Enzymatic Decoration of Prebiotic Galacto-oligosaccharides (Vivinal GOS) with Sialic Acid Using Trypanosoma cruzi trans-Sialidase and Two Bovine Sialoglycoconjugates as Donor Substrates

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ABSTRACT: Decoration of prebiotic galacto-oligosaccharides (GOS) with sialic acid yields mixtures of GOS and sialylated GOS (Sia-GOS), novel products that are expected to have both prebiotic and antiadhesive functionalities. The recombinantly produced trans-sialidase enzyme from Trypanosoma cruzi (TcTS), an enzyme with the ability to transfer (α2–3)-linked sialic acid from sialoglycolysis to asialoglycoligysans, was employed to catalyze this sialylation. As sialic acid acceptor substrates, Vivinal GOS and derived fractions of specific degree of polymerization were taken. As sialic acid donor substrates, bovine κ-casein-derived glycomacropeptide [≥99% N-acetylneuraminic acid (Neu5Ac); <1% N-glycolylneuraminic acid (Neu5Gc)] and bovine blood plasma glycoprotein mixture (45% Neu5Ac; 55% Neu5Gc) were selected, yielding potential food and feed products, respectively. High-pH anion-exchange chromatography, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, and nuclear magnetic resonance spectroscopy were used for product analysis.

KEYWORDS: galacto-oligosaccharides, sialic acid, trans-sialidase, Trypanosoma cruzi, bovine blood plasma glycoprotein

INTRODUCTION

Over the past decades, the combined results of many studies have unravelled the composition of human milk to a great extent. After lactose (∼70 g/L) and fat (∼41 g/L), oligosaccharides constitute the third largest fraction in human milk, reaching levels up to 20–25 g/L in colostrum and 5–15 g/L in mature milk. The amounts of casein/serum proteins are ∼8 g/L.1–3 Nowadays, the structures of more than 120 human milk oligosaccharides (HMOs) have been elucidated.4–7 Given their enormous diversity in terms of composition and structural arrangement, it is not surprising that HMOs fulfill different physiological roles following their uptake in the digestion system of the infant, including prebiotic, antiadhesive, immune-modulating, and brain-developmental activities.8–11 An important subclass of the HMOs (10–20%), that is, the N-acetylneuraminic acid (Neu5Ac)-containing oligosaccharide fraction, is of special importance in preventing pathogen binding, thereby reducing the risk of infections.8,12

The situation with respect to the constituents of bovine milk is quite different. Here, lactose (∼48 g/L), fat (∼37 g/L), and protein (∼32 g/L; mainly casein proteins) occur in comparable amounts.2 The amount of oligosaccharides in mature bovine milk is only ∼0.05 g/L and in colostrum, ∼1 g/L.13 So far, the structures of about 50 bovine milk oligosaccharides (BMOs) have been assigned.4,14–22 Of the low amounts of BMOs, ∼70% has been reported to be sialylated with Neu5Ac or N-glycolylneuraminic acid (Neu5Gc). As a consequence, the relatively high abundance of sialylated oligosaccharides (SOSs) in human milk is not reflected in infant formula based on bovine milk for bottle-fed babies. Because of this disparity, there is a clear demand for human milk SOS, such as Neu5Ac-lactose, or SOS-analogues, which ideally have similar functionalities and can be produced on an industrial scale.

