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Mass Spectrometric Fragmentation Analysis of Oligosialic and Polysialic Acids

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Oligosialic and polysialic acids (oligo/polySia) are characterized by high structural diversity, because of different types of sialic acids and glycosidic linkages. Although several methods have been described for the analysis of oligo/polySia, only high-performance liquid chromatography (HPLC) analysis in conjunction with 1,2-diamino-4,5-methylenedioxybenzene labeling, fluorometric C7/C9 detection, Western blotting, and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) of lactonized oligo/polySia species, require submicrogram amounts of analyte. Since these methods do not provide detailed structural information, this study is focused on the characterization of oligo/polySia by tandem mass spectrometry (MS/MS). MALDI-TOF-MS/MS and electrospray ionization tandem mass spectrometry (ESI-MS/MS), employing up to three cycles of ion isolation and fragmentation in an ion trap, have been used for the characterization of nonderivatized glycans, oligoSia species modified at their reducing or nonreducing ends, as well as partially O-acetylated oligoSia derivatives. The obtained spectra were dominated by simultaneous cleavage of glycosidic linkages and the corresponding lactone ring, whereas classical cross-ring fragments were of minor abundance. However, the combined use of the two different types of fragmentation analysis allowed a sensitive and detailed characterization of both short-chained oligoSia and long polySia species. Furthermore, oxidation of the nonreducing end sugar moiety enabled sequence determination and localization of acetylated and nonacetylated sialic acid residues.

Sialic acids represent a family of more than 50 different α -keto acids that comprise a nine-carbon backbone. Their structural diversity mainly arises from acetylation, phosphorylation, methylation, lactylation, and/or sulfation of the three major isoforms occurring in nature: *N*-acetylneurameric acid (Neu5Ac), *N*-glycolylneurameric acid (Neu5Gc), and 2-keto-3-deoxy-D-glycero-D-galacto-nonoic acid (KDN).^{1,2} Sialic acids are mainly present as

terminal monomers at the nonreducing ends of glycoprotein- or glycolipid-glycans, but also may be linked to each other, thus building up disialyl units or linear oligosialic and polysialic acid (oligo/polySia) chains.³ These chains may exhibit a high structural diversity, because of the presence of different sialic acid compounds, as well as intersialyl linkages and a varying degree of polymerization.^{4–8} The various types of oligo/polySia chains are widely distributed in nature, ranging from neuroinvasive bacteria and fish eggs up to humans, and play diverse important roles in all these species.^{3,7,9} In vertebrates, α 2,8-linked Neu5Ac polymers are predominantly attached to *N*-glycans of the neural cell adhesion molecule (NCAM) in a developmentally regulated manner, thus influencing cell adhesion, signaling, migration, and plasticity in the central nervous system.^{10–12} The polysialylation status of NCAM is strictly controlled, which allows a fine-tuning of NCAM functions.^{13,14} Recently, not only the distribution and the biological role of oligo/polySia but also its use as a biomaterial in medicine have gained considerable interest.^{15–19} Especially, the therapeutic application of polySia in humans requires powerful tools for the characterization and quality control of the polymers.

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So far, several methods have been reported for the characterization of oligo/polySia,^{3,20} among which, however, only derivatization with 1,2-diamino-4,5-methylenedioxybenzene (DMB) followed by high-performance liquid chromatography (HPLC) and fluorometric detection, fluorometric C7/C9 analysis, and Western blotting were of sufficient sensitivity. Moreover, all these methods have several disadvantages, as discussed previously.^{3,21} Therefore, we have recently developed an ultrasensitive method employing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for the characterization of oligo/polySia, offering a number of advantages, in comparison to the traditional techniques.²¹

Efficient ionization requires an acid-induced lactonization of oligo/polySia molecules, which leads to a six-membered ring by esterification of the carboxyl group at C-1 and the hydroxyl group at C-9 of the preceding sialic acid residue.^{21,22} To minimize the amount of analyte required, we performed lactonization directly on the sample carrier. Our results demonstrated that the MALDI-TOF-MS-based method was considerably more sensitive than the DMB-HPLC approach. Moreover, polySia chains with more than 100 sialic residues were easily detectable, and their exact compositions could be calculated because of unambiguous mass determination. In addition, the DMB-HPLC method could be combined with MS analysis when peaks of interest were collected and subsequently analyzed by MALDI-TOF-MS. Because of different lactonization characteristics, it was further possible to distinguish between α 2,8- and α 2,9-linked oligo/polySia.

In the present study, we have established methods for a more detailed structural analysis of, in part, *O*-acetylated oligo/polySia derivatives by MALDI-TOF-MS/MS and electrospray ionization tandem MS (ESI-IT-MSⁿ), which allow an identification of the monosaccharide sequence and allocation of *O*-acetyl substituents.

EXPERIMENTAL SECTION

Materials. Endoneuraminidase (EndoN) from bacteriophage K1F was purified as described previously.²³ Colominic acid was purchased from EY Laboratories (San Mateo, CA). All reagents used were of analytical grade.

Preparation of oligoSia. Colominic acid (10 mg) was degraded using 1 ng EndoN at 37 °C for 2 h in 1 mL of 20 mM Tris/HCl buffer, pH 8.0. The resulting oligoSia comprised a maximal chain length of 7 sialic acid residues. For isolation of bacterial oligoSia *Escherichia coli* (*E. coli*) K1 strains, isolated from a healthy individual (*E. coli* K1 D17, OAc-) and a patient suffering from urinary tract infection (*E. coli* K1 D40, OAc+), were cultivated and harvested as described previously.²¹ Dried pellets were resolved in 10 mM Tris/HCl buffer, pH 8.0, and incubated with EndoN as described elsewhere.²⁴ Resulting oligoSia species were harvested with the supernatant after centrifugation (14 000g) at room temperature.

DMB Labeling of oligo/polySia. To label oligo/polySia with DMB (Dojindo, Kumamoto, Japan), samples were dissolved in

80 μ L of DMB reaction buffer and incubated at 50 °C for 1 h, as outlined elsewhere.²⁰ The samples were neutralized via the addition of 50 μ L of 125 mM NaOH.

