MALDI-FTMS Characterization of Oligosaccharides Labeled with 9-Aminofluorene

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9-Aminofluorene (9AmFL) was investigated as an oligosaccharide label. The label was amenable to high UV detectability but did not interfere with mass spectrometric analysis. The 9AmFL label has high molar absorptivity ($\varepsilon = 1.4 \times 10^4$ L cm^{-1} mol^{-1} at $\lambda = 267$ nm), is chemically stable, and adds easily in reductive amination to the aldehyde terminus of oligosaccharides. Various linear and branched oligosaccharides were labeled with 9AmFL and the products were purified by chromatography on porous graphitized carbon (PGC). The derivatization reaction gave excellent yields (>95%). Up to 100-fold increase in UV sensitivity at $\lambda = 206$ nm, compared to the corresponding alditol, was observed. Mass spectra were recorded for the labeled compounds. In the presence of sodium dopant, series of Y- and B-fragments were observed. Protonation of the labeled compounds prior to mass spectrometric analysis resulted in simplified spectra (Y-fragments only) and allowed for complete sequence analysis. The retention of the positive charge at the label in the protonated species was consistent with the basicity of the amine. The smallest amount of labeled sugar to be detected by photo-diode array (PDA) was 5 pmol ($\lambda = 267$ nm). (J Am Soc Mass Spectrom 2001, 12, 1254–1261) © 2001 American Society for Mass Spectrometry

Oligosaccharides from natural sources are frequently difficult to isolate in sufficient quantity for structural elucidation by nuclear magnetic resonance experiments. Because of its high sensitivity, mass spectrometry has become the method of choice to analyze small quantities of oligosaccharides. However, the heterogeneity of native oligosaccharide samples often requires several levels of chromatographic separation. The inherent difficulty of detecting oligosaccharides is due to the absence of strong chromophores. This problem has been addressed in the past by introducing UV active or fluorescent labels into the sugar prior to chromatographic separation [1–6]. The vast majority of common labeling reagents for oligosaccharides is composed of aromatic amines. Among the most frequently used labels are 2-aminopyridine [2, 3, 4], biotinylated aminopyridine (BAP) [4, 5], 2-aminobenzamide [1], 3-aminoquinoline [1], 2-aminoacridone [1], 4-amino-N-(2-diethylaminoethyl)-benzamide [1], esters of 4-aminobenzoic acid [1, 2], and 1-phenyl-3-methyl-5-pyrazolone [6]. The labeling reaction is based upon reduction of the Schiff's base formed between the amine and the sugar aldehyde to yield the aminomethyl derivative. Unfortunately, aromatic amines have reduced nucleophilicity because of resonance stabilization of the free pair of electrons at the nitrogen atom. Phenylhydrazines, though more nucleophilic, suffer from the problem of overoxidation of aldoses (osazone formation). BAP was introduced into small chain neutral oligosaccharides with 75–80% yield [5], however, in biological samples, the yield of attachment is smaller (65–70%).

The use of benzylamine as an oligosaccharide labeling reagent has been described only twice in the literature [7, 8]. Broberg et al. used benzylamine to label several oligosaccharides [7], which were subsequently quaternized with excess methyl iodide. The sequestered charge at the nitrogen resulted in a 10-fold increase in MALDI-TOF mass spectral sensitivity compared to the underivatized oligosaccharide. As low as 50 fmol of the derivative could be detected. Post source decay (PSD) data of the quaternized derivative of maltoheptaose showed Y- and cross ring fragments. However, PSD spectra of branched oligosaccharides such as LNFP 1 and LNDFH 1 showed only Y- and B-fragments. Domon and coworkers investigated benzylamine as a labeling reagent for linear and branched oligosaccharides [8]. PSD MALDI-TOF data of the protonated species gave sufficient information to determine the saccharide sequences.

Benzylogous amines are as UV active as aromatic amines, but they are more nucleophilic than aromatic amines. Unlike in aromatic amines, the free pair of electrons at the nitrogen in benzylogous compounds is not delocalized into the aromatic system increasing nucleophilicity. The high reactivity and the high UV

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activity prompted us to investigate benzylogous amines such as benzylamine and 9-aminofluorene (9AmFL) as oligosaccharide labels. The fluorene core of 9AmFL is superior to benzylamine in UV absorptivity (1.4×10^4 L cm^{-1} mol^{-1}, λ_{max} = 267 nm compared to 1.3×10^3 L cm^{-1} mol^{-1}, λ_{max} = 248 nm) and promises to improve the detection of minor components during HPLC separation of complex oligosaccharide mixtures from biological sources. We focused our attention on 9AmFL, which is commercially available as an inexpensive and stable hydrochloride. The hydrochloride could be easily converted in situ into the free amine by the addition of $NaHCO_3$ (see Experimental). The new label was envisioned to combine low cost, high nucleophilicity, and high UV sensitivity with no adverse effect on fragmentation characteristics during mass spectrometric analysis. To the best of our knowledge, 9AmFL has not been described in the literature as an oligosaccharide label.

Experimental

Unless stated otherwise, the chemicals used in the derivatization reaction were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Solvents were of HPLC grade. Oligosaccharides were purchased from Oxford Glycosystems (Rosedale, NY). Evaporation of small amounts of solvent was done on a Centrivap Concentrator (Labconco Corporation, Kansas City, MO) at 45 °C in vacuo. To evaluate the separation of oligosaccharides labeled with 9AmFL, HPLC was performed on a Hewlett Packard instrument (1100 series) with a Hypercarb PGC column (100 × 2.1 mm, Thermoquest, Hypersil Division). Water and 60% acetonitrile (vol/vol) in 0.05% trifluoroacetic acid (vol/vol) was used as eluant at a flow rate of 0.25 mL/min with a gradient of 20:80 to 4:96 over 40 min, isocratic for 10 min, and 4:96 to 20:80 over 10 min. To purify the labeled oligosaccharide, the reaction mixture was passed through a PGC cartridges (Alltech Associates, Inc., Deerfield, IL).

