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Influence of borate complexation on the electrophoretic behavior of 2-AA derivatized saccharides in capillary electrophoresis

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ABSTRACT

A complete separation with baseline resolution of the 2-AA derivatized saccharides, including mono-, di-, and oligosaccharides, was achieved using 50 mM sodium phosphate–150 mM borate solution, pH 7.0 as running buffer by capillary electrophoresis. It was thought to be a result of the inclusion of 150 mM borate in the running electrolyte solution. The formation of borate complexes was observed by means of ^{11}B and ^{13}C NMR spectroscopy and the electrophoretic mobilities of the various derivatives were calculated. It was found that steric factors play an important role in the stability of the formed borate complexes, which depends strongly on the configuration of the three vicinal hydroxyl groups at C-2, C-3, and C-4. 2-AA-Glc mainly forms stable 1,2-diester complexes with borate and 2-AA-Mal can form stable 1,2-monoesters. In turn, for 2-AA-Rib the formation of complexes is difficult to take place. The results implied that the configurational difference between the hydroxyl groups could cause the difference in formation of borate complexes leading to significant difference among saccharide molecules in their migration time on CE analysis.

Many complex carbohydrates, which exist naturally as glycoconjugates, are known to have important biological functions.[1–3] Increasing evidence has shown that carbohydrate moieties of glycoproteins are often important as recognition motifs in receptor–ligand or cell–cell interactions, in the modulation of protein folding and in the regulation of protein bioactivity. Any changes in the biological activity of a glycoprotein are often associated with alterations in its glycosylation either through site variations or changes in the structure of the oligosaccharide occupying a particular site. The elucidation of the structures of these carbohydrate moieties has therefore become increasingly important. Thus we have previously described a rapid, effective, and highly sensitive analytical method[4,5] using CE for the determination of 2-AA derivatized mono- and oligosaccharides. The underlying reasons for separation of 2-AA-Man and 2-AA-Glc or 2-AA-Rib and 2-AA-Xyl with the same molecular weight were deduced.[6] The inherent configurational difference between the hydroxyl group, could cause significant difference in Stokes' radii between their molecules and thus could lead to different electrophoretic mobilities.

However, our further research demonstrated that this difference in their electrophoretic mobilities was limited. Insufficient selectivity was achieved in separating major monosaccharides found in the glycoprotein, if only sodium phosphate buffer was used as running electrolyte solution. The 2-AA-aldohexoses were taken as an example. When 2-AA-Gal was added, it peaked between 2-AA-Man and 2-AA-Glc. The three 2-AA-aldohexoses co-migrated (figure not shown). It implied that the selectivity for separating the stereoisomeric aldohexoses was insufficient under such circumstance. The difference in their migration time was small. Consequently their peaks overlapped so that baseline separation could not be reached.

Borate buffers could alter selectivity of polyol-containing substances in CE, which are thus commonly used as background electrolytes for the electrophoretic separation of saccharides.[7–11] We further investigated the electrophoretic behavior of saccharide derivatives in borate buffers. Electropherogram of a standard mixture of 2-AA derivatives of mono-, di-, and some oligosaccharides by CE, using 150 mM borate–50 mM sodium phosphate at pH 7.0 as a running electrolyte solution is shown in Figure 1. Indeed these derivatives, which could not achieve complete separation in phosphate buffer, could be baseline separated in this borate-containing buffer within 12 min. It was clearly demonstrated that resolution and efficiency of the electrophoretic separation for these saccharide derivatives were dramatically improved, when their borate complexes formed.

Abbreviations: 2-AA, 2-aminobenzoic acid; CE, capillary electrophoresis; NMR, nuclear magnetic resonance.