Reduction of oligo/polySia. Dried oligoSia (10 μ g) was dissolved in 25 μ L of 40 mM sodium acetate buffer, pH 5.5, and 5 μ L 3% ethylene glycol. The sample was incubated at 0 °C overnight with 35 μ L of 0.2 M sodium borohydride in 0.2 M sodium borate buffer, pH 8,²⁵ and purified by dialysis using tubes with an exclusion volume of 500 Da (Carl Roth, Karlsruhe, Germany).

Oxidation of oligoSia. Dried oligoSia (10 μ g) was dissolved in 25 μ L of 40 mM sodium acetate buffer, pH 5.5, prior to addition of 2 μ L of 0.25 M sodium metaperiodate.²⁵ The mixture was incubated in the dark at 0 °C for 3 h and dialyzed as described previously.

Anion Exchange HPLC for Separation of oligo/polySia. For separation, a Knauer (Berlin, Germany) HPLC system was used equipped with a DNAPac PA-200 column (Dionex, Idstein, Germany). Milli-Q water (E1) and 3 M ammonium acetate (E2) were used as eluents at a flow rate of 0.5 mL/min. A fluorescent detector was set at 372 nm for excitation and 456 nm for emission to monitor the HPLC run. Elution was performed by the following gradient: $T_{0\text{ min}} = 0\text{ (v/v) E2}$; $T_{15\text{ min}} = 7\text{ (v/v) E2}$; $T_{25\text{ min}} = 14\text{ (v/v) E2}$; $T_{40\text{ min}} = 20\text{ (v/v) E2}$; $T_{55\text{ min}} = 24\text{ (v/v) E2}$; $T_{115\text{ min}} = 35\text{ (v/v) E2}$. Fractions were collected and lyophilized.^{14,26}

MALDI-TOF-MS/MS Analysis. The oligo/polySia sample (3.5–500 ng in 1 μ L) was loaded onto a stainless steel MALDI-TOF target and allowed to dry. For lactonization, the dried sample was dissolved in 1 μ L of 0.5% phosphoric acid, incubated for 30 min at room temperature, and dried again. The lactonized sample was dissolved in 1 μ L of a saturated solution of 6-aza-2-thiothymine (ATT; Sigma) in 50% acetonitrile containing 0.5% phosphoric acid, thus approaching a ratio of analyte to matrix of ~3–1000 ng oligo/polySia/40 μ g ATT.²¹ An outline of the sample preparation protocol, as well as the diameters of the sample and laser beam, are shown in the Supporting Information (see Figure S1). MALDI-TOF-MS/MS analysis was performed on an Ultraflex I instrument (Bruker-Daltonik, Bremen, Germany) equipped with a nitrogen laser and a LIFT-MS/MS facility in the laser-induced dissociation (LID) mode, as described previously.²⁷ The instrument was operated in the negative-ion reflectron mode.

ESI-IT-MSⁿ Analysis. Lactonization of 1 μ g oligo/polySia was induced in glass vials by 10 μ L 1 N HCl for 2 h at room temperature.²² Samples were dried and suspended in 10 μ L of 75% acetonitrile containing 0.1% formic acid. After centrifugation, the clear supernatant was transferred to a spray capillary (Proxeon, Odense, Denmark) and analyzed using an Esquire 3000 ESI-ion trap-mass spectrometer (Bruker-Daltonik) in the off-line negative-ion mode. In tandem mass spectrometric applications, helium was used as collision gas at a partial pressure of 4×10^{-6} mbar.

RESULTS AND DISCUSSION

Analysis of oligo/polySia by MALDI-TOF-MS and ESI-IT-MS. To establish the proper MALDI-TOF-MS conditions, commercially available colominic acid (α 2,8-linked Neu5Ac-polymer)

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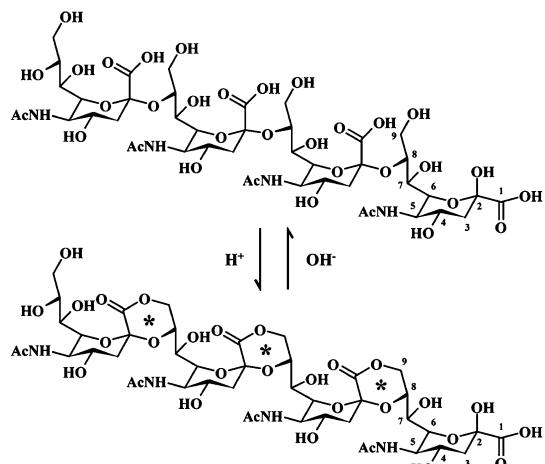
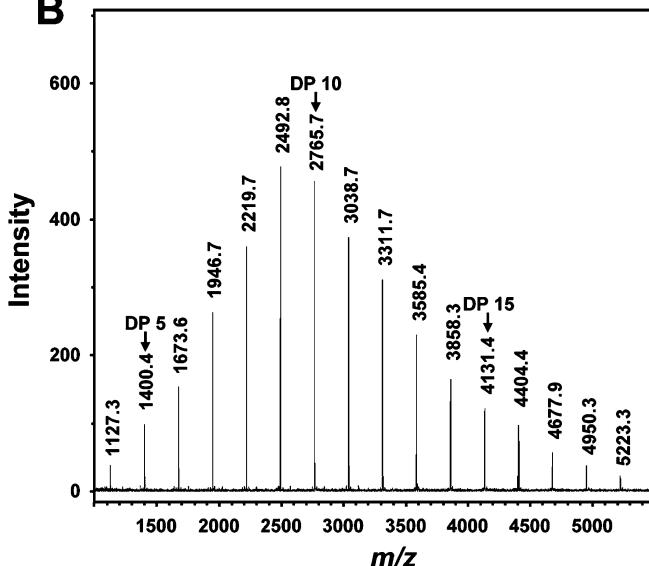
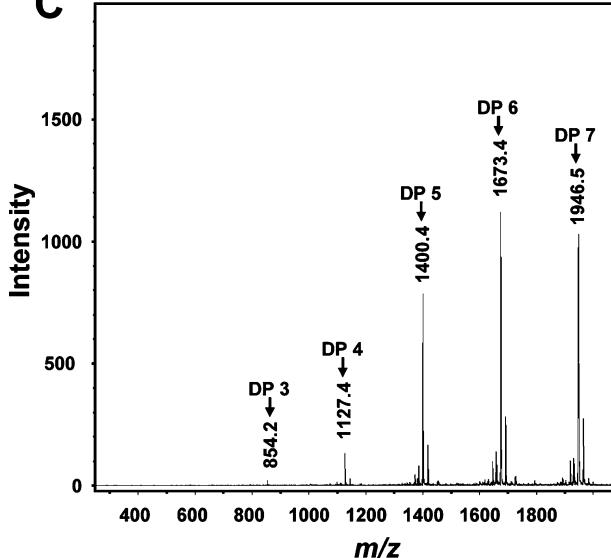
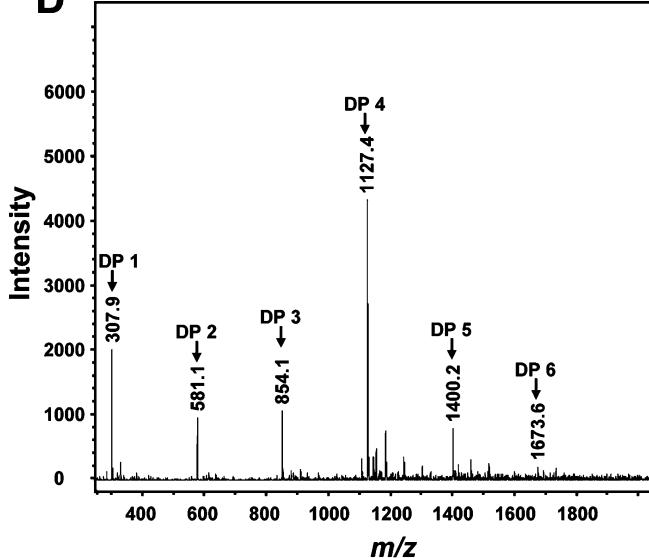
A**B****C****D**

