Negative-ion MALDI-QIT-TOFMS* for structural determination of fucosylated and sialylated oligosaccharides labeled with a pyrene derivative

Junko Amano1,2, Daisuke Sugahara2, Kenji Osumi3, and Koichi Tanaka4

1Laboratory of Glycobiology, The Noguchi Institute; 2Laboratory of Glyco-organic Chemistry, The Noguchi Institute, 1-8-1, Kaga, Itabashi, Tokyo 173-0003; and 3Koichi Tanaka Mass Spectrometry Research Laboratory, Shimadzu Corporation, 1, Nishinokyo-KuwaBaracho, Nakagyo-ku, Kyoto 604-8511, Japan

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Oligosaccharides have many isomers and MALDI-QIT-TOFMS* analysis is effective for determining their structures. However, it is difficult to elucidate in detail the structures of fucosylated and/or sialylated oligosaccharides that are known to be disease markers because fucose and sialic acid residues are easily released. We have introduced a technique of labeling oligosaccharides with a pyrene derivative prior to negative-ion MALDI-QIT-TOFMS*, and we have established a reliable method using this technique for the analysis of neutral oligosaccharides, such as fucosylated oligosaccharides containing blood group antigens H, Leα, and Leβ. Intense and stable ionization in both positive and negative modes was achieved by derivatization with pyrene. As little as 10 fmol of pyrene-labeled oligosaccharides gave sufficient signals for analysis. Specific A-, D- or Y-type ions that depend on the structures of branching antennae could be detected by MS* and were useful for rapid and easy structural determination. These specific fragmentations resulting from collision-induced dissociation can be used to elucidate the structures of unknown oligosaccharides even if authentic oligosaccharides are not available as standards. By using this method, we identified and quantitated isomeric oligosaccharides with different fucosyl linkages from their mixtures. Moreover, sialylated oligosaccharide was converted to the corresponding neutral oligosaccharide by amidation, and the negative-ion spectrum was shown to be more informative than that of the original acidic oligosaccharide. Structural determination of both fucosylated and sialylated isomers, such as siaIlyl-fucosyllacto-N-hexaose I and monosialyllacto-N-neohexaose, was successful because fragment ions bearing fucose or amidated sialic acid were obtained on negative-MS*.

Keywords: fucosylated oligosaccharides/
MALDI-QIT-TOFMS*/negative ion/pyrene-labeling

Introduction

Fucosylation and sialylation of sugar chains are very important for cell recognition and their levels are constantly regulated in various organs during development, differentiation, and activation. Therefore, altered structures of these oligosaccharides are directly associated with immunity and various diseases, such as certain infections and cancers (Greenwel 1997; Aspholm-Hurtig et al. 2004; Kannagi 2004). Oligosaccharide structures of blood-type antigens containing fucose, such as bearing H, Lewisα (Leα), Leβ, and Leβ determinants, occur naturally on the glycans of glycoproteins and glycolipids and are associated with various biological functions. Identification of fucosylation and sialylation is essential for diagnosis, as well as for pathogenic and pathological studies. However, it is difficult to isolate such oligosaccharides and to determine their structures without complicated separation procedures because the different types of linkages (for example, α-fucosyl 1-2, 1-3, 1-4, and 1-6 linkages) produce many isomers. To overcome these difficulties, we have developed a simple method for structural determination and quantitation of fucosylated oligosaccharides at the low-fmol level by means of MALDI-QIT-TOFMS* analysis using pyrene-labeled oligosaccharides. In this study, we demonstrate that this method does not require prior separation of mixtures of isomeric fucosylated oligosaccharides.

Fragmentation of positive ions, such as [M+H]+ ions and [M+Na]+ ions, from neutral oligosaccharides has been extensively studied in recent years. On the other hand, negative-ion mass spectrometry of neutral oligosaccharides has also gradually been shown to generate useful structural information. Several researchers have reported electrospray ionization (ESI) mass spectrometry with collision-induced dissociation (CID) MS*n of [M−H]− or [M−2H]2− produced from underivatized neutral human milk oligosaccharides and determined the structures of various fucosylated oligosaccharides (Chai et al. 2001, 2002; Pfenninger et al. 2002). Harvey reported ESI-MS of underivatized N-linked oligosaccharides in the presence of ammonium nitrate to give [M+NO3]− and [M+(NO3)2]2− and obtained CID spectra of those ions (Harvey 2005a, 2005b, 2005c). Lebrilla’s group generated [M+HSO4]− from underivatized neutral oligosaccharides doped with dilute H2SO4 using MALDI-FTMS and obtained CID spectra (Wong et al. 1999). To generate negative ions, negatively charged derivatives of oligosaccharides are sometimes used. For example, oligosaccharides derivatized with 2-aminobenzoic acid showed improved sensitivity compared with 2-aminobenzenamide derivatives, which are neutral (Harvey 2005d).

