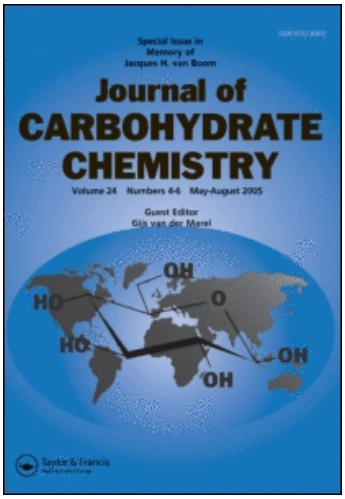

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SYNTHESIS OF GLYCOSYLAMINES: IDENTIFICATION AND QUANTIFICATION OF SIDE PRODUCTS

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ABSTRACT

The synthesis of some glycosylamines (1-amino-1-deoxy-D-glucose, 1-amino-1-deoxy- D-galactose and 1-amino-1-deoxylactose) was carried out by treatment of the corresponding reducing sugars with ammonium hydrogencarbonate in concentrated ammonia. The reaction mixture was first analyzed by capillary electrophoresis with indirect absorbance detection and high performance anion-exchange chromatography with pulsed amperometric detection. Beside glycosylcarbamate, a known reaction by-product, fructose and lactulose were detected during the synthesis of 1-amino-1-deoxyglucose and 1-amino-1-deoxylactose, respectively. Quantification of glycosylamines was carried out by micellar electrokinetic chromatography with UV detection of their 9-fluorenylmethyloxycarbonyl (Fmoc) derivatives; lactulosylamine was thus detected in the synthesis mixture of 1-amino-1-deoxylactose. The Fmoc-glycosylamines were easily purified from the other components of the crude synthesis mixtures.

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INTRODUCTION

Glycosylamines are currently used for the preparation of glycopeptides, detergents, *N*-hydroxysuccinimidyl esters and for the synthesis of glycopolymers.[1–3] There are two main drawbacks related to the preparation and use of glycosylamines: their low stability, due to rapid hydrolysis in neutral or weakly acidic solutions,[1–4] and the formation of *N*-glycosylcarbamate and of diglycosylamine as secondary reaction products.[1–3,5–8] Besides the interest to identify and quantify reaction side-products, it is important to provide an accurate evaluation of product yields in order to guide studies for reaction optimization. Furthermore, amine by-products might interfere when glycosylamines are used to synthesize substances of specific biological relevance. Among the reported methods for glycosylamines synthesis,[1,4,5] we investigated that performed with ammonium hydrogencarbonate in concentrated ammonia solution at 42°C, which is claimed to give quantitative yields with negligible amounts of side-products.[1] Capillary electrophoresis/indirect UV detection (CE/indirect UV), anion-exchange chromatography/pulsed amperometric detection (HPAEC-PAD), micellar electrokinetic chromatography (MEKC)-UV and ion spray mass spectrometry allowed for the quantification of many components in the synthesis mixtures: the starting reducing sugars and their isomerization products, glycosylcarbamates, glycosylamines and amino side-products. A method for purification of glycosylamines is also presented.

RESULTS AND DISCUSSION

Analysis of the Crude Synthesis Mixtures

CE/indirect UV [9–11] turned out to be an ideal tool to analyze the untreated synthesis mixtures of glycosylamines. A rapid migration of the anionic analytes was achieved by adding tetradecyltrimethylammonium bromide (TTAB)[12] to the separation buffer. The high pH (12.5) of the medium also prevented the hydrolysis of the formed glycosylamines.[1,4] In Figure 1 the electropherograms of glucosylamine (Glc-1-NH_2) (A), galactosylamine (Gal-1-NH_2) (B) and lactosylamine (Lac-1-NH_2) (C) synthesis mixtures are shown.[13] In each panel, peak 3 refers to the starting sugar. In Figure 1A, peak 2 can be assigned to glucosylcarbamate.[1,3,5] The strong acidity of the carboxylic group induces the short migration time. Moreover, the disappearance of peak 2 was observed on decreasing the sample solution pH (from 10 to 4). At the same time an increase in the glucose signal occurred. Those evidences can be accounted for by the low stability of carbamates under acidic conditions.[14] Similarly, peaks 2 in panels B and C were assigned to galactosylcarbamate and lactosylcarbamate, respectively. Due to the high alkalinity of the synthesis medium, peaks 3′ in Figure 1 were attributed to fructose (panel A) and lactulose (4-*O*-β- D-galactopyranosyl- D-fructofuranose) (panel B), resulting from isomerization of glucose and lactose, respectively.[15–17] Such assignments were confirmed by comparison with standards.