Figure 1. Analysis of oligo/polySia by MALDI-TOF-MS and ESI-IT-MS: (A) lactonization of α 2,8-linked oligo/polySia under acidic conditions (the positions of lactone rings are marked with asterisks); (B) colominic acid (500 ng) was on-target lactonized and analyzed by MALDI-TOF-MS in the negative-ion reflectron mode; (C) following endoN treatment, oligoSia (500 ng) was similarly lactonized and analyzed; and (D) for ESI-IT-MS, oligoSia (500 ng) was lactonized in a reaction tube, dried, redissolved in 75% acetonitrile containing 0.1% formic acid, and analyzed in the negative-ion mode. Monoisotopic masses $[M-H]^-$ and the corresponding degrees of polymerization (DPs) are given.

was lactonized directly on-target and analyzed in the negative-ion mode.²¹ The spectrum displayed the expected ladder-like series of signals with mass differences of 273.1 Da, reflecting complete lactonization of the molecules (see Figures 1A and 1B). In parallel, colominic acid was treated with endoN, cleaving polySia to short chains of, at most, 7 sialic acid residues,²³ and similarly analyzed. Thus, oligoSia chains with 3–7 lactonized Neu5Ac units could be visualized (see Figure 1C).

ESI-IT-MS analyses required a more sophisticated sample preparation, in that lactonization had to be performed in a reaction tube using a volatile acid. After the removal of acid and evaporation to dryness, the sample had to be resolved in a typical spray solvent such as methanol, acetonitrile, and water and transferred into an electrospray needle. Because polySia forms a precipitate upon lactonization,²² which is insoluble in these solvents, no signals of polySia were obtained by ESI-IT-MS. Using short-chained oligoSia

samples after endoN treatment, however, signals of monomeric up to hexameric Neu5Ac chains were easily obtained (see Figure 1D).

Analysis of oligo/polySia by MALDI-TOF-MS and ESI-IT-MS clearly demonstrated the advantages and disadvantages of both MS techniques. Short chains with four or less sialic acid residues were detectable with higher sensitivity by ESI-IT-MS, whereas in MALDI-TOF-MS, matrix signals interfered in this mass region. Because of their insolubility after lactonization, however, long-chained oligomers as well as polymers could be detected only via MALDI-TOF-MS. Although the sensitivity of detection of the individual species may, in part, also be dependent on the amounts of available material, the results clearly demonstrate that short-chained glycans are preferentially detected via ESI-IT-MS, whereas oligomers that are comprised of more than six sialic acid residues seem to be only registered via MALDI-TOF-MS.

