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Carbohydrate Research 339 (2004) 221–231
Carbohydrate RESEARCH

Neutral, acidic, and basic derivatives of anthranilamide that confer different formal charge to reducing oligosaccharides ☆

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Received 6 August 2003; received in revised form 16 October 2003; accepted 21 October 2003

Abstract—To facilitate the use of oligosaccharides as analytical tools in biological studies, we have designed, synthesized, and conjugated to maltosaccharides a novel series of homologous small fluorescent moieties that differ in formal charge. These moieties are amide derivatives of anthranilic acid: uncharged *N*-(2-aminobenzoyl)glycinamide (ABGlyAmide; **2**), acidic *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine (ABGlyDIMED; **3**), and basic *N*-(2-aminobenzoyl)glycine (ABGly; **1**). Routes for synthesis and optimal reaction conditions for glycoconjugation by conventional reductive amination are presented, as is the compatibility of these adducts with common analytical and preparative chromatographic methods, including RP-HPLC and HPAEC-PAD. These novel anthranilic acid derivatives confer both fluorescence and defined charge to oligosaccharides, and so enhance the repertoire of chromatographic and analytical methods for which anthranilic acid can be used. Furthermore, because glucosaccharides have rigid solution structure, these small fluorescent adducts with different formal charge are ideal tools for molecular sizing studies of membrane pores.

Keywords: Maltosaccharide; Anthranilic acid; Channel; Reductive amination; Carbohydrate; Glycoconjugate

1. Introduction

Carbohydrates are excellent tools for biological and biophysical investigation, owing to the vast number of structural combinations and permutations available from a small number of monosaccharides. The usefulness of underivatized carbohydrates as investigative tools is hampered by the specialized methodologies required for their high-resolution detection and analysis. This problem can be overcome by derivatization with fluorescent moieties, which enables easy and quantitative detection. However, for some applications it would be advantageous to make additional modifications, such as addition of charge, as part of the same derivatization. We describe here the synthesis and characterization of novel anthranilic acid derivatives that are used to generate acidic, basic, and neutral glycoconjugates. These adducts are easily detectable by fluorometry, readily purified, and the conjugations do not significantly alter the structure or dimensions of the oligosaccharides.

☆ Charged derivatives of anthranilic acid amide.
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doi:10.1016/j.carres.2003.10.020

To characterize the permeability pathway of large membrane channels, it is desirable to have a size-indexed set of fluorescent, unreactive molecules of known dimension and similar chemistry. The rigidity conferred upon maltosaccharides by internal hydrogen bonding makes them particularly good candidates for this application when labeled with a small fluorescent group. For channel studies it is also desirable to utilize charged as well as uncharged probes. Therefore our goal was to develop a set of compounds easily conjugated to reducing oligosaccharides that would confer both fluorescent detectability and defined charge.

Reductive amination is a common method for derivatizing reducing sugars. The feasibility of reductive amination for carbohydrate labeling was first demonstrated using 2-aminopyridine to separate a homologous series of glucosaccharides by RP-HPLC.[1] Such derivatized oligosaccharides are uncharged at neutral pH and have been used to determine the pore diameter of intercellular membrane channels.[2] Under strongly acidic conditions 2-aminopyridine carries a weak positive charge, enabling the same series to be separated by CE according to their charge–mass ratio.[3] Additionally, stoichiometric labeling of oligosaccharides offers an excellent method for quantitation while the aglycone provides stability against strongly acidic and alkaline conditions, under which free saccharide may degrade.

However, 2-aminopyridine cannot be easily modified to carry positive or negative charge, and so an alternative moiety was desired. Other charged and uncharged labels[4] have been developed, or applied, to variously enable the separation and analysis of carbohydrate by chromatographic (LC, CE), electrophoretic (FACE), and ion-mass (MS, NMR) techniques. Of those labels characterized, anthranilic acid[5] fulfills most of the desired criteria, being inexpensive and commercially available,[6] highly fluorescent,[7] and rapidly conjugated to reducing saccharide under weak acidic conditions that are not suitable for conjugation of many primary amines.[5,7] Most important, anthranilic acid can be modified to have a single positive or negative charge. Furthermore, anthranilic acid is compatible with the majority of chromatographic techniques employed for carbohydrate separations.

The availability of a new homologous series of small fluorescent labeling compounds, neutral, acidic, and basic amide derivatives of anthranilic acid, will improve the repertoire of chromatographic and analytical methods for which anthranilic acid can be used with minimal disturbance to isolation, purification, and structural determinations of mono- and oligosaccharides, and that will find immediate application in studies of membrane protein channels.

2. Results

2.1. Preparation of anthranilamide derivatives[‡]

The designed 2-aminobenzamide compounds (anthranilamide derivatives) were prepared from 2-nitrobenzoic acid via 2-nitrobenzoyl chloride, as summarized in Figure 1. Starting from 2-nitrobenzoic acid was more satisfactory than having to *N*-protect the amino group of anthranilic acid. Briefly, coupling of 2-nitrobenzoic acid with glycine benzoyl ester gave *N*-(2-nitrobenzoyl)glycine benzyl ester (**4**, 72%). Compound **4** was converted into its corresponding glycinamide **5** with 82% yield, through treatment with concentrated ammonium hydroxide in methanol. Coupling of 2-nitrobenzoyl chloride with *N*,*N*-dimethylethylenediamine provided *N*,*N*-dimethyl-*N*′-(2-nitrobenzoyl)ethylenediamine (**6**, 67%). Finally, palladium-catalyzed hydrogenation of the 2-nitro derivatives **4**, **5**, and **6** gave the corresponding 2-amino derivatives **1** (ABGly; *N*-(2-aminobenzoyl)glycine), **2** (ABGlyAmide; *N*-(2-aminobenzoyl)glycinamide), and **3** (ABGlyDIMED; *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine) in 55, 88, and 91% yields, respectively. Structures were confirmed by NMR (Fig. 2).

2.2. Absorption and fluorescence emission spectra of the free and conjugated anthranilamides

Free label (**1**–**3**) absorbance wavelength maxima were between 312.5 and 317 nm (median 314.8 nm) in water and the range shifted to longer wavelengths, 314.5–318.5 nm (median 316.5 nm) in 6% (v/v) acetic acid. Absorbance in 6% (v/v) acetic acid was attenuated to 30–48% of that in water. Optimal fluorescence wavelength for the excitation of free label in water was 325 nm with emission wavelengths at 410, 415, and 420 nm for the anionic, neutral, and cationic labels, respectively. Labeled oligosaccharide mixtures in water exhibited maximum absorbance wavelengths that were red-shifted (range 330.5–336.5 nm, median 333.5 nm) relative to those of the free labels in solution. Absorbance increased for the labeled maltosaccharide mixtures in water relative to the free labels (36% for negative labeled (**1**, ABGly), 5% for neutral labeled (**2**, ABGlyAmide), 34% for positive labeled (**3**, ABGlyDIMED)); Similarly, optimal fluorescence excitation of the conjugated labels red-shifted (range 342.5–344.5 nm) with emission maxima around 430 nm (range 431.5–432.5 nm) (Table 1).

