FOCUS: MASS SPECTROMETRY IN GLYCOBIOLOGY AND
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Analysis of Sulfate Patterns in Glycosaminoglycan Oligosaccharides by MSⁿ Coupled to Infrared Ion Spectroscopy: the Case of GalNAc4S and GalNAc6S

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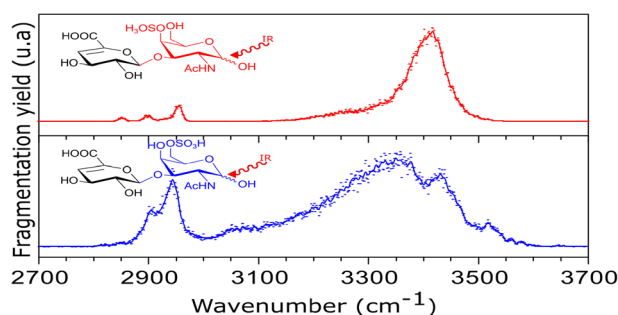
Abstract. We report distinctive spectroscopic fingerprints of the monosaccharide standards GalNAc4S and GalNAc6S by coupling mass spectrometry and ion spectroscopy in the 3-μm range. The disaccharide standards CSA and CSC are used to demonstrate the applicability of a novel approach for the analysis of sulfate position in GalNAc-containing glycosaminoglycans. This approach was then used for the analysis of a sample containing CSA and CSC disaccharides. Finally, we discuss the generalization of the coupling of mass spectrometry with ion spectroscopy for the structural analysis of glycosaminoglycans on a tetrasaccharide from dermatan sulfate source.

Keywords: Glycosaminoglycans, Chondroitin sulfate, Dermatan sulfate, Ion spectroscopy, Infrared spectroscopy, IRMPD, Mass spectrometry, Structural characterization, Sulfate patterns

Introduction

Glycosaminoglycans (GAGs) are negatively charged linear carbohydrates, consisting of a repetition of disaccharides composed of a monosaccharide (D-glucuronic acid or L-iduronic acid or galactose) and an *N*-acetyl hexosamine (*N*-acetyl glucosamine or *N*-acetyl galactosamine). They are expressed on the surface of mammalian cells or are present in the extracellular matrix. Depending on the nature of the monosaccharides and the way the disaccharides are connected to one another, GAGs are classified into five families that are divided into two groups: the sulfated group: heparins (Hep) and heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS) which are bound to proteins

and the non-sulfated group: hyaluronic acid (HA) which is free. These polysaccharides play an important role in many biological processes such as infections, [1] coagulation, [2] cellular interaction and development, [3, 4] inflammation [5, 6], and cancer. [7, 8]

Despite this seemingly simple structure, GAGs are a sugar family that is difficult to analyze. In particular, two structural features must be elucidated: the nature of hexuronic acids and the sulfate pattern. The latest is extremely dynamic and varies considerably depending on the type of cell analyzed, its level of activation or differentiation or its physio-pathological state. The sulfate pattern of GAGs influences their biological activities, [5] it is thus of crucial importance to elaborate analytical strategies to decipher them.

Methods based on mass spectrometry (MS) are increasingly popular for the analysis of carbohydrates. [9] MS analysis of GAGs primarily relies on the availability of standards, which allow establishing libraries of MS and MS/MS signatures, [10, 11] in particular using collision-induced dissociation (CID). A major limitation of this approach is the frequent lack of diagnostic fragments. Indeed, the main fragments obtained by CID

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often correspond to the dissociation of the glycosidic bond, and do not bear direct structural information on the position of sulfated groups. The analysis of the sulfate pattern is particularly challenging in the case of chondroitin sulfate, which may bear a sulfate function on the neighboring four and six positions of the GalNAc unit. In such situation, it is generally accepted to rely on the pattern of relative intensities of the fragments to identify an unknown GAG by comparison with a library of GAG standards. However, the robustness of this strategy is questionable, as several authors have reported conflicting MS/MS results in early studies, which suggests that the relative intensity of the fragments strongly depend on the experimental conditions. [12–14] The influence of the collision energy, in particular, has been extensively examined by Zaïa et al. for CS oligosaccharides. [12] Zaïa et al. later proposed a multistage CID strategy, which appears more robust. [15] Original CID approaches based on the chemical modifications of the oligosaccharides prior to MS have also shown great potential for the identification of the sulfate position in chondroitin sulfate. These include replacement of the sulfate functions by acetyl functions [16] and regioselective enzymatic 6-O-desulfation. [17]

