since sulfo group loss effectively competes with glycosidic and through-ring cleavage, this results in a loss of sequence-informative fragmentation. In conclusion, MS/MS analysis can provide complete sequence coverage for highly sulfated GAG oligosaccharides if the precursor molecular ion has all, or all but one, of its acidic groups deprotonated through a combination of charging and Na^+/H^+ exchange.

■ ASSOCIATED CONTENT

Supporting Information

Additional FTICR tandem mass spectra, Orbitrap data, isotope labeling results, and mass-intensity tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Fax: 706-542-9454. E-mail: jamster@uga.edu.

Present Address

¶Agilent Technologies, Santa Clara, CA 95051

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

National Institutes of Health Grant GM38060 is gratefully acknowledged for supporting this work.

■ REFERENCES

(1) (a) Blackhall, F. H.; Merry, C. L.; Davies, E. J.; Jayson, G. C. *Br. J. Cancer* **2001**, *85*, 1094−1098. (b) Kjellen, L.; Lindahl, U. *Annu. Rev. Biochem.* **1991**, *60*, 443−475, DOI: 10.1146/annurev.-bi.60.070191.002303.

(2) Vlodavsky, I.; Friedmann, Y. *J. Clin. Invest.* **2001**, *108*, 341−347.

(3) Liu, D.; Shriver, Z.; Qi, Y.; Venkataraman, G.; Sasisekharan, R. *Semin. Thromb. Hemost.* **2002**, *28*, 67−78.

(4) Hilgard, P.; Stockert, R. *Hepatology* **2000**, *32*, 1069−1077, DOI: 10.1053/jhep.2000.18713.

(5) Higashiyama, S.; Abraham, J. A.; Klagsbrun, M. *J. Cell Biol.* **1993**, *122*, 933−940.

(6) Rabenstein, D. L. *Nat. Prod. Rep.* **2002**, *19*, 312−331.

(7) Thanawiroon, C.; Rice, K. G.; Toida, T.; Linhardt, R. J. *J. Biol. Chem.* **2004**, *279*, 2608−2615, DOI: 10.1074/jbc.M304772200.

(8) (a) Capila, I.; Gunay, N. S.; Shriver, Z.; Venkataraman, G. In *Chemistry and Biology of Heparin and Heparan Sulfate*; H. G. Garg, C. A. Hales, R. J. Linhardt, Eds.; Elsevier Science: Amsterdam, 2005, pp 55−77. (b) Chi, L.; Amster, J.; Linhardt, R. J. *Curr. Anal. Chem.* **2005**, *1*, 223−240.

(9) Naggar, E. F.; Costello, C. E.; Zaia, J. *J. Am. Soc. Mass. Spectrom.* **2004**, *15*, 1534−1544.

(10) Jones, C. J.; Beni, S.; Limtiaco, J. F. K.; Langeslay, D. J.; Larive, C. K. *Ann. Rev. Anal. Chem.* **2011**, *4*, 439−465, DOI: 10.1146/annurev-anchem-061010-113911.

(11) Zaia, J.; Costello, C. E. *Anal. Chem.* **2003**, *75*, 2445−2455, DOI: 10.1021/ac0263418.

(12) (a) Wolff, J. J.; Amster, I. J.; Chi, L.; Linhardt, R. J. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 234−244. (b) Wolff, J. J.; Chi, L.; Linhardt, R. J.; Amster, I. J. *Anal. Chem.* **2007**, *79*, 2015−2022, DOI: 10.1021/ac061636x. (c) Wolff, J. J.; Leach, F. E.; Laremore, T. N.; Kaplan, D. A.; Easterling, M. L.; Linhardt, R. J.; Amster, I. J. *Anal. Chem.* **2010**, *82*, 3460−3466, DOI: 10.1021/ac100554a.

(13) (a) Wolff, J. J.; Laremore, T. N.; Busch, A. M.; Linhardt, R. J.; Amster, I. J. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 790−798. (b) McClellan, J. E.; Costello, C. E.; O'Connor, P. B.; Zaia, J. *Anal. Chem.* **2002**, *74*, 3760−3771, DOI: 10.1021/ac025506+.

(14) Saad, O. M.; Leary, J. A. *Anal. Chem.* **2003**, *75*, 2985−2995, DOI: 10.1021/ac0340455.

(15) Taylor, C. J.; Burke, R. M.; Wu, B.; Panja, S.; Nielsen, S. B.; Dessent, C. E. H. *Int. J. Mass Spectrom.* **2009**, *285*, 70−77, DOI: 10.1016/j.ijms.2009.04.009.

(16) Petitou, M.; van Boeckel, C. A. A. *Angew. Chem., Int. Ed.* **2004**, *43*, 3118−3133, DOI: 10.1002/anie.200300640.

(17) (a) Rosenberg, R. D.; Lam, L. *Proc. Nat. Acad. Sci.* **1979**, *76*, 1218−1222. (b) Noti, C.; Seeberger, P. H. *Chem. Biol.* **2005**, *12*, 731−756, DOI: 10.1016/j.chembiol.2005.05.013.

(18) Kuberan, B.; Lech, M. Z.; Beeler, D. L.; Wu, Z. L.; Rosenberg, R. D. *Nat. Biotechnol.* **2003**, *21*, 1343−1346; http://www.nature.com/nbt/journal/v21/n11/suppinfo/nbt885_S1.html.

(19) (a) Zaia, J.; Li, X.; Chan, S.; Costello, C. *J. Am. Soc. Mass. Spectrom.* **2003**, *14*, 1270−1281, DOI: 10.1016/s1044-0305(03)00541-5. (b) Hitchcock, A. M.; Costello, C. E.; Zaia, J. *Biochemistry* **2006**, *45*, 2350−2361, DOI: 10.1021/bi052100t.

(20) (a) Gilar, M.; Belenky, A.; Wang, B. H. *J. Chromatogr., A* **2001**, *921*, 3−13, DOI: 10.1016/s0021-9673(01)00833-0. (b) Fountain, K. J.; Gilar, M.; Gebler, J. C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1295−1302, DOI: 10.1002/rcm.1481. (c) Laremore, T. N.; Leach, F. E. III; Solakyildirim, K.; Amster, I. J.; Linhardt, R. J. *Methods Enzymol.* Minoru, F., Ed.; Academic Press, 2010, Vol. *478*, pp 79−108.