[‡] The formulas in Figures 3–7 all imply the D-*gluco* configuration in the sugar portion.

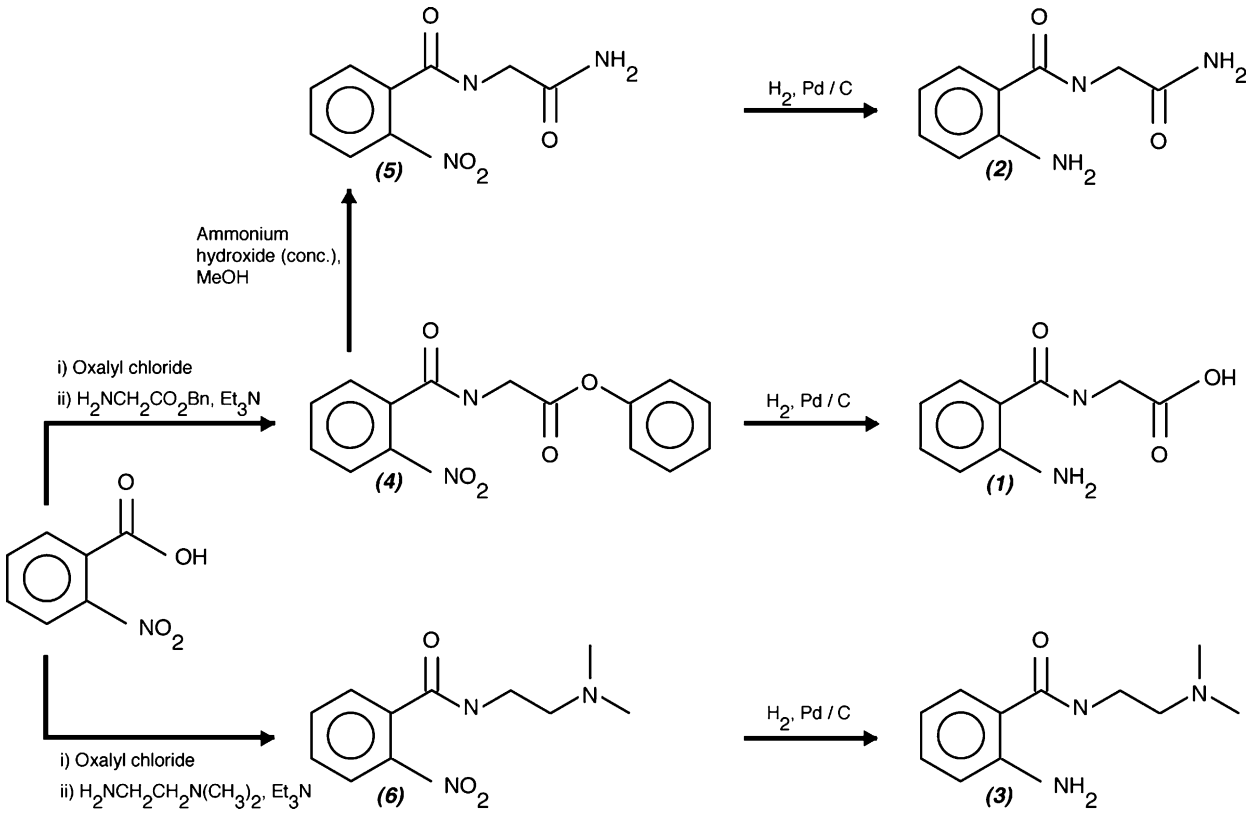

Figure 1. Reaction scheme. The new 2-aminobenzamide derivatives were prepared from 2-nitrobenzoic acid via 2-nitrobenzoyl chloride. Hydrogenation of the 2-nitro derivatives gave the corresponding 2-amino-derivatives **1** (ABGly; *N*-(2-aminobenzoyl)glycine), **2** (ABGlyA; *N*-(2-aminobenzoyl)glycinamide), and **3** (ABGlyDIMED; *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine).

2.3. Oligosaccharide labeling

A maltose through maltohexaose mixture ([4)-α-D-Glc*p*-(1-]$_{2–6}$) labeled with the anionic ABGly-labeled (**1**, *N*-(2-aminobenzoyl)glycine) or uncharged ABGlyAmide-labeled (**2**, *N*-(2-aminobenzoyl)glycinamide) oligosaccharides completely separated from the excess labeling reagents that eluted more slowly through a Sephadex G-10 column (Fig. 3). The cationic ABGlyDIMED (**3**, *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine) derivatives were not as easily separated from free label by this method, however fractions could be judiciously chosen to contain labeled maltosaccharides free of unreacted label. Retention times of eluted peaks for single sugar labeling reactions ([4)-α-D-Glc*p*-(1-]$_{n\leqslant 6}$) bracketed the retention times spanned by the labeled oligosaccharide mixture ([4)-α-D-Glc*p*-(1-]$_{2–6}$) for each anthranilamide derivative. Labeling efficiencies for (**1**) ABGly-, (**2**) ABGlyAmide-, and (**3**) ABGlyDIMED-derivatized oligosaccharides were 75, 100, and 50%, respectively, of the theoretical maxima of one-to-four for a fivefold molar excess of label reacted initially with reducing oligosaccharide. Addition of charge-bearing side groups adversely affected labeling efficiency, assuming the quantum efficiency of free label and oligosaccharide-linked label were equal in each case.

2.4. Recovery of anthranilamide glycoconjugates from aqueous solution by amine adsorption

Conjugated maltosaccharides were retained on aminopropyl solid-phase columns equilibrated with high v/v concentrations of acetonitrile in water. Glycoconjugates could be eluted directly with column eluents for chromatographic separations by RP-HPLC and/or HPAEC (Fig. 4). Because of their simplicity, relatively low cost, and high capacity for binding, aminopropyl silica columns are particularly suitable for quickly de-salting label, or for recovery of a wide range of quantity of derivatives, without loss.