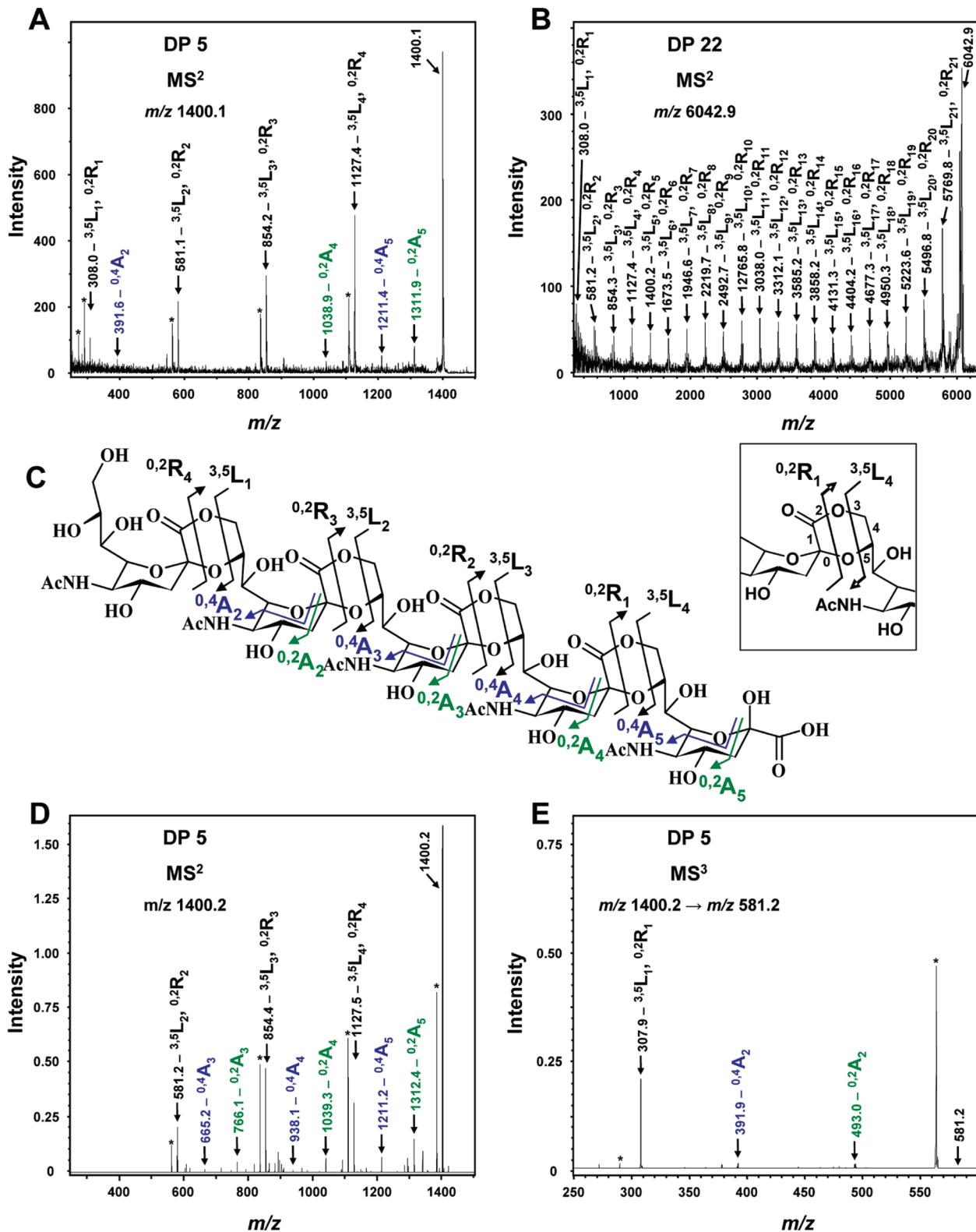


Figure 2. Fragmentation analysis of oligo/polySia by MALDI-TOF-MS/MS(LID) and off-line ESI-IT-MSⁿ. Oligo/polySia was lactonized and analyzed by MALDI-TOF-MS/MS(LID) (panels A and B) and ESI-IT-MSⁿ (panels D and E) in the negative-ion mode: an oligomer consisting of five Neu5Ac residues (80 pmol) (panel A) and a polySia chain consisting of 22 sialic acid residues (500 ng total oligo/polySia) (panel B) were analyzed by MALDI-TOF-MS/MS(LID). Potential fragmentation routes are displayed in the scheme for a Neu5Ac pentamer (panel C). (Fragment ion nomenclature follows the rules of Domon and Costello;²⁸ for ions resulting from cleavage of the lactone ring, an extension of the denotation system has been introduced (see inset). Resulting fragment ions are assigned in the mass spectra.) Panel D shows the ESI-IT-MS² spectrum of the Neu5Ac pentamer, and panel E shows ESI-IT-MS³ analysis of the fragment ion at m/z 581.2. Monoisotopic masses [M-H]⁻ are given for representative signals. In panels D and E, the asterisk (*) denotes the loss of H₂O.