In neonatal calves, lambs, and piglets, infections with enterotoxigenic Escherichia coli strains (e.g., E. coli K99) are known to cause enteric colibacillosis (diarrhea). In vitro experiments with ganglioside and sialoglycoprotein glycan preparations have shown that those containing Neu5Gc as sialic acid have positive effects as antiadhesives,23–25 whereas for BMO preparations the results are conflicting.26 In view of these findings it is interesting to explore the possibilities of producing Neu5Gc-containing products on an industrial scale, as they may be used as feed additive to reduce antibiotic use in livestock. Note that compounds containing high amounts of...
NeuSGc should not be used in human food because of potential immunological problems.27

Galacto-oligosaccharide mixtures (GOS) with degrees of polymerization (DP) from 2 to 9, which are synthesized from lactose by enzymatic transgalactosylation using specific microbial β-galactosidases, have shown to be functional food products with excellent prebiotic functions.28 These ingredients are broadly applied in the infant food sector. Depending on the enzyme used, GOS contains an array of linear and branched (Gal)Glc oligosaccharides with different substitution patterns. For one of these products, prepared with Bacillus circulans β-galactosidase, the ensemble up to DP5 comprises >40 components.29-34

In view of reported functions of both GOS and sialylated oligosaccharides, taking into account the low amounts of sialyl-oligosaccharides in cow’s milk, it is hypothesized that partial sialylation of GOS (Sia-GOS) will generate mixtures of compounds that combine the different properties, meaning novel products with broad functionalities both for the food (Sia = NeuSAc) and feed (Sia = NeuSGc) industries. To this end we have chosen Trypanosoma cruzi trans-sialidase (TcTS), recombinantly produced in E. coli and capable of transferring sialic acid from various artificial and natural Sia((2→3)Gal(β1→x)-glycans (sialogalactoglycans) to Gal(β1→x)-glycans (sialo-galactogalactosylcans), as the biocatalyst (for a review, see ref 35). For the aimed for food and feed products, bovine κ-casein-derived glycomacropeptide (GMP) (>99% NeuSAc; <1% NeuSGc) and bovine blood plasma glycoprotein mixture (BPG) (45% NeuSAc; 55% NeuSGc) were selected, respectively, as sialic acid donor substrates. Using the sialic acid acceptor substrates Neu5Ac; 55% Neu5Gc) were selected, respectively, as sialic acid acceptor substrates Neu5Ac and Neu5Gc, use was made of matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and nuclear magnetic resonance (NMR) studies, on the sialylation of terminal Gal residues of the GOS components 3- and (4-methylumbelliferyl)-α-N-acetylenuraminic acid (4MU-Neu5Ac), and N-acetylenuraminyl((2→3)-lactose (3′-SL) were obtained from Carbo-synth Ltd. (Compton, UK). N-Glycolynuraminyl((2→3)-lactose was a gift of Prof. Dr. T. Urashima (Obihiro, Japan). D,O (99.9 atom % D) was acquired from Cambridge Isotope Laboratories Ltd. (Andover, MA, USA).

TcTS Production and Purification. Recombinant TcTS enzyme batches were produced in E. coli BL21(DE3) (Invitrogen, Carlsbad, CA, USA) using plasmid pTrcTS611/243 (a gift of Prof. Dr. A. C. C. Frasch, Buenos Aires, Argentina), as previously described.42 The specific transglycosylation activity of TcTS was determined as 1.0 μmol/min/mg using 4MU-Neu5Ac and lactose as substrates and quantitative detection of N-acetylenuraminyl((2→3)-lactose using high-pH anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD) analysis.

Isolation of Vivilosal GOS DP Fractions by Size Exclusion Chromatography. Vivilosal GOS was fractionated on a Bio-Gel P-2 Fine (45–90 μm) column (60 × 5 cm) using demineralized water as eluent at a flow rate of 1 mL/min and 40 °C. The separation was followed by an RI-101 Shodex refractive index detector (Showa Denko Europe GmbH, Munich, Germany). Carbohydrate-containing fractions (5 mL) were screened with MALDI-TOF-MS and pooled according to DP.

