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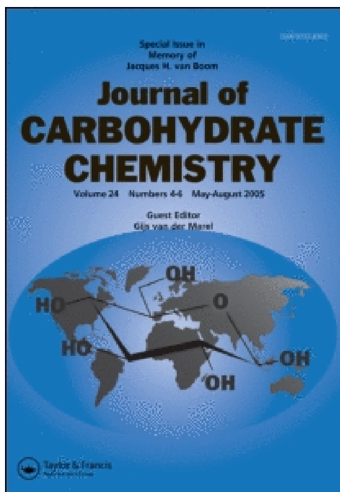

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SIMPLIFIED APPROACH TO HPLC PRECOLUMN FLUORESCENT LABELING OF CARBOHYDRATES: N-(2-PYRIDINYL)-GLYCOSYLAMINES[1]

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ABSTRACT

Reducing sugars were fluorescently labeled by condensation with 2-aminopyridine. The reaction was quantitative and the product was sufficiently stable so that a subsequent reduction step (as in reductive amination) proved unnecessary. The products have been characterized as N-(2-pyridinyl)-glycosylamines. Oligosaccharides labeled in this manner exhibit important analytical characteristics. They: a) are stable under both HPLC separation and prolonged storage; b) have improved chromatographic resolution; c) regenerate original material by weak acid hydrolysis.

INTRODUCTION

The absence of a chromophore in oligosaccharide samples presents a limitation for sensitive detection following high performance liquid chromatographic separation. Detection by refractive index may be employed at high sample concentrations, but refractometers are seriously constrained by the solvent composition changes during gradient operation and their use is limited to isocratic elution. To circumvent these problems a number of reports have described procedures for the introduction of a chromophoric group by pre- or

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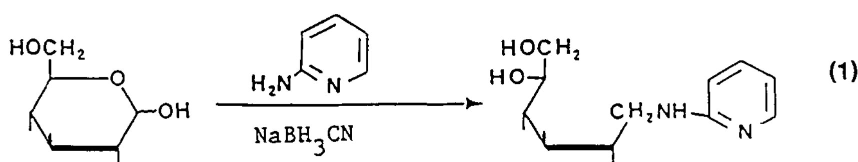
postcolumn derivatization. For those carbohydrate samples possessing a reducing terminus, precolumn derivatization by reductive amination has received considerable attention.[2-6] These procedures involve selective hydride reduction of the intermediate formed from a reducing sugar and an aromatic amine. Many reports have described the use of 2-aminopyridine (AP) as a fluorescent label with sodium cyanoborohydride providing selective reduction of the intermediate (reductive amination) (Equation 1).



Although these procedures have greatly enhanced oligosaccharide detection, the quantitative formation of labeled product has been difficult because of incomplete reduction of the intermediate[5,7] and nonspecific reduction of free oligosaccharides.[6,8] Two alternative derivatives have been introduced which avoid the above complications and have improved sensitivity. These derivatives are formed by reaction with 7-amino-4-methylcoumarin[3] and with 7-amino-1- naphthol.[5] Unfortunately, the former compound is not stable during subsequent linkage analysis that involves permethylation under basic conditions and the latter compound is not easily obtained commercially.

Beyond the improved eluent detectability, introduction of an aromatic group at the reducing terminus provides greater sensitivity and structural understanding when these derivatives are studied by fast atom bombardment mass spectrometry.

As part of our continuing effort to develop better and simpler methods for oligosaccharide separation and detection, we reexamined the reductive amination reaction and noted that the intermediate (before reduction) could be quantitatively formed and was stable during HPLC separation. These findings suggested a simpler fluorescent labeling technique that would

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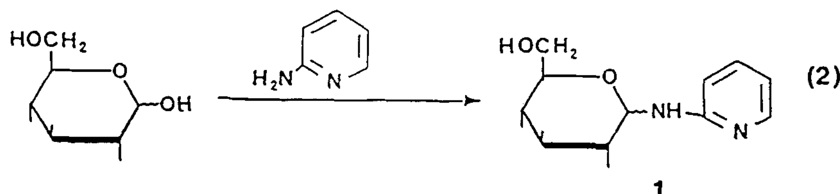
avoid the complications of reduction. This study defines those conditions, describes the N-(2-pyridinyl)-glycosylamine (1) formed, and reports amino column high performance liquid chromatography (HPLC) and fast atom bombardment mass spectrometry (FAB-MS) characteristics.