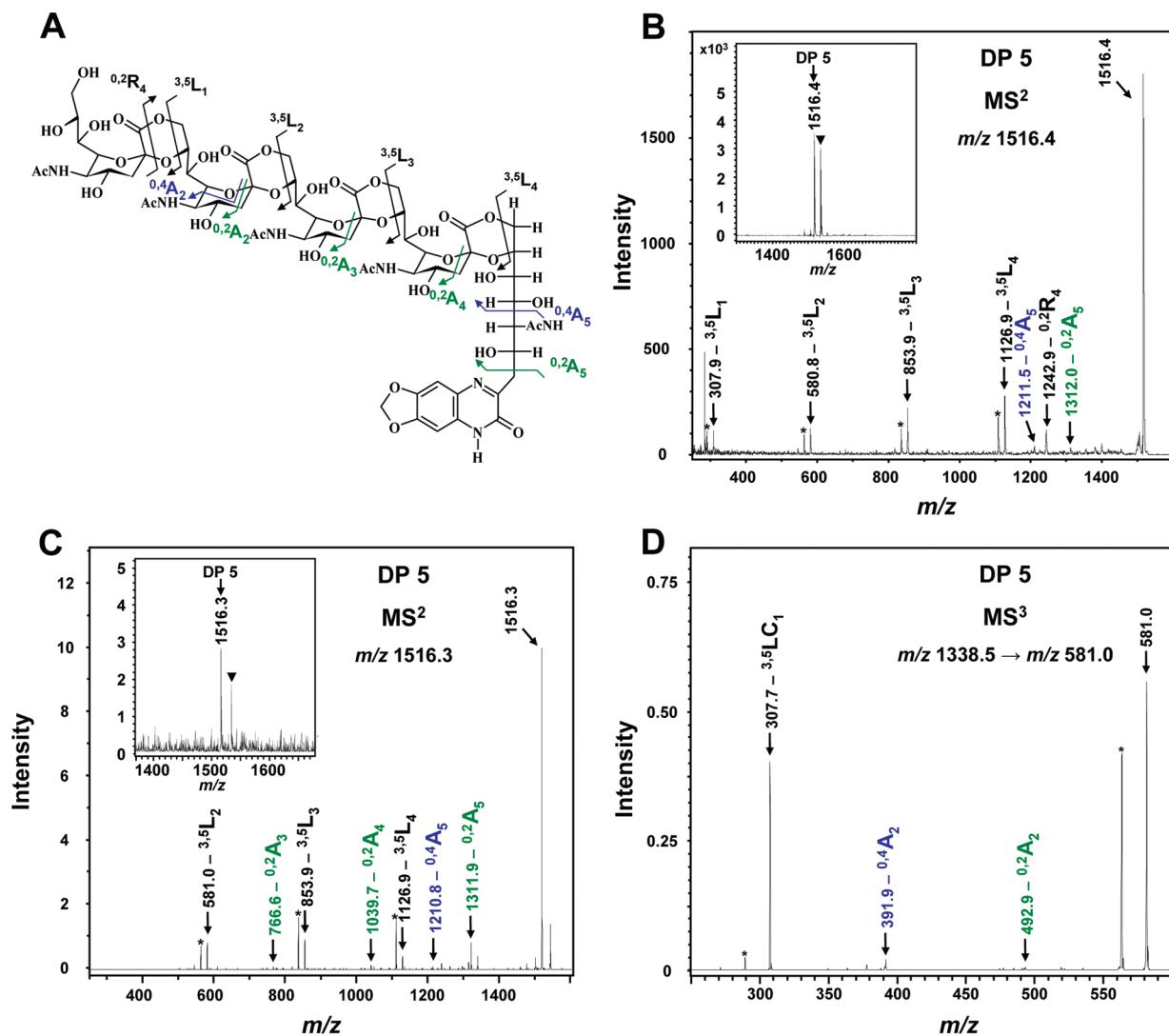


Figure 3. MALDI-TOF-MS/MS(LID) and off-line ESI-IT-MSⁿ analyses of a DMB-labeled Neu5Ac pentamer: (A) schematic representation of a lactonized DMB-labeled pentamer and assignment of observed fragments; (B) MALDI-TOF-MS/MS(LID) and (C) ESI-IT-MS², as well as (D) ESI-IT-MS³, of the fragment ion at m/z 581.0 in the negative-ion mode are shown. Insets in panels (B) and (C), show MALDI-TOF-MS and ESI-IT-MS spectra of the isolated DMB-labeled Neu5Ac pentamer. Monoisotopic masses [$M - H^-$] and corresponding DP values are assigned. In panels (B)–(D), the asterisk (*) denotes the loss of H_2O and the solid inverted triangle (▼) denotes incomplete lactonization.

Fragmentation Analysis of Underivatized oligo/polySia by MALDI-TOF-MS/MS and ESI-IT-MSⁿ. To achieve structural information on oligo- and polySia compounds, mass spectrometric fragmentation analyses were performed. The MALDI-TOF-MS/MS spectra obtained after laser-induced dissociation (LID), i.e., MALDI-TOF-MS/MS(LID) of a Neu5Ac pentamer and a polySia chain comprising 22 monomers, were dominated by fragment ions resulting from simultaneous cleavages of the glycosidic bonds, together with an additional bond within the lactone rings (see Figures 2A and 2B). Furthermore, an additional loss of water has been detected. To designate the new type of fragment ions, which have not been described so far, we have extended the systematic nomenclature of Domon and Costello²⁸ (see Figure 2C). Following their description for cross-ring cleavages of glycans, we numbered the bonds in the lactone ring clockwise, starting with “1” for the C–C bond between the anomeric carbon and the carboxyl group (see the inset in Figure 2C). The C–O bond to the anomeric center is called “0”, analogous to the C–O bond in the glycosidic

ring. Fragments containing the nonreducing end are called “L”-ions, and those including the reducing end are called “R”-ions. In the case of the Neu5Ac pentamer, classical cross-ring fragments such as ^{0,2}A and ^{0,4}A were also observed in addition to L- and R-ions, which have the potential to indicate the position of potential modifications.²⁹ Thus, ^{0,4}A fragment ions could be used to distinguish modifications at C-4 and C-5 from modifications at C-7, C-8, or C-9. Similar results were obtained with 5 pmol of the Neu5Ac pentamer (data not shown). However, none of these cross-ring-fragments could be detected upon fragmentation analysis of long-chained polySia (see Figure 2B).

In parallel, the Neu5Ac-pentamer was also examined by ESI-IT-MS (see Figures 2D and 2E). Compared to the MALDI-TOF-MS/MS(LID) spectrum, the ESI-IT-MS² spectrum displayed, in a reproducible manner, an additional cross-ring fragment (^{0,4}A₄, ^{0,2}A₃, and ^{0,4}A₃), whereas ^{0,2}R₁ or ^{3,5}L₁ fragment ions could not be observed, because of the low mass cutoff of the instrument