Procedure for Labeling Maltoheptaose with Aniline or Benzylamine

Aniline (10 μL) or benzylamine (10 μL) was dissolved in methanol (100 μL). To this solution $NaCNBH_3$ (3 mg, 0.05 mmol) was added followed by glacial acetic acid (5 μL). The resulting solution was added immediately to a solution of maltoheptaose (1 mg) in water (50 μL). The reaction mixture was heated to 80 °C for 2 h. Workup of the product is described below for 9AmFL.

For quaternization, the derivatized oligosaccharide was dissolved in 50 μL of dry ethanol. $NaHCO_3$ was added at room temperature (2 mg) followed by methyl iodide (5 μL). The reaction mixture was heated to 60 °C for 4 h to complete the reaction. The progress of the reaction was monitored by mass spectrometry.

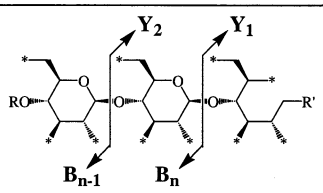
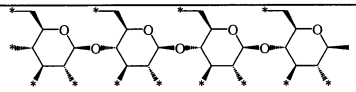
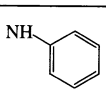
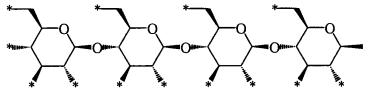
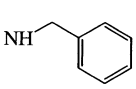
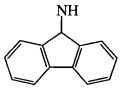
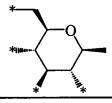
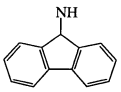
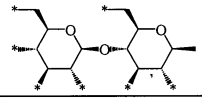
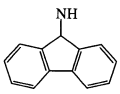
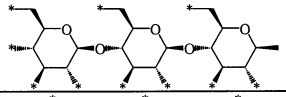
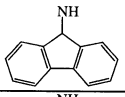
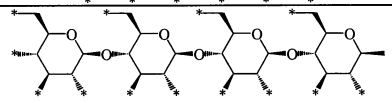
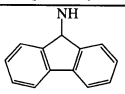
General Procedure for Labeling with 9AmFL

The oligosaccharide (200 nmol) was dissolved in 50 μL of nanopure water in a plastic microcentrifuge tube. In another microcentrifuge tube 9AmFL hydrochloride (2 mol equivalents) was dissolved in water. The solution was treated with $NaHCO_3$ (1 mol equivalent with respect to 9AmFL) to give the free amine as a white precipitate. After ten min at room temperature, the suspension was evaporated in vacuo and the residue was dissolved in 100 μL of methanol. To this solution $NaCNBH_3$ (3 mg, 0.05 mmol) was added followed by glacial acetic acid (5 μL). The resulting reagent solution was added immediately to the above oligosaccharide solution. The reaction mixture enclosed in a 2 mL sealed microcentrifuge tube was heated at 80 °C for 2 h. The reaction mixture was cooled to room temperature and was evaporated in vacuo. Methanol (100 μL) together with glacial acetic acid (2 μL) were added to the tube and evaporated. The evaporation of methanol and glacial acetic acid was repeated seven times, which was found to be sufficient to remove remaining borate contamination completely. The labeled oligosaccharide was dissolved in 100 μL of nanopure water and was desalted as previously described in the literature [13]. A PGC cartridge was washed with aqueous acetonitrile (80%, vol/vol) in TFA (0.05%, vol/vol). The solution of the labeled oligosaccharide was applied and allowed to flow into the cartridge by gravity. The cartridge was washed with nanopure water to remove the salts and by acetonitrile (60%, vol/vol) in TFA (0.05%, vol/vol) to elute the product. The UV active fractions collected were concentrated in vacuo and were used for mass spectrometric analysis.

Mass Spectrometric Analysis (MALDI-FTICR)

Mass spectra were recorded on an external source HiResMALDI (IonSpec Corporation, Irvine, CA) equipped with a 4.7 Tesla magnet and with a LSI 337 nm nitrogen laser [9–12]. 2,5-Dihydroxy-benzoic acid was used as matrix (5 mg/100 μL in ethanol). To obtain sodium coordinated species, a 0.01 M solution of NaCl in methanol was used as sodium dopant. For protonated species, an ammonium resin was used to remove the alkali metals. A solution of the labeled oligosaccharide (1 μL) was applied to the MALDI probe followed by either sodium dopant (1 μL) or ammonium resin. The matrix solution was then applied and the sample was dried under a stream of air. For all CID experiments, the appropriate isolation pulses were programmed starting at 3 s after the initial ionization and followed by sustained off-resonance irridiation (SORI) excitation at 6 s (1 s, 5 V base to peak, +1000 Hz off-resonance). At a background pressure of 10^{-10} torr, argon gas was administered through a pulsed valve at 6 and at 6.5 seconds (peak pressure 5×10^{-5} torr). Final excitation for detection was performed 12 s after the initial laser pulse.

Table 1. Structures of Compounds **1–7**

* = OH			
R =		**R' =**	**Cpd. #**
			**1**
			**2**
H			**3**
			**4**
			**5**
			**6**
			**7**

Results and Discussion

The structures of the compounds synthesized and discussed are depicted in Table 1. In order to compare mass spectral data of oligosaccharides derivatized with an aromatic amine to those derivatized with a benzylogous amine, aniline labeled maltoheptaose (**1**) was prepared and quaternized as described in the Experimental section. The MALDI-FTMS spectra are shown in Figure 1.