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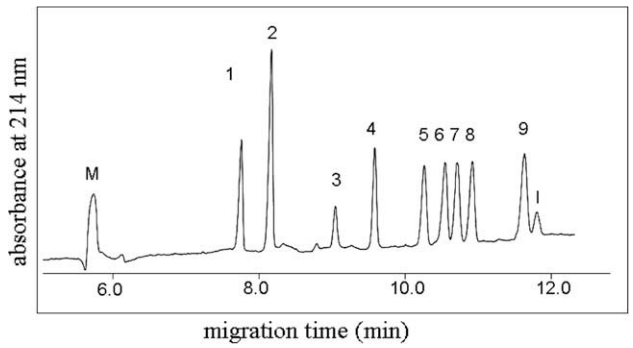

Figure 1. Electropherogram of standard mixture of saccharide derivatives in 50 mM sodium phosphate–150 mM borate running electrolyte solution at pH 7.0. Conditions: capillary, fused silica capillary 50 μm i.d. × 60 cm.; injection, 5 s at 100 mbar; temperature, 25 °C; applied potential, 20 kV; detection, λ = 214 nm. Concentrations: cellopentaose, 75 μM; cellotetraose, 150 μM; others, each 45 μM. Peaks assignment: (1) 2-AA-Cellopentaose; (2) 2-AA-Cellotetraose; (3) 2-AA-GlcN; (4) 2-AA-Rib; (5) 2-AA-Fuc; (6) 2-AA-Man; (7) 2-AA-Xyl; (8) 2-AA-Gal; (9) 2-AA-Glc; M refers to neutral marker (benzyl alcohol); I refers to impurity from reaction solution.

To make a comparison of the electrophoretic performance achieved between the two separation systems in 50 mM phosphate running buffer (pH 7.0) and 50 mM phosphate–150 mM borate running buffer (pH 7.0), resolution R between critical pairs of 2-AA-aldohexoses (Man, Gal, and Glc) or between 2-AA aldopentoses (Rib and Xyl) was calculated by the equation as

$$R = 2({}^{t}r2 - {}^{t}r1)/(W1 + W2),$$

where ${}^{t}r2$ and ${}^{t}r1$ are migration time of two critical pairs, $W1$ and $W2$ are width of their peaks in terms of migration time. Values of ${}^{t}r2$, ${}^{t}r1$, $W1$, $W2$ can be obtained from the separation electropherograms in Figure 2 in our previous paper[6] and in Figure 1 in this paper. The final results are listed in Table 2. It has been shown that all values of R between 2-AA-aldohexoses and between 2-AA-aldopentoses in 50 mM phosphate–150 mM borate running buffer are greater than 1.5, which means that they could be completely separated with high resolution. Comparatively, their corresponding values of R in phosphate running buffer are smaller than 1.0, which demonstrates that they could not be well resolved. It can be deduced that separation of these derivatives with high resolution in the borate-containing buffer may be attributed to their complexation with borate. The addition of borate to running electrolyte solution may result in formation of saccharide–borate complexes (Fig. 4), which thereby enhanced differences in their electrophoretic mobility.

The effect of borate on the mobility of a mixture of 2-AA derivatives, including oligo-, tri-, disaccharide, triose, tetrose, pentose, and hexose was further investigated by varying the concentration