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RESULTS AND DISCUSSION

Fluorescent derivative formation was studied by FAB-MS. The product obtained from maltotriose was detected, $(M+H)^+$, m/z 581, soon after heating at 65 °C. The time course for the reaction was studied and the yield calculated from the ion current ratio of unreacted to derivatized trisaccharide (FIG. 1). The yield was greater than 90% after 3 hours at 65 °C. It has been noted that samples of larger molecular weight take longer for complete reaction. As expected, the reaction rate increases with temperature. The effect of pH on derivative formation was also studied (FIG. 1). The reaction rate was relatively fast at slightly acidic and neutral conditions and decreased under basic conditions. In general, slightly basic conditions are recommended because neuraminyl residues may be lost under acidic conditions. Additionally, acidic conditions may induce Amadori product (2) formation, especially with aromatic amines possessing electron donating groups.[9]

The derivative formed in this labeling procedure is the dehydration product of an aromatic amine and the glycosyl hemiacetal group. It is known that the products formed by reacting selected aromatic amines with aldohexoses give rise to cyclic (glycosylamines, 1) rather than open-ring structures (Schiff bases, 3). For a recent review of this topic see reference 10. Observations in this work which support the glycosylamine (cyclic) structure are the following: 1.)

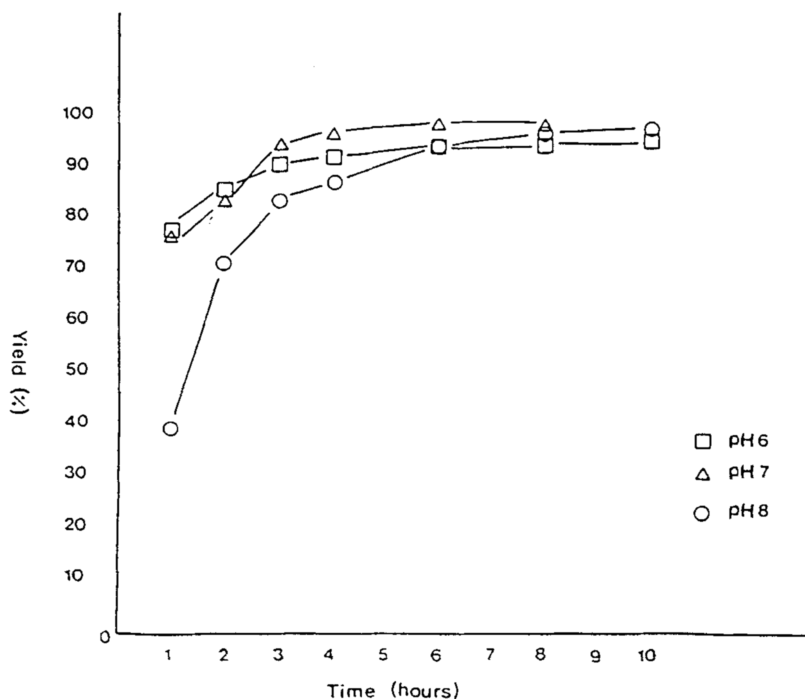

FIG. 1: Time and pH studies of N-(2-pyridinyl maltotriosylamine formation.

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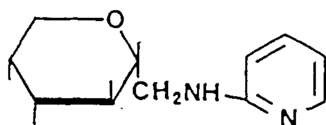
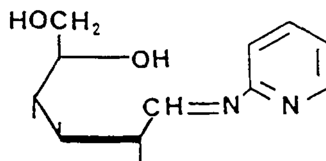
fluorescently labeled (AP) glucose shows anomeric protons when studied by NMR (FIG. 2); 2.) FAB-MS molecular weight analysis of the peracetyl AP-glucose derivative indicated the incorporation of four acetyl groups (five expected if the product was the acyclic Schiff base); 3.) the amino group of the AP derivative of glucose can be hydrolyzed by acetic acid. An Amadori product (**2**), if formed, is stable to acid hydrolysis.[11]



