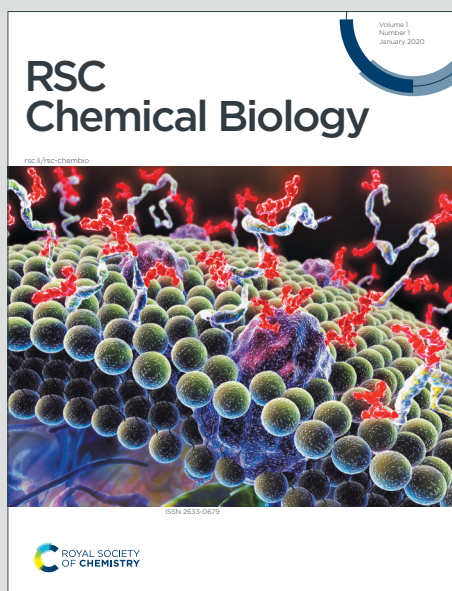


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# Trans-sialylation: a strategy used to incorporate sialic acid into oligosaccharides

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Sialic acid, as component of cell surface glycoconjugates, plays a crucial role in recognition events. Efficient synthetic methods are necessary for the supply of sialosides in enough quantities for biochemical and immunological studies. Enzymatic glycosylations obviate the steps of protection and deprotection of the constituent monosaccharides required in a chemical synthesis. Sialyl transferases with CMP-Neu5Ac as activated donor were used for the construction of  $\alpha$ 2-3 or  $\alpha$ 2-6 linkages to terminal galactose or *N*-acetylgalactosamine units. Trans-sialidases may transfer sialic acid from a sialyl glycoside to a suitable acceptor and specifically construct a Sia $\alpha$ 2-3Galp linkage. The trans-sialidase of *Trypanosoma cruzi* (TcTS), which fulfills an important role in the pathogenicity of the parasite, is the most studied one. The recombinant enzyme was used for the sialylation of  $\beta$ -galactosyl oligosaccharides. One of the main advantages of trans-sialylation is that it circumvents the use of the high energy nucleotide. Easily available glycoproteins with a high content of sialic acid as fetuin and bovine  $\kappa$ -casein-derived glycomacropeptide (GMP) have been used as donor substrates. Here we review the trans-sialidase from various microorganism and describe their application for the synthesis of sialooligosaccharides.

## 1 Introduction



Sialic acid is a crucial family of monosaccharide component of glycoproteins and glycolipids usually located at the surface of cells. They were named neuraminic acids for their presence in brain neurons (1). The most abundant member of this family of about 50 molecules with a common non-2-ulo-pyranosonic structure in mammals is *N*-acetylneuraminic acid (Neu5Ac or NANA) (Fig. 1). Derivatives formed by O-acetylation are part of this family, being the 9-O-acetyl-*N*-acetylneuraminic acid the most frequent one. Acetylation of O-4 or of the exocyclic O-9 hydroxyl group takes place either to provoke or to prevent the interaction with cell receptors (2,3). Another frequent modification is the hydroxylation of the N-acetyl group, giving rise to *N*-glycolylneuraminic acid (Neu5Gc) which, although it is common in the animal kingdom, the corresponding hydroxylase activity is absent in humans (4). Neu5Gc behaves as an exoantigen when incorporated with a meat diet (5). Sialic acids are usually linked  $\alpha$ 2-3 or  $\alpha$ 2-6 to galactopyranose units in  $\beta$  configuration ( $\beta$ -D-Galp).

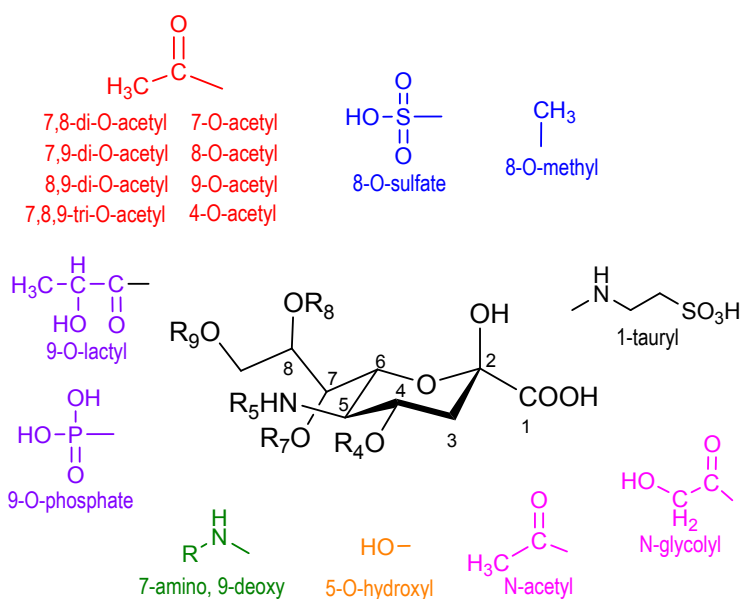


Figure 1 Family of naturally occurring sialic acids. Adapted from (3)

The electronegative charge provided by the carboxyl group of sialic acids in external location may be responsible for cell-cell repulsions, cation binding and masking the antigenicity of the glycoconjugate. A classic example of the last process is the desialylation of serum glycoproteins which uncovers the next galactose in the glycan and allows its uptake by hepatocytes (6). Opposite to masking, sialic acid may be recognized by microbial lectins including viruses in pathological processes. The role of sialic acid in the infection was recently reviewed (7).



It is known that influenza viruses link to host sialic acid (SA) during the infection process. Most corona viruses (CoVs) recognize 9-O-acetyl-SAs (Fig. 1), but switched to recognize the 4-O-acetyl-SA form during evolution of CoVs (8). Although the glycobiology related to the recently emerged SARS-CoV-2, the agent of the current Covid 19 pandemic, was yet not fully elucidated, recent publications show that the viral spike S protein recognizing sialic acid contribute to host tropism (8). This first adhesion facilitates later steps in virus spreading. A diagnostic test based on the interaction of the spike glycoprotein with Neu5NAc was proposed (9). Understanding the protein-carbohydrate interactions in Covid-19 infection may help to the design of inhibitors for therapeutic treatment. The spike protein is heavily glycosylated, mainly in N-glycosylation sites, and recently, O-linked glycans were also described. Sialic acid decorates both types of glycans (10). Virus glycoproteins undergo N- and O-glycosylation using the glycosylation machinery of the host cells and, therefore, the structures vary with the cell type where viral replication takes place (11). The sialoglycans at the surface of the partners interacting during infection are a matter of study for the developing of inhibitors. The synthetic glycans are a necessary tool for these studies, as the natural glycans would not be available in enough quantities.

Chemical synthesis is usually a cumbersome process, since the polyhydroxylated nature of monosaccharides requires the use of protection of the non-participating groups in the glycosylation steps and the consequent deprotection to afford the glycan. This problem may be overcome by enzymatic syntheses which are usually very specific for the construction of glycosidic linkages.

Enzymes involved in the biology of glycosides may be categorized as hydrolases or transferases (Fig. 2) (12). Hydrolases that catalyze the removal of a glycosidically linked sialic acid are sialidases, also called neuraminidases, and can be found in viruses, bacteria, fungi, protozoa (13) and vertebrates, including mammals (14). Sialyltransferases, also present in microorganisms and mammals, synthesize sialosides mainly  $\alpha$ 2-3 and  $\alpha$ 2-6 linked to galactose or *N*-acetylgalactosamine (15–18). Some of these enzymes are multifunctional and are able to construct both types of linkages and also to hydrolyze them (19). Less frequently, other linkages may be found, for instance, sialic acid  $\alpha$ 2-8 linked to another sialic acid was described in glycoproteins (20–24).



Also sialic acid  $\alpha$ 2-9 linked to sialic acid was identified in glycoconjugates (25–27). In bacteria, the well-known colominic acid is a polysialic acid with repeating Neu5Ac $\alpha$ 2-8Neu5Ac units (28,29).

Other rear linkages of sialic acid have been detected and extensively reviewed (30). Bacterial sialyltransferases have been used for the synthesis of sialooligosaccharides using the activated nucleotide CMP-Neu5Ac as the donor (31). Also, multistep enzymatic cascades using *in situ* formation of the CMP-sialic acid donor have been reported (32). Reverse sialylation was described using a mammalian sialyl transferase (ST3Gal-II) and 5'-CMP, which is sialylated *in situ* by a sialoglycoconjugate donor. The CMP-Neu5Ac obtained, is then used to sialylate another acceptor using the same enzyme or other sialyltransferases such as ST6Gal-I and ST6GalNAc-I (33).

