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Structural Analysis of Chromophore-labeled Disaccharides and Oligosaccharides by Electrospray Ionization Mass Spectrometry and High-performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry

D. T. Li and G. R. Her*
Department of Chemistry, National Taiwan University, Taipei, Taiwan

Disaccharides and linear oligosaccharides were labeled with *p*-aminobenzoic ethyl ester (ABEE) chromophore and analyzed by negative ion electrospray ionization mass spectrometry (ESIMS). The formation of glycosylamines rather than reductive amination in the labeling reaction produced many characteristic fragment ions under in-source collision-induced dissociation (CID). These ions provided unambiguous assignment of the position of the glycosidic linkages. This approach was extended to the analysis of linkages and the sequence of the linkages of several linear oligosaccharides. Additionally, the anomeric configuration of ABEE-labeled 1–3-, 1–4- and 1–6-linked glucose disaccharides could be differentiated according to the relative abundance of characteristic ions. Disaccharides with the same linkage but different monosaccharide compositions could be analyzed by on-line coupling of high-performance liquid chromatography with ESIMS. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: disaccharides; chromophore; linkage; anomeric configuration; high-performance liquid chromatography/electrospray ionization mass spectrometry

INTRODUCTION

A considerable part of the biological functions of glycoproteins and glycolipids depends on their carbohydrate moiety; for example, cell-to-cell interactions, differentiation of cells and metastasis are associated with the structure of oligosaccharides present on the cell surface. The structural elucidation of complex carbohydrates requires the determination of the sugar sequence, sugar branching, monosaccharide composition, interglycosidic linkages and anomeric configuration. The most common method in linkage analysis is the permethylation procedure, where the saccharide is methylated at the free hydroxyl groups, total hydrolysis to monosaccharides is brought about, the carbonyl groups are reduced and finally the hydroxyl groups liberated are derivatized. The mixture obtained is usually analyzed by gas chromatography/mass spectrometry (GC/MS).[1] This well standardized technique is, in general, time-consuming, labor extensive and requires larger sample amounts.

Mass spectrometric techniques, especially the combination of soft ionization methods such as fast atom bombardment (FAB) with collision-induced dissociation (CID), have been used for linkage determination of underivatized oligosaccharides.[2–9] Electrospray ionization mass spectrometry (ESI-MS) is another technique which has been reported to allow structural analysis of underivatized oligosaccharide. Garozzo *et al.*[10] used negative-ion ESI-MS and in-source fragmentation to differentiate the linkage position of reducing disaccharides. Traeger and co-workers[11] extended the study of Garozzo *et al.* to anomeric isomer analysis using selected diagnostic peaks and their relative intensities. Fura and Leary[12] used ESI-MS/MS to investigate the branching of Ca^{2+}- and Mg^{2+}-coordinated branched trisaccharides. From the CID spectrum of the intact cationized species, the branching point located on the reducing moiety of an oligosaccharide could be determined.

High-performance liquid chromatography (HPLC) has been a fundamental technique for the separation and recovery for oligosaccharides.[13] It has been recognized that MS may be used as a universal and sensitive detector for HPLC. On-line HPLC/MS and HPLC/MS/MS have been implemented and shown great potential for the analysis of underivatized oligosac-

* *Correspondence to*: G. R. Her, Department of Chemistry, National Taiwan University, Taipei, Taiwan
e-mail: grher@mail.ch.ntu.edu.tw
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charides, especially using ESI.[14,15] Although MS may be powerful for oligosaccharide analysis following HPLC separation, it is common to find separation conditions with a UV detector prior to MS detection. However, the UV sensitivity for most sugars is generally poor because of the lack of a chromophore in oligosaccharide samples. To solve this problem, precolumn derivatization at the reducing end with UV-absorbing or fluorescent chromophores has received considerable attention.[16–21]

Reductive amination is the most popular labeling procedure. This procedure reduces the reducing sugar and an open-ring derivative is produced. In addition to the very popular reductive amination procedure, the formation of glycosylamine in labeling reactions has demonstrated its great potential in HPLC separations and in linkage analysis.[22] Unlike in reductive amination, reducing sugars maintained a closed ring structure in the glycosylamine approach. Based on our previous study,[22] closed-ring derivatives (glycosylamines) provided much more linkage information than open-ring products under FAB/CID conditions. In this study, disaccharides and linear oligosaccharides were derivatized using the glycosylamine approach and were studied by ESI-MS and HPLC/ESI-MS. The merits of this approach are discussed.

EXPERIMENTAL

Chemicals

Table 1 lists the sugars analyzed in this study. Panose, sophorose and lactose were purchased from Tokyo Kasei (Tokyo Japan), Serva (Heidelberg, Germany) and Aldrich Chemical (Milwaukee, WI, USA), respectively. All other sugars were obtained from Sigma Chemical (St Louis, MO, USA). *p*-Aminobenzoic acid ethyl ester was purchased from Aldrich Chemical.

Preparation of chromophore-labeled oligosaccharides

Closed-ring (glycosylamine) derivatives were prepared. The procedure of Reinhold and co-workers[16] was adopted for the preparation of ABEE derivatives. For ABEE glycosylamines, the sugars (up to 1 mg) were dissolved in 20 μl of water and 80 μl of reagent solution (prepared by dissolving 1 g of ABEE in 0.5 ml of methanol at 60 °C) and reacted at 80 °C for 6 h. The solution was washed with 2 ml of ethyl ester to remove the excess ABEE reagent before the HPLC separation.