So far, negative-MALDI-MS analysis has not been successful for small amounts of neutral oligosaccharides although...
derivation by 2-aminopyridine and 4-aminobenzoic acid ethyl ester has been used for positive-MS. Pyrene-labeled oligosaccharides have been developed for highly sensitive analysis of structures and functions, e.g., with HPLC, ELISA, or fluorescence polarity measurement, as pyrene derivatives have strong fluorescence with a long lifetime (Sugahara et al. 2003). In the present study we have demonstrated that pyrene-labeled oligosaccharides, even when uncharged, improve the production and stability of negative ions, as well as positive ions, by MALDI and enable MS² analysis with QIT-TOFMS.

Results and discussion

Enhancement of ionization of pyrene-labeled oligosaccharides

Initially, to investigate the effect of labeling with pyrene butanoic acid hydrazide (PBH) on ion generation from labeled oligosaccharides, we compared pyrene-labeled oligosaccharides with nonlabeled and 2-aminopyridylated oligosaccharides using lacto-N-fucopentaose III. The relative signal intensities of positive ions [M+Na]⁺ were 1:25:100. Generally, in MALDI-QIT-TOFMS of neutral oligosaccharides, positive-ion species, but not negative-ion ones, are well observed. For example, no significant peak of a deprotonated molecular ion [M−H]⁻ was detected in the case of native oligosaccharides at 400 fmol, or even up to 10 pmol. In contrast, [M−H]⁻ ions were generated from labeled oligosaccharides. In particular, pyrene-labeled oligosaccharides produced a strong signal in the negative-ion mode as well as the positive-ion mode, even though the sample was a neutral oligosaccharide pyrene derivative, in the presence of 2,5-dihydroxybenzoic acid (DHBA) matrix without any additives. A significant signal of [M−H]⁻ ion with a signal-to-noise ratio of 5 could be obtained from 10 fmol of pyrene-labeled oligosaccharides (data not shown). This indicates that labeling with a pyrene derivative enhances vaporization and ionization, and stabilizes ions in both positive- and negative-ion modes. One of the reasons why pyrene derivatization is more effective than other derivatization is that the pyrene group easily accepts energy from the matrix due to its strongly aromatic nature. It is noteworthy that a neutral pyrene derivative produces negative-ion species from neutral oligosaccharides because MALDI-MS usually generates only mono-charged ions. If a labeled compound has a negative charge, MS² spectra will exhibit prominent Y and Z ions which contain the labeled compound at the reducing terminal, but not A, B, C, and Z ions. This is not helpful to determine the structures. In contrast, as described later, pyrene-labeled oligosaccharides generate many kinds of fragment ions with and without the pyrene derivative.

Loss of a fucose residue due to in-source and post-source decay on MALDI-QIT-TOFMS was kept at a significantly lower level in the negative-ion mode than in the positive-ion mode. This marked stability of fucosyl linkages in negative ions is also seen in the MS² spectra, as described later.

