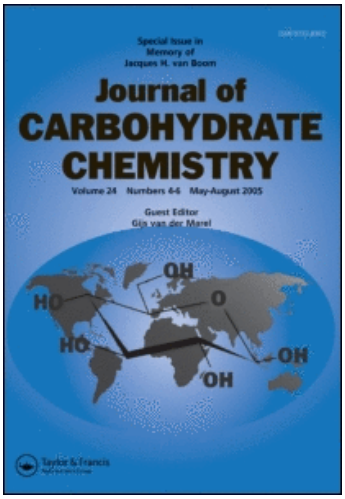

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Derivatization Procedures for Reducing Oligosaccharides, Part 4: Use of Glycosylamines in a Reversible Derivatization of Oligosaccharides with the 9-Fluorenylmethoxycarbonyl Group, and Hplc Separations of the Derivatives

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DERIVATIZATION PROCEDURES FOR REDUCING OLIGOSACCHARIDES[1], PART 4: USE OF GLYCOSYLAMINES IN A REVERSIBLE DERIVATIZATION OF OLIGOSACCHARIDES WITH THE 9-FLUORENYLMETHOXYCARBONYL GROUP, AND HPLC SEPARATIONS OF THE DERIVATIVES

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ABSTRACT

A new, reversible derivatization procedure for reducing oligosaccharides was developed. Reaction of the model compound lacto-*N*-tetraose (β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc) with aqueous ammonium bicarbonate gave the corresponding β-glycosylamine, which was reacted with fluorenylmethoxycarbonyl chloride to give an *N*-fluorenylmethoxycarbonyl β-glycosylamine (FMOC derivative). The free oligosaccharide could be recovered in high yield from the FMOC derivative by brief treatment with 15 % aqueous ammonia. Complex oligosaccharide mixtures from human milk were derivatized with the new procedure, and excellent separations of the derivatives on straight-phase silica HPLC columns were achieved.

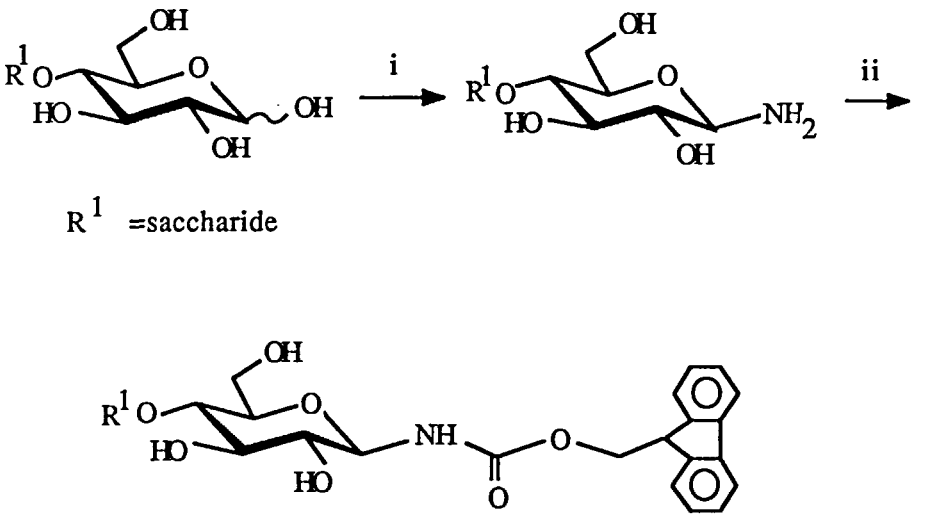
INTRODUCTION

Mixtures of complex oligosaccharides can be obtained from a variety of sources, such as human milk, urine, or hydrolyzates of glycoproteins or glycolipids. Analytical or preparative fractionation of such mixtures by chromatography is often complicated by the fact that each oligosaccharide gives rise to two peaks, due to the slow rate at which the α and β hemiacetal forms interconvert (this fact also complicates the NMR spectra of free oligosaccharides). Therefore, various chemical derivatization procedures have been developed,[2-13] nearly all of them involving irreversible functionalization at the reducing end of the oligosaccharide.