Table 1. List of sugars analyzed

Sophorose	Glcβ1–2Glc
Nigerose	Glcα1–3Glc
Laminaribiose	Glcβ1–3Glc
	Manα1–3Man
Maltose	Glcα1–4Glc
Cellobiose	Glcβ1–4Glc
	Galα1–4Gal
	Galβ1–4Man
Isomaltose	Glcα1–6Glc
Gentiobiose	Glcβ1–6Glc
Melibiose	Galα1–6Glc
Maltotriose	Glcαα–4Glcα1–4Glc
Isomaltotriose	Glcα1–6Glcα1–6Glc
Panose	Glcα1–6Glcα1–4Glc
Maltopentaose	Glcα1–$(4Glc\alpha1)_3$–4Glc

Instrumentation

HPLC separation was performed with a system consisting of two Microtech pumps (Microtech Scientific, Sunnyvale, CA, USA), a Rheodyne (Cotati, CA, USA) model 7725 injector with a 5 μl sample loop, and a Linear (Reno, NV, USA) UVIS 200 variable-wavelength detector. A Spherisorb 3-NH_2 column (25 cm × 2 mm i.d.) was used for the separation of the chromophore-labeled disaccharide mixture. The flow rate on-column was 300 μl min^{-1} and this flow was split post-column. A splitting ratio of 1:1 delivered 150 μl min^{-1} into the electrospray ionization source. Before the mobile phase flowed into the ionization source, 2-propanol–water (1:1) containing 0.5% ammonia was added at a flow rate of 20 μl min^{-1} to increase the negative ion ESI sensitivity. Negative ion ESI mass spectra were collected on a Fisons Platform mass spectrometer (VG Bio Tech, Altrincham, UK). The potential of the capillary was set to 2.5 kV and the counter electrode was held at 0 V. The ion source temperature was set to 90 °C. In-source CID mass spectra were acquired with the cone voltage set to a specified value. A mass range between *m/z* 150 and 550 was scanned and the scan time was set to 2 s. The absorbance at 254 nm was monitored as the remaining 150 μl min^{-1} eluent flowed through a UV cell. The amounts of sample used in HPLC/MS and CID analysis were ~10 and 1 nmol, respectively. The analytical conditions for the separation are given in the figure captions.

RESULTS AND DISCUSSION

Linkage analysis of di- and linear oligosaccharides

Disaccharides with different linkages were derivatized with ABEE and then studied by ESI-MS. Under in-source CID condition, many fragment ions were observed. The most prominent fragments in the positive-ion mode mass spectra resulted from the cleavage of the glycosidic bond, which made the mass spectra very similar and therefore not useful for the differentiation of linkages (data not shown). Unlike positive-ion spectra, the negative-ion mode yielded many characteristic fragment ions (Fig. 1). These fragments appeared to be cross-ring cleavage ions on reducing end monosaccharides. Disaccharide isomers can be seen as a monosaccharide with another monosaccharide as the substituent at different positions, and therefore cross-ring cleavage of the reducing monosaccharide is very helpful for the differentiation of these isomers (Fig. 1). The characteristic fragment ions are listed in Table 2. Since the only difference among the four disaccharides resided in the glycosidic linkage, the difference observed

