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Analysis of Chain and Blood Group Type and Branching Pattern of Sialylated Oligosaccharides by Negative Ion Electrospray Tandem Mass Spectrometry

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We previously reported sequence determination of neutral oligosaccharides by negative ion electrospray tandem mass spectrometry on a quadrupole-orthogonal time-of-flight instrument with high sensitivity and without the need of derivatization. In the present report, we extend our strategies to sialylated oligosaccharides for analysis of chain and blood group types together with branching patterns. A main feature in the negative ion mass spectrometry approach is the unique double glycosidic cleavage induced by 3-glycosidic substitution, producing characteristic D-type fragments which can be used to distinguish the type 1 and type 2 chains, the blood group related Lewis determinants, 3,6-disubstituted core branching patterns, and to assign the structural details of each of the branches. Twenty mono- and disialylated linear and branched oligosaccharides were used for the investigation, and the sensitivity achieved is in the femtomole range. To demonstrate the efficacy of the strategy, we have determined a novel complex disialylated and monofucosylated tridecasaccharide that is based on the lacto-*N*-decaose core. The structure and sequence assignment was corroborated by methylation analysis and ^{1}H NMR spectroscopy.

Awareness of the biological function of oligosaccharide chains in glycoproteins, glycolipids, and proteoglycans has intensified as an increasing number of examples have been reported which reveal that carbohydrate structures participate in various biological events as well as in modifying protein function. One of the early indications of carbohydrates in recognition was the binding of the influenza virus to red blood cells via sialic acid,[1] and it was shown later by work on the chemical basis of antigenicity of the well-known ABO (H) blood group system in which specificity is determined by oligosaccharides.[2,3] Specific oligosaccharide sequences, such as the type 1 (Galβ1−3GlcNAc)/type 2 (Galβ1−4GlcNAc) chains and the blood group related antigens bearing the H (Fucα1−2Galβ1−3/4GlcNAc), Lewis a [Le^a, Galβ1−3(Fucα1−4)GlcNAc], and Lewis x [Le^x, Galβ1−4(Fucα1−3)-GlcNAc] determinants, occur naturally on the carbohydrate chains of glycoproteins and glycolipids and compose the recognition motifs for cell−cell and cell−matrix interactions.

Sialic acids are typically found at the peripheries of oligosaccharide chains of glycoproteins and glycolipids and, frequently, constitute an important part of recognition motifs for carbohydrate-binding proteins (e.g., the selectins,[4] the siglecs,[5] and influenza viral proteins).[6] Various forms of sialic acid have been identified. Its carboxylic acid group can be esterified to form a lactone with a hydroxyl group of an adjacent saccharide residue. Commonly, the amino function at the 5-position is substituted by an *N*-acetyl group (NeuAc) and this can be hydroxylated to give *N*-glycolylneuraminic acid (NeuGc). The 5-amino group can be replaced by a hydroxyl group forming 2-keto-3-deoxy-D-glycero-D-galactonononic acid (KDN). Also, the 4-, 7-, 8-, and 9-hydroxyl groups of NeuAc, NeuGc, and KDN can be substituted by acetylation, methylation, sulfation, and phosphorylation. Diversity in the sequences of sialylated oligosaccharides is generated by the presence of different forms of sialic acid and their different linkages to the saccharide backbones (e.g., 3- or 6-linked to a galactose (Gal) residue or 6-linked to a *N*-acetylgalactosamine

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(1) Gottschalk. A. *Yale J. Biol. Med.* **1954**, *26*, 352−64.
(2) Watkins, W. M. *Blood-Group Specific Substances*; Gottschalk. A., Ed.; Elsevier: Amsterdam, 1972.
(3) Kabat, E. A. *Am. J. Clin. Pathol.* **1982**, *78*, 281−92.
(4) Ley, K.; Kansas, G. S. *Nat. Rev. Immunol.* **2004**, *4*, 325−35.
(5) Blixt, O.; Collins, B. E.; van den Nieuwenhof, I.; Crocker, P. R.; Paulson, J. C. *J. Biol. Chem.* **2003**, *278*, 31007−19.
(6) Blixt, O.; Head, S.; Mondala, T.; Scanlan, C.; Huflejt, M. E.; Alvarez, R.; Bryan, M. C.; Fazio, F.; Calarese, D.; Stevens, J.; Razi, N.; Stevens, D. J.; Skehel, J. J.; Van Die, I.; Burton, D. R.; Wilson, I. A.; Cummings, R.; Bovin, N.; Wong, C. H.; Paulson, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 17033−38.

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(GalNAc) residue).[7] Sialic acids on glycoconjugates have been found almost exclusively in the α-anomeric form.[8] However, free sialic acid occurs mostly in the β-anomeric form and nucleotide-bound sialic acid (e.g., CMP−NeuAc) also occurs in a β-configuration.[8] It is not clear if β-sialic acid is present in oligosaccharide chains, or if it has been largely ignored or escaped detection because of its very low concentration. It is therefore important to develop a sensitive method for sequencing sialylated oligosaccharides and for detection and characterization of other unusual forms of sialic acids including the potential β-linked sialic acids. The importance of such a method has been recently demonstrated by the detection of *N*-unsubstituted sialic acid (neuraminic acid, Neu).[9,10]

We have developed a strategy using negative ion electrospray tandem mass spectrometry with collision-induced dissociation (ES-CID-MS/MS) for determination of important structural features on underivatized neutral oligosaccharides, including differentiation of chain types,[11,12] blood group types,[11−15] and branching patterns,[13−15] that has high sensitivity (low picomole to high femtomole sensitivity) and does not require derivatization. The results contrast with long held views that free neutral oligosaccharides do not ionize efficiently by protonation or deprotonation[16] and that derivatization or metal ion complexation/cationization is a prerequisite for high-sensitivity detection and generation of structurally informative fragmentation in ESMS. In our method the derivation of detailed structural information is based on the presence of several characteristic fragmentations. A double glycosidic D-type cleavage[12,13] is unique to 3-linked GlcNAc and Glc residues, whereas ^{0,2}A-type cleavages[17] only occur with 4-linked GlcNAc and Glc. These fragment ions indicate features of type 1 and type 2 chains or blood group types. For a 3-linked GlcNAc or Glc, the D-type cleavage occurs at the glycosidic bonds at both the reducing and nonreducing sides of the residue from combined C- and Z-type cleavages. For a 3-linked GlcNAc, a fragment at m/z 202 is obtained. If it is further substituted by a Fuc at the 4-position (e.g., in the case of a Le^a determinant), a fragment at m/z 348 (202 Da + 146 Da) results, whereas if the 4-position is substituted by Gal (e.g., in the case of a Le^x determinant), a unique fragment at m/z 364 (202 Da + 162 Da) is produced. Thus, a D-fragment at m/z 202 indicates a type 1 chain, while an ^{0,2}A-ion doublet at m/z 281/263 indicates a type 2 chain. D-ions at m/z 348 or 364 are characteristic of either terminal Le^a or Le^x determinants, respectively.[12]

A method was also established for core branching pattern analysis using CID-MS/MS of singly and doubly charged molecular ions.[13] Complimentary structural information is obtained from the two product ion spectra. Fragment ions from the 6-linked branch dominate, and those from the 3-linked branch are absent in the CID spectra of $[M - H]^-$, whereas in the product ion spectra of $[M - 2H]^{2-}$ fragment ions from both branches are present. This permits the fragment ions derived from either the 3- or 6-branches to be distinguished, the branching pattern to be deduced, and other structural details of 3- and 6-branches to be assigned. An intense and characteristic D-fragment ion at the 3,6-branched core residue, carrying the core residue together with the 6-branch, also assists in the assignment of branching details.

Using these mass spectrometric approaches, we have recently assigned six novel structures isolated from human milk and urine, including a difucosylated hexasaccharide based on the linear core lacto-*N*-neotetraose[11] and non-, di-, and trifucosylated large oligosaccharides based on the branched *iso*-lacto-*N*-octaose[14] and lacto-*N*-decaose[15] cores.

Sialylated oligosaccharides have been considered difficult to analyze as sialic acid is labile and preferentially lost during the mass spectrometric ionization process, thus preventing determination of the location and linkage of the sialic acid from the mass spectra of underivatized sialylated oligosaccharides.[18] Metal ion ligation[18,19] or conversion of sialic acid residues into their amide forms[20] has been proposed to help to stabilized the sialic acid and to produce linkage information. Nevertheless, Wheeler and Harvey[21] were able to demonstrate that negative ion ESMS/MS can be used effectively for analysis of sialylated oligosaccharides without derivatization. In the present report, we extend our negative ion ESMS/MS strategies to sialylated oligosaccharides for linkage analysis of NeuAc residues, including 2−3/6 and α/β on the oligosaccharide backbones, and sequence determination for chain and blood group typing together with branching pattern analysis. Twenty mono- and disialylated linear and branched oligosaccharides were used for the investigation, and a novel sequence of a complex disialylated and monofucosylated tridecasaccharide, based on the lacto-*N*-decaose core, was determined using the strategy.

