Complete synthesis of 3′-sialyl-*N*-acetyllactosamine by regioselective transglycosylation

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Abstract Transglycolytic synthesis of 3′-sialyl-*N*-acetyllactosamine by sequential use of β-galactosidase from *Bacillus circulans* and trans-sialidase from *Trypanosoma cruzi* was described. These reactions depicted the first complete synthesis of a biologically important oligosaccharide with high regioselectivity avoiding use of glycosyltransferases and NDP sugars.

Key words: Transglycosylation; Trans-sialidase; 3′-Sialyl-*N*-acetyllactosamine; Sialyl-Lewis^x

1. Introduction

Sialylated oligosaccharides participate in many important biological events. Mainly as components of glycoproteins and glycolipids, they act as antigenic determinants, receptors for proteins, viruses or bacteria and mediate cell-cell recognition and interactions [1,2]. In particular, the trisaccharide 3′-sialyl-*N*-acetyllactosamine (3′-SLN) has been found in free form in normal and pathological human urine and in human milk where it plays an important biological role [3–5]. Furthermore, α-(1,3)-fucosylation at the D-Glc*p*NAc residue of 3′-SLN gives sialyl-Lewis^x, identified as a functional ligand for E-, P- and L-selectin. It mediates trafficking and recruitment of blood-borne leukocytes to endothelial cells during normal and pathological inflammatory responses to injury and infection [6,7]. The multi-step chemical synthesis of this biologically important trisaccharide has been described [8], but it is very laborious. In order to avoid multi-step chemical synthesis, enzymatic methods have been developed using glycosyltransferases as catalysts [9–11]. Moreover, the latter enzymes require activated sugars which, in spite of recent improvements in production by biochemical recycling [12], still are costly and industrially non-attractive substrates.

In the present work we report the first complete synthesis of 3′-SLN by exclusive use of transglycosylation activities of *Bacillus circulans* β-galactosidase and *Trypanosoma cruzi* trans-sialidase.

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Abbreviations: NDP sugars, nucleoside diphosphate sugars; 3′-SLN, 3′-sialyl-*N*-acetyllactosamine; D-Glc*p*NAc, 2-acetamido-2-deoxy-D-glucopyranose; D-Glc*p*, D-glucopyranose; BcG, *B. circulans* β-galactosidase; TcTs, *T. cruzi* trans-sialidase; [^{14}C]lactose, [D-(glucose)-1-^{14}C]lactose; PBS, phosphate buffer saline; MU-NeuAc, 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; MU, 4-methylumbelliferone

2. Materials and methods

2.1. Materials

Lactose, D-Glc*p*NAc, 3′-*N*-acetylneuraminyl-lactose (3′-sialyllactose) and 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-NeuAc) were purchased from Sigma (St. Louis, MO, USA). 3′-SLN was from Dextra Laboratories Ltd (Reading, UK). [^{14}C]Lactose (50 μCi, 60 mCi/mmol) was from Amersham (Buckinghamshire, UK). β-Galactosidase from *B. circulans* was a kind gift of Daiwa Kasei Co. (Osaka, Japan). *E. coli* strain carrying plasmid pTS154cat containing the gene for *T. cruzi* trans-sialidase was a kind gift of Dr. Dan Eichinger (Department of Medical and Molecular Parasitology, New York University School of Medicine). Bio-Gel P2 was from Bio-Rad (Richmond, CA, USA). QAE-Sephadex A25 was from Pharmacia-Biotech (Uppsala, Sweden). Ni-agarose was from QIAGEN (Hilden, Germany). Centriflo membrane cones CF25 were from Amicon (Beverly, MA, USA). Sep-Pak C18 cartridge were from Waters (Milford, MA, USA). LiChrosorb-NH_2 HPLC column and TLC Silica Gel 60 plates were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Preparation of *T. cruzi* trans-sialidase

Extensive information on the enzymatic characterization of the *T. cruzi* trans-sialidase is given elsewhere [13,14].