Analysis of Sialic Acid Content and Linkage Type of Bovine κ-Galactosidase-Derived GMP and Bovine BPG. To determine the sialic acid content of GMP and BPG, samples were incubated with 0.1 M HCl for 1 h at 80 °C, then neutralized with 1 M NaOH, and, after dilution with dimethyl sulfoxide, subjected to HPAEC-PAD analysis. For quantification of released Neu5Ac and Neu5Gc, use was made of calibration curves of known concentrations of both sialic acids. Following the same analysis protocol, the ratios of ((2→3)- and ((2→6)-linked sialic acid in GMP and BPG were determined by comparing the release of sialic acid by linkage-type nonspecific sialidase A (Protzyme, Hayward, CA, USA) and ((2→3)-specific sialidase S (Prozyme). Enzymatic incubations were carried out according to the manufacturer’s protocols.

Enzyme Incubations. All TcTS incubations were performed in 50 mM sodium citrate buffer (pH 5.0) containing 30 mM NaCl at 25 °C. Reactions were stopped by placing on ice or at −20 °C until further processing. Samples for HPAEC-PAD analysis were diluted with 95% dimethyl sulfoxide.

TcTS incubations with 3.5 mg/mL Vivilosal GOS (DP1→DP9; average DP of 3, corresponding to 2 mM) and 67.5 mg/mL GMP (corresponding to 5 mM ((2→3)-linked Neu5Ac) were carried out for 24 h. Sample aliquots were evaluated by HPAEC-PAD and MALDI-TOF-MS.

TcTS incubations with different GOS DP fractions (each at 3 mM) and 85 mg/ mL GMP (corresponding to 6 mM ((2→3)-linked Neu5Ac) were carried out for various times, depending on the application. Sample aliquots were evaluated by HPAEC-PAD, and incubation mixtures were further subjected to weak anion-exchange chromatography (AEX) on Resource Q, followed by 1H NMR analysis, MALDI-TOF-MS, or NaBH₄ reduction, as described below.

TcTS incubations with 65 mg/mL BPG (corresponding to 2 mM total sialic acid) and 4 mM lactose, GOS DP3, or GOS DP4 were carried out for various times, and samples were taken for evaluation by HPAEC-PAD. Obtained sialyl-lactose mixtures (24 h) were subjected to preparative HPAEC-PAD analysis to yield pure Neu5Ac((2→3)-lactose and Neu5Gc((2→3)-lactose. Obtained Sia-GOS products (48 h) were separated into mono-Sia-GOS and di-Sia-GOS fractions by AEX on Resource Q, and purified sialylated products were desalted prior to 1H NMR analysis, as described below.

Isolation of Sialylated GOS Products by Weak AEX. To remove sialic acid donor glycoconjugates, incubation mixtures were filtered on Amicon Ultra 15 mL filters (10000 MWL membrane) (Millipore) following the manufacturer’s protocol. Then, samples were desalted on CarboGraph solid-phase extraction (SPE) columns (300 mg; Grace, Breda, The Netherlands). After washing with 0.1% trifluoroacetic acid, oligosaccharides were eluted with 25% aqueous acetonitrile, containing 0.1% trifluoroacetic acid as eluent, and...
lyophilized. (Sialylated) oligosaccharide mixtures were fractionated on a 1 mL Resource Q anion-exchange column (GE Healthcare, Uppsala, Sweden) fitted in an Äkta Performance Fast Protein Liquid Chromatography system (GE Healthcare), equipped with a UV detector (214 and 280 nm) and a conductivity cell. The column was subsequently eluted with 3 mL of water, a linear gradient of 0–8% 0.5 M NaCl over 5 min, and a linear gradient of 8–50% 0.5 M NaCl in 6 min, at a flow rate of 1 mL/min. Neutral, monocharged, and dicharged oligosaccharide fractions were collected, desalted on CarboGraph columns (see above for conditions), and subjected to structural analysis of monocharged fraction of the Vivinal GOS/GMP/TcTS reaction mixture, showing [M – H]^+ quasi-molecular ions for mono-Sia-GOS DP2–DP7.