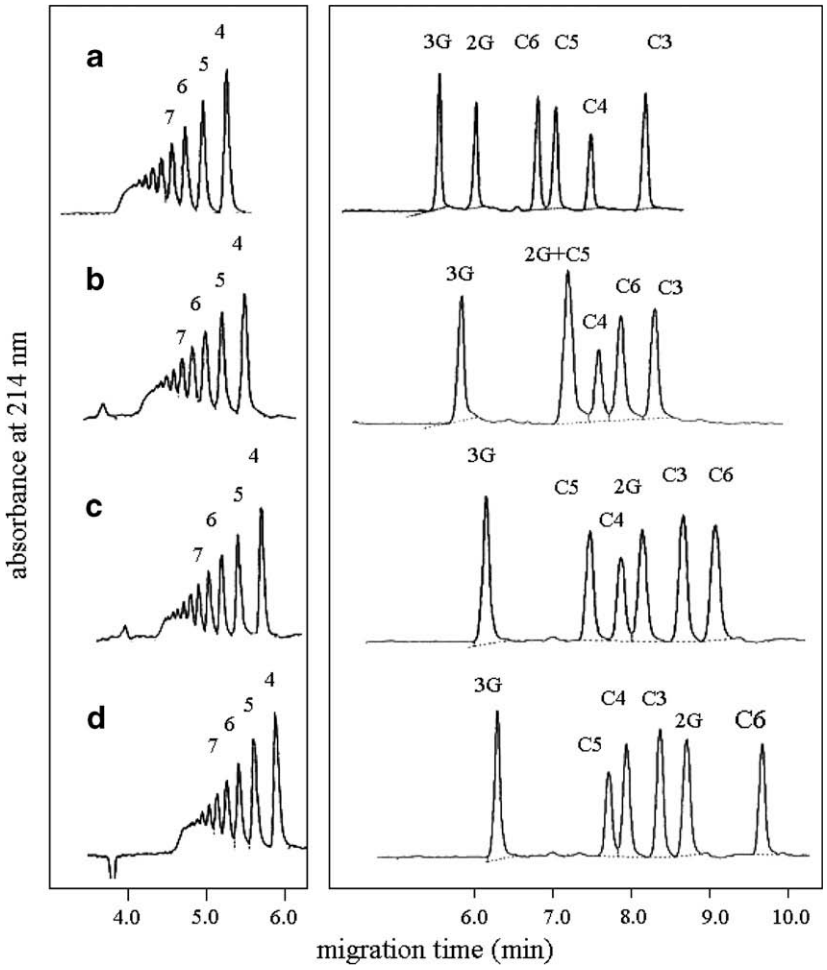

Figure 2. Effect of borate concentration on mobilities of saccharide derivatives. Running buffer: 50 mM sodium phosphate plus 0 mM (a), 50 mM (b), 125 mM (c) and 150 mM (d) of boric acid, respectively, with pH adjusted to 7.0. Conditions: capillary, fused silica capillary 50 μm i.d. × 50 cm; injection, in the hydrostatic mode 10 cm for 15 s; temperature, 25 °C; applied potential, 20 kV; detection, λ = 214 nm. Peaks assignment: left: glucose oligomer ladder (4–10); right (1) 3G refers to 2-AA-Isomaltotriose; (2) 2G refers to 2-AA-Mal; (3) C6 refers to 2-AA-Glc; (4) C5 refers to 2-AA-Rib; (5) C4 refers to 2-AA-Erythrose; (6) C3 refers to 2-AA-Glyceraldehyde.

Table 1
Characteristic values of ^{11}B chemical shifts of borate complexes[12,14–16]

Ester type	^{11}B chemical shift range (ppm)		
	B^- L	B^- L_2	$(B^-)_2L$
1,2-Bidentate[a]	−12.6 ~ −14.9	−7.7 ~ −11.9	−13.0 ~ −18.7
1,3-Bidentate[b]	−17.9 ~ −18.5	−18.4 ~ −19.0	
1,3,5-Tridentate	−18.1 ~ −19.4		

[a] Borate esters formed at vicinal 1,2-diol functions, which is also called α,β-bidentate.
[b] Borate esters formed at vicinal 1,3-diol functions, which is also called α,γ-bidentate.