Nevertheless, cross-ring cleavages bearing direct structural information are sought after, which stimulated the exploration of alternative fragmentation methods. EDD (electron detachment dissociation) of carbohydrates was first proposed by Linhardt et al., who reported abundant formation of both cross-ring and glycosidic cleavages on multiply deprotonated heparan sulfate tetrasaccharides [18] and dermatan sulfate oligosaccharides. [19] NETD (negative electron transfer dissociation), which has the advantage of being compatible with most mass spectrometers, was shown to produce similar fragments. [20] Singly charged heparan sulfate and dermatan sulfate tetrasaccharides could be addressed using EID (electron induced dissociation). [21] These approaches are particularly adapted to the disambiguation of hexuronic acid epimers. To hinder sulfate loss and simplify the interpretation of EDD patterns, Amster et al. explored the influence of the charge state, and in particular the replacement of protons with sodium cations. [22] The formation of diagnostic fragments was also proposed in the positive mode—and greatly enhanced by the formation of metal adducts—using ECD (electron capture dissociation) [23] or high-energy collision-induced dissociation (HCD). [24]

Hyphenated MS-based methods have also been proposed to provide multidimensional fingerprints of the ions of interest, combination of the MS (or MS/MS) signature and additional structural information on the precursor. Such approaches include ion mobility spectrometry (IMS), which can be used both as a separative and structural method and provides the collision cross section of the ions. Discrimination of 4-sulfate and 6-sulfate isomers in CS disaccharides [25, 26] and synthetic CS oligosaccharides [27] were reported. Besides, ion spectroscopy in the IR and UV domains has been proposed to disambiguate GAG-related monosaccharide and disaccharide ions: Cagmat et al. have reported distinctive IRMPD spectra for hexuronic acid isomers. [28] Racaud et al. have reported wavelength-dependent UV photodissociation of heparan disaccharides, [29] and Khanal et al. have reported the IR signatures of a series of GAG disaccharides standards using cryogenic messenger-tagging spectroscopy. [26]

In this context, we have conducted in-depth structural analysis of GAG-relevant compounds using a combination of IRMPD spectroscopy and quantum chemistry, including N-acetyl hexosamine, [30] sulfated monosaccharides [31, 32], and sulfated disaccharides. [33] We have recently proposed an original strategy based on the combination of MS analysis and ion spectroscopy for carbohydrate sequencing, [34] which was successfully applied to the identification of the hexuronic acid content in a HA tetrasaccharide [35].

Here, we report the first application of this spectroscopic sequencing approach to the identification of GalNAc4S and GalNAc6S in GAG oligomers. First, we discuss two situations where IRMPD spectroscopy of the precursor ion does not provide sufficient structural information, which justifies the recourse to ion spectroscopy of the fragment ions. In the second section, we establish the reference spectroscopic fingerprints of the GalNAc4S and GalNAc6S monosaccharide standards, and we validate that the position of the sulfate group can be retrieved by spectroscopic analysis of sulfated fragments. This approach is used to identify the content of a mixture of CS disaccharide standards. Finally, we verify that sequential fragmentation followed by spectroscopic analysis of the fragments can be used to identify the sulfate pattern of a tetrasaccharide.

Methods

Samples Preparation

GalNAc4S (N-acetyl-D-galactosamine-4-O-sulfate) and GalNAc6S (N-acetyl-D-galactosamine-6-O-sulfate) monosaccharide standards were purchased from Carbosynth, UK. Chondroitin sulfate disaccharide standards, CSA [ΔUA (unsaturated uronic acid) – GalNAc4S] and CSC [ΔUA – GalNAc6S] and Dermatan sulfate tetrasaccharide DS-dp4 [ΔUA – GalNAc4S – IdoA (L-iduronic acid) – GalNAc4S] were purchased from Iduron, UK. The mixture of CSA and CSC disaccharide standards was purchased from Dextra, UK. Samples were prepared for mass spectrometry at a concentration of 10 μg/mL in H_2O-MeOH (50/50) solution. Acetic acid (0.1%) was added to the solution to promote electrospray ionization. Although acetic acid is generally used to promote protonated ions, it is found to be favorable for the production of GAGs in the negative mode in our experimental conditions.

Mass Spectrometry

MS analysis was carried out in a commercial mass spectrometer (Thermofinnigan LCQ Classic) equipped with an electrospray ionization source set in negative mode (production of deprotonated ions) and a quadrupolar 3D ion trap analyzer. Collisional activation was performed by resonant activation of the trapped ions in a helium buffer for MS^n analysis.