Figure 1. (A) HPAEC-PAD profile on CarboPac PA-1 of compounds in the Vivinal GOS/GMP/TcTS reaction mixture (incubation at 25 °C and pH 5.0) at t = 0 h (dotted line) and t = 24 h (solid line). The regions of formed mono-Sia-GOS and di-Sia-GOS are marked. (B) MALDI-TOF-MS spectrum (negative-ion mode) of the monocharged fraction of the Vivinal GOS/GMP/TcTS reaction mixture, showing [M – H]^+ quasi-molecular ions for mono-Sia-GOS DP2–DP7.

RESULTS AND DISCUSSION

TcTS Catalyzed Formation of Sia-GOS from GMP and Vivinal GOS. For the sialylation reaction of Vivinal GOS, the following characteristics of the donor GMP and the acceptor GOS have been recently documented. The used GMP preparation (MW 10 kDa) has a total sialic acid content of 3.6% (w/w), corresponding to 0.12 mmol/g dry matter; the ratio of NeuSAc and NeuSgc is 99:1.42 The O-glycans of GMP contain both (α2–3)-linked and (α2–6)-linked NeuSAc; the percentage of (α2–3)-linked NeuSAc is 59% (w/w).42 The used Vivinal GOS preparation contains free Glc (18.5%), free Gal (1.7%), DP2 products (42.5%), DP3 products (23.6%), DP4 products (10.2%), DP5 products (3.0%), and DP6–DP9 products (<0.5%). At present, the structures of >40 components have been elucidated (>98% of the oligosaccharides), being mainly linear and branched reducing (major) and linear nonreducing (minor) (Gal),Glc oligosaccharides (Supporting Information Scheme S1). The common small basic structural fragments, which are nearly only elongated by Gal(β1–4) residues, are five disaccharides, namely, Gal(β1–2)Glc, Gal(β1–3)Glc, Gal(β1–4)Glc, Gal(β1–6)Glc, and Glc(α1–1)Gal, and four branched trisaccharides, namely, Gal(β1–2)[Gal(β1–6)]Glc, Gal(β1–3)[Gal(β1–6)]Glc, Gal(β1–4)[Gal(β1–6)]Glc, and Gal(β1–2)[Gal(β1–4)]Glc.33,34

A mixture of GOS (3 mM) and GMP (80 mg/mL; containing 6 mM (α2–3)-linked NeuSAc) was incubated with TcTS (1 μg/mL) for 24 h at 25 °C and pH 5.0. The chosen time, temperature, and pH are based on the optimal

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conditions established earlier for the transfer of Neu5Ac from 4MU-Neu5Ac to lactose, yielding Neu5Ac(α2−3)lactose.42 Comparison of the HPAEC profile on CarboPac PA-1 (PAD detection) of the obtained reaction mixture after \(t = 24\) h with that of GOS at \(t = 0\) h showed a quite different peak pattern, reflecting a complex mixture of GOS and Sia-GOS components (Figure 1A). Clearly, the peaks in the higher retention time region reflect negatively charged Sia-GOS components. Closer inspection of the HPAEC profile of the reaction mixture revealed that two product pools were distinguishable on the basis of their retention times: a major pool of products with retention times between 10 and 15 min, expected to be mono-Sia-GOS components, and a minor pool of products with retention times between 16 and 20 min, expected to be
UV detection of Cmolar ratio of mono-Sia-GOS and di-Sia-GOS, based on the794.40, 956.58, 1118.69, 1280.58, 1442.22) (Figure 1B). The series of quasi-molecular [M−H] ions for monosialylated oligosaccharides (data not shown). MALDI-TOF-MS analysis of the monocharged fraction revealed a series of quasi-molecular [M−H] ions for monosialylated GOS (mono-Sia-GOS) of GOS DP2−DP7 (m/z 632.09, 794.40, 956.58, 1118.69, 1280.58, 1442.22) (Figure 1B). The molar ratio of mono-Sia-GOS and di-Sia-GOS, based on the UV detection of C==O in Neu5Ac at 214 nm, taking into account the number of Neu5Ac units (one or two) in the detected molecules, was determined to be 97:3. The relatively low percentage of di-Sia-GOS is caused by the relatively high percentage of GOS DP2 (42.5%, w/v; i.e., lactose and other GOS DP2 components), which cannot form di-Sia-GOS structures.