E. coli strain carrying plasmid pTS154cat containing the gene for *T. cruzi* trans-sialidase was grown, induced and pelleted as described [15]. Bacterial lysis and enzyme purification were performed according to the following partly original procedure, which nevertheless is based on published methods [15,16]. The cells were resuspended in 1 ml of permeabilization buffer consisting of 100 mM Tris-HCl pH 7.8 containing 32 mM NaH_2PO_4 and 4% v/v of Triton X-100. Lysis was carried out by adding polymyxin B sulfate to give a final concentration of 200 μg/ml. After incubation for 30 min at room temperature and 30 min on ice, insoluble debris was pelleted and imidazole was added to the supernatant to a final concentration of 10 mM. Purification of trans-sialidase was carried out on a column of Ni-agarose (1.2×2 cm) equilibrated with 100 mM Tris-HCl pH 7.8 containing 32 mM NaH_2PO_4 and 10 mM imidazole. After washing with this buffer, the enzyme was eluted in steps, with 10×1 ml of PBS containing imidazole 33 mM, 67 mM, 125 mM and 250 mM, respectively. Each fraction collected was analyzed for enzyme activity, which revealed that only the fractions corresponding to the elution with an imidazole concentration of 67 and 125 mM were active. SDS-PAGE of the two fractions confirmed that each of them contained a single protein band. The active fractions were pooled and the volume reduced by centrifugation on a Centriflo membrane cone CF25.

2.3. Assay of *T. cruzi* trans-sialidase

Enzymatic activity was assayed according to the procedure reported in [17], which had been demonstrated to ensure linearity between reaction velocity and amount of enzyme. Occasional modification of the previous procedure to follow that reported in [14], i.e. increasing the concentration of [^{14}C]lactose by a factor 10, did not impair the previous conclusions about enzymatic activity.

2.4. Synthesis of *N*-acetyllactosamine

Regioselective synthesis of *N*-acetyllactosamine was obtained from lactose as donor, D-Glc*p*NAc as acceptor and β-galactosidase from *B. circulans* as the transglycosylating enzyme as described in [18].