Table 2
Resolution between some 2-AA-Monosaccharides in two separation system

2-AA-Monosaccharide pairs	R	
	In 50 mM phosphate running buffer (pH 7.0)	In 50 mM phosphate–150 mM borate running buffer (pH 7.0),
2-AA-Man and 2-AA-Gal	<0.36[a]	1.6
2-AA-Gal and 2-AA-Glc	<0.36[a]	3.8
2-AA-Rib and 2-AA-Xyl	0.67	6.3

[a] 0.36 is the resolution between 2-AA-Man and 2-AA-Glc.

of borate added to the running electrolyte. In the absence of any added borate with no complexation taking place, bigger molecules such as tri- and oligosaccharides are among the derivatives detected fast (left of Fig. 2). The bigger the oligosaccharide, the more rapidly it migrated. The elution order of tri- and oligosaccharides did not change in the presence of borate. But these analytes were observed to migrate slower and slower with the concentration of running electrolytes increasing. The increase of running electrolytes increasing. concentration should be accompanied by the decrease of electroosmotic flow velocity, and as a result they migrate slower with the concentration of running electrolytes increasing. Different behaviors can be observed for small saccharides. Interestingly, with borate concentration increasing, elution order of di- and monosaccharide derivatives alternately changed (right of Fig. 2). The migration time and elution order of these derivatives and selectivity in electrophoresis were apparently influenced by the presence of borate. It seems that their separation on CE was not only due to their inherent characteristics but also due to the formation of complexes with tetrahydroxyborate. Especially in the case of 2-AA-Glc and 2-AA-Mal, it is obvious that with borate concentration increased from 0 to 150 mM, 2-AA-Glc and in succession, 2-AA-Mal migrated slower and slower.

Boric acid and borate are known to form esters with hydroxyl compounds in aqueous solution,[12–16] which are illustrated in Figure 3. ^{11}B and ^{13}C NMR spectroscopy has previously been applied to the characterization of such complexes, where ^{11}B chemical shifts range of different types of complexes was given as shown in Table 1.[12,14–16] To determine the binding sites for borate in the saccharides, ^{13}C NMR was often applied. Upon borate ester formation, change in the chemical shift of 3–10 ppm to higher frequency was usually observed for the ^{13}C nuclei in the borate ester ring. Figure 4 demonstrates the ^{11}B NMR spectra of a few selected derivatives from our analytes. The results showed that NMR signal of 2-AA-Mal-Boron or 2-AA-Glc-Boron was strong, but that of 2-AA-Glyceraldehyde-Boron or 2-AA-Rib-Boron was weak. 2-AA-Glc mainly formed stable 1,2-diester with borate and hence exhibits the highest electrophoretic mobility among all investigated derivatives. 2-AA-Mal can form stable 1,2-monoester. In turn, it implies that for 2-AA-Rib and 2-AA-Glyceraldehyde, the formation of com-

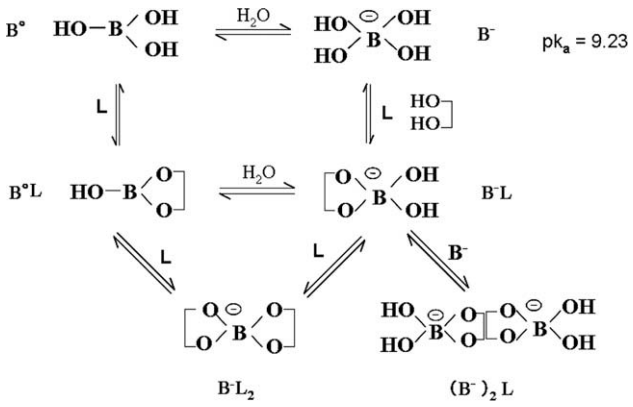

Figure 3. Equilibria between boric acid, borate, and a derivatized saccharide compound in aqueous medium. The indices '−' do not stand for the actual charge of the esters, but refer to the charge of borate anion. L refers to a polyol-containing substance.

plexes is difficult to take place with a weak signal in ^{11}B NMR spectra. It demonstrated that saccharides with different structural characteristics formed different types of esters with borate anion.