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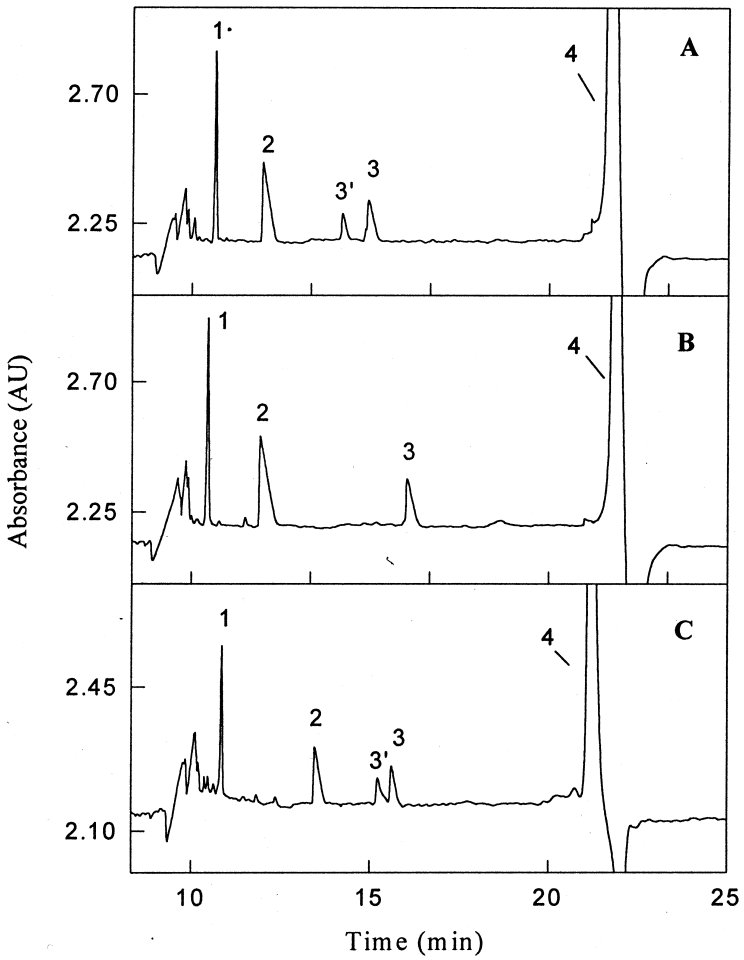

Figure 1. CE/ indirect UV of untreated freeze-dried synthesis mixtures (5 mg in 1 mL of 0.1 mM NaOH) of: (A) Glc-1-NH_2: (1) 0.7 mM galacturonic acid (i.s.), (2) glucosylcarbamate, (3′) fructose, (3) glucose, (4) contribution from glucosylamine; (B) Gal-1-NH_2: (1) 0.7 mM galacturonic acid (i.s.), (2) galactosylcarbamate, (3) galactose, (4) contribution from galactosylamine; (C) Lac-1-NH_2: (1) 0.7 mM galacturonic acid (i.s.), (2) lactosylcarbamate, (3′) lactulose, (3) lactose, (4) contribution from lactosylamine.