2.5. Separation using GCC and Carbopac PA-1 column

C_{18} columns do not retain anthranilamide-derivatized saccharides and therefore a more hydrophobic chemistry, namely, a graphitized carbon column (GCC), is required for their separation. Optimal running condition, of several organic solvents and acid/base additives tested, was 30% (v/v) acetonitrile containing 0.05% (v/v) TFA. Labeled oligosaccharide series with different degrees of polymerization (DP) could be separated for the negatively charged (**1**, ABGly) and neutral (**2**,

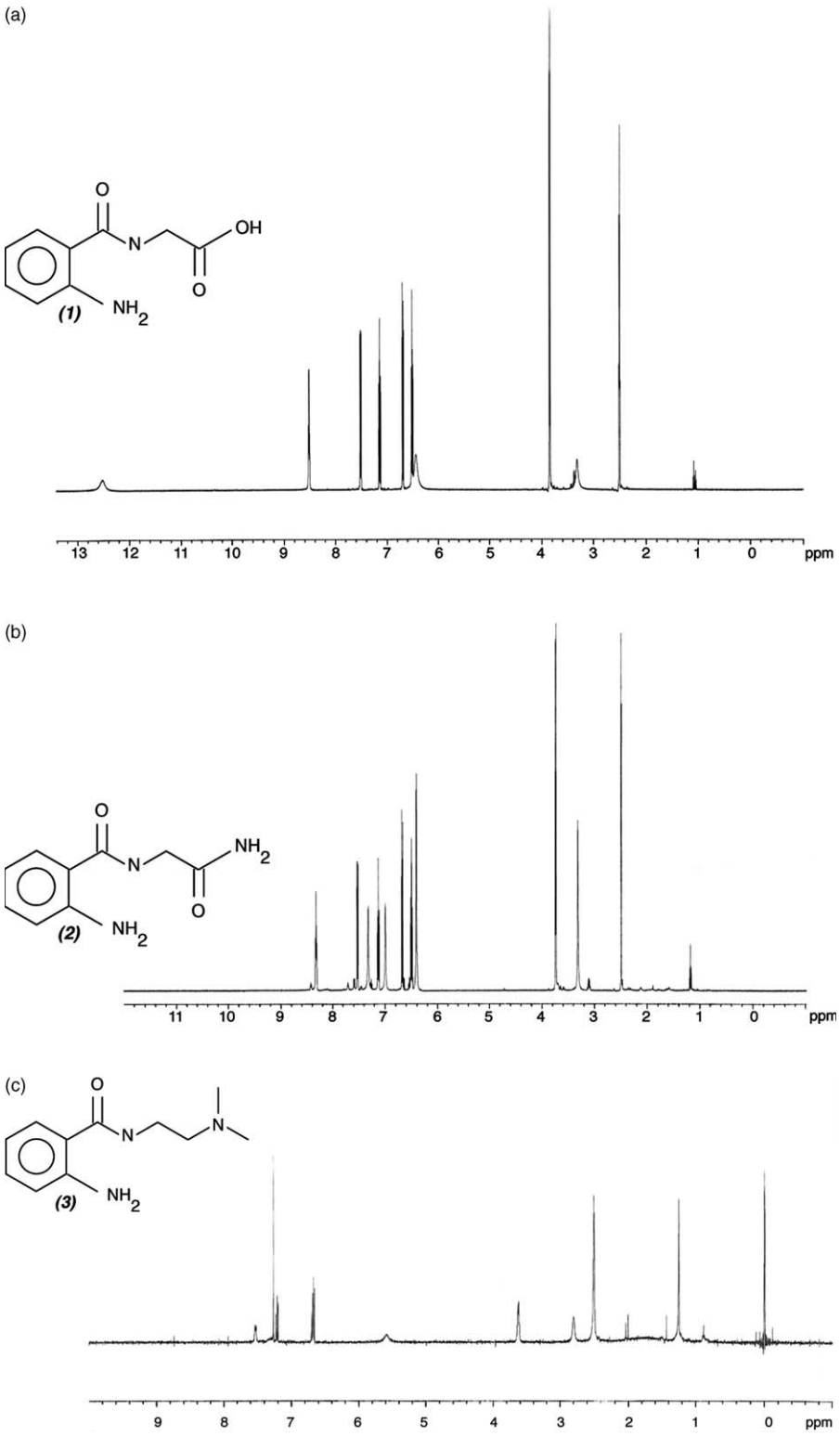

Figure 2. NMR spectra. Preparations as summarized in Methods and Figure 1 (Reaction scheme). In (a), ABGly (**1**), *N*-(2-aminobenzoyl)glycine, an off-white solid: ^{1}H NMR (300 MHz, Me_2SO-d_6 + 5% D_2O + 0.03% Me_4Si): δ 7.510 (d, 1H, *J* 9.93 Hz, H-6), 7.137 (t, 1H, *J* 9.65 Hz, H-4), 6.691 (d, 1H, *J* 9.69 Hz, H-3), 6.521 (t, 1H, *J* 9.69 Hz, H-5), 3.836 (s, 2H, *J* 19.18 Hz, NCH_2). In (b), ABGlyAmide (**2**), *N*-(2-aminobenzoyl)glycinamide, an off-white solid. ^{1}H NMR (300 MHz, Me_2SO-d_6 + 5% D_2O + 0.03% TMS): δ 8.320 (d, 1H, *J* 10.30 Hz, H-6), 7.511 (t, 1H, *J* 10.89 Hz, H-4), 7.123 (T, 1H, *J* 11.43 Hz, H-5), 6.991 (d, 1H, *J* 11.06 Hz, H-3), 3.743 (s, 2H, *J* 26.65 Hz, NCH_2). In (c), ABGlyDIMED (**3**), *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine, a colorless syrup: ^{1}H NMR (300 MHz, $CDCl_3$ + 2% D_2O + 0.03% Me_4Si): δ 7.534 (d, 1H, *J* 5.31 Hz, H-5), 7.222 (t, 1H, *J* 4.35 Hz, H-4), 6.702–6.661 (m, 2H, H-3, 5), 3.635 (t, 2H, *J* 10.58 Hz, NCH_2), 2.801 (t, 2H, *J* 10.42 Hz, CH_2NMe_2), 2.504 [s, 6H, *J* 28.34 Hz, $N(CH_3)_2$].

Table 1. Absorbance and fluorescence emission spectra for the three anthranilamide labels in de-ionized water and acetic acid

Free label	nM	Solvent	λ_{max} (nm)	A_{max} (OD)	$10^{-3} \times \varepsilon_{max}$ ($cm^{-1}\,M^{-1}$)	$\lambda_{excitation}$ (nm)	$\lambda_{emission}$ (nm)
ABGly (**1**)	138	Water	312.5	0.3579	2593	325	410
ABGly (**1**)	138	0.1 M acetic acid	315.0	0.1117	809		
ABGlyAmide (**2**)	138	Water	315.5	0.3888	2817	325	415
ABGlyAmide (**2**)	138	0.1 M acetic acid	314.5	0.1179	854		
ABGlyDIMED (**3**)	138	Water	317.0	0.3078	2230	325	420
ABGlyDIMED (**3**)	138	0.1 M acetic acid	318.5	0.1468	1064		
Glycoconjugate	mg/mL						
ABGly (**1**)	0.1	Water	330.5	0.4856	3519	342	431
ABGlyAmide (**2**)	0.1	Water	336.5	0.4067	2947	343	432
ABGlyDIMED (**3**)	0.1	Water	335.5	0.4128	2991	344	433