In the course of derivatization, the pyranose or furanose ring with the reducing end of saccharides was opened and labeled with 2-AA with saccharides moiety in the open chain form. At the selected pH in phosphate buffer, all of the native derivatives of saccharides are in an identical charge status, that is, carrying one negative charge by their carboxylic anion on the benzoic ring of 2-AA. The partial separation achieved is attributed solely to the small differences in Stokes' radii of the 2-AA-Saccharides. However, due to differential degrees of complexation of the saccharides studied with the borate anion in borate-containing electrolytes, a complete separation of all selected analytes was achieved with increased resolution and efficiency. It is attributed to more pronounced changes in both their overall net charge and ion sizes (Stokes' radii) of the borate complexed saccharides as compared to the uncomplexed form. 1,2-Bidentate diester (three negative charges) carries much more negative charges than 1,2-bidentate monoester (two negative charges) and native derivatives (one negative charge). The more saccharide is complexed, the more negatively they charge and the more slowly they migrate toward the detector. It must be the charge rather than the size, which is responsible for the decrease in mobility. As stated before,[6] the net migration velocity of an analyte υ_s equals to $\upsilon_{eo} + \upsilon_{ep} = (\mu_{eo} + -\mu_{ep})E$. The electrophoretic mobility μ_{ep} of a particle is characterized by $\mu_{ep} = (1/6)q\pi^{-1}\eta^{-1}r^{-1}$, where q is the net charge on the particle and r is Stokes' radius of the analyte molecule. All saccharides in the same injection are driven to the cathode at a uniform electroosmotic mobility μ_{eo} toward the outlet. But their μ_{ep} is different and in the reverse direction because of their negative charges. Therefore, the apparent velocity, υ_s equals to $(\mu_{eo} - \mu_{ep})E$. The more saccharide is complexed, the more negatively they charge and the more slowly they migrate toward the detector with bigger μ_{ep} value. Saccharide with lower value of electrophoretic mobility (smaller value of q) had bigger υ_s and was thereby detected faster. Thus 2-AA-Glc and 2-AA-Mal migrated more slowly than other derivatives on CE analysis. These results also showed that the borate concentration influences the complexation. The higher the borate concentration is, the more saccharide is complexed. This leads to an increase in their negative charge. So 2-AA-Glc and in succession, 2-AA-Mal migrated slower and slower with borate concentration increased.

Table 3 summarizes some selected mono-, di-, and trisaccharide derivatives' electrophoretic mobilities together with their