EXPERIMENTAL SECTION

Oligosaccharides. Twenty sialylated oligosaccharides were used for the present study (Table 1). Sialylated oligosaccharides 3′- and 6′-sialyllactose (SL), 3′- and 6′-sialyl-*N*-acetyllactosamine (SLN), and sialyllacto-*N*-tetraose (LST) a, b, and c were purchased from Dextra Laboratories (Reading, U.K.). 6′-Sialyllacto-*N*-fucopentaose VI (6′-SLNFP VI) was obtained from BioCarb (Accurate Chemical, New York). 3-Sialyllacto-*N*-fucopentaose II (3′SLNFP II) and III (3′SLNFP III) were gifts from G. Strecker (Universite des Sciences et Technologies de Lille, France) and A. Hasegawa (Gifu University, Japan), respectively. Trisaccharides 3′- and 6′-sialyllactose with β-linkages (βSL) and sialyllactose with *N*-deacetylated sialic acid were synthesized by modifications of

(7) Varki, A. *Glycobiology* **1992**, *2*, 25−40.
(8) Brooks, S. A.; Dwek, M. V.; Schumacher, U. *Functional and Molecular Glycobiology*; BIOS Scientific Publishers: Oxford, 2005.
(9) Galustian, C.; Lawson, A. M.; Komba, S.; Ishida, H.; Kiso, M.; Feizi, T. *Biochem. Biophys. Res. Commun.* **1997**, *240*, 748−51.
(10) Chai, W.; Leteux, C.; Westling, C.; Lindahl, U.; Feizi, T. *Biochemistry* **2004**, *43*, 8590−99.
(11) Martin, M. J.; Feizi, T.; Leteux, C.; Pavlovic, D.; Piskarev, V. E.; Chai, W. *Glycobiology* **2002**, *12*, 829−35.
(12) Chai, W.; Piskarev, V.; Lawson, A. M. *Anal. Chem.* **2001**, *73*, 651−57.
(13) Chai, W.; Piskarev, V.; Lawson, A. M. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 670−79.
(14) Kogelberg, H.; Piskarev, V. E.; Zhang, Y.; Lawson, A. M.; Chai, W. *Eur. J. Biochem.* **2004**, *271*, 1172−86.
(15) Chai, W.; Piskarev, V. E.; Zhang, Y.; Lawson, A. M.; Kogelberg, H. *Arch. Biochem. Biophys.* **2005**, *434*, 116−27.
(16) Reinhold, V. N.; Reinhold, B. B.; Costello, C. E. *Anal. Chem.* **1995**, *67*, 1772−84.
(17) Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397−409.
(18) Leavell, M. D.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 528−36.
(19) Von Seggern, C. E.; Zarek, P. E.; Cotter, R. J. *Anal. Chem.* **2003**, *75*, 6523−30.
(20) Sekiya, S.; Wada, Y.; Tanaka, K. *Anal. Chem.* **2005**, *77*, 4962−68.
(21) Wheeler, S. F.; Harvey, D. J. *Anal. Chem.* **2000**, *72*, 5027−39.

Table 1. Sialylated Oligosaccharides Used for ES-CID-MS/MS

Oligosaccharides		Sequences	Chain types[a]	B-g determinants[b]
1.	3'-αSL	NeuAcα2-3Gal-4Glc	–	–
2.	6'-αSL	NeuAcα2-6Gal-4Glc	–	–
3.	3'-βSL	NeuAcβ2-3Gal-4Glc	–	–
4.	6'-βSL	NeuAcβ2-6Gal-4Glc	–	–
5.	3'-SLN	NeuAcα2-3Gal-4GlcNAc	–	–
6.	6'-SLN	NeuAcα2-6Gal-4GlcNAc	–	–
7.	3'-deAc-SL	Neuα2-3Gal-4Glc	–	–
8.	6'-deAc-SL	Neuα2-6Gal-4Glc	–	–
9.	LST-a	NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	1	–
10.	LST-b	NeuAcα2-6 GlcNAcβ1-3Galβ1-4Glc Galβ1-3	1	–
11.	LST-c	NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	2	–
12.	3'SLNFP II	Fucα |1-4 NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	1	Le^a
13.	3'SLNFP III	Fucα |1-3 NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	2	Le^x
14.	6'SLNFP VI	Fucα |1-3 NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	2	–
15.	MSLNnH	Galβ1-4GlcNAcβ1-6 Galβ1-4Glc NeuAcα2-6Galβ1-4GlcNAcβ1-3	2; 2	–
16.	MSLNH	NeuAcα2-6Galβ1-4GlcNAcβ1-6 Galβ1-4Glc Galβ1-3GlcNAcβ1-3	2; 1	–
17.	MSMFLNnH	Fucα |1-3 Galβ1-4GlcNAcβ1-6 Galβ1-4Glc NeuAcα2-6Galβ1-4GlcNAcβ1-3	2; 2	Le^x
18.	MSMFLNH	Fucα |1-3 Galβ1-4GlcNAcβ1-6 Galβ1-4Glc NeuAcα2-3Galβ1-3GlcNAcβ1-3	2; 1	Le^x
19.	DSMFLNH	Fucα |1-3 Galβ1-4GlcNAcβ1-6 Galβ1-4Glc NeuAcα2-3Galβ1-3GlcNAcβ1-3 |2-6 NeuAcα	2; 1	Le^x
20.	DSMFLND	Fucα |1-3 Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-6 Galβ1-3GlcNAcβ1-3 Galβ1-4Glc NeuAcα2-3Galβ1-3GlcNAcβ1-3 |2-6 NeuAcα	2; 1	Le^x

[a] For branched sequences, figures indicate chain types on 6- and 3-branch, respectively. [b] For branched sequences, figures indicate blood group (B-g) determinants on the 6-branch.

literature procedures.[22] In brief, a thioethyl sialic acid donor was activated with *N*-iodosuccinimide and triflic acid in dichloromethane at −40 °C, in the presence of the appropriate lactose acceptor. The use of CH_2Cl_2 as solvent was essential to allow formation of both the α- and β-anomers of the trisaccharide; when CH_3CN was used, formation of the β-anomer was no longer evident. The anomeric trisaccharides were then separated via column chromatography on silica gel using hexane/$CHCl_3$/propan-2-ol as solvent. The separated anomers were deprotected according to literature procedures.[22]

The branched monosialyllacto-*N*-hexaose (MSLNH) and monosialyllacto-*N*-neohexaose (MSLNnH), their monofucosylated (MSMFLNH and MSMFLNnH, respectively) and disialylated (DSMFLNH) analogues, and monosiallyllacto-*N*-decaose (DSMFLND) were isolated from human milk from a healthy 25 year old woman (written consent was obtained from this volunteer for analysis of the milk sample), blood group B secretor, Le^b positive, giving negative reaction in hepatitis B and HIV tests. Fat was removed by centrifugation at 4 °C (5000 g, 30 min), and the proteins were removed by precipitation with cold acetone. The oligosaccharide fraction was desalted on a Sephadex G-25 column (5 × 90 cm), and then neutral oligosaccharides were separated from the acidic ones on a DEAE-Sephadex A-25 column (2.6 × 15 cm, acetate form) with elution by water. A crude "monosialo" fraction was obtained by elution with 0.1 M pyridine−acetic acid buffer (pH 5.0), and the "disialo" fraction was obtained by elution with 0.5 M buffer (the same one). The monosialo fraction was further fractionated as described[23,24] with minor modification using a Dowex 1 × 2 column (2.6 × 90 cm, 400 mesh, acetate form) and isocratic elution with 50 mM pyridine−acetic acid buffer (pH 5.0). A total of 22 fractions were obtained. MSLNH, MSLNnH, MSMFLNH, and MSMFLNnH were isolated from fractions 4 and 5 by ion pair reversed-phase HPLC using a Zorbax octadecyl column (10 × 250 mm) by elution with 5 mM triethylamine−acetate buffer (pH 5.5),[23] whereas DSMFLND was from fraction 22. Further purification was carried out by HPLC on a Supelco LC-NH_2 column (4.6 × 250 mm) with elution by a gradient of acetonitrile/15 mM potassium phosphate (pH 5.2) as described.[25]

Electrospray Mass Spectrometry. Negative ion ES-MS and collision-induced dissociation (CID) MS/MS were carried out on a Micromass Q-Tof mass spectrometer (Waters, Manchester, U.K.). Nitrogen was used as the desolvation and nebulizer gas at a flow rate of 250 L/h and 15 L/h, respectively. The source temperature was 80 °C, and the desolvation temperature 150 °C. The capillary voltage was maintained at 3 kV. A cone voltage of 50 V was used for CID-MS/MS of both singly charged ions $[M - H]^-$ and doubly charged ions $[M - 2H]^{2-}$ except for the SLNFP series where 30 V was used for $[M - 2H]^{2-}$. A scan rate of 1.5 s/scan was employed for CID-MS/MS experiments, and the acquired spectra were summed for presentation. In quasi MS^3 mode, cone voltage fragmentation was used to produce the first generation fragment ions which were then subjected to CID-MS/MS. A cone voltage of 30 V generated desialylated ions (*m/z* 706) from the LST series, 50 V for $C_{3\beta}$ and $D_{2\beta-4}$ ions from the branched LNH/LNnH, and 80−90 V for $D_{3\beta-4}$ (*m/z* 672) and $C_{3\beta}$−SA (*m/z* 673) from the longer branched oligosaccharides. These ions were used as precursors for further fragmentation.

Product ion spectra were obtained from CID with argon as the collision gas at a pressure of 1.7 bar. The collision energies for fragmentation were as follows: $[M - H]^-$ of the trisaccharides, 35 V; LST, 50 V; SLNFP, 55 V; MSLNnH/MSLNH, 75 V. For $[M - 2H]^{2-}$, 25 V was used for MSMFLNnH/MSMFLNH, whereas 45 and 57 V were used for DSMFLNH and DSMFLND, respectively. The energies used for quasi MS^3 of the fragment ions of the longer branched oligosaccharides generated by cone voltage fragmentation were as follows: $C_{3\beta}$ (*m/z* 673), 33 V; $D_{2\beta-4}$ (*m/z* 817), 38 V; $D_{3\beta-4}$ (*m/z* 672), 16 V; $D_{3\beta-5}$ (*m/z* 1402), 45 V.

For analysis, oligosaccharides were dissolved in CH_3CN/2 mM NH_4HCO_3 (1:1), typically at a concentration of 10 pmol/μL, of which 5 μL was loop-injected. Solvent (1:1 CH_3CN/2 mM NH_4HCO_3) was delivered by a Harvard syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 5 μL/min.