We have previously reported[2,3] reductive amination of oligosaccharides with *p*-trifluoroacetamidoaniline as a *reversible* derivatization procedure. The obtained oligosaccharide derivatives showed good chromatographic properties, and the oligosaccharides could be recovered by treatment of the derivatives with hydrogen peroxide.[3] We now report another reversible derivatization procedure for oligosaccharides, using milder reaction conditions. Human milk oligosaccharides were derivatized (via their glycosylamines) with the 9-fluorenylmethoxycarbonyl (FMOC) group. The chromatographic properties of the obtained derivatives were examined, and a procedure for efficient recovery of the free oligosaccharides was developed.

RESULTS AND DISCUSSION

The FMOC group[14] has been used extensively for protection of primary amines, especially in peptide chemistry. In the present work, it was introduced into the oligosaccharides by conversion of these to their



SCHEME 1. i = aqueous ammonium bicarbonate, ii = FMOC-chloride/sodium bicarbonate/water/dioxane

corresponding glycosylamines by treatment with aqueous ammonium bicarbonate,[15,16] followed by acylation with 9-fluorenylmethoxycarbonyl chloride in water-dioxane using sodium bicarbonate as buffer (Scheme 1). In a model reaction with pure lacto-*N*-tetraose, the yield for this two-step conversion was 66 %. Only the β–glycosidic derivative was observed. The obtained *N*-(9-fluorenylmethoxycarbonyl)glycosylamine (FMOC derivative) was more stable than the previously reported[3] *p*-trifluoroacetamidophenyl derivatives towards air oxidation. Thus, the FMOC derivative of lacto-*N*-tetraose (LNT) could be kept in aqueous solution for more than a month (room temperature, pH 7) without showing signs of degradation when analyzed by TLC. The chromatographic properties of oligosaccharide FMOC derivatives were next examined.

A pre-fractionated pool of human milk oligosaccharides was derivatized with the FMOC group as described above, and the resulting

mixture was analyzed by HPLC, using a straight-phase silica column and chloroform/methanol/water (or aqueous ammonia) mixtures as mobile phase (Fig 2). The use of a straight-phase column was made possible by the higher lipophilicity of the FMOC derivatives. Excellent separation of the components was obtained. Interestingly, the retention times of the oligosaccharide FMOC derivatives were influenced by small amounts of ammonia or acetic acid in the eluant. Thus, the retention time for LNF I could be varied from 52 min (60:35:8 chloroform-methanol-1.25 M aqueous ammonia) to 17.8 min (60:35:8 chloroform-methanol-water), and it could be further decreased by substituting the water for aqueous acetic acid. This apparent pH-dependence of the retention times is being further investigated in our laboratory.

The chromatogram of the FMOC-derivatized oligosaccharide pool obtained on a straight phase silica column was compared with the best obtainable chromatogram (Fig 1) of a similar underivatized oligosaccharide pool on a Nucleosil C-18 column.[17] The improvement in separation after derivatization can be clearly seen. A similar improvement was obtained with a tetrasaccharide pool from human milk (data not shown).

The homogeneity of the HPLC peaks was ascertained by NMR spectroscopy. The structures were verified by comparing the ^{1}H NMR spectra with spectra of the underivatized sugars, and also by FAB-MS.