The CID spectrum of the sodiated molecule of Compound **1** showed two series of fragments (Figure 1a). Throughout this paper, the carbohydrate fragmentation nomenclature of Domon and Costello [14] applies. The Y-series (*m/z* 1090 [Y_6], 928 [Y_5], 766 [Y_4], 604 [Y_3], and 442 [Y_2]) represent fragments that retain the aniline label. The B-series (*m/z* 995 [B_6], 833 [B_5], 671 [B_4], 509 [B_3], and 347 [B_2]) comprise fragments that do not retain the aniline label. The unspecific coordination of sodium to polyol segments caused fragmentation to occur from both ends of the linear saccharide chain.

Quaternization of **1** could be monitored at room temperature (Figure 1b). The peak at *m/z* 1252 represents the sodiated starting material. The sodium doped N-methylated Compound **1a** gave a signal at *m/z* 1266, whereas the quaternized product **1b** with a localized charge at the nitrogen gave rise to the peak at *m/z* 1258. Unfortunately, CID experiments with **1b** did not produce many structurally meaningful fragments. The majority of fragment ions corresponded to low molecular weight species that gave little structural information. Even careful adjustment of the excitation energy did not lead to the usual glycosidic bond cleavages (Figure 1c).

Compound **2** is an example of an oligosaccharide derivatized with benzylamine. It was prepared from maltoheptaose and quaternized as described in the Experimental section. The MALDI-FTMS spectra are shown in Figure 2.

As with **1**, the Y- and the B-series of fragments were also observed in the CID spectrum of sodiated Compound **2** (*m/z* 1266, Figure 2a). The Y-series (*m/z* 1104 [Y_6], 942 [Y_5], 780 [Y_4], 618 [Y_3], and 456 [Y_2]) represented fragments that retained the benzyl label. The Y-series were consistent with one additional CH_2-group in Compound **2** ($\Delta m = 14$ u) compared to Compound **1**

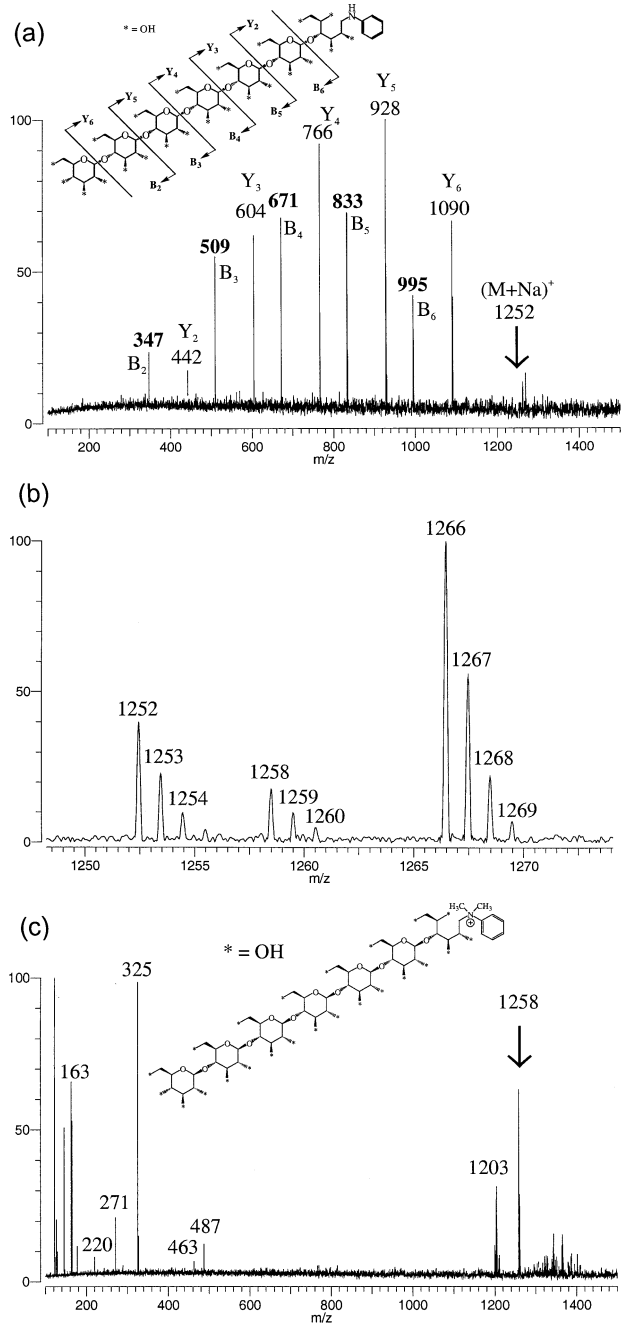


Figure 1. (**a**) MALDI-FT-ICR CID spectrum of **1** (*m/z* 1252, $[M + Na]^+$). (**b**) MALDI-FT-ICR mass spectrum of reaction mixture of quaternization of **1** after 20 min at 60 °C. (**c**) CID spectrum of quaternized product **1b** (*m/z* 1258). Note: The arrows in all spectra indicate where the molecular ion used to be prior to CID.