No isomers of galactose in the Gal-1-NH_2 mixture were found. Due to the scarce ionization, glycosylamines contributed to the peak 4. Unfortunately, since the signal did not re-attain the baseline, an accurate quantification of glycosylamines was precluded. CE/indirect UV monitored formation of side-products. Figure 2A shows the molar percentages of glucose, fructose and glucosylcarbamate as a function of the Glc-1-NH_2 synthesis time.

Fructose was detected only after 12 hours. Analogous data for the crude Lac-1-NH_2 mixture showed that lactulose could be observed after 4 hours (Figure 2B). These results were confirmed analyzing the untreated synthesis mixtures by

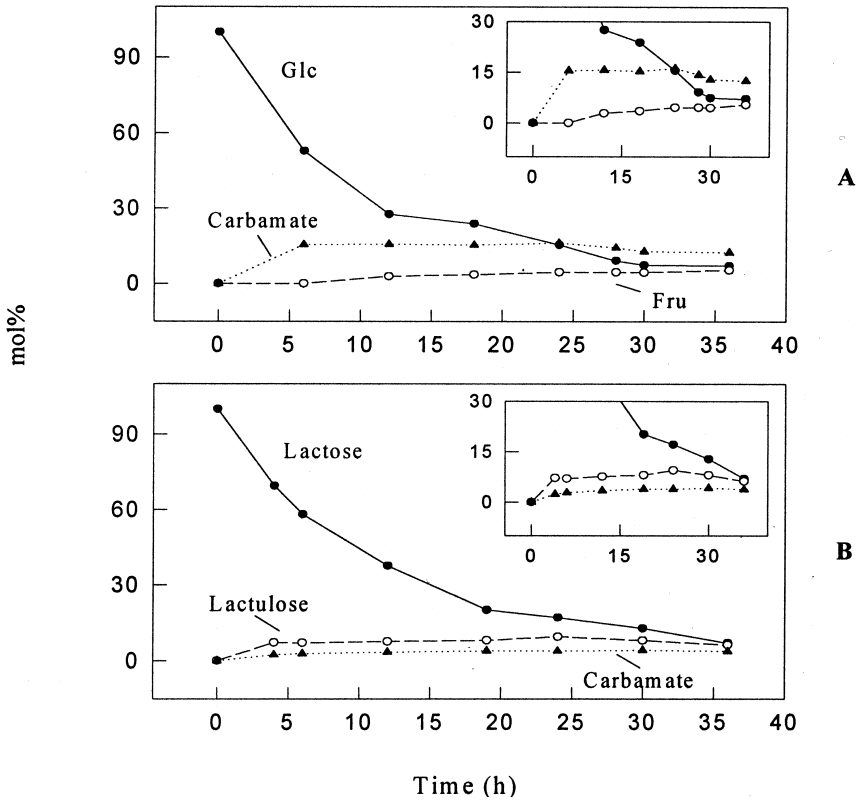

Figure 2. Glycosylamine synthesis monitored by CE/indirect UV: (A) mol percentages of glucose, fructose and glucosylcarbamate, [(mol(x)$_{36h}$/mol(Glc)$_0$)×100], as a function of the Glc-1-NH$_2$ synthesis time; (B) mol percentages of lactose, lactulose and lactosylcarbamate, [(mol(x)$_{36h}$/mol(Lac)$_0$)×100], as a function of the Lac-1-NH$_2$ synthesis time (percentages of glycosylcarbamates have been indirectly evaluated by the yield of the reactions, the identity of the products and the mass balance). In the inserts the magnifications of the corresponding graphics are reported.

HPAEC-PAD[18–20] (Figure 3). Quantification of glycosylamines was again precluded, their retention time being close to the dead time. In crude Glc-1-NH$_2$ mannose was also detected (about 6% of the amount of glucose), as expected on the basis of glucose isomerization occurring in alkaline conditions.[16] The presence of carbamate derivatives (not shown here) was detected by HPAEC-PAD using highly alkaline eluents, as reported elsewhere.[21]

In order to verify whether or not those isomers could form glycosylamines during the syntheses of Glc-1-NH$_2$ and Lac-1-NH$_2$, acidification of the crude Glc-1-NH$_2$, Gal-1-NH$_2$ and Lac-1-NH$_2$ solutions was carried out (Table 1). As expected,[1–4] a net increment of glucose, galactose and lactose percentages was observed, due to the hydrolysis of the corresponding glycosylamines. The amount of fructose remained unchanged and only lactulose increased from 6.2% to 11.6%, suggesting that lactulosylamine could be formed during the lactosylamine synthesis.