Methylation Analysis. DSMFLND was initially reduced with $NaBD_4$ before it was methylated, hydrolyzed, reduced, and acetylated as described previously.[26] GC/MS analysis of the products, partially methylated alditol acetates, was performed on a ThermoQuest Trace system (ThermoElectron, Hemel Hempstead, UK) using a 15 m RTX-5 capillary column. The initial column temperature was 50 °C programmed to 100 °C at 25 °C/min, to 220 °C at 5 °C/min, and to 310 °C at 10 °C/min.

NMR Spectroscopy. DSMFLND (250 μg) was taken up in D_2O 99.9% (Apollo Scientific, Stockport, UK), exchanged by lyophilization, redissolved in 0.3 mL D_2O, and transferred to a 5 mm small-volume NMR tube (Shigemi, Tokyo, Japan). NMR spectra were recorded at 600 MHz, 30 °C, on a Varian Inova spectrometer, using pulse sequences supplied by the manufacturer for 1D and 2D TOCSY spectra.

RESULTS AND DISCUSSION

Sialic Acid Linkage Analysis of Trisaccharides α-SL and β-SL. Trisaccharides of NeuAc−lactose series with α or β and 2−3 or 2−6 linkages show different fragmentation patterns in negative ion ES-CID-MS/MS, from which each isomer can be readily identified (Figure 1 and Table 2). Each of the four spectra is distinguishable from the rest. A specific ion, $^{0,4}A_2-CO_2$ (*m/z* 306),[21] together with C_2 (*m/z* 470) and $^{0,2}A_2$ (*m/z* 410), appears only in the spectrum of NeuAc2−6-linked lactose, whereas $^{2,4}A_3-CO_2$ (*m/z* 468) and B_2-CO_2 (*m/z* 408) ions only appear in those of 2−3-linked lactose. These ions readily differentiate the 2−3 and 2−6 linkages. The difference between the α- and β-linkages is also apparent and can be used for the assignment of these anomeric isomers (e.g., B_1-CO_2 (*m/z* 246) only in the analogue with a β2−3 linkage (Figure 1c) but not α2−3 (Figure 1a), while $^{0,4}A_2$ (*m/z* 350) in β2−6 (Figure 1d) but not α2−6 (Figure 1b)). The sequences of the trisaccharides can be deduced from the presence of the B or C ions together with their decarboxylated ions. It is interesting that B_2 and C_2 ions can be found in the 2−6-

(22) Komba, S.; Yamaguchi, M.; Ishida, H.; Kiso, M. *Biol. Chem.* **2001**, *382*, 233−40.
(23) Gronberg, G.; Lipniunas, P.; Lundgren, T.; Erlansson, K.; Lindh, F.; Nilsson, B. *Carbohydr. Res.* **1989**, *191*, 261−78.
(24) Gronberg, G.; Lipniunas, P.; Lundgren, T.; Lindh, F.; Nilsson, B. *Arch. Biochem. Biophys.* **1992**, *296*, 597−610.
(25) Kitagawa, H.; Nakada, H.; Kurosaka, A.; Hiraiwa, N.; Numata, Y.; Fukui, S.; Funakoshi, I.; Kawasaki, T.; Yamashina, I.; Shimada, I. *Biochemistry* **1989**, *28*, 8891−97.
(26) Chai, W.; Hounsell, E. F.; Cashmore, G. C.; Rosankiewics, J. R.; Bauer, C. J.; Feeney, J.; Feizi, T.; Lawson, A. M. *Eur. J. Biochem.* **1992**, *203*, 257−68.

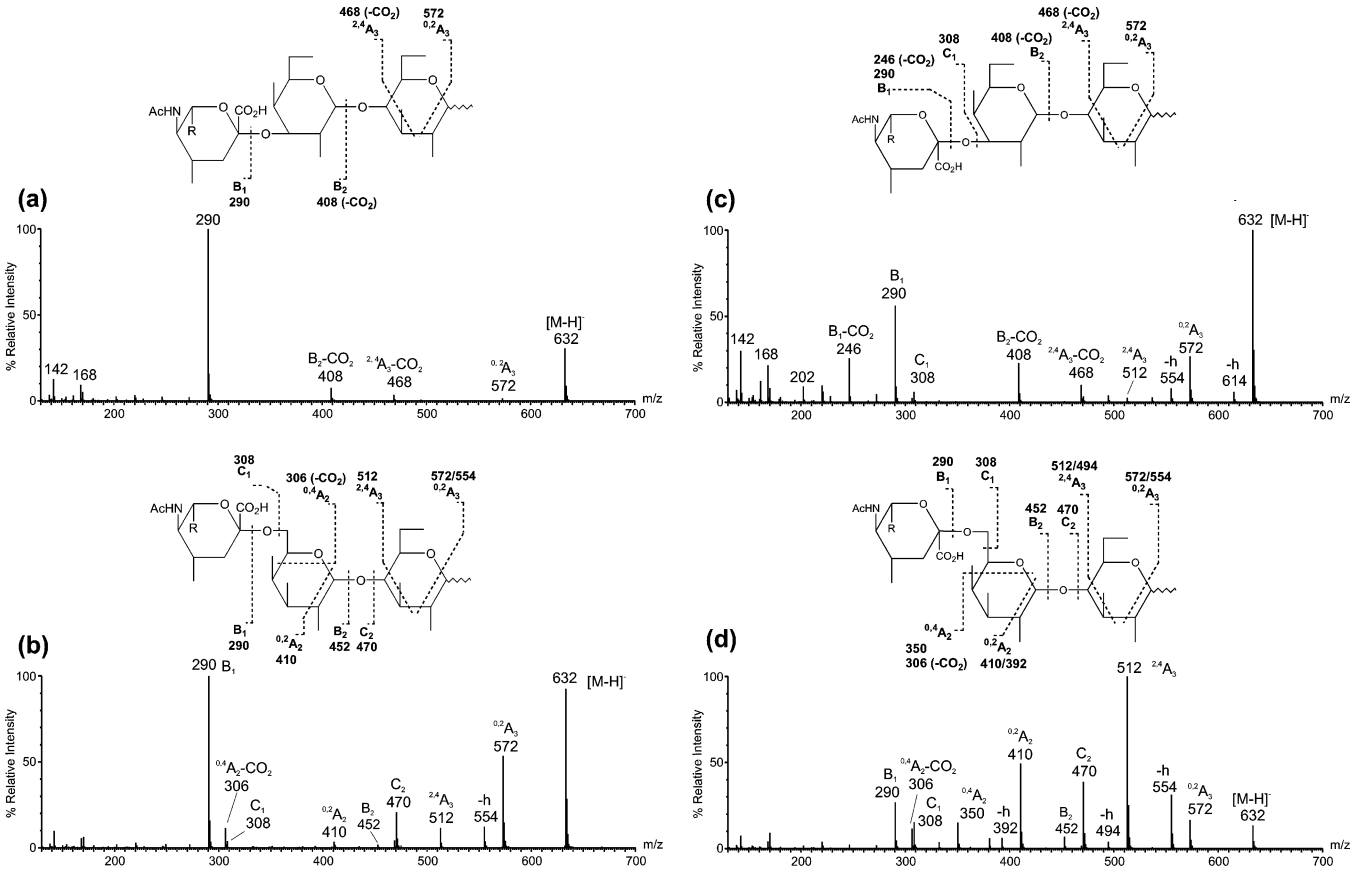


Figure 1. ES-CID-MS/MS spectra of sialyllactose: (a) 3′-αSL, (b) 6′-αSL, (c), 3′-βSL, and (d) 6′-βSL. Structures are shown to indicate the proposed fragmentation.

Table 2. Summary of Fragment Ions Observed in ES CID MS/MS of Sialylated Trisaccharides[a]

samples	$[M-H]^-$	$^{0,2}A_3$	$^{2,4}A_3$	C_2	B_2	$^{2,4}A_3-CO_2$	$^{0,2}A_2$	B_2-CO_2	C_1	$^{0,4}A_2$	$^{0,4}A_2-CO_2$	B_1	B_1-CO_2
3′-βSL	632 (100)	572	512	–	–	468	–	408	308	–	–	290	246
6′-βSL	632 (12)	572	512	470	452	–	410	–	308	350	306	290	–
3′-αSL	632 (22)	572	–	–	–	468	–	408	–	–	–	290	–
6′-αSL	632 (75)	572	512	470	452	–	410	–	308	–	306	290	–
3′-SLN	673 (22)	572	–	–	–	468	–	408	–	–	–	290	–
6′-SLN	673 (35)	572	512	470	452	–	410	–	308	–	306	290	–
3′-dAcSL	590 (12)	530	–	–	–	426	–	366	–	–	–	248	–
6′-dAcSL	590 (28)	530	470	428	410	–	368	–	266	–	264	248	–

[a] Dehydrated secondary fragment ions are not listed. A – denotes either absence or very low intensity. Relative intensities (in parenthesis) are only shown for the $[M-H]^-$ ions.

linked trisaccharides (Figure 1b and 1d, and Table 2), whereas only decarboxylated ions are present in the 2−3-linked analogues (Figure 1a and c, and Table 2).

The stability of the sialic acid linkages is reflected in the relative intensities of the deprotonated molecules $[M-H]^-$. The order of stability is established as follows: β2−3 > α2−6 > α2−3 > β2−6. It is unexpected that the β-linkages do not follow the stability order of the α-linkages in which the 2−3 linkage of sialic acid is more labile than the 2−6 linkage.[21] The difference in precursor ion abundance can also be used as supporting evidence for the differentiation of pairs of isomeric oligosaccharides with 2−3 and 2−6 linked NeuAc.