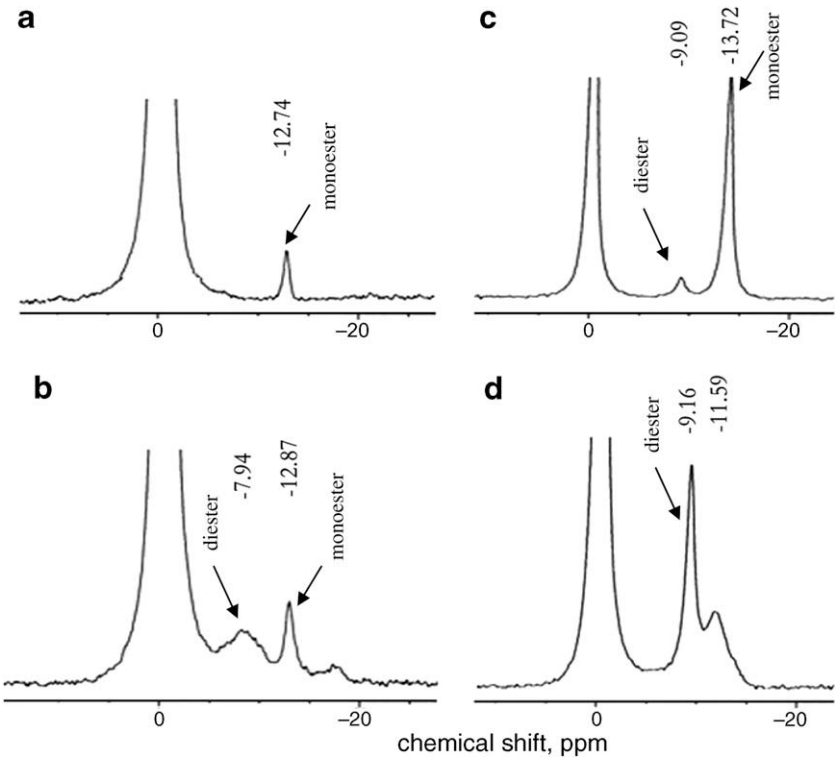

Figure 4. ^{11}B NMR spectra of derivatized saccharide–borate complexes. The spectra were recorded at 25 °C with 0.1 M boric acid as the external reference. Peaks assignment: (a) 2-AA-Glyceraldehyde-Boron (monoester, 12.74 ppm); (b) 2-AA-Rib-Boron (monoester, 12.87 ppm); (c) 2-AA-Mal-Boron (monoester, 13.72 ppm); (d) 2-AA-Glc-Boron (diester, 9.16 ppm).

Table 3
Electrophoretic mobilities and structural characteristics of some investigated derivatives

Carbohydrate derivatives	Electrophoretic mobility[a] $\times 10^{-4}$ cm^{-2} V^{-1} s^{-1}	Configuration of OH on C2–C3	Configuration of OH on C3–C4	Configuration of OH on C4–C5
2-AA-Mal	2.70	*threo*	/	/
2-AA-Glc	3.04	*threo*	*threo*	*erythro*
2-AA-Man	2.67	*erythro*	*threo*	*erythro*
2-AA-Gal	2.72	*threo*	*erythro*	*threo*
2-AA-Rib	2.23	*erythro*	*erythro*	/
2-AA-Xyl	2.82	*threo*	*threo*	/
2-AA-Erythr	2.31	*erythro*	Terminal	/
2-AA-Glycer	2.51	Terminal	/	/
2-AA-Fuc	2.57	*threo*	*erythro*	*threo*
2-AA-GlcN	1.44	/	*threo*	*erythro*
2-AA-GalN	1.25	/	*erythro*	*threo*

[a] Mobility was calculated under the CE condition of 20 kV, L = 60 cm, l = 50 cm in 150 mM borate–50 mM sodium phosphate (pH 7.0).

structural characteristics. Electrophoretic mobilities were calculated using benzyl alcohol as a neutral marker for the determination of the electroosmotic velocity. Table 4 shows ^{11}B and ^{13}C chemical shifts of 2-AA-Saccharides and its borate complexes. It is apparent that trisaccharide and oligosaccharides are among the derivatives detected faster. They have lower mobilities than most of monosaccharides because of their lower complex stability resulting in a lower charge density. It seems that hexoses should migrate faster than pentoses as a result of the higher probabilities of the formation of borate complexes with the increase in the number of hydroxyl groups, but our results did not support it. Therefore it implies that steric factors play an important role in the stability of complexes, which depends strongly on the configuration of hydroxyl groups. This is rendered apparent by relating values of electrophoretic mobility to the configuration of the three vicinal hydroxyl groups at the carbons C2 to C4, and to a lesser extent at the carbons C3 to C5, we conclude that the tendency of three vicinal hydroxyl groups of derivatives to complex with borate appears to increase in the following order: e–e (adjacent *erythro* pairs; an *erythro*–*erythro* grouping), e–t (an *erythro*-diol adjacent to a *threo*-diol), t–e (a *threo*-diol adjacent to an *erythro*-diol), and t–t (adjacent *threo* pairs). In accordance with this sequence, which corroborates a previous finding that *threo* pairs are energetically more favorable than terminal, *erythro* diols for complex formation,[14] 2-AA-Glc forms the strongest complexes with borate and, hence, exhibits the highest electrophoretic mobility among all investigated derivatives. 2-AA-Xyl exhibits the higher μ_{ep} and whereas 2-AA-Rib gives the lowest value of μ_{ep} among the investigated pentose due to its adjacent *erythro* pairs. The effect of a substitute on electrophoretic mobility is apparent from the values calculated for galactosamine and glucosamine, which are significantly lower than those obtained for 2-AA-Gal and 2-AA-Glc. This example also illustrates the great importance of a free hydroxyl group at C2.