Infrared Ion Spectroscopy

The setup used for IR ion spectroscopy analysis was previously described by Schindler et al. [31] In brief, the mass spectrometer was modified to inject a laser beam produced by a YAG-pumped tunable IR OPO/OPA inside the ion trap, in order to irradiate the ion cloud. This enables in situ gas-phase spectroscopy of the trapped ions. Owing to the low density of ions (typically few hundreds of ions within 1 mm^3, that is equivalent to fM concentration), traditional absorption spectroscopy is not feasible. Instead, the alternative action spectroscopy scheme IRMPD (infrared multiple photon dissociation spectroscopy) is used. Upon resonant laser excitation of a vibrational mode, the internal energy of the ion increases and quickly relaxes by fragmentation, as other rapid de-excitation paths are not available in the gas phase. The photo-fragmentation yield is calculated using the formula: IRMPD yield $= -\log[I\text{parent} \div (I\text{parent} + I\text{fragment})]$, where I_{parent} is the intensity of the parent ion and I_{fragment} is the total intensity of fragments detected after laser irradiation. The fragmentation yield is then monitored as a function of the wavenumber to retrieve the absorption spectrum of the analyte. As compared to spectroscopy in the condensed phase, this MS/IR approach has two advantages. Firstly, owing to the MS selection of the ions prior to spectroscopy, it is possible to record the optical signature of mass-selected components in complex samples. Thus, the purity of the sample is less critical. Secondly, gas-phase IR spectroscopy offers a better structural resolution than condensed phase spectroscopy. Indeed, in the absence of intermolecular interactions, vibrational modes in the 3-μm range are better resolved and give a refined picture of the intra-molecular interactions. All spectra shown thereafter were sampled as follows: each data point corresponds to the photo-fragmentation yield averaged five times without further treatment of the data. A smoothing FFT Filter averaging is then overlayed in solid line to guide the eyes. The acquisition time of a spectrum is 45 min and requires about 250 μl of sample.

Results and Discussion

Spectroscopic Analysis of Sulfated GAG Standards

Schindler et al. have recently demonstrated the resolving power of gas-phase IR spectroscopy in the case of positional isomers of sulfated monosaccharides and disaccharides. [33] Here, we further explore the performance of this approach for the resolution of sulfate patterns in systems of increasing complexity, i.e., a DS tetrasaccharide standard featuring two sulfate groups and a mixture of CSA and CSC disaccharide standards. Their IRMPD spectra are displayed in Fig. 1a, b, respectively.

The IRMPD spectrum of the doubly deprotonated precursor ion of dermatan sulfate tetrasaccharide shows a broad region of vibrational activity from 3250 to 3550 cm^{-1}, which corresponds to an unresolved ensemble of OH and NH stretching modes. It also shows two weak bands at 2900 and 2950 cm^{-1} in the CH stretching range. The IRMPD spectrum of the singly
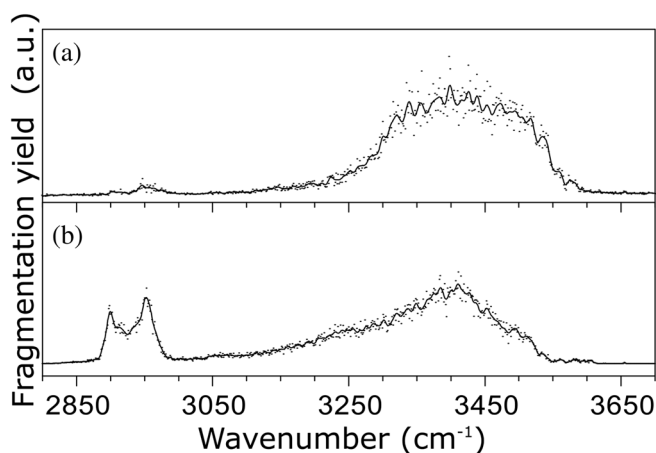

Figure 1. IRMPD signatures of GAG di- and tetra- saccharides. (**a**) IR spectrum of doubly deprotonated precursor ion DS-dp4. (**b**) IR spectrum of singly deprotonated precursor ion mixture of CSA and CSC disaccharides

deprotonated precursor ion of the second sample also displays a broad and unresolved pattern in the OH stretch region, with an apex at 3400 cm^{-1}. Its CH stretch pattern consists of a set of unresolved bands with two maxima at 2900 and 2950 cm^{-1}.