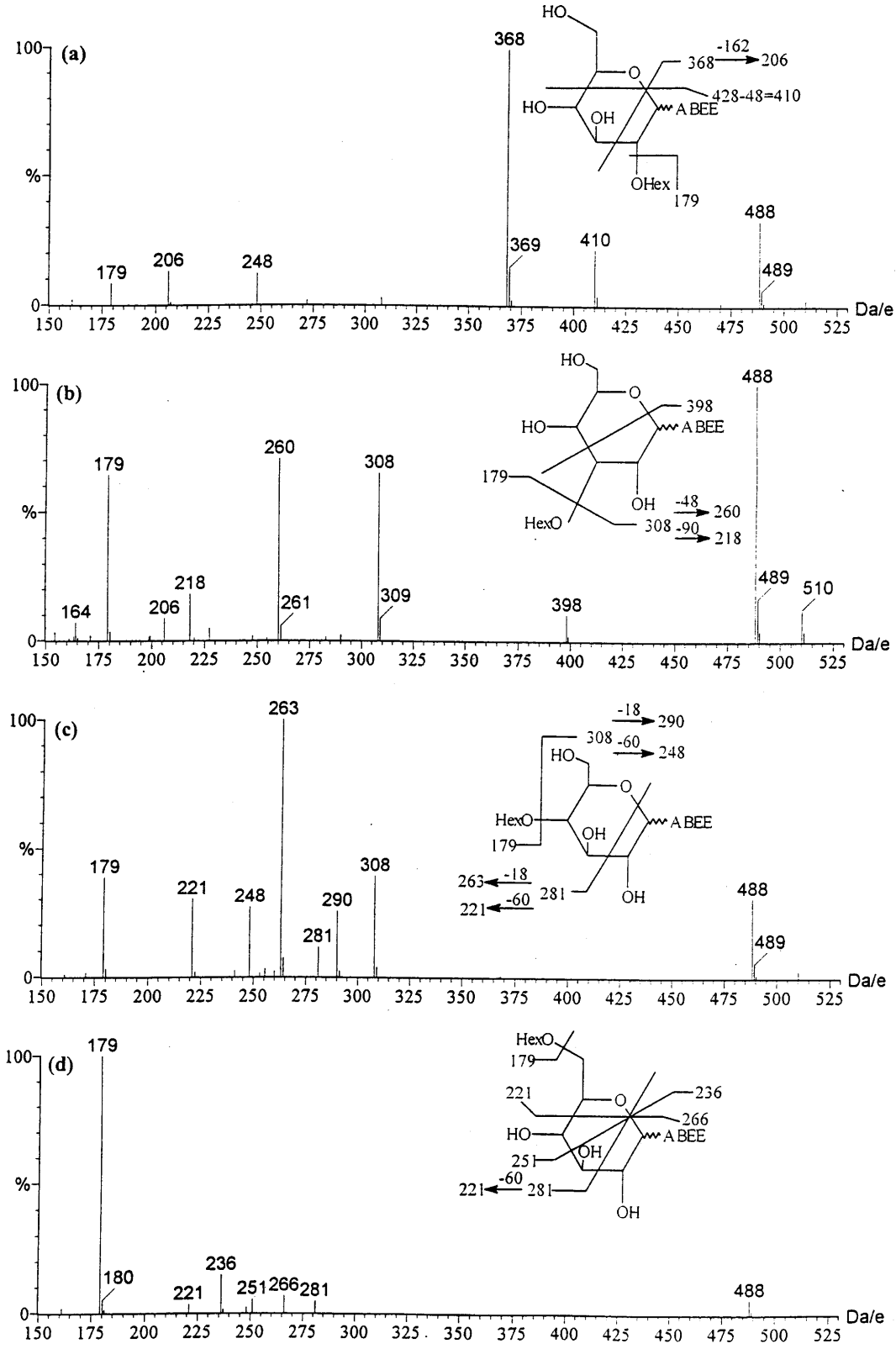

Figure 1. Negative-ion in-source CID ESI mass spectra of ABEE-labeled (a) sophorose, (b) laminaribiose, (c) cellobiose and (d) gentiobiose.

in the negative-ion ESI mass spectra could be used to establish the linkage position in disaccharides. The FAB technique in combination with high-energy CID has been reported for the linkage determination of ABBE labeled disaccharides.[22] Although the difference in collision energies was significant, the ESI mass spectra recorded at 30 V were in general similar to the FAB/high-energy CID product ion spectra. A notable difference was the m/z 281 ion in β-1–4- and β-1–6-linked disaccharides. Except at a lower cone voltage, the

Table 2. Specific fragment ions for ABEE-labeled disaccharides (*m/z*)

Glcβ1–2Glc								368		410
Glcβ1–3Glc	218			260					398	
Glcβ1–4Glc					263		290			
Glcβ1–6Glc		236	251			266				

prominent ion at *m/z* 281 observed in FAB/CID was detected as a minor ion in 1–6- and 1–4-linked disaccharides (Fig. 1(c) and (d)). Fortunately, the *m/z* 281 ion was not critical for the determination of linkages.

UV or fluorescence sensitivity has been the most important factor in the selection of labeling reagents. In recent years, attempts have been made not only to achieve sensitive UV or fluorescence detection but also improve the ionization efficiency in ESI analysis by labeling oligosaccharides with chromophores of high proton affinity or preformed ions.[23–26] The reportedly successful derivative obtained by labeling with 4-aminobenzoic acid 2-(diethylamino) ether ester[23] was not chosen in this study mainly because it was designed for positive ionization and the labeling product was not a closed-ring but an open-ring (reductive amination) derivative. Little information on linkages was obtainable from the ESI mass spectra.

Several derivatives including PA (2-pyridinamine), ABEE and ABBE (*p*-aminobenzoic acid butyl ester) were labeled with glycosylamine and studied by negative-ion ESI. ABEE and ABBE had similar sensitivities which were ~5–10 times more sensitive than