Sialic Acid Linkage Analysis of Trisaccharides SLN and deAc-SL. Four α-linked trisaccharide analogues were analyzed to verify conclusions drawn from the spectra of the NeuAc-lactose series. The product ion spectra of two de-*N*-acetylated analogues, deAc-SLs (Table 2 and Figure S1) are almost identical, in terms of fragment ions and their relative intensities, to the respective NeuAc-lactose anomers except for a mass shift of 42 Da (equivalent to an acetyl group). Similarly, α2−3 and α2−6 sialylated *N*-acetyllactosamines (SLNs) also produce comparable fragmentation patterns to the respective α-SLs linkages (Table 2 and Figure S2). These results are in agreement with the fragmentation assignments made in the α-SL spectra.

Analysis of Sialic Acid Linkage and Chain Type of Pentasaccharides LST. Pentasaccharides LST-a, b, and c have isomeric sequences, with differences in the NeuAc linkages and in the tetrasaccharide backbones. The locations and linkages of NeuAc together with their sequences can be deduced from their CID-MS/MS spectra. Sialic acid residues on Gal in LST-a and LST-c can be assigned from the weak fragment ions B_2 at m/z 452 (Figure 2a and 2c), and the sialic acid linkage can be assigned

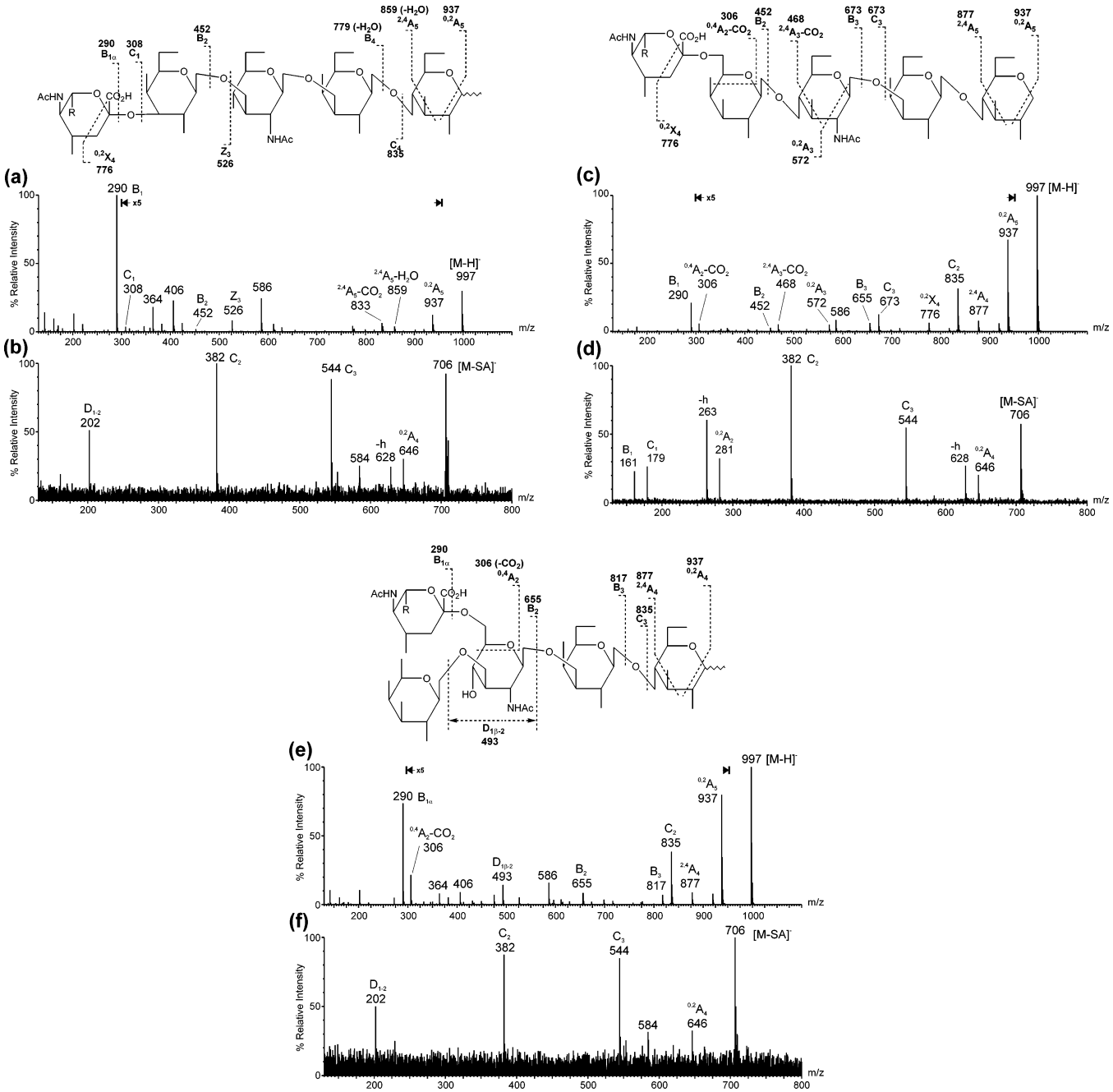


Figure 2. ES-CID-MS/MS spectra of sialyllacto-*N*-tetraose: (a) LST-a, (b) desialylated LST-a produced by CVF, (c) LST-c, (d) desialylated LST-c produced by CVF, (e) LST-b, and (f) desialylated LST-b produced by CVF. Structures are shown to indicate the proposed fragmentation.

from the relative intensities of $[M - H]^-$: a stable $[M - H]^-$ at m/z 997 indicating a α2−6 linkage for LST-c (Figure 2c), and a weaker $[M - H]^-$ indicating a α2−3 linkage for LST-a (Figure 2a). In addition, $^{0,4}A_2-CO_2$ (m/z 306) specific for a α2−6 linkage, as discussed above, only appears in the spectrum of LST-c (Figure 2c), further confirming the assignment. Differences in backbone sequences (type 1 vs type 2 chains) can be unambiguously determined by quasi MS^3 using as precursors the desialylated product ions (m/z 706) created by cone voltage fragmentation.[12] The spectrum obtained from the desialylated ion of LST-a (Figure 2b) is identical to the product ion spectrum of the tetrasaccharide LNT,[12] with the unique m/z 202 fragment ion resulting from a double cleavage of the 3-linked GlcNAc, indicating a type 1 chain. Similarly, the spectrum from LST-c (Figure 2d) is identical to that of LNnT[12] with the characteristic ion doublet $^{0,2}A_2$ (m/z 281/263) for a 4-linked GlcNAc,[12] indicating a type 2 chain.

In LST-b, it can be deduced that the sialic acid is linked at GlcNAc from the double cleavage ion $D_{1\beta-2}$ (m/z 493, Figure 2e) produced from the 3-linked GlcNAc. Its 2−6 rather than a 2−3 linkage is supported by the presence of $^{0,4}A_2-CO_2$ (m/z 306). Again its backbone as a type 1 chain is readily assigned by the product ion spectrum of desialylated ion m/z 706 (Figure 2f) which is identical to that of LST-a.

Analysis of Chain and Blood Group Type of Hexasaccharides SLNFP. The chain and blood group types of sialylated fucosylpentasaccharides are readily distinguished by their CID MS/MS spectra from the unique D- and ^{0,2}A-type double cleavage ions.[12] In the $[M - H]^-$ product ion spectrum (Figure 3a) of