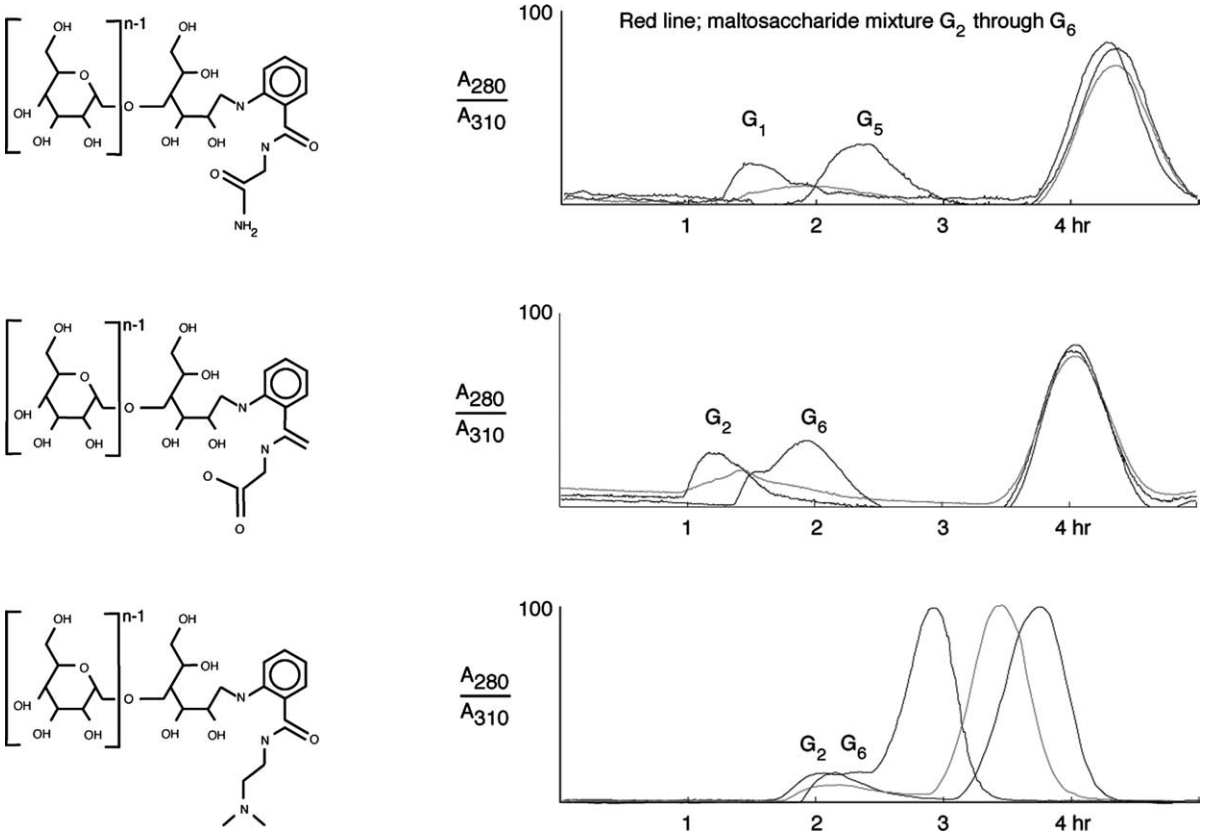

Figure 3. Purification of reductive amination products by gel filtration. Trace shows the elution profile (h) after application of G_2, G_6, and G_2–G_6 reductive amination reaction mixture (entire reaction volume of 0.2 mL diluted to 2 mL with 6% (v/v) acetic acid) to a Sephadex G-10 column (1.5 × 50 cm). Samples were eluted with 6% (v/v) acetic acid. The asymmetric peak of labeled maltosaccharides (left) eluted before free, unreacted label (right peak). Reaction efficiency was determined from the ratio of the areas under the chromatogram peaks that represented the fluorescence of labeled oligosaccharide and unreacted free label.

ABGlyAmide) series; labeled sugars were eluted in order of increasing DP from the GCC. The separation of the positively charged (**3**, ABGlyDIMED) derivatives was not satisfactory and there was no significant improvement in resolution upon heating or cooling the column. Resolution of ABGlyDIMED derivatives (**3**) were improved by using 30% (v/v) acetonitrile including 4–10 mM sodium hydroxide, although quantification of the peaks with different DP was not as reproducible as when using acidified acetonitrile. Tailing of the peaks observed were likely due to the alkali incompatibility of the HPLC system used (Fig. 5) and by the heterogeneous ionic charges that were suppressed under acidic conditions. Free fluorescent reagents remained bound to the GCC columns and their elution required increasing concentrations of acetonitrile.