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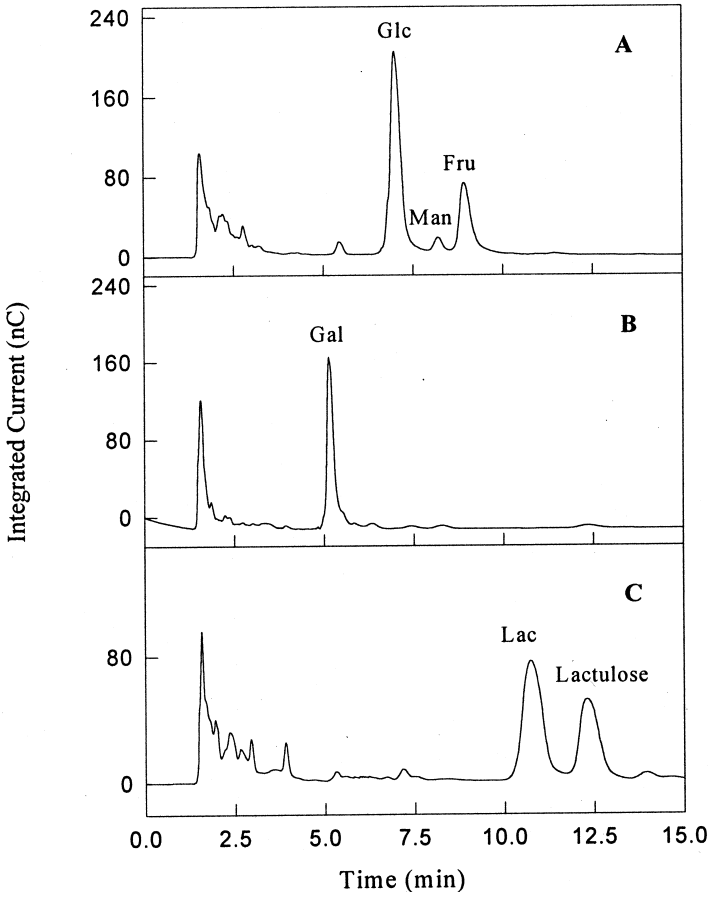

Figure 3. HPAEC-PAD analysis of untreated freeze-dried synthesis mixtures of glycosylamines dissolved in 0.1 mM NaOH: (A) Glc-1-NH_2 (4.0 mg/mL); (B) Gal-1-NH_2 (5.0 mg/mL); (C) Lac-1-NH_2 (3.0 mg/mL).

Table 1. Mol Percentages of Sugars in the Synthesis Mixtures of Glycosylamines[a, b] at Different pH

	Glc-1-NH_2		Gal-1-NH_2	Lac-1-NH_2	
pH	*Glc (%)*[c]	*Fru (%)*[d]	*Gal (%)*[e]	*Lac (%)*[f]	*Lactulose (%)*[g]
12	7.1	5.4	6.5	7.0	6.2
8	7.2	5.2	6.8	7.3	6.5
6	20.2	4.9	23.9	19.9	10.3
4	47.0	5.0	54.6	56.0	11.6

a. injections of freeze-dried reaction mixtures (2.0 mg/mL) were performed 30 min after stable pH readings were attained; b. quantification of each species was achieved using calibration curves of corresponding genuine standards (data consistent with literature9); c. [mol(Glc_{36h})/mol(Glc_0) × 100; d. [mol(Fru_{36h})/mol(Glc_0)] × 100; e. [mol(Gal_{36h})/mol(Gal_0)] × 100; f. [mol(Lac_{36h})/mol(Lac_0)] × 100; g. [mol($Lactulose_{36h}$)/mol(Lac_0)] × 100.