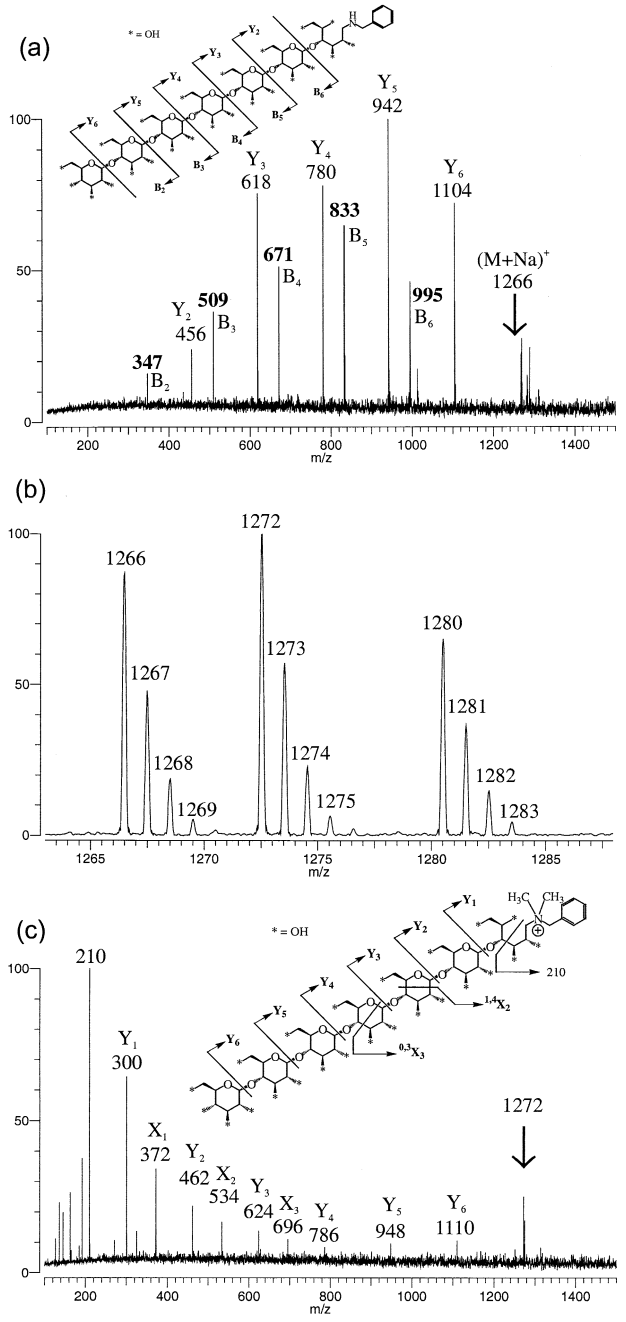


Figure 2. (**a**) MALDI-FT-ICR CID spectrum of **2** (*m/z* 1266, $[M + Na]^+$). (**b**) MALDI-FT-ICR mass spectrum of reaction mixture of quaternization of **2** after 20 min at 60 °C. (**c**) CID spectrum of quaternized product **2b** (*m/z* 1273). Cross ring cleavages ($^{1,4}X$ or $^{0,3}X$) were observed.

(Figure 1a). The B-series (*m/z* 995 [B_6], 833 [B_5], 671 [B_4], 509 [B_3], and 347 [B_2]) comprised fragments that did not retain the benzyl label.

Treatment of Compound **2** with methyl iodide afforded the corresponding quaternized Product **2b** via the mono-methylated Compound **2a** (Figure 2b). Under the same reaction conditions used for Compound **1**, the reaction proceeded faster by a factor of two based on the intensities of the product ions (Figures 1b and 2b, *m/z* 1258 and 1272, respectively) relative to the starting material (Figures 1b and 2b, *m/z* 1252 and 1266, respectively). Figures 1b and 2b were obtained after the same reaction period. As expected, the increased nucleophilicity of the benzylamine label made it more reactive. The CID experiments with Compound **2b** (Figure 2c) yielded the Y-series ions (*m/z* 1110 [Y_6], 948 [Y_5], 786 [Y_4], 624 [Y_3], 462 [Y_2], and 300 [Y_1]). Cross ring cleavages were also observed yielding the X-series fragments

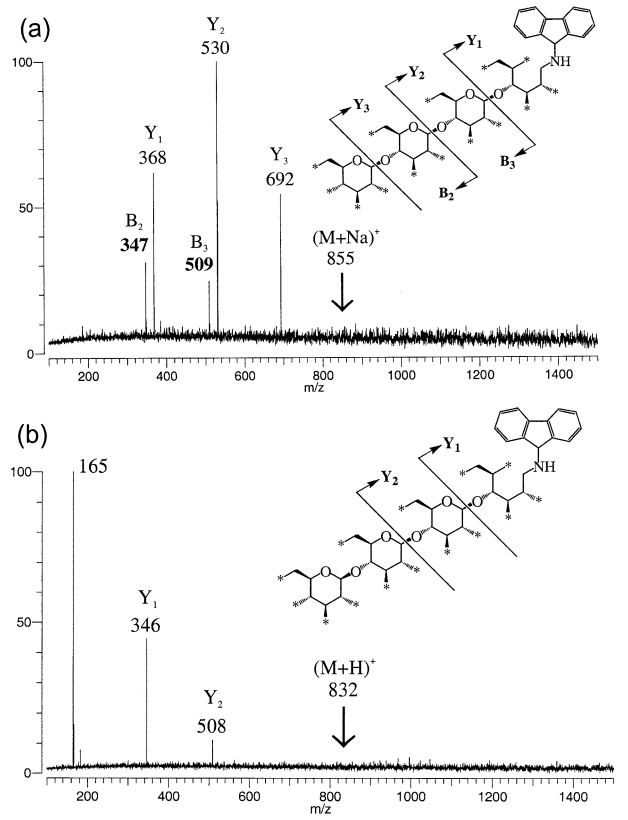


Figure 3. (**a**) CID spectrum of **4** (*m/z* 854, $[M + Na]^+$). (**b**) CID spectrum of **4** (*m/z* 832, $[M + H]^+$). Note: The arrows in all spectra indicate where the molecular ion used to be prior to CID.