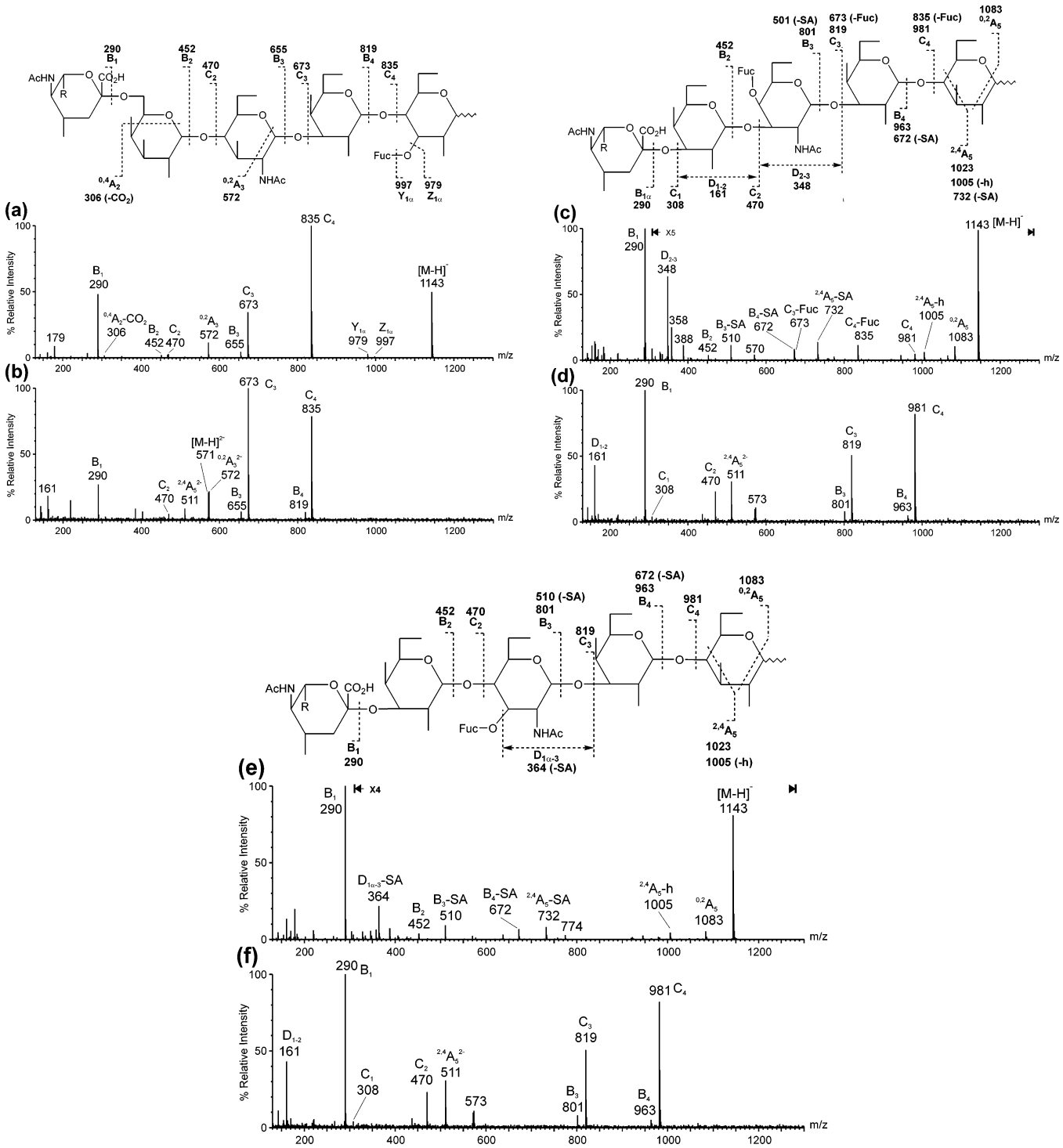


Figure 3. ES-CID-MS/MS spectra of sialyllacto-*N*-fucopentaose. 6′SLNFP VI: (a) $[M - H]^-$, (b) $[M - 2H]^{2-}$; 3′SLNFP II (c) $[M - H]^-$, (d) $[M - 2H]^{2-}$; and 3′SLNFP III (e) $[M - H]^-$, (f) $[M - 2H]^{2-}$. Structures are shown to indicate the proposed fragmentation.

6′SLNFP VI, the sequence ions B_1 (m/z 290), C_2 (m/z 470), C_3 (m/z 673), and C_4 (m/z 835) clearly show its sequence, whereas $^{0,2}A_3$ (m/z 572) indicates a type 2 chain by the presence of a 4-linked internal GlcNAc.[12] A weak ion at m/z 306 ($^{0,4}A_2-CO_2$) identifies a 2−6-linked SA.

The $[M - H]^-$ spectra of 3′SLNFP II (Figure 3c) and 3′SLNFP III (Figure 3e) differ considerably from that of 6′SLNFP VI. The absence of $^{0,4}A_2-CO_2$ ions (m/z 306) and the relatively weak $[M - H]^-$ (note the magnification factor of 5 employed in Figure 3c and e) indicate 2−3-linked NeuAc. A D_{2-3} ion at m/z 348 typical of a Le^a determinant is present in the spectrum of 3′SLNFP II. However, in the spectrum of 3′SLNFP III (Figure 3e), only the desialylated D-type ion is formed ($D_{1\alpha-3}$-SA, m/z 364) which is indicative of a Le^x determinant. Because 2−3-linked NeuAc is labile, the $[M - H]^-$ spectra of 3′SLNFP II and III are complicated by desialylation as shown in Figure 3c and e.

Product ion spectra acquired from $[M - 2H]^{2-}$ as precursors require much lower energy[13] for fragmentation than for those of $[M - H]^-$ precursors. It was anticipated that in the product ion spectra of $[M - 2H]^{2-}$ desialylation should be minimized. The

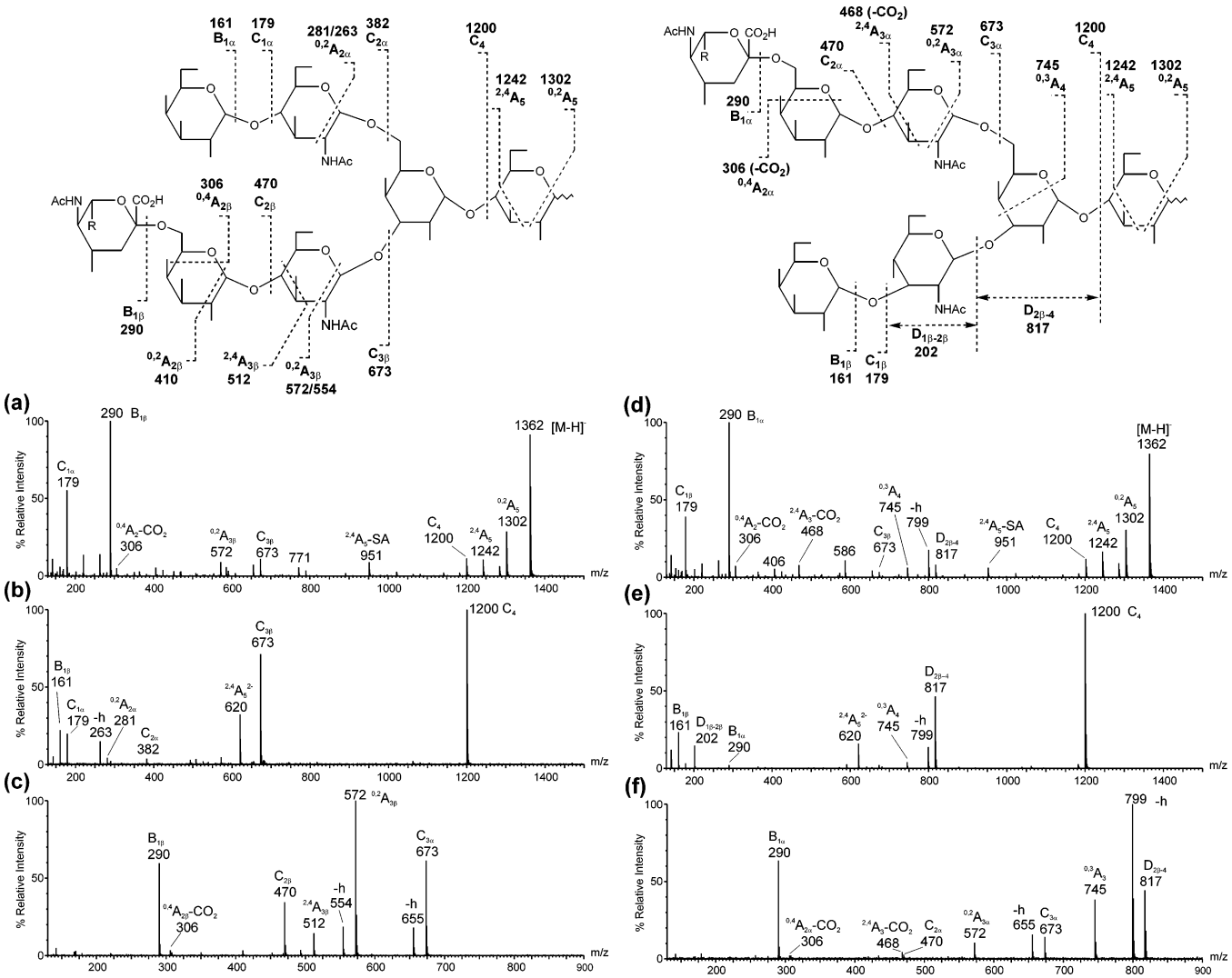


Figure 4. ES-CID-MS/MS spectra of branched monosialylated hexaose: MSLNnH (a) $[M - H]^-$, (b) $[M - 2H]^{2-}$, and (c) $C_{3\beta}$ (*m/z* 673) produced by CVF; MSLNH (d) $[M - H]^-$, (e) $[M - 2H]^{2-}$, and (f) $D_{2\beta-4}$ (*m/z* 817) produced by CVF. Structures are shown to indicate the proposed fragmentation.

spectra obtained demonstrate this by the presence of intact sequence ions: B_1 (*m/z* 290), C_2 (*m/z* 470), C_3 (*m/z* 673), and C_4 (*m/z* 835) in the spectrum of 6′SLNFP VI (Figure 3b) and B_1 (*m/z* 290), C_2 (*m/z* 470), C_3 (*m/z* 819), and C_4 (*m/z* 981) in the spectra of 3′SLNFP II and III (Figure 3d and f, respectively). However, at the lower energy, the D-type fragmentation, which is useful for differentiation of a Le^a or Le^x blood group determinant, is absent.

Further MS/MS experimentswere carried out with sodiated molecules of 3′SLNFP II and 3′SLNFP III as precursors as it has been suggested that an acidic monosaccharide residue can be stabilized by a metal cation.[27] The product ion spectra (Figure S3) obtained from their $[M - 2H + Na]^-$ ions clearly show the improved stability of the sialic acid linkage: intact sequence ions containing the sodiated sialic acid residues C_1 (*m/z* 330), C_2 (*m/z* 492), B_3 (*m/z* 823), and $^{2,4}A_5$ (*m/z* 1045) from which the sequence and the location of Fuc can be deduced. The unique D-type ions induced by the 3-substitution are also present: *m/z* 348 indicating Le^a and *m/z* 677 indicating Le^x determinants in the spectra of 3′SLNFP II and 3′SLNFP, respectively. The $D_{1\alpha-3}$ ion (*m/z* 677) in the spectrum of 3′SLNFP III (Figure S3b) is intact and carries the sialic acid residue.

Analysis of Branching Pattern of Heptasaccharides MSLNnH and MSLNH. Product ion spectra of $[M - H]^-$ of MSLNnH and MSLNH each show similar fragment ions in the low- and high-mass regions (e.g., the C_1 (*m/z* 179), B_1 (*m/z* 290), C_4 (*m/z* 1200), $^{0,4}A_5$ (*m/z* 1242), and $^{0,2}A_5$ (*m/z* 1302) (Figure 4a and d)). These ions identify the nonreducing terminal NeuAc and a reducing terminal 4-linked Glc residue. In both spectra, the stable $[M - H]^-$ and unique $^{0,4}A_2-CO_2$ ion (*m/z* 306)[21] indicate 2−6-linked sialic residues. The major distinctive fragment ions are the $D_{2\beta-4}$ (*m/z* 817) and $^{0,3}A_4$ (*m/z* 745) ions from MSLNH (Figure 4d) confirming the NeuAc−Gal−GlcNAc sequence at the 6-position of a branched Gal. This D-type ion has been observed previously as a unique ion indicating the branching point of neutral oligosaccharides.[13−15] The $^{0,3}A_4$ ion supports this assignment.