Quantification and Purification of Glycosylamines

Direct quantification of glycosylamines and of other amino compounds present in the synthesis mixture was accomplished by a derivatization of the reaction mixtures with *N*-fluorenyl-methoxycarbonyl (Fmoc) chloride.[22] Figure 4 shows the MEKC-UV electropherograms [23,24] of Glc-1-NH_2 (A), Gal-1-NH_2 (B) and Lac-1-NH_2 (C) reaction mixtures treated with Fmoc-chloride. For Glc-1-NH_2 the formation of detectable amounts of fructosylamine was excluded. In the electropherogram of Fmoc-Lac-1-NH_2 synthesis mixture (Figure 4C), the small signal was ascribed to lactulosylamine, as confirmed by comparison with the mobility of Fmoc-lactulosylamine *ad hoc* synthesized.

Although the detected amount of lactulosylamine was only about 8% with respect to the main amino-sugar, its interference in the subsequent reactions of lactosylamine cannot be excluded. No diglycosylamines were detected as confirmed also by the analysis of the Fmoc- treated mixture by ion-spray mass spectrometry (data not shown). The methods used allowed for identification of the

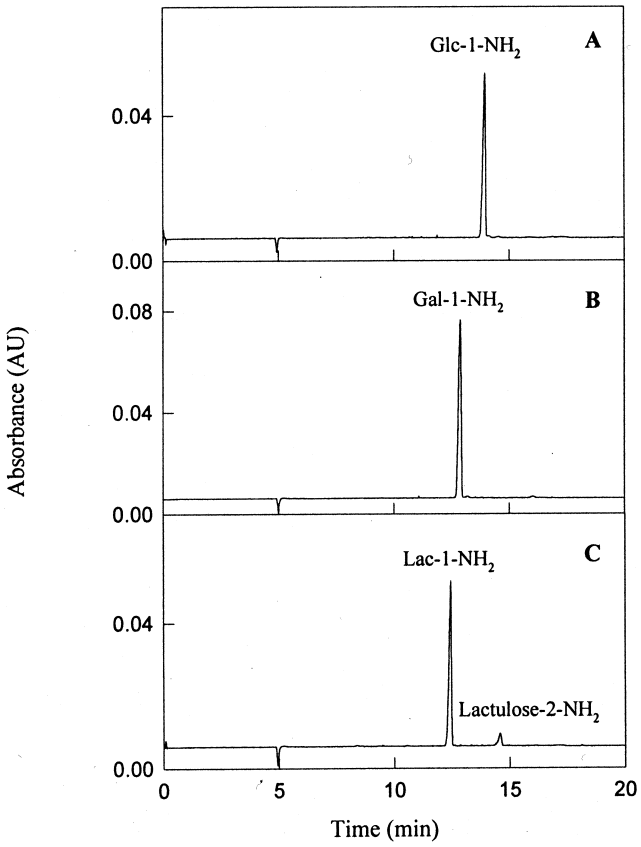

Figure 4. Electropherograms of crude glycosylamines derivatized with Fmoc; (A) Glc-1-NH_2 (0.8mg/mL); (B) Gal-1-NH_2 (1.26 mg/mL); (**C**) Lac-1-NH_2 (1.0 mg/mL).