2.5. Sialylation using 3′-sialyllactose as donor

Equimolar quantities (7.8 μmol) of 3′-sialyllactose and *N*-acetyllac-

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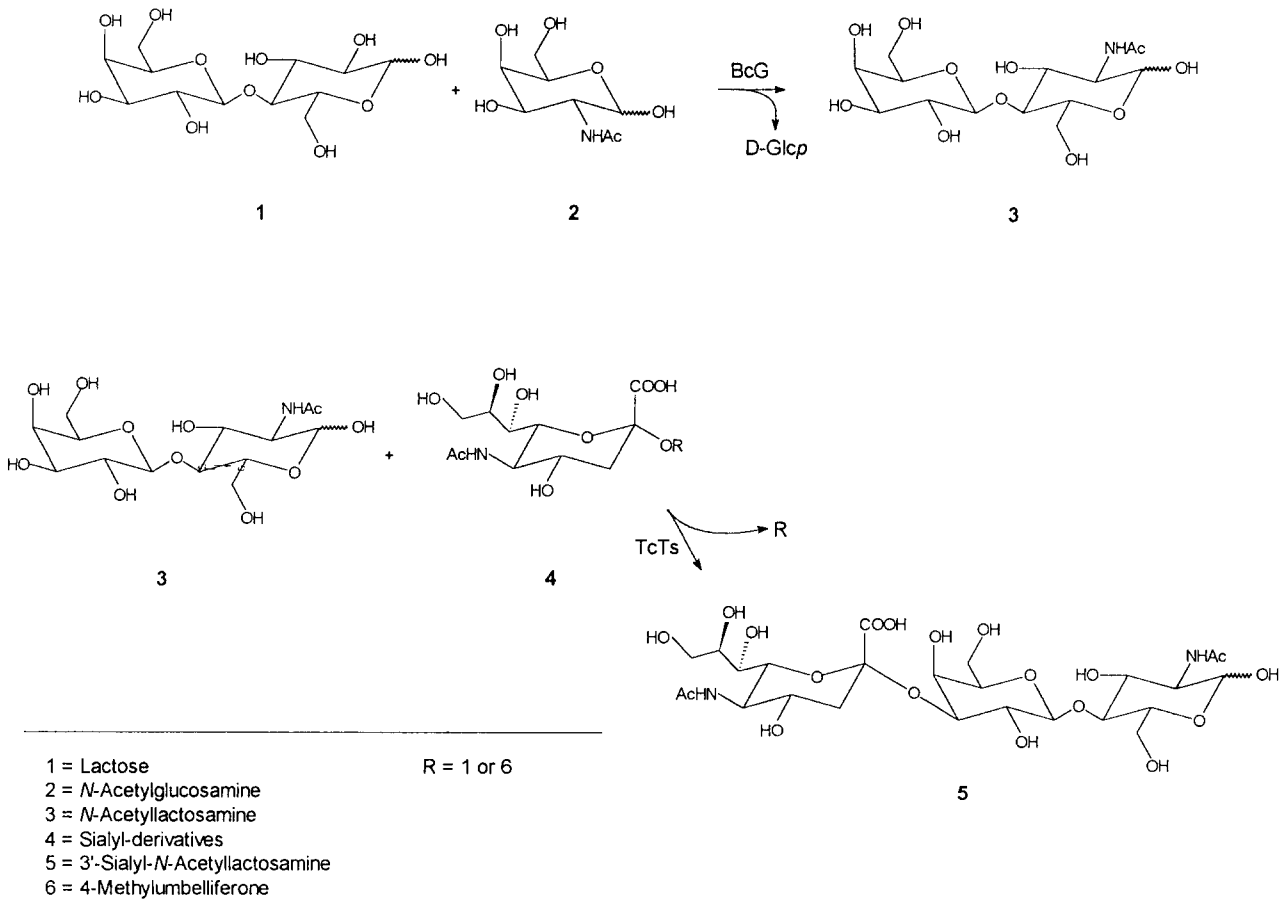

Fig. 1. Schematic representation of the synthesis of 3′-SLN. BcG: *B. circulans* β-galactosidase; TcTs: *T. cruzi* trans-sialidase.

tosamine were dissolved in 400 μl of 100 mM PBS, pH 7.2 containing 0.2% of BSA, then 13 U (100 μl) of *T. cruzi* trans-sialidase were added. After incubation for 24 h at 37°C the mixture was heated in a boiling water bath for 10 min and then immediately cooled in ice. After centrifugation at 11 000 rpm for 5 min, the clear supernatant was diluted 20 times with water and applied on a column of QAE-Sephadex A25 (15×1.5 cm) equilibrated in water. Elution by washing was performed with water for neutral compounds and with 1 M ammonium formate for sialylated compounds. Elution was followed by TLC developed with *n*-propanol/1 N ammonia/water (6:2:1) and detected with 0.2% orcinol in 2 M H_2SO_4. Fractions containing 3′-sialyllactose and 3′-SLN were pooled and concentrated under vacuum. This pool was further purified by gel permeation chromatography on two serial columns (2.0×100 cm each) of Bio-Gel P2 equilibrated in water. The elution was followed by use of R-403 Waters differential refractometer (RI). The fractions were tested for the presence of 3′-SLN by TLC and those containing the trisaccharide were pooled and freeze-dried.

2.6. Sialylation using MU-NeuAc as donor

The same experimental approach as described before was used for sialylation using equimolar quantities (10 μmol) of MU-NeuAc and *N*-acetyllactosamine, but for the following small modification. After incubation and inactivation, the mixture was purified on two serial Sep-Pak C18 cartridges. In this way polar compounds like unreacted *N*-acetyllactosamine and 3′-SLN were eluted with a polar solvent (water) while less polar compounds like MU and MU-NeuAc were eluted with a nonpolar solvent (methanol). The water-eluted fraction was pooled and further purified by gel permeation chromatography on a column (2.0×100 cm) of Bio-Gel P2 equilibrated in water. The elution was followed by use of the R-403 Waters RI detector. The fractions were tested for the presence of 3′-SLN by TLC and those containing the trisaccharide were pooled and freeze-dried.