Table 4
^{11}B and ^{13}C chemical shifts of 2-AA-Saccharides and its borate complexes

Derivatives	^{11}B chemical shift (ppm)		^{13}C chemical shift (ppm)						
	B^-L	B^-L_2		C-1	C-2	C-3	C-4	C-5	C-6
2-AA-Glc	−11.6	−9.2	L	46.3	71.6	71.4	72.2	71.8	63.5
			$B^-L + B^-L_2$	46.4	71.5	75.1	76.9	72.1	63.7
2-AA-Rib	−12.9	/	L	46.7	70.4	72.9	73.9	63.4	/
			B^-L	46.1	70.7	72.9	74.0	63.3	/
2-AA-Glycer	−12.7	/	L	47.7	70.5	64.4	/	/	/
			B^-L	47.1	74.1	70.7	/	/	/

1. Experimental

All CE experiments were performed on a Beckman Coulter P/ACE MDQ capillary electrophoresis system. UV absorption was recorded at 214 nm, which was the maximum absorption wavelength of 2-AA and 2-AA-Saccharides. A reagent-grade sample of 2-aminobenzoic acid was purchased from Sigma (St. Louis, MO, USA). All saccharides were in the D form and were purchased from Sigma. Sodium cyanoborohydride and D_2O were purchased from Aldrich Chemical (Milwaukee, WI, USA). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). The chemicals used were of analytical grade. Running electrolyte solutions were prepared by dissolving an appropriate amount of sodium dihydrogen phosphate and boric acid (Sigma) in the Milli-Q water to yield a final concentration indicated. The pHs for all the solutions were adjusted to 7.0 with 0.5 M NaOH.

Derivatization of saccharides with 2-AA was performed as described before.[5,6] To prepare the CE analysis sample, an aliquot (30 μL each, except 50 μL for Cellotetraose) of a mixture of saccharide standards (15 mM each, except 12 mM for Cellopentaose) was placed in a microtube and lyophilized. Two hundred microliters of the reagent solution were added to the residue in the microtube. Dissolution of the saccharides was facilitated by gentle vortexing. The resultant solution was then heated at 65 °C for 2–4 h with the tube capped tightly. After cooling to ambient temperature, the saccharide derivatives were diluted with CE running buffer to 50-fold and mixed vigorously on a vortex mixer.

For NMR measurement, excess 2-AA and derivatization reagents must be removed prior to preparation. Saccharide (0.5 mmol) was dissolved in 5 mL of reagent aqueous solution and heated at 65 °C for 2 h. After reaction was completed, the reaction mixture containing labeled saccharides and excess reagent 2-AA was then purified with Waters Sep-Pak Vac 10 g C_{18} cartridges described as in our previous publication.[6] The purity of derivatives was assayed by CE to confirm that excess 2-AA had been completely removed. The same operation was repeated for several times (8–10 times) so that enough amount of purified derivatives was obtained for the NMR measurement. A JEOL JEM-A500 spectrometer was used for NMR measurement. ^{13}C and ^{11}B NMR spectra were recorded at 25 °C with a JEOL JEM-A500 spectrometer at 125.65 MHz and 160.35 MHz, respectively. 0.1 M boric acid was used as the external reference. All spectra were first recorded at a total boron concentration $C_B = 0.1$ M and $C_L = 0.1$ M of purified derivatives. The samples were prepared by dissolving appropriate amounts of boric acid and derivatives in D_2O. The pH was adjusted with 2 M NaOH in H_2O and the total volume of each sample was 2 mL. Depending on the saccharides used, the time to achieve stable pH value can vary from seconds to hours.

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