It is evident that these spectra are poorly resolved, and as a consequence do not offer highly distinctive fingerprints for analytical purpose. This raises the question of the limitation of ion spectroscopy for the structural characterization of sulfated carbohydrates. On the basis of these observations, two general effects altering the spectroscopic resolution are discussed thereafter.

On one hand, the IRMPD spectrum of the tetrasaccharide shows less structure than these previously reported for sulfated monosaccharides and disaccharides. This is essentially due to the increasing congestion of the OH spectral range as the number of OH groups increases. Although the resolution can be enhanced by a pertinent choice of counter-ion, [33] the loss of structural information as the size of the oligosaccharide increases is a general trend, which was also observed for other classes of carbohydrates. [36] To gain more spectroscopic resolution, promising cryogenic spectroscopic schemes have been reported by Rizzo [26] and Pagel. [37] However, such alternative schemes have not yet been tested on negative ions, for which the spectroscopic resolution might be very different. [33]

On the other hand, IRMPD can readily deconvolute the optical responses of several species present in a sample provided they have different m/z values, it is not the case for mixtures of isomers. Here, although CSA and CSC disaccharide standards show distinctive fingerprints, [30] the optical signature of the mixture remains convoluted. From such spectroscopic data, it is difficult to retrieve the composition, not to mention the relative amounts present in the sample. Note that ion mobility spectrometry was also proposed for the discrimination of positional isomers of sulfated GAG disaccharides. [26, 27] While diagnostic drift times have been obtained for standards, the lack of baseline resolution augurs for similar limitations for future analysis of heterogeneous samples.

In the next section, we demonstrate how a hyphenated MS[n]-IR strategy can be used to overcome these limitations.

MS[n]-IR Analysis of Sulfate Patterns

The MS[n]-IR analysis has two advantages. Firstly, by recording the fingerprints of carbohydrate fragments rather that the fingerprint of the precursor ions, one produces a larger set of spectroscopic data related to the carbohydrate of interest. Secondly, by reducing the precursor into smaller sub-units, the spectroscopic resolution of each piece of spectroscopic data increases. Finally, it was recently reported that sequential fragmentation of oligosaccharides, combined with spectroscopic analysis of the monosaccharide fragments, can be used to retrieve the primary sequence of the precursor. [34] This strategy is applied to the analysis of the two aforementioned samples.

Reference Spectra of Sulfated N-Acetyl Galactosamine Standards

The MS[n]-IR strategy relies on pre-existing and distinctive spectroscopic fingerprints of pertinent monosaccharide standards, which are used as references for the spectroscopic analysis of the MS fragments. For the elucidation of the sulfate patterns in CS and DS oligosaccharides, the pertinent monosaccharide standards are GalNAc4S and GalNAc6S. Note that upon sequential collision-induced fragmentation, both intact monosaccharide fragments and their dehydrated counterparts may be produced. Therefore, not only the spectra of the monosaccharide standards but also the spectra of the dehydrated monosaccharide standards must be referenced.

The reference IRMPD spectra of singly deprotonated precursor ions of GalNAc4S and GalNAc6S monosaccharide standards (*m/z* 300) are displayed in Fig. 2a They are distinct, allowing unambiguous identification of the two positional isomers. Specifically, GalNAc4S shows three minor peaks at 2850, 2898, and 2954 cm^{-1} and an intense feature centered around 3410 cm^{-1}. In contrast, GalNAc6S presents an intense doublet of partially resolved bands at 2905 and 2942 cm^{-1} and a broad active region from 3050 to 3550 cm^{-1} with an apex at 3350 cm^{-1} and two partially resolved bands at 3429 and 3520 cm^{-1}.

The reference IRMPD spectra of singly deprotonated dehydrated standards (*m/z* 282) also have distinctive IR signatures in the 3050–3600 cm^{-1} range, as shown in Fig. 2b The gas-phase IR spectrum of GalNAc4S-H_2O shows a broad unresolved active region between 3050 and 3500 cm^{-1} with an apex at 3385 cm^{-1} and a small band at 3574 cm^{-1}. In contrast, GalNAc6S-H_2O shows an intense feature centered around 3300 cm^{-1}.