Fragmentation of fucosylated hexose isomers in positive-MALDI-QIT-TOFMS²

Three kinds of fucosylated hexose isomers, monofucosyllacto-N-hexose-I (MFLNH-I), II (MFLNH-II), and III (MFLNH-III), which differ in the linkage position of fucose residue, were analyzed in positive-MALDI-QIT-TOFMS after pyrene-labeling. These isomers express blood group antigens of H antigen (MFLNH-I), Leα antigen (MFLNH-II), and Leα antigen (MFLNH-III), and were detected in MS² spectra by selectively monitoring each [M+Na]⁺ ion as a precursor (Supplementary Figure 1). Since defucosylation prominently occurred and the common hexose ion at m/z 1381.5 and tetraose ion at m/z 1016.4 were obtained with high intensity, there was no significant difference in the observed product ion species and relative signal intensities. In addition, various oligosaccharides with one, two, and three fucose residues were measured in positive-ion mode on MALDI-QIT-TOFMS². Fucα1-2Gal, Fucα1-4GlcNAc, and Fucα1-3GlcNAc linkages were easily cleaved in MFLNH-I, II, and III, but the Fucα1-6GlcNAc linkage in N-glycans was stable (data not shown). In contrast, when these oligosaccharides were examined with negative-MS² using the same laser power and CID energy, fucosyl linkages were not cleaved, as described below.

Fragmentation of fucosylated hexose isomers in negative-MALDI-QIT-TOFMS²

MS² spectra of [M−H]⁻ ions from the above samples were obtained in negative-ion mode. It should be noted that fucosyl linkages are very stable in negative ions [M−H]⁻, unlike positive ions [M+Na]⁺. As shown in Figure 1, no production that had lost only a fucose residue (m/z 1357.5, indicated with arrows) was detected. The fragmentation of pyrene-labeled oligosaccharides produced many types of ions containing A, B, and C fragments in addition to Y and Z fragments (fragmentation nomenclature according to Domon and Costello (1988)) that retained their pyrene moieties (Figure 2). This variety of fragmentation makes structural determination of isomers easy.

In contrast, negative-ion ESI-MS² of underivatized oligosaccharides and MALDI-QIT-TOFMS² of pyrene-labeled oligosaccharides with a nonreduced hydrazone (Supplementary Figure 2) mainly generated A, C, and C/Z (D), but rarely Y and Z ions. The similarities between the kinds of product ions seem to be due to the presence of ring-closed Glc (Cheng and Her 2002). 0.2A- and 2.3A-type ions were produced by cleavage at the terminal glucose. This glucose links through the hydrazone form and seems to resemble a ring-closed form rather than a ring-opened form. 0.2A-type ions were also observed in ESI-MS² of oligosaccharides, which were labeled with p-aminobenzoic ethyl ester and had glycosylamines due to their nonreduced state (Cheng and Her 2002). Instead of 0.4A-type cleavage of pyrene-labeled and reduced oligosaccharides, 0.3A-type cleavage in Gal substituted at the 6-position occurred. These kinds of A-type cleavage have been reported in some structural studies of underivatized oligosaccharides using negative-ESI MS² (Chai et al. 2001, 2002; Pfenninger et al. 2002). Signal intensity of [M−H]⁻ ions from pyrene-labeled oligosaccharides without reduction was less than with reduction. However, pyrene-labeled oligosaccharides without reduction had higher sensitivity than intact oligosaccharides and desalting procedures were not necessary.

Discrimination of three kinds of monofucosylated hexose isomers in negative-MALDI-QIT-TOFMS²

As shown in Figure 1, several different product ion species were observed among the three isomers. For example, the ion at m/z 1177.5 from MFLNH-I, the ion at m/z 1159.5 from MFLNH-II, and the ions at m/z 1138.4 and at m/z 364.1 from MFLNH-III...
were never seen from other isomers. The Z ion at $m/z$ 1177.5 was produced by loss of Fuc-Gal disaccharide, as shown in Figure 2A, and this ion occurs in oligosaccharides with H antigen. The Z/Z ion at $m/z$ 1159.5 was produced by release of fucose and galactose, as shown in Figure 2B, and this ion is specific for oligosaccharides with Le$^a$ antigen. The C/Z ion at $m/z$ 364.1 results from the Le$^e$ structure, as shown in Figure 2C. These fragment ions can help to identify blood group determinants, as described in the accompanying article. In ESI-MS$^3$ of underivatized fucosylated oligosaccharides (Pfenninger et al. 2002), the C ion at $m/z$ 325.1 from the type 1 H structure, the C/Z ion at $m/z$ 348.1 from the Le$^e$ structure were obtained although the C/Z ion at $m/z$ 364.1 from the Le$^a$ structure was found and the ion was produced by our method. The Y ion at $m/z$ 1138.4 was obtained when the 3-branch was released in MFLNH-III (Figure 2C). It is interesting that the Y ion at $m/z$ 1138.4 was not produced from other isomers, although Y ions at $m/z$ 992.4 were found with all isomers. An important point to emphasize is that production of Y ions at $m/z$ 992.4 is not the result of defucosylation, as demonstrated later.