Amadori product
2



Schiff base
3

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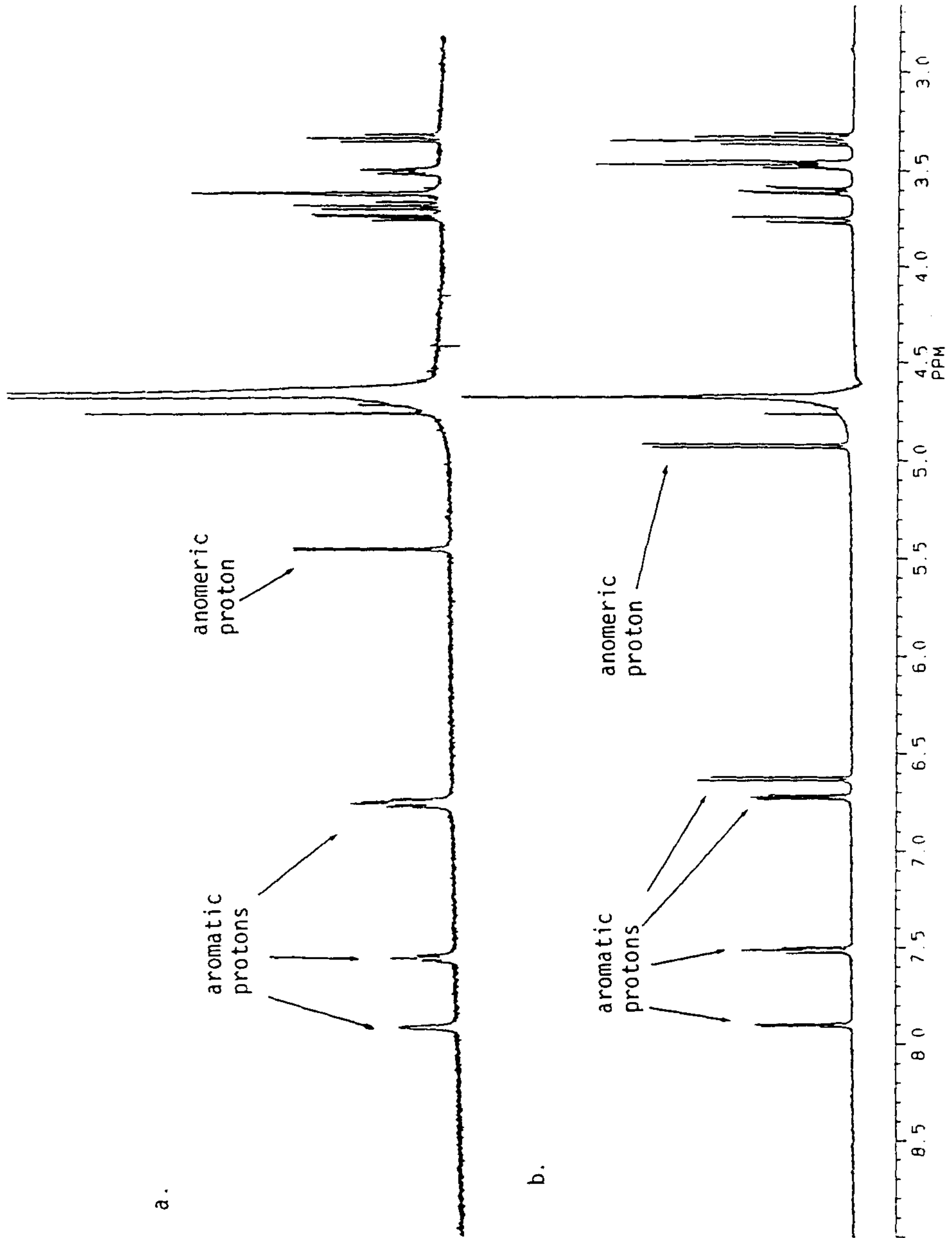

FIG. 2: NMR spectra of AP labeled glucose a.)minor product and b.)major product.(D_2O as solvent).

The AP group was found to be resistant to hydrolysis under mildly basic or neutral conditions and showed no signs of hydrolysis after two days in 50% aqueous acetonitrile solution. After the solvent was evaporated, the glycosylamine (1) could be stored in a refrigerator for a month with no evidence of deterioration. There was no detectable change in the sample after storage for one week in aqueous triethanolamine (pH = 12, 25 ^{o}C). However, under acidic conditions the AP group is gradually hydrolyzed. A 2% aqueous acetic acid solution brought about complete hydrolysis of the AP group in two days at 65 ^{o}C. This provides a way to regenerate the underivatized carbohydrate after the HPLC separation. The reversibility of this derivatization process is important in cases where the AP group may interfere with the biological activity, or complicate further chemical analysis of the material of interest.