The free oligosaccharides could be recovered from the FMOC derivatives by treatment with aqueous ammonia followed by mild acidification (Scheme 2). A typical yield for this reaction was 93 % (for the lacto-*N*-tetraose derivative). Thus, the FMOC derivatization is reversible, which makes it more suitable for preparative separations. To investigate the suitability for small scale derivatizations, the spectroscopic properties of the FMOC derivatives were also briefly examined. Typical molar extinction coefficients

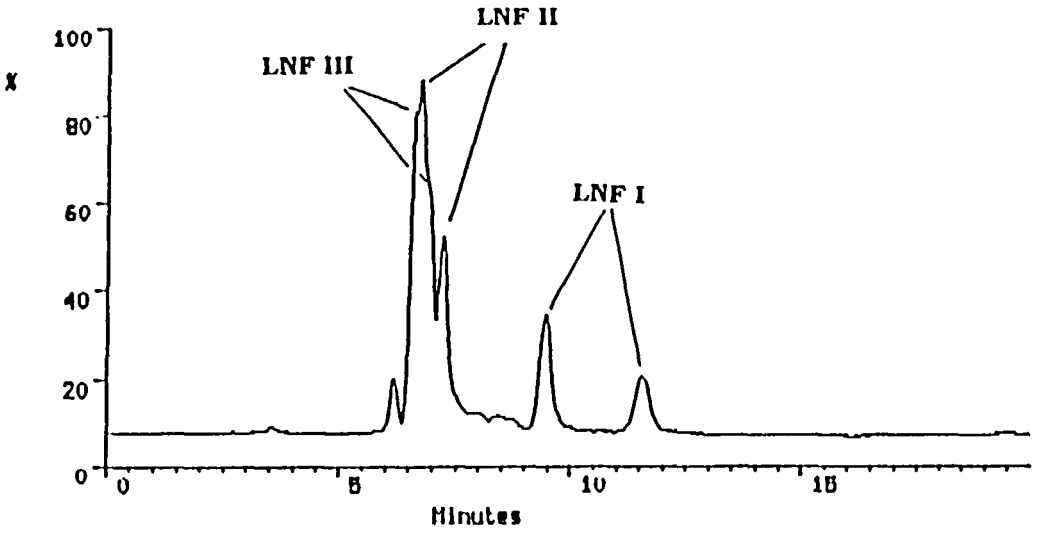


FIG 1. Analytical HPLC chromatogram of a pool of human milk oligosaccharides containing neutral pentasaccharides. Column: Nucleosil C-18, eluant: water, detection: refractive index. Abbreviations:
LNF I = α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc
LNF II = β-D-Gal(1→3)-[α-L-Fuc-(1→4)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc
LNF III = β-D-Gal(1→4)-[α-L-Fuc-(1→3)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc

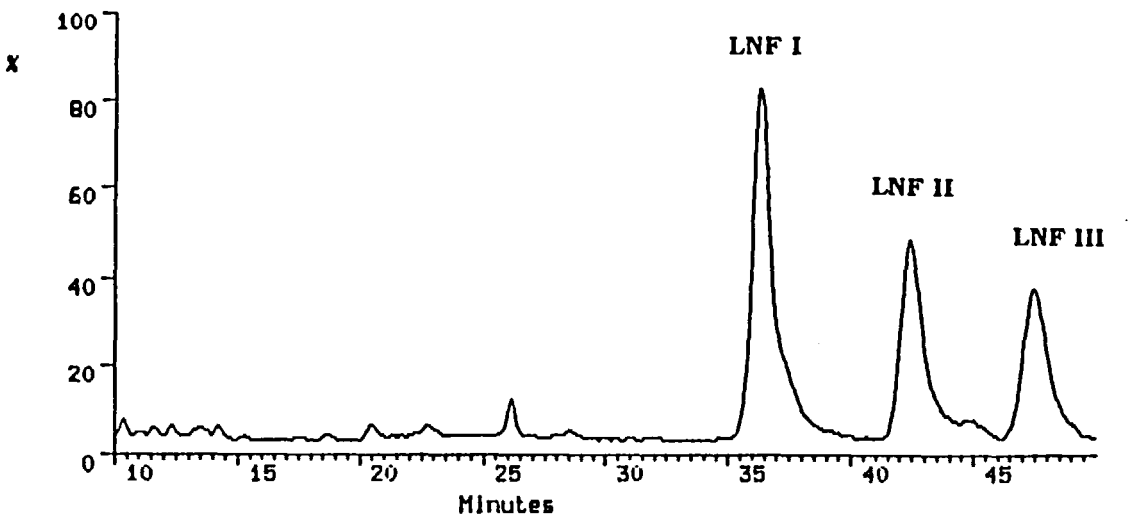
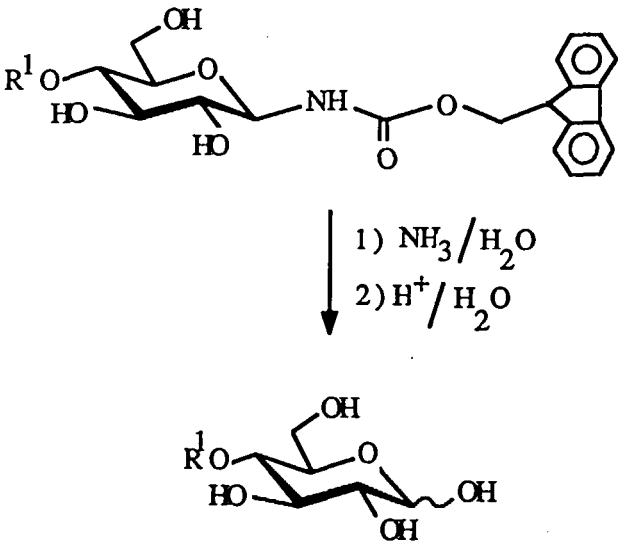