CID of the doubly charged molecules $[M - 2H]^{2-}$ requires less energy (25 V compared with 75 V for the singly charged ion) and gives simpler product ion spectra (Figure 4b and 4e) and complementary information. As described previously for the

(27) Zaia, J.; Costello, C. E. *Anal. Chem.* **2003**, *75*, 2445−55.

branched neutral oligosaccharides, product ion spectra of $[M - 2H]^{2-}$ show major features of both branches, whereas the product ion spectra of $[M - H]^-$ show mainly the fragment ions from the 6-linked branch. In the $[M - 2H]^{2-}$ product ion spectrum of MSLNH (Figure 4e), the D-ion derived from 3-linked GlcNAc at the 3-branch gives a distinctive ion $D_{1\beta-2\beta}$ at m/z 202. $B_{1\beta}$ (m/z 161) and $C_{1\beta}$ (m/z 179) identify a Gal1−3GlcNAc1 sequence, while a C_4 ion at m/z 1200 indicates a branching sequence. The 6-linked branch can be readily assigned by cone voltage fragmentation of the $D_{2\beta-4}$ ion (m/z 817) as the precursor. As shown in Figure 4f, $B_{1\alpha}$ (m/z 290), $^{0,4}A_{2\alpha}-CO_2$ (m/z 306), $C_{2\alpha}$ (m/z 470), $C_{3\alpha}$ (m/z 673), and $^{0,2}A_{3\alpha}$ (m/z 572), which is characteristic of a 4-linked GlcNAc,[13] clearly indicate a NeuAc2−6Gal1−4GlcNAc1 sequence on the 6-linked branch, while $^{0,3}A_4$ at m/z 745 further confirms this sequence at the 6-position of the branched Gal. The ion at m/z 468 has been observed in NeuAcα2−6Gal−GlcNAc sequences[21] resulting from $^{2,4}A_3-CO_2$, and the reason for its presence in this case is unknown.

The CID spectrum of the doubly charged ion $[M - 2H]^{2-}$ of MSLNnH, where the sialic acid is on the 3-linked branch, show apparent differences in fragmentation (Figure 4b) to that of MSLNH (Figure 4e). The 3-branch related the $D_{2\beta-4}$ ion (m/z 817) is absent while the 6-branch related $C_{3\beta}$ (m/z 673) is prominent (Figure 4b). The absence of an ion at m/z 202 indicates that there is no 3-linked GlcNAc in either of the branches. Instead, the doublet at m/z 281/263 is consistent with 4-linked GlcNAc and is present in both branches. Further MS/MS by cone voltage fragmentation (Figure 4c) of the $C_{3\beta}$ ion (m/z 673) gave $B_{1\beta}$ (m/z 290), $^{0,4}A_{2\beta}-CO_2$ (m/z 306), $C_{2\beta}$ (m/z 470), $C_{3\beta}$ (m/z 673), and $^{0,2}A_{3\beta}$ (m/z 572) indicating the sequence of the 3-linked branch as NeuAc−6Gal−4GlcNAc.

The product ion spectra of the sample pair MSLNnH/MSLNH show that when the sialic acid is on the 6-linked branch, a unique double cleavage D-type ion ($D_{2\beta-4}$ at m/z 817 for MSLNH) containing the 6-branch is produced, a characteristic feature of fragmentation previously described for neutral oligosaccharide.[13] However, for MSLNnH, where a sialic acid residue is absent on the 6-linked branch but present on the 3-linked branch, this characteristic $D_{2\beta-4}$ ion carrying the 6-linked branch is very weak, whereas the $C_{3\beta}$ ion representing the sialic acid-containing 3-linked branch is prominent. This is the result of a sialic acid residue being the preferred site to take the negative charge and the neutral 6-linked branch being unable to compete with the sialic acid-containing 3-branch for charge localization.

Analysis of Branching Pattern and Blood Group Type of Octasaccharides MSMFLNnH and MSMFLNH. Product ion spectra of their singly charged molecule ions $[M - H]^-$ are not clearly informative and dominated by the cleavage of sialic acid. However, from the relative intensities (RI) of ions of the intact molecule and the cleaved sialic acid residue, the stability and therefore the linkages of the sialic acid can be derived. A stable $[M - H]^-$ ion (m/z 1508, RI 100%) and a weak B_1 ion (m/z 290, RI 50%) in the singly charged product ion spectrum (Figure S4a) of MSMFLNnH indicates a 2−6-linked NeuAc, while a labile $[M - H]^-$ ion (m/z 1508, RI 30%) and an intense B_1 ion (m/z 290, RI 100%) in the spectrum of MSMFLNH (Figure S4b) suggests a 2−3-linked NeuAc. Similar to MSLNnH and MSLNH, the doubly charged product ion spectra of MSMFLNnH and MSMFLNH (Figure 5a and d) are simpler and more informative. Both monosialylated and monofucosylated octasaccharides give similar doubly charged product ion spectra with dominant $C_{3\beta}$ ions at m/z 673, indicating that the NeuAc residues in both oligosaccharides are almost certainly located on the 3-linked branch as no $D_{2\beta-4}$ ions are present that are distinctive of a NeuAc-containing 6-linked branch as discussed above (Figure 4e). Also the unique ion $D_{2\alpha-3\alpha}$ at m/z 364 identifies a Le^x determinant in each oligosaccharide, and their branch location can be assigned as described below. The gap in the spectrum between the fragment ions $C_{3\beta}$ and C_4 of 673 Da indicates a difference of dHex.Hex.HexNAc (511 Da) together with the branching residue Hex (162 Da).

Further MS/MS on the $C_{3\beta}$ ions (m/z 673) give two distinct product ion spectra. The spectrum derived from MSMFLNnH (Figure 5b) is identical to the one obtained from MSLNnH (Figure 4c), strongly suggesting that the NeuAc residue is on the 3-linked branch with a sequence of NeuAc2−6Gal1−4GlcNAc1 as indicated by the $B_{1\beta}$ (m/z 290), $C_{2\beta}$ (m/z 470), $C_{3\beta}$ (m/z 673), and $^{0,2}A_{3\beta}$ (m/z 572/554) ions. Also, the NeuAc is linked α2−6 as confirmed by the presence of a weak ion m/z 306 ($^{0,4}A_{2\beta}-CO_2$). The m/z 572/554 doublet is unambiguously indicative of a 4-linked GlcNAc.[12] The product ion spectrum of $C_{3\beta}$ from MSMFLNH (Figure 5e) shows simpler fragmentation compared with that obtained from MSMFLNnH. The absence of a typical $^{0,2}A_3$ ion doublet at m/z 572/554 for a 4-linked GlcNAc and the presence of a weak m/z 202 indicate a 3-linked GlcNAc.[12] The absence of a weak ion of $^{0,4}A_{2\beta}-CO_2$ at m/z 306 is also indicative a 2−3-linked rather than 2−6-linked NeuAc, a linkage assignment supported by the information obtained from the product ion spectrum of $[M - H]^-$ described above. The sequence at the 3-branch of MSMFLNH is therefore NeuAc2−3Gal1−3GlcNAc1.

Very weak $D_{3\beta-4}$ ions at m/z 672 are found in the mass spectra obtained with relatively high cone voltage. CID-MS/MS of m/z 672 in both MSMFLNnH and MSMFLNH gave identical product ion spectra (Figure 5c and 5f) with intense $D_{2\alpha-3\alpha}$ (m/z 364) together with $C_{1\alpha}$ (m/z 179) and $^{0,3}A_4$ (m/z 600) ions, unambiguously pointing to a Le^x determinant at the 6-branch in both samples.

Analysis of Disialylated DSMFLNH. Disialyated and monofucosylated DSMFLNH was used to assess structural information obtainable from disialylated branched oligosaccharides. Initial mass spectra indicated that mono-desialylation is a very favored process, and it dominated the product ion spectra. For example, the mono-desialylated ion m/z 1508 and sialic acid cleavage ion m/z 290 ($B_1\beta$) are intense in the product ion spectra of $[M - H]^-$ (Figure S5) and $[M - 2H]^{2-}$ (Figure 6a), respectively (note the magnification factors used for specific regions). Of these spectra, that of $[M - 2H]^{2-}$ is the simpler and more informative. The unique D-ions m/z 364 ($D_{1\alpha-2\alpha}$) and m/z 493 ($D_{2\beta-3\beta}$) indicate a Le^x and 2−6-linked NeuAc, respectively (Figure 6a).