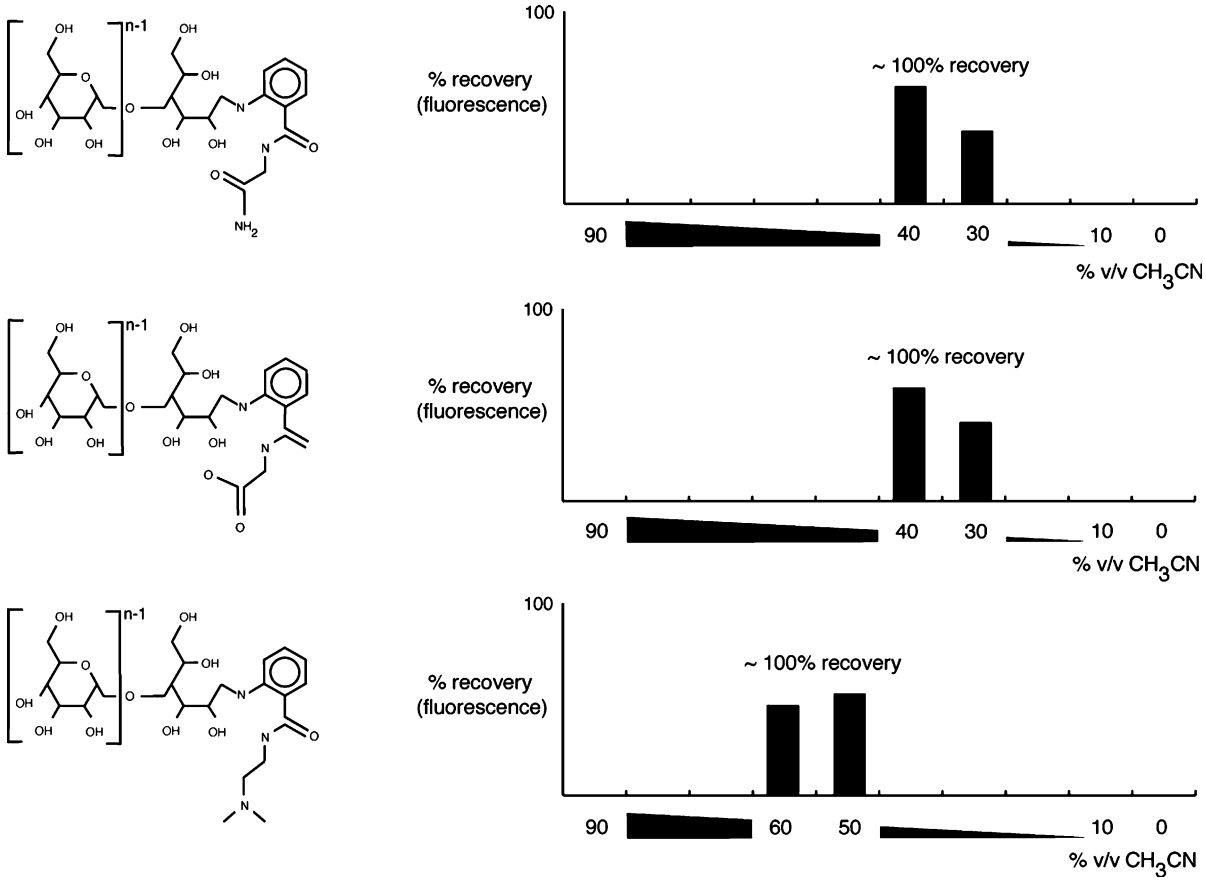

Figure 4. SPE recovery of glycoconjugates on aminopropyl resin. Free label and/or derivatized glycoconjugates can be purified in short time and with excellent recovery by solid-phase extraction using an amine-modified resin. Bindings are in pure or at high acetonitrile concentrations. The molecular basis is likely hydrogen bonding, which can be disrupted through increasing concentrations of water by 10% (v/v) increments.

High-resolution separation of homologous maltosaccharides has been obtained under strongly alkaline conditions by HPAEC with peculiar anion-exchange resins. Methods for separation of neutral (**2**, ABGlyAmide), negatively (**1**, ABGly), and positively charged (**3**, ABGlyDIMED) maltosaccharides were developed using HPAEC and the Carbopac PA-1 column. Labeled sugars were eluted in order of increasing DP from the Carbopac PA-1 column. Neutral (**2**) and positively charged (**3**) derivatives were well separated using a linear (2–25% (v/v) 1 M, 25 min) acetate gradient (Fig. 6), however baseline separations of the negatively charged (**1**) derivatives could only be achieved when pushing agent was substituted by nitrate (10–35% (v/v) 0.2 M, 25 min), which has a higher resolution with this column over acetate gradients for separation of polysialic acids that have large amounts of negative charge (Fig. 7). Free fluorescent labels eluted early from the column.

3. Discussion

Recent developments in carbohydrate detection after analytical separations brings analytical limits for carbohydrate in line with current sensitivity in molecular biology. The full potential of these analyses are being realized by GC and CE with their coupling to mass spectrometry and NMR, and also include electrophoretic FACE analysis for simple and complex carbohydrates. Each strategy has benefited from, and can be hindered by, the ability of carbohydrates to undergo derivitization reactions with photometric or fluorometric labels. Labeling by reductive amination using a combination of aromatic amines or monosubstituted aminobenzene derivatives and reducing reagents is most frequently employed. For many of these downstream methodologies, the labels confer the charge required for their effective resolution, which makes it possible to either adjust mobility in the electrophoretic process or provide greater increases in sensitivity following MALDI and electrospray ionization.

This work describes the synthesis of neutral (**1**, ABGlyAmide; *N*-(2-aminobenzoyl)glycinamide), negative (**2**, ABGly; *N*-(2-aminobenzoyl)glycine), and positively charged (**3**, ABGlyDIMED; *N*,*N*-dimethyl-*N*′-(2-amino-benzoyl)ethylenediamine) derivatives of anthranilic acid, one of the smallest and most widely applicable coupling reagents. These new labeling compounds show

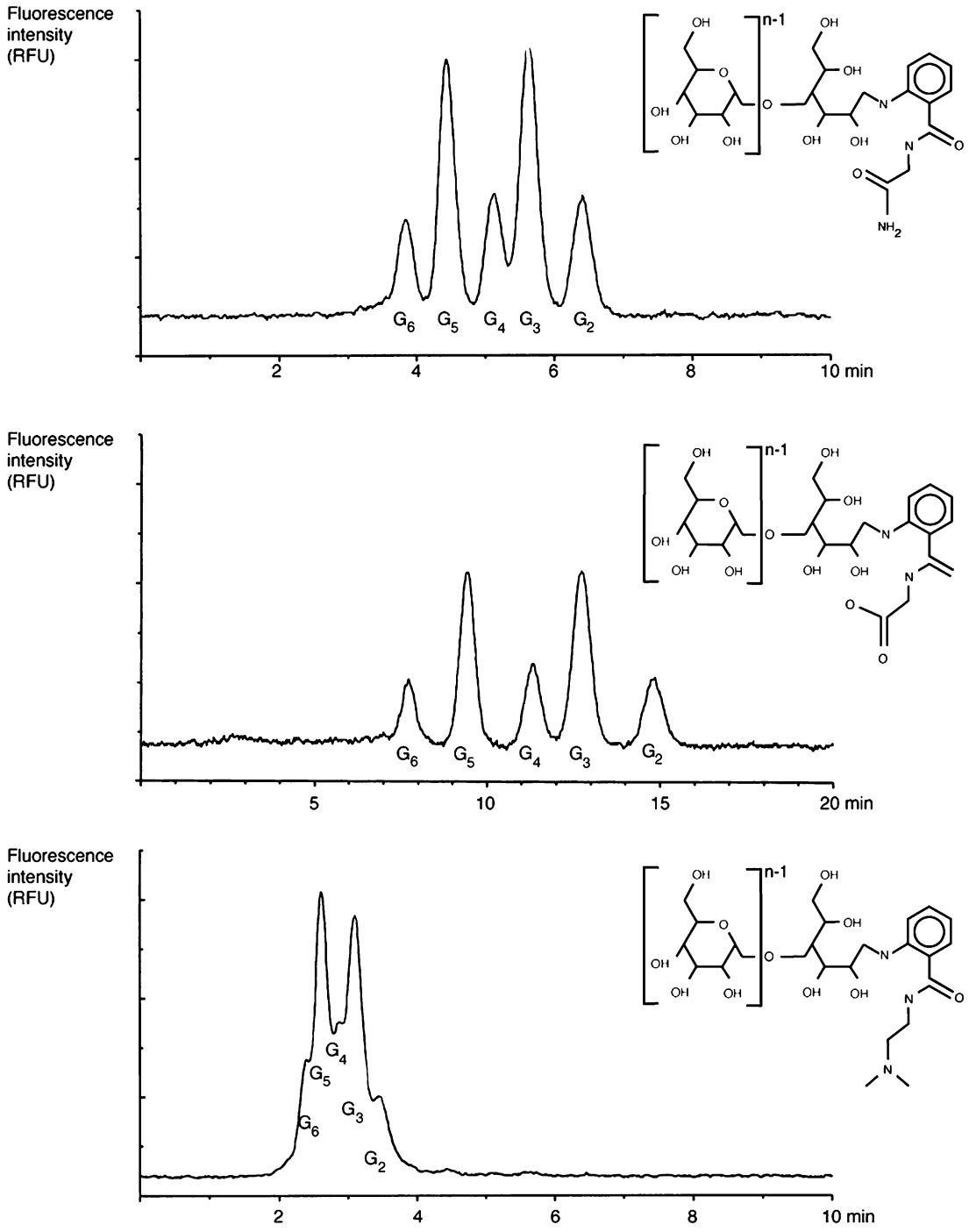

Figure 5. Separation of glycoconjugates using HPLC-GCC. Separation of the glycoconjugate homologues is accomplished isocratically within 20 min using a routine HPLC system and a graphitized carbon column. The order of resolution was negative (**1**, ABGly) > neutral (**2**, ABGly-Amide) > positive (**3**, ABGlyDIMED) derivatives with labeled sugars eluted in order of decreasing DP.