**Table 2. Percentages of Conversion of GOS into Sia-GOS and Mono-Sia-GOS/Di-Sia-GOS Ratios (Determined from Resource Q Chromatograms Using UV 214 nm Responses), Obtained from TcTS Incubations for 32 h at 25 °C with GMP and GOS DP2−DP9**

<table>
<thead>
<tr>
<th>DP GOS</th>
<th>conversion (%)a GOS → Sia-GOS</th>
<th>mono-Sia-GOSb (%)</th>
<th>di-Sia-GOSb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP2</td>
<td>56 ± 5</td>
<td>100</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>DP3</td>
<td>36 ± 2</td>
<td>90 ± 3</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>DP4</td>
<td>35 ± 4</td>
<td>87 ± 4</td>
<td>14 ± 2</td>
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<td>DP5</td>
<td>37 ± 5</td>
<td>86 ± 2</td>
<td>11 ± 4</td>
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<tr>
<td>DP6</td>
<td>36 ± 5</td>
<td>89 ± 4</td>
<td>16 ± 1</td>
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<tr>
<td>DP7</td>
<td>19 ± 1</td>
<td>84 ± 1</td>
<td>19 ± 4</td>
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<tr>
<td>DP8</td>
<td>14 ± 2</td>
<td>81 ± 4</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>DP9</td>
<td>20 ± 3</td>
<td>79 ± 2</td>
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</tbody>
</table>

*aConversion percentages were determined in duplicate. bMono- and di-Sia-GOS percentages were measured in triplicate (DP3−DP6) and duplicate (DP7−DP9).*

disialylated-GOS (di-Sia-GOS) components. The latter structures can be formed from GOS components containing two terminal Gal units, which are both targets for sialylation by TcTS or from 6′-galactosyl-lactose (here terminal and internal Gal can be sialylated) present in GOS.33,34,42 Note that mono-Sia-GOS components can originate from GOS structures with one and two terminal Gal units (and from 6′-galactosyl-lactose). After working up the reaction mixture, indeed AEX on Resource Q, yields two charged fractions for all DPs ≥3 (for DP4, see Figure S2). MALDI-TOF-MS analysis (negative-ion mode) of the charged fractions prepared from GOS DP2−DP6 confirmed in each case, except DP2 (only mono-Sia-GOS), the presence of both mono-Sia-GOS and di-Sia-GOS components (Table 1). When incubation experiments were followed over time, the total Sia-GOS and di-Sia-GOS formations were found to be maximal after 32 h of incubation at 25 °C (Table 2). The conversion of GOS to Sia-GOS was the highest for DP2 (about 56%), about 36% for medium-length GOS chains (DP3−DP6), and about 17% for long-chain GOS (DP7−DP9), respectively, indicating that TcTS has a preference for shorter GOS acceptors.

As the GOS preparation contains reducing linear (one terminal Gal unit) and branched (two terminal Gal units) oligosaccharides and nonreducing linear oligosaccharides (two terminal Gal units), di-Sia-GOS can originate from reducing branched as well as from nonreducing linear oligosaccharides. To make these two groups of di-Sia-GOS components visible, the di-Sia-GOS fractions obtained from GOS DP4 and DP5 were incubated with NaBH4, whereby the reducing branched oligosaccharides were converted into their corresponding oligosaccharide-alditols. In HPAEC-PAD analyses on Carbo-Pac PA-1, oligosaccharide-alditols have shorter retention times than their corresponding oligosaccharide-alditols. Therefore, it was decided to separate GOS in its DP fractions and to incubate them separately with GMP and TcTS. To this end Vivinal GOS was fractionated on Bio-Gel P-2, yielding GOS fractions ranging from DP1 to DP9 (Supporting Information Figure S1). Each GOS DP fraction (3 mM), except DP1, was incubated with GMP [80 mg/mL; containing 6 mM (α2−3)-linked Neu5Ac] and TcTS (1 μg/mL) for 40 h at 25 °C and pH 5.0. HPAEC-PAD analysis on Carbo-Pac PA-1 (Figure 2) showed for all DP fractions the formation of product peaks with increased retention times, indicating that GOS DP2−DP9 were all suitable acceptor substrates for TcTS.