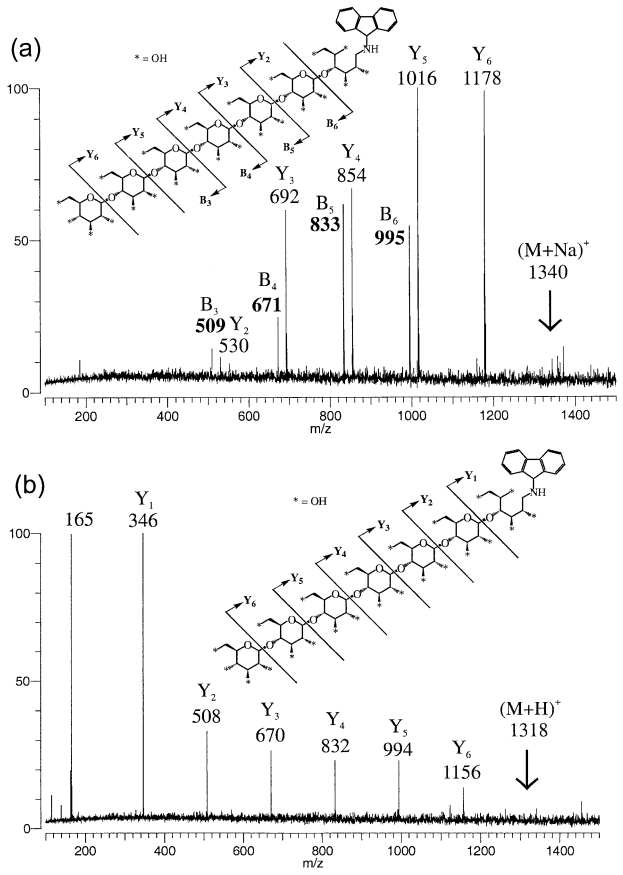


Figure 4. (**a**) CID spectrum of **7** (arrow indicates where *m/z* 1340, $[M + Na]^+$ used to be prior to CID). (**b**) CID spectrum of **7** (arrow indicates where *m/z* 1318, $[M + H]^+$ used to be prior to CID).

(*m/z* 696 [$^{1,4}X_3$ or $^{0,3}X_3$], 534 [$^{1,4}X_2$ or $^{0,3}X_2$], 372 [$^{1,4}X_1$ or $^{0,3}X_1$]). The specific ring cleavage ($^{0,3}X$ or $^{1,4}X$) could not be determined because the resulting fragments had identical masses. The base peak at *m/z* 210 was attributed to cleavage between carbon 3 and carbon 4 of the labeled terminus. These results imply that a localized charge produces cross ring cleavages in accordance with previously published results from MALDI-TOF data in post source decay experiments [7]. It is important to note that under low energy CID conditions, fragments due to cross ring cleavage are not typically observed for oligosaccharides derivatized at the reducing terminus. The fact that cross ring cleavage was produced from Compound **2b**, where the charge is localized, but not from Compound **2** can be rationalized in terms of the higher energy needed to fragment the former. The high energy and the localized charge opened other reaction channels, such as charge remote fragmentation, to take place.

Compound **4** was prepared by reductive amination of maltotetraose with 9AmFL. In the mass spectrum of the sodium doped species, Y-fragment ions (*m/z* 692 [Y_3], 530 [Y_2], and 368 [Y_1]) as well as B-fragment ions (*m/z* 509 [B_3] and 347 [B_2]) were observed (Figure 3a). Again, the unspecific coordination of sodium cations to the oligosaccharide caused fragmentation to occur from both ends of the sugar chain. Multiple fragmentation pathways yielding additional structural information are evident in the CID spectrum. Reduction of the number of fragment ions without loss of structural information was obtained with the protonated molecule. The CID spectrum of **4** (Figure 3b) showed only Y-fragment ions (508 [Y_2] and 346 [Y_1]). The base peak at *m/z* 165 represented the fluorenyl label, which was easily lost due to the formation of a strongly resonance-stabilized cation. The observed fragments in Figures 3a and b could be used to completely assign the sequence of the native sugar.

In this case, the quaternization of the 9AmFL label proceeded four times slower as with Compounds **1b** and **2b** under the same reaction conditions. The reason for this result is currently under investigation.

The series of linear oligosaccharides **3**, **5**, **6** (spectra not shown), and **7** (Figure 4) behaved similarly to Compound **4**. In each case, the sodium doped sample gave rise to fragmentation from both ends of the sugar chain (Figure 4a). The Y-fragments were consistently more abundant than the B-fragments. This implies that the sodium cation was slightly better retained by the labeled terminus than by the other terminus upon fragmentation. This was expected because of the better chelating properties of the modified open chain terminus. In each case, the protonated molecule resulted in a

Table 2. Typical fragment ions observed in CID experiments with sodiated or protonated molecules from Compounds **1–7**

	1	2	3		4		5		6		7	
	Na^+	Na^+	Na^+	H^+	Na^+	H^+	Na^+	H^+	Na^+	H^+	Na^+	H^+
Isolate	**1252.432**[a]	**1266.445**	**692.251**	**670.271**	**854.312**	**832.319**	**1016.365**	**994.389**	**1178.464**	**1156.426**	**1340.528**	**1318.475**
Y_1	—	—	368.146	346.166	368.148	346.164	368.149	346.166	—	346.165	—	346.165
Y_2	442.169	456.185	530.198	—	530.203	508.217	530.205	508.221	530.206	508.219	530.203	508.218
Y_3	604.223	618.239	—	—	692.259	—	692.262	670.278	692.263	670.273	692.260	670.271
Y_4	766.278	780.293	—	—	—	—	854.319	832.333	854.324	832.325	854.319	832.324
Y_5	928.334	942.350	—	—	—	—	—	—	1016.383	994.389	1016.376	994.377
Y_6	1090.393	1104.410	—	—	—	—	—	—	—	—	1178.435	1156.430
B_1	—	—	—	—	—	—	—	—	—	—	—	—
B_2	347.095	347.095	347.094	—	347.096	—	347.097	—	347.096	—	—	—
B_3	509.149	509.149	—	—	509.151	—	509.153	—	509.154	—	509.153	—
B_4	671.204	671.205	—	—	—	—	671.208	—	671.210	—	671.211	—
B_5	833.257	833.259	—	—	—	—	—	—	833.269	—	833.264	—
B_6	995.315	995.316	—	—	—	—	—	—	—	—	995.325	—

[a] Given are *m/z* values of fragments.

simplified CID spectrum with only fragments of the Y-series consistently (Figure 4b). A summary of fragment ions observed in the CID experiments with both sodiated and protonated molecules is given in Table 2. Analysis of the CID data from sodiated and protonated molecules made the determination of the complete sequence of the linear derivatized oligosaccharides possible.