FIG 2. Analytical HPLC chromatogram of an FMOC-derivatized pentasaccharide mixture containing the same saccharides as the mixture in fig 1. Column: Nucleosil Silica (30 cm), eluant: 60:35:8 chloroform-methanol-0.15 M aqueous ammonia, detection: UV (260 nm).



SCHEME 2.

(ε) of 22 500 at 256 nm were found, which gives a reasonably low detection limit. Fluorescence properties were not examined.

In conclusion, a mild and efficient reversible derivatization procedure has been developed, that facilitates the chromatographic separation of oligosaccharide mixtures. The procedure should be applicable to most sugars with a reducing end. Another aspect of the *N*-(9-fluorenylmethoxycarbonyl) glycosylamines described here is that, as temporarily protected oligosaccharides, they are potentially useful in further synthetic transformations. This possibility is currently under investigation.

EXPERIMENTAL

General Procedures. Concentrations were performed at <40 °C (bath). Optical rotations were recorded at 21 °C with a Perkin-Elmer 241

polarimeter. NMR spectra were recorded at 27 °C with a Bruker AM 500 instrument, using acetone (δ 2.225, ^{1}H NMR; 32.2, ^{13}C NMR) as internal standard. Only selected NMR data are given. The FAB-MS spectra were recorded with a VG ZAB-SE mass spectrometer. The primary beam consisted of xenon atoms with a maximum energy of 8 keV. The samples were dissolved in thioglycerol and the positive ions were extracted and accelerated over a potential of 10 kV. Thin layer chromatography was performed on silica gel 60 F_{254} (Merck, Darmstadt, FRG) using the following eluant systems: A, 4:3:3:2 ethyl acetate-acetic acid-methanol-water, B, 10:5:1 chloroform-methanol-water, C, 11:9:2.5 chloroform-methanol-water. The spots were visualized by charring with 5 % aqueous sulfuric acid and by UV detection (254 nm). Reversed phase silica gel chromatography was performed on Bond Elut C-18 cartridges (Analytichem International, Harbor City, USA). 9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl) was purchased from Fluka AG (Buchs, Switzerland). Organic solvents were of p.a. quality.