Separation of the reaction mixtures by AEX on Resource Q (UV detection at 214 nm) yielded two charged fractions for all DPs ≥3 and three independent measurements for the mono- and disialylated products, respectively. As is evident from the above experiment, incubation of GOS with GMP and TcTS leads to a highly complex mixture of GOS + Sia-GOS components with concomitant overlap in retention times on CarboPac PA-1 using HPAEC-PAD. Therefore, it was decided to separate GOS in its DP fractions and to incubate them separately with GMP and TcTS. To this end Vivinal GOS was fractionated on Bio-Gel P-2, yielding GOS fractions ranging from DP1 to DP9 (Supporting Information Figure S1). Each GOS DP fraction (3 mM), except DP1, was incubated with GMP [80 mg/mL; containing 6 mM (α2−3)-linked Neu5Ac] and TcTS (1 μg/mL) for 40 h at 25 °C and pH 5.0. HPAEC-PAD analysis on Carbo-Pac PA-1 (Figure 2) showed for all DP fractions the formation of product peaks with increased retention times, indicating that GOS DP2−DP9 were all suitable acceptor substrates for TcTS.
shown), showed that the majority of the di-Sia peaks shifted to shorter retention times, in agreement with the finding that nonreducing oligosaccharides form only a very minor part of GOS.34

As a typical example of the NMR analysis of the different Sia-GOS DP fractions, a short discussion of the spectra of mono-Sia-GOS DP4 (MALDI-TOF-MS: [M − H]−, 956.60) and di-Sia-GOS DP4 (MALDI-TOF-MS: [M − H]−, 1247.38) is presented. The 1H NMR spectrum of the DP4 GOS acceptor [a mixture of at least 15 DP4 structures in which the d-Glc unit is mono or disubstituted by d-Gal residues (monomeric, dimeric, or trimeric fragments); see Scheme S134] is shown in Figure 3A. It shows α-anomeric peaks at δ 5.449 and 5.406, reflecting the GOS DP4 components with 2-, 2,4-, 2,6- and 1,2-O-substituted α-D-Glc residues; the α-anomeric signals in the range δ 5.25−5.22 reflect the GOS DP4 components with 1-, 3-, 4-, 6-, and 4,6-O-substituted α-D-Glc residues, whereas the signals at δ 4.221 and 4.164 correspond with H-6a signals of 6-O-substituted d-Glc residues (i.e., β- and α-anomeric forms of reducing d-Glc, respectively) present in part of the GOS DP4 ensemble. The signals in the range of δ 4.75−4.40 stem from β-d-Glcp and β-d-Galp H-1 atoms, and those in the range of δ 4.20−4.17 from β-d-Galp H-4 atoms of GOS DP4 components containing 3- (very minor) and/or 4-O-substituted (major) β-d-Galp residues.33,34 Comparison of the 1H NMR spectrum of mono-Sia-GOS DP4 (Figure 3B) with that of GOS DP4 (Figure 3A) shows, besides several signals shifting in the β-anomeric region, three clear additional signals that have similar peak areas. The new signals at δ 2.763 and 1.799 belong to the Neu5Ac H-3e and H-3a atoms of Neu5Ac(α2−3)Gal regions: H-3e, δ 2.75−2.76; H-3a, δ 1.79−1.8045] and the new signal at δ 4.112 is the Gal H-3 signal of 3-O-substituted β-D-Galp units (a terminal Gal residue in GOS), in accordance with the Neu5Ac(α2−3)Gal sequence. Note that Neu5Ac(α2−6) H-3e and H-3a atoms of the Neu5Ac(α2−6)Gal sequence resonate in the regions δ 2.67 and 1.70−1.72, respectively.45 The monosialylation follows from a comparison of the H-1 surface areas of the α- and β-anomeric Glc/Gal residues with those of the Neu5Ac H-3e signal, showing a 3:4:1 ratio for Glc/
Gal:Neu5Ac, fitting to a monosialylation of DP4 structures. Following a similar reasoning, the $^1$H NMR spectrum of di-Sia-GOS (Figure 3C) can be interpreted [Neu5Ac(α-2→3) H-3ε, δ 2.760; H-3α, δ 1.794]. The disialylation is reflected by a 1.6:1 ratio for Glc/Gal:Neu5Ac, fitting to disialylated DP4 structures. Note that β-anomeric signals close to the HOD peak suffer from cosuppression, resulting in underestimation of the Glc/Gal amount in this approach.

