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New Derivatization and Separation Procedures for Reducing Oligosaccharides

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4-Trifluoroacetamidoaniline was reacted with reducing oligosaccharides in the presence of sodium cyanoborohydride to give aminoalditol derivatives, useful for linkage to proteins or solid matrices. A mixture of reducing oligosaccharides, difficult to separate by HPLC, was treated in the same way. The resulting derivatives were easily separated by HPLC.

Oligosaccharides covalently linked to proteins, lipids, or solid matrices are useful as antigens or as affinity chromatography ligands. Using reducing oligosaccharides isolated from natural sources, conjugates [1, 2] with molecules containing amino groups have been prepared by reductive amination [3], aldonate coupling [4], or by a route [5, 6] involving reductive amination to form an oligosaccharide derivative of 4-aminophenylethylamine. This derivative is then, after conversion to the isothiocyanate, coupled to amino groups. Although the latter method involves an extra step, it is often preferred for making oligosaccharide-protein conjugates, since isothiocyanates react fast and in high yield with amino groups in proteins.

We have examined the products formed on reductive amination of lacto-*N*-tetraose with 4-aminophenylethylamine. Different compounds were obtained depending on the conditions used. These results suggested the use of other amines as "linkers". We have used 4-trifluoroacetamidoaniline (TFAN) to prepare reductive amination derivatives of several oligosaccharides isolated from natural sources. These derivatives could be converted to isothiocyanate derivatives when desired by treatment with aqueous ammonia followed by thiophosgene. It was also found that reductive amination of a complicated mixture of reducing oligosaccharides with TFAN gave derivatives which were easily separated by HPLC.

Abbreviations: TFAN, 4-trifluoroacetamidoaniline; lacto-*N*-tetraose, $LcOse_4$; lacto-*N*-fucopentaose I, IV^2Fuc-$LcOse_4$; lacto-*N*-fucopentaose II, III^4Fuc-$LcOse_4$; lacto-*N*-fucopentaose III, III^3Fuc-$nLcOse_4$; lacto-*N*-difucohexaose I, IV^2Fuc,III^4Fuc-$LcOse_4$; lacto-*N*-hexaose, $II^6Gal\beta1$-4GlcNAc-$LcOse_4$; 3′-sialyllactose, II^3NeuAc-Lac; chitotriose, GlcNAcβ1-4GlcNAcβ1-4GlcNAc; A-tetrasaccharide, GalNacα1-3 [Fucα1-2]Galβ1-4Glc.

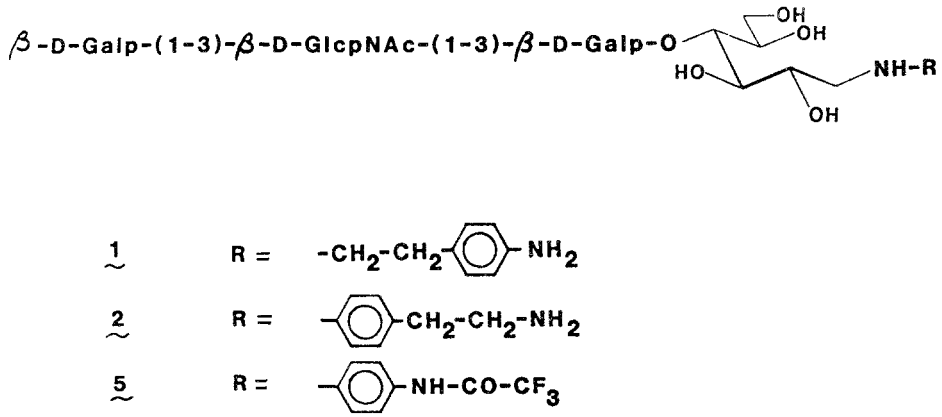
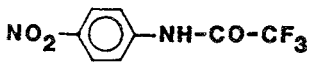


Figure 1. Structures of lacto-*N*-tetraose derivatives.