2.7. Structural identification methods

HPLC analyses were carried out on a Beckman HPLC system (GOLD Model) equipped with a UV-visible detector, monitoring at 210 nm (*N*-acetyl group), and a LiChrosorb-NH_2 column (5 μm, 250×4.00 mm I.D.). The column was eluted under isocratic conditions using acetonitrile:15 mM KH_2PO_4 (50:50) as mobile phase.

^{13}C- and ^{1}H-NMR decoupled spectra were measured on a Bruker AC 200 (^{13}C 50.3 MHz, ^{1}H 200 MHz) spectrometer using a multinuclear 5 mm probe. 500 μl of deuterated water was used for all the measurements with a typical sample concentration of 5 mg/ml for ^{1}H measurements and 30 mg/ml for ^{13}C measurements. The spectra were obtained at 37°C.

Ion spray mass spectrometry data were recorded on a API-I PE-SCIEX quadrupole mass spectrometer connected to a syringe pump for the injection of the samples. The sample was dissolved in 50% aqueous acetonitrile 5 mM ammonium acetate and injected at a flow rate of 7 μl/min. Spectra were recorded in positive ion mode using a step size of 0.1 amu, an orifice potential of 90 V and an ion spray voltage of 5000 V.

3. Results and discussion

Two consecutive transglycosylation reactions were used for the synthesis of 3′-SLN. The *N*-acetyllactosamine produced by the first transglycosylation reaction [18] was used as substrate for the sialylation by use of the specific reaction of trans-sialidase from *T. cruzi* (Fig. 1). Molecules of different kind were used as donors; one of them can be considered the 'natural' substrate (i.e. 3′-sialyllactose) for the other. In all cases we were able to obtain the desired product, although