Sialylation of Lactose with BPG as Sialic Acid Donor and TcTS as Catalyst. For the decoration of GOS with both Neu5Ac and Neu5Gc, a bovine BPG preparation was selected as sialic acid donor. Blood plasma glycoprotein preparations are a rich source of N- and O-glycans terminated by (α2→3)- and (α2→6)-linked sialic acids. Total sialic acid analysis of BPG, after mild acid hydrolysis, by HPAEC-PAD yielded a content of 0.7% (w/w), of which 45% was Neu5Ac and 55% Neu5Gc. By comparing the amount of sialic acid released by the linkage-type nonspecific sialidase A and the (α2→3)-specific sialidase S, the percentages of (α2→3)-linked Neu5Ac and Neu5Gc in BPG were determined to be 60 and 40%, respectively.

To test the sialic acid transfer reaction with BPG as donor, first lactose was applied as acceptor. To this end BPG [65 mg/mL; corresponding with 0.54 and 0.44 mM (α2→3)-linked Neu5Ac and Neu5Gc, respectively] was incubated with TcTS (1 μg/mL) and lactose (4 mM) for 24 h at 25 °C and pH 5.0. When compared with the HPAEC profiles of the starting materials, HPAEC-PAD analysis showed in both cases above 10 min the formation of two nearly identical product pools, suggesting that both Neu5Ac(α2→3)GOS and Neu5Gc(α2→3)GOS were formed in similar ratios [Figure 5; compare with Figure 2B (DP3) and 2C (DP4) for only Neu5Ac(α2→3)GOS]. The sialylated GOS DP3 and DP4 reaction mixtures were separated by AEX on Resource Q and the ratios of the mono-Sia-GOS and di-Sia-GOS fractions were determined to be 97.3 and 98.2, respectively (not optimized). The mono-Sia-GOS fractions were collected and subjected to $^1$H NMR analysis (Figure S4). The NMR spectra showed overlap of the Neu5Ac and Neu5Gc signals; assignment of the chemical shifts led to the conclusion that both mono-Sia-GOS fractions contain (α2→3)-linked Neu5Ac (H-3ε, δ 2.761; H-3α, δ 1.798) and (α2→3)-linked Neu5Gc (H-3ε, δ 2.778; H-3α, δ 1.820; NGc, δ 4.121). Under the applied conditions, it was found that 33% of total Neu5Ac and 20% of total Neu5Gc could be transferred to lactose, which means that the percentage of transfer of (α2→3)-linked Neu5Ac and (α2→3)-linked Neu5Gc amounted to 55 and 50%, respectively.