The labeling reaction was extended to Lewis tetrasaccharide b (LSTb) (Compound **8**) as an example of a branched anionic sugar. The MALDI-FTMS spectrum of the protonated molecule is shown in Figure 5a. We would like to point out that the assignment of the exact stereochemistry of the fragments lost in the following discussion was based upon the known identity of the starting material and not upon mass spectrometric data. It was difficult to obtain the protonated molecule more abundantly at *m/z* 1164 because of the facile loss of N-acetyl neuraminic acid [15] from the protonated molecule to give a base peak at *m/z* 873. Subsequently, this fragment ion was isolated and subjected to CID with the results shown in Figure 5b. Fragmentation in the side chain resulted in a $Y_{3\alpha}$ fragment at *m/z* 711. Further loss of N-acetyl glucosamine (*m/z* 508, Y_2) and of galactose (*m/z* 346, Y_1) was also observed.

Branched neutral oligosaccharides were examined by synthesizing derivatives of LNDFH I and LNDFH II (Compounds **9** and **10**, respectiveley). CID spectra of the sodiated and protonated molecules are shown in Figure 6. The CID spectra of the sodium doped species showed a higher degree of complexity (Figures 6a and b), while the CID spectra of the protonated species were relatively simpler (Figures 6c and d). Despite apparent similarities in the spectra of the sodium doped species, significant differences were observed. Loss of one or two fucose units from the sodiated molecule of **9** gave $Y_{4\alpha}$ and $Y_{3\beta}$ fragments (*m/z* 1041 and 895, Figure 6a). Loss of fucose + galactose was also observed (*m/z* 879). The Y_2 fragment was observed at *m/z* 530. The ion at *m/z* 714 was attributed to a $Y_{4\alpha}/C_4$ event, the ion at *m/z* 696 to either $Y_{4\alpha}/B_4$ or $Z_{4\alpha}/C_4$ cleavages. The ion at *m/z* 714 could lose a galactose (*m/z* 552), a fucose (*m/z* 406), and an N-acetyl-glucosamine (*m/z* 203). The ion at *m/z* 696 lost a fucose (*m/z* 550) and a galactose (*m/z* 388). The reverse combination (*m/z* 534 and 388) was also observed.

The important difference between the CID spectrum of sodiated **9** (Figure 6a) and sodiated **10** (Figure 6b) was the presence of the fragment ion at *m/z* 676 ($Y_{2\alpha}$) for

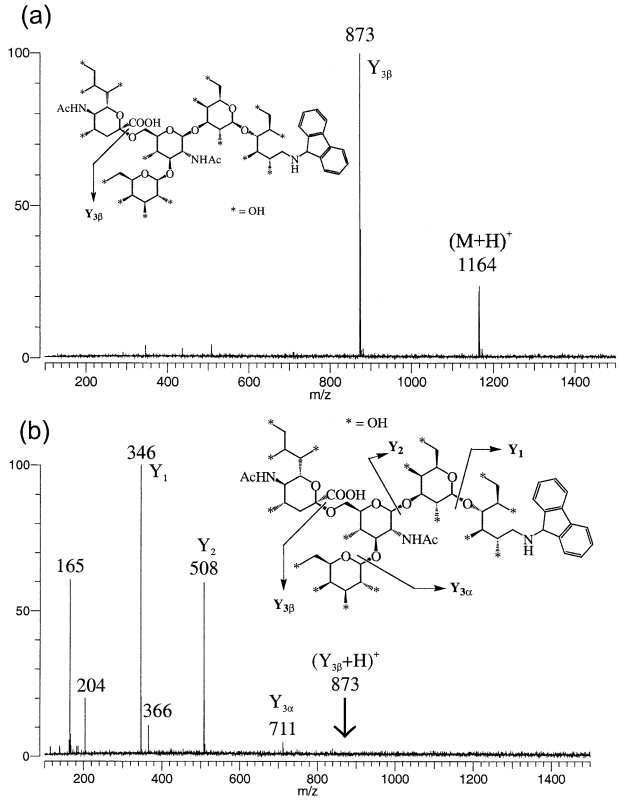


Figure 5. (**a**) MALDI-FT-ICR mass spectrum of **8** ($[M + H]^+$). (**b**) CID spectrum of **8** (arrow indicates where *m/z* 873, $[M + H - NeuNAc]^+$ used to be prior to CID).