Analysis of the CSA and CSC Disaccharides

The MS/MS spectra of CSA, CSC, and the two components mixture are shown in the top layer of Fig. 3b, c, d, respectively. The singly deprotonated precursor ions are observed at *m/z* 458. The fragments of interest, i.e., the sulfated GalNAc moiety (Y1 ion) and its dehydrated counterpart (Y1B2 ion), are the
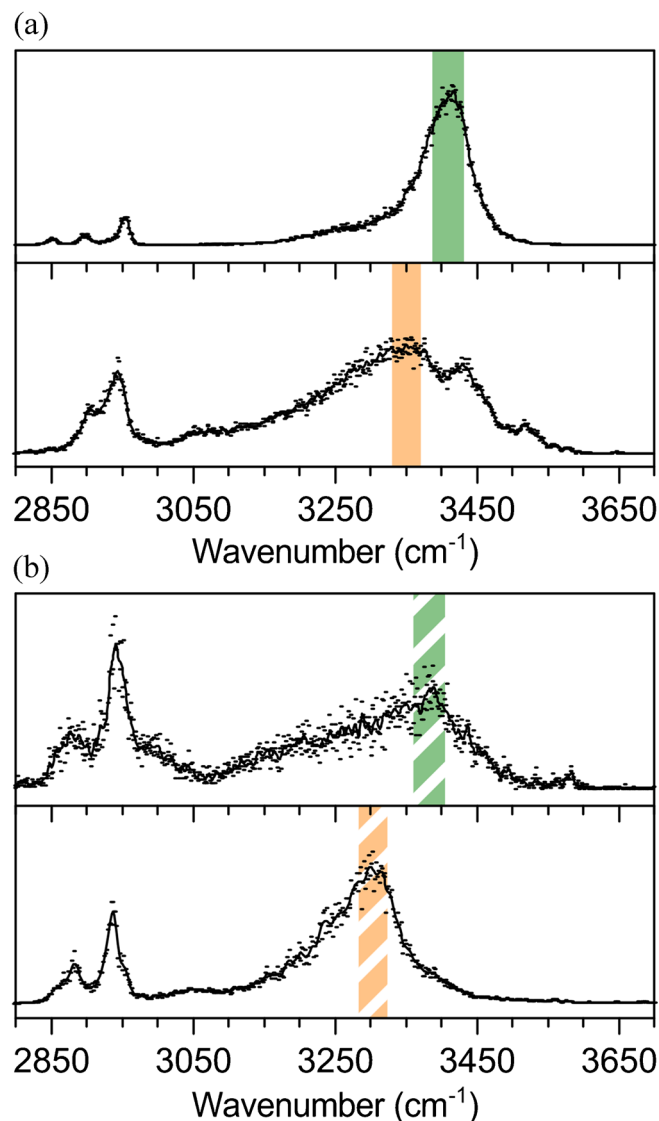

Figure 2. IRMPD signatures of sulfated GalNAc monosaccharide standards. IR spectra of singly deprotonated precursor ion and its dehydrated counterpart. (**a**) Top layer: GalNAc4S with apex highlighted in solid green, lower layer: GalNAc6S with apex highlighted in solid orange. (**b**) Top layer: GalNAc4S-H_2O with apex highlighted in dashed green, lower layer: GalNAc6S-H_2O with apex highlighted in dashed orange