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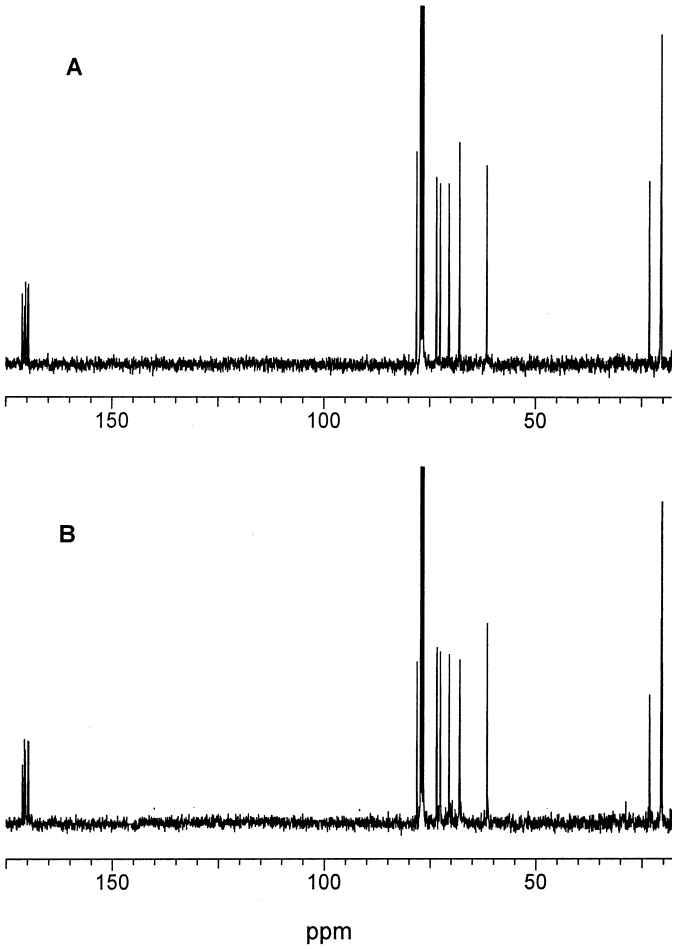

Figure 5. ^{13}C NMR spectra of: (A) peracetylated Glc-1-NH_2 from crude synthesis mixture purified with flash chromatography; (B) peracetylated Glc-1-NH_2 purified as Fmoc-derivative. Peak assignments (ppm): 20.57–20.71 ($CO\underline{C}H_3$), 23.38 ($CO\underline{C}H_3$), 61.60 (C-6), 68.09 (C-4), 70.58 (C-2), 72.65 (C-5), 73.51 (C-3), 78.20 (C-1(β)), 169.53–171.06 ($\underline{C}OCH_3$).

amino components contributing to the unresolved peak 4 in Figure 1C. As the reactions with Fmoc were quantitative, the yields of the synthesis of glycosylamines could be calculated by measuring the amount of the Fmoc-glycosylamines formed:[25] the yields were equal to 75%, a value lower than that reported.[1] The obtained Fmoc-derivatives were readily separated from the reducing sugars on a C-18 cartridge, and the free glycosylamines were then recovered by simple treatment with aqueous ammonia. [22] The success of the purification procedure was confirmed by ^{13}C NMR, as clearly shown by the coincidence of the spectra reported in Figure 5: panel A is relative to peracetylated Glc-1-NH_2 separated from the crude amination reaction and panel B refers to peracetylated Glc-1-NH_2 after Fmoc removal.

CONCLUSIONS

Reliable methods have been developed for the analysis of glycosylamines and of side-products formed during their preparation. In the synthesis mixtures of glucosylamine, the side-products glucosylcarbamate, fructose and mannose were identified and quantified; in the synthesis of lactosylamine, the side-products lactosylcarbamate, lactulose and the corresponding lactulosylamine were detected. In the production galactosylamine, only galactosylcarbamate was found as a side-product.

EXPERIMENTAL

Chemicals

D-glucose, D-galactose, lactose, *N*-fluorenylmethoxycarbonyl chloride, sodium dodecylsulfate and sodium tetraborate were from Sigma (St. Louis, MO, USA). Tetradecyltrimethylammonium bromide and the 50% (w/w) NaOH solution used for the eluent preparation in HPAEC-PAD were from Aldrich (St. Louis, MO, USA). Reversed phase chromatography was performed on Sep-Pak® C18 cartridges (Waters, Milford, USA).