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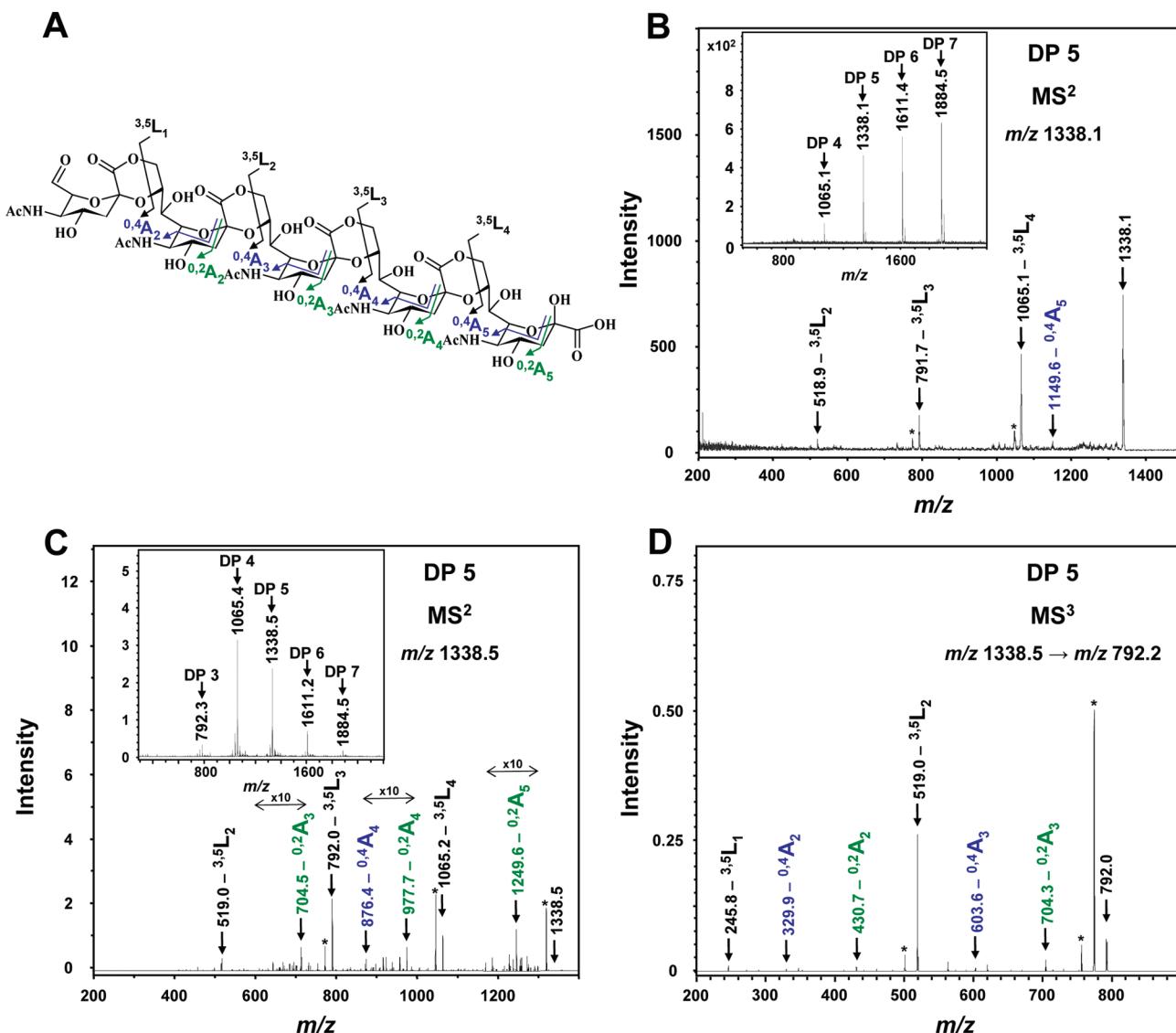


Figure 4. MALDI-TOF-MS/MS(LID) and off-line ESI-IT- MS^n analysis of an oxidized Neu5Ac pentamer. (A and insets) oligoSia was oxidized, resulting in a C₇Neu5Ac residue at the nonreducing end and analyzed by MALDI-TOF-MS and ESI-IT-MS. The modified pentamer was analyzed by (B) MALDI-TOF-MS/MS(LID) and (C and D) ESI-IT- MS^n after lactonization. Selected signals of monoisotopic masses [M-H]⁻ and corresponding DP values are assigned in insets. Fragment ions are defined in panel (A) and are allocated in the mass spectra shown in panels (B)–(D). The asterisk (*) denotes the loss of H₂O.

(see Figure 2D). To visualize these fragment ions, an additional fragmentation cycle of the ^{0.2}R₂/^{3.5}L₂ ion at m/z 581.2 was performed. The obtained MS³ spectrum displayed the expected ^{0.2}R₁ or ^{3.5}L₁ fragment ions and two additional cross-ring fragments (^{0.2}A₂ and ^{0.4}A₂) (see Figure 2D). In conclusion, ESI-IT- MS^n revealed more cross-ring fragments than MALDI-TOF-MS/MS(LID).

The observed fragmentation routes of the Neu5Ac pentamer in MALDI-TOF-MS/MS(LID) and ESI-IT- MS^n can start either from the reducing end or from the nonreducing terminus of the chain resulting in fragment ion pairs of ^{0.2}R₁ and ^{3.5}L₁ or ^{0.2}R₂ and ^{3.5}L₂, which cannot be distinguished. To unambiguously identify which type of ion is formed in each case, labeling of the terminal sialic acid residue at either end was performed.

Fragmentation Analysis of DMB Labeled oligoSia by MALDI-TOF-MS/MS(LID) and ESI-IT- MS^n . As described recently, MALDI-TOF-MS can be combined with the DMB labeling of colominic acid.²¹ Because DMB is covalently linked

to the carboxyl and keto groups of the reducing end sialic acid moiety, thus adding a mass increment of 116.2 Da to the labeled species, it might be a useful marker for fragment ions that comprise the reducing end (see Figure 3A). In the MALDI-TOF-MS profile spectra, strong signals resulting from the addition of one water molecule typically accompanied the signals of the respective molecular ions (cf. inset in Figure 3B). Upon fragmentation, again, fragment ions resulting from the simultaneous cleavage of the glycosidic linkages and the lactone linkage, as well as cross-ring-fragments, were observed (see Figure 3B). The mass values of most of the fragment ions obtained from the DMB-labeled Neu5Ac pentamer were identical to those obtained from the native species, representing either L_n ions encompassing the nonreducing end of the chain or products of multiple cleavages (L/R fragments). Only the signal at m/z 1242.9 could be unambiguously identified as an R-type fragment ion. In addition, two minor signals resulting from the cleavage of C–C bonds of the DMB-bearing Neu5Ac residue were registered.