main fragments formed in our experimental conditions, and observed in variable relative amounts at *m/z* 300 and *m/z* 282, respectively. (Note: the peak at *m/z* 282 is very minor and is not visible in Fig. 3b, see Supp. Info for a zoom). These two fragments are present for both CSA and CSC standards; therefore, they are not directly diagnostic of the precursor. Their relative intensity however is strongly contrasted, and it is tempting to use such patterns for the identification of the precursor. Yet, several authors have reported conflicting fragmentation patterns for sulfated *N*-acetyl galactosamine-containing oligosaccharides [12–14] and it appears that fragmentation patterns are highly dependent on the experimental conditions—in particular on the collisional energy, as
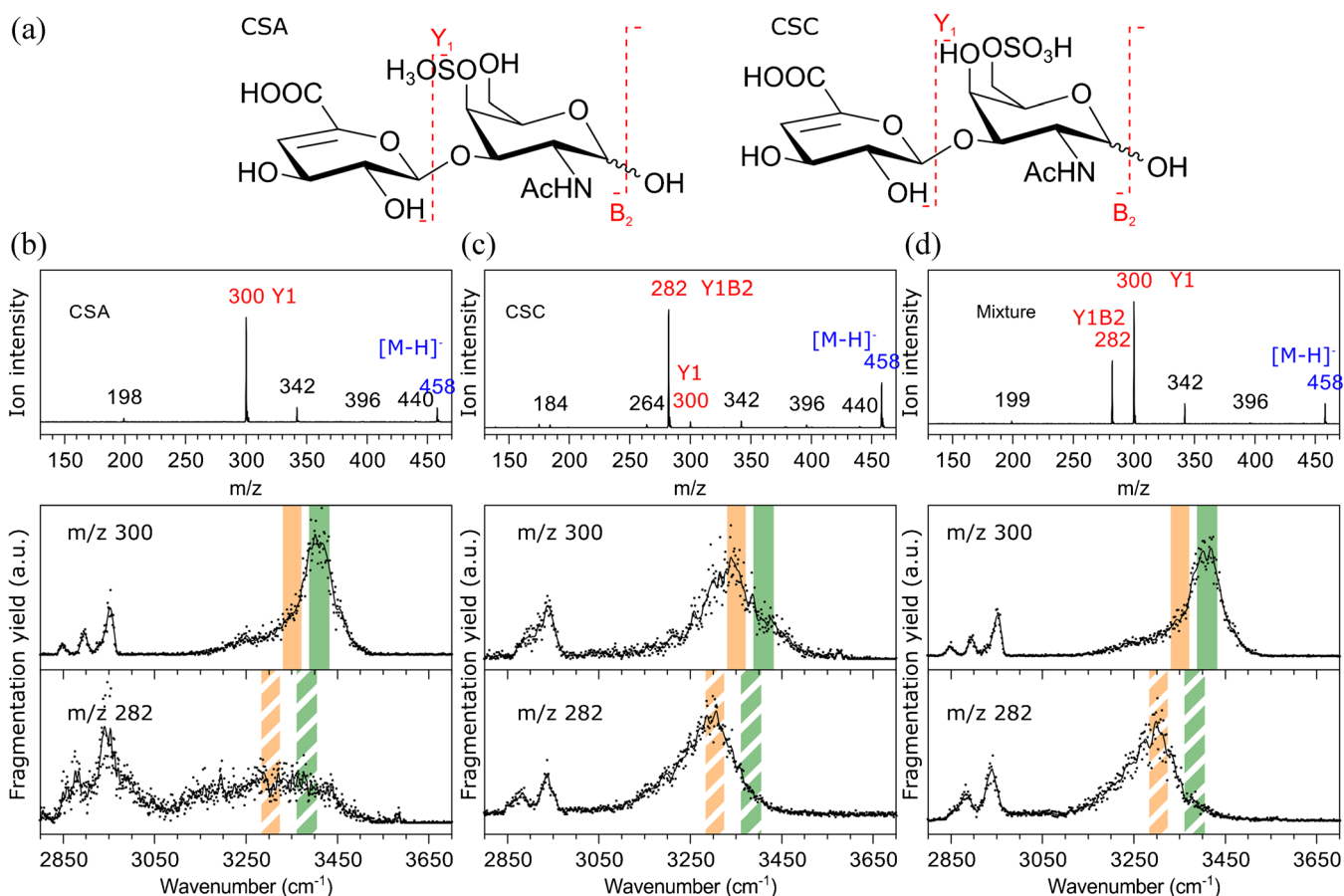
Figure 3. Analysis of the sulfate pattern of CS disaccharides. (**a**) CS disaccharide molecules and their main fragments (left: CSA, right: CSC). Spectroscopic analysis of the sulfated GalNAc moiety of: (**b**) CSA, (**c**) CSC, and (**d**) two components mixture of CSA and CSC. Top panels: CID spectra of singly deprotonated precursor ions (in blue), fragments of interest are marked in red. Center panels: IRMPD spectra of singly deprotonated Y1 ions. Lower panels: IRMPD spectra of singly deprotonated Y1B2 ions. Diagnostic bands of the GalNAc4S (orange) and GalNAc6S (green) standards are overlayed for facile structural identification of the fragments

extensively documented by Zaïa et al. [12] Therefore, in absence of diagnostic fragments, MS/MS analysis must be carried out with extreme caution.

The vibrational spectrum of a molecular ion on the other hand, is an intrinsic property, which does not depend on the MS conditions. The spectroscopic approach is thus expected to offer a robust alternative to MS/MS analysis. Figure 3b, c, d (center panels) shows the IRMPD spectra of singly deprotonated Y1 ion of CSA, CSC, and the two components mixture, respectively. The lower panels show the IRMPD spectra of singly deprotonated Y1B2 ion. In order to identify the structure of the precursor, the spectra of the sulfated GalNAc moiety are compared to the reference spectra of sulfated GalNAc standards shown in Fig. 2. For facile visual diagnostic, the apexes of the GalNAc4S and GalNAc6S standards are reported in color in Fig. 3 (dashed colors for the dehydrated standards).