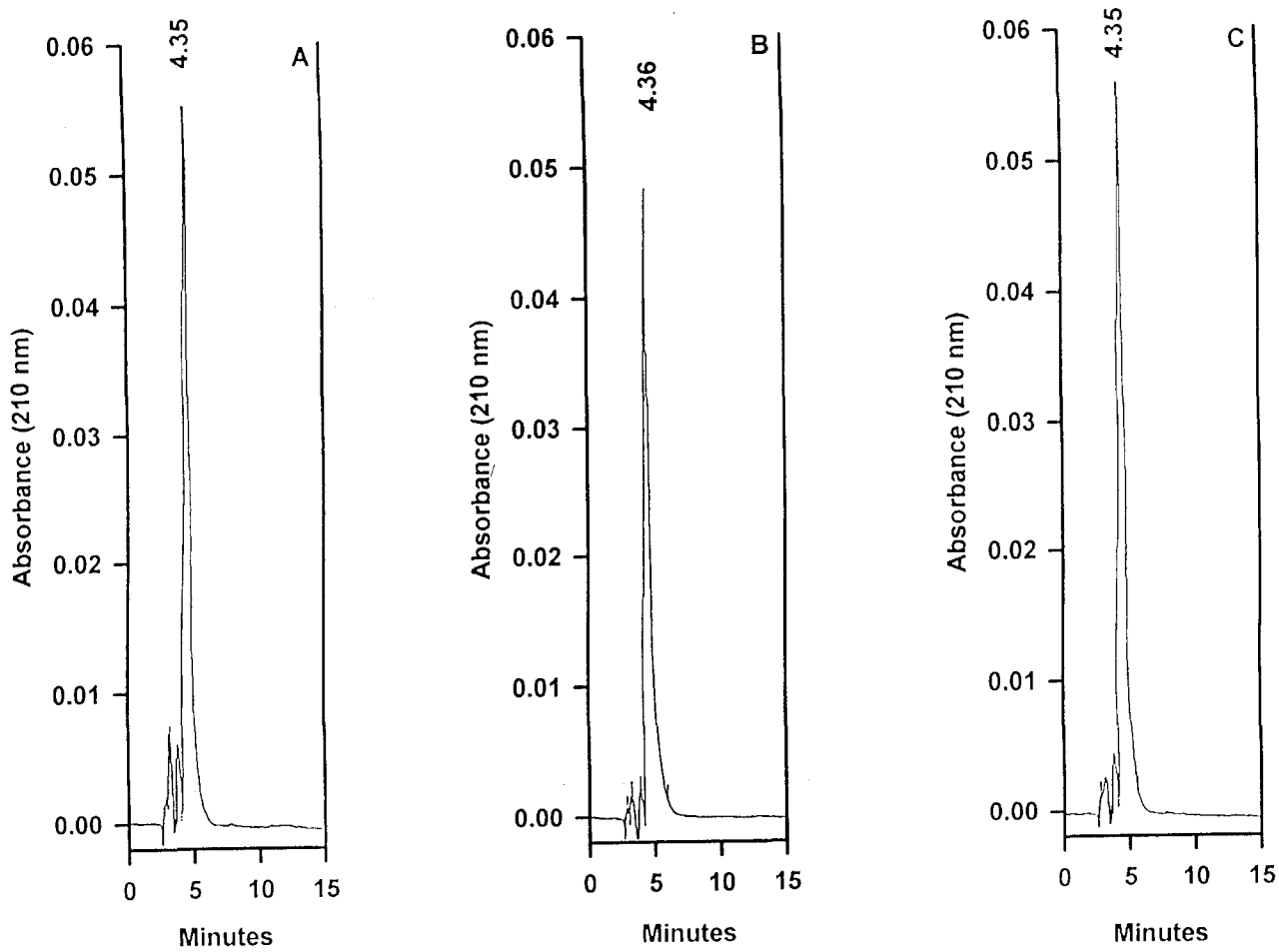

Fig. 2. HPLC profiles of standard (A), transglycosylation product (B) and of a mixture of standard and transglycosylation product (C).

in different yields. Using 3′-sialyllactose as a donor, a yield of 23% (on a molar basis) was obtained, whereas MU-NeuAc gave a molar yield of 60%. From a practical point of view, the use of synthetic substrates such as MU-NeuAc is more advantageous since the procedure of synthesis of this substrate is well described [19] and the aglycone part (4-methylumbelliferone) cannot act as acceptor, thus making the reaction virtually irreversible and shifted towards the synthesis of 3′-SLN. The apolar nature of this substrate facilitates its separation from the reaction products by simple solid phase extraction. In contrast, the 'natural' substrate 3′-sialyllactose is difficult to obtain and/or to synthesize and can easily act as acceptor too.

Table 1
^{13}C chemical shifts and assignments of transglycosylation product

Carbon atom	NeuAcα2-3	Galβ1-4	GlcNAcα	GlcNAcβ
1	175.11	103.90	91.81	96.02
2	101.10	70.62	54.99	57.54
3	40.95	76.43	70.49	73.68
4	69.55	68.78	80.11	79.72
5	53.00	76.81	71.50	76.10
6	74.16	62.25	61.31	61.43
7	69.45			
8	73.01			
9	63.91			
10	176.26			
11	23.19			
CO			176.09	176.09
CH_3			23.15	23.28

Chemical shifts are given in ppm and refer indirectly to tetramethylsilane using 1,4-dioxane as internal standard (67.86 ppm).

The characterization of the trisaccharides synthesized as above described was carried out by HPLC, ^{13}C-NMR spectroscopy and by ion spray mass spectrometry. For both experimental approaches used, the obtained products have been unambiguously identified as 3′-SLN. In fact, the HPLC retention times of both reaction products and of 3′-SLN correspond (Fig. 2); the ^{13}C-NMR assignments of the spectra of both products match perfectly with those reported in the literature (Table 1) [20] and the ion spray mass spectra of both reaction products are identical to each other and to that of a genuine sample of 3′-SLN, showing the ions at *m/z* 675.2, 697.2, and 713.1 corresponding to $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$. A fourth ion at *m/z* 719.1 corresponds to the sodium adduct of the trisaccharide bearing sodium as counterion (Fig. 3). Moreover, for both products obtained using the two different donors the converging evidence of HPLC, NMR and ion spray mass spectrometry indicates that undesired impurities, if present, were below the analytical detection limits.