Preparation of Glycosylamines[1]

The reducing carbohydrate (0.2 M) and ammonium hydrogen carbonate (0.2 M), dissolved in aqueous 16 M ammonia, were left for 36 h at 42°C.

Reaction of Glycosylamines with Fmoc[22]

0.1 mmol of glycosylamine in saturated aqueous sodium bicarbonate (5 mL) were added to Fmoc-Cl (0.4 mmol) in dioxane (5.0 mL). The mixture was stirred overnight at room temperature. A C18 cartridge (Sep-Pak®, 10 g) conditioned in water was first washed with water to remove salt and reducing carbohydrates, and then with methanol. The glycosylamine-Fmoc derivative was recovered after concentration and freeze-drying. The reaction was quantitative (gravimetric test on pure commercial Glc-1-NH_2 and Gal-1-NH_2). Fmoc was removed by stirring glycosylamine-Fmoc (0.2 mmol) in 15% aqueous ammonia (20 mL) overnight. The resulting mixture was filtered (0.45 μm), concentrated and freeze-dried to obtain the glycosylamine (96%). TLC (propanol: 1N ammonia: water, 6:2:1) showed the presence of pure glycosylamine, and no UV-absorbing material.

Capillary Electrophoresis

The system was an Applied Biosystems HPCE Model 270A-HT with Turbochrom Navigator (4.0) software. The fused silica column (72 cm (50 cm to detector) × 50 μm I.D.× 375 μm O.D.) was from Supelco (St. Louis, MO, USA).

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All runs were done at 30°C. Samples were loaded under vacuum at a pressure of 16.9 kPa (1.5 s). Before sample injection, a 2-min conditioning of the capillary with the buffer followed a 2-min washing with 0.1 N NaOH (vacuum pressure 67.6 kPa). CE/indirect UV on glycosylamines solutions in 0.1 mM NaOH was performed using 6 mM sorbate at pH 12.5 + 0.5 mM TTAB [9–12,26] (10 kV; 256 nm at the anode). Positive signals were obtained by reversing the output polarity of the detector. Quantification of Fmoc-glycosylamines was achieved by MEKC using 20 mM tetraborate (pH 9.4) + 25 mM SDS (20 kV, 260 nm at the cathode).

HPAEC-PAD

The system (Dionex, Sunnyvale, CA, USA) consisted of an isocratic pump (IP20), a pulsed amperometric detector (ED40) and PeakNet 5.1 software (10 μL loop). A CarboPac PA1 anion-exchange column (250 × 4 mm I.D.) plus guard column (50 × 4 mm I.D.) were used. The flow-through detection cell contained a gold working electrode (1.0-mm diameter) and an Ag/AgCl reference electrode; the counter electrode was the titanium cell body across the 25 μm thin layer channel from the working electrode. The waveform parameters were $E_{det} = +0.05V$, $t_{det} = 440$ ms ($t_{int} = 240$ ms), $E_{ox} = +0.8V$, $t_{ox} = 180$ ms, $E_{red} = -0.2V$, $t_{red} = 360$ ms, with 1 s response time. The eluent was 0.01 M NaOH + 2 mM $Ba(CH_3COO)_2$ flowing at 1.0 mL/min.

Mass Spectrometry

Mass spectra were recorded on an API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray connected to a syringe pump for sample injection. The solvents were acetone or 50% aqueous methanol containing formic acid (0.1%). The injection flow rate was 0.1 mL/h; the ionspray voltage was 5600 V.

Nuclear Magnetic Resonance

^{13}C NMR spectra were recorded on a Jeol EX400 (100.6 MHz) spectrometer. All experiments were carried out in $CDCl_3$; chemical shifts are reported in ppm downfield from tetramethylsilane. The glycosylamines were peracetylated before their NMR analysis, to achieve stable derivatives.[27] The peracetylated glycosylamines in the crude synthesis mixture were separated from the peracetylated reducing sugars by flash chromatography (ethyl acetate).

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