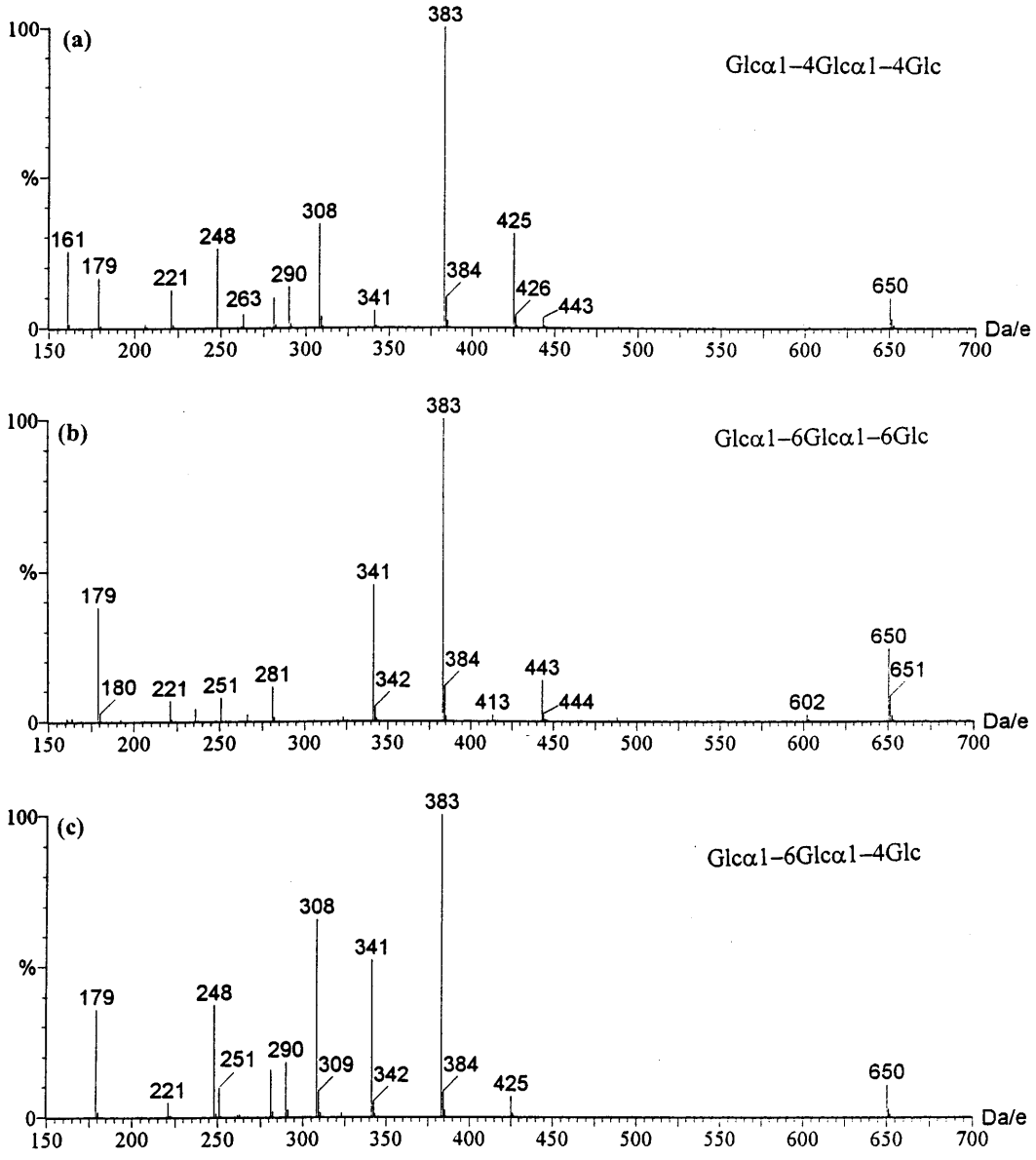

Figure 2. Negative-ion in-source CID ESI mass spectra of ABEE-labeled (a) maltotriose, (b) isomaltotriose and (c) panose.

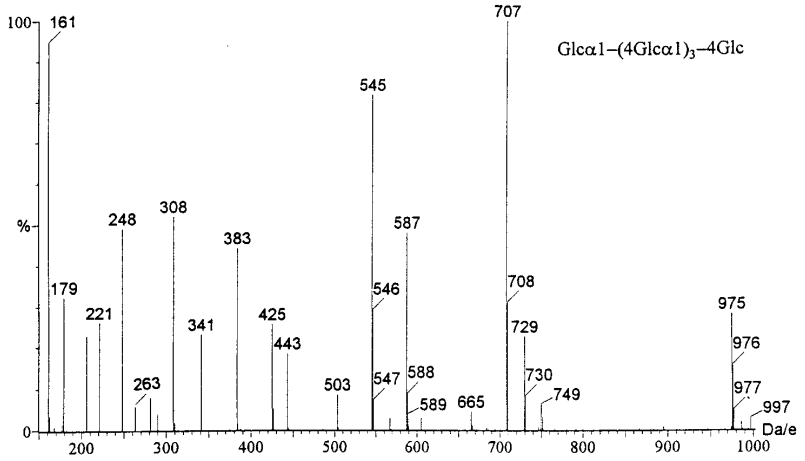

Figure 3. Negative-ion in-source CID ESI mass spectra of ABEE-labeled maltopentaose.

underivatized disaccharides. The resolution of PA sugar was not as good as that of ABEE or ABBE. ABEE was chosen instead of ABBE in this study mainly because it provided better results on anomeric configuration for some disaccharides.

The linkage of disaccharides without precolumn derivatization could also be determined from the ESI mass spectrum.[10,11] However, because of the lack of reducing end fragments, it is often not possible to analyze mixtures with more than one linkage by direct infusion analysis. For example, the characteristic ions for 1–2-, 1–3- and 1–4-linked disaccharides are at *m/z* 221, 263, 323; 221, 281; and 221, 263, 281, respectively;[11] the observation of *m/z* 221, 263, 281 and 323 ions might represent one of the following three possible combinations: 1–2 + 1–4, 1–2 + 1–3 and 1–2 + 1–3 + 1–4. In contrast, every linkage-isomeric disaccharide had its specific ions (Table 2) in this approach, and therefore linkage positions in a mixture containing more than one linkage could be unambiguously assigned by direct infusion analysis.