A ions and C/Z (D) ions are formed as fragments containing a 6-branch and provide direct evidence of fucosylation. The C/Z (D) ion at $m/z$ 526.2 together with its dehydrated ion at $m/z$ 508.2 from MFLNH-I and II, which have 6-branches without fucose,

were produced by cleavage at both reducing (Z) and nonreducing (C) sides of the 3-linkage of 3,6-substituted Gal. MFLNH-III gave the C/Z (D) ion and corresponding dehydrated product at $m/z$ 672.2/654.2 (526.2/508.2 + 146) because fucosylation was present on the 6-branch. Other unique ions at $m/z$ 424.2 and $m/z$ 570.2 (for fucosylated forms) produced by $^0\text{A}$-type ring cleavage in Gal substituted at the 6-position were observed and are also useful for structural determination.

Furthermore, selective cleavages are characterized by negative ions. All the isomers produced the ion at $m/z$ 992.4, and this is interpreted as representing deprotonated Hex-HexNAc-Hex-Hex-PBH. The MS$^3$ spectrum revealed that the ions at $m/z$ 992.4 from MFLNH-I and II had the same structure, but had a different structure from the ion at $m/z$ 992.4 from MFLNH-III. As shown in Figure 3A, the ion at $m/z$ 992.4 from MFLNH-I and MFLNH-II produced two prominent fragments via C-type ($m/z$ 382.1) and A-type ($m/z$ 424.2) cleavages. This fragmentation is the same as that of authentic Gal$\beta\text{1-4GlcNAc}\beta\text{1-6Gal}\beta\text{1-4Glc-PBH}$ (data not shown). The occurrence of the ion at $m/z$ 424.2 indicated that this ion at $m/z$ 992.4 contains the 6-branch. In addition, there is evidence that only the $^2\text{Y}_{2\beta}$ ion at $m/z$ 998.4, but not the defucosylated $^2\text{Y}_{2\alpha}$ ion at $m/z$ 992.4, was obtained from MFLNH-I and MFLNH-II with $[1,2,3,4,5,6]^2\text{C}$-galactose on the 6-branch (data not shown). In contrast, these isomeric oligosaccharides in
Fig. 2. Proposed fragmentation of pyrene-labeled MFLNH-I (A), II (B), and III (C), selecting [M−H]− at m/z 1503.6 on negative-ion MALDI-QIT-TOFMS².

positive-MS² gave ions at m/z 1016.4 and at m/z 1022.4, which should be produced by defucosylation. This indicates that cleavage took place at both the 3-branch and 6-branch linkages. The fragmentation of positive ions appears to proceed competitively at several cleavage points.

In MS³ the ion at m/z 992.4 from MFLNH-III mainly gave the Y ion (m/z 627.3) and the Z ions (m/z 812.3 and 447.2), but no A ion at m/z 424.2 (Figure 3B). This fragmentation is consistent with the MS² spectrum of authentic lacto-N-tetraose-PBH (data not shown), and therefore the ion at m/z 992.4 exclusively contained the 3-branch, not the 6-branch. The difference in fragmentation between the m/z 992.4 ions from other isomers can be explained by relocation of the deprotonation site from the glucitol reducing terminal to the nonreducing terminal on the 6-branch, which seems to be in close proximity. In the tetraose with a 3-branch, the nonreducing terminal may be remote and the negative charge remains at the reducing terminal, resulting in production of Y and Z ions. From a mechanistic point of view, in positive ions the sodium ion can interact with several sites in the oligosaccharide molecule and fragmentation should happen almost instantaneously at many points. In negative ions, deprotonation starts from the reducing terminal and transfers to other sites successively. Harvey also pointed out that, unlike positive ion fragmentation, negative ions appeared to be produced by a single pathway, and consequently, these ions are indicative of specific structural features (Harvey 2005c).