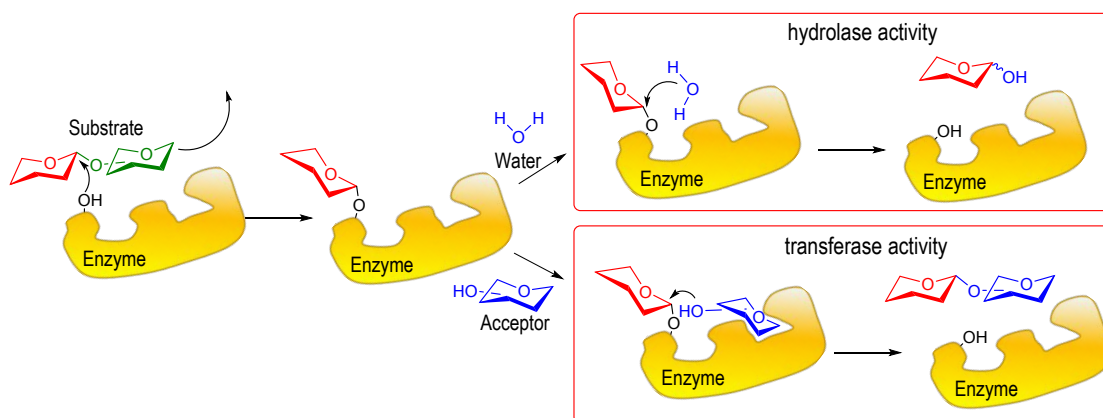


Figure 2 Hydrolase vs. transferase activity in glycosidic enzymes. Adapted from (12)

In the present article we will mainly refer to trans-sialylation, a process used by microorganisms for the incorporation of sialic acid from a sialylated donor, without the need of the activated nucleotide. The use of the trans-sialidase from *Trypanosoma cruzi* (TcTS), the most studied trans-sialidase to date, for the synthesis of biologically important sialooligosaccharides will be described.

## 2 Trans-sialylation

### 2.1 Trans-sialidases in trypanosomatids.

Trans-sialidases were intensively studied in *Trypanosoma cruzi* because they are related to the infectivity of the protozoan, which is the cause of Chagas disease, the American trypanosomiasis (34–36). *T. cruzi* shows a very complex genetic diversity and



its strains have been grouped into six lineages or DTUs (Discrete Typing Units) (37–39). Accordingly, TcTS is a family expressed by around 1400–1700 genes, depending on the *T. cruzi* strain, even though many of them express proteins lacking enzymatic activity (40–43). The sole replacement of Tyr 342 by Hys produces inactive mutants (iTcTS) which, however, act as lectins binding to the glycotope Sia $\alpha$ 2-3 $\beta$ Gal $\rho$  (44). The structural similarity with the reactive TS is evidenced by its recognition by a neutralizing antibody against the enzymatic pocket (45). iTcTS genes were only identified in strains belonging to the lineage classified as DTU II and to the hybrid DTUs TcV and TcVI (46). TcTS expression depends on the parasite's phylogenetic group and increases in the trypomastigote stage (47). This is in agreement with its role in infection and the observation that the invasion of mammal cells depends on their content of sialic acid (36,48). During infection TcTS transfers sialic acid from the host sialoglycoconjugates to the terminal  $\beta$ -linked galactose residues in mucins of the parasite (Fig. 3) and this process is crucial for the infectivity of bloodstream trypomastigotes (49). The reaction is specific and results in the functional unit, sialic acid $\alpha$ 2-3 $\beta$ Gal $\rho$  (34,35,41,50). The specificity of TcTS was studied using convenient oligosaccharides and gangliosides as substrates (51). The authors concluded that the donors must carry Neu5Ac $\alpha$ 2-3Gal and not the Neu5Ac $\alpha$ 2-6Gal terminal unit, whereas the acceptor galactose in the glycan must be  $\beta$ -linked.

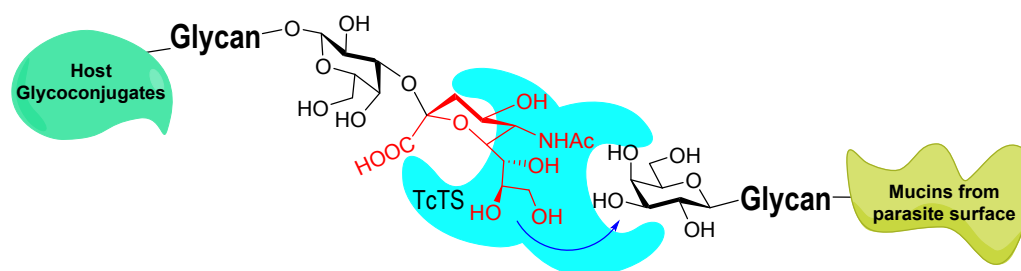
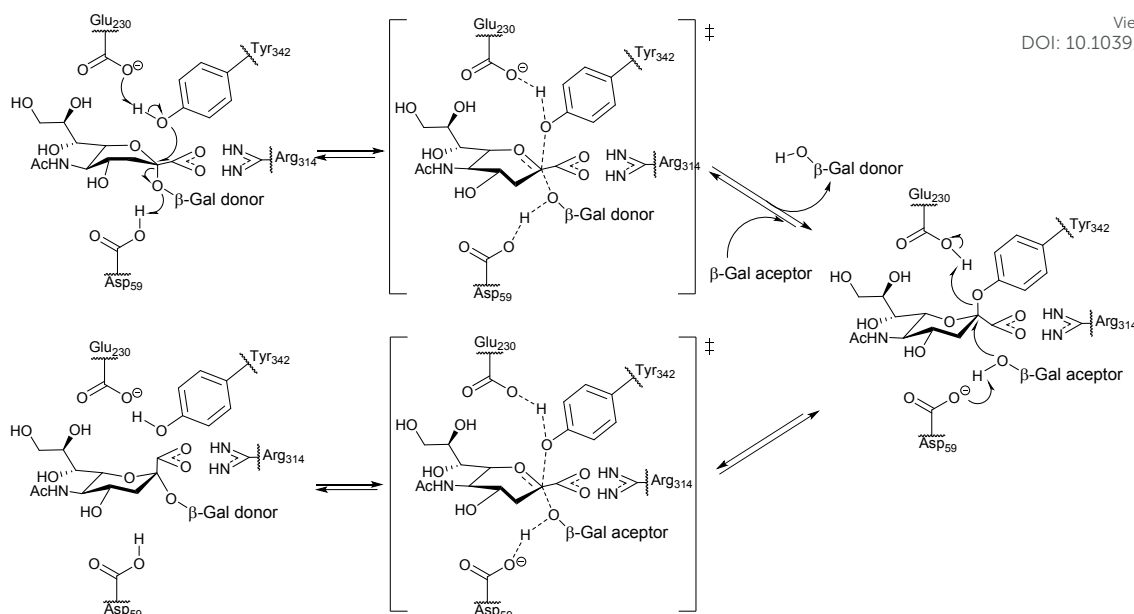


Figure 3 TcTS transfers sialic acid from host glycoconjugates to the *T. cruzi* mucins. Adapted from (52).

Unlike common sialidases, TcTS has two subsites for interaction with the substrates, a subsite for the terminal  $\beta$ -galactopyranosyl unit of the acceptor and another one for the sialic acid donor. Trans-sialylation occurs via a ping-pong mechanism, which starts with formation of a stable intermediate through a covalent bond of sialic acid with Tyr342 of the enzyme, followed by attack of the sialic acid to the hydroxyl group at C3 of a  $\beta$ Gal $\rho$  in the acceptor (Fig. 4) (53).





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Figure 4 Proposed ping-pong mechanism for TcTS activity. Based on (54).

The reaction with different substrates was studied by NMR which showed that the binding of the acceptor to the catalytic side does not take place unless the sialic acid donor is present (55). In the case of TcTS, sialyl transfer is more efficient than hydrolysis, however, when a suitable  $\beta$ Galp-linked acceptor is absent this enzyme behaves as a hydrolase and sialic acid is released (56). Computational simulations suggest that protein flexibility has a role in the transferase/sialidase activity of TcTS (57). Excellent reviews on the structure and function of TcTS have been published (36,42,57–60) as well as reviews on *T. cruzi* trans-sialidase (TcTS) as a synthetic tool (61,62).