The study of several 1–4- and 1–6-linked linear oligosaccharides suggested that with the cone voltage set at a higher value, this approach could be extended to the analysis of linear oligosaccharides. The in-source CID mass spectra of trisaccharides with the cone voltage set to 40 V are shown in Fig. 2. The *m/z* 263 ion in Fig. 2(a) indicates that the non-reducing end is a 1–4-linkage; the ion at *m/z* 425 (162 u above the *m/z* 263 ion) and reducing end fragments at *m/z* 308 and 290 and suggest that the reducing end is also a 1–4-linkage. In Fig. 2(b), the presence of the *m/z* 251 ion indicates that the non-reducing end is a 1–6-linkage; the peak at *m/z* 413 (162 amu above the peak at *m/z* 251) and non-reducing end fragment at *m/z* 236 suggest that the reducing end linkage is also a 1–6-linkage. In Fig. 2(c), the presence of

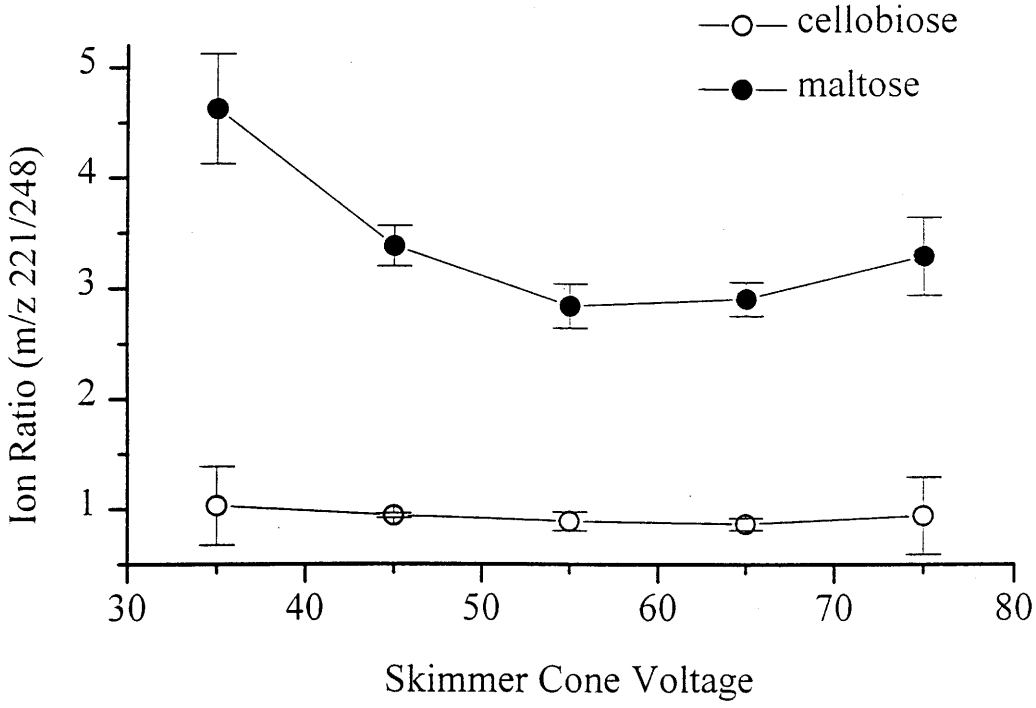

Figure 4. Skimmer cone voltage *vs.* ion ratio of *m/z* 221/248 in ABEE-labeled Glcα1–4Glc and Glcβ1–4Glc. Error bars represent three standard deviations (3σ).

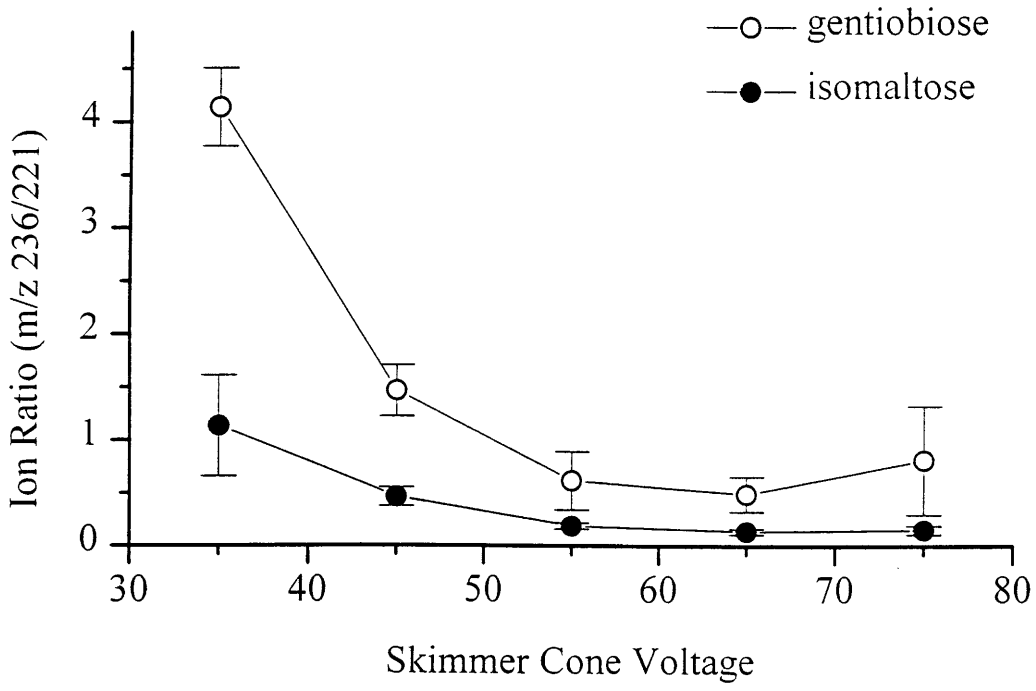

Figure 5. Skimmer cone voltage *vs.* ion ratio of *m/z* 236/221 in ABEE-labeled Glcα1–6Glc and Glcβ1–6Glc. Error bars represent three standard deviations (3σ).

the ion at *m/z* 251 suggests a 1–6-linkage in the non-reducing end and the peaks at *m/z* 425, 308 and 290 indicate that the reducing end is a 1–4-linkage. This method was applicable to higher oligomers. The negative-ion ESI mass spectrum of ABEE-labeled maltopentaose with the cone voltage set to 75 V is shown in Fig. 3. The observation of 1–4-linked diagnostic ions at *m/z* 263, 425, 587 and 749 and reducing end fragments at *m/z* 308 and 290 suggested that the five linkages in maltopentaose were all 1–4-linkages.