Sia-GOS Obtained from TcTS Incubations with BPG and GOS DP3 and DP4. As incubations with BPG would lead to highly complex mixtures of Sia-GOS, built from components of different DP with only Neu5Ac, only Neu5Gc, and in the case of reducing branched oligosaccharides and nonreducing linear oligosaccharides also with both Neu5Ac and Neu5Gc, pilot experiments were carried out with GOS DP3 and DP4.

Both GOS DP fractions (4 mM) were incubated with BPG (65 mg/mL; corresponding with 0.54 and 0.44 mM (α2→3)-linked Neu5Ac and Neu5Gc, respectively) and TcTS (1 μg/mL) for 48 h at 25 °C and pH 5.0. Following a similar reasoning, the $^1$H NMR spectrum of di-Sia-GOS (Figure 4; the relative response factor of Neu5Gc(α2→3)lactose was quantified by HPAEC-PAD on CarboPac PA-1 [Figure 4; the relative response factor of Neu5Gc(α2→3)lactose to Neu5Ac(α2→3)lactose]) is 1.2 times that of Neu5Ac(α2→3)lactose]. MALDI-TOF-MS analysis of the mixture revealed quasi-molecular ions [M + H]$^+$ at m/z 631.88 (Neu5Ac product) and m/z 647.87 (Neu5Gc product). Both products were isolated on CarboPac PA-1 and subjected to $^1$H NMR analysis, showing the typical $^1$H NMR parameters for (α2→3)-linked Neu5Ac (H-3ε, δ 2.759; H-3α, δ 1.801; NAc, δ 2.030) and (α2→3)-linked Neu5Gc (H-3ε, δ 2.777; H-3α, δ 1.820; NGc, δ 4.121). Under the applied conditions, it was found that 33% of total Neu5Ac and 20% of total Neu5Gc could be transferred to lactose, which means that the percentage of transfer of (α2→3)-linked Neu5Ac and (α2→3)-linked Neu5Gc amounted to 55 and 50%, respectively.
substrates GMP and BPG to GOS (total Vivalin GOS or derived specific GOS DPs), thus forming novel sialylated oligosaccharide mixtures (Sia-GOS) in excellent yields. Detailed analytical data for these complex mono-Sia-GOS and di-Sia-GOS mixtures were collected, substantiating the course of the separation reactions. As the used donors contain the two basic members of the sialic acid family, NeuAc and NeuSGc, nearly only (Neu5Ac in GMP) or in an attractive ratio, when focused on NeuSGc (55% NeuSGc) (BPG), they are highly suitable to be further explored for potential applications in both the food and feed industry, respectively. The protocols developed in this study are currently upgraded for the generation of larger batches of Sia-GOS products. When available, these products can be evaluated for food (GOS + Neu5Ac-GOS) and feed (GOS + Neu5Ac/Neu5Gc-GOS) applications in terms of combined prebiotic/antiadhesive properties. This holds for preparations obtained from both total GOS or from specific DP fractions of GOS. Moreover, separation of mono- and di-Sia-GOS fractions generates possibilities for testing them individually.

### Associated Content

#### Supporting Information

Supplemental figures and scheme. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01505.

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**Notes**

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### Abbreviations Used

AEX, anion-exchange chromatography; BMOs, bovine milk oligosaccharides; BPG, bovine blood plasma glycoprotein mixture; DP, degree of polymerization; GMP, bovine κ-casein-derived glycomacropeptide; GOS, galacto-oligosaccharides; HMOs, human milk oligosaccharides; HPAEC-PAD, high-pH anion-exchange chromatography coupled with pulsed amperometric detection; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; 4MU, 4-methylumbelliferyl; NeuAc, N-acetylneuraminic acid; NeuSGc, N-glycolylneuraminic acid; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate—polyacrylamide gel electrophoresis; Sia, sialic acid; SOSs, sialylated oligosaccharides; TcTTS, *Trypanosoma cruzi* trans-sialidase; UV, ultraviolet

### References