Oligosaccharide mixtures were obtained by subjecting human milk to, succesively, centrifugation, protein precipitation, ultrafiltration, and repeated gel filtration.[18] The HPLC apparatus included a Waters M-45 pump and a Waters U6K injector. Detection of FMOC derivatives was performed with a Waters 484 UV detector. The column used was a Nucleosil-Sil (5μm, 300x10 mm, Skandinaviska GeneTec AB, Kungsbacka, Sweden), and elution was carried out with a mixture of chloroform, methanol and aqueous ammonia. The flow rate was 3.0 mL/min and the wavelength for UV detection was 260 nm.

Preparation of oligosaccharide glycosylamines: The glycosylamines were prepared essentially as described,[15,16] except for the fact that the ion exchange procedure was omitted. Briefly, after reacting 100 mg oligosaccharide in 5.0 mL water with excess ammonium bicarbonate for 5

days at room temperature the solution was diluted with water (100 mL) and concentrated to half the volume. This procedure was repeated twice. After lyophilization of the solution, the product was used directly in the next step.

Preparation of FMOC derivatives from oligosaccharide glycosylamines: To a cooled (ice) solution of oligosaccharide glycosylamine (0.1 mmol) in saturated aqueous sodium bicarbonate (5.0 mL) was added FMOC-Cl (0.4 mmol) in dioxane (5.0 mL) dropwise while stirring. The mixture was allowed to attain room temperature, then further stirred overnight. TLC (system A) then showed a single UV-absorbing and charring spot, excess reagent and traces of free reducing sugar. Water (50 mL) was added and the mixture was neutralized with aqueous HCl and washed with diethyl ether (3 x 20 mL). The ether solutions were pooled and extracted with 50 mL water. The combined aqueous phases were concentrated to about 20 mL and applied to a silica gel reversed phase column (Mega Bond Elut C-18, 5 g column) conditioned in water. The column was washed with water (100 mL) to remove salt and reducing oligosaccharide. The column was then eluted with 75% aqueous methanol. Fractions (5 mL) were collected and analyzed by TLC. Appropriate fractions were concentrated and lyophilized.

FMOC derivative of Lacto-N-tetraose (1): Treatment of lacto-*N*-tetraose (100 mg) as described above gave crude glycosylamine (105 mg), which, after treatment with FMOC-chloride (150 mg) and processing, gave pure 1 (87 mg, 66 %); $[\alpha]_D$ -10° (*c* 0.1, water); ^{1}H NMR (acetone-d_6 : deuterium oxide, 2:1) δ 7.95 (d, J =7.5 Hz, ArH), 7.83 (d, J=7.2 Hz, ArH), 7.53 (t, ArH), 7.46 (t, ArH), 4.93 (d, J=8.3 Hz, GlcNAc H-1), 4.89 (d, J=9.3 Hz, Glc H-1), 4.59 (d, J=7.6 Hz, Gal H-1), 4.55 (d, J=7.2 Hz, Gal H-1), 2.13 (s, MeCONH); ^{13}C NMR δ 104.5, 104.0, 103.2 (Gal and GlcNAc C-1), 82.8 (Glc C-1), 55.5 (GlcNAc C-2), 47.5 (ArCH), 23.2 (MeCONH).

Anal. Calcd for $C_{41}H_{56}N_2O_{22}$: C, 53.0; H, 6.1; N, 3.0. Found: C, 51.1; H, 5.9; N, 3.0. A FAB-MS spectrum showed an $[M+1]^+$ ion at m/z 929.

Transformation of Lacto-N-tetraose FMOC derivative (1) into Lacto-N-tetraose: A solution of (1) (20 mg) in 15 % aqueous ammonia (5.0 mL) was stirred at room temperature overnight. Then the mixture was filtered through an 0.45 μm filter, evaporated to half the volume and lyophilized. TLC showed the presence of pure glycosylamine, and no signs of UV-absorbing material. The yield was 14.1 mg (93%). Free reducing lacto-*N*-tetraose was obtained in quantitative yield upon letting a solution of the glycosylamine in water (2.0 mL) and acetic acid (100 μL) stand for 4 h at room temperature, followed by lyophilization of the solution.

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