Analysis of anomeric configuration

The determination of anomeric configuration by a combination of chemical and MS methods has been reported by Khoo and Dell.[27] The determination of anomeric configuration by MS directly is limited, however, mainly because stereoisomers often give very similar mass spectra. Recently, Traeger and co-workers[11] differentiated anomeric configurations by the relative abun-

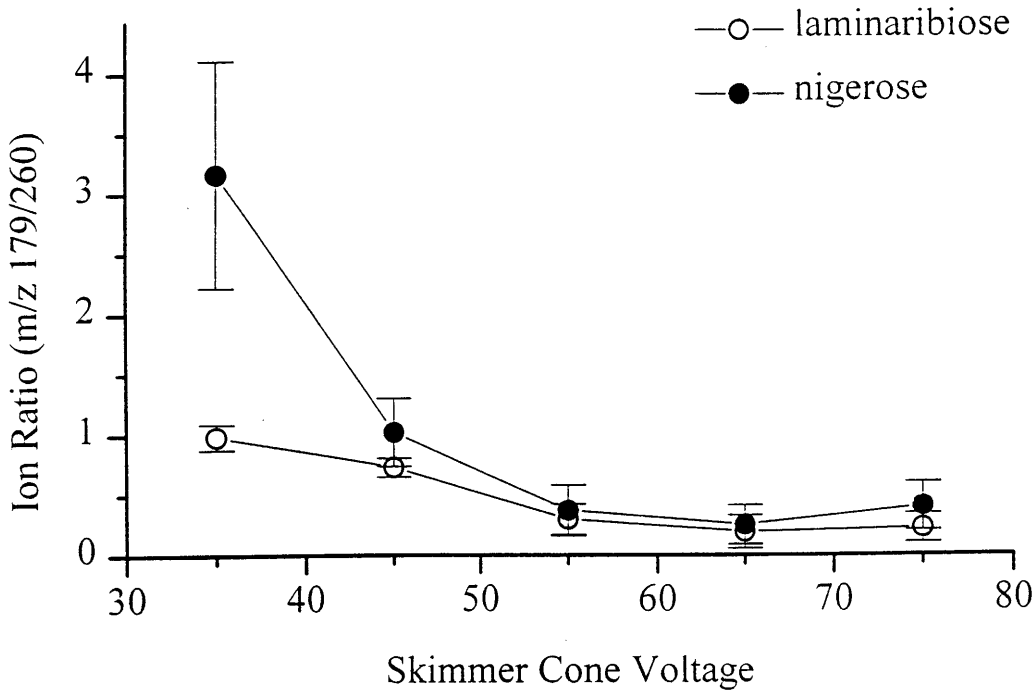

Figure 6. Skimmer cone voltage *vs.* ion ratio of *m/z* 179/260 in ABEE-labeled Glcα1–3Glc and Glcβ1–3Glc. Error bars represent three standard deviations (3σ).