Results and Discussion

Following the procedure of Jeffrey *et al.* [6], lacto-*N*-tetraose was dissolved in 4-aminophenylethylamine and the resulting mixture was then treated with sodium borohydride. The ^{13}C-NMR spectrum of the major product showed, *inter alia*, methylene signals at 31.6 and 49.8 and quarternary carbon signals at 127.9 and 146.0 p.p.m The corresponding signals for 4-phenylethylamine occur at 37.9, 42.9, 131.6, and 144.8 p.p.m. Therefore, since the larger shift changes occur around the aliphatic amino group of the 4-phenylethylamine moiety, the sugar is attached here and the product has the structure **1** (Fig. 1). In contrast, when lacto-*N*-tetraose was reacted with 4-aminophenylethylamine and sodium cyanoborohydride in aqueous solution at pH 8, as described [5], a major product was obtained with a ^{13}C-NMR spectrum clearly different from that of **1**. The signals from the 4-aminophenylethyl moiety were at 32.8, 41.6, 127.9 and 147.5 p.p.m., indicating the structure **2** (Fig. 1) for this compound. Thus, under these conditions, reaction of the aromatic amino group is faster. Experiments using lactose, sodium cyanoborohydride and either aniline or benzylamine verified this conclusion. As judged by TLC, reaction with aniline was completed within 24 h, whereas with benzylamine only 50% of the lactose had been converted after one week.

The facts presented above prompted us to investigate other aromatic amines. A suspension of 4-nitroaniline was reacted with lacto-*N*-tetraose and sodium cyanoborohydride in ethanol-water solution at pH 6. After 24 h, most of the sugar, according to TLC, remained intact, probably due to low solubility and/or low reactivity of the amine. To obtain a more reactive amine, 4-nitroaniline was *N*-trifluoroacetylated with trifluoroacetic anhydride in pyridine and the product, 4-trifluoroacetamidonitrobenzene **3**, was then hydrogenated over palladium to give 4-trifluoroacetamidoaniline [7] (TFAN, **4**). This amine, when reacted with lacto-*N*-tetraose and sodium cyanoborohydride at pH 6, gave



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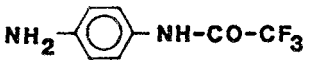

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the aminoalditol derivative **5** (Fig. 1) in 88% yield. Other reducing oligosaccharides were also derivatized in the same way and the yields are shown in Table 1. Selected ^{13}C-NMR data of typical reaction products are shown in Table 2.

The oligosaccharide TFAN derivatives were found to be relatively stable. They were not affected by storage (as lyophilized powders) at room temperature for several months. Aqueous solutions in contact with air gradually became miscolored. However, analysis by TLC of an aqueous solution of the lactose-TFAN derivative showed less than 5% degradation after 48 h in air contact at room temperature. When desired, conversion of the TFAN derivatives into the corresponding isothiocyanate derivatives was effected by brief (3 h, room temperature) treatment with 25% aqueous ammonia followed by evaporation and treatment with thiophosgene [5, 6]. The isothiocyanate derivatives were then coupled to proteins or solid matrices by established procedures [5, 6].

The increased lipophilicity of oligosaccharide-TFAN derivatives suggested that this type of derivatization could be used to simplify the separation of mixtures. The usual HPLC procedures [8-10] for resolving reducing oligosaccharide mixtures into their components give two peaks for each sugar, because of α/β isomerism at the reducing end.

Table 1: Oligosaccharides derivatized with 4-trifluoroacetamidoaniline (TFAN).

Sugar	% Yield
N-Acetylglucosamine	82
Chitotriose[a]	67
Lactose	68
3'-sialyllactose[b]	69
A-tetrasaccharide[c]	77
Lacto-*N*-tetraose	88
Lacto-*N*-fucopentaose I[d]	76
Lacto-*N*-fucopentaose II[e]	86
Lacto-*N*-fucopentaose III[f]	81
Lacto-*N*-hexaose[g]	85
Lacto-*N*-difucohexaose I[h]	86

[a] GlcNAcβ1-4GlcNAcβ1-4GlcNAc
[b] II^{3}NeuAc-Lac
[c] GalNAcα1-3[Fucα1-2]Galβ1-4Glc
[d] IV^{2}Fuc-LcOse$_4$
[e] III^{4}Fuc-LcOse$_4$
[f] III^{3}Fuc-nLcOse$_4$
[g] II^{6}LacNAc-LcOse$_4$
[h] IV^{2}Fuc, III^{4}Fuc-LcOse$_4$

Table 2. Selected ^{13}C-NMR chemical shifts ($^{2}H_2O$ solution, 25°C, internal dioxane = 67.4 p.p.m.) of representative oligosaccharide TFAN derivatives.