The enzymatic synthesis of sialyl oligosaccharides in general, and of 3′-SLN in particular, has already been reported, but in all cases described so far at least a galactosyltransferase [21,22], a sialyltransferase [23] or both [10,24] have been used. When either transgalactosylation [11] or transsialylation was used [11,25,26], low regioselectivity was obtained. The experimental modulation of *B. circulans* β-galactosidase activity used for the synthesis of *N*-acetyllactosamine [18] made it

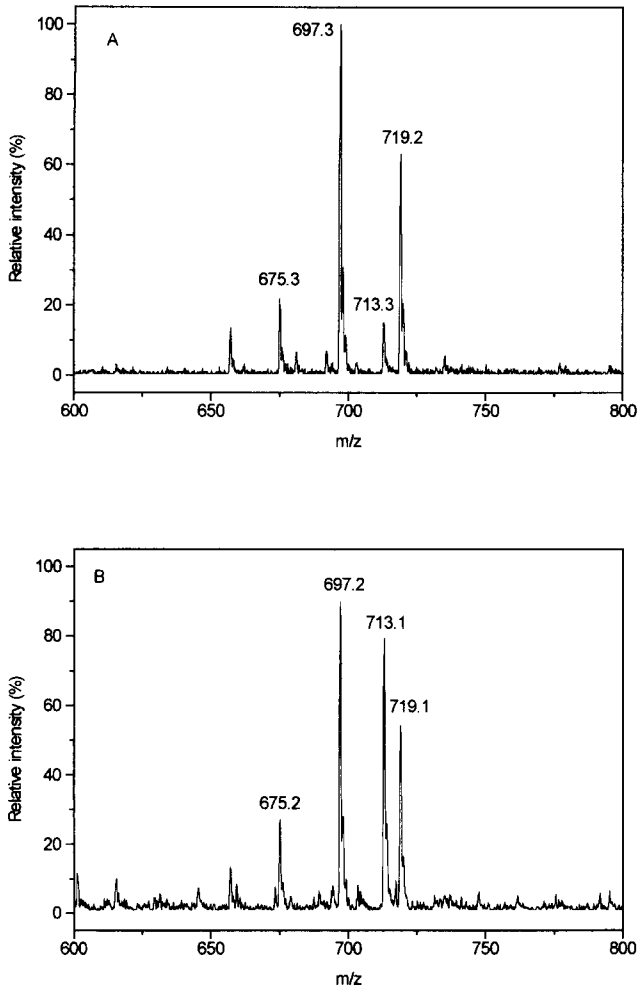

Fig. 3. Positive ion spray mass spectra of standard (A) and transglycosylation product (B).

possible to obtain a high regioselectivity together with a high yield of synthesis; the subsequent use of *T. cruzi* trans-sialidase overcomes the regioselectivity problems of the transsialylation. The latter enzyme shows a better efficiency in transferring sialic acid to saccharides with terminal β-galactose, rather than hydrolyzing it. This reaction results in the formation of the unique α2-3 linkage between the terminal sialic acid and the galactose residue [14,27,28]. Few data have been reported on the use of trans-sialidase for synthetic goals [22,29,30]. Only Nishimura et al. [22] used this enzyme for preparative synthesis of 3′-SLN chemically linked on a water-soluble polyacrylamide, using MU-NeuAc as donor and obtaining only a 35% overall yield on a molar base.

In conclusion, the experimental approaches reported here describe the regiospecific synthesis of the trisaccharide 3′-SLN, confirming the importance and the potential of transglycosylation reactions for the synthesis of biologically important oligosaccharides starting from cheap sugars or their derivatives. In particular the data here presented are the first report on the complete synthesis of 3′-SLN by the exclusive use of highly regioselective transglycolytic reactions.

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