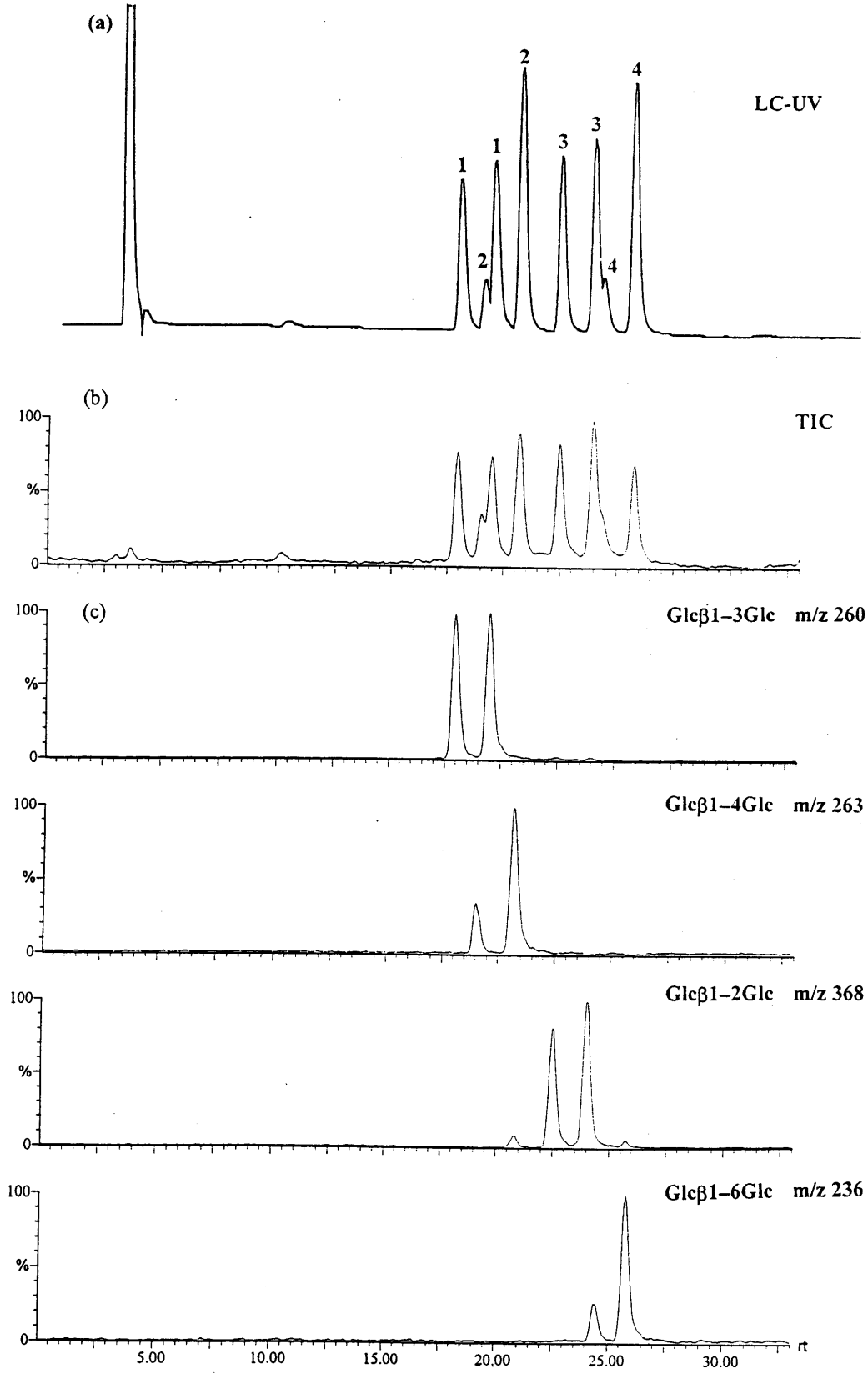

Figure 7. (a) UV, (b) TIC and (c) reconstructed ion chromatograms of four ABEE-labeled disaccharides on an amino column with 5–15% water–acetonitrile as eluent at 300 μl min^{-1} with a 60 min program.

dance of selected ions in a negative-ion ESI mass spectrum.

When we examined the relative abundance of fragment ions in the ESI mass spectra of ABEE-labeled disaccharides, it appeared that the relative abundances of some fragment ions were sensitive to the anomeric configurations. Figure 4 shows the profiles of skimmer cone voltage *vs.* ion ratio of *m/z* 221/248 for Glc*β*1–