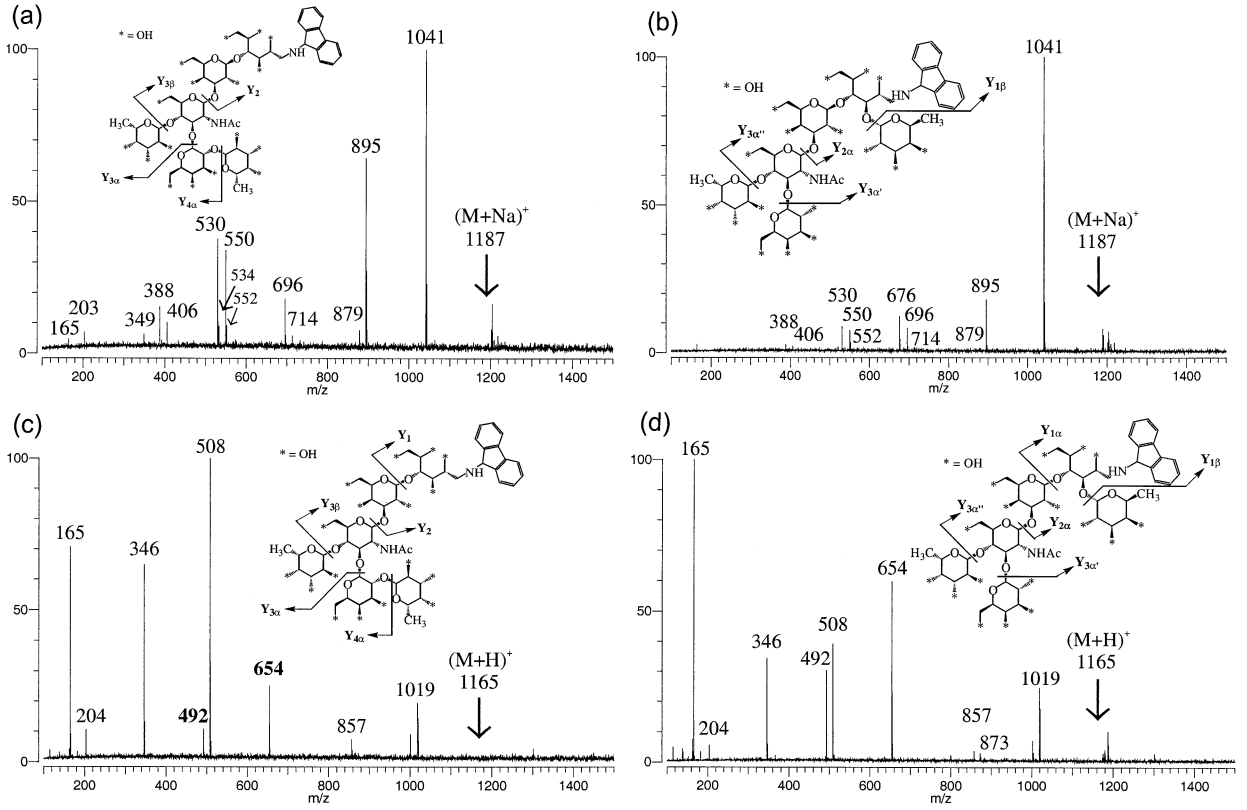


Figure 6. (**a**) CID spectrum of **9** (*m/z* 1187, $[M + Na]^+$). (**b**) CID spectrum of **10** (*m/z* 1187, $[M + Na]^+$). (**c**) CID spectrum of **9** (*m/z* 1165, $[M + H]^+$). (**d**) CID spectrum of **10** (*m/z* 1165, $[M + H]^+$). Note: The arrows in all spectra indicate where the molecular ion used to be prior to CID.

the latter. The absence of this fragment in Figure 6a indicated that the starting material (LNDFH 1) was free of LNDFH 2.

Figure 6c shows the CID spectrum of protonated Compound **9**. The protonated molecule at *m/z* 1165 lost one fucose (*m/z* 1019, $Y_{4\alpha}$) and one galactose (*m/z* 857, $Y_{3\alpha}$). The peak at *m/z* 1001 (not labeled) was due to electronic noise. The fragment ion at *m/z* 857 lost an N-acetyl-glucosamine, a fucose (*m/z* 508, Y_2) and an additional galactose (*m/z* 346, Y_1). The highly resonance-stabilized fluorenyl cation gave rise to the fragment ion at *m/z* 165. Two additional fragment ions (*m/z* 492 and 654) were observed in the CID spectrum. Apparently, the initial loss of L-fucose and D-galactose (*m/z* 857, $Y_{3\alpha}$, vide supra) was followed by a loss of N-acetyl-D-glucosamine (*m/z* 654) with retention of the second L-fucose unit. The second L-fucose was then lost in another event (*m/z* 654 → 508). The fragment ion at *m/z* 654 also lost a galactose to yield *m/z* 492. The possibility of positional isomers with respect to fucose or an internal migration as the source of these unusual fragments is currently being investigated.

For protonated Compound **10**, the fragment ions at *m/z* 654 and 492 were also observed (Figure 6d). However, these fragmentation ions are consistent with the known molecular structure. The protonated molecule (*m/z* 1165) lost a fucose (*m/z* 1019, $Y_{3\alpha'}$), a galactose (*m/z* 857, $Y_{3\alpha'}$) or a second fucose unit (*m/z* 873, $Y_{1\beta}$). Losses of an N-acetyl-glucosamine and a galactose from 857 gave a fragment ion at *m/z* 654 and a fragment ion at *m/z* 492, respectively. Losses of a fucose and a galactose from the ion at *m/z* 654 gave fragment ions at *m/z* 508 and 346, respectively.