good promise in their compatibility for all downstream analytical methodologies. The addition of both a small fluorescent tag and formal charge, if desired, can be achieved without significantly altering the overall size or structure of the labeled oligosaccharides.

One immediate area of application is for the investigation of the permeant properties of protein pores in which the extensive chemical and structural characterization of oligosaccharides (e.g., indexed size, relative nonreactivity) are useful. Several classes of membrane channels are permeable to small mono- and oligosaccharides including mechanosensitive channels,[8,9] certain puringeric receptors,[10–12] and anion channels,[13] bacterial and mitochondrial porins,[14] and intercellular junctions.[2]

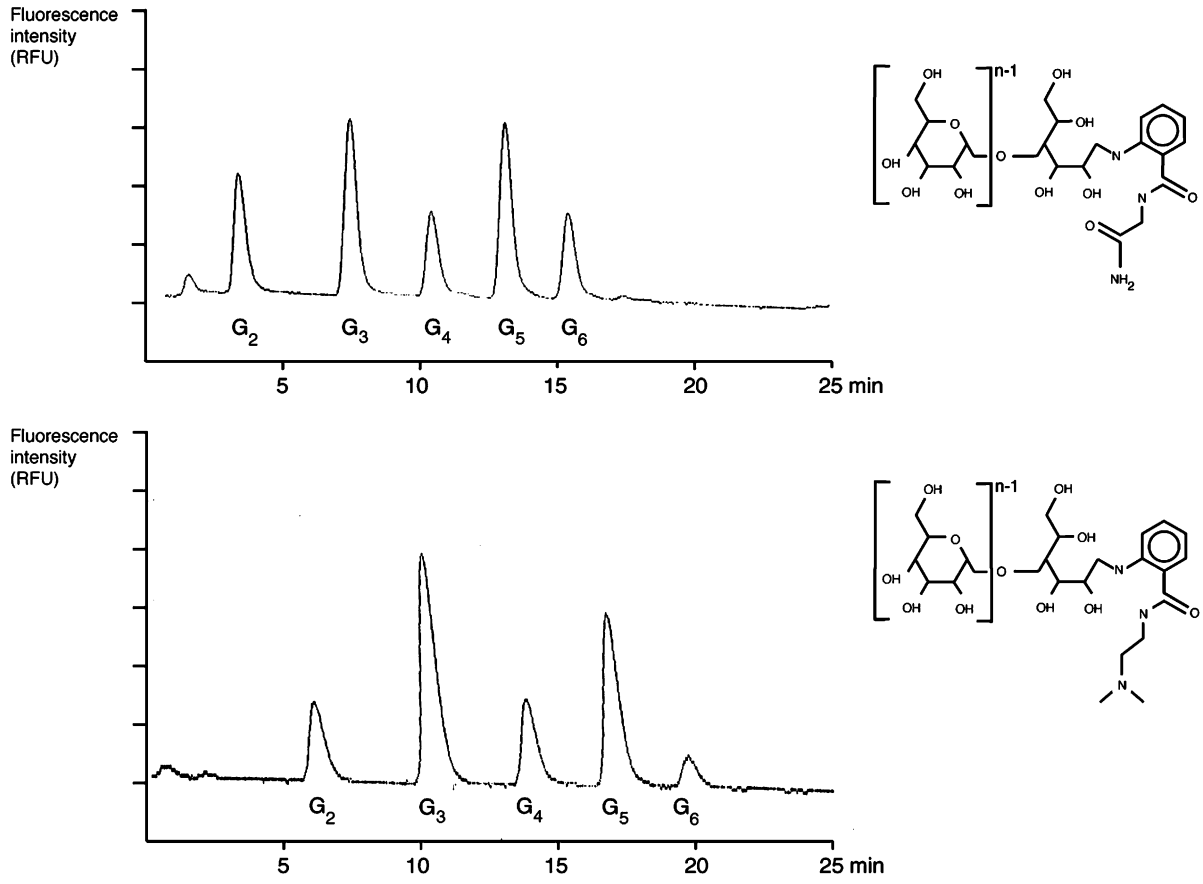

Figure 6. Acetate gradient elution on a PA1 column for ABGly-Amide and -DIMED labeled maltosaccharide. Neutral (**2**, ABGlyAmide) and positively charged (**3**, ABGlyDIMED) derivatives are well separated using a linear acetate gradient within 20 min.

For these channels, determination of pore properties has often relied on the use of fluorescent tracer molecules of diverse size, charge and charge distribution, molecular shape, and chemical structure. Examples include Lucifer Yellow, calcein, fluorescein and its derivatives, biotin and its derivatives, small peptides, propidium iodide, and the Alexa dyes. Because of the differences among these reagents, it has been difficult to attribute differences in their channel permeability to differences in their size, charge, or to their specific chemical interactions with the channel being studied. Meaningful analysis of large pore permeation requires a set of reagents that do not have such variability, and that have known and well-characterized chemical and structural features. The ability to simultaneously confer simple detection (by fluorescence) and to alter charge as desired without significantly altering the overall size and structure allows the use of conjugated oligosaccharide reagents for this purpose.

Indeed, these new charged, fluorescent labels have been used to investigate the pore selectivity of intercellular channels, with intriguing and unexpected results. The derivatized maltosaccharides appear to interact with connexin channels in ways that are substantially different from the 2-aminopyridine glycoconjugates previously used,[2] and may block connexin pores.[15] Thus these reagents will be useful not only as probes of the roles of size and charge in channel permeability, but also as tools for investigating and modulating channel function.

It is hoped that these anthranilamide labels (**1**,**2**,**3**), and the straightforward methods for conjugation, clean-up, and analysis described here will lead to the application of glycoconjugate reagents to a host of biological issues for which appropriate reagents have not been previously available.