TcTS is anchored to the surface of the parasite by a glycosylinositolphospholipid (GPI) (63). An N-terminal signal peptide and a C-terminal peptide indicative of the GPI surface localization may be recognized in all the members of the TS family. Although the lipid of the GPI anchor is cleaved *in vitro* by treatment of TcTS with PI-PLC, microscopical and biochemical studies showed that TcTS is mostly released to the milieu in microvesicles, still linked to the GPI anchor, and not by the action of an endogenous PI-PLC (64,65). Extracellular vesicles (EVs) of trypomastigotes carry more trans-sialidase and show higher adhesion than epimastigote EVs (66). Aside the GPI revealing peptide sequences, a repetitive antigenic sequence was early identified in the soluble TS and called SAPA (shed acute phase antigen) because it is recognized by



sera of patients in the acute phase of the disease (67). SAPA is not present in the TS of epimastigotes (eTcTS), the insect forms of the parasite, and it is not involved in the enzymatic activity. Mice infected with *Trypanosoma cruzi* produce antibodies against the enzymatic domain of TS that inhibit its activity (68). Immunological events caused in Chagas disease by trans-sialylation have been described (64,69–72) and reviewed (73). Campetella and coworkers included an interesting discussion about the humoral response to SAPA in the acute phase of the disease and the detection of neutralizing antibodies in chronic patients (36).

Trans-sialidase and sialidase activities have been investigated in other trypanosomatids. Another health threat is *T. brucei*, the agent of the African trypanosomiasis, known as sleeping sickness in humans and transmitted by the tse tse fly. The TS activity was found in the procyclic stage of the parasite, present in the insect vector, but, unlike TcTS, was not detected in lysates of blood trypomastigotes. The sialic acid acceptor is the glycoprotein procyclin which is characterized by a sialylated GPI anchor (74). Studies of mutants showed that the catalytic site of TcTS and TbTS are similar but not identical (75). Trans-sialidases have been described in another african trypanosome, *T. congolense*, the agent of the disease known as nagana which affects animals (76–78). Trans-sialidase activity was also detected in *T. vivax* which infects cattle in African and South American countries (79).

*T. dionisii*, although genetically related to *T. cruzi* is non-pathogenic to humans (80), however, *in vitro*, metacyclic trypomastigote (MT) forms are able to invade mammalian cells. Its trans-sialidase activity is significantly lower when compared with the same forms of *T. cruzi*. Since it is known that TcTS mediates the escape of trypomastigotes from the parasitophorous vacuole to multiply as amastigotes in the cytoplasm, the intracellular retention of *T. dionisii* and subsequent differentiation into amastigotes within the vacuoles was attributed to the reduced trans-sialidase activity (81).

The sialidase from *T. rangeli* (TrS), although with high identity with TcTS, lacks trans-sialidase activity (82). Mutation of five aminoacids (TrS5) established some activity that increased after six mutations (TrS6). Conformational studies on these mutants, allowed the definition of the aminoacids relevant for trans-sialidase activity (83) and a mutant with 13 mutation was constructed (TrS13) (84). Nevertheless, the mutant showed





promiscuity with respect to the acceptor, since sialic acid could be transferred also to terminal glucose and to melibiose, Gal $\alpha$ 1-6Glc. Seven new variants were obtained by 6-16 amino acid mutations and their trans-sialidase activity to sialylate lactose was studied. The variants with 15 or 16 mutations showed significant trans-sialidase activity (85).

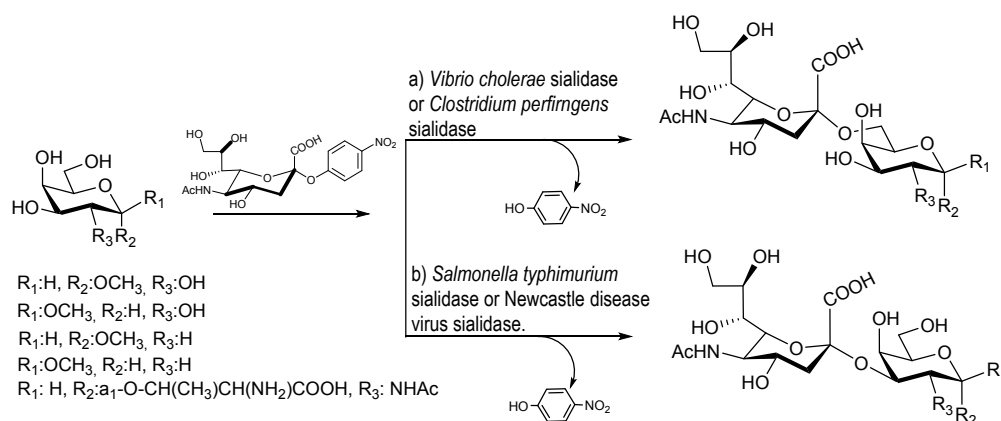
Sialic acid was described as terminal unit of glycoconjugates from the insect stage promastigotes and the mammal amastigotes of several species of *Leishmania*, another genera belonging to the same family as *Trypanosoma* (86). At difference with *T. cruzi*, both,  $\alpha$ 2-3 and  $\alpha$ 2-6 linkages of sialic acid to galactose and the corresponding transferases have been characterized. Although *Leishmania* species may incorporate sialic acid from glycoconjugates, the process is different from the trans-sialylation in *T. cruzi* and was not fully elucidated (7,87).

In an early paper, the trans-sialidase activities of several trypanosomatids were investigated (88). It was reported that whereas *T. cruzi* and *T. conorhini* express mainly trans-sialidase activity, only sialidase activity was detected in *Trypanosoma rangeli* and *Trypanosoma leeuwenhoekii*. Both activities were shown by *Trypanosoma lewisi* and *Endotrypanum* species and none by *Trypanoplasma borreli* and *Leishmania* species.

## 2.2 Trans-sialylation with bacterial sialidases

Bacterial sialidases are less specific in their trans-sialidase activities and afford the sialylated product with lower yields. However, they have the advantage to be easy to express and accept cheap substrates. The *Bacteroides fragilis* sialidase catalyzed trans-sialylation from colominic acid, a homopolimer with Neu5Ac $\alpha$ 2-8Neu5Ac repeating units, to lactose, affording both 3 $\alpha$  and 6 $\alpha$ -sialyllactosides with a total yield of only 0.14% (89). The sialidases from *Vibrio cholerae*, *Clostridium perfringens*, *Salmonella typhimurium*, and Newcastle disease virus were used for sialylation of glycans with average yields of 10-30% (90) (Scheme 1). The new linkages were consistent with the hydrolase activities of the corresponding enzyme, thus, *Vibrio cholera* and *Clostridium perfringens* linked sialic acid  $\alpha$ 2-6 to galactose (91) whereas the other two bacteria showed preference for  $\alpha$ 2-3 formation (92,93).





Scheme 1 Trans-sialylation catalyzed by bacterial sialidases. Adapted from (91)

A truncated mutant of a sialyl transferase from *Campylobacter jejuni*, CstII  $\delta$ 32I53S, showed multifunctionality, including GD3/GT3 oligosaccharide synthase, GD3 oligosaccharide sialidase, and trans-sialidase activities (94). In addition to the  $\alpha$ 2,3 and  $\alpha$ 2,8-sialyltransferase activities reported before for the synthesis of GM3 and GD3-type oligosaccharides, respectively, the CstII  $\Delta$ 32<sup>I53S</sup> has  $\alpha$ 2,8-sialyltransferase activity as evidenced in the synthesis of the GT3 oligosaccharide or in the transfer of a sialic acid from a GD3 oligosaccharide to a different GM3 oligosaccharide. The enzyme showed sialidase or  $\alpha$ 2-8-trans-sialidase activity, depending on the pH of the reaction. The latter activity was observed also in the absence of CMP. It has been used for the synthesis of ganglioside oligosaccharides with flexible donor specificity that included non-natural sialic acids. A strict control of the pH and the reaction time was necessary to obtain good yields (94). In a previous work using a wild strain CstII, CMP was used as activator for the synthesis of  $\alpha$ 2,3-linked sialyllactoside with Neu5Ac pNPh as donor (95).

Another recombinant truncated sialyl transferase was obtained from *Photobacterium damsela* with specific  $\alpha$ 2-6 trans-sialidase activity and was used for the synthesis of Neu5Ac $\alpha$ 2-6Lac $\beta$ MU in good yield using the p-nitrophenyl  $\alpha$ -glycoside of sialic acid (Neu5Ac $\alpha$ pNP) as donor and the methylumbelliferyl  $\beta$ -lactoside (Lac $\beta$ MU) as acceptor (96). The authors claim that this trans-sialidase activity is different from the reported reverse glycosyltransferase activity of some glycosyltransferases which requires the presence of CMP (33) and that kinetic studies showed that the reaction followed a ping-pong mechanism (96). However, the addition of the nucleotide resulted in a modest enhancement of activity. In fact, further work by Mehr and Withers (19)



proved that CMP is required for trans-sialidase activity of bacterial sialyltransferases from glycosyltransferase family 80 and that previous results by other laboratories could be due to impurification of the enzyme with traces of CMP. Only catalytic amounts of the nucleotide are needed to form CMPNeu5Ac for the trans-sialylation reaction.