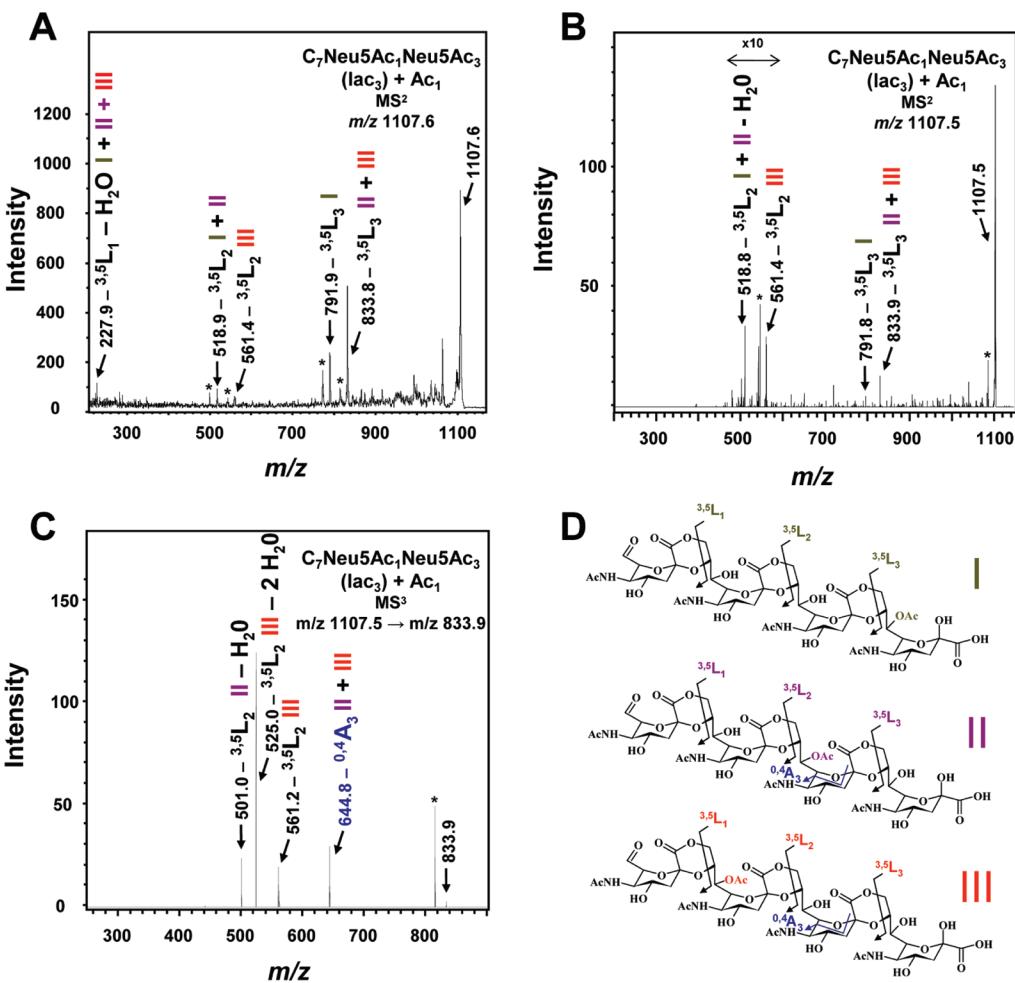


Figure 5. MALDI-TOF-MS/MS(LID) and off-line ESI-IT-MS n analysis of an oxidized and partially O-acetylated oligoSia tetramer. PolySia from *E. coli* K1 (strain D40, OAc+) was digested with endoN, resulting oligoSia chains were oxidized, lactonized, and analyzed by MALDI-TOF-MS (see Figure S3 in the Supporting Information). The mono-O-acetylated tetramer at m/z 1107.6 was analyzed by (A) MALDI-TOF-MS/MS(LID) and (B,C) ESI-IT-MS n . Panel (D) shows a schematic representation of three possible O-acetylated isomeric oligoSia tetramers. Monoisotopic masses [$M - H$] $^-$ are given and key product ions are denoted in the respective colors. The asterisk (*) denotes the loss of H_2O .

The DMB-labeled oligoSia species could be also analyzed by ESI-IT-MS in the negative-ion mode (inset in Figure 3C). The fragment spectrum obtained thereof displayed, in principle, analogous signals of L_n -type fragment ions as MALDI-TOF-MS/MS(LID) (see Figure 3C). Fragment ions including the DMB-labeled sialic acid residue were not detectable. Because of the low mass cutoff of the instrument, $3.5L_1$ fragment ions could only be detected after an additional fragmentation cycle of the $3.5L_2$ fragment ion at m/z 581.0 (see Figure 3D). Similar to native oligoSia, ESI-IT-MS n led to more cross-ring fragments than MALDI-TOF-MS/MS(LID).

To further characterize their fragmentation behavior, oligoSia chains were reduced and analyzed via mass spectrometry. The reduction of oligoSia solely affects the keto group of sialic acid and therefore represents an alternative way to label the reducing end sialic acid residue (see Figure S2A in the Supporting Information). The reduced Neu5Ac pentamer, used as an example, could be easily registered and fragmented either by MALDI-TOF-MS/MS(LID) or ESI-IT-MS n (see Figures S2B and S2C in the Supporting Information). Obtained fragmentation patterns were comparable with the above-described fragment ions of native and DMB-labeled oligoSia species, displaying the same series of L-ions

or internal L/R-ions. However, the loss of a water molecule was much more pronounced in ESI-IT-MS n fragmentation than in MALDI-TOF-MS/MS(LID) analysis. Hence, our results show that the free carboxyl group obviously does not influence the fragmentation behavior and demonstrates that mass spectrometric fragmentation of neither reduced species nor DMB-labeled sialic acid oligomers is a suitable strategy to unequivocally identify the sequence of such linear sialic acid oligomers.

Fragmentation Analysis of Oxidized oligoSia by MALDI-TOF-MS/MS(LID) and ESI-IT-MS n . To modify the nonreducing terminus of the oligoSia chain, we used mild periodate oxidation. In α 2,8-linked Neu5Ac oligomers, solely the nonreducing terminal Neu5Ac residue can be oxidized to the respective C_7 analogue (C_7 Neu5Ac), while internal sialic acid residues remain intact (see Figure 4A).²⁵ The oxidized forms could be detected by both MS techniques exhibiting mass shifts of -62.1 Da, in comparison to the untreated species (see insets in Figures 4B and 4C). The tandem mass spectrum obtained by MALDI-TOF-MS/MS(LID) was again dominated by combined glycosidic and lactone-ring cleavages. Because all of the resulting fragment ions still contained the oxidized C_7 unit at their nonreducing end, they could be unambiguously assigned as L_n -ions (see Figure 4B).