4. Experimental

4.1. Materials

Maltose oligosaccharide mixture, ([4)-α-D-Glc*p*-(1-]$_{2-6}$) was obtained from Pfanstiehl Laboratories (Waukegan, IL, USA). Maltose ([4)-α-D-Glc*p*-(1-]$_2$), G_2, purity 99% w/w by HPAE-PAD), maltotriose ([4)-α-D-Glc*p*-(1-]$_3$), G_3, 98%), maltotetraose ([4)-α-D-Glc*p*-(1-]$_4$), G_4, 95%), maltopentaose ([4)-α-D-Glc*p*-(1-]$_5$), G_5, 97.5%), and

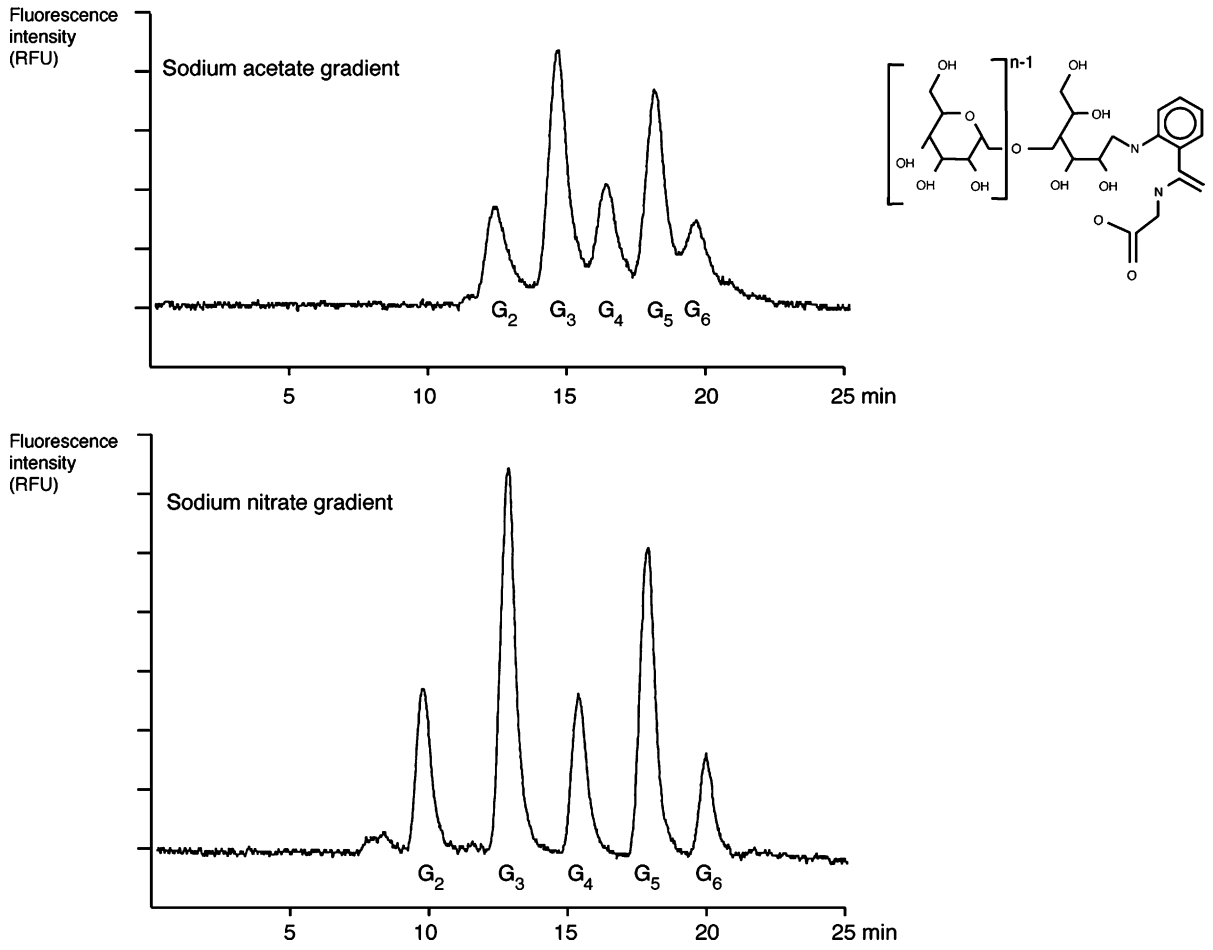

Figure 7. A comparison of acetate versus nitrate pushing agent on the elution of ABGly-labeled saccharide from a PA1 column. High resolution of a homologous series of ABGly derivatives (**1**) is achieved by favoring nitrate over acetate pushing agent.

maltohexaose ([4)-α-D-Glc*p*-(1-]$_6$), G_6, 95%) were from Sigma Chemical (St. Louis, MO). Borane–diethylamine was from Aldrich Chemical (Milwaukee, WI, USA). Sephadex G-10, LH-20 was purchased from Amersham Biosciences (Piscataway, NJ, USA).

4.2. Preparation of labels with different electrostatic charges

4.2.1. ABGly (1), *N*-(2-aminobenzoyl)glycine. To a solution of 2-nitrobenzoic acid (1.7 g, 10 mmol) in anhydrous tetrahydrofuran (10 mL) were added oxalyl chloride (1.75 mL, 20 mmol) and a catalytic amount of *N*,*N*-dimethylformamide (100 μL), sequentially and dropwise. The mixture was stirred at room temperature for 2 h and then the solvent and excess oxalyl chloride removed by evaporation under diminished pressure. The remaining syrup was co-evaporated with toluene (2 × 10 mL) to give the nitrobenzoyl chloride as colorless syrup, which was then used without further purification.

The syrup (1.9 g) and glycine benzyl ester (toluenesufonate salt) (4.0 g, 12 mmol) were successively dissolved in anhydrous tetrahydrofuran (15 mL) and cooled to 0 °C. Triethylamine (2.1 mL, 15 mmol) was added. After reaction for 5 h at 0 °C, the mixture was diluted with EtoAc (80 mL) and washed successively with saturated $NaHCO_3$, 5% (v/v) HCl, and water. The organic layer was dried (Na_2SO_4), filtered, and evaporated. The residue was crystallized from toluene to give *N*-(2-nitrobenzoyl)glycine benzyl ester (**4**) (3.5 g, 72%) as white crystals: mp 76–77 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.107–7.552 (m, 4H, H-3, 4, 5, 6), 7.387 (s, 5H, C_6H_6), 6.401, d, 1H, *J* 5.8 Hz, NH), 5.243 (s, 2H, $CH_2C_6H_5$), 4.319 (d, 2H, *J* 5.8 Hz, NCH_2).

Catalytic hydrogenation of **4** (472 mg, 1.5 mmol) was carried out in 95% EtOH (20 mL) in the presence of 10% palladium on charcoal (100 mg) under a hydrogen atmosphere. After 16 h, the catalyst was removed by filtration through a Celite pad. The filtrate was evaporated to give the crude product (250 mg), which was further refined through a column (1 × 15 cm) of Sephadex LH-20. This column was re-equilibrated and eluted with $CHCl_3$–95% EtOH (1:1, v/v) to afford *N*-(2-aminobenzoyl)glycine (**1**) as an off-white solid: mp 118–120 °C.