The best tool to improve transglycosylation activity and/or diminish hydrolytic activity of sialidases is protein engineering. A screening of bacterial sialidases was performed, looking for the aminoacids that shape the aromatic sandwich proximal to the active site that is considered necessary for the trans-sialidase activity in *T. cruzi*. The candidate was the sialidase from *Haemophilus parasuis*, which after expression proved to be a trans-sialidase (97). A casein glycomacropeptide (GMP) was used as donor of sialic acid and lactose as acceptor. Surprisingly three sialylated products were detected by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD): the expected 3'SL, 6'SL and a third sialylated compound, 3SL, which would be the result of sialylation of the internal glucose. This is the only report on sialylation of an internal glucose by a TS. An endo-sialidase which specifically cleaves the Neu5Ac $\alpha$ 2-8Neu5Ac bond in polysialic acid is expressed by bacteriophages for *E.coli*. (98). A set of oligomeric trifluoromethylumbelliferyl sialosides were prepared using the transferase CstII from *C. jejuni*. The substrate should be at least trimeric, and the cleavage occurred between the aglycone and the sialic acids. In the case of the tetramer, however, two dimers could be obtained. Contrary to all other sialidases, this endo sialidase directly hydrolyzed the Neu5Ac $\alpha$ 2-8Neu5Ac bond by an inverting mechanism to produce the  $\beta$ -hemiketal product.

### 3 Synthesis of sialooligosaccharides

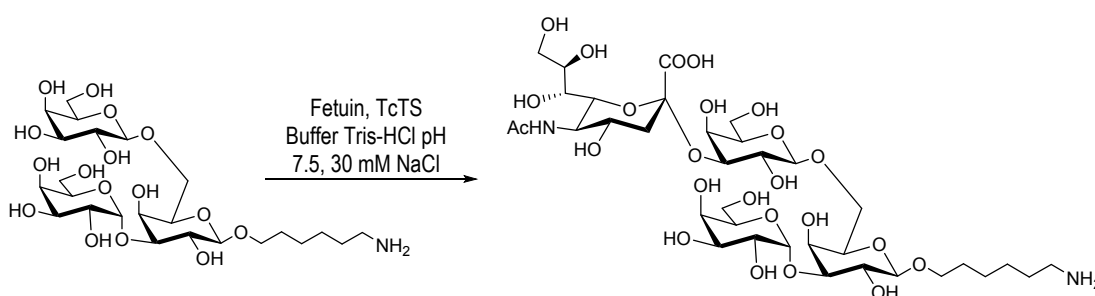
Trans-sialylation reactions are usually analyzed by HPAEC-PAD and the purification of the products is achieved by anion exchange column chromatography. The donors for sialic acid are 3'-sialyllactose (3'SL), or sialyl glycosides like methyl umbelliferyl-*N*-acetyl-neuraminic acid (MUNANA) and *p*-nitrophenyl-*N*-acetylneuraminic acid (Neu5Ac $\alpha$ pNP) with lower activities for the transfer reaction than 3'SL but with the advantage that the reactions are not reversible (61,99). Glycoproteins with an appropriate content of Neu5Ac $\alpha$ 2-3Gal $\beta$  units, like fetuin containing 8.7% of sialic



acids at the non-reducing ends of its oligosaccharides (100), or the casein glycomacropeptide (GMP) with 4-7% sialic acid (101), may be used as donors. Fetuin is a commercially available glycoprotein with a transfer rate to Gal $\beta$ 1-4GlcNAc similar to that of  $\alpha$ 2-3-sialyllactose (102). Fetuin and other glycoproteins have the advantage of simplifying the purification of the sialooligosaccharides by chromatographic techniques. A dialysis step was sometimes included.

The first reports on trans-sialidase activity used a native enzyme obtained from culture derived trypomastigotes (TcTS) (48). Singh *et al.* reported unexpected results in a trans-sialylation study using Neu5Ac $\alpha$ pNP as donor and a recombinant TcTS. The authors reported that methyl  $\alpha$ Galp could be sialylated with a moderate yield and that Galp $\alpha$ 1-6 $\beta$ Galp-OMe was sialylated in the internal galactose to give the branched trisaccharide in 89 % yield. They also found that Galp $\beta$ 1-6 $\beta$ Galp-OMe as acceptor yielded a mixture of three products: the two possible monosialylated products in 88% isolated yield and a bisialylated minor product, but no details on the purification and characterization of these compounds were provided (103).

Giorgi *et al.*, on the other hand, reported the sialylation of the branched trisaccharide, Galp $\alpha$ 1-3(Galp $\beta$ 1-6)Galp, obtained as the 6-aminoethyl  $\beta$ -glycoside (Scheme 2). In this case only one monosialylated compound was detected by HPAEC in agreement with the TcTS specificity. Purification by chromatography on a graphitized carbon column using a step gradient elution of acetonitrile/water afforded the sialylated derivative with a 36% yield (104).



Scheme 2 Sialylation of the 6-aminoethyl  $\beta$ -glycoside of Galp $\alpha$ 1-3(Galp $\beta$ 1-6)Galp by TcTS. Adapted from (104)

Experiments with donors carrying deoxy or methoxy substituted sialic acids led to the conclusion that these modifications did not impair the reaction as long as the changes were at C-9 and not at C-4, C-7 or C-8 (51). Derivatives of MUNANA modified at C-9 were also studied as donors in the TcTS reaction (105).



A polyacrylamide polymer conjugated to 3'SL was prepared using a GlcNAc-bearing polyacrylamide polymer. A  $\beta$ Galp residue was introduced in the first place by means of a bovine  $\beta$ -galactosyl transferase followed by sialylation with TcTS using Neu5Ac $\alpha$ pNP as donor of sialic acid (106).

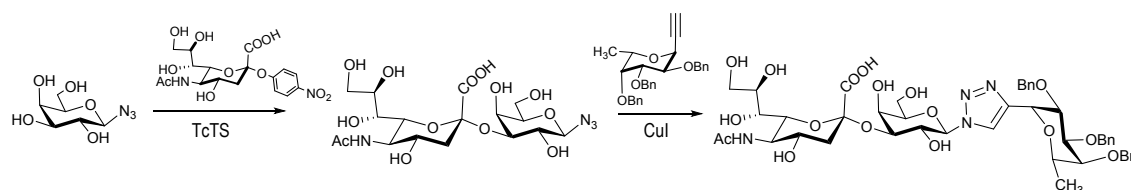
A clone encoding the active N-terminal catalytic domain but lacking the highly immunogenic C-terminal SAPA was expressed in *E.coli* (107,108). Vetere and co-workers used a recombinant sialidase obtained in *E. coli* carrying the plasmid pTS154cat for the synthesis of Neu5Ac $\alpha$ 2-3Galp $\beta$ 1-4GlcNAc (3'-Sialyl-N-acetyllactosamine) by a sequential enzymatic introduction of galactose from lactose and sialic acid from 3'-SL or MUNANA into GlcNAc. In this case, a higher yield (60%) was obtained with the MUNANA donor (109). The same strategy was used for the synthesis of NeuAc $\alpha$ 2-3Gal $\beta$ 1-4Xyl $\beta$ 1-O-p-nitrophenyl, a trisaccharide derivative related to the biosynthesis of glycosaminoglycans. Starting from p-nitrophenyl- $\beta$ -D-xylopyranoside, the trisaccharide was prepared by the sequential action of a  $\beta$ -galactosidase for the incorporation of galactose from lactose and the recombinant trans-sialidase using MUNANA as donor of sialic acid (110). Using the same donor but a specific  $\beta$ -galactosidase for the formation of the Gal $\beta$ 1-3GlcNAc unit, 3'-sialyl-lacto-N-biose (Neu5Ac $\alpha$ 2-3Galp $\beta$ 1-3GlcNAc) was obtained in 35% yield (111). 3'-Sialyl-N-acetyllactosamine was synthesized on the surface of liposomes by a "one-pot" sequential enzymatic modification of a N-acetylglucolipid embedded in the bilayers using a galactosyl transferase with the UDP-Gal nucleotide and TcTS with 3'-sialyllactose as sialic acid donor (112). The coated liposomes could be used for cell recognition studies. Also, vesicles displaying a perfluoroalkyl-tagged lactosyl epitope were sialylated and then recognized by the lectin *Maackia amurensis* leucoagglutinin (113).