Although the neutral 6-linked branch only results in a weak $D_{3\beta-4}$ ion (m/z 672) in the product ion spectrum of the singly charged molecular ion (Figure S5), MS/MS of $D_{3\beta-4}$ (Figure 6b) clearly shows that the Le^x determinant, which produces a $D_{1\alpha-2\alpha}$ ion m/z 364, is on the 6-linked branch. As the disialylated sequence is very labile, the intact $C_{3\beta}$ ion is absent and only the monosialylated $C_{3\beta}$ (m/z 673) is present in the spectrum (Figure S5). This is produced by loss of either 2−3- or 2−6-linked NeuAc,

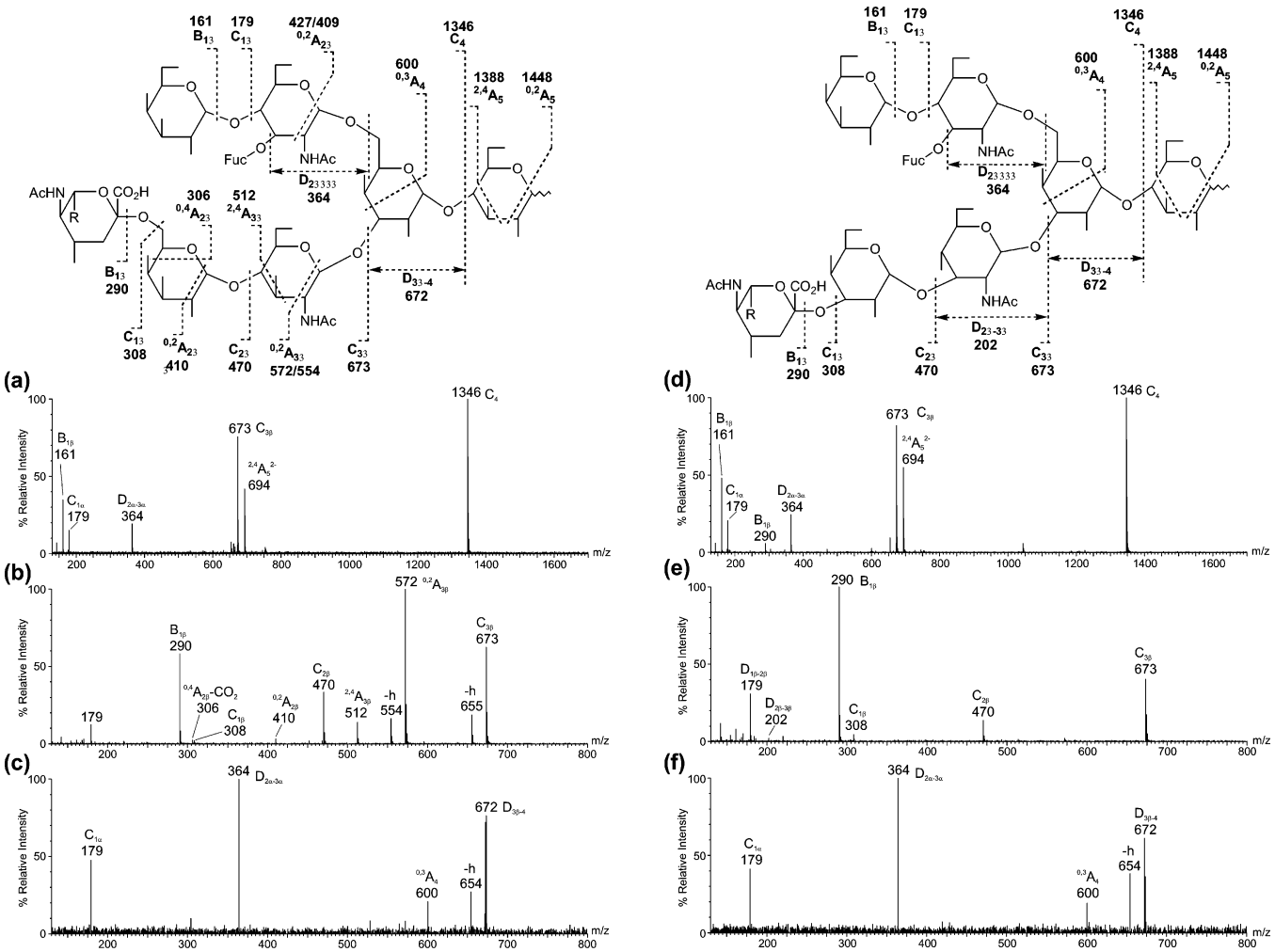


Figure 5. ES-CID-MS/MS spectra of branched monosialylated and monofucosylated hexaose: MSMFLNnH (a) $[M - 2H]^{2-}$, (b) $C_{3\beta}$ (*m*/*z* 673) produced by CVF, and (c) $D_{3\beta-4}$ (*m*/*z* 672) produced by CVF; MSMFLNH (d) $[M - 2H]^{2-}$, (e) $C_{3\beta}$ (*m*/*z* 673) produced by CVF, and (c) $D_{3\beta-4}$ (*m*/*z* 672) produced by CVF. Structures are shown to indicate the proposed fragmentation.

with the 2−3-linkage presumably being more labile. Thus, the product ion spectrum of $C_{3\beta}$ (*m*/*z* 673) (Figure 6c) gives a similar spectrum to that of MSMFLNH (Figure 5e), with $C_{2\beta}$ (*m*/*z* 470) pointing to NeuAc on the Gal residue, but the presence of an additional ion *m*/*z* 493 ($D_{2\beta-3\beta}$) indicates the alternative NeuAc 2−6-linked to the internal GlcNAc.

Hence, although disialylated oligosaccharides are more labile and sialic acid loss is a major feature in the fragmentation, the complete branching pattern can still be assigned with the product ion spectrum of $[M - 2H]^{2-}$ giving the main features and MS/MS of $D_{3\beta-4}$ and mono-desialylated $C_{3\beta}$ giving the details of each branch.

Sequence determination of novel DSMFLND. This oligosaccharide was isolated from human milk and its molecular mass (2350 Da) indicates a composition of $NeuAc_2.Fuc.Hex_6.HexNAc_4$, suggesting a novel sequence. The product ion spectrum of the doubly charged $[M - 2H]^{2-}$ (Figure 7a) is similar to that obtained from DSMFLNH (Figure 6a) apart from a mass increment of 730 Da ($Hex_2.HexNAc_2$) for some fragment ions. Structural features include a Le^x determinant and an internal NeuAc-substituted GlcNAc from the presence of the characteristic ions *m*/*z* 364 ($D_{1\alpha-2\alpha}$) and *m*/*z* 493 ($D_{2\beta-3\beta}$), respectively. The two main branches can be unambiguously assigned by MS/MS of $D_{3\beta-5}$ (*m*/*z* 1402) (Figure 7b) and $C_{3\beta}$−SA (*m*/*z* 673) (Figure 7c), produced by high cone voltage fragmentation, for the 6-branch and 3-branch, respectively. The presence of $D_{2\alpha'-3\alpha}$ (*m*/*z* 672) and $^{0,3}A_{3\alpha}$ (*m*/*z* 600) is evidence that the 6-linked branch contains a further 3,6-branch. The location of the Le^x determinant on the 6-linked sub-branch is indicated by the presence of the unique ion *m*/*z* 364 ($D_{1\alpha-2\alpha}$) in the product ion spectrum of *m*/*z* 1402 (Figure 7b), and this is deduced from the fact that a product spectrum of a singly charged precursor is dominated by fragmentation of the 6-linked branch for a neutral sugar as described previously.[15] This spectrum is very similar to that of the $D_{3\beta-5}$ (*m*/*z* 1402) acquired previously[15] for the nonsialylated MFLND, indicating an identical 6-linked branch. The 3-linked branch is identical to that of DSMFLNH as a very similar product ion spectrum (Figure 7c) to that of mono-desialylated $C_{3\beta}$ (*m*/*z* 673) in DSMFLNH (Figure 6c) is obtained, apart from some additional minor ions, e.g. *m*/*z* 572 which is considered to be from a possible contaminating 4-linked GlcNAc-containing oligosaccharide.

Assignment of the proposed sequence was verified by methylation analysis. The partially methylated alditol acetates obtained clearly indicate the presence of −4Glcol, Fuc1−, Gal1−, −3Gal1−,

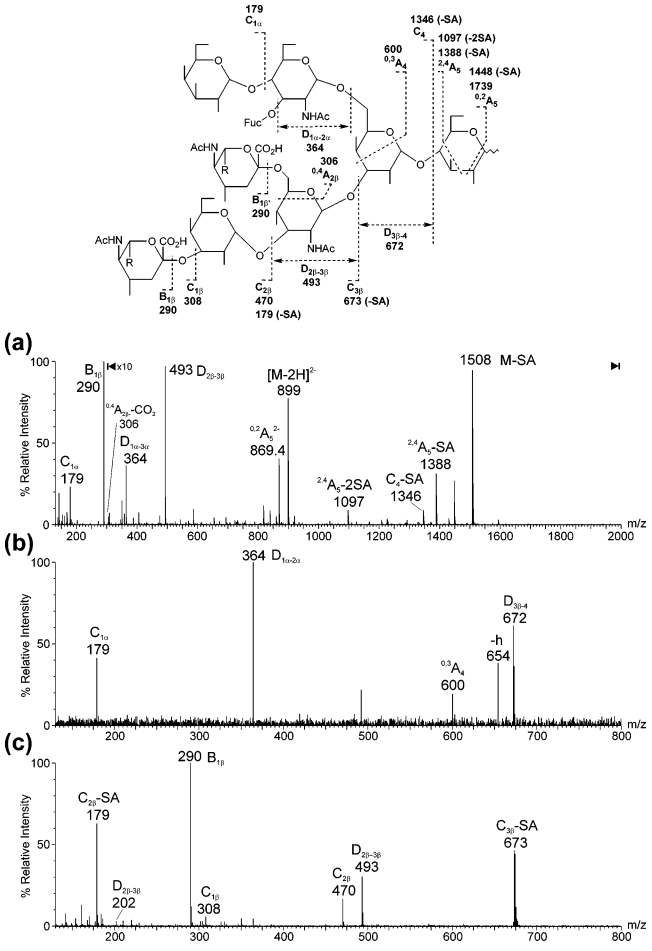


Figure 6. ES-CID-MS/MS spectra of DSMFLNH: (a) $[M - 2H]^{2-}$, (b) $D_{3\beta-4}$ (*m*/*z* 672) produced by CVF, and (c) monodesialylated $C_{3\beta}$ (*m*/*z* 673) produced by CVF. Structure is shown to indicate the proposed fragmentation.

−3,6Gal1−, −3GlcNAc1−, −4GlcNAc1−, −3,4GlcNAc1−, and −3,6GlcNAc1− in an approximate ratio of 1:1:2:1:2:1:1:1:1 (Table S1), in full agreement with the assignment by ES-CID-MS/MS.