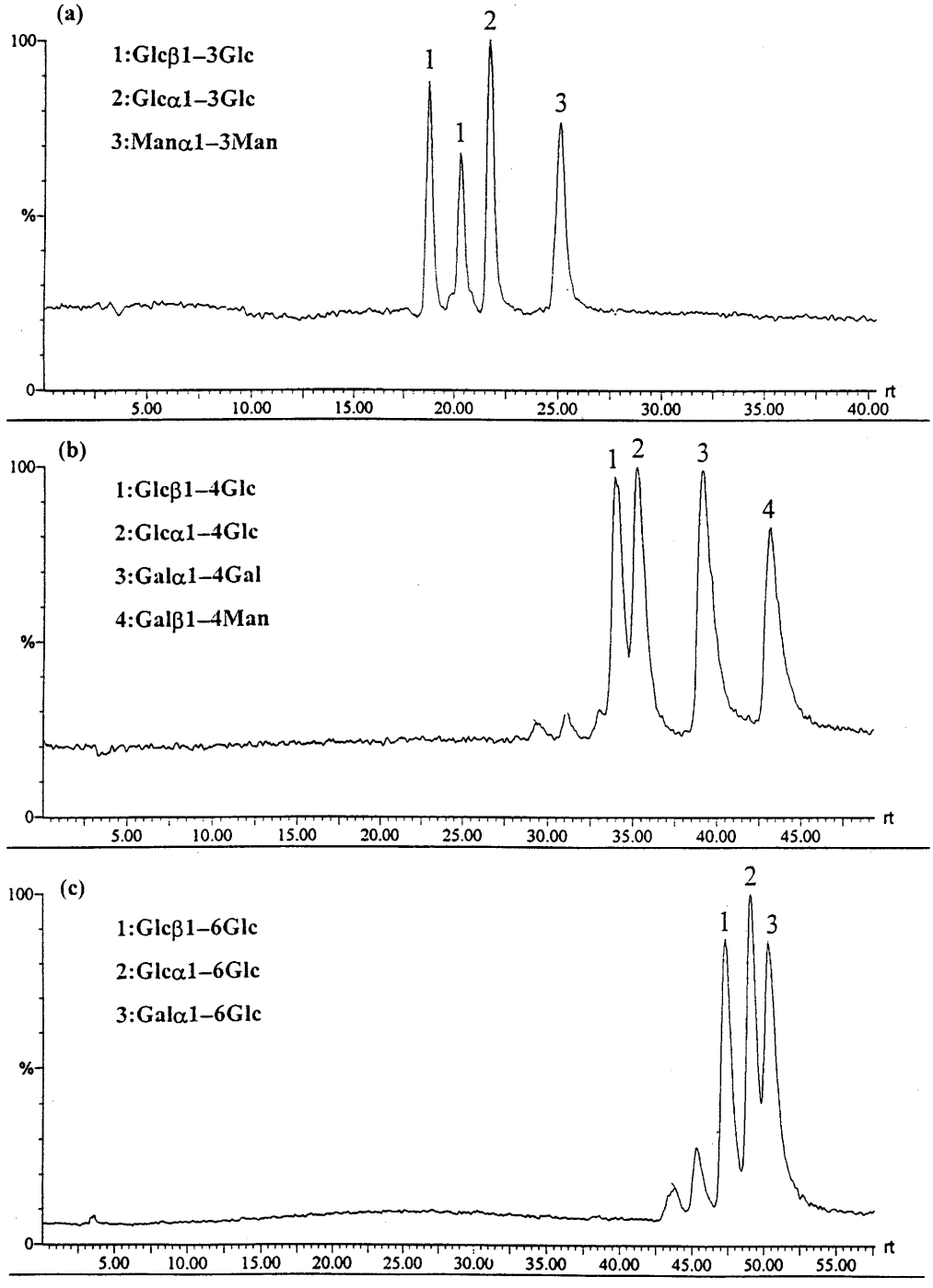

Figure 8. TIC chromatograms of the ABEE-labeled (a) Glcα1–3Glc, Glcβ1–3Glc and Manα1–3Man, (b) Glcα1–4Glc, Galα1–4Gal, Glcβ1–4Glc and Galβ1–4Man and (c) Glcα1–6Glc, Glcβ1–6Glc and Galα1–6Glc mixtures. The HPLC conditions for (a) were the same as in Fig. 7 and for (b) and (c) the eluent was 5–15% water–acetonitrile at 300 µl min^{-1} with a 120 min program.

4Glc and Glcα1–4Glc. Variations in fragment ion abundance were observed; the variation within a week is represented by the 3σ error bars. As can be seen, the anomeric configuration of Glcβ1–4Glc and Glcα1–4Glc could be unambiguously assigned with the skimmer cone voltage set from 35 up to 75 V. For Glcβ1–6Glc and Glcα1–6Glc (Fig. 5), although the anomeric configuration might be determined at both high and low cone voltages, the difference was more significant at lower voltages. The 3σ bars overlap at high voltage for Glcβ1–3Glc and Glcα1–3Glc (Fig. 6), and the anomeric configuration was distinguishable only at low voltage. Owing to the lack of standards, 1–2-linked disaccharides were not studied.

In comparison with the FAB/CID technique,[22] the differences in relative abundance between α- and β-anomers of 1–3- and 1–6-linked disaccharides were much clearer in negative-ion ESIMS.

LC/MS of ABEE-labeled disaccharides

In this study, glycosylamine rather than the popular reductive amination was adopted for labeling the

reducing end of di- and oligosaccharides with an ABEE UV tag. In addition to the fact that the negative-ion in-source CID mass spectrum of a closed-ring (glycosylamine) derivative was more characteristic than that of an open-ring (reductive amination) derivative, closed-ring (glycosylamine) derivatives were reported to show better chromatographic behavior than open-ring derivatives on an amine HPLC column.[16,22]

The separation of a mixture of four ABEE-labeled glucose disaccharides is shown in Fig. 7. Peaks 1, 2, 3 and 4 correspond to laminaribiose, cellobiose, sophorose and gentiobiose, respectively. Disaccharide derivatized with the glycosylamine approach resulted in two anomers,[16] thus a total of eight peaks were observed. Figure 7(a) and (b) are the UV and total ion current chromatograms. In Fig. 7(c), ions at m/z 368, 260, 263 and 236, which represent 1–2-, 1–3-, 1–4- and 1–6-linked disaccharides, respectively, were selected for the reconstructed ion chromatogram. The linkages of these disaccharides can be unambiguously assigned.

Complete structure information on disaccharide includes linkage, anomeric configuration and monosaccharide composition. As discussed above, a direct MS analysis could be used to assign linkages unambiguously and also to provide the anomeric configuration for 1–3-, 1–4- and 1–6-linked glucose disaccharides. Despite the fact that differences in ion abundance with high reproducibility were observed for disaccharides with the same linkage, the same anomeric configuration but different monosaccharide compositions, clear conclusions on monosaccharide composition could not be drawn because of the lack of disaccharide standards with all possible isomeric combinations (linkage, anomeric configuration, monosaccharide composition). On-line coupling of HPLC with ESI-MS might provide a solution to this problem. HPLC was used to separate disaccharide isomers followed by MS detection. Disaccharides with the same linkage but different anomeric configurations and monosaccharide compositions could be resolved on an amine HPLC column. The total ion currents of 1–3-, 1–4- and 1–6-linked ABEE-labeled disaccharides are shown in Fig. 8(a), (b) and (c), respectively. The retention times and resolving power achievable with HPLC are helpful in the analysis of disaccharides. Disaccharide isomers can be identified by the combination of retention times and in-source CID mass spectra.

CONCLUSION

Closed-ring (glycosylamine) derivatization of reducing disaccharides and linear oligosaccharides was analyzed by negative-ion ESI-MS. In comparison with unlabeled disaccharides, a much clearer assignment can be made for the linkages of di- and linear oligosaccharide. The anomeric configuration of 1–3-, 1–4- and 1–6-linked glucose disaccharides can be assigned based on the relative abundances of specific fragment ions. Although monosaccharide composition did play a role in the fragment ion abundance, no clear conclusion could be drawn because of the lack of standard samples. HPLC/ESI-MS could be used for the differentiation of disaccharides with the same linkage but different monosaccharide compositions.

Acknowledgement

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