A communication reported the preparation of  $^{13}$ C-enriched GM3, and sialyl Lewis X oligosaccharides using a recombinant TcTS expressed in *E.coli* with the plasmid pTS-cat7 for the sialylation step (114). The same enzyme and pNP-Neu5Ac as donor were used for the sialylation of lactosides, lactosamide derivatives and Gal $\beta$ 1-3GalNAc $\alpha$ Ser/Thr with yields in the range 20-60% (115). Glycoconjugates containing Neu5Gc may be used for studies related to its antigenic properties in humans (116). The p-NP glycosides of N-acyl modified neuraminic acid donors, among them the N-



glycolyl derivative, have been tested in the TcTS reaction showing that *N*-glycolylneuraminic acid (Neu5Gc) is efficiently transferred by TcTs (117,118). The exocyclic chain of Neu5Ac is not fundamental in the recognition by the enzyme since the C-7 and C-8 analogues of Neu5Ac $\alpha$ pNP obtained by periodate oxidation were donors for the acceptor methyl  $\beta$ -lactoside (119).

The synthesis of Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc, a component of the Thomsen–Friedenreich antigen was reported by Thiem *et al.* (120). Derivatives of Gal $\beta$ 1-3GalNAc modified at the galactose, the *N*-acetylgalactosamine or both residues were prepared to test their ability to act as acceptors with a recombinant TcTS. Mimetics of the sialyl Lewis X tetrasaccharide were prepared by sialylation of a  $\beta$ -galactopyranosyl azide followed by a click reaction with a fucosyl acetylene to afford the 1,4 diglycosylated 1,2,3 triazole (Scheme 3). Also 1,3-diglycosylated indole derivatives were sialylated in the Galp unit. These sialylated mimetics were tested as competitive inhibitors for selectin binding (121).



Scheme 3 Synthesis of a sialyl Lewis X tetrasaccharide mimetic. Adapted from (121).

Recombinant active TcTS was also expressed in eukaryotic cells like the yeast *Pichia pastoris* (122). Also, cells of *S. cerevisiae* were engineered to express enzymatically active TcTS on their walls and the whole cells were used for *in vitro* sialylation of biantennary complex type oligosaccharides previously labeled with a fluorophore to facilitate monitoring the reaction (123). A *T. rangeli* sialidase with six aminoacid mutations, STR6, was expressed in *P. pastoris* at a higher yield (1 g/L) (124) than TcTS (5mg/L in the same expression host) (122).

### 3.1 Synthesis of sialyl galactooligosaccharides (SiaGOS), components of human milk

Sialooligosaccharides in human milk (HMOs) contribute to brain development and prevent bacterial and protozoan attachment to infant mucosal surfaces (125,126). About 150 species of HMOs have been identified in human milk (127). Colostrum, secreted by the mammary gland a few days before and after parturition, is a good



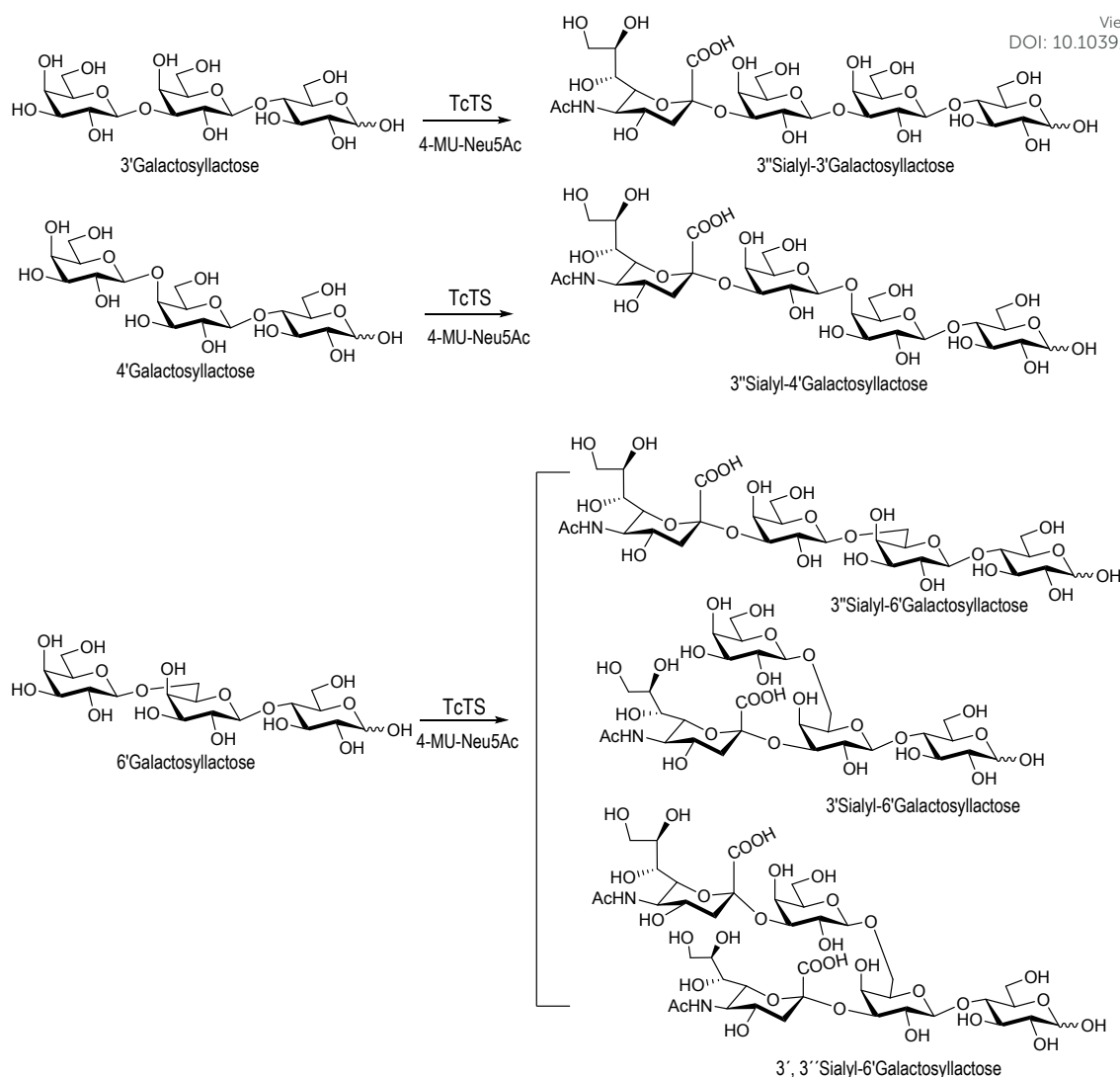
source of oligosaccharides (128). Sialyllactose may be obtained from bovine colostrum in about 500 mg/L (129) and has been used as donor for the synthesis of complex sialylated oligosaccharides (130–136). Since sialyl galactooligosaccharides (SiaGOS) are much less abundant in bovine milk there are several reports describing their enzymatic synthesis with the aim to enrich baby food. The TcTS expressed in *E. coli* was used for the sialylation of galactosyl lactoses with  $\beta$ 1-3',  $\beta$ 1-4' and  $\beta$ 1-6' linkages. Casein glycomacropeptide (GMP), a by-product of the cheese industry, used in this study as donor for the preparation of the sialylated oligosaccharides had a sialic acid content of 3.6% from which 59 % was in  $\alpha$ 2-3 linkage. As expected, only the monosialylated derivative was obtained for the  $\beta$ 1-3'-galactosyl lactose. In the case of Gal $\beta$ 1-6Gal $\beta$ 1-4Glc, two monosialylated compounds corresponding to the sialylation of the external or the internal unit and the disialylated product were obtained. Gal $\beta$ 1-4Gal $\beta$ 1-4Glc, however, was only sialylated in the external galactose residue (Scheme 4) (137). The sialylated galactosyl lactose derivatives, although non-natural HMOs, may be used as functional options

A bovine blood plasma glycoprotein (BPG) containing 0.7% of Neu5Ac and Neu5Gc in similar proportions was used for sialylation of lactose and higher GOS. The products of lactose sialylation, Neu5Ac $\alpha$ 2-3lactose and Neu5Gc $\alpha$ 2-3lactose, were separated by HPAEC and the yield of the sialylated trisaccharides corresponded to a sialic acid transfer of 55 and 50%, respectively, taking into consideration only the  $\alpha$ 2-3 linked Neu5Ac and Neu5Gc in BPG (138).

3'SL and higher oligosaccharides were prepared using GMP as donor and a TcTS expressed in *Pichia pastoris*. The optimal donor:acceptor ratio that minimizes the hydrolase activity was determined to be 1:4 and the conversion yield, considering only the content of  $\alpha$ 2-3 linked Neu5Ac in GMP, was about 64% (101).