In Fig. 3b, the IRMPD spectrum of the main fragment (*m/z* 300) shows an apex at 3410 cm^{-1} and matches the reference fingerprint of the GalNAc4S standard, which is consistent with the sulfate position in the CSA precursor. Despite its very low

ion intensity, the spectrum of the minor fragment at *m/z* 282 could be recorded and its IRMPD spectrum is found to be similar to this of dehydrated GalNAc4S, which further confirms the structure of the precursor. This highlights the excellent sensitivity of IRMPD spectroscopy.

In Fig. 3c, the IRMPD spectrum of the main fragment (*m/z* 282) shows an apex at 3295 cm^{-1} and matches the reference fingerprint of the dehydrated GalNAc6S standard, which is consistent with the sulfate position in the CSC precursor. The minor fragment (intact sulfated GalNAc moiety at *m/z* 300) coincides with the main fragment of the CSA precursor. One could thus question the origin of this fragment: a minor fragmentation pathway of CSC, or a contamination of the CSC sample by a small amount of CSA? Further spectroscopic analysis of this fragment reveals that its IRMPD spectrum matches the reference spectrum of the GalNAc6S standard, with an apex at 3350 cm^{-1}. This unambiguously validates that it originates from a minor fragmentation pathway and is not due to a contamination of the sample.

Finally, the IRMPD spectra of both *m/z* 300 and *m/z* 282 fragments of the mixed sample are shown in Fig. 3d. They
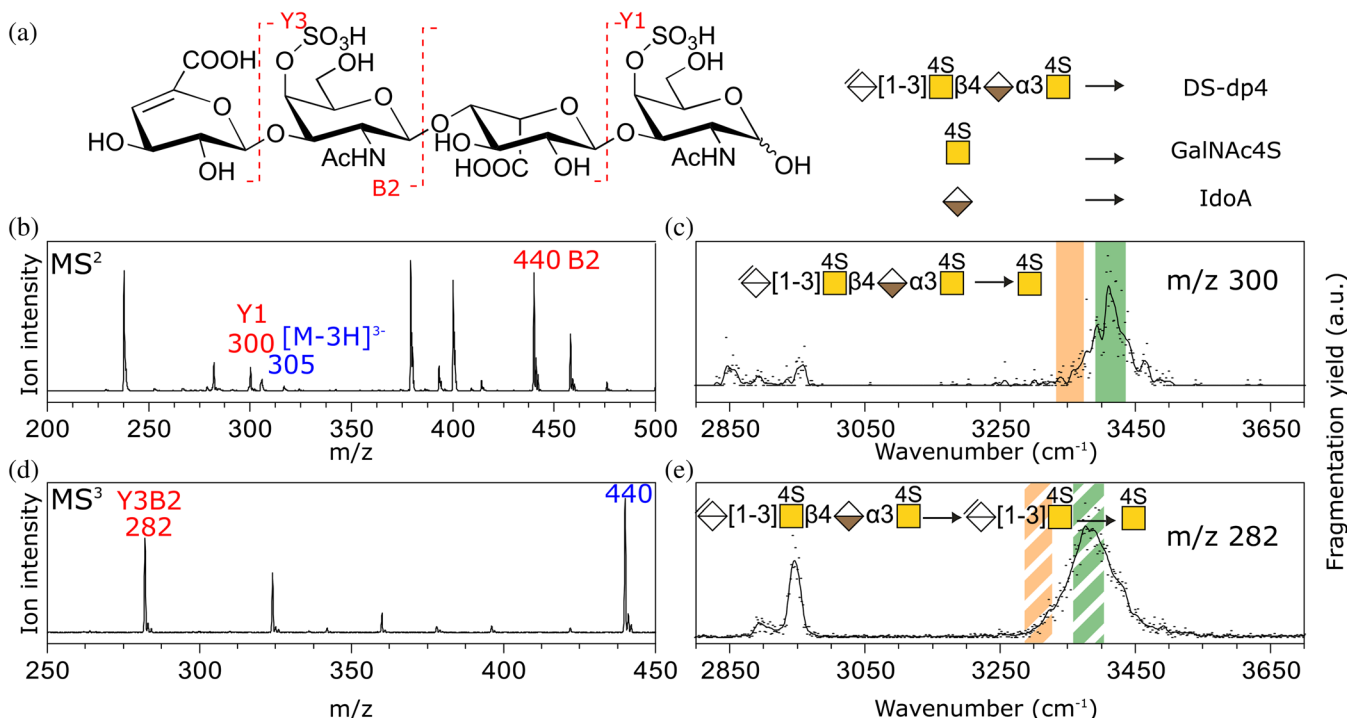
Figure 4. Analysis of the sulfate pattern of DS-dp4. (**a**) DS-dp4 molecule and its main fragments. (**b**) CID spectrum of triply deprotonated DS-dp4, fragments of interest are marked in red. (**c**) IRMPD spectrum of singly deprotonated Y1 ion. (**d**) CID spectrum of singly deprotonated B2 ion, fragment of interest is marked in red. (**e**) IRMPD spectrum of singly deprotonated Y3B2 ion. The diagrams show the fragmentation sequence used to isolate the fragments of interest using the nomenclature from [38]. Diagnostic bands for GalNAc4S (orange) and GalNAc6S (green) standards are superimposed to facilitate structural identification