As DSMFLND is a novel structure,[1]H NMR was also carried out to complete its characterization, with the anomeric configurations being assigned and linkages confirmed. The ^{1}H NMR spectrum of DSMFLND is partially assigned from a TOCSY spectrum by comparison with data for MFLND;[15] chemical shifts are summarized in Table 3. In DSMFLND, residues I−VI and residues IX−XI give signals closely resembling their counterparts in MFLND. Three well-resolved sets of signals are apparent in the spectrum of DSMFLND which are not present in that of MFLND. Two pairs of methylene proton signals between 1.5 and 2.8 ppm are assignable to H3 of NeuAc residues, and their chemical shifts allow differential assignment to α2−6-linked NeuAc (XII) and α2−3-linked NeuAc (XIII) by comparison with literature values.[28] H4 and H5 of the same residues are assigned from TOCSY cross-peaks. The third set of signals, not seen in MFLND, is assignable to a *β*-Gal residue; the signal from H3 is shifted 0.28 downfield from its position in terminal Gal residues VI and X, indicating substitution at C3. These signals are therefore assigned to residue VIII. It is not possible to assign specific signals to the GlcNAc residue VII.

(28) Platzer, N.; Davoust, D.; Lhermitte, M.; Bauvy, C.; Meyer, D. M.; Derappe, C. *Carbohydr. Res.* **2005**, *191*, 191−207.

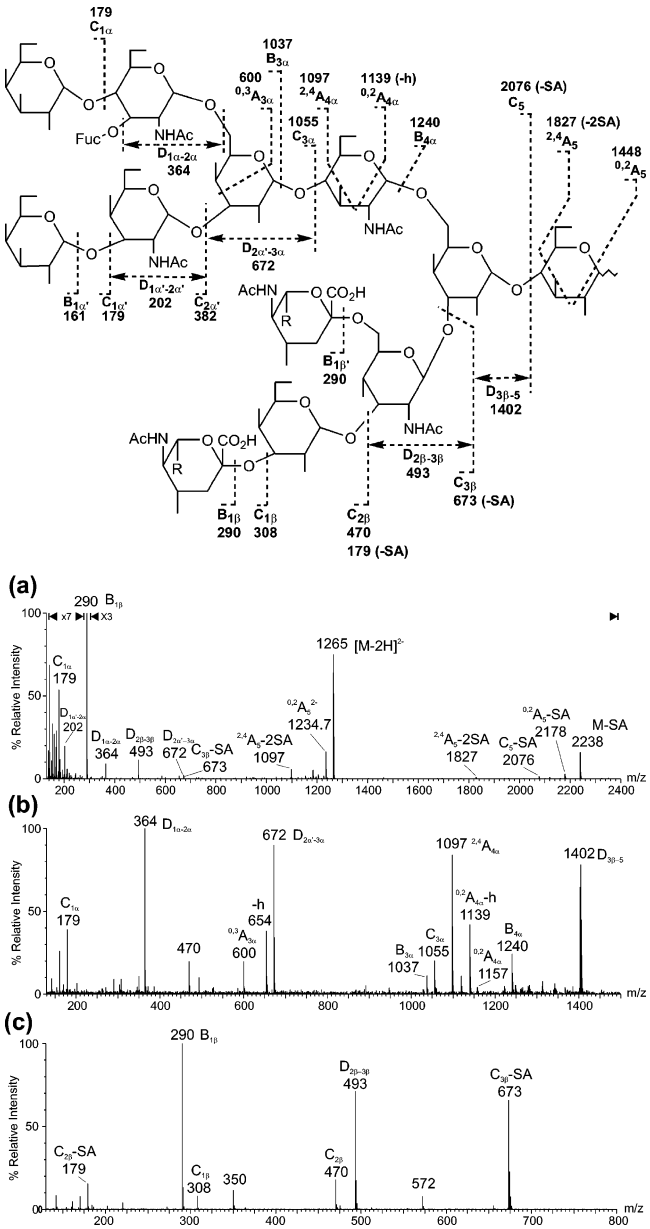
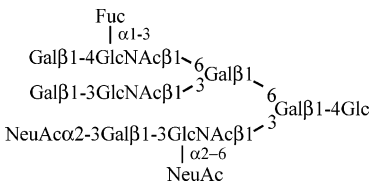


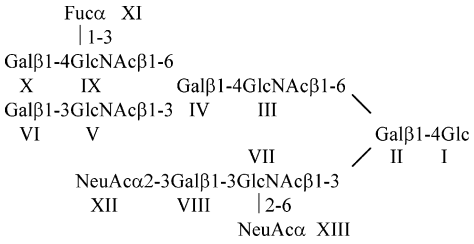
Figure 7. ES-CID-MS/MS spectra of DSMFLND: $[M - 2H]^{2-}$, (b) $D_{3\beta-5}$ (*m*/*z* 1402) produced by CVF, and (c) monodesialylated $C_{3\beta}$ (*m*/*z* 673) produced by CVF. Structure is shown to indicate the proposed fragmentation.

Taken together, the following sequence can be proposed for DSMFLND:



Sensitivity. Detection sensitivity of ES-CID-MS/MS was assessed with 6′-SLN. Sequence information can be readily obtained from a product ion spectrum with 500 fmol for flow injection on a conventional scale (Figure S6a). Further sensitivity improvement can be obtained with nanospray using a capillary needle in which

Table 3. Proton Chemical Shifts in the NMR Spectrum of DSMFLND



residue	H1	H2	H3	H4	H5	H6
I Glcα	5.22	3.60	3.82	3.94		
I Glc*β*	4.66	3.29	3.62	3.60		
II Gal, Gal IV	4.43	3.58	3.73	4.15		
III GlcNAc	4.63	3.73				
V GlcNAc	4.73	3.88	3.81	3.58	3.48	
VI Gal, X Gal	4.44	3.50	3.64	3.90		
VII GlcNAc						
VIII Gal	4.50	3.53	3.92	4.08		
IX GlcNAc	4.61	3.88				
XI Fuc	5.10	3.70	3.88	3.78	4.82	1.16
XII NeuAc			1.68 ax 2.75 eq	3.67	3.82	
XIII NeuAc			1.78 ax 2.77 eq	3.62	3.84	

20 fmol of 6′-SLN (not shown) gives a similar spectrum to the one shown in Figure S6a. Spectra acquired with 5 pmol of 6′-SLN on a conventional scale (Figure S6b) or 100 fmol with nanospray (not shown) show much improved signal-to-noise ratios from which the weak ions *m/z* 306 and 308 are clearly identifiable and can be used for the differentiation of sialic acid linkage.

CONCLUSIONS

We previously reported that sequence, branching pattern, and partial linkage information, including chain and blood group types, of neutral oligosaccharides can be obtained from negative ion ES CID-MS/MS on a quadrupole-orthogonal time-of-flight instrument with high sensitivity and without derivatization.[12,13] In the present report, we extend our strategies to sialylated oligosaccharides. The main feature of the strategy is the unique double glycosidic cleavage induced by 3-glycosidic substitution that produces characteristic D-type fragments which can be used to distinguish the type 1 and type 2 chains, the blood group related Lewis determinants, and 3,6-disubstituted core branching patterns and to assign the structure details of each of the branches. An internal location of a sialic acid on the 3-linked GlcNAc can also be identified by a characteristic D-ion at *m/z* 493. This fragment has further extended the list of specific D-ions for sequence determination: *m/z* 202 for a type 1 chain, *m/z* 348 for a Le^a, and *m/z* 364 for a Le^x determinants.[12−15] Sensitivity achieved is in the femtomole range, with high femtomoles for the conventional flow mode and low femtomoles for nanospray.

Sialic acid is commonly α2−3- or α2−6-linked to an oligosaccharide backbone. As demonstrated here with five pairs of isomers and by a previous report,[21] the two types of linkages can be distinguished by the presence or absence of a unique, although weak (generally <8% in relative abundance), ion *m/z* 306 ($^{0,4}A_2-CO_2$) that is specific for a α2−6-linked sialic acid. The isomeric 2−3- and 2−6-linkages are also reflected in the lability of the intact molecule during ionization. The α2−3-linkage is generally more labile. This provides further evidence to differentiate the α2−3/6 isomers. With the same energy the precursor ions, $[M - H]^-$ and the NeuAc cleavage ions (B_1, *m/z* 290) in CID-MS/MS have different intensities. A weak B_1 and an intense $[M - H]^-$ indicate an α2−3-linkage, whereas an intense $[M - H]^-$ and a weak B_1 indicate an α2−6-linkage. It was unexpected that sialic acid residues with an α- or *β*-linkage give different product ion spectra, and the stability of the linkages were in the order *β*2−3 > α2−6 > α2−3 > *β*2−6.

In some cases, particularly when two or more sialic acids are present, the loss of sialic acid residues is a favored process, and it is difficult to deduce the sequence from the fragmentation. MS/MS of the mono-desialylated ion as precursor gave more informative product ion spectra, whereas in the case of SLNFP series, the sodiated molecules $[M - 2H + Na]^-$ as precursors can prevent the loss of sialic acid residues. For core-branching pattern analysis, a D-ion at the branched 3,6-disubstituted core, bearing the intact 6-linked branch, dominates the product ion spectrum of a neutral oligosaccharide.[13] This is also true for the sialylated oligosaccharides if a sialic acid is on the 6-linked branch. However, if a sialic acid is absent on the 6-branch while present on the 3-branch, a C_β ion derived from the 3-linked branch is prominent while the D-ion is weak. MS/MS scanning of the two ions (D and C_β) as precursors, produced by cone voltage fragmentation, can give unambiguous structural details of each of the branches, as demonstrated by MSLNH/MSLNnH and MSMFLNH/MSMFLNnH. Using the strategy established with nineteen sialylated oligosaccharides, we have determined a novel complex disialylated and monofucosylated tridecasaccharide that is based on the lacto-*N*-decaose core.

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SUPPORTING INFORMATION AVAILABLE

Table of the linkage and composition assignmentof DSMFLND and figures showing ES-CIS-MS/MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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