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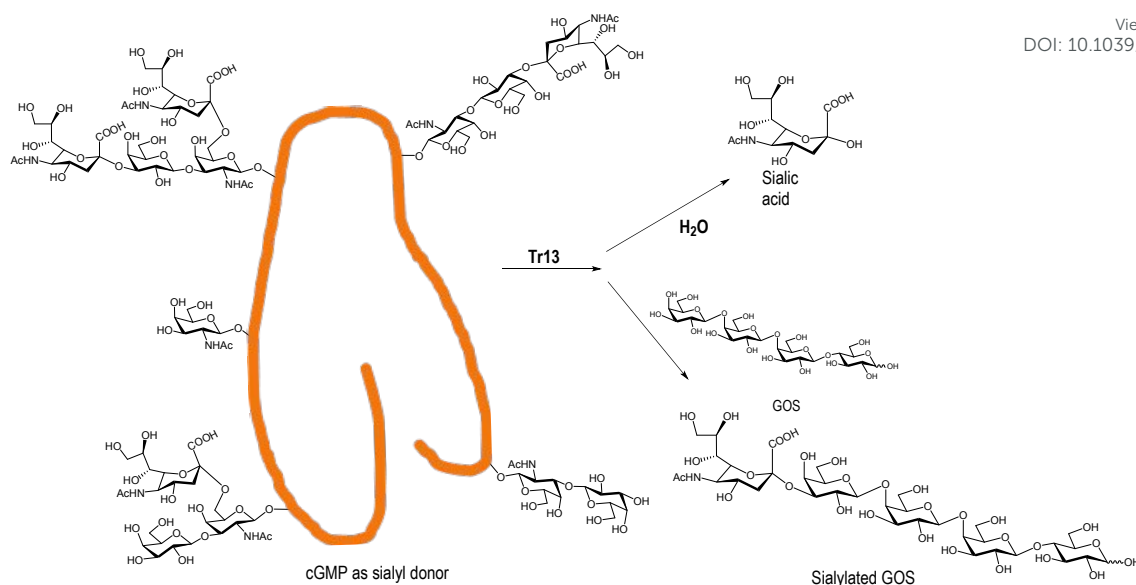


Scheme 4 Synthesis of SiaGOS by TcTS. Adapted from (137)

Engineered trans-sialidases from *T. rangeli*, with multiple mutations, have also been used for the sialylation of GOS (124). A TrS13 mutant could sialylate GOS, in gram scale quantities, independently of their size, showing four times lower hydrolytic activity than the Tr6 mutant (124,139) (Figure 5). Tr15 and Tr16 were used to obtain 3'-SL directly from cow's milk using GMP as donor and the milk lactose as acceptor. With the more efficient Tr15, concentrations of SL similar to those found in breast milk were obtained in a fast reaction (10 min) (140). The use of *T. congolense* TS for sialylation of GOS was patented (141).







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Figure 5 Sialylation of GOS by the *T. rangeli* trans-sialidase mutant Tr13. Adapted from (139).

The sialyltransferase from *Pasteurella multocida* can construct both, Sia $\alpha$ 2-3Gal and Sia $\alpha$ 2-6Gal motifs in a ratio which depends on the reaction conditions. The recombinant enzyme, expressed in *E.coli*, was able to catalyze the synthesis of both 3 $\alpha$  and 6 $\alpha$ -linked sialic acid in higher GOS using GMP as sialic acid donor (142).

### 3.2 Synthesis of sialyl oligosaccharides components of natural glycoproteins

Takahashi and coworkers described the sialylation of N-linked oligosaccharides from human fibrinogen and asialooligosaccharides from fetuin, derivatized as pyridyl 2-amino glycosides, using native TcTS and 3'SL as donor. The sialooligosaccharides were separated by successive HPLC columns and characterized based on their elution times compared with reference compounds and using a three-dimensional mapping technique. Structure assignments were confirmed by digestion with specific exoglycosidases (143).

Trans-sialylation with fetuin as donor and a recombinant TcTS was the last step in the enzymatic preparation of Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-2Man  $\alpha$ -linked to a peptide (Fig. 6). The tetrasaccharide is the most abundant O-mannosyl glycan in  $\alpha$ -dystroglycan ( $\alpha$ -DG), a glycoprotein found in muscle and brain tissue. A one pot enzymatic cascade synthesis was also described (144).



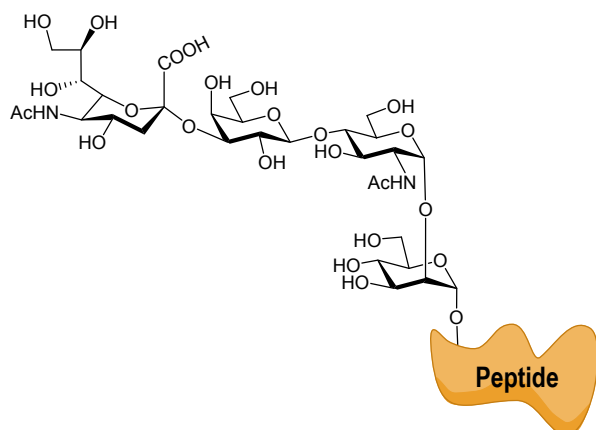
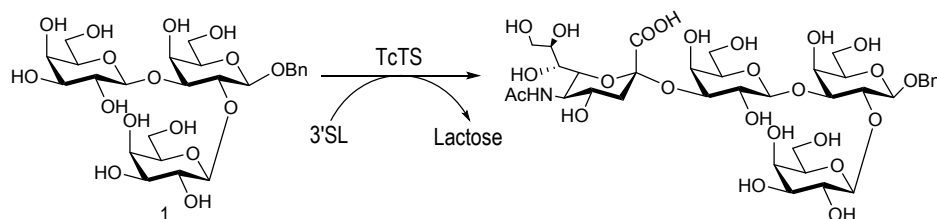


Figure 6 Structure of the most abundant O-mannosyl glycan in  $\alpha$ -Dystroglycan ( $\alpha$ -DG).

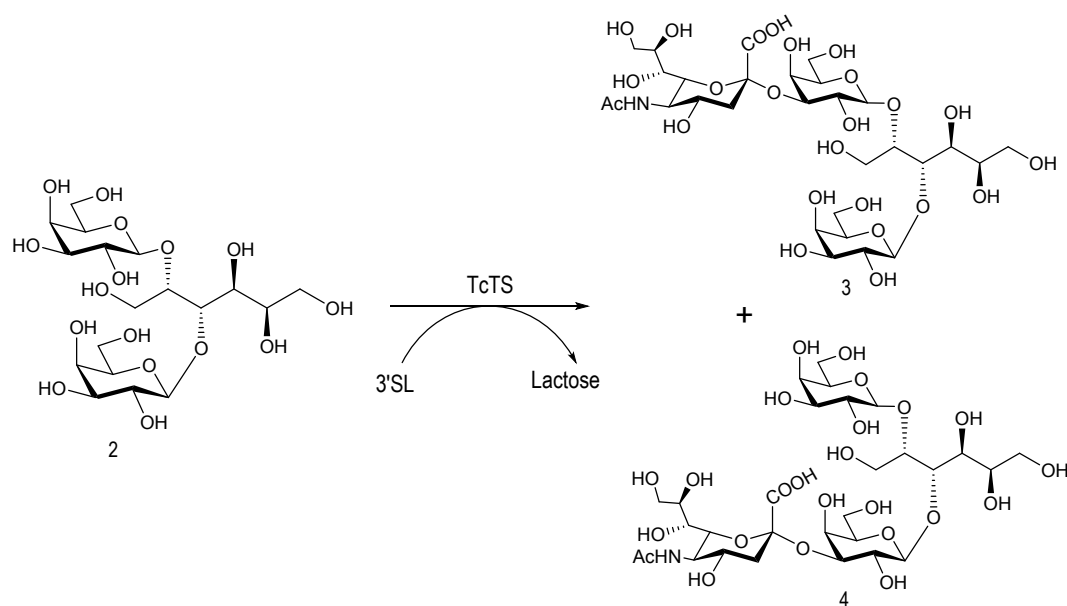
The mucin oligosaccharides of *Trypanosoma cruzi* are the acceptors of sialic acid from the host sialoglycoconjugates, in a reaction catalyzed by TcTS, a crucial process for pathogenesis (35,41,145). In mucins, galactose could appear in the pyranose form or in both pyranose and furanose forms, depending on the strain (52). Galf is not present in any mammal glycan and only appear in some strains of the epimastigotes, one of the insect forms of *T. cruzi* (146). Since both constituents,  $\beta$ Galf and  $\beta$ Galp, may coexist in the same molecule, it was interesting to study their behavior in the TS reaction. The Galf-containing oligosaccharides have been chemically synthesized (132,133,147–151). The trisaccharide unit 2,3-di-O-( $\beta$ -D-Galp)- $\beta$ -D-Galp, with two  $\beta$ Galp for possible sialylation, is the external unit of the three largest oligosaccharides of *T. cruzi* mucins. Reaction of the benzyl glycoside of 2,3-di-O-( $\beta$ -D-Galp)- $\beta$ -D-Galp (**1**, Scheme 5) (152) with TcTS showed selective trans-sialylation from the donor 3'-sialyllactose to the less hindered (1-3)-linked  $\beta$ Galp. Sialylation of the more flexible alditol **2** was not selective and a mixture of compounds **3** and **4** was obtained (Scheme 6), suggesting that the open zig-zag conformation adopted by the alditol turned both galactoses almost equally accessible for TcTS recognition (130). Accordingly, the benzyl glycosides of the pentasaccharide and one of the hexasaccharides of the mucins, compounds **5** and **7** respectively, were also selectively sialylated in the same residue to give **6** and **8**, respectively (Scheme 7) (132,133). All the structures were confirmed by NMR spectroscopy. In the case of sialylation of the benzyl glycoside of the other hexasaccharide, with three terminal  $\beta$ Galp units, two monosialylated compounds and a minor amount of a disialylated product were formed (Fig. 7) (132). A study on the comparative rates of sialylation of the synthetic oligosaccharides, showed that the