Branch-specific fragmentation of pyrene-labeled oligosaccharides in negative-ion mode
From the MS² spectrum shown in Figure 1, it appears that the linkage of GlcNAcβ1-3Gal is easily cleaved, as shown with a thick line in Figure 2, compared with the linkage of GlcNAcβ1-6Gal at a branching Gal residue. Although a large amount of the Y2α ion was clearly obtained from MFLNH-I and MFLNH-II, the Y2α ion at m/z 1138 appeared never to be produced. Regarding MFLNH-III, both the Y2α ion at m/z 1138.5 and the Y2β ion at m/z 992.5 were generated almost equally. After MFLNH-III was digested with β-galactosidase and then incubated with β4-galactosyltransferase and UDP-[1,2,3,4,5,6]13C-galactose, interestingly, only the Y2α ion at m/z 998, but not the Y2β ion at m/z 1138, was obtained from the isomer monofucosyllacto-N-neohexaose (Figure 4A). These results show that the linkage of GlcNAcβ1-3Gal is not always cleavable. This finding raises a question as to what factors affect the fragmentation. We prepared lacto-N-hexaose (LNH) with a 13C-labeled 6-branch and examined which branches were released on CID. The results (Figure 4B) indicated that the Y2β ion at m/z 998, but not the Y2α ion at m/z 992, was generated. This means that 3-linked GlcNAc, but not 4-linked GlcNAc, showed Y-type cleavage at the reducing side. The oligosaccharides with type 1 N-acetyllactosamine or α3-fucosylation, such as LNH, MFLNH-I, MFLNH-II, and MFLNH-III, produce this kind of specific Y-type ion.

As mentioned above, C/Z (D)-type cleavage at a branching galactose residue occurs only at a 3-linkage, but never at a 6-linkage. The specificity of cleavage sites depending on the substituted positions should be useful for structural determination, especially for oligosaccharides with branches (see the accompanying article).

Analysis of polyfucosylated hexaoses on negative-ion MS³
We examined hexaoses which have two or three fucose residues (Figure 5) by negative-MS³ after pyrene derivatization. The oligosaccharides in Figure 5A–C consist of LNH, but the oligosaccharide in Figure 5D has LNnH. On MS² analysis, the fragment ions at m/z 1323 and 1138 were obtained as shown in Figure 5A. The Z ion at m/z 1323 produced by release of Fuc-Gal indicates occurrence of the H structure. In MS³ the ion at m/z 1138, the Z ion at m/z 812 (1323–326) and the C/Z ion at m/z 364 were generated, and the presence of the Le³ structure, besides the H structure, was confirmed. Consequently, m/z 1138 included both Y2α and Y2β ions. The Y ions at m/z 1284 and 1138 and the D ion at m/z 672 were obtained from the oligosaccharide in Figure 5B by MS², and then the B ion at m/z 656 or the C/Z ion at m/z 364 was observed in the MS³ spectrum of the ion at m/z 1284 or m/z 1138, respectively. These results show the presence of the Le³ structure on the 6-branch and the Le³ or Le⁴ structure on the 3-branch. The Y ion at m/z 1138 and the D ion at m/z 672 were produced from both isomeric oligosaccharides shown in Figure 5C and D on MS² analysis. Regarding MS³ analysis of the ion at m/z 1138, the Z/Z ion at m/z 794 (1138–344) and the C/Z ion at m/z 364 were obtained from the oligosaccharide shown in Figure 5C, although only the C/Z ion at m/z 364 was observed from the oligosaccharide

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Fig. 3. Negative-ion MALDI-QIT-TOFMS\(^3\) spectra of pyrene-labeled MFLNH-II (A) and III (B), selecting \(m/z\) 992.4 as a precursor and showing the proposed fragmentation. The spectrum of MFLNH-I is the same as that shown in A.

Fig. 4. Negative-ion MALDI-QIT-TOFMS\(^2\) spectra of \(^1\)C\(_6\)-Gal-labeled fucosylated LNnH (A) and LNH (B) and the proposed fragmentation.
Fig. 5. Proposed fragmentation of pyrene-labeled difucosyllacto-\(N\)-hexose I (A), trifucosyllacto-\(N\)-hexose (B), difucosyllacto-\(N\)-hexose II (C), and difucosyllacto-\(N\)-neohexose (D) selecting [M−H]− at \(m/z\) 1649.6 or \(m/z\) 1795.6 on negative-ion MALDI-QIT-TOFMS2.

shown in Figure 5D. These results confirm that the isomer in Figure 5C contains \(\text{Le}^a\) and \(\text{Le}^b\) structures, but the isomer in Figure 5D has the \(\text{Le}^a\) structure on both branches.