Most interestingly, the AP glycosylamine product showed better HPLC resolution (amino column, H_2O/CH_3CN as eluent) than the derivative prepared by reductive amination, (FIG. 3).

When studied by FAB-MS, the small leading peak (FIG. 3a) had the same molecular weight as the major product and may be an anomer of the minor product. For steric reasons, the anomer having the AP group equatorial is strongly favored. This would force the anomeric proton to the axial position. As a result, the more stable anomer of glucose is H_1 axial-H_2 axial as compared to H_1 equatorial-H_2 axial for the less stable anomer. The NMR spectrum of the major product showed a larger coupling constant ($J_{1,2}$ = 8.9 Hz) when compared to the corresponding coupling constant of the minor product ($J_{1,2}$ = 5.3 Hz) (FIG. 2). This is consistent with the fact that the coupling constant of H axial - H axial should be larger than the coupling constant of H equatorial - H axial. Separation of the AP-glucose oligomers (glycosylamine) is shown in Figure 4. The chromatogram shows baseline resolution up to DP-17.

The use of FAB-MS to support oligosaccharide structural studies has been gaining in popularity over the last few years. The introduction of this pyridinyl group at the oligosaccharide reducing terminus provides two advantages for MS analysis when using FAB. First, due to the high proton

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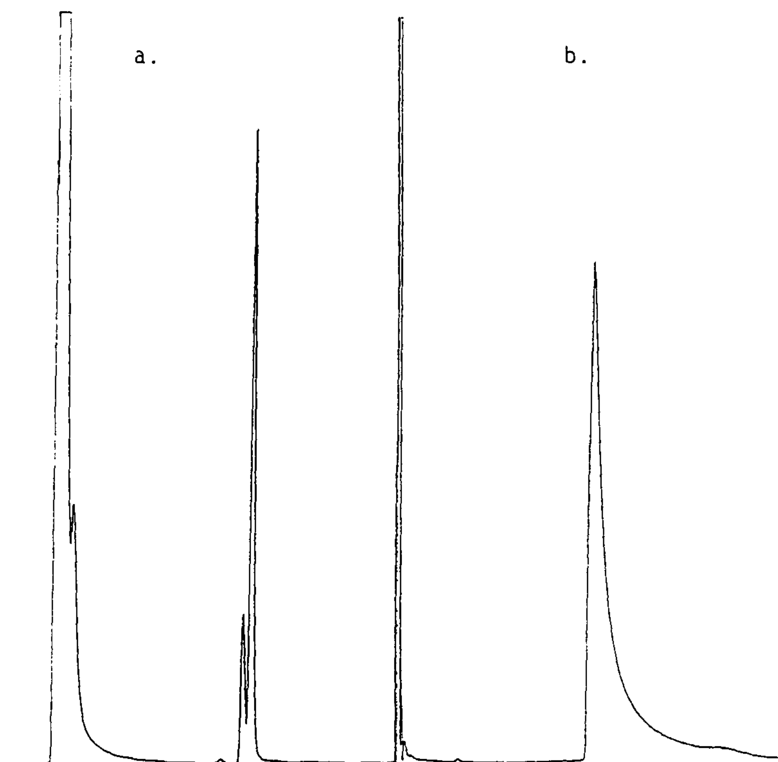

FIG. 3: Liquid chromatography of a.) AP(glycosylamine) maltohexaose and b.) AP(reductive amination) maltohexaose on a column (250 x 4.6 mm id) of amino spherisorb (5μ), in 20% to 60% water-acetonitrile, at 2 mL/min with a 30 minute program.

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affinity of the pyridinyl group, the sensitivity is several times better than underivatized oligosaccharides. Second, the fragment ions are easier to identify since the charge is most likely to reside on the pyridine ring. Figure 5 shows the positive FAB mass spectrum of N-(2-pyridinyl)-maltoheptaosylamine. The molecular ion and sequence ions can be readily identified.