Derivatized oligosaccharide	Aromatic carbons	C-1 (C-2)[a]	C-6 (C-9)[a]	CH_2NH	CH-NH	C-3[a]	NHAc
Lactose	147.7 126.4 124.8 115.4	103.9	62.9 61.6	47.4			
Lacto-*N*-tetraose	146.6 127.0 124.6 116.0	104.2 103.7 103.3	62.7 61.8 61.4 61.2	47.7 55.4			23.0
Chitotriose	146.5 125.4 124.0 114.3	101.5 101.0	61.8 60.6 59.9	45.0 55.5	55.0 50.8		22.0 22.3 22.5
3'-Sialyllactose	136.3 134.7 124.5 122.5	103.2 100.8	63.4 62.7 61.7	52.6 52.5		50.5	22.9

[a] For sialic acid residues

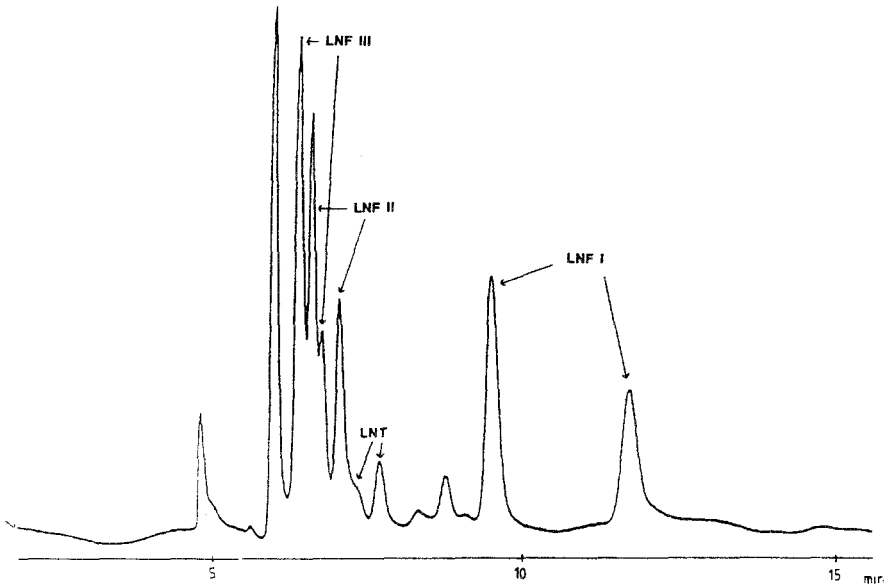

Figure 2. HPLC chromatogram of the underivatized neutral penta- and hexasaccharide pool from human milk (refractive index detection, Nucleosil C18 column, 30 × 1 cm, water as eluant). Abbreviations: LNT=lacto-*N*-tetraose, LNFI-III = lacto-*N*-fucopentaose I-III, LNDI = lacto-*N*-difucohexaose I.

As an example, the penta- and hexasaccharide pool of neutral oligosaccharides from human milk [11] gives the HPLC chromatogram shown in Fig. 2 (Reversed phase C-18 column). This mixture was treated with TFAN and sodium cyanoborohydride as described and was then washed with ether and passed through a short Bio-Gel P 2 column to remove excess reagents. The HPLC chromatogram (silica gel column) of the mixture of TFAN derivatives obtained is shown in Fig. 3. The improvement in separation is clearly seen. Each sugar now gives rise to a single peak, and there is less overlap. It should be noted, however, that to date, there is no known procedure for recovery of the parent sugar from the TFAN derivatives. Therefore, purification of oligosaccharide mixtures by HPLC of TFAN derivatives is useful only in cases where subsequent conjugation of the oligosaccharides is intended.

Experimental

General Methods

Melting points are corrected. Concentrations were performed at 1-2 kPa below 40°C. ^{13}C-NMR Spectra were recorded at 25°C for solutions in $^{2}H_2O$ unless otherwise stated, using a Bruker AM 500 instrument. The signal from internal dioxane (δ 67.4) was used as