**Negative-ion MS2 for sialylated oligosaccharides after amidation**

Since sialyl linkages are also unstable in QIT-TOFMS, Sekiya et al. (Sekiya et al. 2005) converted carboxyl residue to amide and measured positive ions. Because the amidation of the \(\alpha2,3\)-linked sialic acid is incomplete (Toyoda et al. 2008), we chose disialylated bi-antennary \(N\)-linked oligosaccharide containing \(\alpha2,6\)-linked sialic acid. We labeled it with pyrene, amidated the derivative, and then measured negative ions. The original oligosaccharide containing carboxylic acids gave a poor negative-ion MS2 spectrum, which mainly showed desialylated \(Y_6\) ion (Figure 6A). After amidation, the negative-ion MS2 spectrum of this oligosaccharide showed many fragments in addition to the desialylated \(Y_6\) ion, and these ions are very informative (Figure 6B). In particular, the presence of the D (\(C_3/Z_3\)) ion indicates that the 6-linked branch has a modified sialic acid.

Next both sialylated and fucosylated oligosaccharides, monosialyl, monofucosyllacto-\(N\)-hexose I and monosialyl, monofucosyllacto-\(N\)-neohexose, were examined. These oligosaccharides were labeled with PBH and examined with negative-MS2. Almost the same spectra were obtained from both isomers, as shown in Figure 7A and B. The defucosylated ion at \(m/z\) 1649 and the desialylated ion at \(m/z\) 1503 were produced, and the tetrasaccharide ion without sialic acid and fucose at \(m/z\) 992 was observed as the major fragment ion. After amidation, as expected, negative-MS2 spectra did not show the defucosylated ion at \(m/z\) 1648 (Figure 7C and D), although defucosylation should occur on positive-MS3 of amidated oligosaccharides. In MS2 spectra, the 0,4 A ions at \(m/z\) 714 or \(m/z\) 570 clearly indicate the presence of sialic acid or fucose on the 6-branch, respectively (Figures 7C and D, Figure 8A and B). The ions specific to each isomer, such as the ion at \(m/z\) 1468 and the ion at \(m/z\) 1138, were also detected. In addition to the tetrasaccharide ion at \(m/z\) 992, substantial amounts of the fucosylated ion at \(m/z\) 1503 and the ion containing amidated sialic acid at \(m/z\) 1283 were obtained, and therefore, by MS3 analysis of these ions, the positions of the sialic acid and fucose residues could be further confirmed. The MS3 spectrum of the ion at \(m/z\) 1503 in Figure 7C was the same as the MS2 spectrum obtained from LNFH-I, as shown in Figure 1A, and the ions at \(m/z\) 424, 526, and 1177 were detected. In contrast, the ions at \(m/z\) 364, 570, and 672, produced by MS3 of the ion at \(m/z\) 1503 as shown in Figure 7D, indicate the \(\text{Le}^a\) structure on the 6-branch. MS3 analysis of the ion with amidated sialic acid at \(m/z\) 1283 showed many A-type fragment ions from the pentasaccharide ion containing the 6-branch (Figures 7E and 8C). In contrast, Z- and Y-type ions as well as A-type ions were observed in the spectrum of the pentasaccharide ion containing the 3-branch (Figures 7F and 8D).

**Identification and quantitation of mixtures of fucosylated isomers**

We have already demonstrated in this study that isomeric oligosaccharides with various fucosylated antigens, which
should show the same MS spectrum, could be discriminated by negative-MALDI-QIT-TOFMS². Mixtures containing pyrene-labeled MFLNH-I and III in ratios of 80:20, 60:40, 40:60, and 20:80 were prepared, and MS² spectra were obtained by selecting [M−H]⁻ at m/z 1283 in C (E), and monofucosyl monosialyllacto-N-neohexaose (B), after amidation (D) and MS³ spectra of the ion at m/z 1283 in d (F).

Fig. 6. Negative-ion MALDI-QIT-TOFMS² spectra of disialylated biantennary N-glycan before (A) and after (B) amidation, and the proposed fragmentation.