CONCLUSION

Labeling of oligosaccharide samples with chromophoric groups has provided remarkable improvements in HPLC detecting sensitivity. Additionally, terminal labeling has provided

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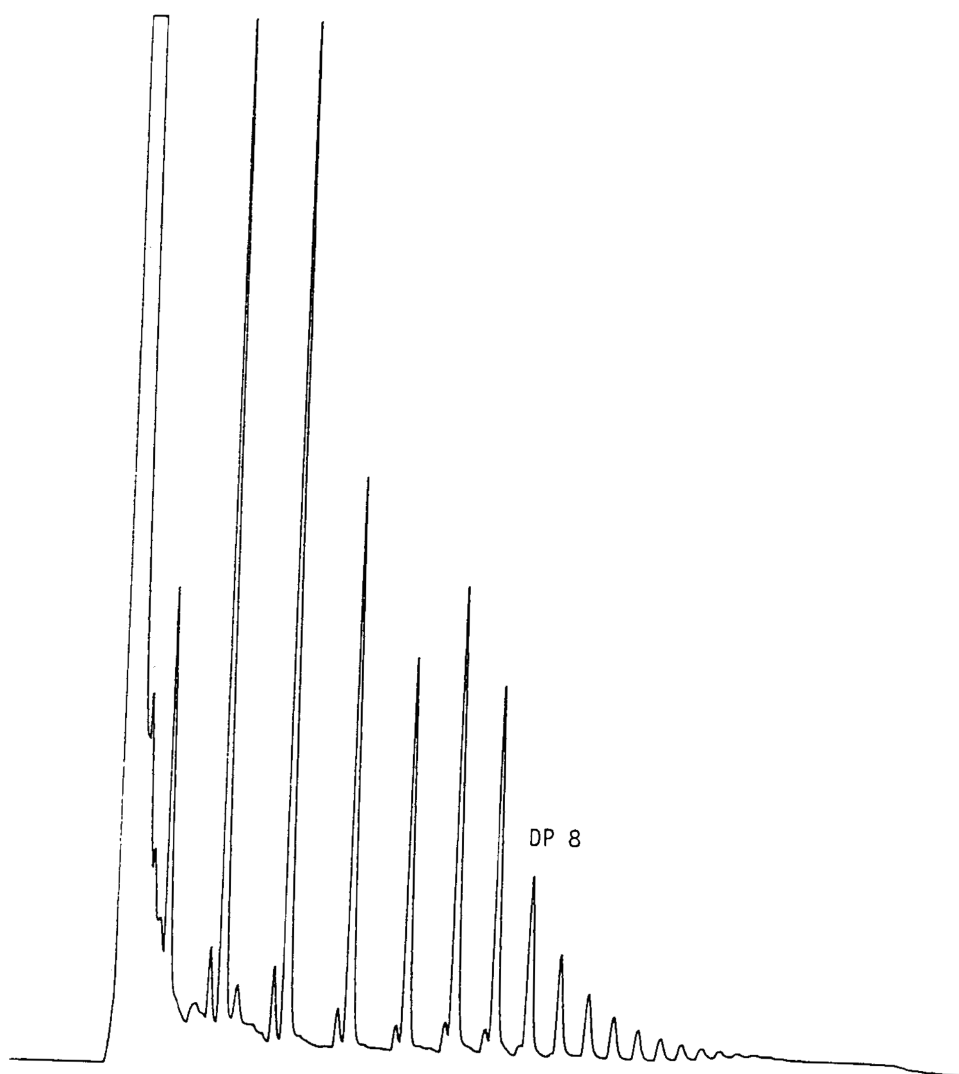

FIG. 4: Liquid chromatography of AP(glycosylamine) Maltooligomers on an amino column. For HPLC conditions, see legend to FIG. 3.