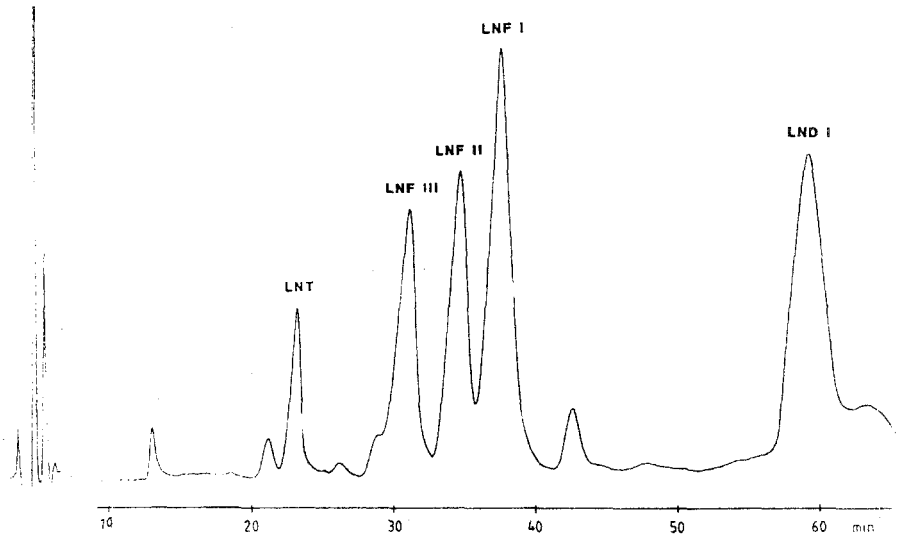

Figure 3. HPLC chromatogram of the TFAN-derivatized neutral penta- and hexasaccharide pool from human milk. Refractive index detection, Nucleosil silica gel column (30 × 1 cm), eluted with ethyl acetate/acetic acid/methanol/water, 10/3/3/2 by vol. Abbreviations: see Fig. 2.

reference unless otherwise stated. Only selected NMR data are reported. TLC was performed on Silica gel F_{254} plates (Merck, Darmstadt, W. Germany) with detection by UV light when applicable or by charring with sulfuric acid. Bio-Gel P-2 (Bio-Rad, Richmond, CA, USA) columns were packed and eluted with 0.1 M pyridinium acetate buffer (pH 5). The fractions were monitored by TLC, using ethyl acetate/acetic acid/methanol/water, 6/3/3/2 by vol, as developing solvent. Waters Associates (Millford, MA, USA) 501 pump and 410 differential refractometer were used in HPLC separations. Commercial 4-aminophenylethylamine (Aldrich, Milwaukee, WI, USA) was used. Lacto-*N*-tetraose (LNT), lacto-*N*-fucopentaose I-III (LNF I-III), Lacto-*N*-difucohexaose I (LND I), lacto-*N*-hexaose (LNH), and 3'-sialyllactose (3'-SL) were obtained from human milk [8-11], chitotriose from partial hydrolysis of chitin with hydrogen fluoride [12, 13], and the A-tetrasaccharide from faeces of breast-fed children [14].

Reductive Amination of Lacto-N-tetraose with 4-Aminophenylethylamine, Method A [6].

Lacto-*N*-tetraose (30 mg) was stirred with 4-aminophenylethylamine (1.0 ml) for 48 h. A solution of sodium borohydride (20 mg) in ethanol (1.0 ml) was added, and stirring was continued. After 4 h, the mixture was diluted with ethanol (1.0 ml), and acetic acid (1.0 ml) was added dropwise. The mixture was concentrated, then co-concentrated with

methanol and water. Gel filtration of the residue on a column of Bio-Gel P-2 gave a major carbohydrate-positive compound (**1**, 34 mg, 97%). ^{13}C-NMR Data: 23.1 (**Me**CO); 31.6 (Ph**C**H_2CH_2); 49.6 (PhCH_2**C**H_2); 50.3 (CHOH**C**H_2NH); 55.5 (C-2‴); 104.3, 103.4, 103.5 (C-1′, C-1″, C-1‴); 117.6, 130.5 (aryl CH), 127.9, 146.0 (aryl C), 175.7 (CO).

Reductive Amination of Lacto-N-tetraose with 4-Aminophenylethylamine, Method B [5]

A solution of 4-aminophenylethylamine (68 mg) and sodium cyanoborohydride (31 mg) in water (1.0 ml) was adjusted to pH 8 with acetic acid, then a solution of lacto-*N*-tetraose (36 mg) in water (0.5 ml) was added. After 16 h, the mixture was concentrated to a small volume and purified by gel filtration on a Bio-Gel P-2 column. A major carbohydrate-positive compound **2** was obtained (40 mg, 96%). ^{13}C-NMR Data: 23.1 (**Me**CO); 32.7 (Ph**C**H_2CH_2); 41.6 (CHOH**C**H_2); 47.6 (CHOH**C**H_2NH); 55.5 (C-2‴); 103.41, 103.7, 104.3 (C-1′, C-1″, C-1‴); 116.2, 130.6 (aryl CH); 127.9, 147.5 (aryl C); 175.7 (CO).