Fig. 7. Negative-ion MALDI-QIT-TOFMS² spectra of sialylfucosyllacto-N-hexaose I (A), after amidation (C) and MS³ spectra of the ion at m/z 1283 in C (E), and monofucosyl monosialyllacto-N-neohexaose (B), after amidation (D) and MS³ spectra of the ion at m/z 1283 in d (F).
Material and methods

Oligosaccharides

Oligosaccharides were purchased from Seikagaku Corp. (Tokyo, Japan) and labeled with pyrene butanoic acid hydrazine (PBH, Molecular Probes) as previously described (Sugahara et al. 2003), followed by reduction with NaBH₄. In this study, pyrene-labeled oligosaccharides refer to the reduced form. Briefly, oligosaccharides (0.1–1 nmol) and PBH (100 nmol) in 20 μL of methanol were heated at 80°C for 20 min. Then, the 1.7 M NaBH₄ solution (30 μL) was added and the reaction mixture was incubated at 40°C for 20 min. The addition of water and CHCl₃ (1:1, V/V) allowed labeled oligosaccharides with or without reduction to be extracted into the aqueous phase. Labeled oligosaccharides were purified by using a small C₁₈ column if necessary and stored at −30°C in the dark until used.

2-Aminopyridylated oligosaccharides were purchased from Seikagaku Corp. Galβ₁-4GlcNAcβ₁-6Galβ₁-4Glc-PBH was prepared from MFLNH-III-PBH by digestion with a mixture of β-galactosidase (Sigma, 10 mU) and β-N-acetylhexosaminidase from jack bean (Sigma, 20 mU) in the 20 mM sodium citrate buffer, pH 5, at 37°C overnight, followed by inactivation by heating, and then digestion with α₁-3,4-fucosidase from Streptomyces sp.142 (Takara Bio, 2 μU) in the 20 mM potassium phosphate buffer, pH 6, at 37°C for 2 h. Stable isotopic labeling of MFLNH-I-PBH and MFLNH-II-PBH oligosaccharides was done by β-galactosidase digestion followed by incubation with β-galactosyltransferase (Takara Bio, 0.8 mU) and UDP-[1,2,3,4,5,6]¹³C-Gal (1 nmol) in the sodium phosphate buffer, pH 7, overnight.

Synthesis of UDP-¹³C₆-D-galactose

UDP-¹³C₆-D-galactose was chemically derived from ¹³C₆-D-galactose (Omicron Biochemicals, South Bend, IN) according to the method reported for the synthesis of UDP-D-galactose (Sabesan and Neira 1992; Wittmann and Wong 1997), with the following modifications. The purification of UDP-¹³C₆-D-galactose was carried out by silica gel chromatography on an
Iatrobeads 6RS-80100 (2.5 × 12 cm, Iatron, Tokyo, Japan) using CHCl₃/MeOH/0.75 M NH₄OH (6:6:1, v/v/v) as an eluent, followed by size exclusion chromatography on a Bio-Gel P2 (2.5 × 90 cm, BioRad, Hercules, CA) using 5% ethanol as an eluent. The purified UDP-¹³C₆-D-galactose was converted to the sodium salt by passage over Dowex 50 W × 8 (1.2 × 3 cm, Na⁺ form, Dow Chemicals, Midland, MI) using water as an eluent.

**Mass spectrometry**

The oligosaccharide samples were dissolved in pure water and 0.4 μL (10–400 fmol) of each sample was spotted on a MALDI target plate. 2,5-Dihydroxybenzoic acid (DHBA, 0.5 μL) was used as a matrix, which was prepared by dissolving 12.5 mg/mL in 40% (v/v) CH₃CN-water. The dried mixture was measured by using MALDI-QIT-TOFMS (AXIMA-QIT, Shimadzu Biotech) in positive- and negative-ion modes in MS, MS², and MS³ modes. All oligosaccharide isomers were measured under the same conditions of laser power and CID energy. The vertical axis of the spectrum in each figure indicates the same scale to allow the comparison of signal intensities.

**Conflict of interest statement**

None declared.

**Supplementary Data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

**Abbreviations**

CID, collision-induced dissociation; ESI, electrospray ionization; PBH, pyrene butanoic acid hydrazide.

**References**