With fragmentation data for 9AmFL-labeled oligosaccharides in hand, we turned our attention to the detection levels of the labeled oligosaccharides in HPLC separation. The maltopentaose alditol was prepared by $NaCNBH_3$ reduction and mixed with the 9AmFL-derivatized maltopentaose for HPLC analysis. Detection was performed with PDA at λ = 206 nm and 267 nm. The integration of the chromatographic peaks showed a 100-fold increase in sensitivity for the 9AmFL-derivatized maltopentaose compared to the alditol (λ = 206 nm). Concentration studies showed that the absorption exhibited nonlinear behavior at λ = 206 nm. However, absorption at λ = 267 nm (λ_{max}) exhibited more linear response and 45 pmol of Compound **9** were detected without a problem. However, we were able to detect as low as 5 pmol. A calibration curve was constructed based on the peak area. The slope of the calibration was determined by standard linear regression of the average measurements of three consecutive chromatographic runs (r^2 = 0.99). The error associated with the amount of sugar derivative calculated from the calibration curve was ±5 pmol. Labeling of 10 pmol of LNDFH 1 starting material was performed to investigate the suitability of

the label for very small amounts of oligosaccharide. The labeled product could be detected at *m/z* 1187 with a S/N of 2:1. A control experiment was performed in which a larger amount of LNDFH 1 (10 μmol) was derivatized and 10 pmol of this product was analyzed by mass spectrometry. The resulting S/N was 10:1 implying that unwanted side reactions during labeling play a significant role with small amounts of starting material (10 pmol or less). However, it should be emphasized that oligosaccharide pools from natural sources normally contain several components with total sample amounts well above 10 pmol.

Conclusion

A new labeling reagent (9-aminofluorene) was investigated. The label could be efficiently introduced into neutral and anionic oligosaccharides of biological interest. The reaction had high synthetic yields (>95%). UV sensitivity of the derivatized sugar at λ = 206 nm increased by 100-fold in comparison to the alditol. The detection limit of derivatized sugar by the PDA was 5 pmol for 9AmFl-labeled LNDFH 1. Thus, the label promises to be of value in the separation, detection, and quantitation of small amounts of biologically important oligosaccharides.

The CID spectra of the investigated linear oligosaccharides allowed for complete sequence analysis. Oligosaccharide derivatives with the 9AmFL label could be efficiently ionized and investigated as both sodiated and protonated molecules in MALDI-FTMS experiments. We believe that the 9AmFL label will be of general applicability in separation, detection, and quantitation techniques such as HPLC in combination with mass spectrometry.

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References

1. Harvey, D. J. Electrospray Mass Spectrometry and Fragmentation of N-linked Carbohydrates Derivatized at the Reducing Terminus. *J. Am. Soc. Mass Spectrom.* **2000,** *11,* 900.
2. Mo, W.; Sakamoto, H.; Nishikawa, A.; Kagi, N.; Langridge, J. I.; Shimonishi, Y.; Takao, T. Structural Chracterization of Chemically Derivatized Oligosaccharides by Nanoflow Electrospray Ionization Mass Spectrometry. *Anal. Chem.* **1999,** *71,* 4100.
3. Zeleny, R.; Altmann, F.; Praznik, W. Structural Characterization of the N-linked Oligosaccharides from Tomato Fruit. *Phytochemistry* **1999,** *51,* 199.
4. Toomre, D. K.; Varki, A. Advances in the Use of Biotinylated Diaminopyridine (BAP) as a Versatile Fluorescent Tag for Oligosaccharides. *Glycobiology* **1994,** *4,* 653.
5. Rothenberg, B. E.; Hayes, B. K.; Toomre, D.; Manzi, A. E.; Varki, A. Biotinylated Diaminopyridine: An Approach to Tagging Oligosaccharides and Exploring their Biology. *Proc. Natl. Acad. Sci. U.S.A.* **1993,** *90,* 11939.
6. Shen, X.; Perreault, H. Characterization of Carbohydrates Using a Combination of Derivatization, High-Performance Liquid Chromatography and Mass Spectrometry. *J. Chromat. A* **1998,** *811,* 47.
7. Broberg, S.; Broberg, A.; Duus, J. Ø. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry of Oligosaccharides Derivatized by Reductive Amination and N,N-Dimethylation. *Rapid Commun. Mass Spectrom.* **2000,** *14,* 1801.
8. Lemoine, J.; Chirat, F.; Domon, B. Structural Analysis of Derivatized Oligosaccharides Using Post-Source Decay Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *J. Mass Spectrom.* **1996,** *31,* 908.
9. Cancilla, M. T.; Wong, A. W.; Voss, L. R.; Lebrilla, C. B. Fragmentation Reactions in the Mass Spectrometry Analysis of Neutral Oligosaccharides. *Anal. Chem.* **1999,** *71,* 3206.
10. Tseng, K.; Hedrick, J. L.; Lebrilla, C. B. Catalog–Library Approach for the Rapid and Sensitive Structural Elucidation of Oligosaccharides. *Anal. Chem.* **1999,** *71,* 3747.
11. Cancilla, M. T.; Penn, S. G.; Lebrilla, C. B. Alkaline Degradation of Oligosaccharides Coupled with Matrix-Assisted Laser Desorption/Ionization Fourier Transform Mass Spectrometry: A method for Sequencing Oligosaccharides. *Anal. Chem.* **1998,** *70,* 663.
12. Tseng, K.; Lindsay, L. L.; Penn, S. G.; Hedrick, J. L.; Lebrilla, C. B. Characterization of Neutral Oligosaccharide–Alditols from *Xenopus laevis* Egg Jelly Coat by Matrix-Assisted Laser Desorption Fourier Transform Mass Spectrometry. *Anal. Biochem.* **1997,** *250,* 18.
13. Packer, N. H.; Lawson, M. A.; Jardine, D. R.; Redmond, J. W. A General Approach to Desalting Oligosaccharides Released from Glycoproteins. *Glycoconj. J.* **1998,** *15,* 737.
14. Domon, B.; Costello, C. E. A Systematic Nomenclature for Carbohydrate Fragmentations in FAB-MS/MS Spectra of Glycoconjugates. *Glycoconj. J.* **1998,** *5,* 397.
15. Papac, D. I.; Wong, A.; Jones, A. J. S. Analysis of Acidic Oligosaccharides and Glycopeptides by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Anal. Chem.* **1996,** *68,* 3215.