The oxidized pentamer was also subjected to ESI-IT-MSⁿ (see Figures 4C and 4D). In agreement with MALDI-TOF-MS/MS(LID), the ESI-IT-MS² spectrum revealed the loss of sialic acid residues, starting at the reducing end, thus providing the same series of L_n ions (see Figure 4C). A further fragmentation step of the L₂ ion at *m/z* 519.0 also produced cross-ring fragment ions and the corresponding L₁ ion (see Figure 4D). Our results clearly demonstrated that mild periodate oxidation of oligoSia chains provides a versatile tool for distinct definition of fragment ions and, thus, can be useful for sequence analysis of an unknown, potentially substituted oligo/polySia chain.

Fragmentation Analysis of Partially O-Acetylated oligoSia from *E. coli* K1. To ascertain the potential of oxidation for clear structural definition of oligoSia, partially O-acetylated capsular polySia from *E. coli* K1 was digested with endoN and mildly oxidized as described above. Our previous studies already demonstrated that additional O-acetyl groups reside primarily at C-7 or C-9 in these capsular polysaccharides.²¹ In agreement with these studies, the MALDI-TOF-MS spectrum of the oxidized species displayed a high variety of species differing in the number of O-acetyl groups and lactone rings. Each mass increment of 42 Da reflects an additional O-acetyl residue and each mass increment of 18 Da reflects a lacking lactone ring (for a detailed view, see Figure S3 in the Supporting Information).

As an example, the signal of the oxidized, completely lactonized Neu5Ac tetrasaccharide at *m/z* 1107.6 containing one additional O-acetyl group and three lactone rings (C₇Neu5Ac₁Neu5Ac₃(lac)₃ + Ac₁) was chosen for further fragmentation by MALDI-TOF-MS/MS(LID). The presence of an acetyl residue at C-7 of the nonreducing Neu5Ac could be excluded, because this would have prevented oxidation of the terminal residue. Consequently, the O-acetyl group could be solely located at one of the remaining three sialic acid residues, resulting in the isomeric structures I, II, and III that are depicted in Figure 5D. The obtained MS/MS spectrum clearly displayed a greater number of fragments than the unsubstituted species (cf. Figures 4B and 5A). The signals at *m/z* 791.9 and *m/z* 833.9 represented ^{3,5}L₃ fragment ions without or with an additional O-acetyl group. Therefore, the signal at *m/z* 791.9 could only originate from isomer I carrying an O-acetyl group at C-7 of the reducing Neu5Ac residue. In contrast, the fragment ion at *m/z* 833.9, still bearing the additional acetyl group, could equally result from isomers II or III (see Figure 5D). In agreement with this assumption, an analogous pair of respective ^{3,5}L₂ fragment ions without or with an additional O-acetyl group appeared at *m/z* 518.9 and *m/z* 561.4 clearly verifying the presence of isomer III. MALDI-TOF-MS/MS(LID), thus, definitely demonstrated the presence of isomers I and III, whereas fragments corroborating the presence of isomer II were not obtained.

The same O-acetylated tetramer was analyzed by ESI-IT-MSⁿ. In agreement with MALDI-TOF-MS/MS(LID), ESI-IT-MS² analysis confirmed the presence of structures I and III. The signal at *m/z* 791.8 represented the ^{3,5}L₃ fragment ion of structure I, whereas the signal at *m/z* 561.4 exhibited the coexistence of structure III (see Figure 5B). The remaining fragment ions could be products of more than one isomer. For more-detailed analysis, a further cycle of ion isolation and

fragmentation was performed. The resulting ESI-IT-MS³ spectrum of the ^{3,5}L₃ ion at *m/z* 833.9 clearly visualized a signal at *m/z* 501.0, which could be assigned as ^{3,5}L₂ fragment ion of structure II. In addition, the signals at *m/z* 525.0 and *m/z* 561.2 were again indicative for the presence of structure III. Moreover, the MS³ spectrum also displayed ^{0,4}A₃ cross-ring cleavage products.

In conclusion, the chosen example of a partially O-acetylated oligoSia derivative demonstrated that fragmentation analyses by MALDI-TOF-MS/MS(LID), as well as ESI-IT-MSⁿ, are powerful strategies for the prediction and localization of a substituent within an oligoSia chain. However, the unambiguous confirmation of the three different species was only possible after oxidation, thus allowing a discrimination between reducing end-derived and nonreducing end-derived fragments.

CONCLUSIONS

MALDI-TOF-MS/MS(LID) and ESI-IT-MSⁿ are universal techniques that are suitable for detailed structural characterization of oligoSia. Both techniques allow fragmentation analyses of individual oligoSia units, thus verifying the composition of the analyte. Although sialic acid dimers, trimers, and tetramers are detected with higher efficiency using the electrospray approach, fragmentation analyses of polySia are only possible using MALDI-TOF-MS/MS. The obtained fragment ion spectra visualized both fragment ions resulting from cleavage of the lactone rings (^{3,5}L fragment ions) as well as classical cross-ring fragments such as ^{0,2}A and ^{0,4}A. In addition to underivatized oligoSia and polySia, such analyses can be similarly applied to DMB-labeled, reduced, or oxidized sialic acid chains, as well as O-acetylated oligoSia species. The monosaccharide sequence of partially substituted oligoSia chains can be easily determined after oxidation of the sialic acid residue at its nonreducing end. So far, such structural information could be only obtained by NMR, which, however, requires much greater amounts of material.

ACKNOWLEDGMENT

This study was financially supported by the Deutsche Forschungsgemeinschaft (Ge 527/3 and SFB 535, Teilprojekt Z1) and the 6th Research Framework Program of the European Union (RIDS Contract No. 011952).

SUPPORTING INFORMATION AVAILABLE

Figures describing on-target lactonization of oligo/polySia samples before MALDI-TOF-MS analysis (Figure S1), mass spectra obtained by MALDI-TOF-MS/MS(LID) and off-line ESI-IT-MSⁿ of a reduced oligoSia pentamer in the negative-ion mode (Figure S2), and MALDI-TOF-MS of oxidized, partially O-acetylated oligoSia chains (Figure S3). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Received for review December 10, 2009. Accepted January 19, 2010.

AC902809Q