match the reference spectra of GalNAc4S and dehydrated GalNAc6S standards, respectively. This clearly indicates that the sample contains both CSA and CSC, which was not clearly established by mere IRMPD spectroscopy of the precursor ion, as seen in Fig. 1b This result validates that the MS^n-IR approach is appropriate to overcome the congestion of IRMPD spectra in the case of complex samples.

Analysis of the Sulfate Pattern of a DS Tetrasaccharide

As seen above, the IR signature of the DS tetrasaccharide mostly consists of a broad, unresolved feature ranging from 3250 to 3550 cm^{-1} and must be analyzed through spectroscopic analysis of its fragments. The singly deprotonated Y1 fragment (intact GalNAcS monosaccharide) is observed at *m/z* 300 in the CID spectrum of the triply charged precursor (*m/z* 305) shown in Fig. 4b. Its IRMPD spectrum is shown in Fig. 4c and its apex at 3410 cm^{-1} matches the GalNAc4S reference. Figure 4b also shows the singly deprotonated B2 fragment ion at *m/z* 440. This ion is further isolated and fragmented by CID in an MS^3 stage to form the singly deprotonated Y3B2 ion (dehydrated GalNAcS monosaccharide) at *m/z* 282, as seen in Fig. 4d. Its IRMPD spectrum is shown in Fig. 4e and shows an apex at 3385 cm^{-1}, which matches the dehydrated GalNAc4S reference. Both GalNAc units of the tetrasaccharide are thus 4-O sulfated, which is consistent with the structure of the DS-dp4 standard.

This example validates the relevance of MS-IR coupling sequencing for the elucidation of sulfate patterns in GAG oligosaccharides, and highlights two advantaged of the methodology. On one hand, the structural resolution is significantly enhanced by the MS-IR sequencing approach, as compared to the mere spectroscopic analysis of the precursor ion. On the other hand, while the availability of standards is generally a major bottleneck for MS analysis of GAGs, only a reduced set of relevant monosaccharide references is required here for the analysis of an oligosaccharide.

Conclusion

The structural resolution of ion spectroscopy was previously established for the disambiguation of positional isomers of sulfated monosaccharides and disaccharides, which opens the way to new strategies for the analysis of glycosaminoglycans. [33] Here, we further reported diagnostic spectroscopic fingerprints of GalNAc4S and GalNAc6S, a major pair of positional isomers present in chondroitin sulfate and dermatan sulfate. Limitations of the ion spectroscopy approach can be foreseen however and were illustrated in two different situations that result in a loss of spectroscopic resolution: a two components mixture of isomers and a GAG standard of larger size. In order to overcome these limitations and to generalize ion spectroscopy as an effective tool to dissect GAG structure, we proposed to apply a combination of MS^n analysis and ion spectroscopy.

This strategy was first validated using disaccharide standards of CSA and CSC, and was used in turn to confirm the purity of the latest. Then, the approach was applied to the elucidation of the structures present in two components mixture, offering an interesting alternative to the traditional analysis of MS/MS patterns which are known to be strongly condition-dependent. Finally, the feasibility of sulfate pattern analysis using sequential fragmentation was established using a DS tetrasaccharide standard. As the analysis only requires the availability of reference spectroscopic data for GalNAc4S and GalNAc6S monosaccharide standards, it is readily applicable to other DS and CS oligosaccharides. More generally, we expect that the analysis of sulfate pattern, together with the previously reported analysis of the hexuronic acid content, [35] can be generalized to the sequence analysis of all groups of GAGs. IRMPD spectra also constitute valuable data for the exploration of the structure of carbohydrate fragments by quantum chemistry modelization. [39] An interesting prospective of this work is thus the fundamental understanding of the fragmentation mechanisms taking place in glycosaminoglycans.

Funding Information

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