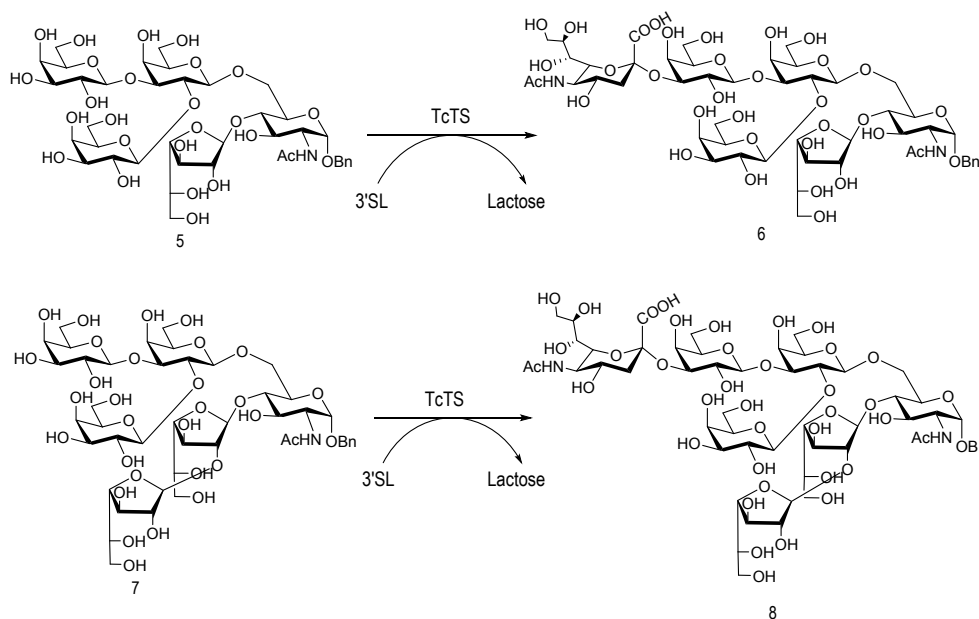
presence of Galf did not impair the reaction. Thus, the diminished virulence of the strains that contain Galf is not related to interference of sialylation by Galf (153).



Scheme 5 Selective trans-sialylation of the  $\beta$ -benzyl glycoside of 2,3-di-O-( $\beta$ -D-Galp)- $\beta$ -D-Galp. Adapted from (130)



Scheme 6 Sialylation of 2,3-di-O-( $\beta$ -D-Galp)- $\beta$ -D-Galp alditol by TcTS. Adapted from (130).



Scheme 7 Trans-sialylation of the  $\beta$ -benzyl glycosides of the pentasaccharide and one of the hexasaccharides from *T. cruzi* mucins. Adapted from (132,133)



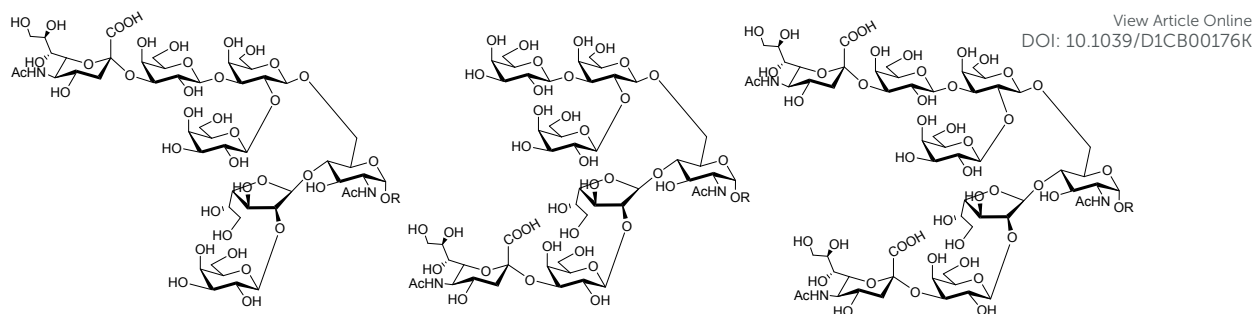
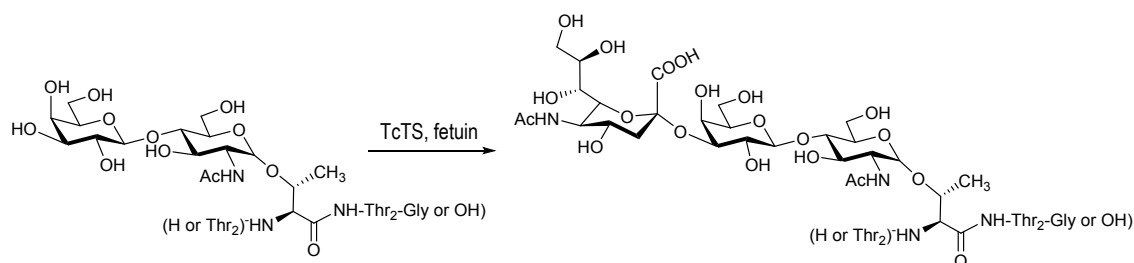


Figure 7 Proposed structures for the sialyl derivatives obtained by trans-sialylation of the  $\beta$ -benzyl glycoside of a hexasaccharide from *T. cruzi* mucins. Adapted from (132).

Based on the aminoacid sequences in the *T. cruzi* mucins, the glycopeptide Thr-Thr-[LacNAcThr]-Thr-Thr-Gly, was synthesized using a chemoenzymatic strategy and further sialylated by *T. cruzi* trans-sialidase using fetuin as donor (Scheme 8) (154).



Scheme 8 Sialylation of Thr-Thr-[LacNAcThr]-Thr-Thr-Gly with TcTS. Based on (154)

Bacterial sialidases have also been used for the synthesis of natural oligosaccharides. The sialidase of *S. typhimurium* was used for the synthesis of the Lewis tetrasaccharides (Fig. 8) (92). Thiem and coworkers used the sialidases of *Vibrio cholerae*, *Clostridium perfringens*, *Salmonella typhimurium*, and Newcastle disease virus for the synthesis of several oligosaccharides, among them the epitopes of the T-tumor antigens (Thomsen-Friedenreich). The reactions were regioselective according with the selectivity of the corresponding enzyme and the yields obtained were between 10-30% (90).

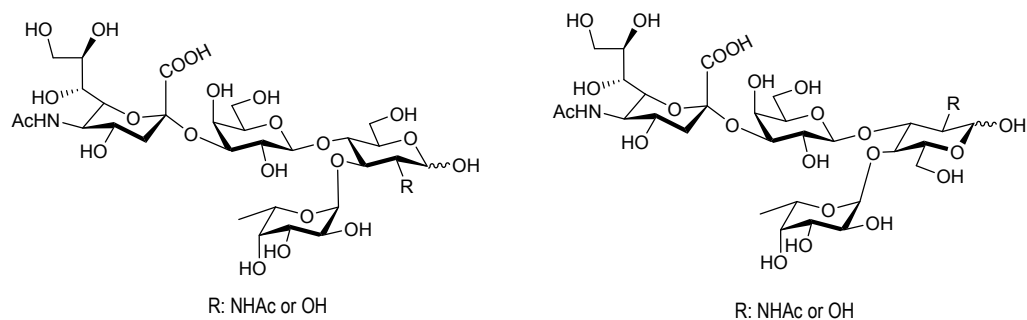


Figure 8 Structures of the Lewis tetrasaccharides synthesized with *S. typhimurium* trans-sialidase. Adapted from (92)



### 3.3 Sialylation of non-natural oligosaccharides

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Cyclic pseudo-galactooligosaccharides dimers and trimers were synthesized by “click chemistry” and analyzed as TcTS substrates using MUNANA as donor of sialic acid to give disialylated and trisialylated products with moderate yields (Fig. 9) (155).