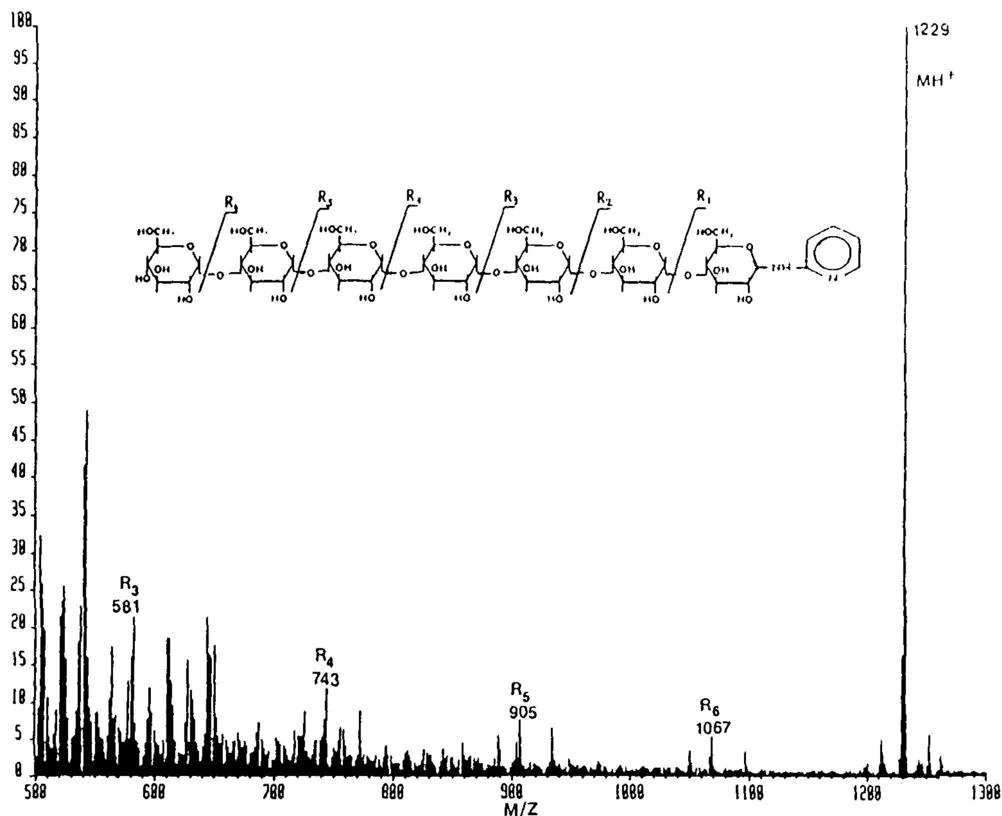

FIG. 5: FAB Mass spectrum of N-(2-pyridinyl) maltoheptaosylamine.

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oligosaccharide samples which show improvements in HPLC resolution and an enhancement in MS structural detail.

The N-(2-pyridinyl)-glycosylamine derivative of reducing oligosaccharides can be prepared simply and quantitatively. The product is stable during chromatographic analysis and equally stable under neutral and basic conditions. This facile labeling procedure provides a fluorescent tag for highly sensitive sugar detection and the original sample can be regenerated under mildly acidic conditions following HPLC separation.

EXPERIMENTAL

Materials. 2-Aminopyridine, and $NaCNBH_4$ were obtained from Aldrich Chemical Company Inc. (Milwaukee, WI 53201);

solvents for HPLC were purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI 49442); 5 micron Spherisorb Amino Columns and the amino column packing materials were obtained from Phase Separation Inc.; maltotriose, maltohexaose, and maltoheptaose were purchased from Sigma Chemical, Inc. (St. Louis, Missouri); maltooligomers were obtained from V-labs Inc. (Covington, LA 70433).

Instrumentation. Liquid chromatographic separations were performed with equipment from Waters Associates, Milford, MA 01757). This equipment consisted of two M6000A pumps, a U6K injector, model 660 solvent programmer, and model 450 variable wavelength detector. Fluorescence was detected with a model FS970 spectrofluorometer from Kratos Analytical Instruments (Ramsey, NJ 07446). The HPLC conditions are described in Figure legends. Fast atom bombardment mass spectra were recorded with a VG ZAB-SE (VG Analytical, Manchester, UK) double focusing mass spectrometer. Thioglycerol was used as the matrix. Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker 500 MHZ NMR spectrometer with D_2O as the solvent.

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Preparation of Fluorescent Derivatives. Oligosaccharides (10 ug to 1 mg) were dissolved in 50 ul of aqueous 2-amino-pyridine solution at pH 7 (prepared by dissolving 1 g of 2-aminopyridine in 0.8 mL constant boiling hydrochloric acid (6N) and 1.6 mL water). The solution was heated to 65 ^{o}C for 10 hours. Aliquots of this solution were directly injected into the HPLC for analysis. Oligosaccharide samples, labeled by reductive amination, were synthesized by previously described procedures.[2,6]

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