Reductive Amination of Lactose with Aniline and Benzylamine

Lactose (68 mg) and sodium cyanoborohydride (75 mg) were reacted, according to method B, in water (6 ml) at pH 6 with either aniline (92 mg) or benzylamine (107 mg). Analysis of the mixture by TLC (ethyl acetate/acetic acid/methanol/water, 6/3/3/2 by vol) showed that with aniline all the lactose had been converted to a faster-migrating, UV-absorbing compound after 12 h. With benzylamine, only traces of product were detected by TLC after this reaction time, but after one week, approximately 50% conversion of lactose into product was detected.

Reductive Amination of Lacto-N-tetraose with 4-Nitroaniline

Lacto-*N*-tetraose (72 mg), p-nitroaniline (70 mg) and sodium cyanoborohydride (62 mg) were reacted in 4 ml water/ethanol, 1/1 by vol, for two days at pH 6. Only traces of product were detected by TLC.

*4-Trifluoroacetamidonitrobenzene (**3**)*

Trifluoroacetic anhydride (5.0 ml) was added, dropwise at 0°C, to a stirred solution of *p*-nitroaniline (2.76 g) in pyridine (25 ml). After 2 h at room temperature, the mixture was poured into ice-cold water (500 ml). The mixture was stirred for 1 h, then filtered. The solid was recrystallized from methanol-water to give **3** (4.3 g, 92%).

*4-Trifluoroacetamidoaniline (**4**) [7]*

Hydrogenation of **3** (2.0 g) in ethanol (30 ml) over Pd/C (50 mg) at atmospheric pressure gave, after filtration and concentration, a residue, which was recrystallized from ether-hexane to give **4** (1.51 g, 87%), m.p. 118°C (118°C [7]).

General Procedure for Reductive Amination of Reducing Sugars with 4-Trifluoroacetamidoaniline

A solution of **4** (75 mg, 0.365 mmol) and sodium cyanoborohydride (25 mg, 0.40 mmol) in 1.5 ml ethanol/water, 1/2 by vol, was adjusted to pH 6 with acetic acid. A solution of the sugar (0.05 mmol) in water (0.75 ml) was added, and the mixture was stirred at room temperature (15-48 h). It was then washed with ether, concentrated, and the residue was purified by gel filtration on a Bio-Gel P-2 column. The yields with various oligosaccharides are shown in Table 1; selected ^{13}C-NMR data of the derivatives are given in Table 2.

Reductive Amination with TFAN of an Oligosaccharide Mixture, and Separation of the Derivatives Obtained

The neutral penta- and hexasaccharide pool from human milk [8-11] (1000 mg) was treated as above with **4** (1670 mg) and sodium cyanoborohydride (560 mg) for 48 h. Purification as described gave a mixture of derivatives (745 mg), 380 mg of which was loaded on a Nucleosil 100-5 semi-preparative Silica gel HPLC column (diameter: 10 mm, length: 300 mm, particle size: 5 μm) in 10 mg portions. The chromatogram obtained when eluting with ethyl acetate/acetic acid/methanol/water, 10/3/3/2 by vol, at a flow rate of 5 ml/min is shown in Fig. 3 (refractive index detection). The fractions were identified by comparison of the ^{1}H-NMR spectra with the spectra of underivatized milk oligosaccharides. The yields of TFAN derivatives were as follows: lacto-*N*-tetraose (19 mg), lacto-*N*-fucopentaose,III (48 mg), lacto-*N*-fucopentaose II (59 mg), lacto-*N*-fucopentaose I (87 mg), lacto-*N*-difucohexaose I (88 mg). The total yield of minor, as yet uncharacterized fractions was 65 mg.

Conversion of Oligosaccharide TFAN Derivatives to the Corresponding Isothiocyanates

A solution of the oligosaccharide TFAN derivative (0.032 mmol) in 25% aqueous ammonia (1 ml) was kept at room temperature under nitrogen for 3 h, then concentrated and co-concentrated twice with water (2 × 10 ml). The residue was dissolved in 5 ml water/ethanol/0.1 M sodium phosphate-buffered 0.15 M saline pH7, 1/1/1 by vol, and a freshly prepared solution of thiophosgene (0.010 ml) in acetone (1.0 ml) was added dropwise at 0°C, while maintaining the pH at 6-7 by simultaneous addition of aqueous 1M sodium hydroxide. After 10 min, the mixture was washed with ether, the aqueous layer was concentrated to half the volume and then used directly in couplings to proteins [5, 6] or solid matrices.

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