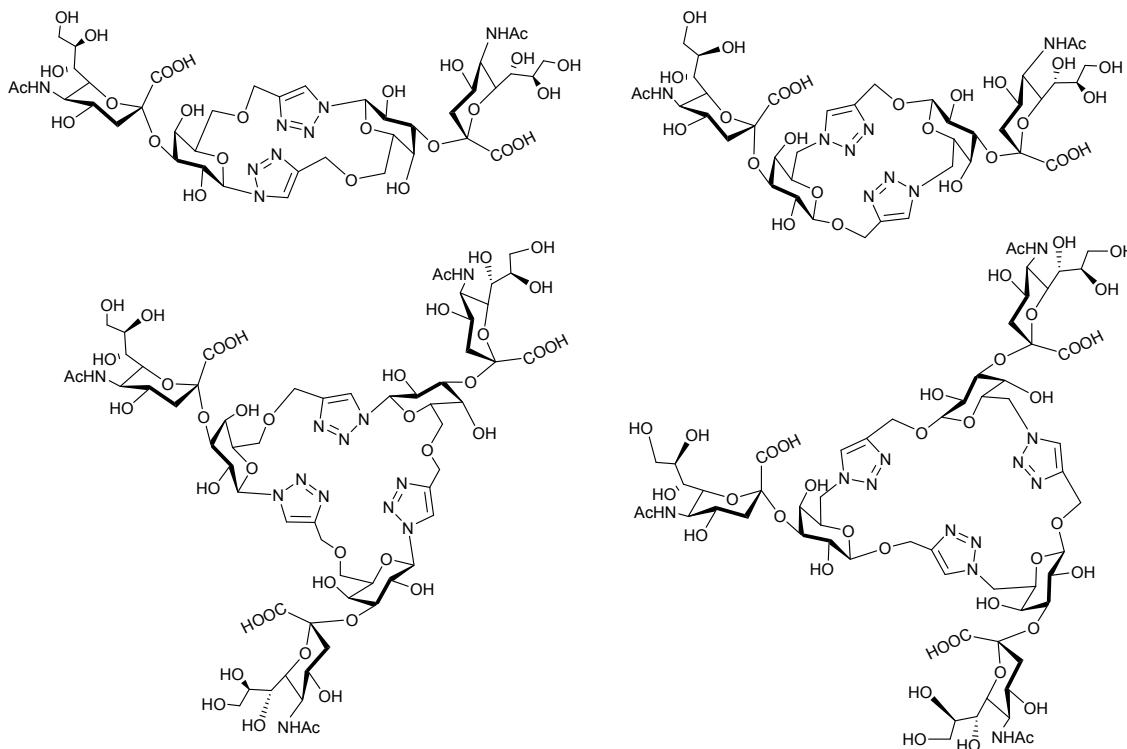
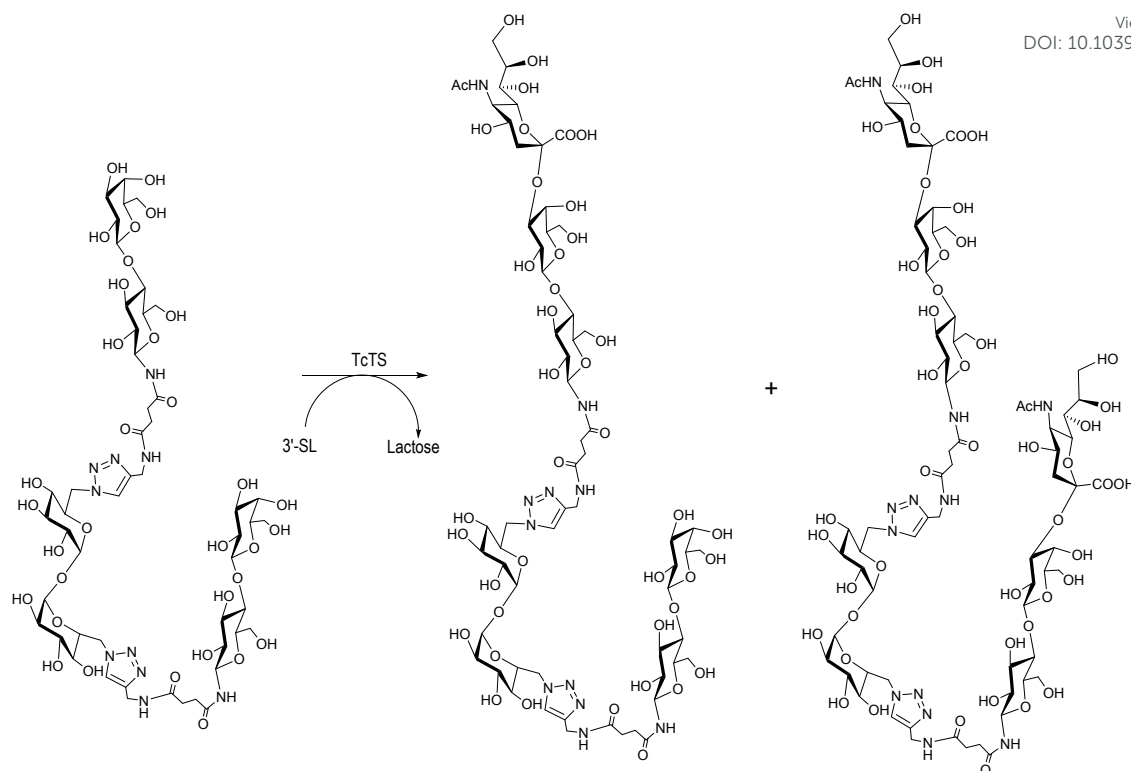


Figure 9 Sialylation of galactomacrocycles by TcTS. Adapted from (155)

Multivalent ligands of  $\beta$ -thio- and  $\beta$ -N-lactosides are also acceptors of sialic acid in the TcTS reaction using 3'-SL as donor (131,156). A divalent  $\beta$ -N-lactoside yielded the monosialylated derivative as the major product with minor amounts of the disialylated product (Scheme 9) as observed by HPAEC analysis and later confirmed by mass spectrometry. TcTS efficiently transferred sialyl residues to di, tri, tetra and octa  $\beta$ -thiolactosides (131). A preparative reaction with a tetravalent  $\beta$ -thio-glycocluster gave a mixture of monosialo, disialo and trisialo species that could be separated with an anion exchange resin and their degree of sialylation confirmed by MALDI-MS. The possibility of multisialylation of ligands suggests their use as competitive inhibitors of sialylation and anti-adhesion agents for microbial infections (157).





Scheme 9 Mono and disialylation of divalent  $\beta$ -N-lactosides synthesized by click chemistry. Adapted from (156)

## 4 Conclusions

The role of sialic acid in the infection by microorganisms is well known. The spike glycoprotein of SARS-CoV-2, the agent of the current Covid 19 pandemic carries N- and O-glycans decorated with sialic acids. Future studies on interaction of the glycans with host cells could lead to the design of inhibitors to the penetration of the virus. With this purpose chemoenzymatic synthesis of the glycans would be desirable.

Selected trans-sialidases are a convenient tool for the synthesis of sialooligosaccharides. The reaction is regiospecific for the construction of the non-reducing unit Neu5Ac $\alpha$ 2-3 $\beta$ Gal $p$ . One of the main advantages is that the donor Neu5Ac-CMP may be replaced by a glycoprotein with a convenient content of sialic acid such as fetuin or GMP, an easily available by-product of the cheese industry. In addition to being less expensive their higher molecular weights facilitate separation of the excess donor from the newly obtained sialooligosaccharide. The commercial sialosides, Neu5Ac $\alpha$ pNP and Neu5Ac $\alpha$ MU (MUNANA) have much lower activities than 3'SL for the transfer reaction (61,99), but have the advantage that the reactions are not reversible and the nonpolar aglycone can be easily isolated from the reaction



products. Sialidases have been engineered to act as trans-sialidases, with an activity that depended on the reaction conditions, like temperature, pH, water activity, time of reaction. Transglycosylation was favored when higher acceptor concentrations were used. Recombinant TcTS was used for preparative synthesis of sialooligosaccharides with potential application in the elaboration of supplement for baby formula, based on the beneficial effect of these sugars present in human colostrum. The enzyme is not commercially available yet, however, extension of its use may encourage its commercial production.

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**Authors contributions.** RM L organized the manuscript, RML, MEG and RA drafted the manuscript, MEG prepared the Figures. All authors revised the manuscript and approved the final version of the manuscript

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