4.2.2. ABGlyAmide (2), *N*-(2-aminobenzoyl)glycinamide. *N*-(2-Nitrobenzoyl)glycine benzoic ester (**4**) (472 mg, 1.5 mmol) was dissolved in MeOH (8 mL) and treated with concentrated NH_4OH (8 mL) for 10 h at 20 °C. The solution was evaporated and the solid residue crystallized from ethanol to give *N*-(2-nitrobenzoyl)glycinamide (**5**) (276 mg, 82%) as a white solid: mp 193–195 °C; ^{1}H NMR (300 MHz, Me_2SO-d_6+5% D_2O): δ 8.012 (d, 1H, *J* 7.8 Hz, H-3), 7.788 (t, 1H, *J* 7.3 Hz, H-5), 7.707–7.364 (M, 2H, H-4, 6), 3.800 (s, 2H, NCH_2). Catalytic hydrogenation of **5** was performed as for the preparation of **1** to afford *N*-(2-aminobenzoyl)glycinamide (**2**) (170 mg, 88%).

4.2.3. ABGlyDIMED (3), *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine. 2-Nitrobenzoyl chloride, prepared as described previously from 2-nitrobenzoic acid (1.67 g, 10 mmol), was reacted with *N*,*N*-dimethylethylenediamine (0.92 mL, 13 mmol) and Et_3N (2.1 mL, 15 mmol) in tetrahydrofuan (10 mL) for 5 h at 0 °C. The reaction was evaporated and the residue partitioned between chloroform (50 mL) and aqueous $NaHCO_3$ (10 mL). The organic layer was washed with water (2×10 mL), dried (Na_2SO_4) sodium sulfate, and evaporated. The solid was crystallized from hexane–EtOAc to give *N*,*N*-Dimethyl-*N*′-(2-nitrobenzoyl)ethylenediamine (**6**) (1.59 g, 67%): ^{1}H NMR (300 MHz, $CDCl_3$ + 2% D_2O): δ 8.063 (d, 1H, *J* 7.8 Hz, H-3), 7.688 (t, 1H, *J* 7.4 Hz, H-5), 7.643–7.523 (m, 2H, H-4, 6), 3.562 (t, 2H, *J* 5.6 Hz, NHCH2), 2.623 (t, 2H, *J* 5.6 Hz, CH_2NMe_2), 2.317 [s, 6H, $N(CH_3)_2$].

Catalytic hydrogenation of **6** (474 mg, 2.0 mmol) was performed in EtOH (15 mL) as described for the preparation of **1** and the product was purified by column chromatography on silica gel using 7:1 (v/v) $CHCl_3$–EtOH as the eluent to give *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)ethylenediamine (**3**) (369 mg, 91%) as a colorless syrup.

4.3. Labeling of oligosaccharides by reductive amination

Approximately 1 mg of sugar, individually or a ([4)-α-D-Glc*p*-(1-]$_{2-6}$) maltosaccharide mixture, were dissolved in 100 μL of 6% (v/v) acetic acid by heating at 60 °C, followed by vortexing. A fivefold molar excess of the labeling agent was added as solid, heated, and vortexed into solution. The borane reductant (10 μmol) was added, vortexed, and the reaction allowed to proceed for 2 h at 60 °C, after which the reaction mixture was cooled to room temperature.

A Sephadex G-10 column (1.5×50 cm), equilibrated in 6% (v/v) HOAc, was used to resolve unreacted free label from conjugate at an elution flow rate of 0.5 mL/min. Three mL fractions were collected and 200 μL aliquots monitored by absorbance ratio at 280 and 310 nm. Fractions eluting early that contained labeled sugars were pooled and evaporated under reduced pressure using a rotary evaporator at 60 °C with repeated addition of MeOH. The residue was dissolved in double-distilled, nylon-filtered water to a final concentration of ~10 mg/mL and frozen.

Where required, chart tracing of column eluents were digitized using Un-Scan-It (Silk Scientific, Orem, UT, USA). PeakFit v3.18 (Jandel Scientific, Sausalito, CA, USA) was used to fit independent, nonsymmetric Haarhoff Van der Linde functions to the data using the Marquardt–Levenburg NonLinear Least Squares Algorithm. All fits successfully converged.

4.4. Absorption and fluorescence emission spectra

Absorption and fluorescence emission spectra were measured for the labeled oligosaccharides (Table 1) using a Varian Cary 3C UV vis spectrometer and an Eclipse spectrofluorometer (Varian, Palo Alto, CA, USA).

4.5. Separation of labeled oligosaccharides using graphitized carbon column (GCC) chemistry

Separation with a porous graphitic carbon column (Shandon Hypercarb 100×4.6, 7 μm; Alltech Associates, Deerfield, IL, USA) was performed using a HPLC system consisting of Gilson piston pumps model 302 (Gilson Medical Electronics Inc., Middleton, WI, USA) and a Rheodyne 7125 injector. Detection was by a model LS 40 fluorescence detector (Perkin–Elmer Ltd., Beaconsfield, Buckinghamshire, UK).

A 30% (v/v) acetonitrile (J.T. Baker Inc., Philipsburg, NJ, USA) solution containing 0.05% (v/v) TFA (Fluka Chemical Corp., Ronkonkoma, NY, USA) was used as the mobile phase. Separation was performed isocratically under ambient temperature for all oligosaccharide derivatives of neutral, acidic, and basic anthanilamides.

4.6. Separation of labeled oligosaccharides using Carbopac PA-1 chromatography

Separation was achieved by high-performance anion-exchange chromatography (HPAEC) using a Dionex Bio LC 4000 system and Carbopac PA-1 (250×4 mm) column (Dionex Corp., Sunnyvale, CA, USA). Detection was made with either a pulsed amperometric detector (PAD) equipped with a gold working electrode or with a LS 40 fluorescence detector, or by both detectors in tandem. The mobile phase buffers contained 0.1 M NaOH and one of the following pushing agents; 1 M sodium acetate in a linear gradient from 2% to 50% (v/v) over 25 min for oligosaccharides labeled with neutral and basic anthranilamide derivatives, or 0.2 M sodium nitrate for negatively charged maltosac-

charides using a linear gradient of 10–35% (v/v) over 25 min.

4.7. Solid-phase recovery on aminopropyl column

A simple method for the efficient recovery of anthranilamide derivatives was through binding to aminopropyl solid-phase extraction cartridges (Sep-Pac; Waters Corp., Milford, MA, USA) that were pre-equilibrated in high acetonitrile concentrations (aq) to facilitate binding. Elution from the solid-phase resin was accomplished by lowering acetonitrile concentrations to below ~40% (v/v) (aq), or with water elution.

Acknowledgements

This work was supported by National Multiple Sclerosis Society grant RG3324A1/T to CGB and NIH grants GM36044 and GM61406 to ALH and DK009970 to YCL.

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